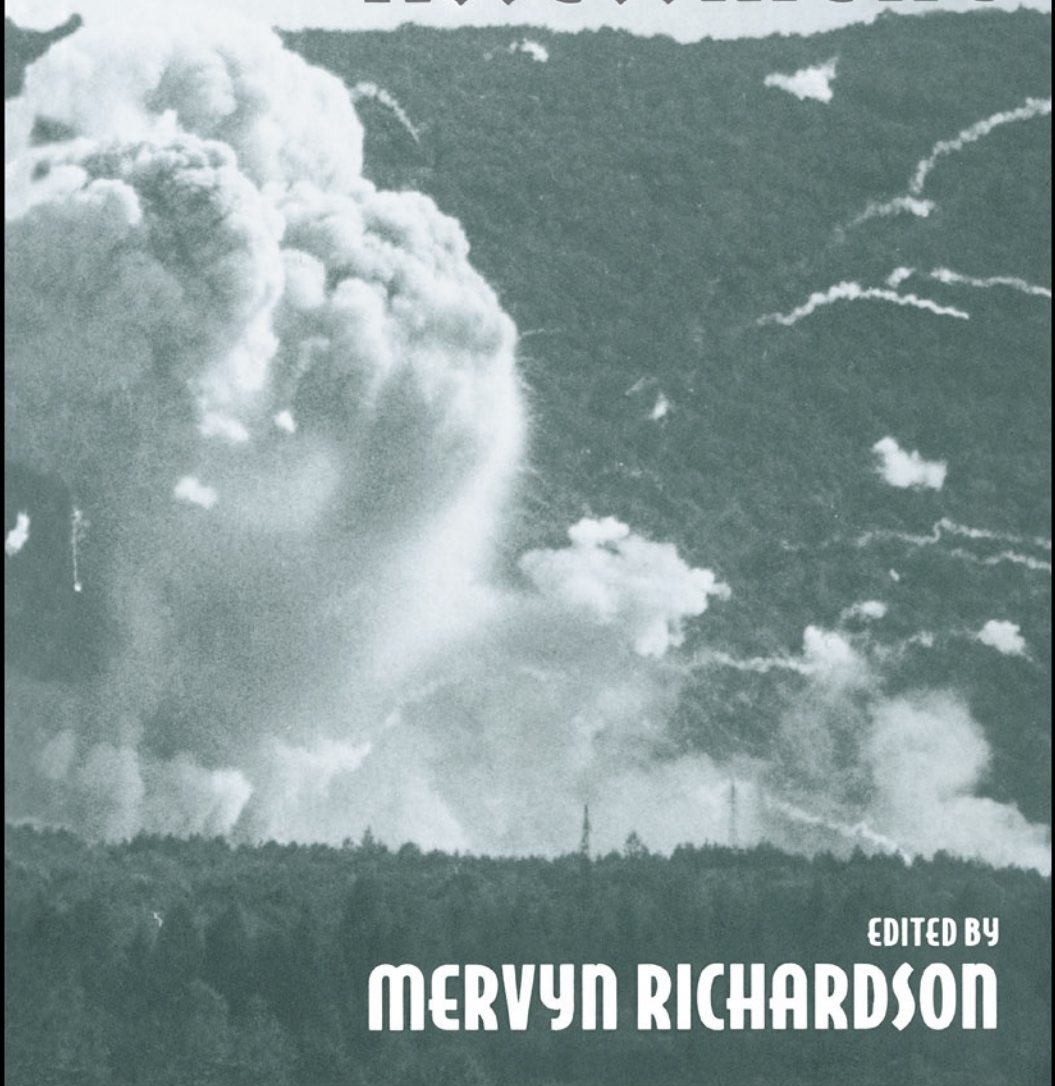
Environmental Toxicology Assessment



EDITED BY

MERVYN RICHARDSON

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Front cover illustration: An explosion following the intentional ignition of an ammunition stockpile at Oštarije, Croatia, 1992.

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Preface

Mervyn L.Richardson

The preservation of the species, *Homo sapiens,* on our planet earth is now more than ever dependent on the environment. The exploitation of our natural environment (air, soil and water) cannot continue unabated if the health of mankind and that of future generations is to be maintained. Environmental toxicology is concerned predominantly with the harmful effects of substances in the natural environment including effects on populations and ecosystems.

During my missions for the United Nations' agencies in Asia and in Central and Eastern Europe, I observed at first hand some of the horrendous consequences of man's activities and the disruptions to the natural environment and the resultant consequences to human health.

Through World Aid agencies remediation work in a number of countries is already in hand. Hence, it is particularly gratifying that this volume contains chapters from the Islamic Republic of Pakistan, including the inaugural address from her President on the occasion of the opening of the Ecotoxicology Research Centre on 27 March 1994 in Islamabad. This is supported by the address from the Federal Minister for Food, Agriculture and Livestock and the address welcoming delegates to the workshop which corresponded to the opening of the Centre by the Chairman of the Pakistan Agricultural Research Centre followed by a statement from the United Nations Industrial Development Organization representative. These laboratories were funded by the United Nations Development Programme in conjunction with DANIDA and will serve the requirements for the South-East Asian Region. This programme is due to the foresight and continuing work of the United Nations Industrial Development Organization in Vienna.

Chapters from internationally eminent scientists who delivered papers at the Islamabad workshop include those by Vollner, Sugavanam, Baloch, Masud, Wyn Ellis, with myself presenting the plenary lecture which, in turn, is the basis of my introductory chapter. The role of pesticide application in developing countries is of paramount importance and this is stressed in a number of chapters.

This volume includes new and exciting ecotoxicological techniques such as Microbic's chronic toxicity test, the umu-C assay and research on DNA probes, etc. Some of the effects of man's pillage of the environment are referred to in the two case studies by the Croatian authors, one on the environmental toxicological effects of explosives (used in warfare), the other relating to 500 years of pollution from mercury mining.

The chapters written by scientists from 12 countries stress how international scientific collaboration is proceeding. Whilst many of the ravages caused to the environment will require expensive remediation processes, it should be remembered

that *prevention is better than cure*—a statement made by Bernardino Ramazzini (1663–1714).

Toxicology, in a variety of specialized and primitive forms, has been part of the history of man; one of the earliest reference works being the Ebers papyrus (~1500 BC).

In Rome, poisoning seemed to take on epidemic proportions in ~400 BC, in addition to the environmental toxicology problems assigned to lead leaching into piped drinking water supplies.

The pragmatic assessment of the toxicology of chemicals, whether man-made or natural, in the environment and their consequences, is of concern to everyone. This volume addresses many of the current problems and details corrective means. Only by means of proper environmental toxicology assessments can a nation's economic future be guaranteed.

As is common with multi-author works, some overlap between chapter content is inevitable. These were reduced to a minimum during editing except on those occasions where it was deemed that overlap would enhance the topic.

The Editor is indebted to Taylor & Francis for their support, especially to Janie Curtis, who had the vision to promote this topical subject. Great appreciation is also expressed to Pauline A. Sim of Gascoigne Secretarial Services, High Wycombe, who retyped the whole book and attended to all administrative matters. Sincere thanks must be extended to my wife Beryl for her general support and who patiently tolerated the editing of this volume, the mountains of paper, faxing of whole chapters and telephone calls on a global basis at all hours of the day and night.

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'At all times, people and the capacities of ecosystems to support life is at the center of the focus of Environmentally Sustainable Development. The existence of acute poverty in the World and the degradation and contamination of ecosystems are related critical issues and essential concerns in environmentally sustainable development.'

The World Bank Annual Report 1994, p. 42

بِسۡمِ اللّٰہِ الرَّحۡمٰنِ الرَّحِیۡمِ

THE ISLAMIC REPUBLIC OF PAKISTAN

Farooq Ahmad Khan Leghari ISLAMABAD

Inaugural Address by Sardar Farooq Ahmed Khan Laghari
President of Islamic Republic of Pakistan

Workshop on Ecotoxicology, Islamabad, Pakistan, March 27–31, 1994

Mr. Minister, Excellencies, Distinguished Delegates, Ladies and Gentlemen!

Cliches are distasteful because they are oft repeated, but in renewing my contacts with agricultural scientists I can say that it is a matter of pleasure and great privilege. Pleasure because, I have, and I am in the primary production system and my heredity avocation has been and is agriculture. It is a matter of privilege because one feels that it is with scientific vigor that we can make this world a better place to live in. Today's agenda is very relevant to the one world we have, to the cause of humanity and to our very breathing. Since technological progress is a continuous and never ending process, there is a need and desire to implement this in our part of the world. This present Ecotoxicology Workshop forms part of this new dimension. It has been rather late in starting but I hope that in the years to come it will make up its belated entry through extra vigor and energy.

In 1980 when the shift in the pesticide's sector was made, from the public to private sector, a number of incentives were provided to the multinationals by providing them a level field and a propitious environment. That was carried out in order to ensure that productivity in the agriculture sector increased substantially. The use of pesticides did indeed improve this productivity and an analysis of facts provides substantial evidence. But then, somewhere along the line, the balance was lost and today you see resistance has developed in certain pests where the use of pesticides has been inappropriately high. It is visible in the cotton area, in the Brown hopper population in the rice fields. In using excessive pesticides, we have endangered the balance nature has brought between predators, parasitoids, and parasites. The emergence of white fly as a primary parasite is indicative of this effect.

Although the agriculture sector in Pakistan has made good progress during the last decade but it did not fulfil its promise because of natural calamities. Biotic and abiotic stresses have reduced seriously production of crops which cannot be overcome without adequate research and development efforts. I am informed that generally almost 33% of crop productivity is lost to pest attack. For example, during the year 1993, when cotton crop was victim of cotton leaf curl virus, a decline of 27% in cotton production was experienced, while during 1994, the loss is anticipated to equal 33% of production. The banana crop due to Banana Bunchy top virus has been reduced to almost half. Thus we should focus our attention on change to broaden the base of pest management. We must analyze ecological relationships in our agro-ecosystem which, in turn, will require much more research and supervision directed at development of integrated pest management system with a rational use of pesticides. Allocation of substantial resources has to be made for upgrading research and development infrastructure for crop protection. I am concerned that research findings be transferred to the end-users effectively to bring an effective change.

The environment is to be saved at all cost, and I hope that the toxic residues that have been left in the soil by excessive use of pesticides would be monitored, its impact evaluated and prescriptions provided to regenerate the natural life balance. I am already aware that the adverse effects are manifested in the phased elimination of wildlife in our world. Wildlife provides a very important balance in the natural scheme and we must very jealously protect what has been provided to us by nature.

I must state that the Government of Pakistan accords a very high priority to the issue of safe use of pesticides and trust that you will suggest the needed recommendations and support for follow-up activation.

I understand the Government of Denmark and the United Nations Industrial Development Organization (UNIDO) have helped us in initiating this Institute of Pesticide Ecology of Ecotoxicology Research Centre. This venture has become a challenge for future generations.

I wish to thank all those who initiated the establishment of this Institute. I hope I have contributed in some little way by taking this first step of inaugurating this Institute. I am also pleased to inaugurate this workshop.

I wish you well and God speed! **Pakistan Paendabad**

(Sardar Farooq Ahmed Khan Laghari)

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GOVERNMENT OF PAKISTAN
MINISTER FOR FOOD, AGRICULTURE
AND LIVESTOCK
ISLAMABAD.

Date 27 March 1994

Nawab Muhammad Yousuf Talpur

Address by Nawab Muhammad Yousaf Talpur
Federal Minister for Food, Agriculture & Livestock
Islamabad, Pakistan

Workshop on Ecotoxicology, Islamabad, Pakistan, March 27–31, 1994

Mr. President, Distinguished Delegates, Ladies and Gentlemen

It is my proud privilege to address this gathering of scientists. On behalf of the Government of Pakistan and on my own I extend a hearty welcome to the delegates attending the 'Ecotoxicology Workshop'. Their agenda is relevant to humanity and the environment. This is of significance to the world in general and the developing countries in particular.

Factor productivity analysis would have us believe that the scientific model constitutes the be all and end all of productivity. When this model was being developed in the mid 1960s in Pakistan, it had promise, as yields had been stagnant and population increases were a cause for concern. One component of this model was the use of pesticides—and these were used indiscriminately, thus endangering everyone. The world can ill afford this luxury of a toxic laden world. No matter who is involved or who he is involved, the repercussions are wide ranging.

The emergence of pests is of economic importance, e.g. white-fly, jassids, bollworm in cotton, and brown hopper in rice. The natural world of predators, parasitoids and parasites was destroyed. Once again, man had done what he should have guarded—the natural ecological balance.

Several constraints pose challenge to agriculture growth in Pakistan. 1 Pests, insects, diseases and weeds are major calamities which devastate crops. During the last 3 years our agriculture has been in the grip of viral diseases affecting seriously the production of cotton, banana, tomatoes and chillies. Obviously, this is a warning that we have decided to accord a very high priority to the pest management system in

general, and safe use of pesticides in particular. Outbreaks of viral diseases are reported to have assisted the development of resistance in their insect vector hosts (hoppers, white-fly and aphids). The understanding of the dynamics of pesticide residues in the ecosystem is a difficult task. The assessment of their risks must be based on knowledge of complex factors. Pesticides kill indiscriminately, contaminate food, water, soil, micro- and macroorganisms, invertebrates and vertebrates, leaving very persistent residues. This is especially true in African and Asian countries.

We are in the process of establishing facilities for studying the toxic effects of agrochemicals in nature. The establishment of the Ecotoxicology Research Centre in Pakistan is the first step towards promoting our plans. I may add that the Pakistan Government has high priority for sustainable agriculture. A considerable amount of thinking has been given to drawing up requirements in the Task Force report on Ariculture. This Government hopes that structural and directional changes will be developed to make agriculture more meaningful.

Our thanks for establishing these facilities are due to the Government of Denmark, United Nations Industrial Development Organization (UNIDO), and the United Nations Development Programme (UNDP). I hope this support will continue.

I once again thank you all for your participation, particularly the President of Pakistan, the delegates to the workshop, and the Pakistan Agricultural Research Council.

(Nawab Muhammad Yousaf Talpur)

Government of Pakistan
Ministry of Food, Agriculture and Cooperatives

Secretary

Chairman
Pakistan Agricultural Research Council

Islamabad, the 27 March 1994

Welcome Address by Dr. Zafar Altaf

Workshop on Ecotoxicology, Islamabad, Pakistan, March 27–31, 1994

Excellency, the President of the Islamic Republic of Pakistan, Honourable Minister, Distinguished Delegates, Ladies and Gentlemen!

In welcoming your excellency, Mr. President, I am conscious of welcoming someone who has a deep and lasting affection for agriculture and the environment. The current situation in pesticide use, in ecotoxicology is known to you. Surprisingly enough, we had nothing to monitor regarding what was happening in the country. What was the impact of all the toxic materials which we throw around at random? This impact had to be studied. Disturbing news had to be curbed not by ignorance but by a reasonable approach. So this new intervention was considered and in this the Danish Government and UNIDO/UNDP help in establishment of the Ecotoxicology Centre is gratefully acknowledged.

In fact here at National Agricultural Research Centre, this is one of the new interventions that has been made. There have been a number of other areas such as the plant genetics institute, water resources development, horticulture institute, where new interventions have been made. There are now adequate technologies available on the shelf. Scientific endeavor is creating more of technology now than ever before. All it requires is interjection into the macro-economic framework. This would enable some of the variables to move positively.

Excellency, agriculture is at a crossroads. It is time for not only new directions but also new dimensions. It is a time for consideration and striking forth. Vigilance is required at all times whether these are good or bad. But the interventions because of these vigilances at different times are to be different. In cotton the present time will try men's souls. It is time for understanding and deep thought.

Reason, Mr. President, must dictate the logic of institutions. It is the institutional logic that we are putting in place, but it is experience that will make

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this institution work. This experience has been brought forward from international sources. We have initiated the present system, in a miniature form and we hope that in time this will increase in magnitude. The effort, however, for any new intervention to grow and work is exhausting. From unconcerned attitudes to unreasonable rebellions—in short, everything under the sun is accosted. Under such circumstances how is principle to be combined with practice? This present activity is finally coming into its own, after roughly two years of effort, UNIDO/UNDP efforts have substantially helped us in overcoming systemic rigidities.

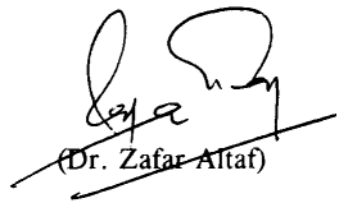
The compulsion of research are not easily understood. Sometimes the time factor and the gestation period is so distant in time, that ordinary minds are unable to comprehend. Much time and effort has been lost in persuading unpersuasive minds. The effort and exhaustion was well worth the end result. But if research is to make a march then the inner discords in research are also to be resolved. For along with ability certain other parameters are to work in harmony. Wherewithal and faith in the scientist would help. But there is something over and above that needs to be put in place. Is it high motivation? The scientist, your excellency, has to pitch against himself. Only then will he transcend his own self. The task is Herculean and yet the cure is in the self.

Our endeavor is to reincarnate the spirit of science. And this spirit of science is not of form but of substance. This spirit is manifest in a fiercely independent belief and attitude system. It is our firm conviction that belief and attitude form the central core to any action plan that we might have. This will only be internalized if the scientist is free of from externalities.

In this effort the workshop organized here in collaboration with UNIDO will be of great help. Not only will it help improve awareness, it will also be able to indicate the many interfaces of this subject. Health, environment, industry, the multiple institutions whose actions will need to be coordinated, will be identified here. A common frame of mind-thought will have to be developed.

The research in this centre will be seminal in nature. Starting from scratch means making marks on snow. Much will depend on how they perform, how they improve the system, how mankind will benefit from this work. The nature of this work in essence will be preventive rather than curative. We are in process, we will never have prescriptions. That is what makes this more challenging.

Mr. President, we are grateful to you for your graciousness in being here with us. On behalf of the scientists I thank you!



Statement from the UNIDO Representative

Workshop on Ecotoxicology, Islamabad, Pakistan, March 27–31, 1994

Balasubramanyan Sugavanam

Your Excellency, the President of the Islamic Republic of Pakistan, the Resident Representative of the United Nations Development Programme, Ladies and Gentlemen

It gives me great pleasure to come to Pakistan to participate in the Ecotoxicology Workshop and on behalf of the Director General of UNIDO, Mr. Mauricio de Maria y Campos I welcome you all to this first Workshop on Ecotoxicology. I would like to express our appreciation and gratitude to the Government of Pakistan, especially to the Ministry of Agriculture for agreeing to host this workshop and for providing all the facilities for the workshop. We are especially honored. Your Excellency, by your gracious presence which clearly indicates the importance given by your Country to this project.

We are all well aware of the fact that there has been a revolution in agriculture in Asia during the last quarter of a century, and many chronic food deficit countries in this region became either self-sufficient or even food exporting countries. In addition, the quality of their agricultural produce, especially rice, perishables and cash crops has improved vastly so as to meet export requirements. This is due to the constructive agricultural policies adopted by the governments of the various countries of the region which paved the way in facilitating application of technologies as they developed. We are well aware that, following the green revolution the supply of fertilizers in the region had improved during the 1960s and the 1970s. Above all, pesticides played a significant role in protecting crop losses due to infestation by pests during pre- and post-harvest stages, and also to improving public health standards.

However, major advances made in the industrialized world during the last two decades and the research and development work carried out in these countries paid high dividends due to the introduction of highly active and more selective pesticides. This decreased drastically the amount of pesticides needed per ha from the kg level to <100 g ha^{-1}. These developments also introduced high technology in formulation and application of pesticides. Despite these successes in pushing the frontiers of science and technology. Your Excellency, there is a great concern with regard to safety in the production and use of pesticides. This is more pronounced in the developing countries where many of these advances in technology have yet to make inroads into agriculture. In the majority of cases use of an older generation of pesticides, outdated methods of application and ineffective methods of disposal are

practised and create great concern regarding their negative impacts on the environment. Even with newer pesticides, it is not possible to be fully aware of their effects on the environment. Added to this, increased production and use of other chemicals including fertilizers, has led to many persistent chemicals, their metabolites and heavy metals contaminating the soil, ground and surface waters thereby causing transboundary pollution. All of these problems create the necessity to monitor and follow their fate and effects of these chemicals on the environment. This needs coordination between the industry, the users, the government, the public, governmental and non-governmental organizations, and international organizations. If the benefits of modern technology are to be utilized fully, especially in chemical and allied industries the countries should have the capacity to monitor the movement and the fate of toxic chemicals entering their environment from direct and indirect sources. Additionally, the companies themselves should become transparent regarding their operations within the given limits of confidentiality.

Many developing countries with increased production and consumption of chemicals do not have the capacity to monitor the presence and fate of these chemicals in their ecosystems which could lead to catastrophic effects. For example, some recent news from Ecuador claims that improper and inadequate use of fungicides in banana plantations have ruined the shrimp exporting industry thus affecting the nation's economy. Such a catastrophe could have been averted if the nation had the capacity to monitor the fate of toxic chemicals leaving the source, and could have taken proper precautions in hazard identification, reducing risks and managing acceptable risks.

In this respect UNIDO assisted by UNDP and the Government of Denmark is providing the catalytic effect on a regional and national basis to promote the necessary mechanism to strengthen the capacity in the region for hazard identification, assessment of hazards and eliminate, reduce or mitigate risks associated with toxic chemicals entering the ecosystem either from chemical and allied industries, or due to their use in agriculture or in other outlets. In this we are very thankful to UNDP for their support to the Regional Network on Pesticides for Asia and the Pacific (RENPAP), in which various aspects related to safe developments of pesticide production and use are addressed on a regional basis. This is the first time the project has given full support to ecotoxicology and thanks to the long standing efforts of the National Coordinator of RENPAP for Pakistan, Dr. Baloch, and the efforts of the Regional Coordinator, Dr. Dhua, support was obtained from the Government of Pakistan, the Government of Denmark and the UNDP. Thanks to their generous contribution in cash and kind, today we are in a position to commence the first stage of the Ecotoxicology Research Centre. In this respect I would like to thank in particular the Pakistan Agricultural Research Council (PARC), for providing the local facilities and highly qualified staff. This Centre has just started and is expected to commence research and development projects during the course of the next couple of years when they will be in a position to assist the other countries of the region in monitoring toxic chemicals in the environment to avert major pollution hazards.

Even though the project has just started, it is pleasing that PARC, even at this early stage of implementation, has agreed to host this workshop with the hope of exchanging ideas and experience as a North—South interaction to promote awareness and action to reduce/eliminate problems associated with toxic chemicals.

The *Aide Memoir* for the workshop stressed the importance of the topic to various ministries, industries and non-governmental organizations and an excellent response, due to the importance of the subject, was obtained. Unfortunately, due to budgetary constraints, a limit had to be placed on participation from member countries.

We are also very fortunate to have international experts who are active in the field who can guide us in discussing various issues, taking into account the requirements of the developing countries of the region. We are also fortunate to have delegates from FAO and GIFAP, and I extend a warm welcome to all participants of this workshop. The purpose of this workshop is to provide a platform to participants from member countries to discuss various aspects related to ecotoxicology and how the UN system could provide the necessary assistance in risk reduction in handling toxic chemicals on the basis of chapter 19 of Agenda 21 dealing with environmentally sound management of chemicals, especially toxic and hazardous chemicals. This workshop will hopefully set the scene to increase the capability of Member Countries in monitoring the movement and the fate of toxic chemicals in their ecosystems. Once again, I sincerely thank the Government of Denmark, the Government of Pakistan and the UNDP for providing the necessary support to bring together experts from the North and the South to discuss the important issues of ecotoxicology. I extend a warm welcome to the country delegates who are representing the member countries of RENPAP and who will all give inputs in guiding the workshop in making recommendations to increase the capability of the region in risk reduction—an essential consideration, due to toxic chemicals entering the ecosystem.

I again thank Your Excellency, the President of the Islamic Republic of Pakistan, for sparing your valuable time to grace this occasion to inaugurate this workshop and open the Ecotoxicology Centre, and also thanks to the Government of Pakistan and especially the project authorities for making this workshop possible. I wish you all fruitful deliberations during this five day workshop.

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Abbreviations Used for United Nations and Other International Agencies

AAP	Algal assay procedure
AChE	Acetylcholinesterase inhibition
ADR	European Agreement Concerning the International Carriage of Dangerous Goods by Road
AFE	Alkaline filtration elution
ANOVA	ANalysis Of VAriance
AP	Ascorbate peroxidase
APTAC	Agricultural Pesticide Technical Advisory Committee
AOX	Absorbable halogens
AWQMS	Austrian water quality monitoring system
BCF	Bioconcentration factor
BaP	Benzo(a)pyrene
BOD	Biological oxygen demand
CBE	Carboxyl esterase
CEC	Commission of the European Community
CEFIC	European Chemical Industry Council
CEN	Comité Europeen de Normalisation
CFA	Colony forming assay
CPU	Colony forming unit
CHIP	The Chemicals (Hazard Information and Packaging) Regulations
COD	Chemical oxygen demand
DANIDA	Danish International Development Agency
DIG	Dissolved inorganic carbon
DIN	Deutsches Institut für Normung
DNA	Deoxyribonucleic acid
DNB	Dinitrobenzene
DNT	Dinitrotoluene
dpm	decompositions per min
DOC	Dissolved organic carbon
dw	dry weight
EAC	Emergency Action Code
EB	Ethidium bromide
EC	European Community
ECETOC	European Centre for Ecotoxicology and Toxicology of Chemicals
ECOD	7-Ethoxycourmarin *O*-deethylase
EDTA	Ethylenediaminetetraacetic acid
EEA	European Economic Area
EEM	Environmental effect monitoring
EFTA	European Free Trade Association
EIFAC	European Inland Fisheries Advisory Commission
EINECS	European Inventory of Existing Commercial Chemical Substances

ELST	Early Life Stage Test
EMPA	Swiss Federal Laboratories for Material Testing and Research
ER	Endoplasmic reticulum
EROD	7-Ethoxyresorufin O-deethylase
ESCAP	Economic and Social Council for Asia and the Pacific
ESID	Ecologically Sustainable Industrial development
EU	European Union
EUCLID	European Chemical Information Database
FAO	Food and Agricultural Organization
FARM	Farmer-Centred Agricultural Management
FBS	Fetal bovine serum
FDA	Fluorescein diacetate
FEA	Federal Environment Agency
FITC	Fluorescein isothiocyanate
FONSI	Finding no significant impact
FRG	Germany
FSC	Forward angle light scatter
FWMR	Federal Water Management Register
GAC	Granulated activated carbon
GC	Gas chromatography
GIFAP	Groupement International des Associations Nationals de Fabricants de Produits Agrochemiques
GLP	Good laboratory practice
GR	Glutathione reductase
GSF	National Environmental Research Center (München)
GST	Glutathione S transferase
GT	Glucosyltransferase
GTZ	Deutsche Gesellschaft für Technische Zummenarbeit
GWP	Greenhouse warning potential
HEDSET	Harmonized Electronic Data Set
HMX	Octahydro-1, 3, 5, 7-tetranitro-1, 3, 5, 7-tetrazocine
HPLC	High pressure liquid chromatograph
IARC	International Agency for Research on Cancer
ICAO	Technical Instructions for the Safe Transport of Dangerous Goods by Air
ICES	International Council for the Exploration of the Seas
ICP	Inductively coupled plasma
ILO	International Labour Office
IMDC	International Maritime Dangerous Goods Code
IPM	Integrated Pest Management
ISO	International Standards Organization
JECFA	Joint Expert Committee on Food Additives
JRC	Joint Research Centre
LSC	Liquid scintillation counting
MCFOD	Methoxycarbonylfluorescein O-demethylase
MFO	Mixed function oxidases
MRL	Maximum residue limits
MS	Mass spectrometry
MITI	Ministry of International Trade and Industry
MPMMG	Marine Pollution Monitoring Management Group

MT	Metallothionein
NAFTA	North American Free Trade Association
NFPA	National Fire Protection Association
NGO	Non-government organization
NOEC	No observed effect concentrations
NOEL	No observed effect limit
OECD	Organization for Economic Cooperation and Development
PAPA	Pakistan Agriculture Pesticide Association
PARC	Pakistan Agricultural Research Council
PAHs	Polycyclic aromatic hydrocarbons
PARCOM	Paris Commission
PCR	Polymerase chain reaction
PEC	Predicted environmental concentration
PI	Propidium iodide
PNEC	Predicted no effect concentration
POX	Peroxidase
QSAR	Quantitative structure-activity relationships
QSBR	Qualitative structural-biodegradability relationships
QSPR	Quantitative structure-property relationships
RAP	Remedial action plan
RDX	2, 4, 6-Trinitro-1, 3, 5-triazine
RID	Regulations Concerning the International Carriage of Dangerous Goods by Rail
RIVPACS	River Invertebrate Prediction and Classification System
RENPAP	Regional Network on Pesticides for Asia and the Pacific
ROS	Reactive oxygen species
SAF	Submerged aerated filter
SAR	Structure-activity relationships
SCA	Standing Committee of Analysts
SCAS	Semi-Continuous Activated Sludge
SCE	Sister chromatid exchange
SDS	Safety data sheet
SOD	Superoxide dismutase
Sp	Sponja (Idrija)
SPR	Structure-property relationships
SPT	Solid phase test
SUP	Safe use projects
TB	Trypan blue
TDN	Total oxidized nitrate
TGHDP	Thai-German Highland Development Project
THM	Trihalomethanes
TLC	Thin layer chromatograph
TNT	Trinitrotoluene
TOC	Total organic carbon
TPA	Thai Pesticide Association
TR	Toxicity ratio
TRE	Toxicity reduction evaluation
TT	Toxicity threshold
UN	United Nations
UNDP	United Nations Development Programme
UNEP	United Nations Environment Programme

UNIDO	United Nations Industrial Development
USA	United States of America
USEPA	United States Environmental Protection Agency
WHO	World Health Organization
WWTW	Wastewater treatment works

SECTION 1: PROLOG

1 Prolog

Mervyn Richardson

1.1 THE PRINCIPLES

To understand the principles involved in assessing the effects of chemicals in the natural environment and their subsequent effects on humans, it is necessary to appreciate the meaning of a number of terms, some of which can be confusing. These terms are shown in Table 1.1.

Environmental toxicology should only be applied to the study of direct effects of environmental chemicals on human beings, whereas the term ecotoxicology should be used only for the study of the effects of chemicals on ecosystems and their nonhuman components. However, it should pointed out that in many cases this distinction can be artificial. Human beings are not isolated from the natural environment; they are at the top of many food chains and there are now few ecosystems in which the human species is not involved.[1]

Whilst the main thrust of preventive toxicology is in the arena of human health, it is becoming increasingly evident that human health is connected intimately with conditions in the natural environment. Chemicals released into the environment far from human habitation may become a health hazard for humans through food chain accumulation. Other chemicals may adversely affect crop growth or kill economically important fish stocks or bird life.[2] It should be remembered that neither poisonous gas clouds nor contaminants in river or oceanic waters recognize natural boundaries. Often, adverse effects of chemicals on wildlife may be the initial indication of an early warning of hazards to human beings.

In the consideration of environmental toxicology, the manner in which the effects of the hazards of chemicals present in any environmental media, ie, air, water, or soil, must be taken into consideration. Environmental and ecotoxicology monitoring[3] is an important step in the assessment of the risk to be attributed to environmental effects. In simplistic terms, risk is the integral of hazard and exposure or dose, ie, any measurement obtained by monitoring either continuously or from single samples. The assessment of these effects, the subject of this book, illustrates the range of disciplines and personnel involved in the decision-making process, ie, on the use and, perhaps even more importantly, in the disposal of chemicals in such a manner as to safeguard human health.

The problems and scope involved with environmental toxicology is so great that the topics had to be selected very carefully, either because of their intrinsic importance, or because they provided a basis from which general principles may be derived. Hence the fundamental environmental toxicological topics, including the use of

animals, plants, algae and bacteria in toxicological assessment, the basis of biotransformation and degradation of chemicals, molecular mechanisms, etc. have to be considered. Additionally, it is necessary to consider the general principles involved in ecological monitoring, with particular reference to the aquatic environment.[2]

Table 1.1 *Terms*

Ecotoxicology
The study of the toxic effects of chemical and physical agents on all living organisms, especially on populations and communities within defined ecosystems; it includes transfer pathways of these agents and their interactions with the environment.

Ecosystem
The grouping of organisms (microorganisms, plants, animals) interacting together, with and through their physical and chemical environments, to form a functional entity.[4,5]

Environment
The aggregate at a given moment of all external conditions and influences to which a system under study is subjected.[4,6]

Environmental chemistry
Chemistry dealing with the origins, transport, reactions, effects, and fate of chemical species in the water, air, terrestrial, and living environments.

Toxicological chemistry
Chemistry of toxic substances with emphasis on their interaction with biological tissue and living systems.

Biochemistry
A branch of chemistry concerned with the chemical properties, composition, and biologically-mediated processes of complex substances in living systems.

Toxic
Able to cause injury to living organisms as a result of physiochemical interactions; whereas **toxicity** is:

(i) Capacity to cause injury to a living organism defined with reference to the quantity of substance administered or absorbed, the way in which the substance is administered (inhalation, ingestion, topical application, injection) and distributed in time (single or repeated doses), the type and severity of injury, the time needed to produce the injury, the nature of the organism(s) affected, and other relevant conditions;
(ii) Adverse effects of a substance on a living organism defined as above; or
(iii) Measure of incompatibility of a substance with life: this quantity may be expressed as the reciprocal of the absolute value of median lethal dose ($1/LD_{50}$) or concentration ($1/LC_{50}$).

The natural environment is amazingly complex and the environmental toxicologist is beset with the basic problem that there is often no real information at a particular site as to what is normal, acceptable or tolerable. Hence, as it is almost

impossible to predict exactly what will happen when a chemical is released to such environments. Measurement or monitoring is the obvious choice of strategy, but the form that such monitoring should take is a matter for most careful consideration.

1.2 MEASUREMENT

If the discharge is one of inorganic ions eg, heavy metals, chemical analysis is on the whole reasonably easy and straightforward as compared to discharges of organic wastes. However, great care is necessary in considering the techniques used in sample preparation. Should this be total, ie, digestion in *aqua regia* or in more realistic terms by leachability into say ammonium acetate solution? An excellent example of this is given by Miko et al[7] in describing perhaps the worst contamination of the natural environment in Europe in recent years by the explosion at the ammunition depot at Oštarije, Croatia in October 1991.

The major problem associated with organic substances is that no matter how sophisticated the analytical chemical technique used for the identification, and even less so for the quantification, of substances in a complex effluent stream, effluvia or leachate, only under the best of conditions is it possible to achieve ~20% success. This is unlikely to include the enormous range of chemicals produced by chemical interaction, biological transformation, etc.

Biological measurement techniques for environmental toxicology have been increasing in availability and scope over the past ten years and their attraction is of obvious advantage and such techniques, unlike chemical methods, provide an indication of the toxicity of the sample investigated. Many of these techniques, eg, the use of *Vibrio fischerii* (formerly known as *Photobacterium phosphoreum*) NRRL B-11177, the Microtox® and Mutatox® tests are robust and certainly with the former are extremely rapid and portable, therefore the laboratory can be taken to the contaminated site rather than the more usual submission of samples to laboratories involving transportation problems, delays, possible contamination, etc.[3] Whilst such techniques will not indicate which substances in such complex mixtures are toxic, they do provide a very rapid indication of the environmental toxicity of a sample.

A number of new and exciting variations of such biological measuring techniques are now in the course of development, eg, EROD, cytochrome P-450 activation, umu assay, spindle unwinding, DNA probes, etc, and the development of these techniques will surely lead to the concept of *toxic insult*. As such techniques are rapid and in the majority of cases can be used in the field, they lead to a relatively simple assessment of the toxicity of the environment condition. One now has to question whether further development of sophisticated analytical chemical techniques is the preferred strategy for the 21st century.[8] It is vital to stress that measurement, whether discrete or generic, is essential so that the hazard can be assessed for risks and that techniques displaying risk management can be applied in a pragmatic manner so that a decision can be made regarding chemical (biological or physical) safety.[9] There cannot now be a return to the days of the hunter-gatherer as

we are all totally dependent on chemicals and such benefits are related to attempts to answer the question *how safe is safe enough*?[10]

1.3 TOXIC INSULT

In turn, the concept of *toxic insult* will play a major role in an environmental impact assessment which is the possible environmental consequences of a past, ongoing, or planned action, resulting in the production of an environmental impact statement or 'finding of no significant impact' (FONSI).[4] An environmental impact statement which is the report emanating from an environmental impact assessment can thus be prepared. During these processes it is important to consider all aspects of environmental hygiene or sanitation which is the predicted control measure used to improve the basic environmental conditions affecting human health, eg, clean water supply, human and animal waste disposal, protection of food from biological contamination, and housing conditions, all of which are concerned with the quality of the human environment.[4,11]

It is essential to stress that adverse effects to the natural environment lead to a high cost in human wellbeing, as now indicated by lower life expectancy, higher infant mortality, and higher incidence of respiratory diseases, cancers, birth defects, especially congenital malformations, and other illnesses.[12] These are not the only costs of environmental degradation in some areas; without a base of functioning water, land and air resources, industrial productivity, economy and growth, are hampered. Only by adequate regard for the environment can a country enjoy a prosperous economy. The decline in the forestry and tourism industry, eg, as an aftermath of the war in Croatia,[13] due to damaged forests, crop yield deficiencies due to contamination, damage to cultural buildings, damage to water installation by warfare, or pipe corrosion by polluted water or other causes, are but a few of the examples of the real cost of having an inadequate regard for environmental consequences.

1.4 CRADLE TO GRAVE APPROACH

Both chemical manufacturers and their customers must demonstrate clearly their responsibility for the total life of their products.[9,14] The researcher who synthesizes a new pharmaceutical chemical, agrochemical or dyestuff is the first in this chain of events and it is at the primary stage that the *toxic insult* has now surely to be considered. Whilst responsible chemical manufacturers are now rightfully concerned over their discharges to air, land and water, and their major customers likewise, are their ultimate downstream customers so vigilant? This was a matter raised by The Royal Society of Chemistry at their symposium held at the University of Surrey in July 1992 and it was resolved that if a responsible manufacturing company was not able to assure that his ultimate customer could manage the use and disposal of his products then he should cease to supply. This concept now needs to be implemented by the assurance that all industrial users of chemicals are able to demonstrate to all

concerned that they have undertaken an *environmental toxicology assessment*. This will need to be extended to the use of chemicals in the domestic environment. Perhaps the greatest care is necessary in the use of the 'cradle to grave' concept in the use of pesticides. This is important as increasingly the developing centuries turn from the use of natural materials, eg, manure (itself a pollutant in some circumstances),[14] to sophisticated modem agrochemicals.[15] Hence, it is gratifying that these principles are being given due attention in South-East Asia by the new facilities in Islamabad and by the Regional Network on Pesticides for Asia and the Pacific (RENPAP) as a result of UNIDO's Farmer-Centered Agricultural Management (FARM) sub program.[16]

It was also apparent that during both the missions to Croatia in 1993[17,18] and the Ostrava, Czech Republic for the 'Interagency Workshop on Environment and Health Management in Industry and Government' that agrochemical usage in Central and Eastern Europe has declined, largely because of economics. This lack of use in the longer-term is poor economics as crop yield, and especially quality, have both declined.

The resurgence in the use of pesticides will need to be undertaken with a knowledge of the full consequences gained from an *environmental toxicology assessment*. This will need the supply of high level expertise in both environmental measuring, ideally by generic techniques, and toxicological assessment in all aspects of industrial and domestic pollutants.

1.5 THE FUTURE

The future must lie in the most appropriate application of *environmental toxicology assessment* involving techniques such as:

- Risk reduction;
- Cleaner techniques and technologies;
- Human resource development;
- Industrial safety;
- Waste management; and
- Use of renewable resources.

This book has been divided into the following sections.

1.5.1 New measuring techniques

This includes the limitations of new technical approaches, especially in biomarkers, use of luminescent bacteria for chronic and genotoxicity measurements, applications of the use of DNA probes and cytochrome P-450, applications in community analyses in the field taking into account biochemical, physiological and cellular methods, test design and data interpretation and the use of radiolabeled techniques.

1.5.2 Use of plants and algae

Within the aquatic environment naturally occurring plants and algae have obvious advantages. Aquatic plants are able to bioconcentrate organic (and inorganic pollutants) and provide biochemical responses. Quantitative structural activity relationships (QSARs) have a great future, especially when applied to interspecies relationships. New techniques on mobility inhibition using microplates and flow cytometry are exciting new prospects.

1.5.3 The application of measuring techniques

These are illustrated by the action being taken by the international agencies within developing countries, especially in the fields of agroecotoxicology, monitoring of industrial effluents and in the application of biodegradation testing.

1.5.4 Case studies

These range from water quality monitoring and management to a bromate survey in European water supplies. Bromate in potable water supplies is perhaps one of the most important issues to beset water undertakers who use chlorine or ozone to disinfect both groundwaters and surface water. The aquatic chemistry of bromate formation will be a challenge to the analytical chemist, water chemists and toxicologist for the next decade at least. Pesticide residues in foodstuffs, especially in developing countries, is of growing importance as foodstuffs, once only available locally, are now transported for thousands of kilometers so as to be available to all.

The problems associated with inhibition of nitrification processes in wastewater plants by an increasing number of chemicals has severe economic consequences and is a matter which should be objectively redressed at source as part of a discharger's policy on a 'cradle to grave' and 'pollution prevention pays' basis. As earlier indicated, the decline of communism has led to many environmental toxicological adverse consequences which will require the initial use of simplistic audit techniques. The future will bring the use of additional applications of QSAR and related techniques from substances having similarities, eg, drugs and explosives.

The final case study proves that pollution is not new and that pollution from mercury mining started at least 500 years ago.

1.5.5 Risk assessment, s afety and economics

The application of risk assessment techniques in Europe and elsewhere for both new and existing chemicals is vital so as to ensure that chemicals are manufactured, used, transported, packaged and disposed of in a regulated manner. The integration of public and private sector cooperation in pesticide safety standards in developing countries in particular are essential to ensure adequate environmental toxicological assessments.

As spillages and accidents are inevitable, no matter how adequately a country's regulations may be, it becomes vital that any ensuing emergency can be handled effectively, speedily and efficiently. Finally, economics, whether in wastewater treatment or for other environmental assessments, is a major consideration. This aspect, especially macro-economics, will necessitate improvements in risk reduction at source.

1.6 CONCLUSIONS

Lasting economic growth has to be based on managing natural resources in a sustainable manner. It is essential that the business world recognizes that sustainable development and production is 'good for business'. There has to be much greater effort to reduce waste, recycle waste materials, control pollution, and conserve all resources including energy requirements, which in turn will lead to lower production costs. Hence, in an environmental assessment, not only has there to be a full regard of the toxicology of substances emitted to any environmental media, or indeed species, but a study of the probable changes in the various socioeconomic and biophysical characteristics of the environment which may result from a proposed or impending action. Such activities should never be adversarial.

1.7 ACKNOWLEDGEMENTS

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SECTION 2: NEW MEASURING TECHNIQUES

2 The Potential and Limitations of New Technical Approaches to Ecotoxicology Monitoring

Peter-Diedrich Hansen

2.1 INTRODUCTION

It is impossible to detect the 100,000+ anthropogenic pollutants in waterways by means of chemical detectors Biological effects are suitable for monitoring purposes, and should be an important parameter for assessing the survival of individuals, a population or species. 'Environmental effects monitoring' approaches provide 'signals' in frequencies and amplitudes to understand changes in the environment in advance. Also, they are capable of recording the integral effects of substances released to the environment. To understand the complexity of the processes, efforts have to be directed to promote rapid and cost-effective biochemical and cytotoxic testing methods in addition to 'on-line monitoring systems (biosensors)'. As with physico-chemical methods, biological and biochemical methods are of increasing importance for the integral monitoring of pollutants. As an example, the hepatic mixed function oxygenases (MFO) activity, as indicated by 7-ethoxyresorufin O-de-ethylase (EROD) and other measurements of Cytochrome P-450 1A1, is a very sensitive indicator of the ability of fish to detoxify certain pollutants. The MFO activity can be related quantitatively to the extent of pollutant exposure, and can be used as a bioassay to identify the effects of pollutants. There is in existence a vast amount of expertise in the area of environmental mapping of MFO-activities (Quality Status Report of the North Sea 1993)[1] and genotoxic potential (ie, DNA-unwinding and umu-C assay). Similarly, phagocytosis (immunological defence activity of organisms) has such a potential. The use of *in situ* bioassays provides a good opportunity to evaluate 'signals' and to understand in advance changes in the environment. The 'signals' from such environmental monitoring investigations are helpful to promote an environmentally sensitive and sustainable use of waterways and coastal zones for ecosystem health management.

This chapter outlines the application of the currently available biomonitoring tools to generate information on early warning signals on ecosystem damage due to both manmade and natural pollution. Emphasis is made on the use of recently developed 'on-line monitoring' approaches as a potential biotechnological technique to assess environmental damage and manage ecosystems health.

2.2 ENVIRONMENTAL REQUIREMENTS

In order to establish effective pollution control measures for water conservation, both emission and their levels must be taken into consideration so that a better understanding of ecological responses to these environmental impacts can be obtained. Knowledge concerning small temporal dimensions of individual physicochemical processes is essential for developing large model development.

Principles associated with different scales of biochemical processes relating to ecosystem functions are given in Table 2.1 which shows the structural and functional hierarchies of biological interactions as they relate to ecosystem complexity. In order to understand these complex ecosystem interactions, it is necessary to break down these functional and structural components into their respective parts in order to understand the studies as indicated by McIntosh[2] as compared with studies using Odum's holistic methods[3]. However, in order to understand fully the overall ecosystem function and ecosystem health, it is necessary to apply both concepts.

Table 2.1 outlines how biological systems respond to sublethal levels of environmental pollutants at suborganism, organism, population and ecosystem levels with their endpoints.

Table 2.1 *Timescalc responses in biological systems (suborganism to ecosystem levels) to sublethal pollutants*

Response by the biological system		
Minutes		**Ecosystem level**
10^6	> 2 y	Alteration in ecosystems – redevelopment of the system's elements and structure
	0.4–1 y	**Population level**
	0.5–1 y	Population dynamics
		– self organization
		– reorganization
10^5	1–12 m	Change in growth
		– adaptation of the system
	20–120 d	**Organism level (exposure): '*in situ* bioassays'**
		– growth, reproduction, ELST, accumulation,
10^4		biotransformation: MFO, reaction with macromolecules, DNA damage, repair, mutagenesis
		Organism/suborganism level
	1–3 d	Symptoms in individuals: detoxification and regulation processes
10^3		– biochemical response ('MFO', AChE, phagocytosis)
		– changes in behaviour
10^2		
10^1	10 min	Early Warning Systems
		– On-line monitoring
		– 'Biosensors'
10^0		**Input of pollutants (sublethal level)**

Generally the rapid reaction level is initiated at the suborganism level (1–3 days), where symptoms relating to initial stages of detoxification and regulatory processes are observed. Examples of impact monitoring tools to assess biochemical responses at this level include mixed function oxygenases (MFO), acetylcholinesterase-inhibition (AChE) and immunosuppression (phagocytosis). This level of functional component effects at the organism level can be shown to overlap when organisms are exposed for longer periods to these environmental pollutants. Effects at this level would result eventually in contaminant genotoxic effects at the macromolecular levels such as DNA-damage, mutagenesis etc. Since environmental genotoxicity assessment approaches provide information on such effects they can be considered as potential environmental monitoring tools.

Table 2.2 provides information on the consequences of DNA damage at the organizational levels of biological systems.

Table 2.2 *Consequences of DNA damage of different organisational levels of biological systems*

Level of biological organization	**Effects**
DNA	– Mutations
Cell	– Cell death – Disordered proliferation and differentiation – Neoplastic transformation
Tissue/Organ	– Functional defects – Malformations – Tumors
Organism	– Reduced viability – Reduced fertility
Population	– Reduction of population size – Extinction
Ecosystem	– Reduction of species diversity

Detoxification responses to such substances within organisms occur mainly in liver tissues and kidneys. Measurements of hepatic MFO activity is a sensitive indicator of the organisms' ability to detoxify these pollutants.

These enzyme systems are located in the endoplasmatic reticulum (ER) of the hepatic cells. There are two phases in the metabolism of these xenobiotic substances:

(i) Phase I includes hydroxylation, deethylation, dealkalization and deamination. Primary oxidation products from phase I are excreted or transformed into water soluble products by the series of conjugating phase II enzymes[4];
(ii) Phase II is the real detoxification stage where the polarity of these substances increase.

However, the main process is the oxidation reaction of phase I products.

In addition to the detoxification systems, there are other systems such as phagocytosis which measures and quantifies the immunological defence activities. This procedure allows the establishment of the stress of the immunological defence mechanism of organisms exposed to chemical pollutants. Similarly, information on deoxyribonucleic acid (DNA) damaging activity provides information on genotoxic effects of these pollutants through macromolecular reactions (Figure 2.1).

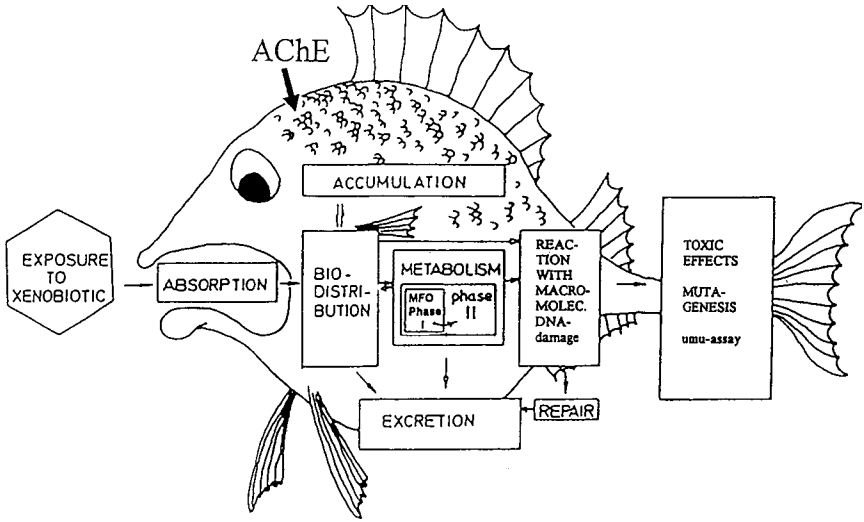

Figure 2.1 *Responses by biological system and endpoints ie, AChE=acetylcholinest erase inhibition, MFO=mixed function oxygenases activity (detoxification), DNA damage.*

2.3 BIOMARKERS

2.3.1 Enzymatic inhibition (cholinesterase)

The basic mechanism for the toxic action of organophosphates and carbamates includes accumulation of these pollutants at the nerve endings with the eventual disruption of nerve functions. Essentially, recovery of acetylcholinesterase activity (AChE) in an organism that survives acute effects is dependent on the spontaneous, but slow dephosphorylation of the inhibited site and the synthesis of new AChE. This

monitoring approach is both helpful in identifying the hot spots and pollution gradients near inputs and also to characterize the impact of pollutants (EEM= environmental effect monitoring). The effects on these substances as detected by cholinesterase inhibition are quantified as paraoxon equivalents and the inhibition constant (Herzsprung et al).[6]

The phosphorus pesticides and insecticide carbamates inhibit cholinesterases at different levels. The well known cholinesterases are acetylcholinesterase and butyrylcholinesterase. The former were isolated from bovine and human erythrocytes and from the electric eel *(Electrophorus electricus),* the latter was isolated from both horses and human serum. The strength of the inhibition effect is expressed as the inhibition constant. The inhibition constants of the most important phosphorus pesticides and insecticide carbamates have been published by Herzsprung *et al.*[6] A standard DIN protocol (DIN 38415 Part 1) is currently available[7] which outlines the details of a procedure applicable to water samples and with slight modifications for samples from organisms such as fish, worms, etc.

2.3.2 Enzymatic i nduction (detoxification activity MFO)

This parameter MFO (EC-No. 1.14.14.1) is widely used as a monitoring tool to detect impacts of pollutants on fish[8,11] has also been used more recently in mussels[12] It is a sensitive indicator to detect the ability of an organism to detoxify certain pollutants. MFO activity can be related quantitatively to determine the extent of exposure to pollutants and is used as a bioassay to detect sublethal effects.

The most common substrates used for the determination of the MFO induction are 7-ethoxyresorufin (EROD) and 7-ethoxycoumarin (ECOD). The end-product is determined as resorufin and umbelliferone.

Factors affecting MFO-induction in fish for example include their physiology (sexual maturity and/or reproductive status, nutritional status) and other environmental factors such as pH-values, temperature and oxygen. The salinity apparently has no effect on MFO induction.[13]

Figure 2.3 the elevated MFO activity near input sources which declines with distance. Such data supports the usefulness of the parameter as biomonitor tool monitoring MFO activity. Figure 2.3 shows the usefulness of MFO-activity as a screening tool for detecting the areas where fish are induced due to anthropogenic impact.

This approach is an ideal strategy for determine impact effects and managing ecosystem health. A standard protocol for MFO-induction is currently available including data on interlaboratory comparisons.[14] A measurement parameter that can provide information about the effects of multiple environmental components are of great importance in environmental monitoring processes. Such a parameter would need to be standardized for uniform application.

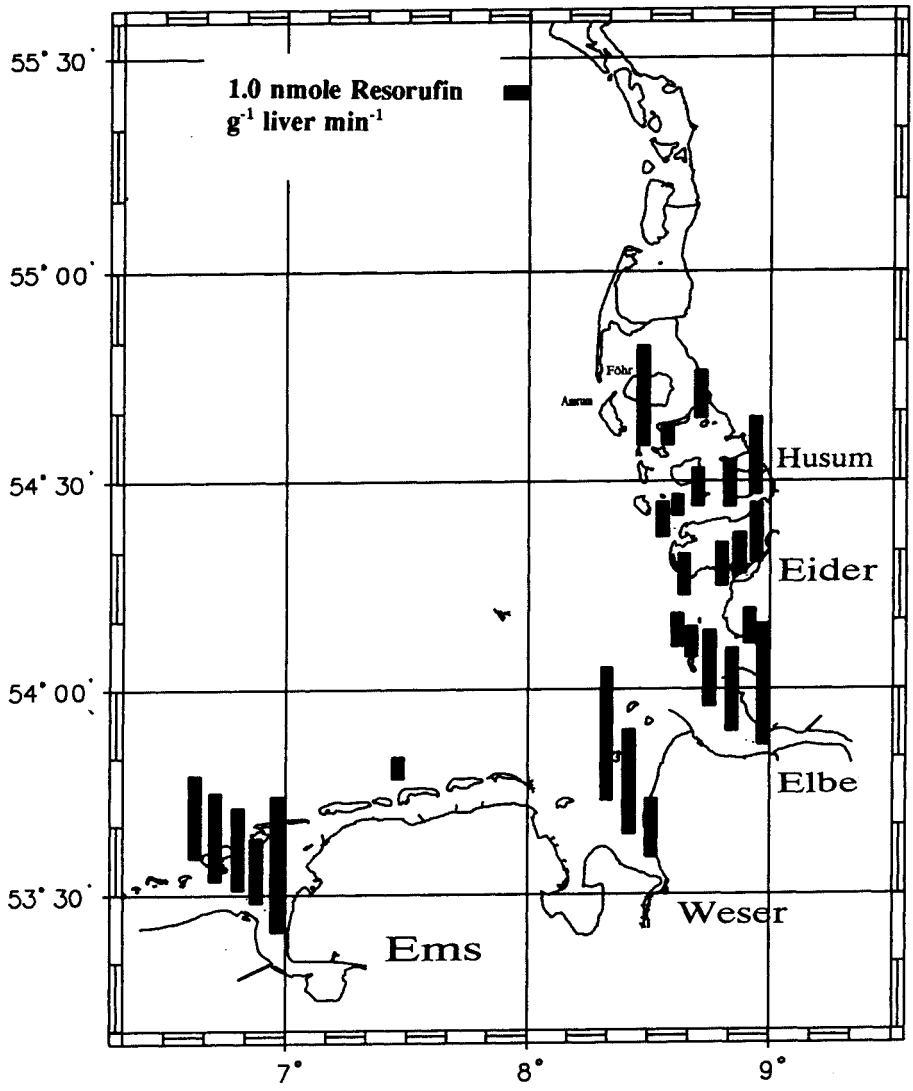

Figure 2.2 *MFO activity (detoxification)—EROD—in flounder (Platichthys flesus). Sampling sites in the German Wadden Sea and the estuaries, August 1989*

2.3.3 Immunosuppression

The resilience of an organism is influenced by changes in the environment which result from both natural and manmade pollution.[11,15] By recording immunological resistance (phagocytosis) in terms of quality and quantity, it is possible to detect

pollution effects on organisms. Through phagocytic activity foreign particles and attached pollutants are digested.[16,18] A luminescent bacteria, *Photobacterium phosphoreum,* is used as a detection system. The hemocytes of the mussel feed on the luminescent bacteria which are treated as foreign particles and attached pollutants. The phagocytic activity is directly related to a decrease in luminescence. Hemocytes play a major part in the immunological defence system of many invertebrates. Measurement of phagocytic activity offers ample opportunities for detecting unknown biotoxins by their influence on the mussel immunology. A good example for application of the phagocytic bioassay is the detection of algal toxins in marine and freshwater environments. The number of blooms of toxic algae has increased in recent time because of eutrophication of freshwater and marine ecosystems; these blooms pose a threat not only to natural ecosystems and fisheries but also to human health. Since biotoxins excreted by algae have not been identified, a bioassay system such as phagocytosis could be extremely useful for detecting their effects. Environmental effects monitoring by this assay to record the integrated effects of biotoxins would be exceedingly useful.

Experiments involving feeding toxic algae *(Chrysochromulina polylepis)* and non-toxic algae *(Isochrysis galbana)* to mussels and the resulting phagocytosis analysis is reported by Krumbeck *et al.*[18] For these experiments the phagocytosis was performed using a microplate technique. Currently, developments in this area are under way using FITC-conjugated yeast cells and the final measurements of the phagocytosis index using a fluorescence microplate reader. This procedure has potential to become an on-line immunotoxicity biosensor system.

2.3.4 Genetic damage

Monitoring the effects of environmental toxic and genotoxic substances has gained increased importance in recent times. Assessment of environmental samples for the presence of genotoxins (carcinogens and mutagens) have become a valuable approach for evaluating genotoxic potentials of environmental pollutants. Currently, biomonitoring approaches are in existence. In Germany there are three protocols for environmental genotoxicity assessment:

(i) DNA unwinding;[19–22]
(ii) AFE (alkaline filtration elution) technique;[23] and
(iii) Umu-assay.[24]

The German Institute for Standardisation (DIN) has recently adapted the umu-C assay as an official protocol (DIN 38415 Part 3) for monitoring environmental genotoxicity. Figure 2.3 indicates the principles of the assay. The umu assay has been submitted to ISO as an ISO document (ISO/TC 147/SC 5 WG9N).

For monitoring the genotoxic potential in the environment the DNA unwinding assay is a leading tool. The DNA unwinding assay has been shown to be a promising tool for detecting DNA damage due to environmental genotoxins in aquatic animals. Extensive work is currently under way to develop a basis for uniform application of

this bioassay for environmental samples. Recent work (Figure 2.4) on the application of this protocol indicates clearly its usefulness as genotoxic monitoring tool[20].

In the future environmental monitoring approaches should include measurements of DNA damage in fish and mussels and umu assay in other environmental samples.[25] Finally, environmental mapping of hot spots for the genotoxic potential should be made mandatory for assessing and maintaining an ecosystem's health.

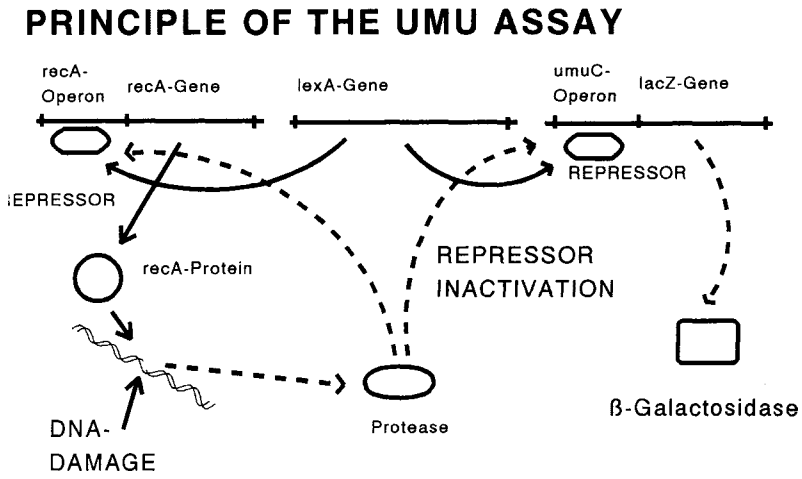

Figure 2.3 *Principle of the umu assay*

2.4 ECOLOGICAL RELEVANCE OF BIOLOGICAL RESPONSES

Biological responses to environmental stress in ecosystems could provide information that signals potential damage. If perceived at an early stage these responses from ecosystems could prevent eventual deterioration. Conversely, once damage to an ecosystem has occurred, the remedial action process for their recovery could be expensive and pose certain logistical problems. However, the remedial action plan (RAP) has been used in certain instances to recover the damaged water bodies. Prevention of ecosystem deterioration is always better than a cure. Ideally 'early warning signals' in ecosystems using biosensors[26] could be used to indicate both the initial levels of damage and these signals could provide answers to develop control strategies (precautionary measures). This results in:

(i) Acute toxicity results in organism selection;
(ii) Genotoxicity results in mutagenicity; and
(iii) Physiological impairments (genetic disease syndromes).

Induction of MFO (biochemistry) shows that fish which are induced with elevated detoxification levels provides data on the effects of specific chemical species (warning signals). However these indicators do not have high ecological relevance such as information from immunosuppression (phagocytosis). Similarly, information from immunosuppression assay do not provide signals of environmental damage similar to

data from population levels. Genotoxic damage endpoints have high ecological significance as they relate to the ability of reproduction. Stress responses at population levels have direct ecological implications even though they exhibit low specificity. Therefore, there should be a harmonized ecosystem assessment approach where the overall information (high specificity to how specificity) should be considered in parallel for adquete ecosystem health management.

In considering the impact of either natural stress or manmade stress, detoxification, disese defence, regulation and adaptation processes occur. This situation makes the assessment approach rather complicated. Alternatively, symptoms analysis, even when including fuctional (behavior, activity and metabolism) and structural changes in the organism (cellular, tissue and organs), have ecological assessment potential.

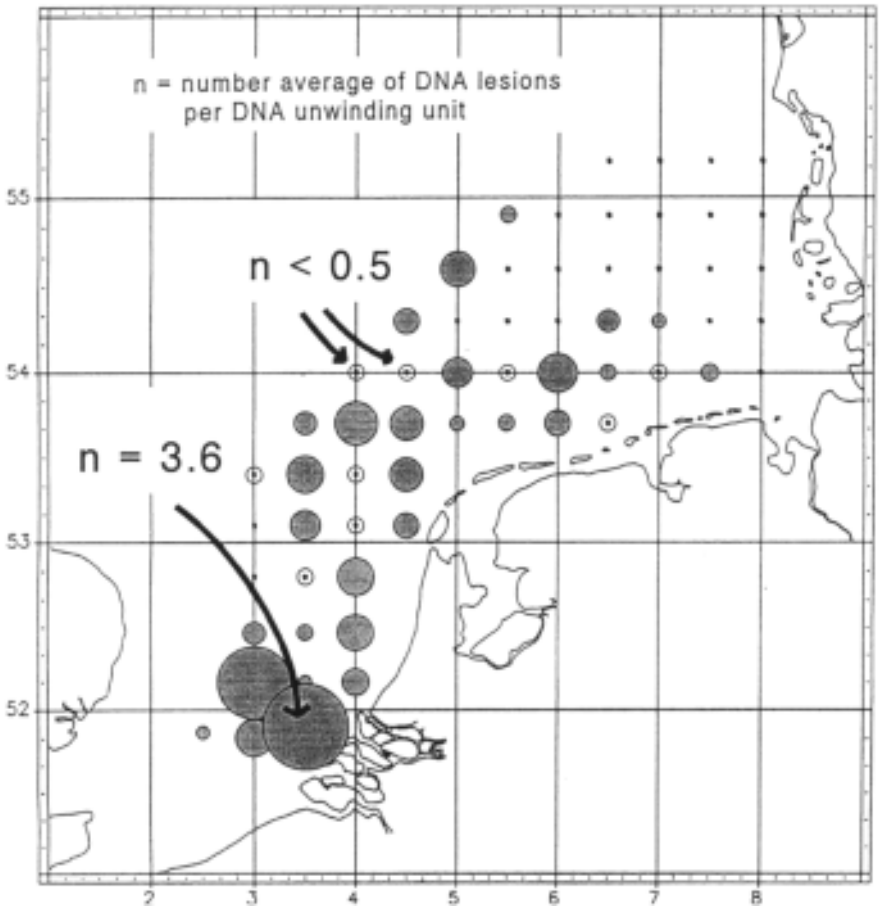

Figure 2.4 *DNA Damage in fish (dab) liver from the North Sea (March 1993)*

2.5 DERIVATION OF QUALITY OBJECTIVES FOR THE PROTECTION OF AQUATIC ECOSYSTEMS

Substances both proved and suspected to be dangerous to an ecosystem's health and human health, must, in so far as is possible, be excluded from the environment and away from waterways. The concept of establishing water quality objectives can be applied when the goal is to manage the water in such a manner that it is available for a wide variety of uses in addition to being a source of potable water, including commercial and sport fishing and general recreation.

Water management practices and objectives play a crucial role in determining the extent to which individual surface waters, or parts thereof, are to be protected. This chapter directs its attention to the importance of establishing water quality objectives which can serve as water management tools for protecting the waterways as a valuable natural resource supporting both commercial and sport fishing and outdoor recreation in general ecosystem health.

Water quality objectives are defined as having specific orientation values which serve to specify the water quality which should be attained in order to sustain the uses deemed worthy of protection. The quality objectives based on this concept are also important as a planning instrument for setting use priorities for the given waterway and in establishing water quality standards to be attained and thereafter enforced. They place in the hands of water management officials the objective means by which regulations and ordinances pertaining to water quality can be established and enforced.

Criteria is defined as being concentrations of substances in water or organisms which, when reached or exceeded, can be scientifically proven to cause adverse effects. Criteria serve as the basis for the stipulation of quality objectives.

In relation to dangerous substances, criteria must take into consideration eg, the following:

(i) Use and Aquatic Communities;
- Effects onEaquatic organisms as a result of both short and long term exposure;
- Accumulation in aquatic organisms. By definition a bioconcentration in the organisms are relevant, if the bioconcentration factor is BCF>100;
- Mutagenic effects;
- In a specific water body, or part thereof, a site attached, self reproducing, and self regulating community of plants and animals is to be sustained or restored under as natural a condition as possible;
- Communities include, in particular, bacteria, lower aquatic plants (algae), higher aquatic plants, organisms on which fish feed (eg, water flees), and fish. They participate in the self purification of waters (reduction of residual pollution from effluent discharges like industrial drainage) and maintain the natural biological equilibrium; and

(ii) Use of commercial and sport fishing:
- The management goal in relation to these uses is the maintenance and/or restoration of the diversity of the communities of importance to fisheries such as fish food organisms and site specific fish populations including the conditions for their natural reproduction (see protection of aquatic communities).

Moreover, water quality has to be maintained or restored in such a way that human beings do not suffer any health damage as a result of the pollutant content of the fish they consume.

Results from the following aquatic toxicity tests should be available to serve as the base set for the derivation of quality objectives.

The data required in order to protect aquatic organisms and fish populations are the so-called no observed effect concentration (NOEC) data:

(i) NOEC values from studies on a primary producer (eg, green algae in a test over several cell generations—72 h) using a recognized test method;
(ii) NOEC values from studies on a primary consumer (eg, the water fly *Daphnia magna* in a 21 d reproduction test), using a recognized test method; and
(iii) NOEC values from studies on a secondary consumer (eg, test on one fish species of at least 28 d duration, including reproduction, alternatively a fish early life stages test), using a recognized test method; or
(iv) NOEC values from studies on a reducer (eg. the bacterium *Photobacterium phosphoreum* or the bacterium *Pseudomonas putida* in a test over several cell generations—16 h), using a recognized test method.

Recognized test methods include methods developed by internationally recognized agencies such as DIN, ISO, CEN, OECD or EU. As a general principle, the lowest test result for the most sensitive species is to be used as the starting point for the risk assessment and for the derivation of the water quality objectives in relation to manganese. There are some reports in the literature on the effects of manganese on metabolism and thus such data should also be taken in account. The toxicity data used for the risk assessment have to be examined critically with respect to validity and relevance.

A compensation factor F has been introduced to take into account the uncertainty associated with extrapolating results to the real environment. F_1=0.1, if NOEC values are available for each of the trophic stages (primary producer, primary consumer, secondary consumer, reducer). Furthermore, a compensation factor F_2 (F_2 =0.1) can be used, if additional risk factors exist such as bioaccumulation and genotoxic potential or mutagenicity.

For the evaluation of the toxicity of anthropogenic dangerous substances the water quality objectives are listed in Tables 2.3–2.5.

In order to achieve the goal of having a water supply which is safe for human consumption, it is necessary to establish quality objectives for waterways (as laid down in public health guidelines and ordinances, eg, Germany's Drinking Water Ordinance).[28, 29]

The values of the quality objectives (QO) for waterways are to be determined as follows: Using the limits value for drinking water (TG) and the reduction factor F:

$$QO = F \cdot TG$$

For the xenobiotic substances the reduction factor F may not exceed a value of 0.5; because the concentrations of substances in water bodies vary, depending on the discharge and other factors, such as substance-specific seasonal (summer/winter) fluctuations. The quality objectives must be the more stringent in those cases in which the substance can not be eliminated by means of near natural water treatment processes.

Table 2.3 *Water Quality Objectives (QO) for the protection of inland waters against dangerous substances (n.r.=not relevant; r=relevant, but no data available; dw=dry weight)*

	Aquatic communities ($\mu g\ l^{-1}$)	Fishery ($\mu g\ l^{-1}$)	Drinking water supply ($\mu g\ l^{-1}$)	Sediments ($\mu g\ kg^{-1}$ dw)	Water quality objectives ($\mu g\ l^{-1}$)
2-Chloroaniline	0.1	n.r.	1.0	n.r.	0.1
4-Chloroaniline	0.01	n.r.	0.1	n.r.	0.01
3,4-Dichloroaniline	0.1	r.	0.1	n.r.	0.1
1,4-Dichlorobenzene	10	r.	1	n.r.	1
Hexachlorobenzene	0.001	0.001	0.1	40	0.001
Hexachlorobutadiene	0.01	r.	1	n.r.	0.01

To establish quality objectives aimed at safeguarding commercial and sport fishing, a tiered procedure is used as follows:

Where legally binding guide or limit values (maximum permissible quantities= W_F, see Table 2.4) for the pollutant content in fish, notably in their muscular system, have been established to protect human health, these are employed together with the bioaccumulation factors (BCF) to derive quality objectives (QO) using the following equation:

$$QO\ (\mu g\ l^{-1}) = \frac{W_F\ (mg\ kg^{-1})}{BCF\ (l\ kg^{-1})}$$

The BCF values are to be obtained from the literature or determined in laboratory experiments, using OECD Guideline 305 E. BCF values<100 are not taken into account; this means, such substances are not assumed to accumulate in fish to any dangerous degree.

Further intensification of the research and international cooperation with respect to the derivation of quality objectives for the various uses (see Table 2.3–2.5) is urgently required. However, in view of the many thousands of substances reaching water bodies from both anthropogenic and natural sources, the application of quality objectives has to remain confined to a manageable, finite number of individual substances. Therefore, the monitoring and evaluation of water quality has to be

supplemented by appropriate parameters and especially by suitable biotests covering not only acute, but also longer-term effects on aquatic organisms. For the derivation of quality objectives using NOEC data from the laboratory and the field—some longer-term effects data are listed in Table 2.6.

Table 2.4 *Maximum permissible pesticide residues (Pflanzenschutzmittel-Höchstmengen-Verordnung (PHmV)) and the maximum permissible quantities by ordinance on maximum permissible pollutants residues in foodstuffs (Schadstoff-Höchstmengenverordnung (SHmV)), bioconcentration factor (BCF) and the water quality objectives for fisheries*

	Maximum permissible quantities PHmV / SHmV (mg kg l^{-1})	Bioconcentration factor (BCF)	Water quality objectives and fishery (mg l^{-1})
Lindane	2.0	350	0.6
α, β, δ – HCH	0.5	100,000	0.0005
Endrine	0.01	3,000	0.003
Heptachloroepoxide	0.01	2,000	0.005
PCB IUPA – Nr.			
28	0.2	100,000	0.002
52	0.2	100,000	0.002
138	0.3	100,000	0.003
180	0.08	100,000	0.0008

Table 2.5 *Derivation of water quality objectives for the protection of inland surface waters against heavy metals.[28] Use and aquatic communities: use and commercial and sport fishing; use and drinking water supply; use and sediments (n.r.=not relevant)*

	Aquatic communities (mg kg^{-1} dw)	µg l^{-1}	Fishery µg l^{-1}	Drinking water supply (µg l^{-1})	Sediments (suspended solids) (mg kg^{-1} dw)
Pb	100	3.4	5	40	100
Cd	1.2	0.07	1	5	1.5
Cr	320	10	n.r.	50	100
Cu	80	4	n.r.	50	60
Ni	120	4.4	n.r.	50	50
Hg	0.8	0.04	0.1	1	1
Zn	400	14	n.r	3000	200

Table 2.6 shows clearly that there is a safety buffer between laboratory and field data. The determination of the NOEC data or threshold values serve to secure the fundamental functions of ecosystem health such as metabolism, growth, reproduction and regulatory performance.

Table 2.6 *Quality Objectives for the protection of aquatic communities (concept for the derivation of quality objectives for the protection of inland surface waters) in comparison with NOEC data from laboratory and field investigations*

	Azinphos methyl	**Parathion ethyl**
NOEC – Laboratory	0.1	0.002
NOEC – Field	0.25	0.1
FRG – Water quality objectives	**0.01**	**0.0002**
Netherlands Maximum tolerable risk level		
– Limit value (guideline)	0.0007	0.00005
– Intervention value	0.02	0.005
US EPA – Extrapolation	0.01	0.002

The range of validity of the water quality objectives cannot be applied universally but is to be examined for each individual water body. The water quality objectives are for testing wether or not sufficient measures have been taken to protect the individual water body and especially its valuable and exploitable resources from 'dangerous substances'. In order to secure the functioning and stability of a water body (ecosystem) or to actually re-establish these qualities, concrete ecological knowledge is necessary concerning the interactivity of functions and structures of organisms, in addition to nutrient cycles and energy fluxes in temporal succession. This is the only way possible of recognizing the multifactorial burden on a ecosystem which has been caused by 'dangerous substances', and of establishing which restoration measures should be taken by eg, water management.

Where water quality objectives can be implemented by way of water management measures, eg, water management plans, this is a national task. If implementation can only be achieved through substance-related regulations, eg, prohibition of substances, this is primarily a task to be tackled internationally.

2.6 RECOMMENDATIONS

From the practical standpoint there are several concepts which can be used as powerful tools for management of ecosystem health and deal with the day by day problems. There are principle grounds for determine the water quality requirements to protect

inland surface waters against dangerous substances. These water quality requirements have to be derived from scientific approaches, for example NOEC data. There is currently a battery of test approaches available for assessing the trophic levels of food chain using bacteria, algae, crustacean and fish. In addition, there is a need to apply other assessment concepts which include effects parameters such as AChE-inhibition, MFO-induction, phagocytosis-index etc. Among the existing genotoxicity assessment approaches, the umu-C assay has become a DIN standard (DIN 38415 Part 3) and will become part of the Federal Regulatory Programme within the Federal Water Act (§ 7a WHG).

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3 Environmental Toxicity Assessment Using Luminescent Bacteria

Antony Bulich and Gerald Bailey

3.1 INTRODUCTION

As the year 2000 approaches, and chemists continue to synthesize an increasing number of chemicals of ever growing complexity, there is a requirement to be able to control the environmental effects of these chemicals. This number of xenobiotics is dwarfed by the number of natural substances which have now been identified. Chemical Abstracts now include data from a few basic physico-chemical details, through to methods of identification, synthesis, including patents, use, means of disposal and in a minority of cases numerous abstracts on environmental effects.

Chemical synthesizers and, perhaps even more so the users of these products in pure chemical terms, have little knowledge of the composition of the chemicals present in their atmospheric emissions, discharges to water bodies or in their solid or semi solid wastes. It is not pragmatic to consider detailed analyses of such emissions by techniques such as gas chromatography-mass spectrometry, etc. and then attempt to assess the toxicity either to man or the environment of the chemicals identified. This has been know for almost a century and was one of the reasons for the development of simple chemical tests, eg, chemical oxygen demand, permanganate

Table 3.1 *Availability of effects data for high production volume chemicals*

End point	Percentage
Acute toxicity	90
Sub-acute toxicity	30
Carcinogenicity	10
Mutagenicity	50
Fertility	10
Teratogenicity	30
Acute ecotoxicity to fish or *Daphnia*	50
Short-term ecotoxicity to algae	5
Toxicity to terrestrial and soil-dwelling organisms	< 5

value etc., which are simple and fast, and the much slower biological oxygen demand test.

Whilst lexicological testing of chemicals and especially the high volume chemicals, have been in hand for a few decades, the availability of data is not considerable, especially that for toxicity to terrestrial and soil-dwelling organisms (see Table 3.1).

With these deficiencies in mind there was a need to develop rapid, robust and peer-reviewed techniques based on biochemical mechanisms to assist toxicological parameters. Bacteria and enzyme systems have great potential; certain species of marine luminescent bacteria, in particular *Vibrio fischeri* (formerly known as *Photobacterium phosphoreum*), are useful surrogates for toxicological assessment. Some other techniques include DNA probes, induction of cytochrome P-450 1A1, 7-ethoxyresorufin-O-deethylase, etc. (see Chapters by Hansen and Gagné and Blaise) and the use of microalgae (see Chapters by Blaise and Kusui and Hänninen and Roy).

3.2 BASIC CHARACTERISTICS OF *VIBRIO FISCHERI*

Luminescence in *Vibrio fischeri* is dependent upon both cell density and actively growing bacteria which undergo induction of the *lux* system during the late stage of exponential growth. This 'autoinduction' phenomenon is attributed to the accumulation of a specific cell product (N-3-oxo-hexanoyl)-L-homoserine lactone) in the culture medium.[1]

During the past decade several researchers have furthered the understanding of the regulation of bioluminescence in *Vibrio flscheri* and the association of the luciferase pathway with the growth and physiology of the cell population.[2]

The regulation of the *V.fischeri lux* system depends on the binding of the inducer with the *lux R* regulatory protein, a step which is mediated by the heat shock protein GroESL which is required to stabilize the *lux R* protein prior to binding with the inducer.[3]

3.3 DEVELOPMENT OF THE CHRONIC TEST PROCEDURE

Only from a detailed understanding of the *lux* gene transcriptional control system was it possible to develop a chronic test system using *V, flscheri*. The development of a reliable test was based on the formulation of a defined media. The minimum requirements for this test included several cell divisions and complete induction of the luciferase system to provide adequate light to quantify the test end point. The plan for the development of a chronic test with luminescent bacteria focused upon the concept of the activation of as many metabolic pathways as is possible within the test organisms to provide as large as possible selection of enzymes or enzymic pathways to be available for inhibition. Figure 3.1 represents the relative metabolic activity in those cells and with the Microtox® acute test, whilst Figure 3.2 represents all those pathways associated with actively dividing/growing cells similar to those used with the Microtox® chronic test.

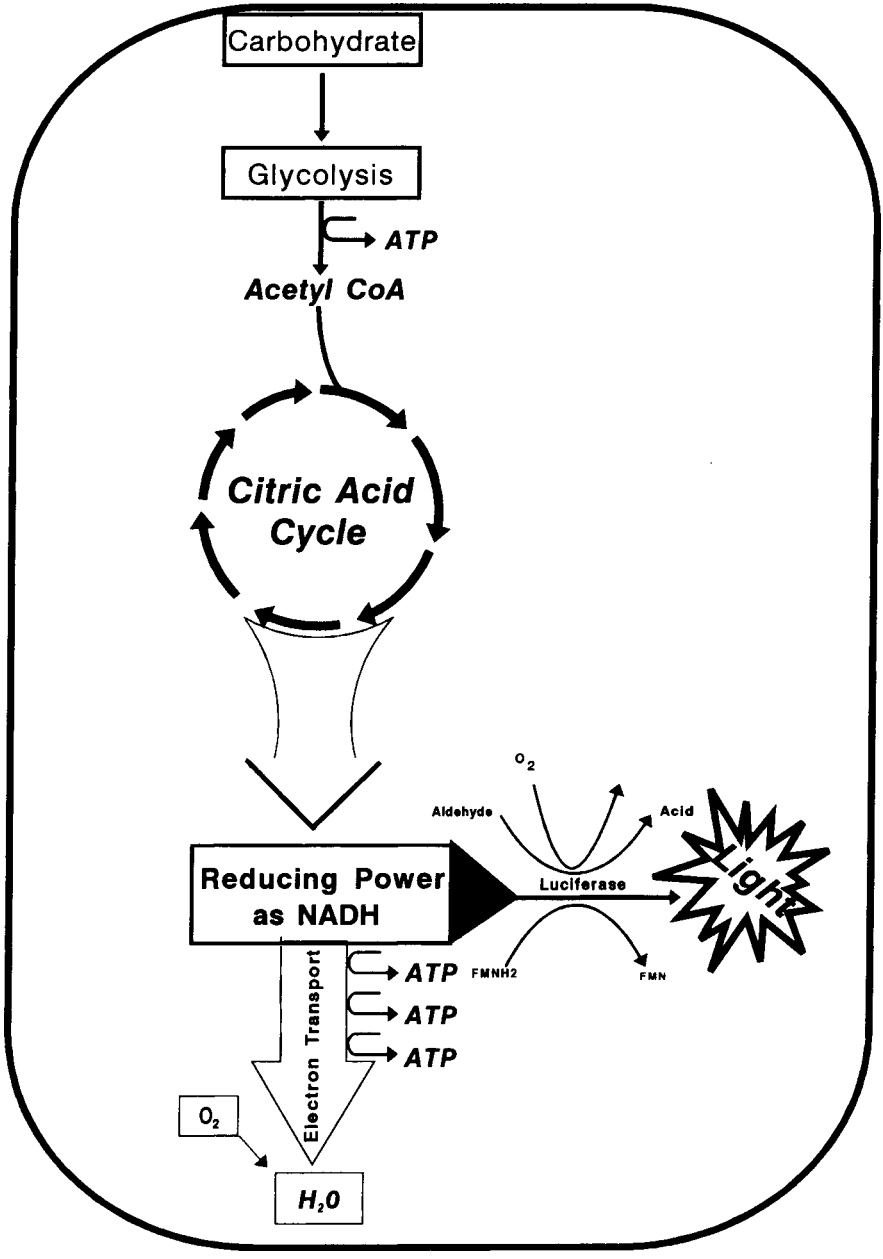

Figure 3.1 *Relative metabolic activity in those cells used for the Microtox* ***acute*** *test*

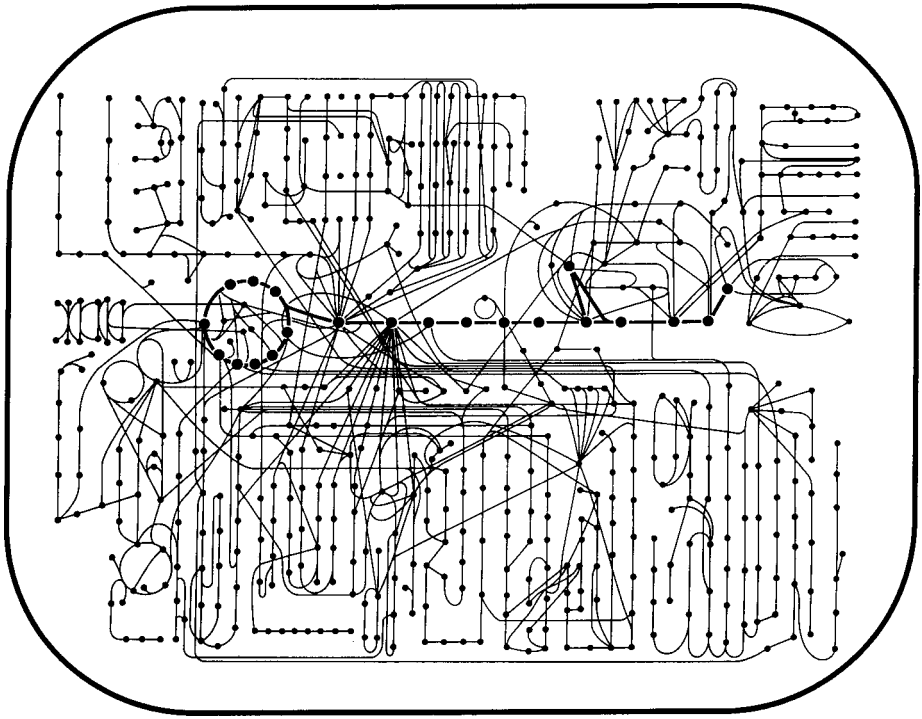

Figure 3.2 *Pathways associated with one actively dividing/growing cells similar to those used in the Microtox* ***chronic*** *test*

Table 3.2 indicates chronic test data from samples containing cadmium tested in duplicate and read after 21 and 24 h. The results (in bold) indicate those concentrations where the light output is at leat two standard deviations less than the average control value; such concentrations are interpreted to be the lowest observable effect concentration (LOEC).

Figure 3.3 indicates chronic test data where the light levels were recorded every 2.5 h and which represents the light induction kinetics and the corresponding inhibition in the cuvettes containing inhibitory concentrations of cadmium. Figure 3.3 also shows the cell counts of the test population and demonstrated that there is a three-fold increase in cell numbers during the test period.

Initial evaluation of this chronic test method included a comparison with the *Ceriodaphnia dubia* chronic test. Table 3.3 indicates Microtox chronic test sensitivity data with *C. dubia* pure compound LOEC literature values.[4,5]

Table 3.4 includes a number of Microtox chronic LOEC values. Some of these data are compared with Microtox EC_{50} values and Table 3.5 shows that the chronic test is significantly more sensitive than the Microtox acute test.

Table 3.2 *Chronic test data: cadmium*

Control 1 21 h	Control 2 21 h	Test 1 21 h	Test 2 21 h	Control 1 24 h	Control 2 24 h	Test 1 24 h	Test 2 24 h
310	338	281	299	352	404	241	340
394	327	268	282	446	237	189	380
579	363	192	162	518	385	135	160
341	352	153	142	343	357	130	185
310	391	140	50	289	262	479	105
358	312	101	80	323	227	693	53
338	205	190	22	252	241	252	31
314	378	23	20	319	303	17	1
389	358	1	1	237	326	1	1
231	240	1	1	326	337	1	1
X = 356	X = 326			X = 340	X = 308		
sd = 91	sd = 60			sd = 85	sd = 64		

Highest concentration tested = 4 ppm; 1:2 dilution series

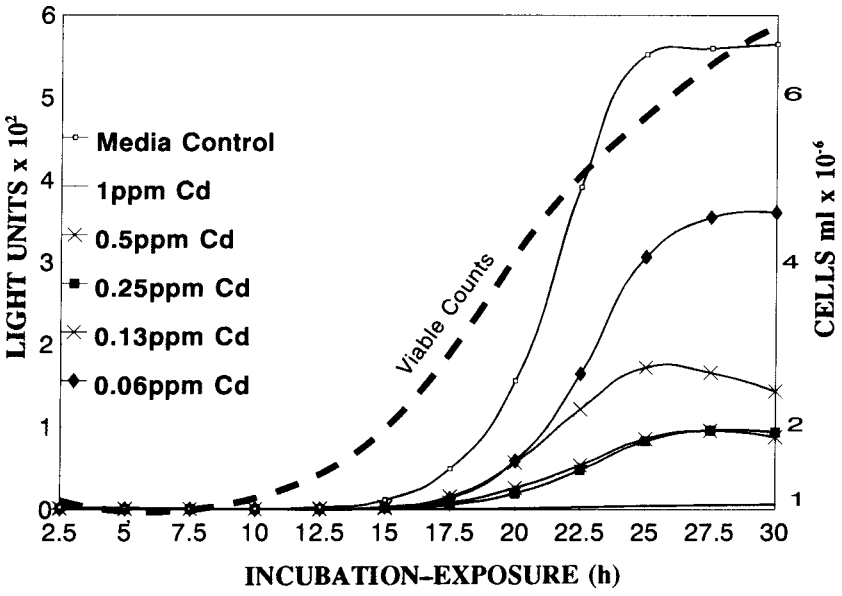

Figure 3.3 *Chronic test-kinetics*

Table 3.3 *Ceriodaphnia vs. Microtox chronic*

Compound	*C. dubia* LOEC (ppm)	Microtox chronic LOEC (ppm)
Cadmium	0.003	0.06
Chromium	3	0.035
Copper	0.04	0.019
2,4-D	40	0.3
Diazinon	0.03	0.75
Lead	0.1	1
Methoxychlor	0.01	0.18
Nickel	0.01	0.17
Zinc	0.1	0.1

Table 3.4 *Chronic test LOEC data (ppm)*

Compound	LOEC	Compound	LOEC
Acetone	6300	Dimethyl sulfoxide	800
Aflatoxin	1.56	2,4-D	0.3
Aluminium	1.57	3,5-Dichlorophenol	0.2
Aroclor 1242	0.16	Ethanol	6300
Aroclor 1254	0.29	Lead	1
Atrazine	6	Mercury	0.05
2-Aminoanthracene	0.098	Methoxychlor	0.18
Benzene	25	Methanol	10,000
Benzo(a)pyrene	0.16	3-Chloro-4-(dichloromethyl)-5-hydroxy-2(5H)-furanone	0.0015
Butanol	7.81	Nickel	0.17
Cadmium	0.06	Phenol	10
Chloroform	1250	Sodium azide	0.047
Chromium	0.035	Sodium lauryl sulfate	0.098
Copper	0.019	Pentachlorophenate	0.06
Diazinon	0.75	Trichloroethene	0.2
Dibromochloromethane	10	Zinc	0.1

Table 3.5 *Microtox acute vs chronic*

Compound	Acute EC_{50}	Chronic LOEC
Butanol	7240	7.81
Chromium	22	0.035
2,4-D	65	0.3
3,5-Dichlorophenol	7.3	0.2
Diazinon	64	0.75
Sodium lauryl sulfate	2.5	0.098
Sodium pentachlorophenate	2.8	0.06
Zinc	6.1	0.1

One of the primary environmental applications of the chronic test method is whole effluent testing. Consequently, the validation program for this new test included (Table 3.6) a comparison of effluent sample results using *C. dubia* and the Microtox chronic test.There is good agreement between the two methods based on this small sample test. It is important to note that many more effluent samples were actually tested with both methods. Much of the *C. dubia* data was invalidated because of excessive mortality of the control organisms.

Table 3.6 *Comparison between C. dubia and Microtox chronic test on effluent samples*

Sample No.	*C. dubia* LOEC	Luminescent Bacteria LOEC
1	100	100
2	100	50
3	50	50
4	50	50
5	50	100
6	25	12.5
7	25	12.5
8	6	6
9	6	3
10	0.2	0.2

LOEC values reported as percent of effluent sample tested

Additional studies are in progress to increase this comparative database to include both a wide selection of samples and industry types.

3.4 GENOTOXICITY MEASUREMENT IN ENVIRONMENTAL SAMPLES

A number of the methods available currently for estimating the genotoxicity of environmental samples have practical limitations. These include high cost each test and complex protocols which require highly trained personnel to obtain reproducible test data.

Luminescent marine bacteria in the form of the Microtox® test have been used since 1982 to measure the acute toxicity of pure chemicals and environmental samples. This technology has been extended to utilize a dark strain of these bacteria to detect the presence of genotoxicity in environmental samples.[6] This test—the Mutatox® test using a dark mutant (M169) of *V. fischeri* that exhibits light production only when grown in the presence of sub-lethal concentrations of genotoxic agents. Details of the procedure have been outlined in detail[6] and results obtained have been compared with the Ames test and carcinogenic data.[7]

The test strain M169 is the dim variant of *V. fischeri*. The primary genetic lesion responsible for the low light of this strain has yet to be identified completely. Current information indicate that the GroESL activity in M169 is altered, which is a critical component of the *lux* gene regulatory system.

Several studies have focused on the use of Mutatox® to measure the genotoxicity of water and solvent extracts from a series of sediment samples taken from several sites in the Great Lakes.[8,9] When compared with results using the *Salmonella* reverse mutation assay there was an overall agreement of 91% for the 55 samples tested.

In a more practical study, Mutatox was used to measure genotoxic activity in soils contaminated with polycyclic aromatic hydrocarbons (PAHs). Extracts from two sample types were tested:

(i) Clean soil artificially contaminated with a mixture of PAHs; and
(ii) Soil excavated from a hazardous waste site known to be contaminated with PAHs.[10]

One of the major trends indicated by the test data was that removal of the original contaminants as measured by gas chromatography was not an accurate measure of complete remediation of the waste site. Figure 3.4 shows that the genotoxicity decreased initially with an decrease in PAH concentration. Only after a significant increase in genotoxicity followed by a longer period of biodegradation did the genotoxicity of the soil extracts diminish to background levels. These data suggest the importance of genotoxicity monitoring during bioremediation of a hazardous waste site to ensure complete degradation of contaminants and biodegradation by-products.[10]

Ho and Quinn[11] have outlined a bioassay technique after fractionation of organic compounds by organic solvent extraction of a contaminated estuarine sediment from Black Rock Harbor, Connecticut. They used the M169 dark mutant of *V. fischeri* and found that two mutagenic fractions did not contain any known mutagenic components

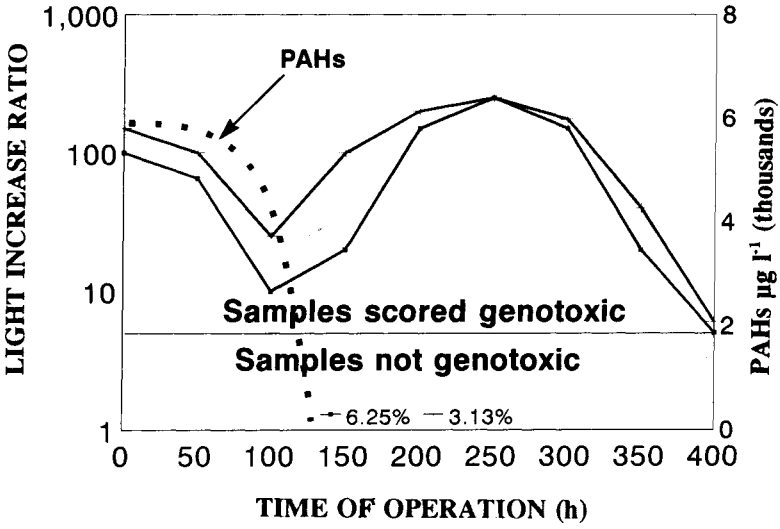

Figure 3.4 *Genotoxicity data: Soil column effluent samples (%)*

detectable by gas chromatography-mass spectrometry and found the Mutatox® test to be a fast and convenient bioassay that has the potential for use in this type of work.

Sun and Stahr[12] report on good correlations between the Mutatox® test and the Ames test. Their study showed that methyl-imidazo-quinoline and tryptophan pyrolysates were genotoxic in the presence of S-9 activation; aflatoxin B_1 epoxide and fumonisin B_1 showed direct genotoxic activity and aflatoxin B_2 and ochratoxin A were not genotoxic. They reported that the Mutatox® test is much easier to conduct and involved minimum personnel training and laboratory facility requirements. By example, aseptic techniques are required for the Ames test. For the Mutatox® test the dark mutant responds only to the genotoxic agent with restoration of luminescence. The test is a rapid general screening test which can be used to any large sample number of both pure chemicals and complex samples and results were obtained in 24 h. Similar dilutions of the samples can be used to provide dose-response data.

Legault *et al.*[13] compare the SOS chromotest kit, Mutatox® with the *Salmonella* plate incorporation (Ames test) and fluctuation tests for screening genotoxic agents. They used three criteria for performance assessment:

(i) Concordance (interprocedural and previous literature data);
(ii) Accuracy; and
(iii) Sensitivity.

The Mutatox® test performed well for all three.

Their results can be summarized:

(i) The presence (or absence) of genotoxicity for the SOS chromotest and Mutatox® test in relation to the Ames test were 86 and 93% respectively for the 14 chemicals tested;
(ii) The capability to identify known animal (non) carcinogens, the SOS chromotest. Ames test and Mutatox® were accurate to 64, 73 and 82% respectively for the 11 chemicals assessed;
(iii) Qualitative concordance from their comparative results matched the literature results well: Ames test 85%, SOS chromotest 77% and 67% for the Mutatox® for 13, 13 and 9 chemicals respectively; and
(iv) The Ames test had the greatest sensitivity of 6/9 chemicals than either the Mutatox 3/9 chemicals or the SOS chromotest 0/9 chemicals for the detection of the lowest active concentration for the 14 chemicals, especially for those requiring metabolic activation.

Ho *et al.*[14] used four bioassays: Microtox®, Mutatox®, sister chromatid exchange (SCE) and metabolic cooperation to analyze marine sediment extracts. They were able to conclude:

(i) Different fractionation methods for the same sediments, providing different and sometimes conflicting results for the same bioassay;
(ii) The genotoxicity was found only in S-9 activated substances; and
(iii) The Mutatox® test indicated good comparison with the SCE and *Salmonella* assays for complex mixtures.

Rowe *et al.*[15] reported that the Mutatox® test provided reasonable sensitivity and specificity in detecting mutagens when compared to the microbial mutagenicity assay; however, when compared to the DNA alkaline elution assay it was neither sensitive nor specific. Six non-microbial mutagens which were positive in the Mutatox® assay+S-9 had no apparent structural relationship. Two of these were negative in the Mutatox® S-9 and one was positive in the DNA alkaline elution assay.

The Mutatox® test detected mutagenicity in four compounds with known antibiotic properties and in at least two clones of chemicals known to be genotoxic, nitro-aromatics and hydrazines (see also Chapter by Rendić *et al.*).

Rowe *et al.*'s studies has a number of limitations:

(i) The small number (22) of chemicals tested;
(ii) Their structural diversity; and
(iii) Both assays are inaccurate predictors of genotoxicity.

3.5 CONCLUSIONS

Both the chronic test and Mutatox® test use variants of *Vibrio fischeri* formerly known as *Photobacterium phosphoreum* NRRL B-11177.

The chronic test, whilst in an embryonic stage of development indicates excellent comparability with 96 h results obtained from the *Ceriodaphnia dubia* test.

The Mutatox® test performed well when compared with other genotoxicity test results, eg, Ames *Salmonella* tests, SOS chromotest, SCE, DNA alkaline elution tests, etc.

Both of these tests based on *V. fischeri* have an important role to play in environmental toxicity assessments as they can provide a direct measure of the 'toxic insult' inflicted by chemicals in effluent streams, sediments and other complex samples, invariably of unknown chemical composition.

The acquisition of further data on both pure chemicals and complex samples is a requirement for the future. These tests have a major role to play in such assessments and can be used in a battery of tests using both fish and invertebrates and other bacterial systems, eg, the umu-C-assay (see Chapter by Hansen), DNA probes (see Chapter by Gagné and Blaise), microalgae (see Chapters by Blaise and Kusui and Hänninen and Roy).

3.6 ACKNOWLEDGEMENTS

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4 Fluorescence *in situ* Hybridization *en* Suspension (FISHES) Using Biotin-labeled DNA Probes for Measuring Genetic Expression of Metallothionein and Cytochrome P-450 1A1 (CYP1A1) in Rainbow Trout Hepatocytes Exposed to Wastewaters

François Gagné and Christian Blaise

4.1 INTRODUCTION

Fish comprise a key level in the aquatic biota and hence play a major role in industrial and municipal environmental toxicological assessments as test organisms. In Canada, the rainbow trout *(Oncorhynchus mykiss)* 96 h acute lethality bioassay is employed to evaluate the potential hazards of chemicals, in addition to the regulation of industrial effluents by means of a standardized procedure.[1] Alternative methods, such as fish cell cultures, are also gaining increasing recognition for toxicological assessments. Fish cell systems, in contrast to whole fish testing, can reduce substantially the number of fish required, improve cost efficiency and augment toxicological knowledge of complex mixtures. Several fish cell systems are now recognized that present promise for toxicological investigations.[2,3] Among these are the rainbow trout gonad (RTG-2) and hepatoma (RTH-149) cell lines, primary cultures of rainbow trout hepatocytes or gill epithelial cells,[4] the bluefish fin (BF-2), brown bullhead catfish (BB) and goldfish scale (GFS) cell lines.[5,6] These cell lines have been proposed as suitable candidates for *in vitro* bioassays. The use of primary cultures, such as rainbow trout hepatocytes, offers additional advantages; as that they are often a cellular target during toxic insult and are also responsible for xenobiotic biotransformation of chemicals.[6] Moreover, primary cultures of hepatocytes match more closely *in vivo* tissue characteristics. Finally, cell-based assays are sometimes predictive of similar toxic effects towards whole organisms,[7] thereby displaying relevance as alternative testing systems.

Evaluating sublethal effects in addition to flagrant cell toxicity effects can indicate a more comprehensive profile of toxic aggression. Metallothionein (MT) and cytochrome P-450 1A1 (CYP1A1) induction are well-known cellular defense mechanisms resulting from cell exposure to specific xenobiotics. MT levels can be induced by exposure to divalent heavy metals[8] and possibly to compounds causing oxidative stress.[9] In the same manner, induction of CYP1A1 following exposure to polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), dioxins

and furans[10] represents a cellular defense mechanism. Therefore, appraisal of MT and CYP1A1 levels together with cell toxicity is an important adjunct for toxicological investigations of complex mixtures. Such inductions can be followed at the transcriptional level (mRNA for MT and CYP1A1) or at the translational level (ie, protein and enzyme expression). In the former case, measurement of specific mRNAs may have the advantage of eliminating potential interferences linked to enzyme activity measurement for CYP1A1[11,12] and offer a highly specific measurement of MT and CYP1 Al levels. Quantization of mRNA in single cells is rendered possible by fluorescence *in situ* hybridization using DNA probe hybridization and flow cytometry methodologies. In this work, DNA probes were cloned from reverse-transcribed total trout RNA and amplified using asymmetric polymerase chain reaction (aPCR) methodology.

Therefore, the aim of the present study was to exploit the rainbow trout hepatocyte primary culture model to assess toxicity after cell exposure to environmental samples. MT and CYP1A1 inductions were measured in exposed cells at the transcriptional level using a rapid and simple FISHES methodology and detection was achieved using flow cytometry technology.

4.2 MATERIALS AND METHODS

4.2.1 Chemicals

Phosphate buffered saline (Dulbecco's PBS), calcium chloride, ß-naphthoflavone (ß-NF), cadmium chloride (Cd), benzo[a]pyrene (BaP), collagenase type IV, foetal bovine serum (FBS), gentamycin sulfate, trypan blue, ethylenediaminetetraacetic acid (EDTA), Liebovitz medium (L-15), sodium citrate, tris(hydroxymethyl)-aminomethane (Tris), Tween-20, diethylpyrocarbonate (DEPC), digitonin, salmon sperm DNA, sodium dodecylsulfate (SDS), bovine serum albumin (BSA), polyvinylpyrrolidone (PVP), Ficoll and propidium iodide (PI) were purchased from Sigma Chemical Co. Biotin-16-dUTP, streptavidin-fluorescein, dCTP, dATP, dGTP and dTTP were purchased from Boehringer Mannheim. Generation of DNA probes was achieved by asymmetric polymerase chain reaction (PCR) using the GeneAMP RNA/PCR kit supplied by Applied Biosystems Division of Perkin-Elmer Cetus Corporation. Total RNA from rainbow trout hepatocytes was extracted according to the guanidine thiocyanate method supplied in a kit (Promega, USA).

4.2.2 Production of MT and CYP1A1 DNA probes

DNA probes for MT and CYP1A1 mRNA were prepared by reverse transcription of total cell RNA that was exposed to either 100 ng ml^{-1} Cd (MT) or 100 ng ml^{-1} ß-NF (CYP1A1 mRNA) for 48 h at 15°C, followed by amplification with the polymerase chain reaction. Total cell RNA was extracted by the guanidine thiocyanate method using Promega's protocol. The PCR was asymmetric[13] in that

unequal amount of primers were used during amplification. This resulted in an excess of the complementary strand of the MT or CYP1A1 mRNA. The primers used for reverse transcription and PCR for producing a DNA probe specific to MT mRNA were: sense 5'-ATG-GAT-CCT-TGT-GAA-TGC-3' (0.5 μM) and antisense 5'-TCA-CTG-ACA-ACA-GCT-GGT-3' (10 μM). The primers used for reverse transcription and PCR for producing a DNA probe for CYP1A1 mRNA were: sense 5'-TCC-ATT-CCC-ATC-CTT-GGT-3' (0.5 μM) and antisense 5'-TCT-AGT-TTC-CTG-TCC-TCA-3' (10 μM). For reverse transcription of total RNA (5 μg m l^{-1}) into DNA, only the antisense primers were used. A DNA probe was also produced for evaluating non-specific absorption of DNA within hepatocytes. This probe consisted mainly of the interleukin 1a (IL-1a) mRNA derived from PAW109 plasmid which is supplied with the GeneAmp reverse transcriptase PCR kit. Amplification was performed in the presence of 10 μM of biotin-16-dUTP with 200 μM of nucleotides except that dTTP was at 100 μM. The amount of Taq DNA polymerase was at 5 units ml^{-1} and Mg^{2+} concentration was at 1.5 mM. After mixing and overlaying with 50 μl of mineral oil, the mixture was incubated at 95 °C for 4 min and 40 cycles of the following steps were performed: 94 °C for 50 sec, 58 °C for 50 sec and 72 °C for 25 sec. Afterwards, the mixture was incubated for 5 min at 72 °C and placed in ice. The DNA was extracted with phenol-chloroform-isoamyl alcohol and precipitated with 1 volume of isopropanol at -85 °C for 5 min. The pellet was washed with isopropanol, precipitated as above and resuspended in sterile solution of saline citrate (SSC) 1X (150 mM NaCl and 15 mM citrate, pH 7.2). The purity was assayed (ratio of absorbances at 260 nm and 280 nm) and the concentration calculated at 260 nm with standard solutions of salmon sperm DNA. The specificity of the PCR reaction was verified by electrophoresis on 2% agarose gel containing 1 μg ml^{-1} of ethidium bromide. The electrode and running buffer was 40 mM Trisacetate, pH 8.0, containing 2 mM EDTA and 1 μg ml^{-1} of ethidium bromide. The gels were scanned at 300 nm for densitometric analysis.

4.2.3 Effluent and surface water sample preparation

Industrial effluents were investigated in this study for cell viability effects, in addition to MT and CYP1A1 mRNA induction capabilities. Effluents were representative of several key industrial sectors: pulp and paper (PP), petrochemical (PC), surface treatment (ST) and inorganic chemical production (IC). Each was a 24 h composite sample conforming to Environment Canada's sampling procedure.[14] The composite sample was returned to the laboratory in several Rubbermaid 60 1 containers lined with polyethylene bags and stored in the dark at 4°C. Each effluent sample was recomposed before the bioassays to ensure homogeneity and divided to comply with biological and chemical volumetric requirements. A volume of 20 ml sufficed for bioassays using our cell system.

Surface water samples collected upstream and downstream from a major urban effluent discharge, as well as the discharge itself, were also investigated in this study. The samples (20 ml) were drawn from 5 1 containers and stored at 4 °C in the dark. On arrival in

the laboratory, they were filter-sterilized (0.1 μm) and stored at 4 °C in the dark prior to the undertaking of bioassays.

4.2.4 Preparation of rainbow trout hepatocytes

Primary cultures of rainbow trout hepatocytes were prepared according to the double perfusion methodology of Klauning[15] with some modifications. After a portal vein perfusion with sterile PBS (without calcium), pH 7.5, containing 1 mM glucose, 0.3 mM pyruvate, 50 μg ml^{-1} gentamycin, 2.5 μg ml^{-1} amphotericin B, 2 mg ml^{-1} BSA, 5 mM EDTA and 25 mM Tris-HCl, the livers were perfused with 10 ml of collagenase (100 units in PBS with 1 mM $CaCl_2$ and no EDTA) and were removed from the abdominal cavity. The livers were cut into small slices and mixed in 50 ml collagenase solution for 20 min at room temperature. Afterwards the cells were liberated from the tissue with a cell dissociation sieve kit (Sigma Chemical Co.). The cells were then washed 4 times by centrifuging at 200×g for 2 min and resuspending in sterile PBS without calcium and EDTA. The cells were counted and cell viability was estimated by the trypan blue exclusion test.[15] Typical cell yields were ~2.3 (±0.5)×10^6 cells g^{-1} b.w. with a viability of 95±2% (derived from n =50 isolations). The cells were resuspended in 4 ml of sterile L-15 containing 1% FBS, 25 μg ml^{-1} gentamycin sulfate and 2.5 μg ml^{-1} amphotericin B. For each effluent or surface water sample toxicity evaluation, the cells from five yearling trout were pooled prior to exposure.

4.2.5 Exposure of cells to chemicals, effluents and surface waters

Hepatocytes were distributed in 24-well microplates (cell culture treated) at a density of 1×10^6 viable cells ml^{-1} of L-15 medium as described above. The cells were exposed to several dilutions of effluent (0.001, 0.01, 0.1, 1, 10 and 50% v/v) or surface water samples (0.1, 1, 10, 25 and 50% v/v). The exposure period was 48 h at 15 °C in a humidified atmosphere. Rainbow trout hepatocytes were also exposed to Cd (0, 25, 50, 75, 100 and 200 ng ml^{-1}), BaP (0, 25, 50, 75, 100, 200, 300, 400 ng ml^{-1}) and ß-NF (0, 25, 50, 75, 100 and 200 ng ml^{-1}) under identical conditions.

4.2.6 Cell viability evaluation

Cell viability was assayed by flow cytometry using the PI exclusion test.[16] An aliquot of cells (50×10^4 cells) was centrifuged and, after discarding the supernatant, was incubated with 200 μl of PI (10 μg ml^{-1}) in PBS for 10 min at room temperature. The cells were then analyzed by flow cytometry as described below. The proportion of viable cells (exposed *versus* unexposed) was determined with digitonin, a positive (biocidal) control which permeabilizes cells. The same number of cells was treated with one volume of 0.02% digitonin in PBS for 5 min, incubated with PI for 10 min

and analyzed with flow cytometry. The percentage of live cells was calculated as follows:

where: X is the proportion of stained cells in the exposed group;
A is the proportion of stained cells in the unexposed group; and
B is the proportion of stained cells in the digitonin-treated group.

4.2.7 Cell suspension *in situ* hybridization

After the exposure period, the medium was removed and washed in ice-cold SSC 1 X and centrifuged at 200×g for 2 min at 4 °C. The supernatant was removed and a selected number of cells (200,000) were fixed in 150 µl of PBS containing 4% formaldehyde and 5 mM $MgCl_2$ for 10 min at 22 °C. The cell suspension was transferred to a 96-well polycarbonate microplate. The cells were then centrifuged and, after discarding the supernatant, fixed in 150 µl of 70% ethanol containing 0.05% DEPC for 45 min at 4 °C. The cells were again centrifuged, the supernatant removed and rehydrated/permeabilized with 100 µl of PBS containing 0.1% Tween-20 for 15 min at 22 °C. Afterwards, the cells were centrifuged and, after supernatant removal, were incubated with 500 ng of the DNA probe in 100 µl of SSC 4X containing 0.1% SDS, 0.1% Ficoll, 0.1% PVP and 0.1% BSA. This mixture was overlaid with 50 µl of mineral oil and incubated at 85 °C for 2 min, after which hybridization was allowed to proceed at 58 °C for 1 h. The cells were then washed by centrifugation and resuspended with 150 µl of PBS containing 0.1% BSA and Tween-20 for 5 min. The cells were washed once more by centrifugation and resuspended in PBS containing 10 µg ml^{-1} streptavidin-fluorescein, 0.1% BSA and Tween-20 for 30 min at 37 °C. The cells were washed for one last time in PBS for 10 min, centrifuged and resuspended in PBS containing 10 µg ml^{-1} of PI. The cells were then ready for flow cytometry analysis.

The extent of hybridization of DNA probes to MT and MFO mRNAs in Cd or ß-NF treated cells was compared with that of MT protein levels assayed by the silver saturation assay.[17] CYP1A1 induction measurement was also compared with methoxycarbonylfluorescein O-demethylase (MCFOD) activity assayed in live hepatocytes using flow cytometry.[18]

4.2.8 Flow cytometric analysis

Flow cytometric measurements were achieved with a FASCan flow cytometer (Becton-Dickinson) equipped with an analytical flow cell, argon (blue) laser emitting at 488 nm at 15 mW. The flow rate was maintained at about 13 µl min^{-1} during acquisition of data. For each measurement, data from 5000 gated cells were recorded, analyzed and stored with the Lysis II software. The cells were first gated according to forward (fsc detector) and orthogonal light scatter (ssc detector) characteristics to

isolate them from smaller particles. The fsc detector was set at -1 exponential in log scale and the ssc detector was set at 175 V equally in log scale.

For FISHES measurements, the detector for fluorescein emission (500–550 nm, fl-1 detector) was set at 400 V and the channel number was set at 1024 for maximum resolution. The detector for DNA analysis and cell viability determinations (600–650 nm, fl-3 detector) was set at 275 V and the channel number was set at 256. The cells were aspirated into the flow cytometer and 5,000 cells were collected. A second gating was constructed such that DNA- containing cells could be analyzed for fluorescein emission. The data were reported in a 2-dimensional graph (dot plot) with cell volume (forward light scatter) on the *y* axis and fluorescence emission on the *x* axis. For data analysis, the mean fluorescence of the cell population was measured.

For cell viability measurements (fluorescence analysis of the DNA-PI complex at 620 nm with the fl-3 detector), unexposed cells were aspirated, analyzed and reported in a 2-dimensional plot with forward light scatter on the *y* axis and red fluorescence on the *x* axis. Gating was constructed in order to circumscribe the proportion of cells that became more fluorescent (ie, cells that did not exclude PI dye).

4.2.9 Statistical analysis

Cells were exposed to effluent, surface waters and test compounds in quadruplicate. Flow cytometric data (% of events or mean population fluorescence) were subjected to a Bartlett's homogeneity of variance test and a normality test. When variances were homogenous, data underwent an analysis of variance and critical differences between unexposed and exposed cells were determined with Dunnett's t test. In some cases, variances were not homogenous and data were not normally distributed. In such cases, data were analyzed by non-parametric Kruskal-Wallis analysis of variance and critical differences between control and treatment were appraised by a distribution-free multiple comparison test (Dunn's test). Significance was invariably set at $p<0.05$. For environmental samples, the lowest observable effect concentration (LOEC) and the no observable effect concentration (NOEC), expressed in % v/v, were determined. A toxicity threshold (TT) was then calculated from the following equation:

$$TT = (NOEC \times LOEC)^{1/2}$$

Environmental sample data were further transformed into toxicity units (TU) with the help of the following equation: .

$$TU = 100\%\ v/v \div TT$$

This transformation renders data directly proportional to toxic strength.

4.3 RESULTS AND DISCUSSION

4.3.1 Hybridization of DNA probes in hepatocytes

The DNA probes were produced by asymmetric PCR from reverse transcription of total RNA in cells exposed to either ß-NF or Cd^{2+}. These cells had increased levels of MT and MCFOD at the protein or enzyme levels, respectively (see Figures 4.1

and 4.2). MCFOD activity (Figure 4.2) reflected the induction of CYP1 Al and also of its transcriptional mRNA (Figure 4.2A). Similarly, MT protein units reflected the transcription of MT mRNA (Figure 4.1). Reverse transcription of total cell RNA followed by PCR amplification generally yielded 20 to 100 μg ml^{-1} of total DNA. Gel electrophoresis of the DNA samples after ribonuclease *A* digestion showed bands principally of 200 and 1500 nucleotides for MT and CYP1A1 DNA probes, respectively. However, some faint bands were also observed in these gels. These appeared to be smaller fragments of DNA probes as other nonspecific DNA fragments may be present. Generated probes were tested for their capacity to detect induction of mRNA using known inducers as discussed below. Furthermore, an interleukin-1a (IL-1a) DNA probe (308 nucleotides) was also produced (supplied with the GeneAmp RT-PCR kit) which served as a probe for estimating non-specific absorbtion. This probe did not show any specific binding when compared to cells treated only with streptavidin-fluorescein and PI (Table 4.1).

However, when cells were hybridized with DNA probe for MT or CYP1A1, significant signals were measured in unexposed cells. Significantly higher fluorescence values were again observed in Cd^{2+} or ß-NF treated cells (Table 4.1).

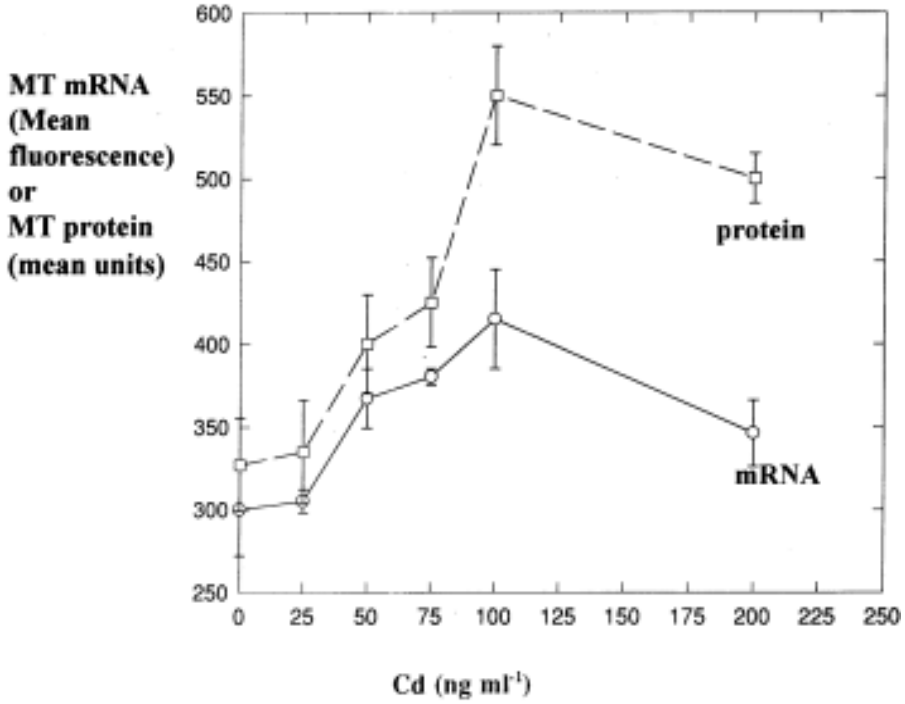

Figure 4.1 *MT induction due to cadmium exposure in rainbow trout hepatocytes. Rainbow trout hepatocytes were exposed to several concentrations of Cd for 48 h at 15 °C. MT levels were determined at the protein (——) and mRNA levels (—) of expression. Data represen the mean with the standard deviation from n=4 replicates.*

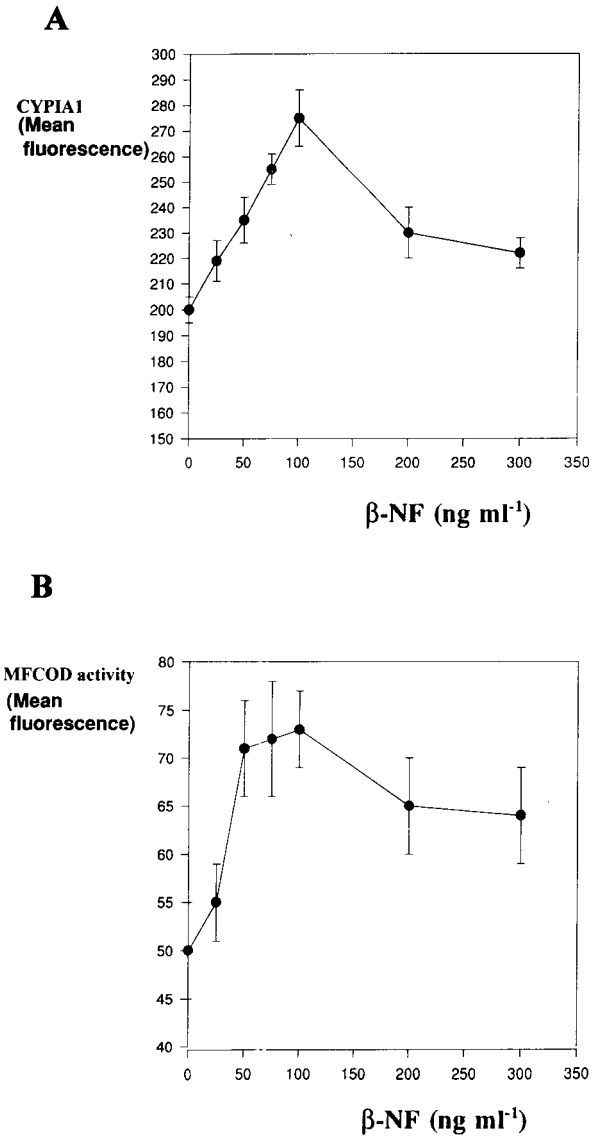

Figure 4.2 *Induction of CYP1A1 due to ß-NF exposure in rainbow trout hepatocytes. Rainbow trout hepatocytes were exposed to ß-NF for 48 h at 15 °C. CYP1A1 levels were assayed at transcriptional (A) and enzyme activity (B) levels of expression. Data represent the mean with the standard deviation from n=4 replicates.*

Table 4.1 *Flow cytometric analysis of cellular MT and CYP1A1 mRNA*

Cell treatment	CYP1A1 (RFU[a])	MT (RFU)
Cells	25±5[b]	30±4
Cells and PI	28±7	32±3
Cells, streptavidin-FITC and PI	39±5	38±4
Cells, DNA probe (IL-1α)[c] streptavidin-FITC and PI	42±4	43±6
Control cells, DNA probe (MT or CYP1A1), streptavidin-FITC and PI	225±20	300±31
Treated cells (Cd or β-NF), DNA probe (MT or CYP1A1), streptavidin-FITC and PI	293±30	400±28

[a]RFU: relative fluorescence units
[b]Data are expressed as the mean RFU of the population with the standard deviation
[c]This probe was used to detect non-specific binding to cellular sites. The IL-α cDNA probe was made in the presence of 10 μM of biotin-16-dUTP during PCR amplification and hybridized to fixed hepatocytes as described in section 4.2

These results indicate that the hybridization cocktail is adequate for reducing nonspecific absorbtion of DNA molecules while favoring DNA probe hybridization to mRNA. The addition of dextran sulfate or formamide in the hybridization cocktail gave poor cell recovery, partly because the density of the cocktail was higher, thereby possibly restricting cell sedimentation during centrifugation and did not decrease the background signals. Inclusion in the hybridization cocktail of transfer RNA from *Escherichia coli* and salmon sperm DNA at 1 μg ml^{-1} and 25 μg ml^{-1}, respectively, also did not diminish background signals and even lowered the signals with the MT and CYP1A1 DNA probes. Formaldehyde fixation followed by ethanol/DEPC fixation was essential for trapping mRNAs within cells, by inhibiting ribonucleases (DEPC) and preserving cell morphology.

Also, DNA probes can potentially hybridize with genomic DNA which also contains these sequences. Because the time allowed for hybridization is not long, this interference can be considered low. In addition, DNA of non-dividing hepatocytes are being considered.[15] Its quantity is constant as suggested by its flow cytometric measurement using propidium iodide in fixed hepatocytes. Therefore, gene expression for MT and CYP1A1 is measurable because of the constancy of total genomic DNA and translation into mRNA is inducible in this cell system.[10,17]

4.3.2 Induction of cellular MT and CYP1A1 mRNA

Rainbow trout hepatocytes were exposed to either Cd or ß-NF which are well known inducers for MT[8] and CYP1A1[10] both *in vivo* and *in vitro* with fish and mammals. After a 48 h exposure, hepatocytes were collected and analyzed for MT and CYP1A1 at the protein (or enzyme) and mRNA levels. In this study, CYP1 Al (at the enzyme level) is expressed as the enzyme activity of MCFOD. When cells were exposed to Cd^{2+}, significant hybridization signal and MT levels were measured (Figure 4.1). Moreover, MT mRNA levels paralleled those of MT protein and a significant and positive correlation was obtained (R=0.830; p=0.04). These results suggest that, in Cd-exposed cells, the DNA probe generated by PCR methodology is of adequate specificity and that post transcriptional regulation of MT mRNA appears not to occur significantly in exposed cells. Therefore, it is deduced that the measurement of MT at the transcriptional instead of at the protein levels could be used as an early warning event for heavy metal exposure using PCR-generated probes. Similarly, cells exposed to ß-NF had higher hybridization signals than untreated cells (Figure 4.2A) and these levels also varied with MCFOD activity (Figure 4.2B), as indicated by a significant and positive correlation (R=0.906; p=0.0129). Here again, as with MT DNA probe, PCR-generated DNA probes appear adequately specific and no significant post-transcriptional activity seems to occur in hepatocytes exposed to ß-NF in the conditions described. These results suggest that CYP1A1 mRNA or MCFOD activity measurements could serve as an early warning system to detect exposure to inducing chemicals such as PAHs, PCBs, dioxins and furans. Hybridization of mRNA with PCR-generated DNA probes in hepatocytes followed by fluorescence detection using flow cytometry is feasible using the experimental conditions described in this chapter. This method demonstrates the means of producing a rapid quantitative measurement of mRNA in single cells. Asymmetric PCR methodology has the advantage of generating a DNA probe of any desired length to improve its specificity without excluding it from the intracellular environment. This study has shown that probe lengths of 200–1500 nucleotides can be used for *in situ* hybridization in rainbow trout hepatocytes.

4.3.3 Cytotoxic effects of a major urban discharge

Water samples were taken above, at and below a major urban wastewater outfall to assess cytotoxicity indicated by cell viability, MT mRNA and CYP1A1 mRNA levels (Figure 4.3). Levels of MT mRNA were induced clearly at the urban effluent outfall and then decreased with distance downstream in the river. This suggests that bioavailable heavy metals are present in the urban effluent and have induced MT in hepatocytes. MT can be induced principally by heavy metals[8] and have also been shown to be induced by compounds responsible for oxidative stress in rat liver.[9] Induction of MT in trout hepatocytes exposed to oxidative stress, however, has yet to be confirmed.

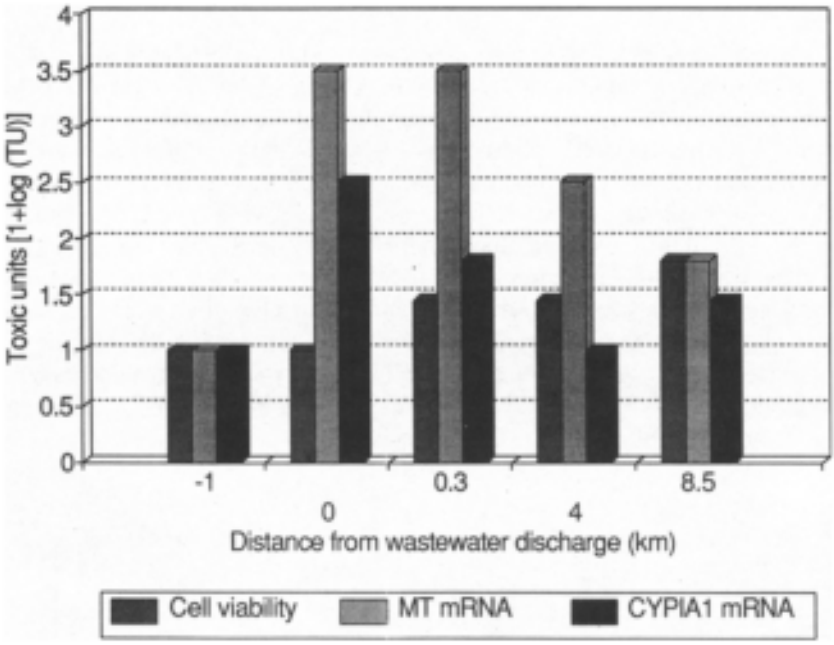

Figure 4.3 *Cytotoxicity of a major urban discharge. Rainbow trout hepatocytes were exposed to several dilutions of a major urban discharge and of its associated upstream/downstream stations for 48 h at 15 °C. Cell viability, MT mRNA and CYP1A1 mRNA were assayed. Data are reported in* $\log_{10}$ *toxicity units.*

CYP1A1 mRNA induction was also detected in the effluent and decreased with distance downstream in the river. Hence, these water samples may contain planar organic compounds such as PAHs or PCBs (or other unknown inducing compounds) that are bioavailable to liver cells. Toxic effects on cell viability were increased somewhat at distances located 0.3, 4 and 8.5 km downstream. These responses appear unrelated to the urban discharge and perhaps suggest that additional sources of toxicants are influential downstream. Levels of PAHs and polychlorinated biphenyls (PCBs) were measured in the urban effluent. Levels of PAHs, obtained from 21 distinct PAHs at the time of sampling, totalled 4 ± 0.3 μg l^{-1} and that of PCBs, obtained from 13 congeners at the time of sampling, totalled 0.6 ± 0.015 μg l^{-1} (results not shown). However, it remains to be seen whether these levels alone are sufficient to induce CYP1A1 mRNA in trout hepatocytes.

Water samples obtained upstream and 8.5 km downstream in the vicinity of the urban effluent plume (not shown) did not produce any significantly detectable effects on cell viability, MT and CYP1A1 mRNA levels. This urban effluent study highlights the sensitivity of the rainbow trout hepatocyte bioassay and its usefulness as a simple screening tool to detect toxic effects along a chemical pollution gradient.

4.3.4 Cytotoxic effects of industrial effluents

Rainbow trout hepatocytes were also exposed to industrial effluents in order to explore effects on cell viability, MT and CYP1A1 mRNA levels (Figure 4.4). All effluents elicited significant responses in one or more of the three toxicity endpoints measured. Reduction in cell viability was highest in effluent ICI, manifest in effluents ST and PC, but less pronounced in the other effluents. Effluents ICI and PC, in particular, proved to be the strongest inducers of MT mRNA, thereby suggesting that they contained bioavailable heavy metal(s). For industrial wastewaters, hepatic MT induction has already been reported for trout exposed to pulp and paper effluents.[11] CYP1A1 mRNA induction, suggestive of the presence of certain classes of high risk organic chemicals, was observed in three of the six effluents tested (ie, PC, PP2 and ST). While MFO activity induction responses have been documented for PP and PC effluents,[11,19] such information for ST-type effluents is not available currently.

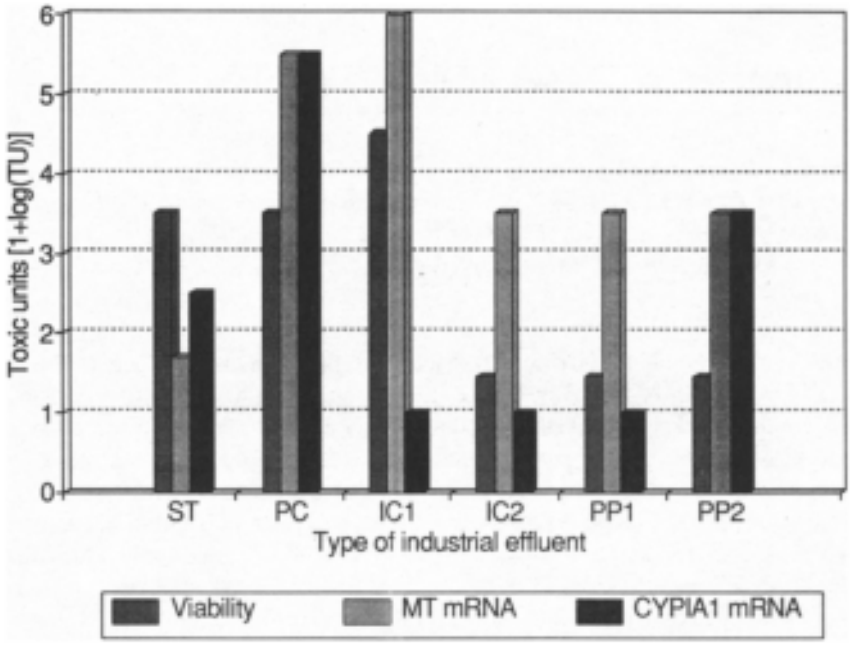

Figure 4.4 *Cytotoxicity of industrial effluents. Rainbow trout hepatocytes were exposed to several dilutions of industrial effluents for 48 h at 15 °C. The industrial effluents were from the following sectors: surface treatment (ST), petrochemical (PC), inorganic chemical production (IC) and pulp and paper (PP). Cell viability, MT mRNA and CYP1A1 mRNA were assayed. Data are reported in* $\log_{10}$ *toxicity units.*

4.4 CONCLUSIONS

A simple and rapid cell suspension *in situ* hybridization methodology using rainbow trout hepatocytes has been developed, whereby hybridized DNA probes within cells can detect transcriptional expression of MT and CYP1A1 mRNA by coupling streptavidin-fluorescein binding to biotin and flow cytometry measurement techniques. In this study, biotin-16-dUTP was used as the nucleotide analogue of thymidine, but other analogues can be used. For example, digoxigenin-12-dUTP[13] and fluorescein-11-dUTP are also suitable analogues of thymidine. The DNA probes used in this study were cloned and amplified from total RNA of hepatocytes exposed either to Cd and ß-NF. This yielded DNA strands which were consistent in size to MT and CYP1A1 mRNAs. Other secondary strands were also generated suggesting that amplification was not entirely specific to the desired mRNAs. However, the presence of these other DNA strands did not markedly hinder measuring mRNAs in cells exposed to known inducers. Significant hybridization signals, confirmed by parallel increases in levels of translation products (MT protein or MCFOD activity), were measured in treated cells as compared with unexposed cells, indicating adequate specificity of the DNA probes generated by asymmetric PCR. The preliminary tests with selected effluents and surface water samples suggest that this methodological approach may eventually prove useful to screen for the presence of relevant (sub)lethal effects triggered by chemical pollutants in various environmental matrices. This will prove to be a highly advantageous tool in environmental toxicological assessment.

4.5 ACKNOWLEDGEMENTS

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5 Ecotoxicology Testing—Effective Use In Field Monitoring

John G.McHenery

5.1 INTRODUCTION

It is frequently forgotten that ecotoxicology is the study of the fate as well as the effect of toxic agents in ecosystems. The fate component has been dealt with in other chapters and is mentioned here only to reiterate the importance of its assessment in any ecological or environmental risk assessment, whilst degradation of materials in the environment usually results in a reduction in toxicity there may be the potential for more toxic agents to be found. The effect caused by any agent will be dependant on both its inherent toxicity, and also on the degree of exposure. In turn his will be dependant how much of the material enters the environment, how it partitions in the environment, and how it persists. From this it can be seen that ecotoxicology is an integrative discipline, combining a number of factors to give, hopefully, an end result. It is this integrative capacity, combining the factors within a temporal framework which lies at the heart of the potential of ecotoxicology as a field monitor for the assessment of environmental exposure. Field deployments of sentinel organisms or collection of animals from the environment creates the possibility for the scientist to obtain a time integrated view of what an organism has been exposed to, how long for, and whether the exposure has resulted in an effect. Whether this potential can be realised will be dependant on whether the organisms can accumulate the materials of concern, whether it persists, whether it has an identifiable and quantifiable effect and whether the scientists know what to look for.

The use of ecotoxicological tests as a means of assessing environmental levels of toxicants or their biological effects is, by definition, a bioassay rather than a toxicity test. In cases where the effects are not calibrated against a standard response the classification of the test type becomes more difficult, unless the untreated control group is viewed as the reference comparator. The distinction between the test types can be further complicated where the ecotoxicity test guideline includes a reference test material against which the test results are compared to facilitate ranking, such as is found in the PARCOM guidelines.[1] From this it can be seen that bioassay and ecotoxicological tests merge depending on procedural use.

The potential of biological effects methods as a means of assessing the hazard posed by materials to life in the environment can be viewed as having developed from work drawn together in the 1970s.[2-4] The use of biological assessment methods, to detect potential harmful materials by their biological effects relies on the recognition that it is the effects of contaminants on life, rather than their levels

per se which is important in nature. This opens the opportunity of using biological test methods to screen for adverse activities in complex mixtures where determination of all components is not practical; to assess the health of the receiving environment; and to screen effluents/materials to determine if there has been a change in composition/activity.

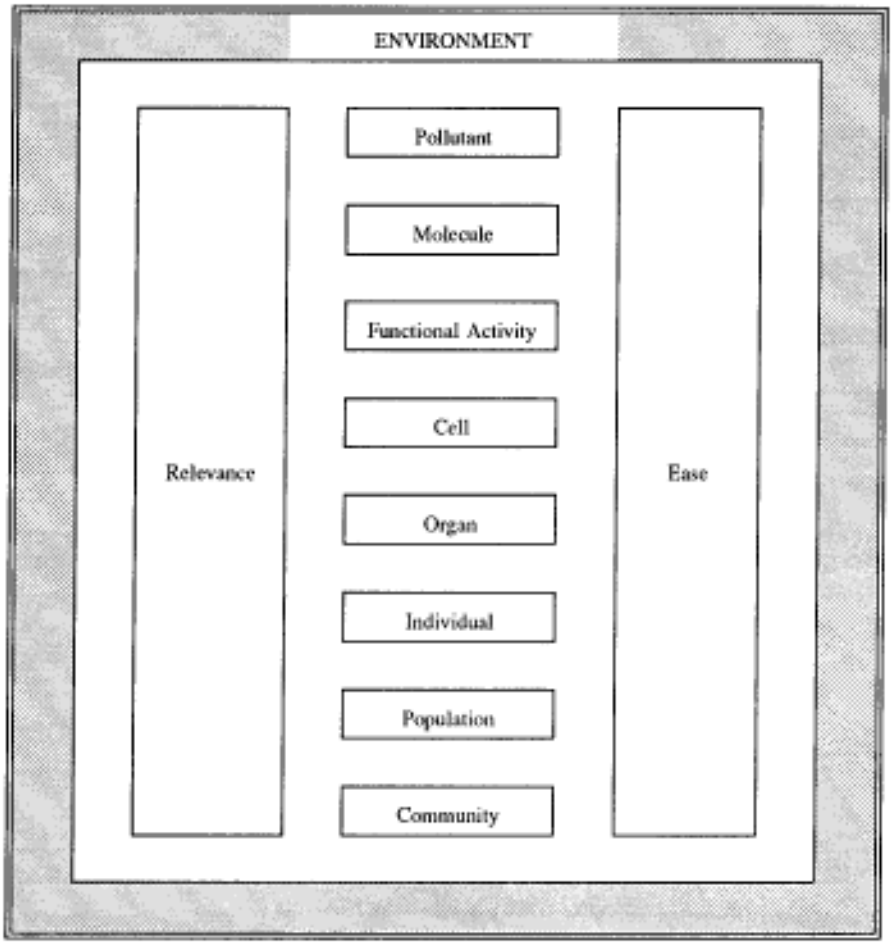

Figure 5.1 *Relationships between biological monitoring methods*

5.2 MOLECULE TO COMMUNITY

Biological effects techniques cover the full spectrum of biological activity and organization from the molecule through to the community. Figure 5.1. It is generally accepted that biological effects measures have a potential as a means of assessing 'biological well-being'. The complexity of biological systems, and the ability of systems to

compensate for 'stress' complicates the interpretation of many biological effects techniques. Whilst it might be considered comparatively straightforward to translate observed changes in communities and populations to a statement on the health of the environment this is not necessarily the case. Problems also arise in translating the results of tests at the molecular to cellular level to statements on the health of the community and environment though it can be easier to attribute effects to specific toxicants. This has been identified in the past as one of the main difficulties in the use of a number of biological effects techniques. Whilst the techniques may be elegant in their aims and design their relevance has not been established, detracting from their usefulness and impeding their wider adoption outside the cognoscenti who are familiar with their scientific basis and to whom the ease of interpretation, and environmental relevance, might be seen as being of less importance.

In the past it has frequently been the case that any change relative to a 'control' group, whether at the sub-cellular or at the community level, has been proclaimed as evidence of a biological effect. In such cases the lack of cause and effect data, demonstrable links to community health and supporting chemical determinations can lead to doubts over the validity of conclusions drawn from limited studies. In too many cases the scientists are not at fault but are suffering from lack of resources which would enable sufficiently comprehensive studies to be undertaken. This said, there were still cases where the scientists appeared to think that those outside their field were not really entitled to question their conclusions.

The use to which a biological effects technique is to be put and the intended use of the results will be critical in deciding the suite of tests to be considered and how they should be applied, particularly where there is a potential that the results may be used in prosecutions and the interpretation subjected to the rigours of the legal system.

5.3 COMMUNITY ANALYSIS

If a toxicant has an effect on the well-being of individuals within a population, impairs their reproductive capacity or makes the environment less desirable so that those that can move do so before they die, then changes can be expected in the population structure and diversity of the community. This typically results in marked increases in the numbers of opportunists and reductions in the numbers and types of other less robust species. This pollution effect can be assessed by monitoring communities and quantified for example by means of biodiversity indices, eg Shannon-Wiener[5] and River Invertebrate Prediction and Classification System (RIVPACS)[6] for which theoretical links to ecological processes have been assessed.[7,8] Diversity within communities, and their indices, can also be affected by other factors such as predation, spatial heterogeneity and time.[9,10]

Whilst caution should be applied when considering the results of an ecological survey it is probably acceptable to say that it is the ultimate form of a bioassay.[11] Whether the results are interpreted on a spatial or temporal scale will depend on the nature and extent of the survey undertaken, whether base line data is available and

whether sufficient data is available to enable a meaningful interpretation to be conducted.

5.4 ECOTOXICITY/ORGANISM TESTS

Whilst laboratory toxicity tests provide a means of comparing and ranking the effects of materials in the laboratory the responses should not be directly interpreted as ecologically significant.[12] The responses in laboratory tests should be related to predicted environmental concentrations.[13] Even in cases where there is an extensive data set on toxicological effects, such as is found for certain pesticides,[13–17] and effects might be expected, eg from use in fish farms, the reported effects are marginal and limited spatially[13,18,19] compared with those that might be expected from laboratory data.[20]

The tiered testing approach for toxicity, ecotoxicity, fate and chemical behavior[20,21] enables an assessment to be made of the environmental compartment and component most likely to be effected by a material. Further testing can then be planned to address the components viewed as being at greatest risk.

This data, together with predicted use and disposal patterns, enables an environmental risk assessment to be conducted.[22,23] A number of computer models are now available, or are being developed, to undertake risk assessments. Central to the conduct of meaningful modelling exercises are reliable data. The data from the toxicity assessments can be used to identify potential monitoring methods which can in turn provide a basis for field assessments and model validations.

5.5 BIOCHEMICAL, PHYSIOLOGICAL AND CELLULAR METHODS

Biomarkers, changes in biochemical, physiological and cellular changes, have been investigated as a means of detecting and predicting the effects of pollutants on organisms and ecosystems. Such activities are central to ecotoxicology.[23]

Many of the biological effects studies which were initially proposed were viewed as a means of testing for the presence of contaminants which were known, or suspected, to have deleterious effects on life but for which chemical determination methods were not sufficiently developed to enable detection or quantification in environmental samples.

Comparatively few of the biological effects measures which have been proposed and developed have offered the degree of specificity and discrimination to enable toxicant specific assessments to be conducted.

Techniques to monitor effects such as mixed function oxidases (MFO) as an indicator of organic chemical contamination[24] have been widely used as general contamination and effects monitors. Whilst disease symptoms can be induced in experimental studies, the complexity of natural systems makes it difficult to demonstrate cause/effect relationships in field studies.[25] A variety of techniques are now available for assessing the effects of toxicants on the DNA and chromosomal integrity of life. These techniques, as with many biochemical and sub-cellular methods, have been adapted from methods

which are widely used in human and mammalian scientific disciplines and can be expected to become more widely used.

Chemical determination has continued to develop and the resolution of traditional methods has been improved, and new methods have become commercial, so that one of the early reasons for developing biological effects measures has been superseded by chemical technology. However, there are a number of examples where biological effects indicators have been found to be sufficiently specific to be used as sensitive indicators of toxicant exposure in the environment eg, Imposex in dog whelks exposed to tributyl tin (TBT)[26] and acetylcholinesterase inhibition due to exposure to organophosphate and carbamate pesticides.[18,27] In such cases the measurement of the biological indicator can provide a cheaper and easier method of determining environmental levels and effects of biologically available toxicant than chemical determination.

5.6 BIOSENSORS AND ON-LINE BIOLOGICAL MONITORS

A major disadvantage with most chemical and biological monitoring systems is the delay between the pollution event occurring and its detection. A number of approaches have been developed to reduce this by using in- and on-line biochemical and physiological responses in organisms and using biocatalysts to measure responses in enzymes and cells.[28,29] Some of the existing techniques provide pollutant specific responses whilst others provide a more general 'pollution event' response, the relative merits of the two approaches being dependant on the nature of the intended use. Disadvantages found with a number of the existing on-line biomonitoring techniques include:

(i) Use of a single species and measurement of a single parameter;
(ii) Level of hardware and supporting computer technology;
(iii) System maintenance and baseline response establishment; and
(iv) Calibration and expertise required to identify responses and their interpretation.

5.7 MONITORING AND ASSESSMENT PROGRAMS

When a monitoring or assessment program, biological or otherwise, is being planned care is required to ensure that it is targeted. The questions to be answered must be identified and the appropriate techniques selected so that there is a reasonable chance that the study will answer at least some of the questions and not merely give rise to others.

Techniques and organisms should be selected which satisfy the '5Rs' for biomonitors,[30] they should be:

(i) Relevant—functionally significant within the ecosystem;
(ii) Reliable—sufficiently common and widely distributed to enable comparison between sites;

(iii) Robust—death should not follow minor exposure unless mortality is the indicator being monitored eg as in community structure monitoring. Transplanted organisms should tolerate deployment;
(iv) Responsive—responses to pollution exposure should be measurable, distinguish between pollution induced and other variability and preferably be quantifiable; and
(v) Reproducible—responses to pollutant exposure should be similar in different situations and certainly where studies are repeated under identical conditions.

The ease with which field responses can be interpreted will be increased where the responses have been calibrated under defined exposure conditions with supporting chemical analysis. In the ideal situation all of the above criteria should be met but in the real world this might not be possible and an acceptable balance of what is desirable and what is practical may have to be accepted. Where a number of factors may influence a response in a given situation it may be necessary to use, multiple response indicators in different organisms. Such an approach has been used successfully where acetylcholinesterase activity has been measured as an indicator of organophosphate exposure from fish farms in sea lochs where reduced salinity due to fresh water run off can kill the primary indicator organisms. By comparing the results for different indicator species and effects it is possible to distinguish between mortalities and effects due to toxicant exposure and other causes.[18,31]

Before any program is implemented the basic biology of the organism should be investigated, and where necessary its suitability determined by experimentation.[32] Where sub-lethal effects monitors are to be used the advantages of using indigenous organisms should be considered. In cases where the methods to be used have not been validated, or are not suitable, for indigenous organisms care should be taken in the selection of suitable organisms for deployment. It should always be considered that suitable organisms may be absent from an area because the environmental conditions, excluding anthropomorphic effects, would not permit the organism to become established or survive.

The methods adopted for any monitoring, be it biological, chemical or both, will be dependant on the availability of appropriate methods, their suitability for the organisms and environment of concern and the nature of the toxicant being considered. In the broadest terms pollutants can be divided into the following types:[33]

(i) Disruptives—exert non-selective effect and cause impact by overwhelming the environment;
(ii) Distributives—have unusual physicochemical activity and partition and accumulate along critical pathways; and
(iii) Directives—molecules with the capacity to direct the future well being of organisms.

In reality, many pollutants will exhibit a mixture of the above characteristics and monitoring programmes will require to be tailored to encompass the characteristics and the environment of concern.

5.8 CURRENT AND FUTURE MONITORING

On-line biological effects monitoring is currently used within existing regulatory frameworks to protect water quality both at drinking water treatment plant intakes and sewage treatment plant effluent discharge points. Examples of the former application include the use of the Water Research centre (WRc) fish monitor, which measures gill ventilation rate, and on-line Microtox® which has recently been introduced to monitor quality of the Seine River.[34]

The use of biological tests in monitoring effluent quality is well established. Such applications include the setting of toxicity limits for effluents in consent conditions set by regulatory authorities within the United Kingdom and United States. Consents of this type typically specify a dilution which will not cause greater than 50% mortality in organisms exposed under defined conditions. These tests are conducted as spot checks to ensure that effluents meet their consented conditions. Such consents typically specify the test conditions and the organisms to be tested which should be representative of the biota in the receiving environment. Biological effects methods are well established in the monitoring of sewage sludge disposal sites and have proven useful in monitoring the assimilative capacity and health of the receiving environments and their recovery.[35,36] Such monitoring program have typically used community analysis as a standard monitor with other methods being developed and compared with them. A similar approach has been adopted in monitoring off-shore oil exploration and production facilities.

A criticism which has been made of community analysis is that it frequently is only conducted after a pollution event and as such has no base line data to enable a comparison to be conducted to enable assessment of recovery. This is further complicated by the natural variability in populations and communities which occurs both temporally and spatially. For this, if for no other reason, trends which are detected should not be viewed in isolation but in the wider environmental context.

The move towards **toxicity based consents** for effluents and **direct toxicity assessment** within the United Kingdom can be seen as linking **substance specific control** of chemicals in effluents and **biological assessment** in the receiving environment, toxicological tests and bioassays with environmental samples supporting the test strategy.[37] It is probable that this approach will gain wider acceptance once it has been fully trialled and results, both good and bad, are reported and available for assessment.

The increase in computer modelling as a means of predicting environmental dispersion, concentrations, effects and risks will increase the need for tests to confirm predictions, validate models and monitor the environment. Undoubtedly this will be required until the regulatory authorities are certain that the output of models realistically reflects the real world and not merely the modellers interpretation of it. The preferred way to assess whether a models prediction of potential environmental effect, or indeed no-effect, will be to deploy sensitive organisms in the environment of concern. Ideally the organism deployed will be the same organisms as those used to provide the data which was used in the model. This approach should eliminate some of the uncertainties associated with models, those due to differences in species

sensitivity. It should also provide information on the uncertainties relating to extrapolation of laboratory to field data. The validity of the model could then be determined under more strictly controlled conditions and its environmental relevance better assessed.

There is a natural, and understandable, concern amongst many who see themselves as being on the receiving end of changes in the manner in which effluents and other materials are monitored and assessed. Whilst the heightened prominence of such tests may cause concern amongst those who have not, and some who have, experienced their application in the past, they should not be viewed as a novel means being adopted by the regulatory authorities to make life more difficult. Instead, they should be viewed as a means of protecting and improving our environment in a practical way. For this to be the case there is a need for industry to be actively involved to ensure that the monitoring effort of the regulators can lead to improvements and does not merely gather data which will enable boxes to be ticked on forms which will be filed and never accessed or acted upon. Worse still, they should not be used merely as a means of bringing prosecutions without a constructive aim. The natural variability of biological systems, populations and tests should ensure that a degree of restraint accompanies the introduction of wider scale toxicity testing of environmental samples to enable all who are involved to come to terms with the changing, and hopefully improving the environment.

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6 Solid Phase Testing of Aquatic Sediments Using *Vibrio Fischeri:* Test Design and Data Interpretation

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6.1 INTRODUCTION

Until recently, most toxicity tests of aquatic sediments used aqueous extracts (elutriates or pore waters) as the exposure medium.[1] Whole sediment, or solid phase, tests are now commonly used.[2,3] Solid phase tests have the advantage of exposing test organisms not only to contaminants dissolved in the pore water, but also to sediment particles which may have contaminants sorbed to them. Most solid phase tests are costly and time consuming, making them unsuitable for preliminary screening of large numbers of samples. Bacterial toxicity tests have been shown to be cost-effective in screening evaluations,[4,5] and are also valuable in adding the choice of another trophic level to multi-trophic level test batteries.[2–4,6,7] It is with these considerations in mind that direct contact, solid phase test procedures using bacteria have been developed.

The basic strengths and weaknesses of toxicity testing with the luminescent bacterium *Vibrio fischeri,* formerly *Photobacterium phosphoreum,* have been reviewed by Warren-Hicks et al[8] and by Ross.[9] The Microtox® test[10] measures toxicity by tracking decreases in the light output of *V.fischeri,* a naturally luminescent marine bacterium. The presence of toxic or bioreactive substances that disrupt or inhibit cellular metabolism will ultimately effect the cell's electron transport system and can be readily quantified by measuring the change in light output of the test cell suspension.

6.2 SOLID PHASE TEST DEVELOPMENT AND APPLICATION

Brouwer et al[11] used a sediment contact assay in which the bacteria are mixed with a slurry of whole sediment sample and diluent medium. After exposure for 15 min, the bacteria are recovered by centrifugation. After recovery, the light output of the cells is measured in a photometer, much as the basic (liquid-phase) test.[7] Brouwer et al used this procedure to screen sediments from 48 sites in Hamilton Harbor (Ontario, Canada).

The direct-contact assay developed by the Microbics Corporation (Carlsbad, CA, USA) is referred to as the Microtox Solid Phase Test, or SPT.[12] The main difference between the SPT and the Brouwer et al procedure[11] is that the SPT uses filtration, rather than centrifugation, to extract the bacteria after exposure. The exposure times

and conditions are also slightly different in the SPT protocol. Tay et al[13] used the SPT to assess toxicity in samples from seven sites in Halifax Harbor (Nova Scotia, Canada), where contamination by heavy metals and organic compounds is known to exist. In this study, the Microtox SPT proved to be the most sensitive of five assays, including two amphipod tests (*Corophium volutator* and *Rhepoxynius abronius*), a polychaete test (*Neanthes* sp.) and the Microtox pore water assay. Hoskin[14] used to SPT screen for toxicity at 34 sites in the Galveston Bay complex and found the test to be sensitive and in good agreement with a more costly Sediment Quality Triad[15] approach. In this chapter the test design and data interpretation are discussed.

6.2.1 Test design

Most applications of the Microtox bioassay are used to test null hypotheses that no difference exists between a number of samples by using statistics. One particularly useful method of analysis is ANOVA (ANalysis OfVAriance)[16] because it can test for differences between a control group and several test groups. ANOVA actually tests the null hypothesis that the variance between the group means is zero, which can only happen if the means are identical. If the null hypothesis is rejected, this indicates that the means are not identical and that the variance between them is not zero. Once the null hypothesis is rejected, then subsequent testing (orthogonal sets, the Tukey test, the Neuman-Keuls test, Dunnett' s test, Scheffe's multiple contrasts) can illustrate where the differences are, and can be used to rank the groups.[16] Whenever the null hypothesis is rejected, there still exists a small quantity, alpha, which indicates the chance of a type I error, or that the result of rejecting the null hypothesis is actually a false positive. Alpha is usually set at 0.01 or 0.05 for most toxicological applications.

If the null hypothesis is not rejected, this indicates that the group means are not different. Whenever the null hypothesis is not rejected, the quantity beta is the chance that the result is actually a false negative. This value can range from very high to very low depending on alpha, variance, sample size, number of groups and the magnitude of the effect in the test groups. Beta is generally less well known than alpha and is rarely calculated. This can cause scientists to draw the incorrect conclusion that there is no effect in the test groups, when actually the chance of a false negative is very high. The statistical power of a test is equal to 1-beta and it indicates the chance that the negative result is a true one.

Alpha is usually set at a small value in toxicological work because the consequences of a false positive could be very expensive. For instance, a false positive result from a site characterization study could mean an unnecessary remediation of that test site. When scientists and resource managers treat an acceptance of the null hypothesis as a verification that no difference exists between groups without considering the power of the test (the chance of a false negative) the cost could be even greater. In the case of a site characterization, this could mean that the site would still be used for human activity when in fact it is not suitable. If a false negative

occurred in a resource management situation, then that could lead to depletion of that resource. The ratio of beta to alpha implies the cost of each type of error. For instance a value of 0.20 beta to 0.05 alpha suggests that the cost of making a type I error is four times greater (0.2/0.05) than the cost of making a type II error.[17] A value of 0.80 power (ie, beta=0.20) has been suggested by some authors as acceptable.[17–19] Others suggest that in some situations beta should be less than or equal to alpha,[18,20,21] Such information concerning alpha and beta is summarized in Table 6.1.

Table 6.1 *Information in relationship to alpha and beta*

Quantity	Alpha	Beta
Means	Chance of false positive	Chance of false negative
Only possible if ...	Null hypothesis is rejected	Null hypothesis is not rejected
Type of error	Type I	Type II
Typical value	0.05	0.2

Unfortunately, the power of statistical tests that accept null hypotheses are rarely calculated in the literature. Peterman[18] reviewed 408 articles on fisheries science and found that 106 papers had at least one instance of accepting the null hypothesis and that 83 of those 106 had at least one assertion of no effect, but that only three of those papers reported values of beta. Hayes[22] undertook a review of the literature on toxicology and only found 19 out of 688 reports had high power in cases that rejected the null hypothesis.

The Microtox system is very conducive to performing high-power research because many tests can be performed with a minimum of sample and expense. The amount of sample needed for an assay is very small compared to most other bioassays: only 0.3 g for a solid phase test, compared to ~150 ml of sediment for the *Rhepoxynius abronius* amphipod bioassay.[23] At the maximum, one Microtox test costs $45.00 per sample, takes no more than one hour to process and requires only moderate labor.[5]

In a pilot study with a relatively small number of Microtox samples and careful application of statistical power analysis, it can be shown that cost effective and statistically powerful experiments can be designed with the Microtox SPT. Samples of the top 2 cm of sediments were collected from a salt marsh near Charleston, South Carolina. While the watershed is mostly unimpacted, one sample came from an area that could be affected by bridge runoff (Site 1). A second sample was obtained three meters away from a bridge runoff downspout (Site 2) and a third sample was collected 10 m away from the bridge runoff downspout (Site 3). Bridge and highway runoff has been shown to contain metals and PAHs and to concentrate these materials in local sediments.[24] SPT assays were run on the samples on the same day that they were collected. The assay generated EC_{50}s for the samples in units of percent sediment. After it was determined that the data conformed to the

requirements of parametric statistics the EC_{50}s were subjected to ANOVA analysis and power analysis.

A posteriori, power can be determined by calculating *phi* as

$$\phi = \frac{(K-1)\ (\text{Between } MS)}{Ks^2} \quad (1)$$

where K is the number of groups, S^2 is the variance of the data (the nine EC_{50}s) and Between *MS* is the between group (sometimes called 'among' or 'groups') mean square from the ANOVA. *Phi* is then cross-referenced in a chart to determine power.[16]

The EC_{50}s and the results of the variance and ANOVA analysis are shown in Table 6.2.

Table 6.2 *Microtox EC_{50}s, F-test for variance and ANOVA results. A probability of 0.15 is greater than the alpha of 0.05 so the null hypothesis is not rejected*

	Microtox EC_{50}s			
	Site 1	**Site 2**	**Site 3**	
	2.6863	1.5833	2.9274	
	2.3368	1.3122	1.9603	
	1.9013	2.0458	2.2159	
	Site 1	Site 2	Site 3	All 3 sites together
Mean	2.3081	1.6471	2.3679	2.1077
Standard error	0.2270	0.2142	0.2893	0.1686
Variance	0.1546	0.1376	0.2511	0.2558
Range	0.7849	0.7336	0.9671	1.6152
F test for difference between two variances, alpha = 0.05				
	F value	Critical F	Probability	Result
Site 1/Site 3	1.6241	39	> 0.50	The variances are equal
Site 2/Site 1	1.1238	39	> 0.50	The variances are equal
Site 2/Site 3	1.8252	39	> 0.50	The variances are equal
Single-Factor ANOVA				
Source of variation	*Sum of squares*	*df*	*Mean square*	*F statistic*	*Probability*
Between groups	0.9600	2	0.4800	2.6501	**0.14968951**
Within groups	1.0867	6	0.1811		
Total	2.0467	8			

With these data, and these experimental design parameters, ANOVA analysis accepts the null hypothesis that the means are equal. The power analysis shows that the power of the ANOVA test was approximately 26% and the chance that this result is actually a false negative, beta, is 74%.

The beta to alpha ratio implies that the cost of a type I error is 15 times (0.74/0.05=14.8) as expensive as a type II error.[17] In this situation this ratio is unacceptable. The salt marsh area is used for fishing, for shellfishing and for human recreation. If these activities continued when the watershed was overly polluted (due to the management acting on a type II error) they could possibly endanger human lives. Conversely, if fishing, shellfishing and recreation were temporarily halted (due to the management acting under a type I error) there might be potential short-term financial hardship for some fishermen and oystermen, but no endangered lives. In this case, a type II error is much more costly than a type I error.

This pilot study information can be utilized to design a powerful study with the Microtox SPT assay of the sediment toxicity in this area. Power of an ANOVA is calculated as a complex function of sample size (n), alpha, variance, the number of groups (K) and the effect size (f).[16,19] There are several ways to increase power by manipulating these experimental design parameters.

One of the easiest ways to increase power is to increase sample size. As sample size increases, power increases, but eventually levels out (Figure 6.1). Increasing the sample size by only two replicates per group, to n=5, practically doubles the power. Acceptable power of at least 80% is reached when n=9. It is not particularly useful to increase the sample size above 10 because there is no significant rise in power after that.

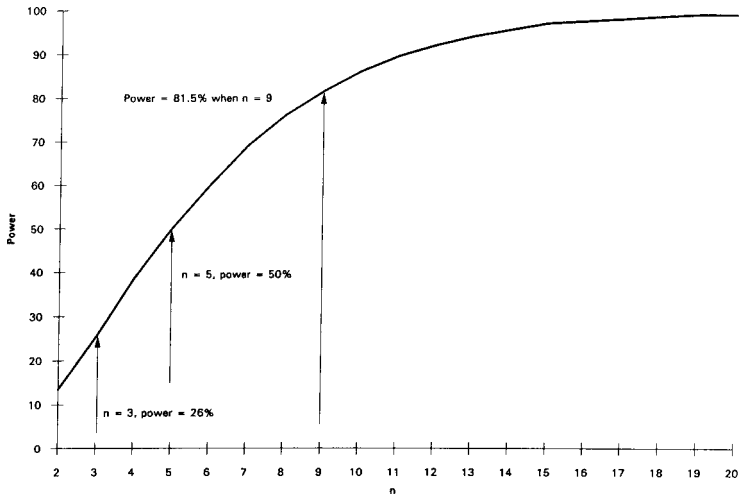

Figure 6.1 *Effect of n on power*

Alpha also has a significant effect on power. As alpha decreases, beta increases (and power, 1-beta, decreases). Figure 6.2 shows the power of a statistical analysis as sample size increases at three levels of alpha. With the current sample size of n =3, power at alpha of 0.05 is 26%, if that alpha is raised to 0.10 the power jumps to 40.5%. If the alpha requirements are made more rigorous (alpha=0.01) the power is 8.5%. This graph also demonstrates that acceptable power of 80% can be reached with only seven samples at an alpha of 0.10.

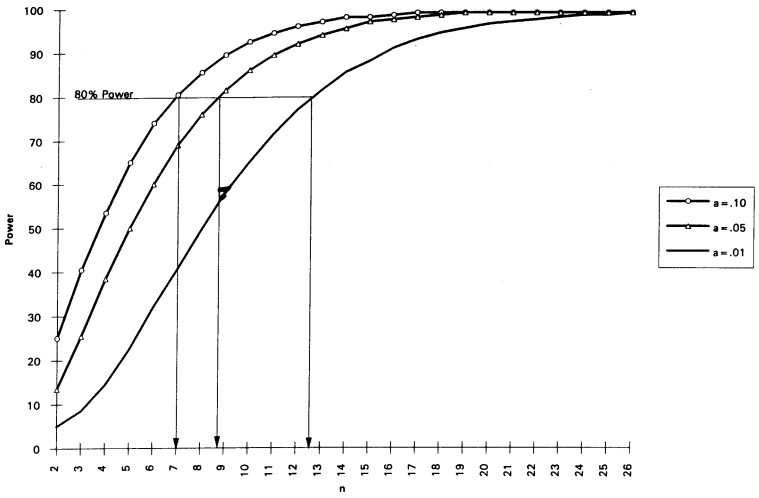

Figure 6.2 *Effect of alpha on powe*

Depending on the purpose of the study, this gain in power might justify raising alpha. Again, graphical analysis shows that once a sufficient sample size is achieved, alpha can be lowered to the most rigorous levels without affecting the power.

The variable that has the most profound effect on power is variance. At the initial sample of n=3, the most dramatic effects of variance occur at unrealistically low levels. Figure 6.3 shows that at a larger sample size, if variance of this data could be reduced from its current value of 0.2558 by as little as 0.05, to a value of 0.20, then the power would rise to generally acceptable levels. Although Microtox variance is typically low, it encompasses the inherent variability in the sediment.[19] Good laboratory practices such as stringent glassware cleaning protocols, good pipetting technique, proper sample storage and proper Microtox reagent storage will reduce the variance. These factors are much easier to control in an assay like the Microtox than in bioassay with larger organisms that must be collected in the field.

Figure 6.4 shows the effect of raising *K* (the number of groups) at sample sizes 3 and 8. As *K* increases, the power decreases, but not rapidly. At n=3 raising *K* from 3 to 4 only results in a 3% loss of power. If there was a reason to add a fourth site in the final experimental design it would not significantly affect power with these parameters. At

larger sample sizes the consequences of adding extra groups are more pronounced. In most cases the loss of power will stabilize after a certain number of groups, so that a graphical analysis will show at what point extra groups can be added without significant loss of power.

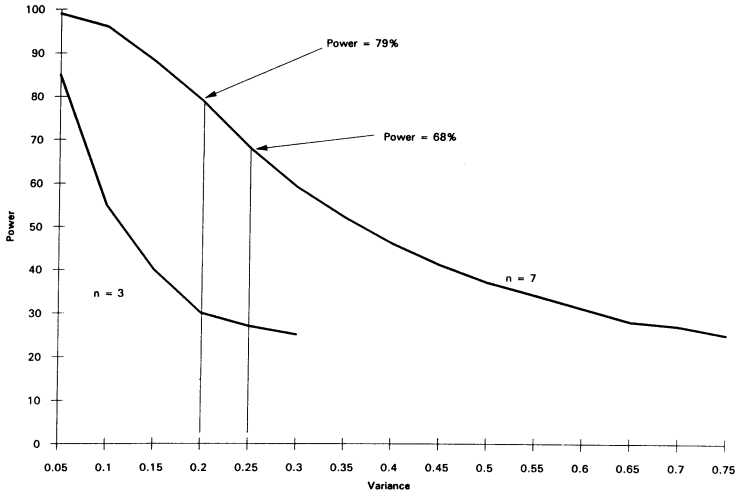

Figure 6.3 *Effect of variance on power*

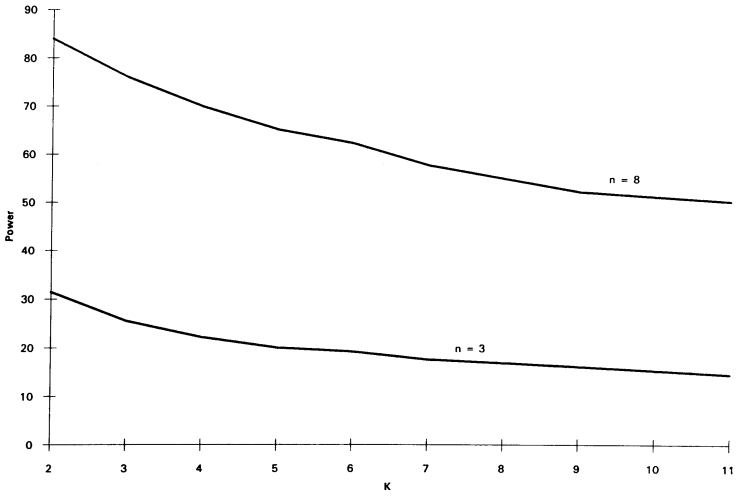

Figure 6.4 *Effect of K on power*

Another factor that influences the power of a statistical analysis is delta, or the smallest detectable difference between group means.[16] The minimum detectable difference means that any populations tested whose means are closer than delta are not statistically different. For example, if a scientist plans an experiment with a delta of 1% sediment, and if the ANOVA does not reject the null hypothesis, it is understood that the means could actually be different by a full percent. As delta increases, because there is a larger area that the researcher has decided to be essentially identical and therefore where accepting the null hypothesis is not a false negative.

As Figure 6.5 shows, increasing delta from 1% to 2% has a profound effect on the power. The biological significance of a few percent difference in a Microtox EC_{50} is highly debatable. If a researcher is willing to accept a delta as high as 2%, this is a good way to increase power. This equation can be rearranged to solve for delta to determine the minimum detectable difference in an *a posteriori* power analysis.[16]

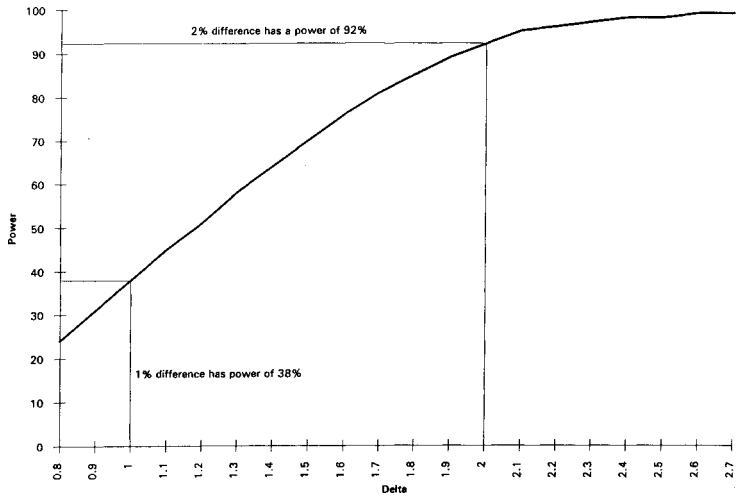

Figure 6.5 *Effect of delta on power*

A more powerful experimental design can be determined using a synthesis of these methods. For instance, using 4 replicates in 4 groups, an alpha of 0.10 and assuming the variance will be no more than 0.20 and that the smallest difference between group means is 1.5% sediment, the power is 89% (*phi*=2.65). These parameters allow the number of groups to rise from 3 to 4 and still have high power. Four sites will give a better indication of sediment characteristics in this area. In this case a type II error is more costly than a type I error, which justifies changing the

Table 6.3 *This contrasts the differences in power and other parameters between a pilot study and a study designed to be more statistically powerful*

	Pilot study	Powerful study
No. of groups or K	3	4
No. of replicates or n	3	5
Variance	0.255	0.2
Alpha	0.05	0.1
Beta	0.74	0.11
Ratio of beta to alpha	14.8	1.1
Power	26%	89%

alpha to 0.10. A contrast of the pilot study and the more powerful study are presented in Table 6.3.

6.2.2 Data interpretation

Care should be exercised in the interpretation of Microtox Solid Phase Test (SPT) results. There are five factors that can contribute to a decrease in the light output of the inoculum of bacterial cells introduced to the test tubes at time T_0 when it is measured at the end of the test:

(i) Natural loss of metabolic activity of cells during the exposure period;
(ii) Loss of cells to the filter material during the recovery process;
(iii) Light attenuation by dissolved color in the final extract;
(iv) Retention of cells by the solid phase matrix (sample) being tested; or
(v) Loss of metabolic activity due to toxic stress.

Due to the combined action of Factors (i), (ii), (iii) and (iv), an entirely uncontaminated sample could have an uncorrected EC_{50}<5%. Clearly, this uncorrected EC_{50} does not connote the same degree of toxicity as a liquid phase sample with an EC_{50}<5%. In order to properly interpret SPT results, it is necessary to separate the effects of Factor (v) (toxicity) from the effects of the other four factors. Factors (i) and (ii) are accounted for in the control tubes, while factor (iii) can be dealt with by color correction procedures.[12] Factor (iv) must be dealt with by creating a reference data file, which the data reduction software then uses to correct test sample results for light reduction due to cell retention.

The choice of the sample used to create the reference data file is thus vitally important to meaningful data interpretation. Ideally, the reference sample material should have cell retention characteristics similar to that of the test material, but should be free of toxicants. The choice of an appropriate reference material is up to the investigator. Some options are:

(i) A sample collected from a location near the site of the test material location, but

known or assumed to be relatively uncontaminated, may be used as the reference sample;

(ii) In large-scale investigations where the array of samples collected exhibits a wide range in degree of contamination, the least toxic sample (that with the highest EC_{50}) may be used as the reference sample;

(iii) In aquatic sediment investigations, a vertical core from an undisturbed deposit will contain strata from different time periods. A stratum visually similar in texture and color to the test sediment, but from the pre-industrial age, may be sectioned and used as an appropriate reference material;

(iv) Standard reference soils or sediments, if similar in physical and chemical make-up, may be appropriate reference materials in some cases;

(v) Synthetic soils or sediments, composed of varying amounts of sand, clay and organic matter, may be mixed to simulate test material characteristics and used as reference material; or

(vi) An investigator may archive useful reference sample data files and then select the most appropriate file for each sample to be tested.

When an SPT data file has been corrected by a reference sample data file, results may then be interpreted with more confidence. The corrected EC_{50} may be used as a raw number and values from various samples may be compared. As an alternative, the relative toxicity of a sample may be graphically derived. The higher the relative toxicity ratio, the more toxic the sample. Ratios may then be compared among samples or between study sites.

6.3 CONCLUSION

Power analysis is an important, yet often overlooked part of experimental design. This study has demonstrated that power analysis of ANOVA is affected by sample size, alpha, variance among the data, the number of groups and the smallest difference between group means that the experimenter wishes to detect. The Microtox SPT assay is a good tool to use in these studies because it has an inherently low variance and a large sample size can be obtained without large expenses or sample volumes. The variables that affect power analysis can be manipulated to design powerful studies, so that if the null hypothesis is not rejected, it is because there is actually no difference between the groups.

Extreme care must be taken in the interpretation of SPT assay data. Raw EC_{50} data can be used for comparisons but are meaningless as absolute numbers. Careful consideration must be given to the choice of a method for reference sample correction.

The Microtox Solid Phase Test has proved useful in several large-scale projects involving the assessment of contaminated bottom sediments. The simplicity and standardized nature of the protocol reduces variance and experimental error. The low cost and rapid assay technique allow for the screening of large numbers of samples. This is crucial in two types of investigations: studies on a large spatial scale, where many samples are needed for a meaningful coverage, and studies of remedial action feasibility, where tight sample grids are necessary for precision in two- or three-dimensional

mapping and cost analysis. To help define the operational characteristics (inter-laboratory variance, sensitivity, discriminatory power, response range) of the SPT, a ring test (round robin test) is currently underway in the United States.

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7 Use of Radiolabeled Chemicals in Agricultural and Environmental Studies: Transport and Degradation of Pesticides in Lower Sand Layers

Lajos Vollner and Dietmar Klotz

7.1 INTRODUCTION

Stable or radioactive isotopic tracers are used extensively and commonly in many fields of research. They are also widely used for studying trace contaminant problems. Suitable isotopic labeling of compounds allows their chemical and physical fate to be followed in the environment, in food and in plant or animal organisms.

The National Environment Research Centre (GSF) near Munich has been using such materials for >30 years in a significant number of experiments in different fields.

This chapter illustrates the cooperation between two institutes, namely Hydrology and Ecological Chemistry, in finding rates of leaching and identifying types of degradation products (metabolites) of various pesticides in lower sand layers.

In recent years drinking water has been found to contain an increasing number of pesticide residues (~50 different pesticides or their degradation products have been identified in groundwaters),[1] the current study is relevant for checking migration rates and types of contamination of groundwater after pesticide application.

Up to now, almost all of the published data in relation to pesticides and soil, describe the fate of these environmental contaminants in the upper soil layers, where low transportation, high adsorption and high microbial degradation occur. In contrast, only few data are available for groundwater layers down to 5 m deep, in filtration media such as sand or stony layers.

Our interest was in the fate of pesticides after passing the thin humus range (30–50 cm). This occurs after heavy rains and erosion or in soils with less humus and in presence of gaps which were created eg, by dryness, plant roots and earthworms (Figure 7.1).

For this purpose our institutes have developed laboratory testing systems that permits examinations close to natural conditions.

These include original sands and stones from relevant groundwater filtration areas, original water from the same areas, measurement of velocity of water migrations. Temperature, pH, exact analysis of sand quality and particle size were determined prior to the experiments.

For producing accurate data of leaching and adsorption, and for finding degradation products more easily, ^{14}C labeled pesticides were used. To ascertain column characteristics, tritium (^{3}H) labeled water was used.

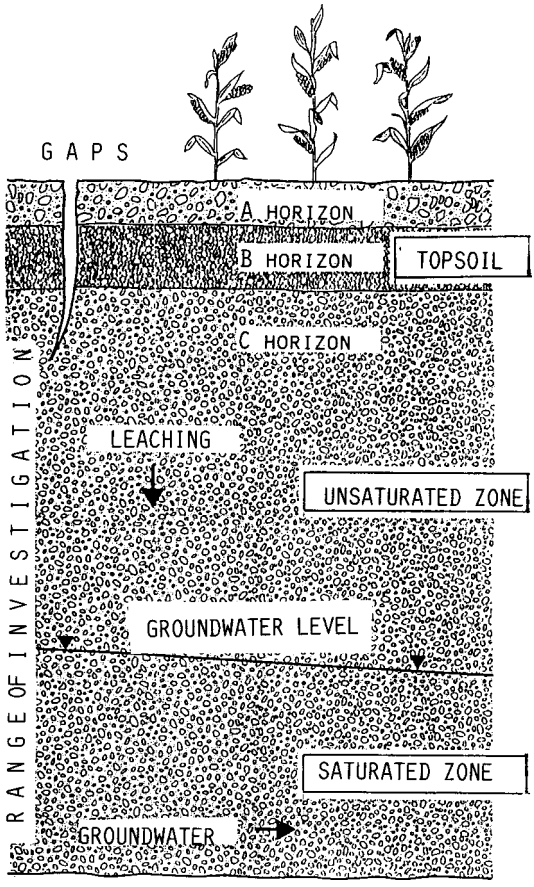

Figure 7.1 *Soil and groundwater layers*

7.2 MATERIALS AND METHODS

Special V2A alloy steel columns, 50 cm long with an internal diameter of 5 cm (ie, a cross section of 19.64 cm^2 and the volume of 981.74 cm^3) were used.[2]

Monitoring of conductivity and of pressure during the experiments was maintained by sensors, installed in holes of column walls, in different places. Constant flow rate was maintained by using peristaltic pumps. The columns were protected by effluent-level containers, in case difficulties with the pumps or leakage of water should occur (Figure 7.2).

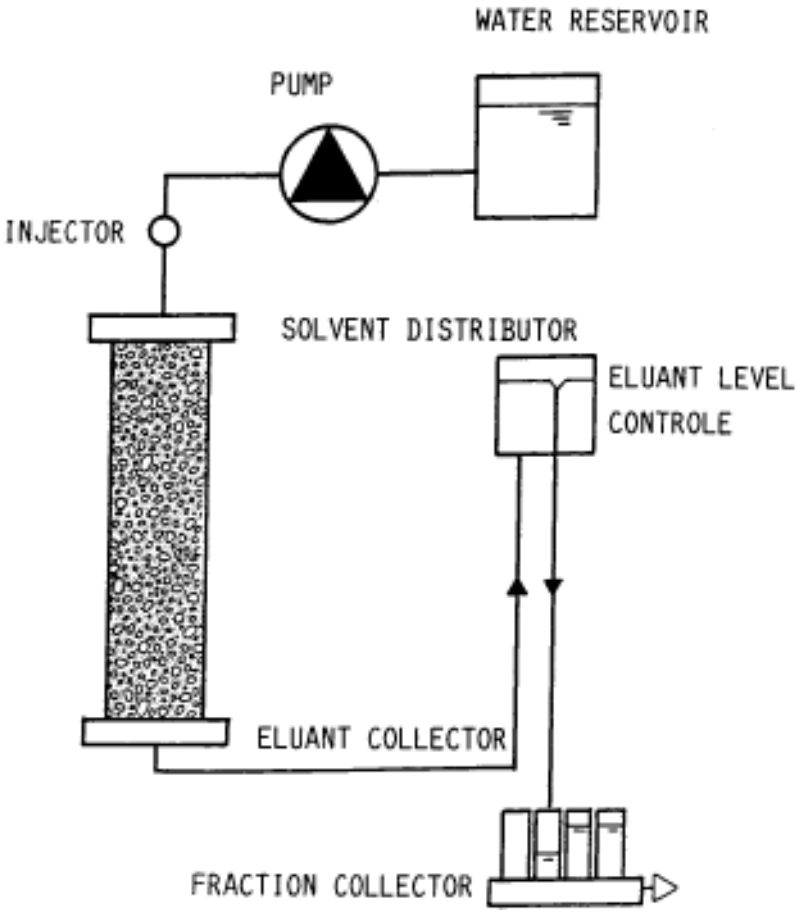

Figure 7.2 *Design of leaching experiment*

The injection of tracers and pesticides was undertaken by continuous flow, using a special injection system with a septum. For uniform application of the injected sample to the top surface of the column, a special solvent distributor was installed.[2]

Two different types of sand that are typical for groundwater filtration in the Munich area were used. These are the so-called Tertiärsand (tertiary sand, tert in this chapter), particle size 0.19–0.31 mm, collected from 5 m below the surface (northern Munich area), and the so-called Quartärkies (quartary gravel, quar in this chapter), particle size 0.67–4.70 mm, collected from 2 m below the surface (eastern Munich area). The mass of these filtration material, the density (dry), the total porosity and volume of the water were determined for every chemical. Table 7.1 gives an average value of these parameters.

Table 7.1 Deposition parameters of sediments in the columns (average of 11 determinations)

Column	mass (g)	density (g cm^{-3})	porosity (n)	water (cm^3)
tert	1600	1.63	0.37	370
quart	2170	2.22	0.18	190

n, the dry porosity is given by the relation of sample volume;

V_s to the total volume V_T, ($n = V_s V_T^{-1}$). V_s is the difference between total volume and sediment volume V_{Se} ($V_S = V_T - V_{Se}$).

Sediments were transferred to the columns by special techniques which had been developed for these experiments.[2] About 5 mm of a silica sand was placed in the columns prior to this procedure and afterwards, when the sediment layers were added (~70 g each) which avoided irregular application and irregular elution of the resolved pesticides.

Groundwater was taken from the same locations and analyzed for ions, prior to the leaching experiments. Table 7.2 shows the analytical data.

Table 7.2 Groundwater analysis prior to leaching experiments

Ions	tert water (mg l^{-1})	quart water (mg l^{-1})
Na^+	–	17.40
K^+	–	1.86
CA^{2+}	38.60	99.80
Mg^{2+}	21.60	25.10
Mn (total)	0.01	–
Fe (total)	0.01	–
NH_4^+	0.05	–
Al^{3+}	–	0.02
HCO_3^-	–	360.00
Cl^-	8.80	47.60
NO_3^-	0.20	31.60
SO_4^{2-}	9.95	20.00
PO_4^{3-}	–	0.05
F^-	0.45	–

The rate of elution was maintained close to the natural movement of water (2×10^{-4} cm s^{-1} and 6×10^{-4} cm s^{-1}); total elution volume was 2.8–3.2 1 in tert columns and 0.8–1.2 1 in quart columns.

Radioactivity in the eluate was analyzed by liquid scintillation counting (LSC), the quality of the substance mixtures (parent compound and degradation products) by thin layer (TLC), gas liquid (GC) and high pressure liquid (HPLC) chromatography, after extraction from the water and from the sand.

The following 11 pesticides have been investigated. **Carbamate insecticide:** carbofuran; **Chlorinated hydrocarbons:** lindane, DDT, DDE; **Phosphoric acid esters:** chlorpyriphos, diazinon, malathion, parathion,; **Phenylurea herbicides:** diuron, monolinuron; **Triazine herbicide:** terbuthylazine.

Stock solutions of 50 μCi and ~10 mg total of each pesticide were used. Aliquots, dissolved in water were applied (see section 7.4).

Eluates were collected every hour and 1 ml aliquot was taken for counting. For qualitative investigations, residues were extracted by solid phase extraction (SPE), using RP-C18 columns (eg, Bond Elut C18).

For investigating the adsorption profile, sand was removed from the columns, layer by layer: every 5 cm. After homogenization of these fractions, aliquots (tert 10 g, quart 15 g) were transferred into liquid scintillation (LS) vials. After adding LS fluids, direct measurement of radioactivity was undertaken. This procedure was verified by sample oxidation of sediments. Since quenching rates were the same for every sample, data could be used without further corrections for the preparation of adsorption curves. The quantity of adsorbed material was determined by the difference between applied and eluted amounts.

For qualitative analytical work, the removed sand layers were extracted first with n-hexane and acetone mixtures, followed by extraction with methanol. After evaporation of the solvents, TLC analysis was carried out. Since the aim of this work was to find out if transportation through the sand layers leaves pesticides either unchanged or degraded, no complex GC- and HPLC-MS investigations were carried out.

7.3 HYDRAULIC PARAMETERS

In general, mobility of pesticides in soils and sediments is related to adsorption and mass flux of the dissolved fractions. Adsorption influences the mass flux, which consists of diffusion, convection and dispersion.[3] Diffusion is a physical process by which molecules move from sites of higher concentration to those of lower concentration. Convection is the passive movement of solutes with the water movement. Dispersion is the distribution of solutes in the moving pure water, which results from different flow velocities of individual water volumes.

To identify all these important parameters and to standardize the columns, tritiated water (tracer) was used prior to each leaching experiment. Table 7.3 gives an example for two of these investigations. A triplicate experiment (eg, with terbuthylazine showed a good correlation of data (Figure 7.3), which proves the reproducibility of the described experiments.

Table 7.3 Hydraulic parameters in the columns

	Column	Q mlh^{-1}	V_f cms^{-1}	V_d cms^{-1}	n_{eff}
DDT	tert	14.20	2.01×10^{-4}	5.6×10^{-4}	0.401
	quart	35.28	4.99×10^{-4}	4.5×10^{-3}	0.110
Parathion	tert	15.27	2.16×10^{-4}	5.62×10^{-4}	0.384
	quart	44.02	6.23×10^{-4}	4.19×10^{-3}	0.149

Q flow rate

V_f velocity of filtration, relation Q to total cross section (CS) of the column, $V_f = QCS^{-1}$

V_d distance velocity of tritium; relation of column length (l), to the retention time (t), of tritium) $V_d = lt^{-1}$

n_{eff} effective porosity (see Table 7.1).

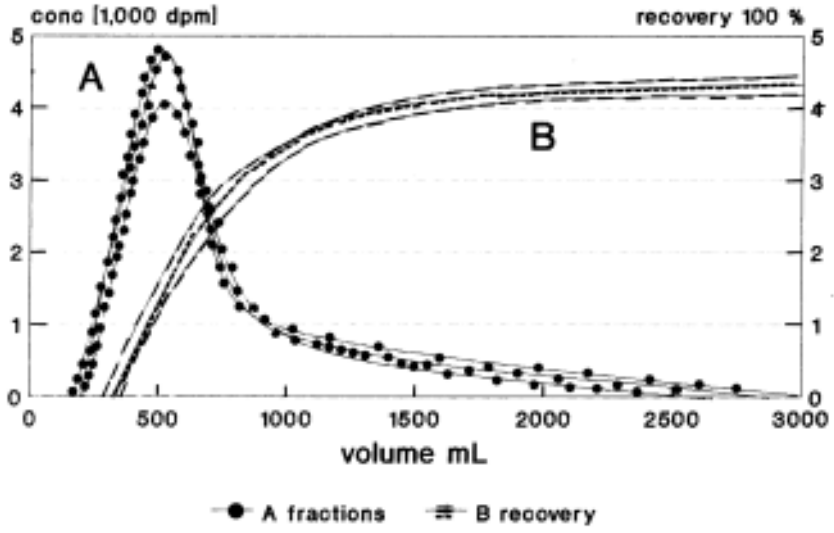

Figure 7.3 Leaching of terbuthylazin

7.4 RESULTS

7.4.1 Leaching results

Table 7.4 *Shows the applied radioactivity, the eluted radioactivity (in dpm), and the recovery of the elution (in %)*

Pesticide	Column	Applied (x 10^6 dpm)	Eluted	R
DDE	t	4.89	0.29	6.0
	q		0.31	6.6
DDT	t	19.60	0.88	4.5
	q		0.56	2.9
Lindane	t	8.90	4.20	47.2
	q		2.78	31.3
Chlorpyriphos	t	16.31	8.63	52.9
	q		7.71	47.3
Diazinon	t	5.50	5.22	95.0
	q	3.79	69.1	
Malathion	t	19.44	13.84	71.2
	q		15.92	81.9
parathion	t	12.50	9.00	72.6
	q		10.87	87.1
Carbofuran	t	16.32	13.18	80.8
	q		13.92	85.3
Diuron	t	25.56	21.57	84.4
	q		21.39	83.7
Monolinuron	t	20.12	14.16	70.4
	q		14.06	69.9
Terbuthylazine	t	8.16	6.96	85.0
	q		7.26	89.0

t = tert, q = quart; 2.2 x 10^6 dpm (1 µCi) corresponds to 0.2 mg.

The preparation of the elution curves (curve A, eg, in Figure 7.4) was performed by relating the elution volume (V) in ml and the concentration of pesticides in the fractions (cone) in dpm. The sum of radioactivity of all fractions gives the total amount eluted (curve B), which is given the name 'recovery of the leaching (R)' (Table 7.4).

Table 7.5 Maxima of elution curves (see Figures 7.3–7.4)

Pesticide	Column	V/V_{eff}	Conc
DDE	t	0.9	2.4×10^4
	q	1.0	1.9×10^4
DDT	t	1.0	1.2×10^4
	q	1.2	4.8×10^4
Lindane	t	1.2	2.0×10^3
	q	6.0	2.3×10^4
Chlorpyriphos	t	6.0	3.2×10^4
	q	12.0	3.1×10^4
Diazinon	t	2.0	2.5×10^3
	q	2.0	1.6×10^3
Malathion	t	1.2	5.8×10^3
	q	1.0	3.2×10^3
Parathion	t	3.0	8.2×10^4
	q	5.0	4.8×10^4
Carbofuran	t	1.1	6.6×10^3
	q	1.0	4.0×10^3
Diuron	t	1.3	7.8×10^3
	q	1.0	2.3×10^3
Monolinuron	t	1.2	3.4×10^3
	q	1.5	2.4×10^3
Terbuthylazine	t	1.2	4.2×10^3
	q	1.3	5.4×10^3

t = tert, q = quart, V/V_{eff} and conc, see in text.

Figures 7.4 and 7.5 show in comparison two significantly different elution curves. In cases of DDT (Figure 7.4) three to four peaks appear (A), indicating different degradation products, but the total elution (B) is very low (only about 5%, see also Table 7.4). In the case of parathion (Figure 7.5) mainly one broad peak appears, but the total elution is very high (~70%).

Although the recovery data of the other individual pesticides are quite similar in the eluants of the two different sand types, the elution curves differ in most cases. This is an indication for different degradation rates and different degradation procedures of the same pesticide, which is clearly to be observed in the different values of the concentration maxima (Table 7.5).

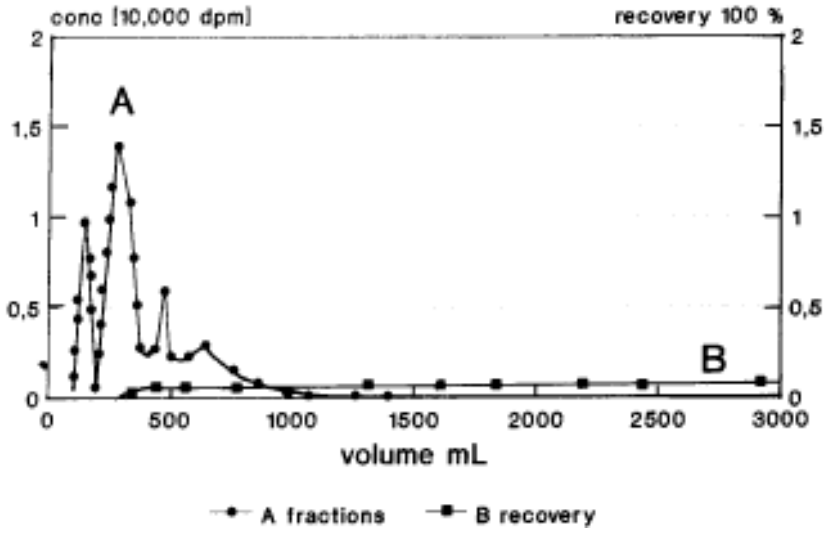

Figure 7.4 *Leaching of DDT*

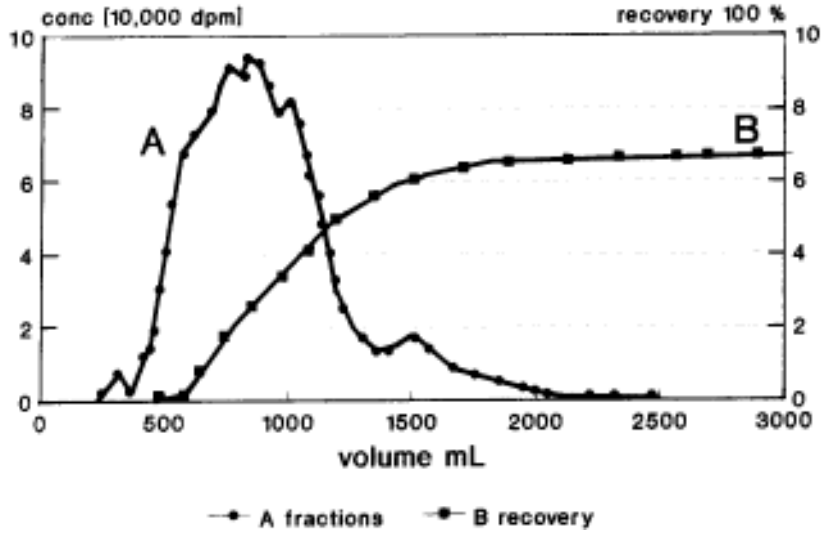

Figure 7.5 *Leaching of parathion*

7.4.2 Adsorption results

The adsorption profile of the different substances in the columns were examined by stepwise removing the sediments as described in section 7.2. Table 7.6 shows these data. (The data in this table includes also extractable and non-extractable values, which were obtained by summarizing all data extracted from the individual layers.)

Table 7.6 *Adsorption of pesticides on sands (in %) (difference of applied and eluted amounts)*

	Column	Adsorbed	Extractable	Non-extractable
DDE	t	94.0	15.0	79.0
	q	93.4	12.1	81.3
DDT	t	95.0	15.2	79.8
	q	97.1	13.5	83.6
Lindane	t	52.8	5.8	47.0
	q	68.7	5.5	63.2
Chlorpyriphos	t	47.1	15.9	31.2
	q	53.7	27.9	25.8
Diazinon	t	5.0	1.4	3.6
	q	3.5	1.7	1.8
Malathion	t	28.8	18.1	10.7
	q	18.1	3.9	14.2
Carbofuran	t	19.2	10.1	9.1
	q	14.7	11.4	3.3
Diuron	t	15.6	4.0	11.6
	q	16.3	1.8	14.5
Monolinuron	t	29.6	3.5	26.1
	q	30.1	2.4	27.7

Figure 7.6 gives an example for the distribution of three different types of pesticides, and Table 7.7 summarizes all the results.

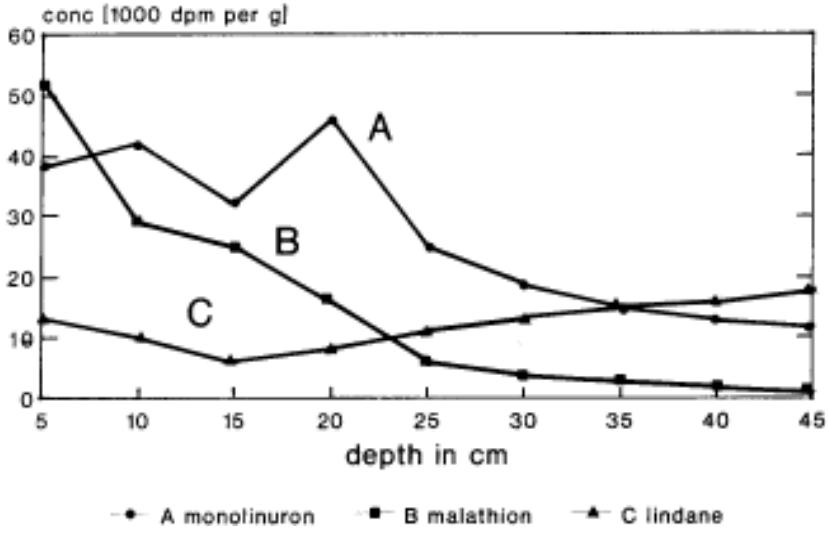

Figure 7.6 *Distribution on sand*

7.4.3 Interpretation of degradation products

The extracted fractions were investigated by TLC to distinguish between parent compounds (applied) and their degradation products. Figure 7.7 shows the investigation of diazinon extracts from eluted waters. The two sand columns show significantly different degradation rates (peak no. 3 is the parent compound).

Table 7.8 summarizes the results of these qualitative investigations for all the pesticides, indicating the rate of unchanged substances and the sum of conversion products (which are usually more polar than the original substance). In almost all cases the non-extractable part was greater than the extractable part. As is well known from soil experiments, these products might be chemically bound to the sediment particles and cannot be easily identified.

7.4.3.1 DDE

As DDT is still widely used worldwide the fate of DDE was investigated. DDE is the main degradation product of DDT with similar properties and frequently appears in residue analysis of DDT.

Table 7.7 *Adsorption of pesticides and their degradation products in sediment layers, in relative %*

		Depth in cm									
		5	**10**	**15**	**20**	**25**	**30**	**35**	**40**	**45**	**50**
DDE	t	58	20	12	3	3	0	0	1	1	1
	q	75	10	7	4	1	1	0	0	0	0
DDT	t	64	12	7	2	3	3	2	2	2	2
	q	57	13	9	4	3	3	4	4	2	1
Lindane	t	63	10	5	5	3	3	2	2	3	3
	q	12	9	13	13	10	10	6	6	10	10
Chlorpyriphos	t	11	9	7	7	11	11	12	12	10	10
	q	11	13	13	15	11	11	5	5	8	8
Diazinon	t	36	13	13	7	6	6	3	3	7	6
	q	34	12	12	10	8	8	4	4	4	4
Malathion	t	37	20	18	11	4	4	2	2	1	1
	q	14	12	10	8	9	9	10	10	9	10
Carbofuran	t	28	21	14	10	6	6	3	3	4	4
	q	32	29	12	7	5	5	3	2	2	2
Diuron	t	63	9	4	5	2	2	3	3	4	4
	q	11	11	11	11	11	11	8	8	9	9
Monolinuron	t	75	16	4	1	0	0	0	1	1	1
	q	62	12	5	3	4	4	3	3	2	2

The minor part of radioactivity, which was eluted with water, is a mixture of further degradation products. Two polar groups of substances can be found. In the quart columns the rate of the more polar material is higher.

In the 0–10 cm layer DDE was decomposed to 60% and in the 15 cm layer no original material could be found.

Degradation products originate in many different ways, which can occur stepwise by eg, dechlorination and hydroxylation reactions, or simultaneously, by chemical or microbial actions. These materials have been studied thoroughly worldwide and are still the subject of many investigations.[4,5]

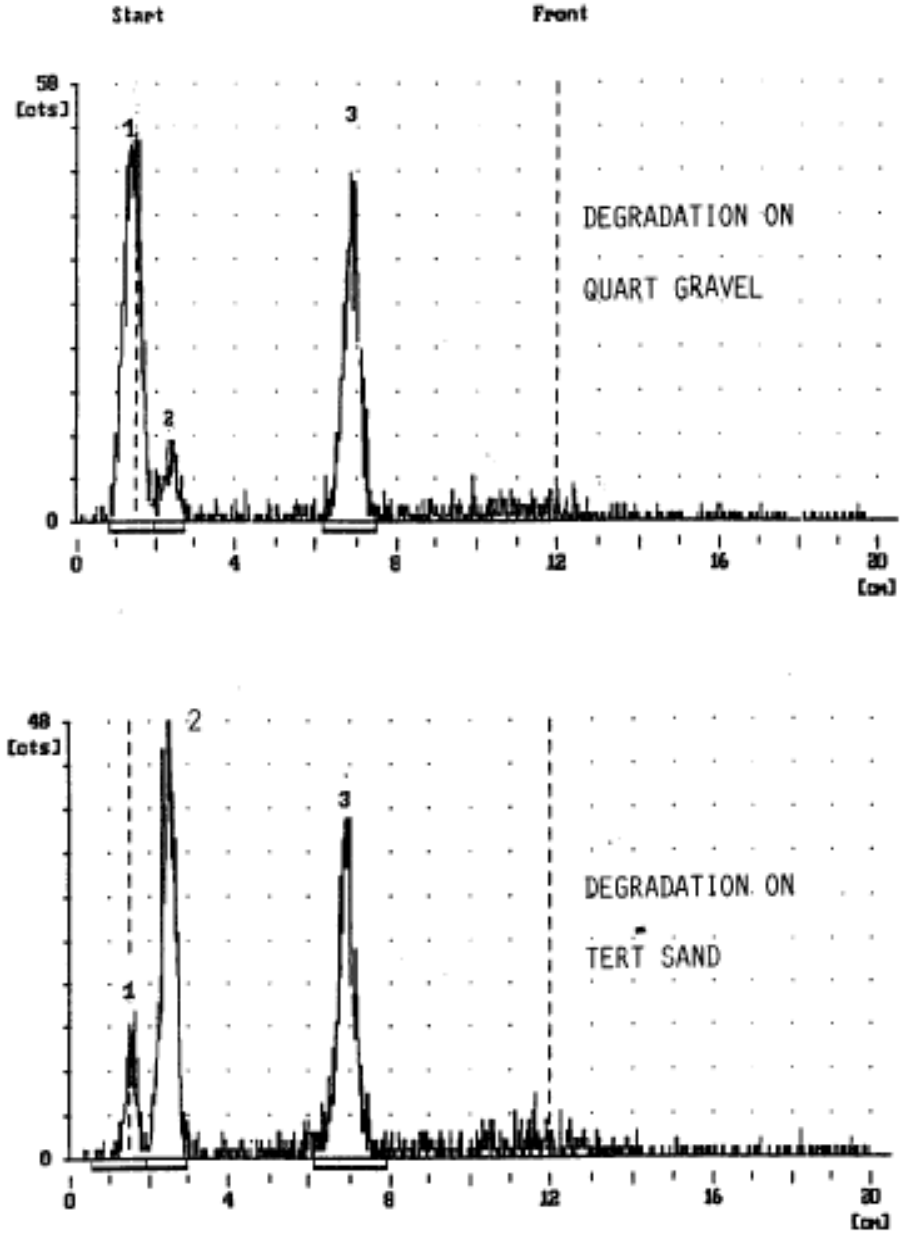

***Figure* 7.7** *TLC investigation of leachate extracts of diazinon*

Table 7.8 *TLC analysis of extracts. Rates of parent compound and conversion products in relative % (the extractable part and the eluted part are assumed to be each 100%)*

	Column	Adsorbed	Eluted
		Parent/conversion	
DDE	t	7/93	0/100
	q	6/94	0/100
DDT	t	11/89	0/100
	q	10/90	0/100
Lindane	t	47/53	0/100
	q	31/69	82/18
Chlorpyriphos	t	36/67	45/55
	q	58/42	0/100
Diazinon	t	20/80	43/57
	q	12/88	39/61
Malathion	t	100/0	0/100
	q	0/100	0/100
Carbofuran	t	40/60	90/10
	q	19/81	30/70
Diuron	t	95/ 5	90/10
	q	98/ 2	100/0
Monolinuron	t	0/100	88/12
	q	15/85	83/17

7.4.3.2 DDT

Similarly to DDE, the eluted part of radioactivity (3–5%) is a mixture of polar degradation products. In the columns, 98% of the original material was already degraded in the 5 cm layer. Beside DDE two other (non-identified) more polar substances at the rate of 1.3% and 6% were found. In deeper layers only DDE and further degradation products could be identified. Finally, DDE was also degraded up to 100% in 15–20 cm depth. For further possible degradation refer to section 7.4.3.1.

Based on these observations it can be concluded that if DDT passed through the humus layer, it would be adsorbed to a significantly greater part on sand layers as a mixture of decomposition products. Only a very small part, which is also a mixture of degradation products would be leached into the groundwater.

7.4.3.3 Lindane

In contrast to DDT the greater part of lindane (tert 47%, quart 31%) was leached. Although the leaching rates are comparable, the pattern of the quality analysis differs significantly. In the case of tert column eluant, lindane was quantitatively degraded, while in the case of quart eluant, 82% of lindane was found unchanged.

The adsorbed material in both cases was a mixture of a lindane and degradation products. Lindane was distributed throughout the whole column, which had been observed in previous investigations.[6] The degradation has created even less polar substances than lindane, which is unusual. The polar part of the degraded compound was higher in the case of the tert column, which explains the quality and the higher leaching rate in the case of tert eluant.

These types of degradation products are known from soil experiments.[7] Dechlorination and dehydrogenization occur at the initial phase of decomposition. Introduction of double bounds results from these reactions. Further oxidation leads to chlorophenols. All these substances are more polar than lindane and are also toxic.

In conclusion, lindane might pass the 50 cm sand layers at higher rates unchanged. It might lead to groundwater contamination together with its degradation products, if sandy soils are concerned, or if thin humus layers should be passed or be by-passed.

7.4.3.4 Chlorpyriphos

High rates (47–53%) of this substance were leached. Forty percent of the unchanged material was present in the eluant of tert sand, but only degradation products could be detected in the eluant of quart gravel.

Similar to these data, 36% chlorpyriphos remained, ie, distributed along the column, on the tert sand, the remainder were polar degradation products. Unchanged material (55%) could be found up to a depth of 10 cm on the quart columns, but below this level only degradation products were detected.

The main degradation products of chlorpyriphos are 3, 5, 6-trichloropyridinol and chlorpyriphos oxon,[8] additionally, dechlorination can occur.

As with lindane, chlorpyriphos and its degradation products move through sand layers at high rates.

7.4.3.5 Diazinon

High elution rates could be observed (95% in tert sand and 69% in quart gravels). Eluants of both sand types contain ~40% of the unchanged molecule, but the degradation patterns are significantly different (see also Figure 7.7).

Data for adsorption patterns on sand are similar. The original molecule is distributed along the whole column.

The main degradation products of diazinon are diazoxon, hydroxydiazinon, pyrimidinol and hydroxy-pyrimidinol.[9] Diazinon moves at a high rate through sand layers and could create ground water contamination if site properties are appropriate.

7.4.3.6 Malathion

Eluants contain about seven different degradation products. Only traces of the parent compound could be detected.

The adsorption and degradation on sand is different. The tert column contains almost unchanged malathion along the whole length. Traces of highly polar degradation products could be found in the layer 15–20 cm, and higher polar substances in the layer 20–30 cm. In the quart column malathion is degraded quantitatively. The main degradation product (~63%) is highly polar and remains in the 10 cm layer of the column, together with the second and less polar substance.

The main polar degradation product is the mono- and di-malathion carboxylic acid. A less polar degradation product is diethyl maleate.[9] Carboxylic acids were also found as highly polar degradation products.

According to these observations the greater part of the degraded molecule, with the polar carboxylic acid groups, will be adsorbed on the higher layers. Only the small degradation products, which are usually biodegradable, will move to the groundwater. Thus, although high rates of radioactivity were leached, groundwater contamination by malathion is unlikely.

7.4.3.7 Carbofuran

The leaching rate of carbofuran is>80%. The tert eluant contains 90% unchanged molecule, while the quart eluant contains only 30%.

The main part of adsorbed substance was found in the layer 0–20 cm. In the case of tert sand, 40% of the carbofuran remains unchanged. A high polar part of 50% and two less polar compounds with ~5% each could be detected. Only 19% unchanged carbofuran was found on the quart gravel. The high polar part is only 21% and the lesser polar part ~50%. A significantly different degradation behavior was also observed.

The main degradation products of carbofuran in aquatic systems are hydroxy and phenol compounds, eg, 3-hydroxycarbofuran, carbofuran phenol, etc.[10] All these compounds contain a major substructure of the original molecule. These high leaching rates of all of these substances could lead to water contamination by carbofuran.

7.4.3.8 Diuron

About 84% of mainly unchanged material was leached in both types of columns. Small amounts (<10%) of polar compounds were detected.

The adsorbed substance is distributed along the whole column, with only small proportions of degradation products. The data are similar in both cases.

The main degradation product of diuron is 3, 4-dichloroaniline, which could also represent the polar component of the mixtures.

According to the high leaching rate of the unchanged molecule, groundwater contamination could easily occur, especially if sand layers are involved.

7.4.3.9 Monolinuron

As with diuron, ~70% of the total radioactivity was eluted. The main part of the eluant (88 tert, 83 quart) is the unchanged substance.

On tert sand, monolinuron was degraded quantitatively, while on quart gravel 15% of unchanged substance could be detected.

Similarly to diuron, chloroanilines and other polar compounds could be present, but the main part of the parent compound remains unchanged.

As mentioned previously, groundwater contamination is likely if the described conditions and soil properties occur.

7.5 CONCLUSIONS

By simulating conditions of groundwater filtration close to natural situations, the following can be concluded:

(i) Behavior of chemicals concerning elution and degradation is different even if they belong to the same class of bioactive compounds;
(ii) The behavior of the same substance differs in different types of sand;
(iii) With the exception of DDT and its corresponding degradation product, DDE, all pesticides investigated move at high rates through the 50 cm thick sand layer;
(iv) Degradation and types of breakdown products are dependant on the stability of the original compound, but in general, high degradation rates on the two types of sand could be observed; and
(v) Because of the low transportation velocity, and following long retention times of pesticides within the sand media, microbial degradation (metabolisms) can also occur.[11]

The significant variation of these findings does not permit a prediction of trends or to forecast groundwater contaminations for other pesticides. However, the results provide a basis for consideration concerning the improved application of the pesticides investigated. Furthermore, these data emphasize the need for investigation of pesticide behavior in any single case, especially as far as groundwater quality is concerned.

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SECTION 3: ESTS SINGPLANTS, ALGAE, ETC.

8 Use of Aquatic Plants in Ecotoxicology Monitoring of Organic Pollutants: Bioconcentration and Biochemical Responses

Sashwati Roy and Osmo Hänninen

8.1 INTRODUCTION

Every year thousands of tonnes of chemicals and their by-products are generated through industrial or agricultural activities. These chemicals are eventually deposited directly or indirectly (eg, via hydrological or atmospheric processes) to the aquatic environment. Organic chemicals constitute a major part of these contaminants.[1] Polyaromatic hydrocarbons (PAHs), chlorobenzenes, polychlorinated biphenyls, polychlorinated dibenzofurans and *p*-dioxins and chlorinated phenols are some of the hazardous and potential carcinogenic components of such mixture.[1] Increasing concern regarding environmental problems from such hazardous contaminants has led to the development of sound approaches for assessing the health and environmental effects that result from exposure to toxic chemicals.[2]

The analysis of water samples from polluted aquatic reservoirs often furnishes inconclusive information. For example, at a given time of sampling the concentration of the target pollutant in water can have transported a few kilometers downstream from the point of discharge rather than just below it. This is a major drawback in ascertaining the precise location of the sources of pollutants. Most of the hydrophobic organic chemicals are readily adsorbed/absorbed onto sediments. Such problems may sometimes be resolved by analyzing the chemical concentration in sediments.[3] However, such an approach does not account for the evaluation of bioaccumulation and subsequent physiological or biochemical consequences of pollutants in living organisms. Thus, the use of organisms in monitoring programs for such environmental contaminants is necessary to estimate:

(i) The exposure and bioavailability of the compounds;
(ii) Fate of xenobiotics in the exposed organism; and
(iii) The exposure levels of the pollutant that can incite significant physiological and biochemical alterations in organisms.

The need for sensitive and reliable methods to assess the impact of chemical pollution on the aquatic environment has generated considerable interest in biochemical and physiological indices of contaminant exposure and effects on aquatic food chain.[4–6]

Plants have a crucial position in the food chain during the biosynthesis of organic compounds and supplying oxygen. Aquatic macrophytes have a great potential for use in the biomonitoring of water borne contaminants because of their potential to

absorb organic and inorganic substances.[5,7] Their stationary nature makes the sampling process very simple and allows the precise location of the pollutant source. Moreover, plants require less maintenance and in some cases have longer lives than their animal counterparts. Aquatic plants are also used in some water treatment systems to remove organic and inorganic substances from the water.[8] The rhizosphere of such plants support large microbial populations that conduct desirable cleaning of water by modifying the nutrients, metallic ions and other organic pollutants.

The initial effects of a toxic compound on these organisms are normally indicated by changes at the biochemical level of the cell function before visible morphological alterations appear.[5,9] Most xenobiotics pass through biotransformation reactions which are catalyzed by a number of intracellular enzymes. Exposure to xenobiotics often causes changes in the function and expression of such enzymes.[5] Generation of free radicals during the course of xenobiotic metabolism and its enhancement via redox cycling of reactive intermediates is well documented.[10] The antioxidant defence system in plants is crucial in scavenging such reactive intermediates. Pollution exposure dependent changes (ie, increase or decrease) in activity and/or concentration of enzymes of xenobiotic metabolism and antioxidant systems in plants and relative extent of such effects may be implicated as suitable tools to evaluate the impact of the chemicals on an aquatic ecosystem's health. Such monitoring of contaminants will also enable an early detection of adaptive responses in exposed organisms before structural changes occur, diseases appear or population shifts take place.[5]

This chapter aims at reviewing the prospects of developing the bioconcentration factor (BCF) and the responses of the enzymes involved in xenobiotic metabolism and antioxidant systems in aquatic plants exposed to pollutants as implements for ecotoxicology monitoring of organic pollutants.

8.2 AQUATIC PLANTS IN ENVIRONMENTAL TOXICOLOGY EVALUATIONS AND BIOMONITORING

The uptake of environmental chemicals into plants is the first step for accumulation of such pollutants in the aquatic food chain.[11] Plants are sedentary organisms. Their need to obtain the full spectrum of nutrients from environment has led to a maximization of their surface area to absorb the raw material.[12] Thus, the anatomical and physiological features that enable plants to accumulate nutrients, water and carbon dioxide also make them vulnerable to contamination by anthropogenic chemicals from air, water, soil or sediment.[13] The study of bioconcentration of environmental chemicals and their metabolites and associated biochemical responses in aquatic vegetation is necessary:

(i) To predict chemical biomagnification through the food chain;
(ii) Analyze phytotoxicity;
(iii) Understand the cycling of organic contaminants by plants in the aquatic environment; and
(iv) Other potential impacts of such chemical release into the aquatic environment.[5–7,14]

Such evaluations will also allow the use of plants as *in situ* biomonitors of aquatic pollution.[7] Aquatic plants have been used widely to monitor aquatic pollution.[15–17] The current literature has made possible the understanding, description and estimation of the principal fate of chemicals in water, soil and atmosphere; whereas despite its importance, the role of plants in the behavior of chemicals is much less understood.[13]

8.2.1 Bioavailability and uptake

The factors that determine primarily the availability of organic chemicals to organisms are rather complex.[2] The form of a given chemical may be modified greatly by physical, chemical and/or biological events eg, partition of the chemical between dissolved and particulate forms. The concentration of many organic pollutants (hydrophobic) with high octanol-water partition coefficient (K_{ow}) values exceeds in sediments by several orders of magnitudes those in the water column. Likewise, modification of the chemical form of contaminants by biological (eg, biotransformation) or physical means (eg, photooxidation), may alter greatly their availability through changes in their solubility or reactivity.[18] Dissolved organic material in natural waters is a major factor affecting bioavailability of organic pollutants. Bioavailability of such organic pollutants have been reported to decrease by increasing dissolved organic matter concentration in water.[19] Hence, the physico-chemical characteristics of the particulate-water interface is crucial in determining the bioavailability and resulting toxicity of a water-borne chemical. Because the biological membranes are composed largely of lipids and most of the organic pollutants are lipophilic, it has been predicted that higher the lipid content of the biological membrane, the greater is the rate of uptake.[20]

Aquatic plants either in submerged or floating conditions, are exposed continuously to water pollutants. The most common mode of exposure is the direct exposure ie, continuous absorption through the exposed surface. The physicochemical properties of the contaminant with anatomical features and physiological process of the exposed organism are crucial determinants of the uptake process.[21] In plants, these chemicals are absorbed by the exposed parts (eg, roots, shoot and leaves) via passive diffusion[14] and then conducted through apoplast (xylem) or symplast (phloem).

8.2.2 Bioconcentration

Bioconcentration of chemicals in aquatic organisms is one of the key parameters that has to be considered when assessing the respective ecotoxicological potentials.[21] Determination of **bioconcentration** is the assessment of the extent to which any environmental chemical will achieve concentrations in biotic organisms.[22] The bioconcentration factor (BCF) has been defined as a proportionality constant relating the concentration of chemical in water to its concentration in aquatic

organism at steady state equilibrium.[21,23] Measured or predicted BCF is a crucial component of environmental risk assessment. In recent years, a number of models have been proposed to predict the BCF of various organic pollutants in terrestrial[13,24,25] and aquatic plants.[7,14]

Rapid uptake and bioconcentration of a number of nonreactive hydrophobic compounds (eg, chlorobenzenes, chlorinated biphenyls etc.) has been observed in aquatic plants.[7,14] Studies have also been carried out to predict chemical accumulation in the shoots of aquatic plants and to determine the chemical exchange between the shoots and water.[14]

A study of the uptake/elimination kinetics and bioconcentration factor (BCF) of pentachlorophenol (PCP), benz(a)anthracene (BaA) and benzo(a)pyrene (BaP) in the aquatic plants *Eichhornia crassipes* and *Fontinalis antipyretica* was undertaken.[26,27] The uptake and elimination of the chemicals by *E.crassipes* and *F. antipyretica* was initially rapid followed by a slower phase. The rapid uptake of the chemicals was suggested to be due to a relatively high $K_{o/w}$ of PCP ($K_{o/w}$=5.01), BaA ($K_{o/w}$=5.5) and BaP ($K_{o/w}$=6.3).[26]

Biphasial trend for the elimination of organic environmental compounds (eg, PCP, BaP, chlorobenzenes) from aquatic plants has been observed[7,26,27] and explained using a multicompartment model. In such a model the plant was divided into separate compartments (eg, leaf, stem and roots[13,14]). Such compartmentation accounts for the differences in chemical and physical characteristics (eg, surface areas available for exchange, lipid content and potential sorption of chemical) of tissues that may influence the diffusive exchange of the compounds between plant and water.[14]

8.2.3 Major enzymes of plant xenobiotic metabolism

Xenobiotic (eg, herbicides, pesticides, environmental chemicals etc.) metabolism and the enzymes participating in such metabolic processes have been studied extensively in terrestrial[28–31] and aquatic[26,27,32] plants. Major enzymes involved in basic processes of xenobiotic metabolism in plants ie, oxidation, hydrolysis, reduction and conjugation are indicated in Figure 8.1).

8.2.3.1 *Peroxidase*

Peroxidases (POX), ubiquitous in the plant kingdom, are the principal enzymes for oxidizing xenobiotics in plants with a wide spectrum of substrate specificity.[29,33] Peroxidases are distributed throughout the plant cell and show high affinities to exogenous substrates with K_m values between 10^{-6}–10^{-4} mol 1^{-1}.[34] They catalyze two general types of reactions, the classical peroxidative reaction that requires hydrogen peroxide and the oxidative reaction that utilizes molecular oxygen.[35]

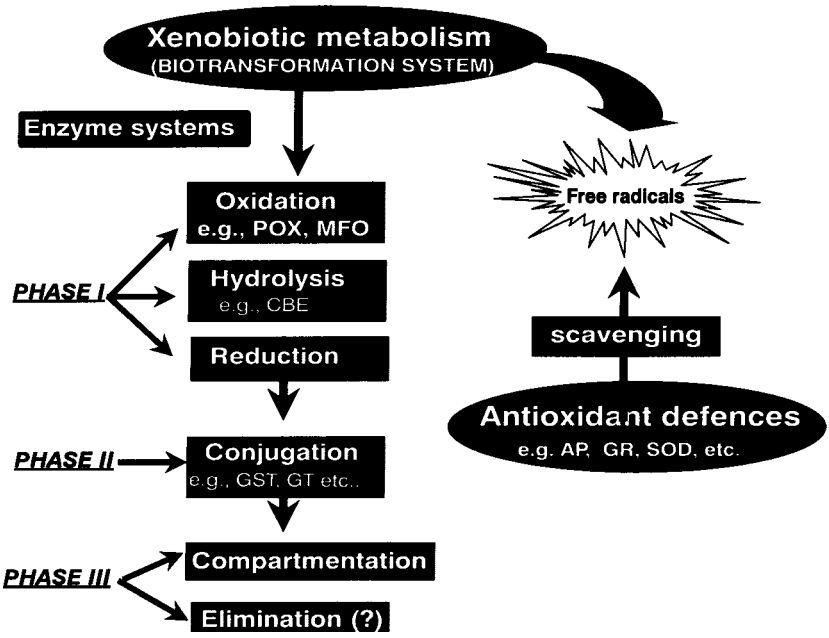

Figure 8.1 *A schematic representation of the major steps of xenobiotic metabolism and antioxidant defenses in plants. Phase I reactions may transform organic pollutants via oxidation, hydrolysis and/or reduction reactions to reactive intermediate sometimes with carcinogenic potentials. Conjugation products are mostly water soluble and compartmentation of such products usually take place in higher plants. Elimination of hydroxylated and conjugated metabolites (hydrophilic) of pollutants may be another major mechanism of detoxification in aquatic plants. Some of the major enzymes of oxidation (eg, peroxidase, POX; mixed function oxidases; MFO), hydroxytation (eg, carboxylesterase, CBE) conjugation (eg, glutathione S transferase, GST; glucosyltransferase, GT) and antioxidant defenses (eg, super oxide dismutase, SOD; ascorbate peroxidase, AP; glutathione reductase, GR) have been indicated in the scheme.*

In addition to oxidative condensations, functions such as decarboxylations, sulfur oxidations, N-demethylation, ring hydroxylations, carbon-halogen bond cleavage and oxidation of aromatic methyl group have been attributed to peroxidases (Table 8.1).

Table 8.1 *Major enzymes in plants catalyzing the biotransformation of xenobiotics*[36]

Enzyme	Type of reactions attributed
Peroxidase	Oxidative condensation, decarboxylation, sulphur oxidation, N-demethylations, ring hydroxylations, carbon-halogen bond cleavage, aromatic methyl group oxidation etc.
Mixed function oxidases	*N*-dealkylation, *O*-dealkylation, alkyl hydroxylation, aromatic hydroxylation, epoxidation, desulfuration, hydrolysis, nitrogen oxidation etc.
Aryl acylamidases	Hydrolysis.
Glutathione S-transferase	Conjugation of xenobiotics with an electrophilic center to glutathione
Glucosyltransferases	Xenobiotic conjugation with glucose

The activity of peroxidases have been used as an indicator of pollution stress in the fine roots of *Pinus sylvestris* and in the macerated leaf tissue of forest trees.[37,38] The extracellular activity of this enzyme has also been suggested to be a reliable indicator of environmental stress.[39]

In aquatic plants, a significant increase in the POX activity in the roots and leaves of *E. crassipes* have been observed when plants were exposed to either unbleached pulp mill effluents or PCP.[26,40] Increased POX activity has been also observed in aquatic mosses that were transplanted near a city harbor area for about one month (unpublished data). The activity of POX has been used as an implement for aquatic macrophyte bioassay for sediment toxicity evaluation[41] and as markers of aquatic pollution.[6]

In an attempt to investigate the relation between peroxidase activity of the plants studied and their degree of tolerance to water pollution chiefly caused by unbleached pulp mill effluents,[32] the activities of this enzyme were compared with the respective pollution tolerance ranks as reported in a previous study.[42] The pollution tolerance abilities were observed to be consistently related to high peroxidase activities in all the floating and submerged aquatic species studied.[32]

8.2.3.2 *Mixed function oxidases*

The hydroxylation capabilities differ significantly among various plant species especially due to the expression of cytochrome P-450 related mixed function oxidases (MFO), that are mainly responsible for these reactions.[28] MFO, isolated from plants typically display very low levels of activity (specific activity in the range of 1–10 nMol product mg protein^{-1} h^{-1}). The low levels of activity may be due to very low cytochrome P-450 levels (0.007–0.025 nMol P-450 protein mg microsomal protein^{-1}) in plants.[28,43]

Induction of cytochrome P-450 in higher plants has been reported following exposure to phenobarbital, ethanol and herbicides.[44] The number of xenobiotics shown to be metabolized by *in vitro* plant MFO is limited especially because of extremely low levels of the enzyme activity that is further complicated by the presence of endogenous inhibitors and the instability of many of these systems.[28]

8.2.3.3 *Aryl acylamidase*

It is suggested that this hydrolytic enzyme has a crucial role in the metabolism of pesticides in plants.[28] The enzyme was found to be stable, required no co-factors and had a pH optimum of 7.5–7.9. However, the involvement of this enzyme in environmental chemical metabolism is poorly understood.

8.2.3.4 *Glutathione S-transferase*

Glutathione S-transferase (GST) is found widely in the plant kingdom.[45] This enzyme catalyzes the conjugation of glutathione to a xenobiotic having an electrophilic center and an appropriate leaving group or, with xenobiotics that can be activated by oxidation or, by some other means to afford an active substrate.[46] Induction of GST following exposure to herbicide antidotes and oxadiazon have been observed in sorghum and chickpeas, respectively.[45,47]

An increase in GST activity have been observed in plants that were exposed to either PCP or BaP.[26,27] Such an observation suggests that the enzyme may have catalyzed the conjugation of the pollutants and their transformed products with glutathione (GSH). Biotransformation of BaP to diol metabolites and subsequent conjugation of such metabolites with glutathione has been reported in green algae.[48]

8.2.3.5 *Glucosyltransferase*

The conversion of certain xenobiotics to glucosides is a common phenomenon in plants exposed to a polluted environment. *Nymphaea Candida,* an aquatic macrophyte with floating leaves and a well developed root system showed glucoside formation when exposed to phenols. Whereas in free-floating plants with poor root system (eg, *Elodea canadensis, Lemna minor* and *Utricularia vulgaris*), no similar glucosides of phenol were observed.[49] These observations suggest that the principal pathways of xenobiotic metabolism in aquatic macrophytes may depend upon their life-forms. Recently, *O*-glucosyltransferase and *O*-malonyltransferase activities were detected in the cultured soybean cells with marked substrate specificities for chlorinated aromatic xenobiotics.[50]

8.3 FREE RADICALS AND ANTIOXIDANT DEFENCE SYSTEM

8.3.1 Free radicals

The generation of free radicals in the environment is enhanced in the presence of organic and/or inorganic pollutants.[51] Free radicals from organic contaminants in aquatic environment are generated mainly by:

(i) Photo-reactions (eg, peroxide and hydroperoxide reactions;[52]; and/or
(ii) Metabolic activation (one- and two-electron oxidation or monooxygenation) to redox cycling within organisms.[10,53,54]

Therefore, aquatic plants are exposed continuously to reactive oxygen species (ROS) in environments polluted with organic contaminants. ROS have been implicated in oxidative tissue damage and free radical pathology.[55] In plants, ROS involve alterations in the physical and compositional properties of cellular membrane.[56] These major alterations include membrane phase separation, changes in lipid composition and increase in electrolyte leakage etc.

8.3.2 Antioxidant defence system

Plant cells have evolved a complex series of antioxidants in various aqueous and membrane compartments to scavenge free radicals. The aqueous enzymatic systems, including superoxide dismutase (SOD), glutathione reductase (GR), ascorbate peroxidase (AP)[35] and catalase, are the best characterized antioxidant enzymes in plants.[57] Hence, the extent to which oxidative damage can occur under normal or exacerbated conditions depends upon the effectiveness of the antioxidant reserve of the organism.[58] Elevation in the enzyme activities of xenobiotic metabolism and antioxidant defence systems in plants have been used as marker of polluted conditions.[59] A positive correlation between the activity of Halliwell-Asada pathway enzymes (SOD, AP and GR)[60] and resistance to herbicide toxicity has been observed.[61] Gupta et at[62] reported responses from cellular antioxidants in populus leaves following ozone exposure. Oxidative stress is known to increase SOD activity in plants including mosses.[63,64] The rapid decomposition of hydrogen peroxide, a strong pro-oxidant is produced following dismutation of superoxides by SOD which is crucial to intercept free radical damage. Ascorbate peroxidase in plants is a specific enzyme involved in the decomposition of hydrogen peroxide.[65] GR is primarily responsible for maintaining a favorable redox status (ie, higher GSH/GSSG ratio) of glutathione. Correlations between the GR activities and environmental/xenobiotic stress have also been reported in maize inbreeds.[66]

The increase in the activities of antioxidant enzymes have been observed in the plants exposed to PCP, BaA and BaP.[26,27] Such an increase may be as an adaptive response to the generation of surplus amounts of reactive oxyradicals as a result of metabolism of the above mentioned compounds. The effects of BaA and BaP exposure on the antioxidant enzymes in plants were more pronounced in light than in dark.[27] Such an effect may be due to enhanced generation of free radicals as a result of photooxidation[67]

in addition to metabolic activation. Involvement of singlet oxygen in photooxidation of PAHs have been reported.[67]

8.4 AQUATIC MOSSES AND 'MOSS BAG' INSTALLATIONS

Because of their relative lack of seasonally, wide geographical and ecological distribution and tolerance to various types of mineral and organic pollutants, aquatic mosses (Bryophytes) have been proven to be effective practical biomonitors especially in temperate regions.[68,69,70] Their stationary nature makes the sampling process very simple and facilitates the precise determination of the pollutant source. The simple morphology of mosses minimizes problems which may arise due to the differentiated organs present in higher plants. Aquatic mosses have been shown to accumulate polychlorinated biphenyls and certain pesticides.[71]

The use of a 'moss bag'—a technique widely adopted for the monitoring of organic[71] and inorganic environmental contaminants using living or non-living mosses, extends the range of sites to those without natural populations of mosses.[72] This approach is also useful to reduce variation in the genetic background and exposure history that can occur in feral samples during the study of biochemical responses in plants exposed to pollutants.[5]

Recently, we studied the accumulation of polycyclic aromatic hydrocarbons (PAHs) and responses of antioxidant enzymes in the aquatic moss, *Fontinalis antipyretica* transplanted as 'moss-bags' around a city harbor[73]. Glass fiber bags containing *F. antipyretica* ('moss bags') were transplanted at five sites on a lake of Finland in summer. Three sites were located south (downstream) and near to a city harbor area and two sites which were located north (upstream) and far from the harbor served as reference sites. PAH accumulation and the responses of the activities of antioxidant enzymes were determined in the transplanted moss following 35 d of exposure to the lake water. Significantly higher levels of total PAHs in moss tissue were observed at the sites close to the harbor compared to the corresponding values obtained from the reference sites. Compared to that observed at reference sites, higher activities of antioxidant enzymes (SOD, POX and AP) were observed in moss transplanted near the harbor. This study introduces a novel approach to investigate the cause-effect relationship between bioaccumulation of aquatic pollutants and the biochemical responses in organisms following exposure to such pollutants in a field setting.[73]

8.5 CONCLUDING REMARKS

The literature reviewed in this chapter shows clearly the crucial role of aquatic plants in environmental toxicology assessments. The uptake/elimination and metabolism of environmental pollutants in aquatic plants are critical to understand the bioaccumulation, transport and transformation of pollutants in the food chain. Such accumulation, transport and transformation of pollutants by plants are of concern

because they may have direct effects on the aquatic ecosystem and also because of their potential to harm indirectly human populations that are a part of the food chain. Accumulation and subsequent release (after death) of pollutants by plants greatly contribute to the recycling of organic chemicals in the aquatic environment. A well developed biotransformation and antioxidant systems, that sensitively respond to the pollution exposures, are present in aquatic plants. Such responses of plant enzymes may be implemented as markers of environmental stress. Furthermore, the need for simultaneous determination of pollutant residues in plants and the biochemical responses incited due to such exposure has been emphasized. Such simultaneous determinations are critical to understand the cause-effect relationship of contaminants. 'Moss-bags' have been suggested as efficient implements to study such cause-effect relationships in a field setting.

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9 QSAR Studies of Algal Toxicity

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9.1 INTRODUCTION

To comprehend the effect of a xenobiotic on the environment, toxicological information is required on all trophic levels that the chemical may come into contact with. It is obvious therefore that for a full toxicological evaluation, primary producers, ie, those organisms converting light into usable energy for higher organisms, must be included. In the aquatic environment, of course, this role is at least partly fulfilled by the algae, and thus there is concern as to how toxicants may affect their viability. Obviously, with these organisms being at the bottom of most food chains, any factor affecting them may affect radically higher trophic levels. Also, it is important to check if algae may act as surrogates of higher species.

Algal toxicity tests are required as part of the regulatory testing for the premanufactory notices on new chemicals within the European Union.[1] In many respects they are unique, being the only aquatic organisms required to be tested that are capable of photosynthesis; thus the toxicological impact of chemicals on these organisms may be different from that on higher organisms which have different physiology and enzyme systems.

This chapter is not intended to be a critical discussion of algal toxicity testing as such (for such a review see Blaise),[2] but reviews the use of algal toxicity data in quantitative structure-activity relationships (QSARs), and the use of algae as potential surrogate species for higher organism toxicity testing. QSARs attempt to relate statistically the biological activity of a chemical to its physicochemical structure. Their methodology and use in ecotoxicology are reviewed elsewhere.[3,4] QSAR analysis of toxicity data allows not only the prediction of toxicities for compounds that have yet to be tested or may not even exist, but also an opportunity to understand the mechanism of toxic action of chemicals. In addition, the study of interspecies relationships of toxicity (also referred to as quantitative activity-activity relationships[5] reveals whether it is possible to extrapolate from the toxicity of a chemical to lower species such as algae to higher organisms such as fish or even mammals.

9.2 QUANTITATIVE STRUCTURE-ACTIVITY RELATIONSHIPS

9.2.1 Non-polar narcosis

For successful QSAR analysis of aquatic toxicological data, accurately measured biological activities and preferably a knowledge of the mode of action of the toxicants are prerequisites. One of the easiest starting points is the analysis of the toxicity of

unreactive organic chemicals such as simple hydrocarbons, aliphatic alcohols and simple substituted benzenes. The toxicity of such chemicals is found to be simply a function of their ability to reach the response site in an organism. Such toxicity is termed 'non-polar narcosis' or 'baseline toxicity' and is considered to be a non-specific physiological effect independent of chemical structure.[6] As such, this toxicity is well correlated to hydrophobicity, ie, the ability of compounds to partition into the lipid phase. Hydrophobicity is usually described by the logarithm of the octanol-water partition coefficient (log *P*) which may be either measured experimentally or calculated.

Könemann[7] was the first worker to establish a QSAR for non-polar narcosis. He found the 14-day LC_{50} in the guppy *(Poecilia reticulata)* for 50 unreactive industrial organic chemicals including chlorobenzenes, toluenes, alcohols and chlorinated alkanes correlated well to log *P:*

$$\log 1/EC_{50} = 1.00 \log P + 1.23, \quad (1)$$

for n=50, r^2=0.976, s=0.237, and F not given, where n is the number of observations; r^2 is the square of the correlation coefficient; s is the standard error of the estimate; and F is the Fisher statistic.

NB. These toxicities and all others in this chapter are expressed in moles per litre.

This phenomenon of non-specific toxicity being well correlated to hydrophobicity has been observed in many species.[8-9] van Leeuwen et al[10] recommend various QSARs for the prediction of toxicity to aquatic organisms. The following QSAR is recommended for the prediction of the EC_{50} for population growth of the alga *Selenastrum capricornutum*[11] (data taken from Galassi et al[12] and Calamari et al[13] for 10 alkyl benzene derivatives). It can be observed that it is very similar to that for fish toxicity given above:

$$\log 1/EC_{50} = 1.00 \log P + 1.23, \quad (2)$$

for n=10, r^2=0.93, s=0.17, and F not given.

Ideally a QSAR should not be taken in isolation and should be compared with those for similar compounds in different species. Such validation is in many ways as important as the statistics associated with regression analysis as it allows for species sensitivities to be noted and may aid the identification of spurious correlations.[8] It is obvious that the two QSARs for non-polar narcosis reported above have similar slopes and intercepts and thus show that despite the vast species difference between the guppy and the alga the toxicities and probably the mechanisms of action are comparable.

The toxicity of chlorobenzene derivatives to the freshwater green alga *Ankistrodesmus falcatus* was measured by Wong et al[14] Chlorobenzenes are considered to act as non-polar narcotics and the following correlation was obtained with log *P:*

$$\log 1/EC_{50} = 0.985 \log P + 0.374, \quad (3)$$

for n=12, r^2=0.971, s not given, and F not given.

It is interesting to note that although the slope is virtually the same as for equation 2, the intercept is much lower. This indicates a decreased sensitivity of this species compared to *Se. capricornutum*. Another study by Figueroa and Simmons[15] investigated the effects of chlorobenzenes on the diatom *Cyclotella meneghiniana* using DNA measurement after 48 h as a toxicity parameter. Good correlations were found for these non-polar narcotics between algal toxicity and both water solubility (log *S*) and log *P*. (Aqueous solubility is clearly a measure of hydrophilicity and thus an inverse relationship is to be expected between log *S* and log *P*.) Good correlations are observed between log *S* and log *P* for organic liquids, but for solids the relationship breaks down due to the entropy of fusion;[16] there is a correlation of 0.897 for the log *P* and log *S* values reported in this study.) Using the data presented in the original paper we found the following QSAR (it should be noted that despite using the same data this is different from that reported in the first reference, also the original paper reports log *P* values from a relatively old report[17] and so may not be as accurate as more modern data):

$$\log 1/EC_{50} = 2.40 \log P - 4.83, \quad (4)$$

for n=12, r^2=0.867, s=0.582, and F=73.0.

Later reanalysis of these data by Hoekman[18] using calculated log *P* values revealed the following QSAR, the slope and intercept of which are not at all similar to equations 2 and 3 above and may indicate that this particular assay is not suitable for these chemicals. Also the slope itself is very high for such a QSAR and may cast some doubt over the validity of the toxicity data:

$$\log 1/EC_{50} = 1.896 \log P - 3.05, \quad (5)$$

for n=12, r^2=0.933, s=0.436, and F not given.

The effect of 24-h exposure to simple alcohols on the retention of a stain, neutral red, has been studied by Schild et al[19] for the marine macroalga *Enteromorpha intestinalis*. Despite this being a marine seaweed, again a good correlation was found with log *P*. It should be noted that there is a decrease in the intercept indicating decreased sensitivity compared with *Se. capricornutum:*

$$\log 1/EC_{50} = 0.879 \log P + 0.13, \quad (6)$$

for n=8, r^2=0.974, s=0.190, and F=268.

9.2.2 Polar narcosis

Veith and Broderais[6] studied the toxic effect of some supposedly narcotic chemicals to the fathead minnow. They found that these compounds had elevated toxicity above that predicted for non-polar narcosis. These compounds were characterized by a strong hydrogen bonding group on the molecule, thus making the compound more polar. This toxicity was termed 'polar narcosis' and although it is greater than that due to non-polar narcosis, there are still good correlations with hydrophobicity albeit with a lower slope and greater intercept than the QSAR for non-polar narcosis. Veith and Broderius[6] found the following polar narcosis QSAR for the toxicity of some phenol and aniline derivatives in the fathead minnow *(Pimephales promelas):*

$$\log 1/LC_{50} = 0.65 \log P + 2.29, \tag{7}$$

for n=39, r^2=0.900, s not given, and F not given.

It would appear however that in algae the correlation of hydrophobicity with the toxicity of potential polar narcotics may not be as good. For instance, Nendza and Seydel[20] found the following correlation with log P for the inhibition of the growth of *Scenedesmus quadricauda* and *Chlorella* sp. by substituted phenols:

$$\log 1/EC_{50} = 0.65 \log P + 2.32, \tag{8}$$

for n=16, r^2=0.67, s=0.55, and F not given.

Unfortunately the source of these data and the units are not detailed exactly in the paper but assuming that they are reported in molar units we can see that there is a decrease in the slope and an increase in the intercept compared with non-polar narcosis.

Kramer and Trümper[21] analyzed the toxicity of monosubstituted phenols (also thought to be acting as polar narcotics) to *Chlorella vulgaris*. However, efforts to develop a strong predictive QSAR were thwarted because they modelled across modes of action or segregated derivatives using position isomers. Jaworska and Schultz,[22] reanalyzing these data, improved on the correlations by combining log P and a further descriptor, the Hammett sigma constant (σ). It was concluded that the improvement of the QSAR after the inclusion of σ was due to the fact that σ accounts for the degree of ionization, since σ controls pK_a. After the removal of 2-nitrophenol as an outlier the following QSAR was obtained:

$$\log 1/EC_{50} = 0.718 \log P - 0.836\,\sigma + 4.451, \tag{9}$$

for n=29, r^2=0.870, s=0.183, and F=87.2.

Shigeoka et al[23] measured the acute toxicity of phenol and its mono- to penta-chlorinated derivatives to two species of algae. Tetra- and pentachlorophenols are thought to act as uncouplers of oxidative phosphorylation,[24] whereas the other chlorophenols are likely to be polar narcotics.[25] An interesting species-specific effect occurs here; the toxicities to *Se. capricornutum* are linearly related to hydrophobicity (note we feel there was an error in the original paper in the reporting of the units which are quoted as being in molar units, whereas the QSAR appears to have been calculated in millimolar units; the following QSARs are quoted for molar units):

$$\log 1/EC_{50} = 0.887 \log P + 1.455, \quad (10)$$

for n=13, r^2=0.960, s not given, and F not given.

In contrast to the linear relationship in equation 10, the toxicity to *Ch. vulgaris* shows a biphasic relationship with log P which was modelled by the authors[23] with a parabolic relationship:

$$\log 1/EC_{50} = 2.326 \log P - 0.272 (\log P)^2 - 0.493, \quad (11)$$

for n=8, r^2=0.925, s not given, and F not given.

The toxicities of the di-, tri-, tetra-, and pentachlorophenol to *Ch. vulgaris* were almost the same which suggests that there is a levelling off of toxicity rather than a parabolic relationship. In this instance the use of the parabolic relationship is not correct as there is no falling off in toxicity. Such a relationship should be modelled using a bilinear equation:

$$\log 1/EC_{50} = 1.450 \log P - 1.566 \log(3.039 \times 10^P + 1) - 2.805, \quad (12)$$

for n=8, r^2=0.942, s=0.245, and F not given.

Aside from the QSAR modelling, several further points must be drawn from these results. Firstly the toxicity of more hydrophobic chemicals to *Ch. vulgaris* was found to be similar, which would suggest that this assay, which was actually performed to the guidelines of the Organization for Economic Cooperation and Development (OECD), is not suitable for testing compounds with a log P>3. *Se. capricornutum,* on the other hand, will provide a reasonable estimate of toxicity up to a log P=5. Also the slope and intercept of the above equation for *Se. capricornutum* are considerably closer to that for non-polar narcosis than are those for polar narcosis reported by Nendza and Seydel.[20] It may be that some of the compounds in the data set of Nendza and Seydel,[20] such as the nitro- and aminophenol derivatives, are acting as reactive toxicants, thus increasing the slope of the equation and also reducing the statistical validity of the QSAR. In conclusion, therefore,

it can be seen that although it can be demonstrated that polar narcosis causes toxicity elevated above that of non-polar narcosis, an accurate hydrophobicity based QSAR (similar to those for fish toxicity) to model polar narcosis has not yet been achieved.

9.2.3 Reactive and specific modes of action

Some chemicals are much more toxic than would be predicted by QSARs for either non-polar or polar narcosis. Of these some may act as electrophiles or proelectrophiles[26] and are commonly termed 'reactive'. Others may irreversibly affect cells by disrupting enzyme action or binding with proteins or DNA. Many of these effects are species-specific and are commonly utilized in pesticides. Such mechanisms of action are loosely termed 'specific'.

Both of these properties can provide considerable problems for the QSAR modelling of such compounds.These are intensified by the lack of readily available physicochemical descriptors for reactivity, and the problems of identifying compounds that may have reactive or specific toxicological properties. In addition, so-called surrogate microbial species may not be suitable to identify these compounds.[27,28] Ikemoto et al[29] demonstrated these problems, showing that chemicals that are specific toxicants in fish and *Daphnia* did not have toxicity significantly greater than baseline toxicity (ie, excess toxicity) in *Ch. vulgaris*. However, 3-(3, 4-dichlorophenyl)-1, 1-dimethylurea (DCMU) was found to be a specific toxicant in the alga as it is known to inhibit photosynthesis system II, which is obviously not present in fish or *Daphnia*. More hydrophobic (log P>5) specific toxicants were apparently less toxic than predicted by non-polar narcosis due to problems with water solubility. Using these data, it was possible to calculate a QSAR for eight non-specific and four specific toxicants. (NB. DCMU and DDT were omitted, the former for the reasons described above, DDT because its toxicity was less than that predicted by non-polar narcosis, probably due to the effect of water solubility):

$$\log 1/EC_{50} = 0.835 \log P + 0.525, \tag{13}$$

for n=12, r^2=0.882, s=0.402, and F=83.4.

The toxicity of nitrobenzene derivatives, presumed to be reactive, to *Ch. pyrenoidosa* was assessed by Deneer et al.[30] Mononitrobenzenes were found to be correlated with hydrophobicity and the Hammett sigma constant (σ-):

$$\log 1/EC_{50} = 0.45 \log P + 1.15\ \sigma\text{-} + 3.00, \tag{14}$$

for n=15, r^2=0.82, s=0.25, and F not given.

Inclusion of toxicity data for dinitrobenzenes into this QSAR significantly worsens the correlation. Deueer et al[30] suggest that most dinitrobenzenes are reactive and their toxicity is governed by the formation of reactive metabolites. Interestingly, for these

reactive compounds a similar pattern of toxicity was observed in *D. magna,* yet toxicity data from the Microtox® test (a bioassay based on the reduction of light emission from the luminescent bacterium *Photobacterium phosphoreum*) for both mono- and dinitrobenzenes were only poorly correlated in QSAR analysis. Furthermore, the toxicities were not related to those of the alga or daphnid suggesting that the alga is a more suitable surrogate species for assessing the toxicity of such compounds.

An important step forward in the prediction of specific toxicity to aquatic organisms is reported by Nendza.[31] Attempts to predict the toxicity of phenylureas are made for several aquatic organisms from QSARs for non-polar and polar narcosis. Whereas these QSARs may predict the toxicity of phenylureas to fish within acceptable limits, narcosis QSARs significantly underpredict their toxicity to algae. This is because phenylureas are specifically phytotoxic. Acceptable predictions of toxicity were however obtained when a QSAR modelling the inhibition of the Hill reaction (photosystem II) in isolated radish chloroplasts by 3- and 4-substituted phenyl-*N*-methoxy-*N*-methylureas[32] was modified:

$$\log 1/EC_{50} = 1.89 \log P - 0.17 (\log P)^2 - 0.65 \sigma + 2.34, \quad (15)$$

for n=38, r^2=0.86, s=0.45, and F not given.

Note that there is a relationship (albeit parabolic) with hydrophobicity, implying that a similar relative (specific) toxic action is occurring, the magnitude of which is governed by transport to the active site. This approach demonstrates not only that toxicity of chemicals with specific mechanisms of toxic action can be modelled adequately, but also the intriguing possibility that enzyme systems may be able to replace *in vivo* toxicity tests for such chemicals.

More detailed analysis of the toxicity of substituted arylureas, arylthioureas, and 1-aryl-2-methyl-isothioureas to *Ch. vulgaris* was performed by Schelenz and Kramer.[33] This revealed, however, a linear relationship with hydrophobicity. The linear, as opposed to parabolic, relationship with log P may be due to more hydrophobic compounds being present in the data of Takemoto et al.[32] Log $P \not> 3$ for the compounds analyzed by Schelenz and Kramer,[33] and it may be noted that Shigeoka et al[23] found compounds with log P>3 had similar toxicity. Perhaps surprisingly the QSAR reported represents toxicity somewhere between that for non-polar narcosis and polar narcosis (as defined by Nendza and Seydel:[20]

$$\log 1/EC_{50} = 0.861 \log P + 1.720, \quad (16)$$

for n=65, r^2=0.910, s=0.214, and F not given.

Algal toxicity tests can also be used to assay phytotoxic activity of chemicals. QSAR analysis of such data will not only have important consequences for the design of more potent specific herbicides, but may allow for an understanding of the mechanisms of action and for the possibility of risk assessment of chemicals to higher

photosynthetic organisms to be made. For instance, Sandmann and Böger[34] reported the following correlation for the phytotoxic activity to interfere with plant pigment formation in the green alga *Sc. acutus* by a congeneric series of 2-phenylpyridazinones:

$$\log 1/I_{50} = 0.90\ \pi + 3.57\ \sigma_{meta} + 5.25, \qquad (17)$$

for n=11, r^2=0.83, s=0.52, and F=19, where π is the Hansch hydrophobic substituent constant and σ_{meta} is the Hammett constant in the meta position.

Surprisingly, with the concern over inorganic compounds in the environment, little QSAR work has been performed on acute aquatic toxicity data for such compounds,[3] algal toxicity data being no .Wong et al,[35] however, without fully quantifying their data, do report that the toxicity of organic tin compounds is related to the carbon chain length, which obviously is related directly to log *P*.

9.3 INTERSPECIES RELATIONSHIPS OF TOXICITY

The study of interspecies relationships of toxicity can provide information as to which species is more sensitive to potential pollutants or, more importantly for the purposes of this review, allow extrapolation of toxicity from lower species to higher ones, ie, the possibility of algae acting as surrogate species. We have already noted that QSARs should, if possible, not be considered in isolation;[8] comment has already been made concerning comparative analysis of QSARs and previous studies have shown them to be useful tools for studying comparative toxicity.[36]

Simple comparative studies of the toxicity of 15 chemicals to 22 freshwater species in (sub)acute tests showed that toxicity to green algae, along with crustacean and fish toxicity, is required to indicate the full toxic potential of chemicals.[37] Analysis of the (semi)chronic toxicity of 11 freshwater species to eight chemicals showed that algae were again important in providing an indication of the toxic potential of chemicals, along with toxicity to a crustacean and an egg-laying fish species.[38] These studies show not only that the evaluation of algal toxicity is essential, but that for a heterogeneous group of chemicals no other species can provide similar information.

Other more detailed comparative studies of toxicity with fewer chemicals and species[39–41] also confirm the importance of algal toxicity testing to provide toxicological information along with that of fish and *Daphnia*. A more recent study,[42] although being rather qualitative in nature, demonstrates that even within the recognized species for toxicity testing there are differential responses of green algal species, in this case to common solvents. *Gleocystis, Scenedesmus, Nannochloris* and *Tetraselmis* proved to be the most tolerant genera to solvents, whereas *Chlorococcus* and *Chlorella* were the most sensitive. In addition, Kirby and Sheahan[43] studied the effects of three herbicides on the alga *Sc. subspicatus* and the macrophyte plant *Lemna minor* (common duckweed). Their findings indicate that toxicities to these species were not strictly comparable and information

should be used from both to assess the phototoxicity of potential environmental pollutants, especially those with herbicidal activity. Other studies have, however, found good correlations between toxicities to species such as *Minutocellus polymorphus* and *Skeletonema costatum,*[44] although these studies are all on single chemicals, van Wijk et al[45] assessed the toxicity of mixtures of three ethylamines to *Se. capricornutum, D. magna* and the guppy and found additivity in the toxicities common in all three species. The whole area of the prediction of mixture toxicity in all species requires more work.

LeBlanc[46] attempted a more quantitative study of the interspecies relationships of toxicity in aquatic organisms. A good correlation between toxicity of non-pesticide organic compounds in the freshwater alga *Se. capricornutum* and the saltwater alga *Sk. costatum* is reported:

$$\log EC_{50(Sk.\ costatum)} = 1.00 \log EC_{50(Se.\ capricornutum)} - 0.28, \quad (18)$$

for n=17, r^2=0.87, s not given, and F not given.

The relationship between fish (bluegill, *Lepomis macrochirus*) LC_{50} and *Se. capricornutum* EC_{50} is worse however:

$$\log LC_{50} = 0.71 \log EC_{50} + 0.17, \quad (19)$$

for n=19, r^2=0.62, s not given, and F not given.

LeBlanc[46] concluded that good correlations between toxicity could be obtained within the same trophic level but that, not unexpectedly, these correlations decreased for more distantly related trophic levels. A better approach to analyzing interspecies relationships is, at least initially, to study either closely related compounds, or those acting by the same mechanism of toxic action. For instance, the chlorobenzenes as described above all appear to act by non-polar narcosis and QSARs are comparable for different species. Therefore, it is not surprising that good correlations for chlorobenzene toxicity to *Se. capricomutum* are reported with *D. magna* toxicities and many fish toxicities; the exception is a poor correlation with rat toxicity, due probably to differences in toxicokinetics.[47] Other studies have demonstrated that a knowledge of the mode of toxic action of chemicals is important in understanding interspecies relationships.[27,28]

Jaworska and Schultz[22] studied the comparative toxicity of monosubstituted phenols thought to be acting as polar narcotics to *Ch. vulgaris* and the ciliate *Tetrahymena pyriformis*. After the removal of 2-nitrophenol as a significant outlier, the following good correlation was obtained, suggesting that for polar narcotics there is good comparative toxicity between the alga and the ciliate:

$$\log EC_{50} = 0.856 \log IG_{50} + 5.397, \quad (20)$$

for n=29, r^2=0.842, s=0.219, and F=144, where log IG_{50} is the 48-h *T. pyriformis* growth inhibition.

A more multivariate approach to the analysis of interspecies relationships was taken by Galassi et al[12] who used partial least squares (PLS) to correlate the toxicities of eight simple aromatic hydrocarbons to fish (salmon and guppy), *D. magna,* and *Se. capricornutum*. PLS proved a successful method for predicting toxicity of all four species when compared to regression analysis, but unfortunately the authors appear to have missed the opportunity to extract more information concerning the comparative toxicities of the chemicals to the test organisms.

Nendza and Seydel[20] used principal component analysis (PCA) to analyze toxicity data of 26 phenol and aniline derivatives to 11 aquatic test systems including the inhibition of the growth of *Sc. quadricauda* and *Chlorella* sp. (both species considered together) and inhibition of algal fluorescence in an undefined species. Although PCA is not a predictive technique, it does help describe large multivariate, collinear data matrices and the interrelationships between data. For these test systems the algal growth inhibition assays were found to be similar (ie, to contain similar toxicological information) to the inhibition of ribulose *bis*phosphate-carboxylase from *Vicia faba* protoplasts, and the minimal inhibitory concentrations to *Escherichia coli* and *Mycobacterium smegmatis*. Interestingly the inhibition of algal fluorescence was significantly separated from the other toxicities, although the authors suggest that this may be due to experimental variability, rather than this test containing different toxicological information.

9.4 CONCLUSIONS

There is a regulatory requirement to provide information concerning the adverse effects of xenobiotics to aquatic biota. Algal toxicity tests provide valuable information concerning the primary producers of an ecosystem. This review demonstrates that algal toxicity data for unreactive organic chemicals acting by the non-polar narcosis mechanism of action are well modelled by QSARs with hydrophobicity as the only parameter. Although there appears to be considerable difference in sensitivity between algal species, algal toxicity data for such compounds are comparable to those for higher organisms such as fish.

There is an elevated biological response by algae to the so-called polar narcotics. These compounds appear to be generally less well modelled by QSARs, and other parameters, such as those describing ionization, are required. For these chemicals algal toxicity tests may not provide good information for other species.

Some xenobiotics have considerable toxicity over that predicted from a non-polar narcosis QSAR. QSAR analysis of such toxicity data is possible when the compounds are acting by a single definable mechanism of action, as is commonly observed for specific pesticides. Caution must be taken, however, as these toxicity data are unlikely to be comparable to those in higher species (and *vice versa*) due to the intrinsic physiological differences. An obvious example is specific toxicity to photosynthetic systems not present in other biota. As a result many analyses of interspecies relationships

of toxicity give poor correlations, although this is commonly because the toxicity data utilized are from a too heterogeneous set of chemicals. Much better interspecies relationships of toxicity will be achieved if compounds with a single mode of toxic action are studied.

Overall, there are few well validated QSARs for algal toxicity and there are many gaps that need to be filled in our knowledge, such as the effect of reactive toxicants. QSAR is a powerful technique for many reasons, not least of which is that irregularities in data are uncovered. This chapter has described some of these (eg, large differences in species sensitivities) and has emphasized the need for more high quality, accurate data to help understand these problems.

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10 Acute Exposure Phytotoxicity Assay Based on Motility Inhibition of *Chlamydomonas Variabilis*

Takashi Kusui and Christian Blaise

10.1 INTRODUCTION

To meet today's increasing demand for hazard and risk assessment, more rapid and simpler biomonitoring methods are required.[1]

In the field of bioassays involving primary producers, flask toxicity testing with the green alga *Selenastrum capricornutum* has been widely employed to conduct both enrichment and toxicity studies since the 1970s.[2–4] More recently, a simpler and more cost-efficient microplate assay has been developed successfully and has shown good agreement with the flask assay in screening the toxic potential of metals, effluents and herbicides.[5,6] One attractive feature of a microplate-based assay is its automation potential, motivating on its own the development of other useful microplate-associated phytotoxicity assays.[7,8] Currently, such microassays tend generally to report chronic measurement endpoints (eg, cell growth inhibition) which require exposure times of 72–96 h. However, from a practical point of view it would certainly be advantageous to obtain toxicity results within one working day.

In order to achieve this goal, novel endpoints capable of detecting rapid responses to chemical insults (eg, biochemical changes or impairment of motility) are worthy of exploration. The development of a new 5-h exposure microplate toxicity assay based on motility inhibition of the flagellated chlorophyte *Chlamydomonas variabilis* which can be initiated easily and completed in a working day is described in this chapter. Experimental objectives are:

(i) To select a suitable algal species for the assay;
(ii) To optimize test conditions; and
(iii) To obtain preliminary insight on how the sensitivity response of this new acute test compared with that of growth inhibition endpoint values generated with a chronic exposure micro-algal assay.

10.2 PRINCIPLE OF IMMOBILIZATION TEST

A variety of toxicity responses, such as growth inhibition, loss of motility, deflagellation and encystment, have been assessed and reported in the literature involving flagellated chlorophytes of the genus *Chlamydomonas* (Table 10.1). While interest for these techniques is unquestionable, they nevertheless suffer from drawbacks which include long exposure times ($\geq$24 h for most tests) and/or tedious microscopic observations.

Hence, it appears unlikely that such tests could ever be adapted for phytotoxicity screening on a routine basis to study large numbers of samples.

Table 10.1 *Algal assays using Chlamydomonas sp*

Species	Endpoint	Exposure time	Toxicant	Reference
C. eugametos	Growth inhibition	48 h	herbicides	9
C. reinhardtii	Cell density, deflagellation	24, 48, 72 h	Cu	10
C. reinhardtii	Cell density, deflagellation and encystment	72 h	Cu	11
C. reinhardtii	Motility	15 min	Polyphenols	12
C. variabilis	Motility	24 h	Pesticides	13

To measure motility inhibition in a simpler and quicker way, it was hypothesized that, while motile *Chlamydomonas* cells are swimming in an aqueous medium, they would start to settle as their motility became impaired under the influence of toxicants. Consequently, the number of floating motile cells would decrease in proportion to the intensity of a toxic effect (Figure 10.1). The number of motile cells in a liquid sample would then be measured by simply enumerating those remaining in the supernatant after exposure. From an ecological perspective, loss of flagellar motility can be consequential, as it could lead to a competitive disadvantage within a food web and thereby affect population growth.[11]

Figure 10.1 *Principle of immobilization test*

To evaluate immobilization quantitatively, the percentage of remaining cells in the supernatant of sample compared to that of the control is calculated from the equation:

$$R_m = \frac{C_s}{C_c} \times 100$$

where:
R_m is the percentage of motile cells;
C_c is the cell concentration remaining in the supernatant of the control; and
C_s is the cell concentration remaining in the supernatant of the sample.

The immobilization testing procedure is illustrated in Figure 10.2.

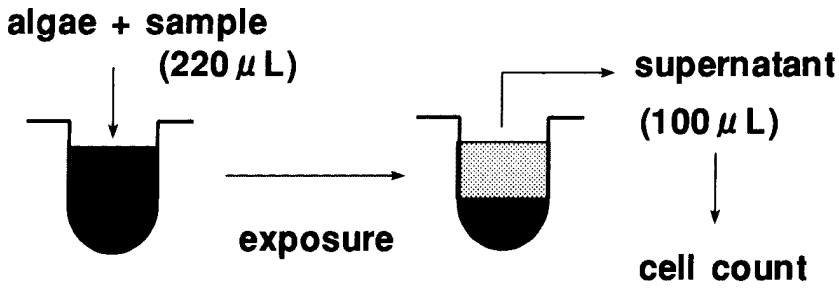

Figure 10.2 *Microplate flagellar immobilization test*

Each assay is undertaken in a 96-well round-bottomed polystyrene microplate (sterile Linbro/Titertek plate, catalog No.76–242–05). The final assay protocol, summarized in Table 10.2, was reached after optimization experiments reported in sections 10.3–10.5 were performed. Essentially, the following simple steps are called for:

(i) Micropipetting of algal inoculum and sample (total volume of 220 μl) into microplate wells;
(ii) Incubation of microplate (acute exposure) under specific temperature and light regimes;
(iii) Careful post-exposure withdrawal of 100 μl of supernatant from each well; and
(iv) Enumeration of algal cells in the supernatant with an electronic particle counter (Coulter Counter model ZM with a 70 μ aperture).

Table 10.2 *Summary of the test procedure*

Test type	: Static, 5 h duration
Test container	: 96-well polystyrene microplate, round-bottomed
Test water	: Reagent or reconstituted water, filtered through a 0.45 μ membrane filter
Lighting	: Continuous overhead 'cool-white' fluorescent illumination with 4.0 klux at the surface of the test container, and a quantal flux between 60 to 80 μE/(m^2.s)
Temperature	: 24±2 °C
Test organism	: *Chlamydomonas variabilis* from a culture that is 3 d-old and in an exponential phase of growth
Concentration method	: Centrifugation (300 g, 5 min)
Algal inoculum	: 500,000 cells ml^{-1}
No. of replicates	: Minimum of three
Observations	: Cell concentration in supernatant at 5 h
Endpoint	: Percentage of remaining cells in supernatant of treated wells compared to control wells (IC_{50}, IC_{20}, NOEC/LOEC)

10.3 SELECTION OF A TEST ALGAL SPECIES

In this work, the performance of three motile algae, *Chlamydomonas variabilis* (UTCC 100), *Chlamydomonas reinhardtii* (UTCC 84) and *Carteria olivieri* (UTCC 83), as potential candidates for an immobilization test were appraised. The use of the first two for toxicity tests involving motility inhibition has already been indicated (Table 10.1).

For practical reasons, most of the culture conditions employed in the assessment of these three algae were identical to those required in the *Selenastrum capricornutum* growth inhibition microtest:[14] lighting (4±10% klux), temperature (24±2 °C), shaking speed (100 rpm) and AAP ('Algal Assay Procedure') growth medium. An additional reason for choosing the AAP medium lies in its relatively low nutrient content (4.2 N mg l^{-1} and 0.18 P mg l^{-1}). For *Chlamydomonas* sp., several media, such as Sager-Granick's medium and Bold's basal medium, are commonly used for growth.[15] However, the higher nutrient content in these media have been reported to lower the sensitivity of *C. reinhartdtii* towards toxicants.[13] Furthermore, the polyphosphate stored intracellularly due to the high P concentration in the medium may increase metal detoxification capacity.[17]

Figure 10.3 shows the growth curve of the three algae in AAP medium. Their initial inoculum concentration was set at 10,000 cells ml^{-1}. *Carteria,* which is known to grow well in Bristol's medium, was obviously unsuited for growth in AAP medium, as confirmed by the total absence of cellular division. In contrast, *C. reinhardtii* and *C. variabilis* displayed adequate growth, as indicated by the onset of a rapid exponential phase followed by a stationary phase reached after 4–5 d. At this phase, maximum cell concentration was ~1,000,000 and 600,000 cells ml^{-1} for *C. variabilis* and *C. reinhardtii,* respectively. In ensuing experiments, these two *Chlamydomonas* species were harvested from 3 d old liquid cultures that were in a logarithmic phase of growth.

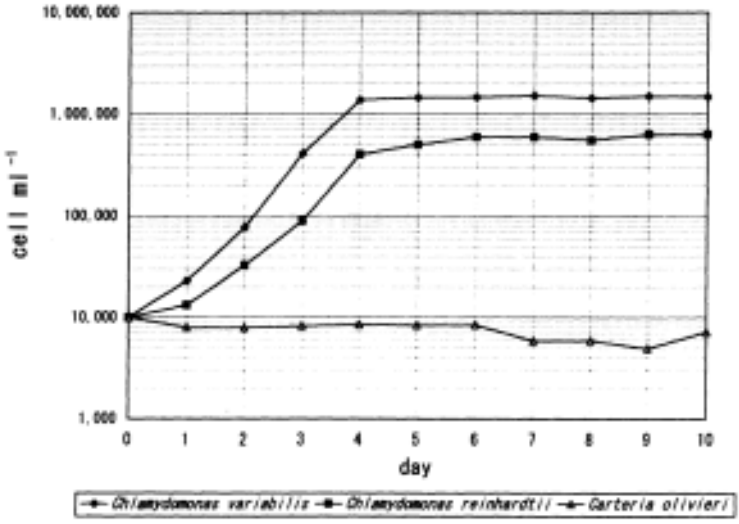

Figure 10.3 *Growth curve of algae in AAP medium*

10.4 ALGAL CONCENTRATION METHOD

Concentration of algal cells is essential so that appropriate inocula can be prepared for exposure (toxicity) tests. Centrifugation methods are usually employed for this purpose. However, such treatment, which can cause mechanical shearing, has been reported to be an initial contributing factor to flagellar loss.[17]

To alleviate potential problems of this nature, the effects that specific gravity (centrifugation) and duration (time) might have on cell recovery and deflagellation under different regimes were examined. To calculate cell recovery, the post-centrifugation supernatant was first discarded and tube volume was adjusted to that of the original. Sedimented cells were then resuspended and cell concentration was measured with an electronic particle counter in the prescribed manner. The percentage of flagellated cells was determined microscopically.

The results of centrifugation experiments with *C. variabilis* are shown in Figure 10.4. It can be seen that as many as 84% of the cells were flagellated before centrifugation and that deflagellation resulted following all treatments (Figure 10.4). When time is held constant (5 min), increasing specific gravity from 300 to 1000 g decreased the percentage of flagellated cells from 84% to 33%. Based on an expected average cell recovery greater than 90%, 300 g for 5 min was selected as the optimal centrifugation condition. After this concentration regime, close to 60% of cells are expected to remain flagellated (Figure 10.4).

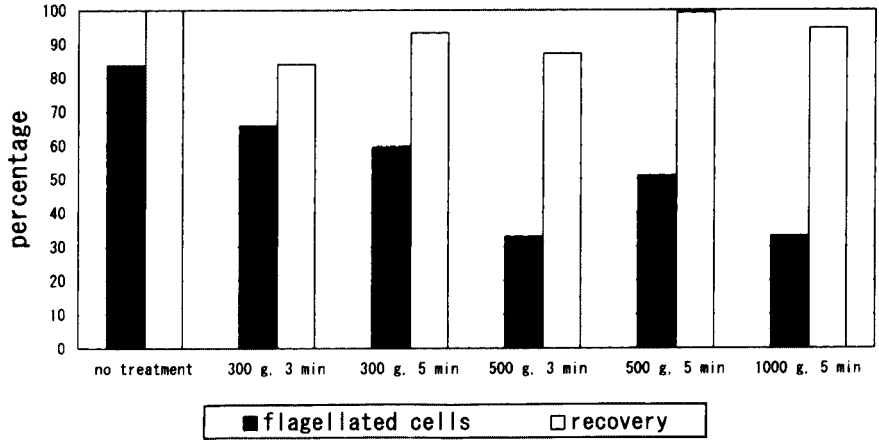

Figure 10.4 *Effect of centrifugation on deflagellation and cell recovery of Chlamydomonas variabilis (mean of three experiments)*

Similar experiments conducted with *C. reinhardtii* demonstrated that this species had relatively low percentages of flagellated cells ranging from 23% to 56% even before centrifugation. Furthermore, *C. reinhardtii* was so susceptible to mechanical shearing that the least severe treatment (ie, 300 g for 3 min) caused a 92–95% flagellar loss. Based on flagellar strength, therefore, *C.variabilis* was finally selected as the favored test species.

10.5 SETTLING BEHAVIOR OF ALGAE IN MICROPLATE WELLS

Before exposure experiments could be undertaken, it was imperative to examine the settling characteristics of *C. variabilis* in microplate wells using initial cell density as a variable.

The temporal variation of cell concentration in the supernatant (100 μl) was measured with inocula varying from 29,200–935,000 cells ml^{-1} (Figure 10.5). Rapid settling occurred within 1 to 2 h and only 20%–28% of initial cells remained in the supernatant after 3 h. It was also observed that cell concentration in some of the

series tended to increase again after 4 to 6 h. This 'recovery' might be partly explained by the fact that full flagellar length is restored within 1–2 h after deflagellation caused by mechanical shearing.[15] Considering that numbers of flagellated cells in the supernatant of wells appeared to stabilize between 4–6 h, it seemed appropriate to select a test exposure time within this timeframe. Based on both test sensitivity considerations (eg, a 5 h test would tend to favor a greater toxicity response than a 4 h test) and logistical ones (eg, when routinely performed, a 5 h test more easily fits in to a person's normal working hours than a 6 h test), a 5 h test exposure was chosen for the final protocol.

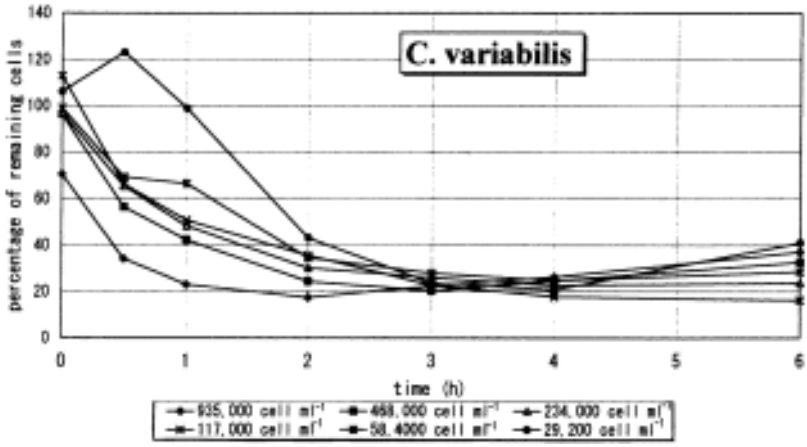

Figure 10.5 *Settling behavior of cells in microplate wells*

There then remained the question of which cell inoculum to introduce into microplate wells. Indeed, with respect to sensitivity, a lower initial cell concentration would imply higher toxicant availability per unit cell at specific exposure concentrations, thereby favoring higher sensitivity of the test system. However, with regard to analytical considerations, too low an inoculum would translate into an insufficient number of cells being processed by the electronic particle counter and this might lead to unacceptable error or variations in reporting actual cell concentrations in microplate well supernatants. As an inoculum of 500,000 cells ml^{-1} in microplate wells would satisfy the enumeration requirements of the particle counter, this concentration was chosen as the starting cell concentration for the test procedure. The latter, with final test conditions selected on the basis of optimization experiments discussed previously, is shown in Table 10.2.

Table 10.3 *Comparison of acute exposure versus chronic exposure sensitivity responses for five heavy metals. Endpoint values are expressed in μg l^{-1} (± 95% confidence intervals)*

	***Chlamydomonas variabilis* immobilization test**				***Selenastrum capricornutum* microplate growth inhibition test[5,6,18,19]**
	LOEC (5 h)		**IC_{20} (5 h)**		**IC_{50} (96 h)**
Cu^{2+}	12.4	(7.31~17.5)[a]	13.5	(6.66~20.3)[a]	48,[5] 66 (61~71)[6]
Cr^{6+}	28.4		23.4		130 (94~170),[6] 170 (91~246)[18]
Cd^{2+}	5.32	(1.84~8.81)[b]	5.12	(2.77~7.40)[b]	50,[5] 56[19]
Zn^{2+}	63.6	(14.2~113)[c]	33.9	(11.0~56.7)[c]	48,[5] 53 (32~73)[6]
Hg^{2+}	22.5	(-11.2~56.2)[d]	3.76		19.5 (7~35)[18]

a,b,c,d mean of nine(a), three(b,d) and two(c) experiments

10.6 METAL TOXICITY TESTING AND SENSITIVITY COMPARISON

Figure 10.6 indicates a series of dose-response curves obtained from 5 h exposure experiments with copper (Cu^{2+}), substantiating the principal hypothesis that flagellar immobilization is influenced by toxicity. However, in some trials dose-response curves appeared irregular and precluded the determination of a precise IC_{50} (ie, that concentration provoking 50% immobilization, corresponding to an R_m=50%). Microscopic observation of 5 h supernatants exposed to high Cu^{2+} concentrations revealed considerable debris, presumably from cells lysed by the effects of this ion. Since some debris were large enough to be counted with the Coulter counter, they may have contributed in reducing the slope of some dose-response curves or in promoting its heterogeneity. Because of these observations, LOECs (lowest observable effect concentration) and IC_{20}s (concentration corresponding to an R_m= 80%) were reported as the preferred endpoints.

Results obtained with five heavy metals, in addition to literature comparisons with IC_{50}s generated with the *Selenastrum capricornutum* growth inhibition assay for the same metals (cadmium, chromium, copper, mercury and zinc),[5,6,18,19] are shown in Table 10.3. Test metal solutions were prepared from $CdCl_2.H_20$, $K_2Cr_2O_7$, $CuSO_4.5H_20$, $HgCl_2$, and $ZnSO_4.7H_20$. All metals, except Hg^{2+}, indicated good agreement in the LOEC and IC_{20} values determined with the 5 h immobilization assay. For these four metals, toxicity decreased in the following order: Cd^{2+}>Cu^{2+} >Cr^{6+}>Zn^{2+}. When compared to previously published results with the *Selenastrum capricornutum* assay, the proposed assay showed equal or greater sensitivity: XI for Zn^{2+}, X5–10 for Cu^{2+}, Cr^{6+} and Cd^{2+}. Although limited to reference metal toxicants

at this time, the present comparison nevertheless suggests that the 5 h immobilization test may eventually have potential as an alternative assay to the chronic growth inhibition microplate assay for toxicity screening purposes.

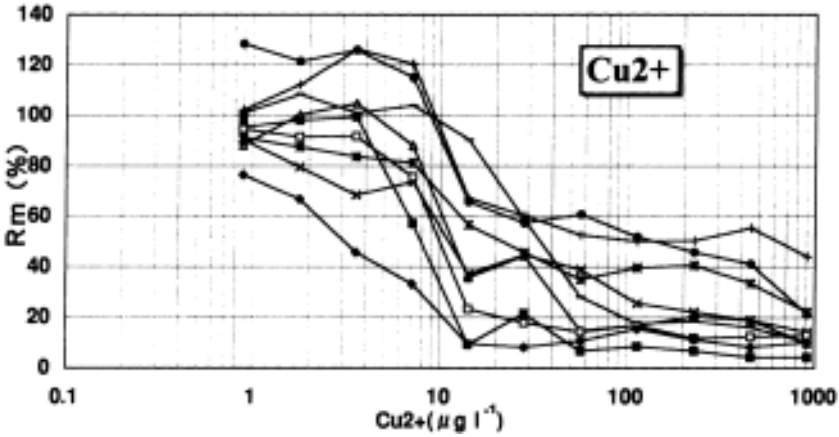

Figure 10.6 *Dose-response curve of immobilization test (R_m=motile cells remaining in supernatant of microplate wells)*

From these observations as well as from literature information, it appears that several factors can contribute to algal (im)mobilization. These include deflagellation, shortening of flagella, beat frequency of flagella, and regeneration of flagella. Some chemicals and physical factors (UV radiation, pH, heat, etc.) are also known to promote specifically and/or inhibit motility.[15] Exposure to metals (Cu^{2+}, Hg^{2+} and Cd^{2+}), in fact, has recently been reported to decrease both swimming velocity and precision of gravimetric orientation of populations of the photosynthetic flagellate *Euglena gracilis*.[20] Clearly, to verify the usefulness of this assay, the effect of all such factors will have to be investigated more fully.

10.7 CONCLUSIONS

A rapid 5 h exposure micro-algal assay based on motility inhibition of the flagellated chlorophyte *Chlamydomonas variabilis* was developed. This simple procedure requires the preparation of liquid sample dilutions in a 96-well microplate and the addition of algal inoculum. After exposure, the percentages of remaining cells in the supernatant of sample microplate wells were compared to those of control wells. Subsequent determination of LOECs and IC_{20}s for five metals (Cu^{2+}, Cr^{6+}, Cd^{2+}, Zn^{2+}, Hg^{2+}) indicated that test sensitivity was commensurate with that of a chronic exposure test

already reported in the literature. Attractive features of this algal assay includes being able to undertake determinations within a normal working day and the possibility of processing large numbers of samples on a routine basis for phytotoxicity screening purposes. Additional investigations are ongoing to assess the potential of this acute test to detect the toxicity of organic classes of chemicals and varied environmental samples.

10.8 ACKNOWLEDGEMENTS

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11 Validation of a Microplate-Based Algal Lethality Test Developed with the Help of Flow Cytometry

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11.1 INTRODUCTION

Microalgae play an extremely beneficial role in nature. Because of their inherent photosynthetic activity, microalgae are highly important primary producers of organic matter in aquatic ecosystems. If microalgae (along with aquatic angiosperms) were to disappear from aquatic environments, notably as a result of multiple manmade stresses, the animal world would be lacking its primary source of food and energy.[1] Accordingly, reliable microalgal lethality assays conducted in the laboratory, as early warning systems enabling decision-making to be proactive instead of reactive to pollution-generating activities, would contribute valuable information to environmental protection programs.[2]

The percentage of living (ie, both viable and fertile) cells in a population of microalgae can be determined using the colony-forming unit (CFU) method. While this technique provides unequivocal evidence of viability, it is labor-intensive, however, and inefficient when high sample throughput is required, because of the long waiting period before colony appearance. In this regard, development of surrogate techniques allowing a rapid assessment of cell viability constitutes a justifiable goal.

One such technique exploiting the fluorochrome fluorescein diacetate (FDA) has been developed and has been the object of many studies. FDA, a lipophilic, non-polar and non-fluorescent molecule, readily penetrates the cell plasma membrane. Once inside the cell, FDA is hydrolyzed by non-specific esterases, yielding the fluorescent anion fluorescein. Fluorescein is hydrophilic and is retained by an intact cell membrane.[3] Accumulation of fluorescent fluorescein within the cell is therefore an expression of intact esterasic activity and cell membrane permeability which, in turn, have been used as cell viability criteria by many authors. Thus, viability of human and murine cells as well as bacteria, fungus, fungal spores, plant protoplasts, pollen and microalgae has been assessed using FDA alone or in combination with a second fluorochrome (eg, propidium iodide (PI) or ethidium bromide (EB)).

The variable used for quantification of viability after staining with FDA is either global green fluorescence emitted by the cell suspension and measured with a spectrofluorometer[4-6] or the proportion of green fluorescing cells in that suspension. The latter is determined either with epifluorescence microscopy[7-13] or flow cytometry.[14-19] Flow cytometry allows the analysis of hundreds of cells s^{-1}, to give a significant statistical representation of the physical and biochemical characteristics of a cell population.[20] Because of this feature, flow cytometry has the advantage of

providing a very rapid, unbiased quantitative assessment of the proportions of different sub-populations, including that of living and dead individuals, in a cell suspension.

Accuracy of the flow cytometric determination of viability using FDA as fluorochrome has been assessed through comparison with the classical trypan blue exclusion (TB) assay and other bioanalyses.[14-18] While good correspondence was obtained on average, these comparative assessments of the flow cytometric FDA assay were not undertaken with reference to reproduction of cells in culture. Comparison with such a clonogenicity technique was the object of only one study according to our (non-exhaustive) literature review. In that study, P388 murine and HL-60 human leukemia cells in culture were used as model systems and cell viability results, obtained by flow cytometric measurement of cellular fluorescence after staining with FDA and PI, approximated those obtained from the soft agar clonogenic assay.[19]

To the best of our knowledge, positive correlation between the percentages of living cells in a microalgal population *per se* determined by the flow cytometric FDA method and those obtained with the colony-forming unit method has not been demonstrated. Therefore, the main objective of this study was to develop a method exploiting FDA-induced green fluorescence, red (chlorophyll) autofluorescence and flow cytometry for a determination of the percentage of living cells in a population of *Selenastrum capricornutum* cells, that is reliable and accurate, but less time-consuming and labor-intensive than the colony-forming unit technique. Further to this goal was the quantification of the correlation between the flow cytometric FDA and traditional plating assays.

11.2 MATERIALS AND METHODS

11.2.1 Cell culture

All *Selenastrum capricornutum* (University of Toronto Culture Collection 37) cells used in this study were obtained from logarithmic phase (ie, 4 to 7 d-old) liquid cultures grown and maintained according to Environment Canada's standard method.[21] Briefly, cells were cultured in 500 ml standard liquid growth media individually contained in 2 l Erlenmeyer flasks. Flasks were incubated with 100 rpm agitation at 24 ± 2 °C under continuous 'cool white' fluorescent illumination in a Psychrotherm™ controlled environment chamber (New Brunswick Scientific Co., Inc., Edison, NJ). Light intensity at the base of the flasks was 4 klux±10%.

11.2.2 Optimal soft growth medium

A preliminary experiment for the determination of an optimal semi-solid growth medium was undertaken prior to the comparative study. A washed algal concentrate was diluted twice in sodium hydrogen carbonate solution (15 mg l^{-1}) to yield a suspension of 100 cells ml^{-1}. From this latter preparation, 1 ml aliquots served to inoculate, in

duplicate, sterile disposable plastic 100×15 mm Petri dishes containing standard growth media solidified with 1, 0.5, 0.25, 0.1 and 0.0625% BiTek™ agar (0138–01–4 Difco Laboratories, Detroit, MI). Inoculated dishes were immediately incubated in a Psychrotherm™ chamber under the same conditions as above except that there was no agitation and illumination intensity was 9.7 klux± 10%. Enumeration of the colonies was made after their appearance, ie, after 9 d of incubation.

11.2.3 Induction of death in algal cells

Several algal suspensions of viabilities ranging from 0 to almost 100% were obtained by their exposure to cupric sulfate. Exposure was performed as described by Environment Canada.[21] Briefly, a cupric sulfate stock solution was obtained by dissolving 0.3 g of $CuSO_4 \bullet 5H_2O$ in 1 l of Millipore SuperQ™ water (Millipore Corporation, Bedford, MA). Inductively coupled plasma (ICP) analysis (Atomscan 25, Thermo Jarrell Ash, Franklin, MA) revealed that the concentration of Cu^{2+} ions in that stock solution was 76.0 μg l^{-1}. Ten test concentrations were then prepared by mixing variable proportions of stock solution and SuperQ™ water directly in a sterile polystyrene 96-well microplate (Flow Laboratories No. 76–242–05; capacity of 0.25 ml $well^{-1}$). Each well received 200 μl of test solution and constituted one replicate. There were respectively three and six replicates for each test concentration and the control, placed in a predetermined pattern in the microplate. A separate quality control (QC) microplate made up of control wells exclusively was prepared simultaneously. This QC microplate served to detect any problems with the experimental procedure. It also served to set the acquisition threshold based on the forward angle light scatter (FSC), as well as the voltages for the green (FL1) and red (FL3) fluorescence detectors, required for the subsequent flow cytometry analysis.

All wells were inoculated with 10 μl of nutrient spike and 10 μl of algal inoculum. Density of algal inoculum was set such that initial cell concentration in each well was ~20,000 cells ml^{-1}. Microplates were then covered with a transparent plastic lid and sealed in a transparent plastic bag to minimize evaporation during the 96 h exposure period. Incubation took place in a Psychrotherm™ controlled environment chamber at 24±2 °C under continuous 4 klux±10% 'cool white' fluorescent illumination (no agitation).

After incubation, all six control replicates from each of the copper-containing and QC microplates, and three replicates for each cupric ion concentration, were used for the comparative assessment. For each of those replicates, 200 μl of supernatant were discarded without prior centrifugation and replaced by 200 μl of $NaHCO_3$ 15 mg l^{-1}. After mixing, a 100 μl aliquot was used to estimate the post-exposure cell concentration. A second 100 μl aliquot was kept for FDA staining and subsequent flow cytometric determination of viability. The remainder (ie, 10 μl) was used for the colony-forming unit (CFU) assay. All of these post-exposure analyses were initiated within 12 h of the end of the incubation period.

Final nominal concentrations to which algal cells were exposed were 22.5, 27.6, 32.8, 38.0, 43.2, 48.4, 53.5, 58.7, 63.9 and 69.1 μg Cu^{2+} l^{-1}.

11.2.4 Post-exposure cell concentration assessment

The 100 µl aliquot removed from each well was poured into a 20 ml plastic cup to which approximately 5 ml of isotonic diluent (Hematall®, Fisher Scientific CS606–20) was added. Cells contained in 100 µl of this latter solution were enumerated using a Coulter Counter® Model ZM electronic particle counter (Coulter Electronics, Inc., Hialeah, FL) fitted with a 70 µm aperture diameter probe. Cell counts were afterwards converted to cell concentrations using the appropriate dilution factor.

11.2.5 CFU determination of viability

The 10 µl aliquots removed from wells were diluted in one or two steps with $NaHCO_3$ 15 mg l^{-1} to generate suspensions of ~100 cells ml^{-1}. From these, 1 ml aliquots were individually injected both on the base and at the centre of sterile disposable plastic 100×15 mm Petri dishes containing the optimal soft growth medium developed earlier (see above), ie, standard growth media solidified with 0.1% BiTek™ agar. Plates were incubated in a Psychrotherm™ chamber at 24±2 °C under continuous 9.7 klux±10% 'cool white' fluorescent illumination. Visible colonies were counted after 9 d of incubation. Inoculation of plates was undertaken with the dishes already placed in the incubation chamber and dishes were not moved until enumeration of colonies was completed. This precaution was essential to prevent colonies from mixing together, which would have rendered enumeration impossible. The percentage of CFU-determined living cells in the algal suspensions was calculated using the following equation:

$$\%\ living\ cells = \frac{no.\ of\ colonies\ formed}{nominal\ no.\ of\ cells\ plated} \times 100 \qquad (1)$$

11.2.6 FDA staining and flow cytometry

A stock solution of FDA 1000 µ*M* was prepared by dissolving 0.0417 g of FDA (Sigma® F-7378, Sigma Chemical Co., St. Louis, MO) in 100 ml of pure acetone. The stock solution was kept in the dark at -20 °C until use. A fresh working solution of FDA was made immediately before staining of the cells. This latter solution was obtained by mixing two parts of stock solution with three parts of SuperQ™ water resulting in an FDA concentration of 400 µ*M*. The 100 µl aliquots taken from wells set aside for flow cytometric analysis (see above) were split into two equal volumes. For all replicates, one of the two resulting 50 µl subsamples was pipetted into a sterile 12×75 mm polystyrene tube containing 345 µl of sterile $NaHCO_3$ 15 mg l^{-1}. Five µl of FDA working solution was then added and cells were left to incubate with FDA for 30 min. In the case of the six control replicates taken from the QC microplate, the

same treatment was applied to the second 50 µl subsample except that addition of FDA was omitted. Cells were analyzed with a FACScan flow cytometer (Becton Dickinson, Mountain View, CA) interfaced with a HP 9000 model 340 computer (Hewlett-Packard Co., Fort Collins, CO) and equipped with a 15 mW argon laser emitting an excitation light of 488 nm. Green (FL1) and red (FL3) fluorescences were measured through 530 nm band-pass and >620 nm long-pass filters, respectively, by photomultiplier tubes positioned at 90° to the laser beam. The acquisition threshold was set using the FSC signatures of the stained QC microplate control populations. FSC, FL1 and FL3 readings were recorded on 2000 cells for each algal population and flow rate was set to 'high' (ie, 60 µl min^{-1}). The LYSYS™ II software program (Becton Dickinson, Mountain View, CA) was used to create regions on FL1 and FL3 histograms and to combine the appropriate regions with logical operators in order to determine the percentage of living cells in the algal suspensions (see details in 11.3 Results and Discussion).

11.2.7 Data analysis

The LC_{50} corresponds to the concentration estimated to cause a 50% reduction in population viability compared to the control. LC_{50} values and corresponding 95% confidence intervals were obtained by inverse prediction from the linear regression of the data located between 10 and 90% effect on the concentration-lethality curve, where viability reduction percentages and nominal Cu^{2+} concentrations constituted Y and X values, respectively. Viability reduction percentages were calculated with the following equation:

$$L = \frac{P_c - P}{P_c} \; x \; 100 \qquad (2)$$

where L is the percentage reduction of algal population viability for a given test-concentration replicate;
P_c is the mean percentage of living cells for the control; and
P is the percentage of living cells in a given test-concentration replicate.

Correspondence in viabilities of algal populations determined by the FC-FDA and CFU assays was assessed with the correlation coefficient of linear regression on the respective percentages of living cells using the SigmaStat™ program (Jandel Scientific, San Rafael, CA). Concordance between the two methods was also evaluated by comparing the respective 96 h LC_{50} values for Cu^{2+} and their associated 95% confidence intervals, as well as the respective concentration-lethality slopes with a Student's *t* test.[22]

11.3 RESULTS AND DISCUSSION

11.3.1 Optimal soft growth medium

While a liquid growth medium is ideal for the reproduction of algal cells, the medium in its liquid state is inadequate for the subsequent enumeration of colonies because it is too vulnerable to shocks. When a Petri dish filled with the liquid growth medium is subjected to even the slightest shock, colonies present spread out and colony overlapping results, rendering enumeration impossible. However, initial plating and incubation of approximately one hundred algal cells on standard growth medium solidified with 1% agar, performed as described by Environment Canada,[21] demonstrated that none of the plated cells generated colonies (results not shown). Green algae being aquatic organisms, low water content of the solid medium obviously became the suspected cause of absence of colony formation. Preliminary development of a growth medium with an intermediate solidity, ie, both providing optimal reproduction conditions and sufficiently resistant to the shocks inherent in the CFU method, was deemed essential. Results on the success of colony formation for the different solidified media assessed are presented in Table 11.1.

Table 11.1 *Effect of agar content in growth medium on the reproduction of Selenastrum capricornutum cells*

		Agar content (g 100 ml^{-1})				
		0.0625	0.1	0.25	0.5	1.0
Nominal quantity of algal cells plated		104	104	97	97	97
No. of colonies formed	rep.1	88	88	(50)[A]	27	0
	rep.2	75	85	(50)[A]	50	0

[A]Approximate values. Actual number of colonies formed could not be determined with accuracy due to overlap in a certain proportion of colonies.

With an agar concentration of 1.0%, none of the 97 algal cells placed generates a colony. Rate of success of colony formation increases as the agar content decreases from 1.0 to 0.25% and reaches a plateau somewhere between agar concentrations of 0.25 and 0.1%. Assuming that colony formation rate of success remains constant within the range of 0.1 to 0% agar, it seems unlikely that growth media of agar content lower than 0.0625% would further stimulate colony formation. For this reason, optimization experiments were not pursued any further. Since the number of colonies formed on

the 0.1 and 0.0625% agar media are similar, the highest agar concentration, ie, 0.1%, was taken for the subsequent comparative assessment in order to decrease the probability of colony overlapping caused by the inevitable shocks encountered during incubation and enumeration. With this agar concentration, the state of the media is more of a creamy liquid than solid type and algal colonies can be detected by the naked eye after approximately 9 d of incubation.

Colony appearance following plating of algal cells on 1% agar growth medium, as performed routinely in our laboratories (results not shown), is likely attributable to the relatively large cell concentration in the inoculum (ie, 100 µl of a 2.5×10^6 cells ml^{-1} stock culture). Quantification of colony formation success under these conditions and verification of the hypotheses that explain it were beyond the scope of this study. Nonetheless, it is possible that cells which eventually give rise to colonies benefit from the protection against dehydration given by the surrounding cells. Assimilation by eventual colony-forming units of water, micro- and macromolecules released by the surrounding cells following their death constitutes another possible explanation. Finally, colony formation potential may be linked to the cell's genetic constitution, ie, only cells of genetic constitution conferring the ability to adapt to the hostile growing conditions will survive. If so, stronger strains might have been isolated accidentally with the successive subculturings made over the years in our laboratories, and assays presently performed might be involving a *Selenastrum capricornutum* strain with a different resistance to toxic substances than that of the original culture. Verification of this latter possibility could be the subject of a future investigation.

11.3.2 Acquisition threshold setting

Selection of cells of interest and discrimination between positive and negative events may constitute a major source of variation in flow cytometric analysis of immunologic markers.[23] Therefore, not only are guidelines essential for obtaining consistency in analytical results, but these guidelines must also remove as much subjectivity as possible. In this study, development of guidelines for gating the sub-population of living cells was undertaken with this concern in mind. Guidelines were defined from histograms of populations not subjected to any test substance, ie, the QC microplate control populations. Control populations of the copper microplate were not used for this purpose because of the possibility of contamination from adjacent test wells, as observed in the past by Thellen *et al.*[24]

When the gain and level of the FSC detector are respectively set to E-01 and 100 on the flow cytometer model used in this study, FSC histograms of FDA-stained QC populations present a bimodal curve (Figure 11.1). The convexity located at the right on the graph corresponds to the algal cells. The portion of the curve located to the left of this convexity is associated with noise and small size particles, including cell debris. A first region (ie, R1) demarcating algal cells was defined so as to exclude the data due

to noise or small size particles. Region R1 was defined from the superimposed FSC histograms of the FDA-stained QC control replicates (Figure 11.1).

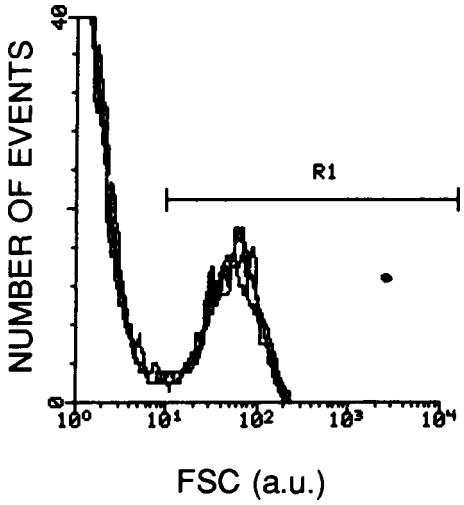

Figure 11.1 *Typical superimposed histograms in logarithmic scale of forward angle light scatter (FSC) as obtained by flow cytometric analysis of QC control algal population replicates (n=6) incubated with FDA. Abscissa values are expressed in arbitrary units (a.u.). All curves were smoothed by a factor of 5. Region R1 boundaries demarcate events that correspond to algal cells (see text).*

Superimposed FSC histograms, rather than only one, were used in order to integrate the variability of the QC control replicates in the demarcation of R1. Furthermore, because fluorescence emitted from cells disrupts the forward angle light scatter,[25] a superimposition made up of a mixture of histograms from both stained and unstained algal suspensions was not suitable. Since the subsequent determination of living cell proportions would bear solely on marked cells, histograms of the FDA-stained QC controls, exclusively, were selected to define R1. Lower limit of R1 was positioned using the 'deepest valley' technique[20] and upper boundary was set beyond the maximal abscissa (Figure 11.1). All algal populations were live gated, ie, only the first 2000 events of each population falling in R1 were stored. Subsequent delimitation of regions R2 and R3 (see below) was therefore accomplished from solely R1 events of FDA-stained QC control replicates.

11.3.3 Algal esterase cleavage of FDA

Mean green fluorescence intensity of a healthy cell suspension increases significantly when cells are stained with FDA (Figure 11.2*(a)* and *(b)*.

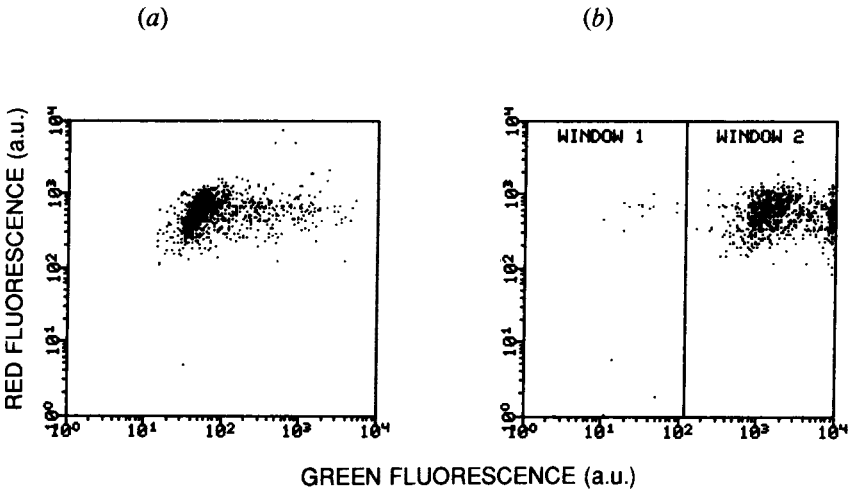

Figure 11.2 *Esterase cleavage of FDA by a control population of S. capricornutum originally contained in the quality control microplate (see text), as shown with dot-plots resulting from flow cytometric analysis, (a) and (b) are dot-plots of the control population before and after staining with FDA, respectively. Each dot-plot contains a total of 2000 events. Cell debris was excluded from the computer analysis by setting an acquisition threshold. Abscissa and ordinate values are arbitrary units (a.u.) of green (530 nm) and algal chlorophyll red (620 nm) fluorescences, respectively.*

Window 1 of Figure 11.2*(b)* contains cells with minimal green fluorescence and represents individuals that did not convert FDA to the green fluorescent compound fluorescein or that did not retain the fluorescein formed in the cytoplasm. These cells possessed no esterase activity and/or an altered membrane that could not hold back fluorescein.[3] In both cases, cell integrity has been affected. Window 2 encompasses cells that converted FDA and retained fluorescein at different levels.[26]

Magnitude of the green fluorescence emitted by window 1 cells ranges from approximately 10 to 100 a.u. This corresponds exactly to the green fluorescence intensity of the majority of control cells before staining with FDA (Figure 11.2*(a)*. The small amount of green fluorescence observed in window 1 cells is thus from background autofluorescence of the photosynthetic pigments.

Moreover, it should be noted that staining with FDA does not modify red fluorescence emission by algal cells as indicated by the similarity between the algal population red fluorescence patterns before and after FDA staining (Figure 11.2*(a)* and *(b)*. This has the advantage of allowing the measurement of red autofluorescence directly on FDA-stained

cells and therefore eliminating the necessity of subjecting unstained treated cells to flow cytometry analysis.

3.4 Effect of Cu^{2+} on optical properties of algal cells stained with FDA

Generally speaking, mean green and red fluorescence intensity of algal cells stained with FDA decreases with increasing concentrations of Cu^{2+}. This phenomenon is illustrated by a dot shift from the upper right to the lower left corner on a green *vs* red fluorescence graph (Figure 11.3).

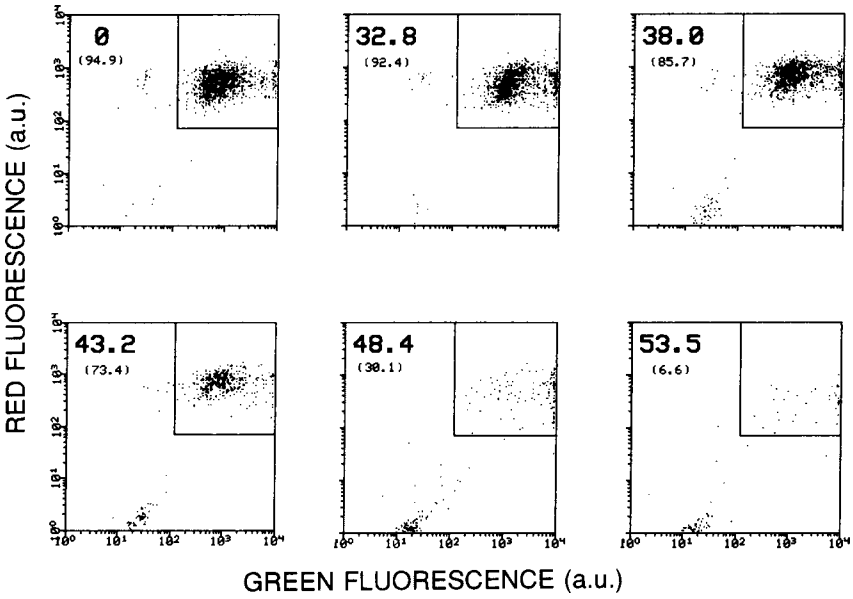

Figure 11.3 *Effect of Cu^{2+} on optical properties of S. capricornutum after a 96 h exposure, as shown with dot-plots resulting from flow cytometric analysis. Each dot-plot contains a total of 2000 events. Cell debris was excluded from the computer analysis by setting an acquisition threshold. Abscissa and ordinate values are arbitrary units (a.u.) of green (530 nm) and algal chlorophyll red (620 nm) fluorescences, respectively, after post-exposure incubation with FDA (see text). Large numbers inside graphs refer to Cu^{2+} exposure concentrations in $\mu g\ l^{-1}$. Events in upper right quadrant are defined as living cells. Percentages of living cells in algal populations are designated by the numbers in parentheses.*

Reduction of green fluorescence by algal cells exposed to Cu^{2+} is probably attributable to the cell's inability to hold back fluorescein since it has been demonstrated that copper, like other heavy metals, affects the permeability of phytoplankton plasma membranes.[27] Moreover, the fact that the number of algal cells emitting low intensity red fluorescence increases as Cu^{2+} concentration augments indicates a gradual disturbance of photosystem II. As such, copper has been shown to affect photosynthesis of algal cells by directly inhibiting photosynthetic activity or causing a loss of photosynthetic pigments.[27] It is notably because of these destructive effects that copper, in its copper sulfate form, is proposed as an algicide to control or prevent algal growths, particularly waterblooms.[28] Regardless of the mechanisms by which copper poisons algal cells, exposure to the inorganic toxicant results in the formation of three sub-populations: cells emitting less intensively solely in the green, both in the green and the red, or solely in the red.

11.3.5 Living cell percentage determination

The strategy employed for the development of the flow cytometric FDA viability assay consisted in defining living cells using optical criteria and of examining whether living cell percentages generated as a result of the given definition correlated with those determined by the CFU method. Since it was suspected that cell viability (and/or fertility) of individuals with reduced green or red fluorescence was affected, a living ceil was defined as a cell capable of emitting green fluorescence, after staining with FDA, and red fluorescence at levels greater than or equal to that given out by standard FDA-stained control cells. With this definition of cell viability, the difficulty of discriminating between the contribution of autofluorescence and fluorescein fluorescence in the green region of the visible spectrum[29] is bypassed. Moreover, addition of the red fluorescence criteria in the viability definition given above reduces the probability of reporting false positives: ie, a non-viable cell that does not accumulate fluorescein but emits strongly in the green and weakly in the red as a result of an alteration to photosystem II,[30] will not be classified as living. Likewise, a non-viable cell that does not accumulate fluorescein but for which chlorophyll-borne red (auto)fluorescence takes some time to fade out after the toxic aggression,[31] will not be classified as living.

Discrimination between living and dead cells was achieved firstly by defining two additional regions (ie, R2 and R3) and combining them with a logical operator. Once again, R2 and R3 were defined from histograms of populations not subjected to any test substance, QC microplate control populations in this case. The methods for discriminating between living and dead cells and for determining living cell percentage of an algal population are described below.

11.3.5.1 *Region R2 demarcation*

Frequency distribution of red, or chlorophyll, fluorescence of QC control populations after staining with FDA is illustrated in Figure 11.4

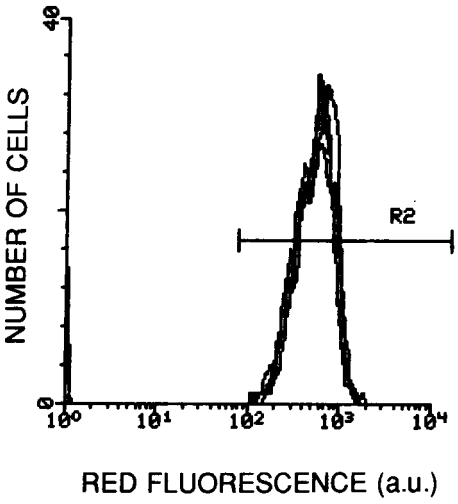

Figure 11.4 *Typical frequency distribution curves of red fluorescence emitted by the QC population replicates after staining with FDA (n=6). Abscissa values are arbitrary units (a.u.) of cellular chlorophyll red (620 nm) fluorescence. Each curve contains a total of 2000 events. Cell debris was excluded from the computer analysis by setting an acquisition threshold. All curves were smoothed by a factor of 5.*

Region R2 demarcation was made from these superimposed histograms. Lower limit of R2 was positioned in the first empty channels located at the left of the superimposed FL3 convexities and, as for R1, upper boundary was set beyond the maximal abscissa (Figure 11.4). Emission of red fluorescence by an algal cell at an intensity lower than the lower limit of R2 indicates that photosystem II has been somehow affected following exposure to the test substance.[32]

11.3.5.2 *Region R3 demarcation*

As mentioned earlier, global green fluorescence emitted by an healthy cell population increases significantly when the individuals that make up that suspension convert FDA into fluorescent fluorescein (Figure 11.5). Lower limit of R3 was fixed from the superimposed green fluorescence signatures of solely FDA-stained QC control replicates in accordance with the same guidelines as those described for R2 (Figure 11.4). Marked cells emitting below the green fluorescence threshold correspond to individuals with impaired cell integrity, ie, for which esterasic activity has been inhibited and/or cell membrane altered.

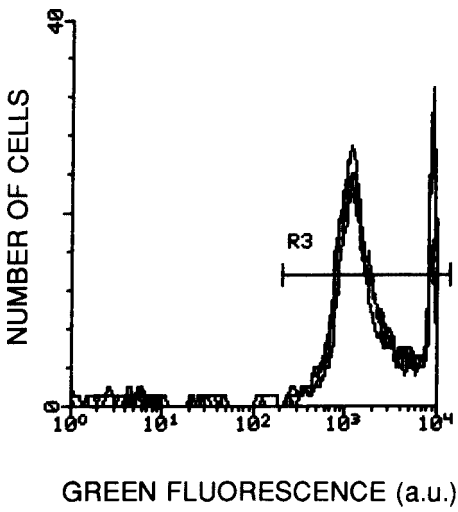

Figure 11.5 *Typical frequency distribution curves of green fluorescence emitted by the QC population replicates after staining with FDA (n=6). Abscissa values are arbitrary units (a.u.) of cellular green (530 nm) fluorescence. Each curve contains a total of 2000 events. Cell debris was excluded from the computer analysis by setting an acquisition threshold. All curves were smoothed by a factor of 5.*

11.3.5.3 *Determination of the proportion of living cells*

Algal population viability was readily quantified with the LYSIS™ II program. It is expressed in 'percent living cells' and corresponds to the percent ratio of the number of living cells on total number of cells. Quantity of living cells was obtained by combining R2 and R3 with the logical operator 'and', and enumerating the number of events in the resulting set intersection. On a green fluorescence *vs* red fluorescence dot-plot, living cells are found in the upper right quadrant whose boundaries correspond to the respective lower limits of R2 and R3 (Figure 11.3).

11.3.6 Flow cytometric FDA assay *vs* CFU method

Selenastrum capricornutum population viabilities obtained with the colony-forming unit and flow cytometric procedures following exposure to different concentrations of Cu^{2+} are shown in Figure 11.6. Greater than 100% values for viability percentages determined by the CFU assay are attributable to the fact that the number of cells plated is a nominal, rather than measured, value. Indeed, CFU viability percentage is derived in part from the number of cells plated, which in turn is derived from the product of the algal concentrate cell concentration and the dilution performed to generate the 100 cells ml^{-1} suspension (see section 11.2 Materials and Methods). Deviations from the 100% value are thus explained by the precision (ie, variability) of the cell enumeration technique

and the magnitude of the dilution factors, the latter of which ranged from 400 to ~11,000. Actual numbers of cells plated were therefore, at random, slightly higher or lower than the estimated quantity.

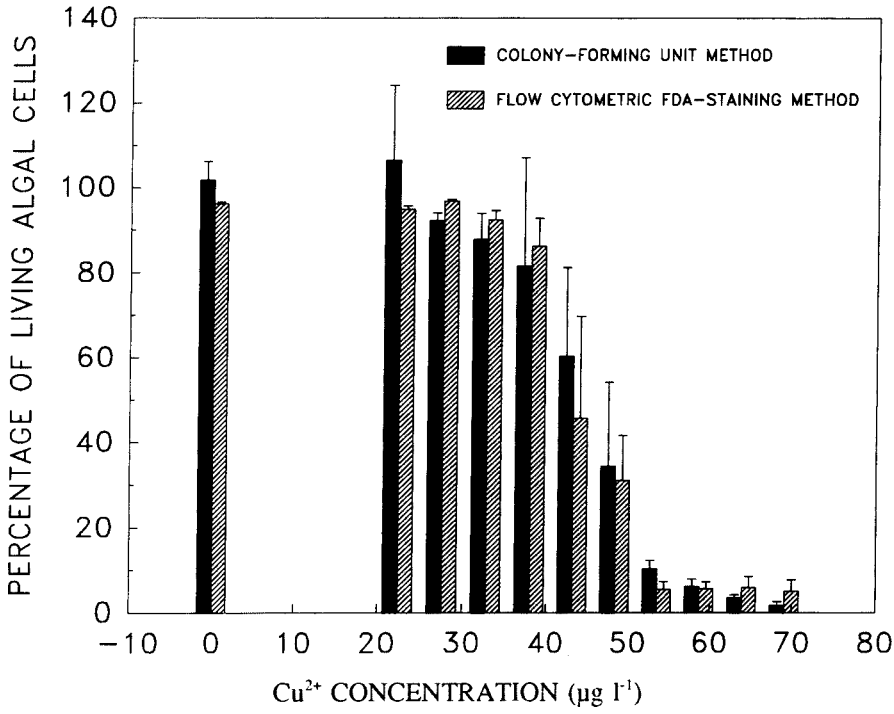

Figure 11.6 *Selenastrum capricornutum population viability as determined with the colony-forming unit* ■ and flow cytometric FDA ▨ *methods following exposure to different concentrations of* Cu^{2+}. *Shown is the mean±standard deviation. In all cases n=3, except for controls where n=6.*

Nonetheless, Figure 11.6 results are indicative of good correlation between both assay procedures in quantifying Cu^{2+} lethal toxicity to *S. capricornutum,* as shown by the similarity in viability percentage values reported. This good correlation is supported by the strong resemblance in LC_{50} values produced by each assay and the overlap in respective 95% confidence intervals, and by the fact that the concentration-response slopes generated by both techniques are not significantly different (Table x.2). Furthermore, linear regression on all CFU and flow cytometric percentage values is illustrated in Figure 11.7.

Table 11.2 *Statistical values for comparison of Cu^{2+} concentration-lethality curves derived from colony-forming unit and flow cytometric FDA assay results*[A]

	Concentration–lethality regression		Slope comparison		
Assay	96 h LC_{50} (95% c.i.[B]) ($\mu g\ l^{-1}$)	r^2 value[C]	| Calculated | t	Critical value of t	Degrees of freedom
CFU	44.9 (35.2–54.1)	0.74			
			0.006[D]	3.622	32
FC–FDA	44.6 (37.8–51.2)	0.84			

[A]Data are from Figure 11.6
[B]c.i.: confidence interval
[C]r^2: coefficient of determination
[D]Indicates that $ß_{CFU}$ is not significantly different from $ß_{FC-FDA}$ at $p<0.001$

The high correlation coefficient obtained provides a clear demonstration of the strong association between the two variables (R=0.968). As can be observed, the density of points between the two extremes of the regression line is slightly low. However, with reference to other method comparison studies that relied exclusively on either totally viable or totally non-viable cell suspensions,[8,13,14] the presence of several points in the middle portion of the regression line in our study increases the strength of the comparative assessment. In this respect, future comparative experiments using a narrower copper concentration interval, in order to increase the number of points in that middle portion, would be useful for confirming the interprocedural correlation demonstrated in this study and for evaluating the repeatability of the flow cytometric assay.

Finally, the 96 h LC_{50} value of Cu^{2+} generated by the flow cytometric FDA assay (Table 11.2) compares favorably with that of Ménard[33] where experimental conditions closely matched our own but where the trigger used for quantifying live and dead cell proportions was solely red (chlorophyll) autofluorescence intensity (LC_{50} of Cu^{2+}=42 $\mu g\ l^{-1}$, 95% confidence interval of 30 to 46 $\mu g\ l^{-1}$, r^2=0.92).

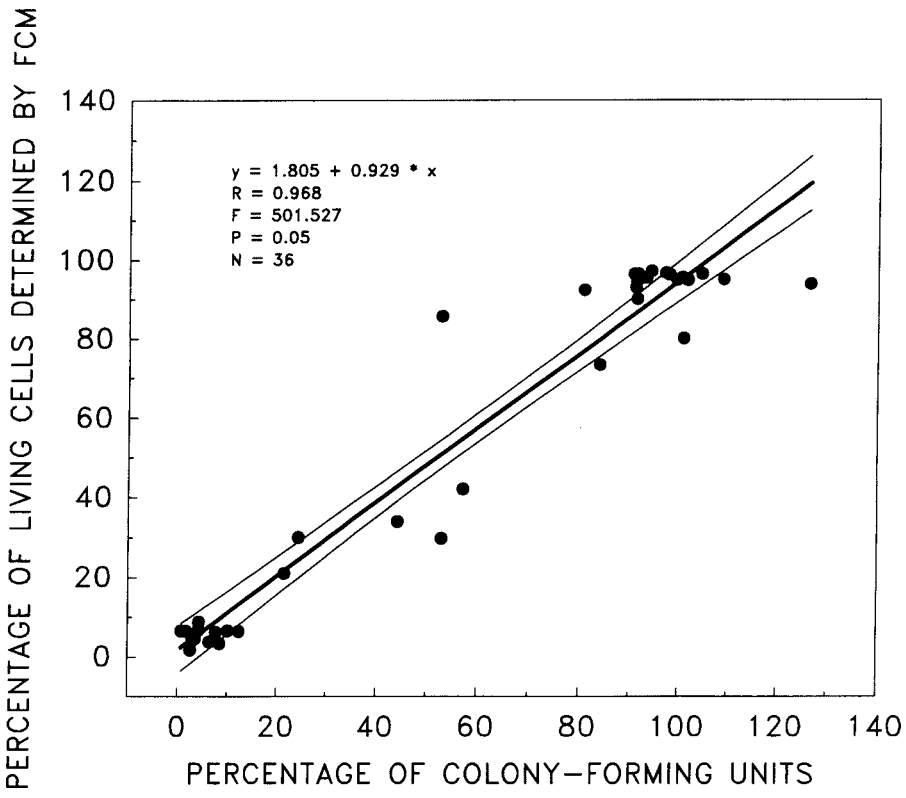

Figure 11.7 *A comparison of colony-forming unit and flow cytometric FDA assay results based on simple linear regression of the Selenastrum capricornutum data presented in Figure 11.6. Regression line and 95% confidence bands are depicted by the heavy and light lines, respectively.*

11.4 SUMMARY AND CONCLUSION

Reliable laboratory assays for assessing the proportion of both viable and fertile individuals in a population of microalgae would contribute valuable information to environmental protection programs. While the colony-forming unit technique provides unequivocal evidence of viability, however, it is labor-intensive and, inefficient when high sample throughput is required, because of the long waiting time before colony appearance. Hence, the search for novel viability assessment procedures capable of increasing sample processing at lower cost is a justifiable objective. Because flow cytometry can provide a fast, unbiased quantitative assessment of the proportions of different sub-populations in a cell suspension, an attempt was made to develop a method exploiting flow cytometry, along with cellular green and red fluorescence emissions after staining with fluorescein diacetate, for assessing microalgal population viability. Living cell percentages generated by this flow cytometric FDA assay were then compared to those produced by the CFU method. The general findings of this study can be summarized as follows:

(i) Initial plating and incubation of approximately 100 algal cells on algal growth medium solidified with standardized agar concentration of 1% demonstrated that none of the plated cells generated colonies;
(ii) Subsequent experiments on the development of an optimal solidified growth medium indicated that the highest agar concentration for which colony development was still optimal was 0.1% and proved to be adequate for cell plating and subsequent enumeration even if the state of the medium was more of a creamy liquid than solid type;
(iii) Specific flow cytometric guidelines relying on both algal red autofluorescence and FDA-induced green fluorescence were developed for objectively discriminating between living (ie, both viable and fertile) and non-living algal cells;
(iv) High correlation between living cell percentages generated by the flow cytometric FDA and CFU methods ($R=0.968$), as well as strong resemblance between respective LC_{50} values for copper (ie, 44.6 and 44.9 $\mu g\ l^{-1}$, respectively) and concentration-lethality slopes ($p<0.001$), indicated excellent interprocedural concordance; and
(v) The interprocedural viability assay comparison described here, although based on only one inorganic reference toxicant, suggests that the more practical flow cytometric FDA assay is a suitable alternative to the traditional colony-forming unit method (2 h *vs* 4 h execution times and 2 h *vs* 9 d measurement endpoint waiting periods, respectively) for evaluating the proportion of viable and fertile individuals in a population of microalgae.

Now that this flow cytometric FDA assay for assessing microalgal population fertility after a 96 h exposure period has been validated, a future study could investigate whether the FC-FDA assay can accurately estimate the number of dead individuals after a shorter exposure to a toxicant (eg, 4 h). If such an investigation proved conclusive, the short-term FC-FDA lethality test could then be helpful for prompt prediction of the recovery of phytoplankton populations exposed to wastewaters, and consequently facilitate decision-making for immediate remedial measures. Likewise, successful adaptation of the FDA-based lethality procedure to microplate fluorometry, by allowing algal lethality quantification at a lower analytical cost, would also be of value.

11.5 ACKNOWLEDGEMENTS

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SECTION 4: APPLICATION OF MONITORING TECHNIQUES

12 The Role of UNIDO in Ecotoxicology Monitoring in Developing Countries

Balasubramanyan Sugavanam

12.1 INTRODUCTION

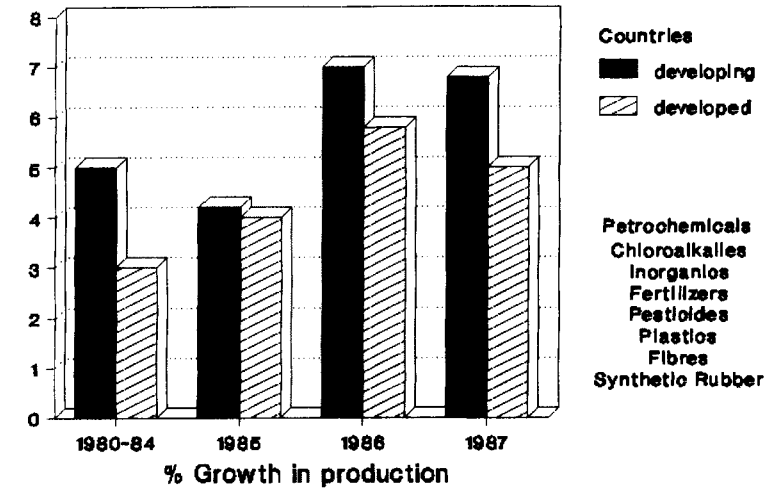
Notwithstanding the criticism regarding the detrimental effects of manmade chemicals in the environment, it is recognized that in any country or community the *per capita* production and consumption of chemicals determine the standard of living and are essential for the basic necessities of life. According to a United Nations Industrial Development Organization (UNIDO) Global Report,[1] in the 1980s the percentage growth of industrial chemicals in developing countries was higher compared to developed countries (Figure 12.1). It is evident that the 1990s, with the expansion of the Chinese economy, the liberalization of the Indian trade and the signing of the NAFTA Treaty,[2] there will be an even greater increase in the production and consumption of chemicals in the developing world, especially in Asia and Latin America. It is necessary to emphasize the ecological problems that could be envisaged with the production of chemicals and their use in various outlets such as agriculture, pulp and paper industries, paint industries, electroplating, leather tanning, textile industries, construction industries, electronic industries, etc.

12.2 MANDATE OF UNIDO

The mandate of UNIDO is to assist developing countries in their aspirations for industrial development. Under this, great stress is given to chemical and allied industries because of their importance to the overall economy. With mounting concern over chemical pollution and fast consumption of the earth's non-renewable resources, UNIDO has embarked on an Ecologically Sustainable Industrial Development (ESID).[3] The main thrust areas are:

- Cleaner technologies
- Waste minimization
- Biotechnology
- Integrated industrial safety
- Energy and value added products from renewable resources
- Ecotoxicology
- Environmental and economic issues.

Industrial chemicals



Other chemicals

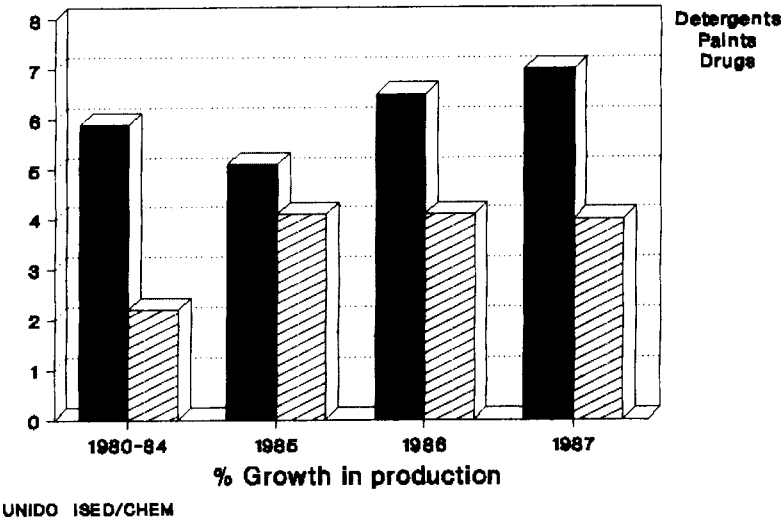


Figure 12.1 *Chemicals' growth in the 1980s*

12.3 CONCEPT OF ECOTOXICOLOGY IN DEVELOPING COUNTRIES

The concept of ecotoxicology is new to developing countries and considering that many developing countries were facing a variety of other problems, ecotoxicology issues due to contamination of the environment by manmade chemicals was low in their priorities. This situation started to change during the 1980s when the consumption of agrochemicals increased significantly in developing countries. For example. Table 12.1 shows the average growth of pesticides worldwide during the last 30 years. In the 1980s most of the increase in consumption was for Asia and Latin America.

Table 12.1 *Average growth of pesticides from 1960–*

	1960 (US$ million)	1990 (US$ million)	Average annual increase (%)
Herbicides	170	11,625	16.6
Insecticides	310	7,655	12.4
Fungicides	340	5,545	11.5
Plant growth regulators, etc	30	1,575	12.1

With the introduction of registration procedures for pesticides in many developing countries, issues related to ecotoxicity are often raised and data are obtained from the manufacturers. Data required for pesticide registration became more stringent over the years as shown in Table 12.2. During the 1990s some countries require toxicology data related to pesticide formulations and on the so-called 'inerts'. The developing countries became increasingly aware of ecological aspects of chemicals due to industrial pollution especially for pesticides.

There are two distinct groups to be considered:

(i) The agrochemicals which are deliberately introduced into the environment for which responsibility lies with the producers and the users; and

(ii) All other chemicals where industry has almost the sole responsibility in controlling discharges at source.

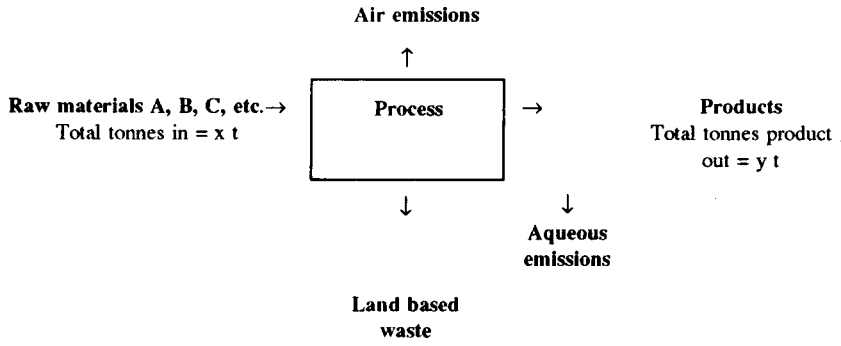
Industrial activity is linked to industrial pollution and if this is not properly handled it will have an immediate and long-term effect on ecosystems. The simple diagram (Figure 12.2) shows the basic mass balance for any industrial process. In many developing countries, especially in medium and small scale industrial operations, this mass balance is largely neglected. It is noticeable in a simple safety review in such industrial premises and hence ecotoxicology problems could be a consequential factor.

In addition to pesticides other industries, especially the metal extracting industries and the pulp and paper industries in the organized sector and the textile dyeing and leather tanning industries in the unorganized sectors, have a poor record in developing countries in meeting effluent limitation standards.

1950	1960	1970
Toxicology		
Acute toxicity 30–90 d rat	Acute toxicity 90 d rat feeding 90 d dog feeding 2 y rat feeding 1 y dog feeding	Acute toxicity 90 d rat feeding 90 d dog feeding 2 y rat feeding 2 y dog feeding Reproduction 3 rat generations Teratogenesis in rodents Toxicity to fish Toxicity to shellfish Toxicity to birds
Metabolism		
None	Rat	Rat, dog and plant
Residues		
Food crops 1 ppm	Food crops 0.1 ppm Meat 0.1 ppm Milk 0.1 ppm	Food crops 0.01 ppm Meat 0.1 ppm Milk 0.005 ppm
Ecology		
None	None	Environmental stability, movement, accumulation. Total effect on all non-target species.

Table 12.2 *Registration requirements for pesticides over three decades*

In the 1970s and 1980s there was a revolution in the invention of highly active pesticides which practically reduced the rates used from kg ha^{-1} to <100 g ha^{-1} and in some cases even to the level of 5–10 g ha^{-1}. The driving force was the progress in the specificity to pests and safety to mammals. However, attention was not given to ecotoxicology and, more recently, to environment toxicology assessment; some of these so-called safe compounds had side effects in ecosystems. In addition to the lack of toxicology knowledge many chemicals that enter ecosystems require appropriate monitoring and necessary measures taken for hazard identification, risk assessment, risk elimination and management of acceptable risks.



Total tonnes in=total tonnes out
x=y+z; z=loss from process;
z=air emissions+aqueous emissions+land based waste

Figure 12.2 *Basic mass balance for process*

12.4 UNIDO ACTIVITIES

With its mandate linked to industrial development UNIDO programs are geared to provide the catalytic effect for promoting safer and cleaner technologies in developing countries. In this, ecotoxicology monitoring is one of the recent activities on which UNIDO is placing great emphasis.

12.4.1 Agrochemicals

With the increasing activity in the agrochemicals sector the major concern for developing countries was ecotoxicology related to the use of pesticides. This was further strengthened when many developing countries in Asia became food exporting countries. The emphasis is now equally on mammalian toxicity and ecotoxicity, in order to provide environmental assessments.

12.4.2 Assistance to China and South Korea

During 1980, the People's Republic of China approached UNIDO to assist in strengthening the Toxicology Center located near Shenyang. With the financial support of the United Kingdom, UNIDO provided international expertise, equipment and training, much of which concentrated on mammalian toxicity for chemicals to be registered in China for agriculture outlets. The Toxicology Center was modelled after An Pyo Center of Japan and was completed in 1987.[4]

Following this. South Korea wished to upgrade their facilities for generating toxicological data for chemicals to be registered in South Korea and also to assist government institutions and the local industry in generating toxicological data applicable to local conditions. The principal objective of the project was to support the national program for the protection of human health and the environment from chemicals and industrial pollutants in Korea. The aim was to upgrade and broaden the capacity of the toxicological research center of Korean Research Institute of Chemical Technology to perform toxicity testing of chemicals at the level of international standards within a few years.

The target beneficiaries were:

(i) The personnel of the Toxicology Research Center who would gain experience and knowledge in conducting toxicity testing according to international standards;
(ii) The industries involved in production of chemicals for various outlets;
(iii) The consumer and general public who would be protected from any hazard associated with the use of chemicals; and
(iv) The environment, protected from any adverse effect due to accumulation of xenobiotics at damaging concentrations.

UNDP/UNIDO's assistance from 1984–1992 consisted of:

- Preparatory assistance to assess requirements and exposure to facilities abroad;
- Preparation of the facilities;
- Recruitment and training of key personnel;
- Sending the staff abroad for training;
- Recruitment of foreign experts;
- Procurement and installation of equipment;
- Undertaking toxicity testing; and
- Setting up the operational systems.

Today, the Institute is one of the best equipped laboratories with highly trained staff in Asia outside of Japan. The Institute from 1989–1993 has tested ~500 compounds according to international or Korean standards. Apart from mammalian toxicity the Institute can test for aquatic toxicity as follows:

Acute toxicity 96 h	Fish	Korean GLP
Acute toxicity 96 h	*Daphnia*	Korean GLP
Acute toxicity 96 h	Algae	Korean GLP
4 w Subacute	Fish	In development
Reproduction 3 w	*Daphnia*	In development
Environmental chemistry		In development

The ultimate aim is to reach GLP standards according to OECD requirements.

12.4.2 Regional network on pesticides for Asia and the Pacific (RENPAP)

In the early 1980s UNIDO, supported UNDP, brought nine Asian countries together which had similar problems in the development of pesticides at that time. This led to the formation of a Regional Network on Pesticides for Asia and the Pacific (RENPAP). In the early days it covered many topics relevant to the region and included the harmonization of data required for toxicity and registration of pesticides. The nine countries were Afghanistan, Pakistan, India, Sri Lanka, Bangladesh, The Philippines, Indonesia, Thailand and South Korea. In this network, South Korea and India were at that time relatively more advanced in pesticide production. During this period the region also witnessed phenomenal growth in the production and use of pesticides.

Due to benefits accrued in the network project more countries joined the network during the late part of 1980 and the early part of 1990. Today the network has 15 member countries covering almost half the world's population which depends on agriculture and 20% of the earth's surface. Obviously the network evolved according to the changes that occurred in pesticide development with emphasis on low risk/low volume pesticides in support of integrated pest management (IPM), industrial safety, waste minimization/waste management, ecotoxicology, etc. While the central coordination unit was established in New Delhi, the technical areas were decentralized to member countries in the form of technical coordination units as shown below:

Pesticide formulation technology Quality control	India
Impurities in technical grade pesticides	South Korea
Bio- and botanical pesticides	Thailand
Industrial safety and waste management	Indonesia
Industrial hygiene and occupational safety	Philippines
Application technology	Malaysia
Ecotoxicology	Pakistan
Pesticide data collection dissemination	Thailand/India

This technical coordination was assigned based on the facilities and infrastructure already available in the countries. For those countries which did not have the infrastructure UNIDO provided the necessary upgrading of the country's facilities. Supported by UNDP, facilities in India on pesticide formulation were upgraded. At the same time Pakistan needed to strengthen their capability within ecotoxicology.

Initially, UNIDO embarked on an appraisal mission and identified the Pakistan Agricultural Research Council (PARC) to be the focal institute. Supported by the Government of Denmark, UNIDO is providing high-level technical advice and training, modernizing the PARC laboratories and also in the selection of suitable research projects on ecotoxicology. Four major laboratories have been created at PARC:

(i) Terrestrial Ecology Laboratory;
(ii) Agrochemicals Microbiology Laboratory;
(iii) Chemical Analysis Laboratory; and
(iv) Sample, storage/Preparation Laboratory.

These laboratories will be fully functional by 1995 and will be able to undertake ecotoxicology and environmental monitoring of pesticides of concern to Pakistan in collaboration with industries and government institutions. The Center was inaugurated by his Excellency the President of Pakistan during March 1994 and in recognition of the event UNIDO organized a workshop on ecotoxicology for the benefit of the Asian region.[5] The program consisted of lectures from leading international experts on ecotoxicology followed by panel discussions on:

(i) Ecotoxicology related to water (aquatic toxicology, groundwater contamination);
(ii) Terrestrial ecology and soil contamination; and
(iii) The need for assistance to Asia and the Pacific countries in monitoring movement and fate of xenobiotics in ecosystems.

The interest shown by the Asian countries was so overwhelming that UNIDO had to restrict the number of participants. The workshop gave an excellent opportunity to member countries of the region to discuss among themselves and with international experts the importance of ecotoxicology monitoring of chemicals causing concern. The workshop concluded that ecotoxicology, being a multifaceted field, would need inter-ministerial coordination and also inputs from international agencies and non-governmental organizations to assist the member countries in hazard identification, risk assessment/reduction and management of risks associated with the large use of agrochemicals and then extend the experience to other industrial pollutants. Among the various recommendations made, one recommendation requested formation of a working group to consider and advise on priorities for work on ecotoxicology in the region and that RENPAP should prepare terms of reference for such a working group which should include representatives of industry.

As previously mentioned, UNIDO assistance should be catalytic and as a result of the Pakistan workshop The Philippines organized a similar workshop on ecotoxicology 17–19 October 1994. Malaysia will be organizing a workshop in 1995 on aquatic toxicology. The fact that the Far East region depends significantly on the fishing industry necessitates studies on aquatic toxicology and soil contamination which affect the waterways. Thus, the region needs appropriate ecotoxicology monitoring to avoid any catastrophe and requires a good database providing information. RENPAP is setting up

a pesticide database in collaboration with the Economic and Social Council for Asia and the Pacific (ESCAP) covering data on toxicology of all pesticides and those formulations used in the region which could be easily accessed by the RENPAP member countries.

12.4.3 Effects of war on the environment

Memories of the Gulf War are still apparent, especially the burning oil wells left behind by the retreating Iraqi army and this brought to the forefront the implications of manmade disasters to the environment. The marine environment is a key resource for Kuwait and other Gulf states; for Kuwait the sea is the principal source of drinking water and supports important fisheries. Potential major threats to the marine environment of Kuwait are the introduction of petroleum hydrocarbons via spills or chronic inputs from land-based sources of offshore activities, including transport and other contaminants from industrial or urban developments in the region. In addition, the Gulf War resulted in the release of large quantities of crude oil to the environment both on land and directly into the sea.

The Kuwait Institute for Scientific Research (KSIR) who were planning a five-year Strategic Plan in 1989 entitled 'Oil Pollution in the Marine Environment' suffered a total loss of their equipment and damage to buildings during the Iraqi occupation. After the war a Transitional Strategic Plan was developed and three areas of research were defined:

(i) Atmospheric and terrestrial;
(ii) Marine and coastal; and
(iii) Environmental risk assessment.

In order to assist Kuwait, UNDP/UNIDO was asked to provide technical assistance to ecotoxicology and marine ecology. The purpose is to assist the Kuwait Institute of Scientific Research to set up long-term research studies for monitoring movement and accumulation of toxic pollutants, their effects upon the environment and carry out model systems to eliminate/minimize the toxic effects to humans, animals, crops and aquatic life. The technical assistance has now commenced and will concentrate on making a bioassay laboratory fully functional to carry out:

(i) Acute and chronic toxicity tests for effluent monitoring and incident assessment; and
(ii) Research into the fate and effects of petroleum hydrocarbon in the Gulf environment, including the coral reefs. The studies would involve assessment of fate and effects and laboratory and field studies using model ecosystems.

In parallel, assistance will be given for a coral reef ecological study to investigate the structural and functional characteristics of Kuwait's coral reefs to natural and anthropogenic stressors. The program will include ecological aspects, ecotoxicology to immature and mature corals, data analysis and interpretation.

12.4.4 Croatia

The newly independent state of the Republic of Croatia, having suffered enormous damage to its infrastructure due to the Balkan War requested UNIDO's assistance to organize an international conference on 'The Effects of War on the Environment' and assess the damage to chemical industries and the consequent environmental damage. On behalf of UNIDO, Mervyn Richardson during early 1993 took the courage to visit a number of sites damaged by the war and prepared a report which was used as the basic document in the international conference held in Zagreb, April 1993. Mr. Richardson's mission on behalf of UNIDO visited 18 towns in Croatia identified ~70 installations damaged in some way by the war and which were a cause of concern to the environment. The chemicals ranged from polyaromatic hydrocarbons, polyhalogenated aromatics, pesticides, heavy metals, burnt oil, organic solvents, many inorganic, domestic and hazardous mixed wastes, etc. Based on the discussions with the Government of Croatia, UNIDO identified ~12 projects and one of them is to strengthen the capability of the country in ecotoxicology and environmental monitoring. Subject to the availability of funds the project to assist Croatia will be taken up by UNIDO.

12.5 EARTH SUMMIT AT RIO AND UNIDO

The Earth Summit convened in Rio de Janeiro, fully ratified Agenda 21 and dealt with two chapters—19 and 20—being specially relevant and concern the sound management of chemicals, especially toxic chemicals and environmentally sound management of hazardous waste.

Under the environmentally sound management of toxic chemicals five different programs have been identified:

(i) Expanding and accelerating international assessment of chemical risks;
(ii) Harmonization of classification and labeling of chemicals;
(iii) Information exchange on toxic chemicals and chemical risks establishment of risk reduction programs;
(iv) Strengthening national capabilities and capacities for management of chemicals; and
(v) Prevention of illegal traffic in toxic and dangerous products.

Under environmentally sound management of hazardous waste four program areas were identified:

(i) Promoting the prevention and minimization of hazardous waste;
(ii) Promoting and strengthening institutional capacities in hazardous waste management;
(iii) Promoting and strengthening international cooperation in the management of transboundary movements of hazardous waste; and
(iv) Preventing illegal international traffic in hazardous waste.

For the implementation of chapters 19 and 20 of Agenda 21 UNIDO, in collaboration with other international organizations, will be placing greater emphasis on ecotoxicology aspects related to chemical pollution in the developing world.

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13 Agroecotoxicology in the Developing Asian Region—Pakistan

Umar Khan Baloch and Muhammad Haseeb

13.1 ASIA AND THE ENVIRONMENT

The population of Asian developing countries doubled from 1.2 billion to 2.6 billion between 1950 and 1985. Currently, the figure is 3.1 billion out of the world total of 5.2 billion and is projected to increase to 3.3 billion by the year 2000. The overall economic growth in Asia has been impressive (>7%) but uneven due to high population growth; the poverty indices in these countries remain 30–45%. Of the >1 billion poor; earning <1 US$ d^{-1} $person^{-1}$ with 900 million living in this region. Over 700 million people in the region still do not have access to adequate food to lead a healthy and productive life.[1,2]

The Asian Region by any standards (economic, cultural, linguistic, geographic) is the most heterogeneous and populous in the world and has become host of 'mosts'. However, in recent years environmental degradation has emerged as a major global issue threatening the very viability of the earth's life-support system and the future of those who depend on it. The damage to the environment began with the industrial revolution and has continued. The environmental problem encompasses several factors:

(i) Rapid population increases;
(ii) Poverty in the developing world;
(iii) Depletion of natural resources;
(iv) Loss of biological diversity;
(v) Pollution of air, land and water;
(vi) Global warming; and
(vii) Ozone depletion.

These interact and amplify each other. Man in his insatiable drive for greater material gain is destroying the very surroundings of which he is a part and without which he cannot exist. The speed of this motivated destruction is much more higher in the heavily populated poor Asian countries.

The earth's pollution is mainly because of the growing release of chemicals, principally from burning fossil fuel and, secondly, from the steady increase in the use of biocides and toxic substances. This is result of economic production or modern technology to meet the needs of modern life style of increasing population. The increased carbon dioxide, dioxin, PCBs and pesticides are the major threats to life and the environment. Most are reported as having the potential to cause cancer and reproductive impairment. The major cause of environmental degradation is the unrestricted consumption of natural resources by the industrialized countries and the consequent waste produced. The population explosion in the third world runs parallel. To rectify this, effects are

underway for sustainable development, defined as 'development that meets the needs of the poorest without compromising the ability of future generations to meet their own needs'.[3] This aims at providing better standards of living without sacrificing the earth's ecosystem and its finite resources.

Associated with the problems of poverty is the equally serious issue of environmental degradation. The environmental management and economic growth must be perused simultaneously and complementary and reflected in their development priorities. Therefore there is greater need for socio-economic sustainability, enhanced conservation of natural resources and balanced demographic situation leading to promoting economic growth, reducing poverty, conserving environment, facilitating population planning with enhanced role of women.

Pakistan today is the world's 8th most populous (120 M) country with two most populous countries of the world as its immediate neighbours (China and India). The population growth rate in Pakistan (3.1%) is the highest among Asian and will double in 24 y as against 125 y for Japan. Wide spread illiteracy, poor health care, low labor force participation (50%), rapid rural to urban migration, deforestation and environmental degradation are the major problems in Pakistan. Pakistan like most of the Asian Regional countries has agro-based economy.[1,4]

13.2 AGROCHEMICALS IN ASIAN AGRICULTURE

Although the pollution is predominantly a problem of the industrialized developed countries but it is growing in the Asian developing countries through increased use of agrochemicals to produce more food to meet the demand of a growing population explosion and six fold annual economic out put (two trillion US$) between 1965 and 1985. In an effort to meet demand to produce more food the use of agrochemicals (fertilizers, pesticides and growth regulators) is predominant and an inevitable component of the crop production system. The use of pesticides and fertilizers plays a vital role in increasing crop yields in this region.

The world output of rice has multiplied to four times, for example an increase from 145 M tonnes in 1948 to 519 M tonnes in 1991 with a yield of 1.68 to 3.5 M tonnes ha^{-1}. Advances in the production of wheat were also envisaged mainly due to gigantic manufacture and use of agrochemicals resulting in a green revolution in the late 1960s when the yield of rice was only 2.23 M tonnes ha^{-1}. The rice yield increase in Japan from 6.5 to 8.0 M tonnes $hectare^{-1}$ is correlated directly to the use of agrochemicals which is highest in the world.

13.3 FERTILIZERS

As with most chemicals, fertilizers pose potential pollution hazards if used excessively or indiscriminately. It is very pertinent to consider the crop requirement for various nutrients and their actual uptake because the unutilized fertilizers can pollute the environment. To produce 5 tonnes of rice or wheat for each hectare ~368 and 305 kg

of nutrient fertilizers, respectively, are required. While production of 8.8 tonnes of grain the yield in a rice-wheat system is 663 kg of nutrients. The fertilizer use pattern in the developing countries of Asia indicate that the use of fertilizer nutrients is not very high and the chances for environmental pollution are remote. However, there is still a potential danger of pollution due to indiscriminate use of nitrogenous fertilizers in certain areas. The possible reason for nutrient environmental pollution are nitrate leaching, emission of gaseous nitrogen, ammonia volatilization and soil erosion.

The fertilizer nutrients used in Asia and the Pacific Region increased from 11 M tonnes (1968–70) to 52 M tonnes (1988–90), while fertilizer consumption in the world increased from 62 to 142 M tonnes during the same period. During 1990–91, China consumed 27 M tonnes of fertilizers ranking first in the world while India consumed 12 M tonnes occupying second position in the region and third in the world. While other major countries (Indonesia, Iran, Republic of Korea and Pakistan) in the Asian region consumed quantities >1 M tonnes of nutrients. The current fertilizer nutrient consumption in Asia averages at ~120 kg ha^{-1}. China and the Republic of Korea have the highest consumption of fertilizer nutrients (>500 kg ha^{-1}) while Indonesia, Malaysia, Pakistan and Bangladesh have ~100 kg ha^{-1} consumption. The fertilizer consumption shows a very steep ratio of consumption in the Asia Region. This has resulted in significant growth in agricultural production particularly major crops including rice, wheat, maize and coarse grain which account for >40% of the world cereal production and almost 90% of world rice production. The fertilizer (nutrient-wise) use in the selected Asian countries during 1990–91 is given in Table 13.1.

Pakistan, with a total geographic area of 79.6 M (M ha), and a 68 M ha of an arid and

Table 13.1 *Fertilizer consumption in selected Asian countries (1990–91)*[5]

Country	N	P_2O_5	K_2O	Total
Bangladesh	609	240	90	938
China	19,450	5,879	1,748	27,077
India	7,566	3,099	1,308	11,973
Indonesia	1,514	575	288	2,377
Iran	516	670	16	1,102
Korea DPR	655	159	18	832
Pakistan	1,472	389	33	1,893
Sri Lanka	94	31	51	174
Thailand	577	318	149	1,044
Vietnam	410	92	20	522
World	77,240	36,130	24,617	137,987

semi-arid region has a low rain fall and evapo-transpiration exceeds precipitation.Thus due to a net upward movement of water, dissolved salts from the lower soil region are being continuously brought upward. The bulk of fertilizers used in Pakistan are nitrogenous (Table 13.2). Urea is the single most used nitrogen source (80%). Most of this is applied at the time of planting and ~30% is top dressed which, after irrigation, moves downward (60 cm) but the majority remains within the 30 cm depth and is absorbed quickly by plants as nitrate.Thus there is very little scope for nitrate leaching.

Table 13.2 *Consumption of fertilizer—Pakistan* (in '000' N/tonnes)

Year	Nitrogenous	Phosphate	Potash	Total	Annual Change
1970–71	251.5	30.5	1.2	283.2	(-)8.0
1975–76	445.3	102.5	2.8	550.6	(+)29.4
1980–81	843.0	226.9	9.6	1079.5	(+)3.4
1985–86	1128.2	350.3	33.2	1511.7	(+)20.6
1986–87	1332.4	408.8	42.6	1783.8	(+)18.0
1987–88	1281.7	393.4	45.1	1720.2	(-)3.6
1988–89	1324.9	390.4	24.5	1739.8	(+)1.1
1989–90	1467.6	382.4	40.1	1890.1	(+)8.6
1990–91	1471.6	388.5	32.8	1892.9	(+)0.1
1991–92	1462.6	398.0	23.3	1883.9	(-)0.5
1992–93	1635.8	388.2	24.1	2148.1	(+)14.0

13.4 PESTICIDES

The major constraints to achieve higher crop yields are a wide range of pests (insects, diseases and weeds). Pakistan, along other Asian developing countries, is blessed with a diverse agroecology capable of growing successfully almost all types of crops. However, these conditions are also ideal in providing an environment conducive to varied types of pests. Crops in Pakistan are highly vulnerable to these enemies resulting in considerable loss of agricultural commodities both in quantitative and qualitative terms.

It has been estimated that on an average almost 30–40% of the crop yield at pre-harvest and 10–30% at post-harvest stage is lost or damaged by different pests and diseases. It is difficult to present statistically correct data due to losses by pests. However, in Pakistan most of references indicate aggregate pest losses at ~50% but the invisible losses are twice those reported. Currently, on the basis of most agreed figures, 50% loss (insects 20%, weeds 15%, diseases 10% and rodents 5%) the economic loss to crops in Pakistan is valued at PRS. 145 billion.

Since Pakistan was created, she faced several serious outbreaks of pests. During the 1950s many fields of rice crop were left unharvested and allowed to be grazed by cattle. As a result of the intensity of rice borer attack (77%) in Sialkot and Sheikhupura districts the damage amounted to PRS 40 million. During the 1960s the attack of *Pyrilla* on

sugarcane in the North West Frontier Province was so high that it reduced the sugar recovery to 5% and some fields were burnt unharvested. Wheat crop was attacked severely by rusts in 1978 and the yield was reduced by about 20%. In 1982, gram blight diseases totally deprived farmers of a gram yield.[5] While the cotton crops faced this crises the situation resulted from insect pest outbreaks (mainly cotton bollworms) resulting in yield losses >40%. The epidemic of Banana Bunchy Top Virus (1990–92) in the lower Sindh, completely destroyed many banana orchards, which were then replaced with sugarcane.

Under the natural ecological balance the natural enemies of pests keep the harmful species under control and reduces the possibility of an outbreak of pests. There are also insect species which predate upon useful insects, however there is balance in their forces. Useful insects continue to prey on harmful insects and pollinating plants but when pesticides are used useful and friendly insects are eliminated and harmful insects achieve superiority to destroy crops. Thus chemical control becomes an economic disaster for crop production. It is also an observed fact that useful insects are more susceptible to pesticides than the pests. Also natural enemies are slow in increasing their population because they contract larger amounts of poisons.[6] In Pakistan the best example of indiscriminate use of pesticides is chemical control of cotton insects whereby the ecological balance for cotton white fly was disturbed through the killing of its natural enemies. The white fly species became more abundant thereby creating crises for cotton production because it is the vector of cotton leaf curl virus. The cotton production during a single year (1992–93) was reduced to 7.8 M bales from 12.5 M bales.

13.4.1 Pesticide consumption

The availability of reliable data on pesticide consumption at the regional level is scanty. However efforts have been underway in the Asia and Pacific Region through Regional Network on Pesticides for Asia and the Pacific (Afghanistan, Bangladesh, People's Republic of China, India, Indonesia, Islamic Republic of Iran, Myanmar, Malaysia, Nepal, Pakistan, Philippines, Republic of Korea, Sri Lanka, Thailand and Vietnam) but so far this data is in the process of being compiled (see also Chapter by Sugavanam). The available figures show (Table 13.3) that the pesticide consumption in most of the regional countries increased substantially and the trends are reported in favor of increased use of pesticides.

In Pakistan the major result in government policy was the withdrawal of subsidies and transfer of pesticides sale and distribution to the private sector in February, 1980. Since then pesticide marketing consumption has shown a remarkable progress. This trend is evident as pesticide usage rose dramatically to 6865 tonnes (active ingredient) worth PRS ~6554 million in 1992 as against 915 tonnes costing only PRS 213 million in 1981 (Tables 13.4 and 13.5). This is about a 7.5-fold increase in tonnage and over 30 fold increase in cost in 11 years.

Table 13.3 *Pesticide consumption trends in Asia Region (tonnes)*

		1988				
Country	**1984**	**Insecticides**	**Herbicides**	**Fungicides**	**Other**	**Total**
India	68,683	60,115	3,111	5,118	714	69,058
Iran	–	7,399	1,609	3,033	–	12,041
Pakistan	2,517	3,197	241	319	2	3,759
China	–	113,434	29,186	21,491	2,087	166,198
R.Korea	21,767	8,497	6,214	10,106	405	25,222
Philippines	3,664	1,844	1,156	177	891	4,068
Thailand	3,710	4,693	1,249	36	54	6,032
Sri Lanka	–	256	316	7	1	580
		199,435	**43,082**	**40,287**	**4,154**	**286,958**

Table 13.4 *Import of agricultural pesticides in Pakistan*[10]

Year	**Quantity (000 tonnes)**	**Value PRS million)**
1952–53	2.0	0.02
1955–56	164.6	0.38
1960–61	4,979.4	19.52
1965–66	882.9	11.26
1970–71	2,248.0	41.77
1975–76	13,758.3	310.42
1980–81	7,105.0	224.72
1985–86	17,498.9	1,416.81
1990–91	13,030.1	1,489.43
1991–92	15,258.3	1,945.98
1992–93	14,434.8	1,730.60

Table 13.5 *Consumption of agricultural pesticides in Pakistan*[11]

Year	**Quantity M tonnes (m^3)**	**Active ingredient**	**Value PRS million**
1980	665	175	39
1981	3,677	915	213
1982	5,000	1,290	320
1983	6,588	1,810	627
1984	9,213	2,517	1,256
1985	12,500	3,458	2,249
1986	14,499	3,980	2,978
1987	14,848	4,554	3,259
1988	13,107	3,918	2,340
1989	14,667	4,460	3,642
1990	17,443	5,296	4,581
1991	20,213	5,920	5,536
1992	23,439	6,865	6,554
1993	20,279	NA	5,385

Despite the pesticides usage, worth PRS billions year^{-1}, consumption in Pakistan is still below that of the developed countries of the world. According to an estimate only 30% of Pakistan's total crop hectarage receive partial pesticide cover, while the remaining 70% (eg, farms) lack a plant protection facility. However, there is a growing awareness amongst farmers for pesticide use to increase production. The major shareholder of the pesticide market is the cotton crop which alone accounts for about 70% of the total pesticide consumption in Pakistan. According to a report submitted to the Federal Pesticide Committee 54% of cotton crop was given plant protection coverage consuming 1430 M tonnes during 1985, 60% of the crop was protected from pest consuming 2010 M tonnes of pesticide during 1986. During the year 1987 to 1990 only 65% of the cotton crop was protected from insect pests consuming ~2236 M tonnes of pesticides.

The report of the Agricultural Commission's sub-group on pesticides estimated that the demand for pesticides will grow by at least 10% y^{-1} to sustain growth in the agricultural sector. In view of a withdrawal of subsidy, the crop coverage through aerial spraying has been reduced substantially and during the past few years it was restricted only to the rice crop in the Punjab, sugarcane in the NWFP and fruits in Baluchistan, where the farmers have used their political influence to force the government to facilitate aerial spraying. However, the spray coverage by ground operation shows an increasing trend (Table 13.6).

Table 13.6 *Spray coverage by aerial and ground operation ('000' hectare)*[11]

Year	Aerial	Ground	Total
1951–52	8	NA	8
1954–55	23	NA	23
1959–60	153	508	661
1964–65	406	1176	1582
1969–70	1106	831	1937
1974–75	1585	1580	3165
1977–78	1315	2615	3930
1978–79	888	2140	3028
1979–80	382	1768	2150
1980–81	250	1505	1755
1981–82	192	2300	2492
1982–83	273	2552	2825
1983–84	250	2680	2930
1984–85	319	3711	4030
1985–86	173	6055	6228
1986–87	294	6297	6591
1987–88	270	7408	7678
1988–89	80	5244	5324
1989–90	80	6996	7076
1990–91	110	8197	8307

13.4.2 Pesticide legislation

Pesticide control legislation have been promulgated in most of the Asian countries. In the developing countries of the Asian region the pesticide legislation were enacted during 1970s as a follow-up of the FAO efforts which provided guidelines for pesticide registration and quality control; however, some of the countries in the region did not enact this legislation until 1980. The Regional Network on Pesticide for Asia and the Pacific (RENPAP) expedited enactment of legislation for pesticide registration during later part of 1980s.

Whilst the legislation exists in almost all these countries, the legislation is mostly ineffective due to the non-availability of manpower and infrastructure.

In Pakistan, the Pesticide Act was promulgated in 1971 and the Pesticide Rules were framed in 1973 on the basis of FAO guidelines issued. These Rules, except time bound changes, are very comprehensive and elaborate because the guidelines were prepared in consultation with international agencies and representatives of the developed and developing countries. However, the facilities for implementation of these Rules were lacking except for the establishment of the Pesticide laboratory, PARC. This Laboratory has been capable of performing only physical and chemical analysis of the products submitted for registration and to meet the quality control requirements in addition to few demand oriented residue studies.

Following legislation the Agricultural Pesticide Technical Advisory Committee (APTAC) and Sub-committee were established comprising of representatives from the various related agencies from the Provincial and Federal Governments and private sector. The Department of Plant Protection, Ministry of Food, Agriculture and Livestock, Government of Pakistan, serves as the secretariat of the Committees and the Director, the Department of Plant Protection is the Registrar. The Subcommittee reviews the results of the bioefficacy tests performed by the various research organizations for the control of pests attacking various crops and makes recommendations while APTA Committee accepts/rejects applications for registration submitted by the pesticide companies for marketing in Pakistan. Currently, this committee has recommended registration of 202 pesticides active ingredients.

Based on international restrictions and local reviews the import of 21 pesticides either in technical grade material or formulation is banned vide notification No.PP.IMP-1/93-REG of 4th May 1993 of the Ministry of Food, Agriculture and Livestock, Government of Pakistan (Table 13.7).

The generic scheme for agricultural pesticides, with the intention to reduce the price of pesticides, which is almost two to four times higher than the international market particularly in the Asia and Pacific Region, was considered in the several meetings of the Agricultural Pesticide Technical Advisory Committee during the years 1989–91 and finally the Pesticide Act/Rules were amended. Under this scheme the bioefficacy testing of pesticides in the various ecologies of Pakistan under field conditions has been dispensed with. Thus the role of APTA Committees on toxicity is ineffective. It is generally believed that in the absence of proper arrangements for pesticides monitoring, the scheme will be used to the advantage of those who can exploit the situation. According to Pakistan Agriculture Pesticide Association (PAPA) the generic scheme

poses a number of potential risks to farmers, the environment and to the country's economy. The generic scheme would be a loophole in the system, creating a pole of attraction for substandard products. Competition on a generic basis will inevitably cause a reduction in field work and extension services. As a result, small farmers in particular will be deprived of information and support which is vital for them in order to improve agricultural production.[12]

Table 13.7 *Pesticides banned/withdrawn in Pakistan*[11]

Binapacryl	Dieldrin
Bromophos ethyl	Disulfoton
Captafol	Endrin
Chlordimeform	Ethylene dichloride + Carbontetrachloride
Chlorobenzilate	Leptophos
Chlorthiophos	Mercury compound
Cyhexatin	Mevinphos
Dalapon	Propergite
DDT	Toxaphene
Dibromochloropropane + Dibromochloropropene	Zineb
Dicrotophos	

13.5 PESTICIDE TOXICITY

Pesticides, a major product and the modern chemical industry, are used for pest control and health because they are toxic. Although pesticides are useful biocides to reap economic rewards they are also toxic to human and the wildlife. The benefits of pesticide use for increased food, fiber and shelter are undeniably great. Pesticide sales globally have grown ten-fold from US$ five billion in 1975 to a projected 50 billion in 1990. However, the adverse effects on human health and wild life are devastating in the long-term. A million cases of poisoning have been estimated worldwide.

Some of the 'dirty dozen' pesticides: aldicarb (Temik), camphechlor (toxaphene), chlordane and heptachlor, chlorine form DBCP, DDT, drins (aldrin, dieldrin, endrin, EDB, HCH/BHC and lindane, paraquat ethyl and methylparathion, pentachlorophenol and 2, 4, 5-T are still in use in some Asian and Pacific countries.

Pesticides are an evil of necessity which at no cost can be excluded from an effective pest management program. Simultaneously, they cause a serious threat to human being and the environment. In the developing world the lavish, unplanned and indiscriminate use of pesticides, accidents during storage, transportation and application in the field which have resulted in hazards after application have created concerns in the minds of those engaged in pesticide research and management. The side effects attributed to the use of these chemicals are the destruction of natural biotic balance, suppression of

biocontrol agents, insect resurgence, pesticide resistance, dangers to pollinators, soil and aquatic fauna, and wild life etc. The toxicity of pesticides is affecting the whole chain of food, air, water and plants. Their indiscriminate use is also showing signs of several serious human ailments, the most common being cancer, liver diseases and hypertension. The health security problems have now become an area of concern to manufacturers, traders and users of these toxicants and also to the community outside of the pesticide sector. With the latest knowledge and technology, it is possible to minimize these losses by applying pesticides systematically[14]

Recently, the World Health Organization estimated that every year due to pesticide poisoning, 500,000 deaths are reported, almost half of this figure is from the developing countries. The recent WHO report further claim another 400,000 cases of poisoning due to pesticide handling and usage. Enhancement in the import of pesticides commenced in 1980 and Pakistan has emerged as a major dumping ground. Thus Pakistan stands second as the largest pesticide consuming country among the 12 Asian and Pacific Regional countries which is a major threat to human health and environment, very often the adverse effect of inert ingredients surpasses that of the active ingredients which are highly toxic to human health, these are usually not included in publicity or shown on labels.[15]

A survey of acute poisoning among agricultural workers in four Asian countries revealed organophosphate compounds to be those mostly responsible for poisoning. The pesticide poisoning in Malaysia was 53.6% of the total poisoning cases registered, in Sri Lanka 69.1%, in Thailand 22.7% with organophosphate insecticides and 25% with bipyridyls. In Indonesia, copper compounds were responsible for 23.4% of the cases and organophosphorus insecticides for 17.8%. In Afghanistan the overall mortality rate was 6.2% due to accidental poisoning among children; organophosphorus insecticide poisoning alone was responsible for 50% of total deaths.[16]

The factory explosion at Seveso, Italy, in July, 1976, spread 2–3 kg of the highly toxic chemical, dioxin, downwind over a considerable distance. The extent of the contamination was not disclosed to the farmers affected and, therefore, domestic animals grazed on contaminated ground, and contaminated milk and eggs reached many people. Similarly, the Bhopal tragedy in India is the latest example of an accident where, in 1984, methyl isocyanide gas leaked from a reservoir in a Union Carbide pesticide factory at Bhopal and contaminated the air in the vicinity, killing ?2500 people and seriously affecting thousands of others.[17–19]

13.6 ECOTOXICITY RISK ASSESSMENT

The discovery of the insecticidal activity of DDT almost 50 years ago initiated a new era in the man's ability to control both pests in agriculture and vector born diseases in public health. Research and development originated ~100s of new chemicals of varying toxicity to control pests to improve the yield of cereals/vegetables and cash crops and elimination of many vector borne diseases. While mankind enjoyed the benefits of the

chemicals, it took almost four decades to understand the side effects of these chemicals in the environment due to normal use and misuse and over production of pesticides.

The adverse effects of these otherwise essential chemicals are primarily residues in food and feed, occupational hazards; pollution of drinking water, contamination of environment both in aquatic and terrestrial habitats affecting non-target beneficial organisms (parasites predators of insect pests, annelids, birds and mammals) and the eventual reduction in biodiversity. These led to the realization that these chemicals are not only doing the job they are intended to do but also causing damage to the environment. Simultaneously, it was also realized that mankind cannot continue to use these chemicals in the future. The answer is to investigate and analyze the complex problems arise due to their use so as to minimize these adverse effects by identifying and recommending use of safe chemicals, better administration and eventually management and safe use of these chemicals.[20]

The environmental and health considerations have now become an area of major concern for public and consequently for scientists forcing them to search for effective measures. It is a scientifically known fact that very small quantities of pesticide applied to the agricultural crops hit the target, while most of it is deposited on the non-target facets of the environment such as adjacent crops, forest areas, water bodies, fallow land and residential areas. This situation generates a variety of toxicological and ecological pollution problems affecting human beings and other forms of life. The danger to human life is largely because of the lack of proper management and training in the use of poisonous agrochemicals.

Asia occupies a disadvantageous position in terms of the safe use of pesticides, as the bulk of farmers is illiterate and untrained in the use of pesticides. This is causing harm to human health particularly in the rural area. There is considerable ignorance as a result of lack of realization at the level of actual user especially the farmer and farm worker. Application practices on farm are often very poor mainly due to inappropriate labelling, illiteracy, poor extension, insufficient dealer/farmer training and poor application equipment resulting into unintended human hazards.[21]

In order to counter the threat posed by pesticides to human health and other living creatures on the earth, it is vital to investigate the rate of degradation/dissipation of various pesticides on different crops. Suspected pesticide residues in commodities have always caused special concern to consumers. Unfortunately, until now there was no regular system and facilities in the poor countries to monitor accurately pesticide residues and their toxic effects in agricultural commodities and the environment. There is a grave shortage of expertise in this area.

The problems of ecotoxicity due to the use of pesticides are of particular concern because of the ever increasing use of pesticides required for adequate pest management with a continuing trend to crop monocultures such as cotton, rice, sugarcane, fruits and vegetables etc. for achieving a better yield (see Chapters by Masud and Hansen). The poor and illiterate farmers having big families with small holdings and cannot afford protective clothing, shoes, gloves, masks etc. and thus maximum exposure of the workers to the adverse effects of pesticides are unavoidable especially during the hot summers for pest control operations on cotton and vegetables. Also, most of the pesticides are emitted into the air, soil, water, plants, animals, various food chains and other

non-target organisms because of and inefficient application technology of pesticides together with adulterated products. Indiscriminate use of these chemicals has created an ecological disruption which warrants preventive educational efforts and best management practices through the use of better application technology.[21]

Although no systematic studies/surveys have been undertaken, scanty data on pesticide residues are available. In spite of limited financial resources, some attempts have been made by the Pakistan Agricultural Research Council to monitor pesticide residues in food commodities (see also Chapters by Masud and Hansen). The results show evidence of pesticide contamination in several samples of fruits and vegetables collected from wholesale market of Karachi which in some cases, exceed the Maximum Residue Limits (MRL) proposed by FAO/WHO for different pesticides/ products. The various studies conducted on pesticide residues in the human blood/ tissue and the environment particularly food commodities are summarised:

(i) In 1973 a study of human adipose tissue (60 samples) from general population of Karachi indicated varying levels of DDT (25 ppm) BHC (0.48 ppm) and dieldrin (0.04 ppm);[22]

(ii) In 1984, 79 samples of cattle drinking water drawn from the Karachi Cattle Colony were monitored for organochlorine pesticides. Nearly 13% of the samples were found to be contaminated with different chlorinated pesticides or their metabolites;[23]

(iii) In 1984 milk samples from the Karachi Cattle Colony were monitored for organochlorine pesticide residues. A total of 79 samples of milk were analyzed out of which approximately 40% of the samples were found to be contaminated with either BHC isomers, pp'-DDT, pp'-DDE, heptachlor epoxide, aldrin or dieldrin. The most frequently occurring pesticide was a-BHC. The presence of aldrin residues in milk indicates that this product is still being used in Pakistan although its sale was banned;[24]

(iv) In animal feed samples from Cattle Colony, Karachi, 79 random samples were screened for lindane, α-BHC, ß-BHC, σ-BHC, endrin, aldrin, dieldrin, pp'-DDT, pp'-DDE, pp'-DDD, dicofol, heptachlor and heptachlor epoxide. Approximately 46% of the samples were contaminated with different pesticides and their metabolites. α-BHC and γ-BHC were found to be present in most of the samples;[25]

(v) During 1986–88 250 samples of cotton seed were collected, 73.60% of these samples showed contamination with 24 different pesticides/metabolites. Out of 24 pesticides, 9 were organochlorine, 8 organophosphorus and 7 pyrethroid compounds (40–64%). These samples exceeded MRL. The most frequently occurring pesticides were cyhalothrin, dimethoate, DDT and its metabolites, endosulfan and monocrotophos;[26]

(vi) During July 1988 and September 1992, a total of 250 samples of fruits and vegetables were screened out of which 214 samples were found contaminated. 79 samples have been found to contain residue above MRLs;[27] and

(vii) In 1993, it was reported that samples of soil at ~1 m depth contained residues of DDT, DDD, DDE, aldrin, dieldrin, monocrotophos, cyhalothrin, dimethoate, c ypermethrin, fenvalerate and profenfos whereas ground water samples at ~1 m were contaminated with monocrotophos, cyhalothrin and endrin. The results

obtained were sufficient to show concern for the potential hazard for drinking water contamination.[28]

Under present circumstances, it is impossible to confirm that all food supplies will be free of objectionable pesticide residues. To obviate the risk of toxic/ persistent pesticides, adequate residue data for pesticides should be generated in Pakistan on an organized, systematic and national scale. This information would be essentially assigning tolerance/ acceptable limits of various pesticides for safety of health of consumers commodities contaminated with these toxicants. More over, in order to protect and promote quality of food stuffs, it is now imperative to establish infrastructure with adequate laboratory facilities in addition to strengthening the existing insignificant facilities.

13.7 CONCLUDING COMMENTS

The use of pesticides in the member countries of the Asia and the Pacific region has shown a distinct and increasing trend and this region has extremes of climate (heat, rainfall, humidity, soil conditions) ranging from arid to irrigated agriculture with a variety of crops grown under distinct agroecological conditions. The fate of pesticides from 'cradle to grave' needs to be closely monitored to ensure overall safety of the mankind and the environment.[21]

However, it is recognised that these chemicals need to be managed properly to ensure sustainable development with emphasis on a high degree of environmental and human health protection. One of the most effective scientific approaches is to have a thorough understanding of the ecotoxicological aspects pertaining to the use of agrochemicals in agriculture and health sectors.

The infrastructure for research and development in the field of toxicology exists only in the developed countries of Asia, particularly Japan, while most of the developing countries lack this facility. Several developing countries in the area, in the recent past, have been considering seriously the establishment of these facilities which are prerequisite for the local manufacture of raw material for new pesticides. The facilities for ecotoxicology research does not exist in most of the Asian developing countries. Pakistan, as a member of RENPAP has taken the lead for the establishment of Ecotoxicology Research Centre.

In Pakistan the work undertaken to protect the environment is still in its embryonic stage. However, tentative steps were taken in the 1980s when the Environmental Protection Ordinance (EPO) 1983 was framed, under which a National Environmental Council with the prime minster as its chairman was formed. An Environmental Protection Agency (EPA) was established. Recently provincial EPAs have been organized, the principal objectives of which are the preparation of a national environmental policy, promulgating national environmental quality standards, administering the environmental ordinance and compiling the Environment Impact Assessment (EIA).

The progress of and implementation of the environmental ordinance during the last eleven years has been so slow that the Council has met only once in ten years, the national environmental quality standards proposed in 1983 were approved by the government a decade later in 1993. The EPA was supposed to assign regulations for the

effective implementation of the EPO in 1983. To overcome the deficiency in the EPO in 1983, a draft Pakistan Environmental Protection Act 1993 has been drafted which has still to be presented to the parliament.

The most significant provision of the ordinance is section 8 which provides for the submission of an environment impact statement to the agency by every person involved in an industrial project. The statement will contain information about the impact on the environment by the proposed industrial activity, the unavoidable adverse environmental effect of the proposed projects and steps to be taken to minimise these adverse effects. However the regulations have still to be approved by the government.

To curtail environmental damage, a new approach integrating environmental preservation with development was adopted in the National Conservation Strategy (NCS), approved by the government in April 1992, and it indicated 14 program areas including irrigation efficiency, protecting watersheds supporting forestry and plantations, increasing energy efficiency, developing and deploying renewable resources and integrating population and environment programmers. The NCS has planned 67 investment programmes costing PRS 150 billion over ten years; 1992–2001.

The Government of Pakistan with assistance from Danish International Development Agency (DANIDA) Government of Denmark through United Nations Industrial Development Organization (UNIDO) has established the Institute of Pesticide Ecology, Ecotoxicology Research Centre which is intended to serve as Centre of Excellence on Ecotoxicology in Pakistan. The Centre, with its satellite laboratories in the various ecological zones of the provinces of Pakistan will provide much needed technical assistance and training facilities for the network countries of Regional Network on Pesticide for Asia and the Pacific including:

(i) Monitoring residues of xenobiotics in food and feed and assessment of residues impact on the soil and water microflora;
(ii) Investigation on the fate of agroindustrial chemicals in the environment; and
(iii) Monitoring of agroindustrial health hazard and creation of awareness amongst concerned occupational groups.

The mandate of the component laboratories are:

(i) Terrestrial Ecology Laboratory (TEL)—Investigation of the impact of chemicals on reproduction, survival and behavior of wildlife particularly mammals, beneficial insects (predators and parasitoids) and terrestrial beneficial macrofauna (eg, e arthworms) in the ecosystem;
(ii) Chemical Microbiological Laboratory (CML)—Assessment of the impact of chemicals on microorganisms in soil, water and evaluate tolerance, detoxification and degradation of chemicals by the predominant associate microflora;
(iii) Chemical Analysis Laboratory (CAL)—Determination of the content of xenobiotics in environmental samples (eg, food, water etc.).

The application of the results obtained from these new facilities will lead to appropriate decisions including risk assessment, the application of risk management techniques through to assessment of environmental toxicology.

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14 Ecotoxicological Monitoring of Industrial Effluents

Thomas Stuhlfauth

14.1 INTRODUCTION

Ecotoxicity tests provide an efficient way to control industrial effluents and to minimize their environmental impact. In the examination of sewage, these tests can replace to a certain extent the routine analyses of chemical substances. For that purpose several biotests are available as standardized procedures. In Germany, biotests are already used to define emission limit values. Fish, daphnia, algae and bacteria were chosen as representative test organisms of the ecological relevant trophic levels. However, the test procedures, with their advantages, their problems, their reproducibility and their ecotoxicological relevance require to be critically reviewed. The application of biotests for an estimation of the ecological quality of surface waters or for the industrial self-control of process water complete the view on the toxicity assessment on industrial wastewaters.

14.2 ADVANTAGES OF BIOTESTING COMPLEX INDUSTRIAL WASTEWATER

At chemical works of medium size, >100 chemical substances are produced in relevant amounts; often these substances are part of >1000 end products. During the production processes, the process water is contaminated in many cases with the reaction products. Afterwards, the process water has to be cleaned by physico-chemical procedures and/or a biological waste-water treatment. The effluent discharged should not cause any ecotoxicological effect. By analytical investigations only a small fraction of the chemical substances can be monitored (Figure 14.1). Even in public sewage, only 3 % of the low molecular weight fraction could be identified using sophisticated analytical equipment.[1]

The analyses of individual substances or summarizing parameters such as chemical oxygen demand (COD), dissolved organic carbon (DOC) or absorbable organohalogenes (AOX) result in concentration data and give no direct information about environmental risk potential. The advantage of biotests is that the total toxicity of wastewater is measured and compared. Thus, the toxic potential of the contaminants is indicated, taking into account bioavailability and synergistic or antagonistic effects. If there is a toxic effect, biotests, however, give no information about the kind of substance that causes this effect. In such a case, the chemical analysis of the effluent may help to identify that

substance. Therefore, biotests can replace the chemical analyses in routine control measurements, when normally no toxic effects are expected.

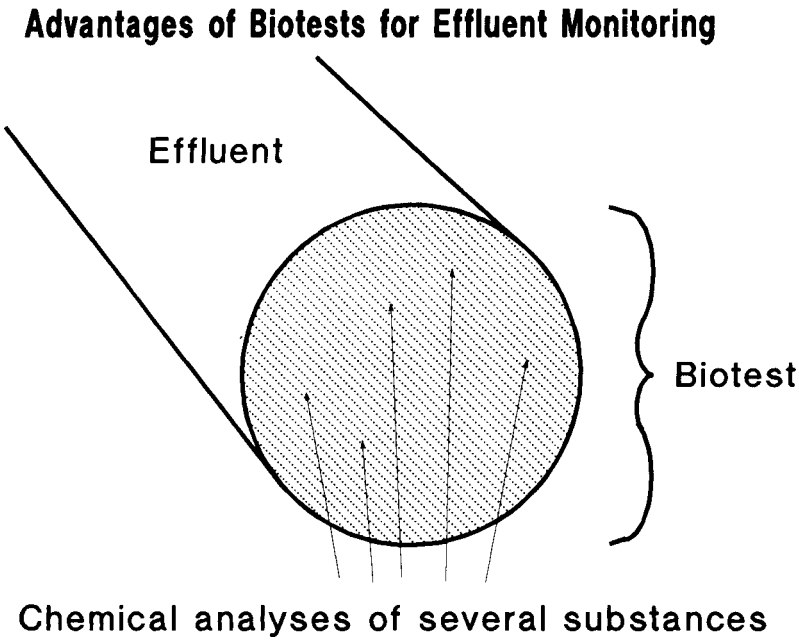

Figure 14.1 *Comparison between an effluent biotest and the chemical analyses of several substances*

14.3 REQUIREMENTS FOR THE BIOTESTING OF EFFLUENTS

There are four basic requirements for ecotoxicological testing:

(i) The test system should be representative and relevant for the community of several 100s of species living in the recipient waters;
(ii) The test results should be reproducible in different laboratories to enable a comparison of results between different regional authorities or industrial control institutions;
(iii) The test system should be simple to enable the frequent control of effluents by short-term testing; and
(iv) The test methods should be cost-effective to fulfil the economic criteria of sustainability.[2]

Several test system hierarchies might be considered for these purposes:

(i) Ecosystem tests (eg, Mesocosm, Microcosm or Biocenosis studies[3,4]);
(ii) Toxicity tests with single species (eg, fish, daphnia, algae); or
(iii) Suborganismic test systems (eg, fish cell tests[5]).

As can be seen from Table 14.1, no test system completely fulfils the above mentioned criteria.

Table 14.1 *Criteria for the selection in environmental toxicity assessment*

	Ecosystem tests	Toxicity tests	Suborganismic tests
Ecosystem relevance	+	±	—
Interlaboratory reproducibility	—	+	+
Frequent control	—	+	+
Cost-effectiveness	—	+	+

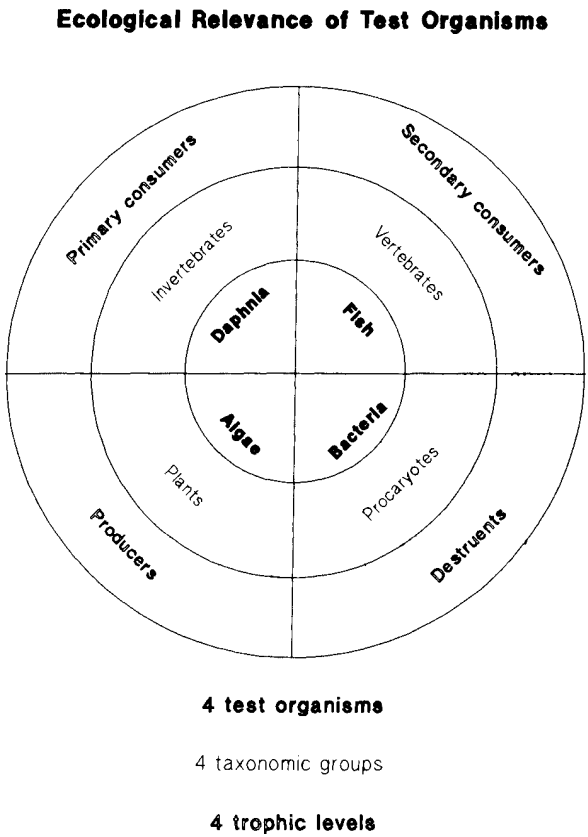

Figure 14.2 *Representation of the trophic levels and taxonomic groups by four test organisms*

Ecosystem tests are of high relevance, because they cover interactions within the biocenosis. Due to the complexity and the weather influence in the field systems, a good reproducibility is only reached with laboratory biocenosis systems. However, even these relatively simple ecosystem tests do not meet the high testing frequency required and are not cost-effective.

Suborganismic tests are cost-effective, reproducible and can be carried out at a high frequency, but, the end-points of these tests are usually not relevant for the ecosystem. This is because the consequences of suborganismic changes for intact organisms and ecosystems, if any, are difficult to assess. However, less animal testing would be required if suborganismic test systems could be used instead. For that reason, a research project will be started in Germany to replace the fish test by a fish cell test if the two test systems give similar results.

Toxicity tests with single organisms cannot perfectly fulfil the expectations for ecosystem relevance. Whereas such a test is representative for the same species and related organisms, a single species test does not reflect basic transactions in the ecosystem. To overcome these difficulties, a system based on four single-species tests has been developed (Figure 14.2). This system represents the four trophic levels in the ecosystem: the producers, the primary, and secondary consumers, and the destruents. Additionally, four relevant taxonomic groups are covered: vertebrates, invertebrates, plants and prokaryotes. Due to this testing scheme, the four basic requirements for the assessment of environmental toxicity can be met.

Principally, this scheme resembles the scheme used for the classification of chemicals according to guideline 91/325/EEC.[6] As bacteria normally are less susceptible to chemicals than other taxonomic groups, the testing of bacteria could be omitted in that guideline.

14.4 COMMON PRINCIPLES FOR THE BIOTESTING OF EFFLUENTS

According to the above mentioned scheme for effluent testing, four biotests were developed by DIN (Deutsches Institut für Normung—German Institute for Standardization)[7]:

- The fish test, DIN 38412 part 31 (formerly part 20)
- The daphnia test, DIN 38412 part 30
- The algae test, DIN 38412 part 33
- The luminescent bacteria test, DIN 38412 part 34 and part 341

In all these tests, the wastewater is examined in a dilution series following the geometric series of 2 and 3 (eg, 2, 3, 4, 6, 8, 12, 16 etc.), which are called '*G*-levels' (dilution levels). A dilution with a wastewater content of 1/4 (25% v/v) is called *G*=4. Generally, time consuming evaluation methods for wastewater effects are avoided by a uniform and effective way of expressing test results: The dilution level *G* of the most concentrated test suspension with no significant inhibitory effect is

reported. The significance limits for that decision are defined in the respective standard.

These dilution levels are used by national and regional authorities to define emission limit values (ELVs). For example, the fish test ELV for the effluent from the wastewater treatment plants of chemical industries in the Frankfurt region is G_F=3 (no fish toxicity in a dilution with 1/3=33% v/v wastewater). Our Hoechst works has voluntarily proposed to reduce this value to G_F=2 (no fish toxicity in a dilution with 1/2=50% v/v wastewater), which would enable savings according to the Federal Waste Water Charges Act (Abwasserabgabengesetz).

14.5 A COMPARISON OF THE BIOTEST STANDARDS

14.5.1 Fish test

This test is carried out with *Leuciscus idus melanotus* (golden orfe). Three juvenile fishes are exposed to wastewater dilutions for 48 h. G_F is defined as the most concentrated dilution level *G* in which no fish is killed. The test fish are limited to a certain size to guarantee a constant range of sensitivity. As the orfe is a European fish that reproduces only once a year, the breeder has to ensure the desired size by keeping the animals in a cold environment at low food supply. Care has to be taken to guarantee healthy test fish after a prolonged time under these conditions. The interlaboratory reproducibility of the fish test has been evaluated in a test series together with our regional authority.[8] Only minor differences of ±1 *G* level were found, variations which are inevitable if the real value is exactly between 2 *G* levels. The costs of a single fish test according to the procedure described are about GB£140.

Summing up, the fish test, which has been carried out since the beginning of the 1980s, can be regarded as an established and reliable procedure.

14.5.2 *Daphnia* test

This is earned out with 10 freshly bred *Daphnia magna*. The test animals are exposed to wastewater dilutions for 24 h. The G_D-value is defined as the most concentrated dilution level *G* in which no immobility occurs. Problems in the breeding of *Daphnia* might occur due to the lack of trace elements in the recommended ISO/DIN medium. A complete synthetic medium called 'M4' was developed by Elendt.[9] The interlaboratory reproducibility has been evaluated in a test series together with our regional authority.[8] Differences of ±1.5 *G* levels were found, a value acceptable especially because different daphnia clones were used in the participating laboratories at that time. To ensure the same sensitivity, the same daphnia clone should be used in all testing laboratories. In Germany the so-called 'clone 5' is used in the meantime. This clone is also recommended for the testing of chemicals in Europe. A single daphnia test costs about GB£100.

14.5.3 Algae test

This is carried out measuring the cell multiplication of the freshwater species *Scenedesmus subspicatus* by fluorescence after 72 h of incubation. The G_A-value is defined as the most concentrated dilution level G in which growth is greater than 80% of the control (<20% inhibition). As algae use light of the whole visible spectrum for growth, colored or turbid samples might cause false positive results by light absorption. An identical illumination of all test and control vessels is essential to achieve comparable growth rates. A photobleaching of the algae during the fluorescence measurement has to be avoided by flow through equipment or pulsed light techniques. The DIN round robin test and a series of interlaboratory comparisons together with our regional authority[8] have shown that differences of ± 2 G levels occur. The algae test costs about GB£240.

14.5.4 Luminescent bacteria test

This measures the bioluminescent radiation from marine photobacteria after a 30 min contact period with wastewater dilutions. The G_L-value is defined as the most concentrated dilution level with >80% bioluminescence as compared to the control. This test enables most rapid investigations. If several samples are to be examined, this test does not last longer than a series of DOC or GC analyses. However, bioluminescence occurs only in a certain physiological stage of the bacteria and it has been shown that some nutrients do inhibit bioluminescence by inducing growth of the bacteria. An additional growth test with luminescence bacteria is currently developed by DIN to differentiate between toxic and growth promoting effects in the luminescence test. Summing up, the luminescent bacteria test, which is offered from different manufacturers as a test kit, is an essential screening test to detect nutritive as well as toxic substances in water. A DIN round robin test and a series of interlaboratory comparisons by our regional authority[8] have shown that differences of ± 3 G levels do occur, whereas the intralaboratory differences were much lower. The luminescent bacteria test costs about GB£120.

14.5.5 Test variability

The variation of biotests is larger than that of most chemical analyses. However, it has to be kept in mind that the result of a chemical analysis, ie, the concentration of a substance in the sample, does not give a direct information relating to the environmental risk potential; biotests with that substance would be necessary to transform the information about concentration into an information about risk potential. For that reason a comparison of variances of chemical analysis and wastewater biotest is not helpful without considering the variance of the supplementary substance biotest. Generally, the direct estimation of an effect on the environment should be preferred to indirect analyses.

The DIN methods for wastewater examination quite closely correspond to international standards and guidelines for the testing of chemicals.

The fish test is similar to the static test of OECD guideline 203; the golden orfe is not recommended as a test fish in that guideline, but seems to be slightly more sensitive than the often used subtropic zebra fish. The test duration of the wastewater test is shorter (48 h) than the duration recommended for the testing of chemicals (96 h).

The *daphnia* test corresponds to the OECD guideline 202; the duration of the wastewater test is shorter (24 h) than the test duration recommended for chemicals (48 h).

The algae test corresponds to the OECD guideline 201; both tests last 72 h, but the wastewater test requires only one measurement instead of three measurements recommended for the testing of chemicals.

The luminescent bacteria test does not correspond to an OECD guideline for the testing of chemicals; an ISO standard is being developed currently.

14.6 EMISSION LIMIT VALUES, PROCESS CONTROL AND ENVIRONMENTAL QUALITY OBJECTIVES

Water is used for many processes in industry. As a result of these processes, the water is contaminated with substances. This wastewater might be purified in a wastewater treatment plant and afterwards discharged to the recipient water.

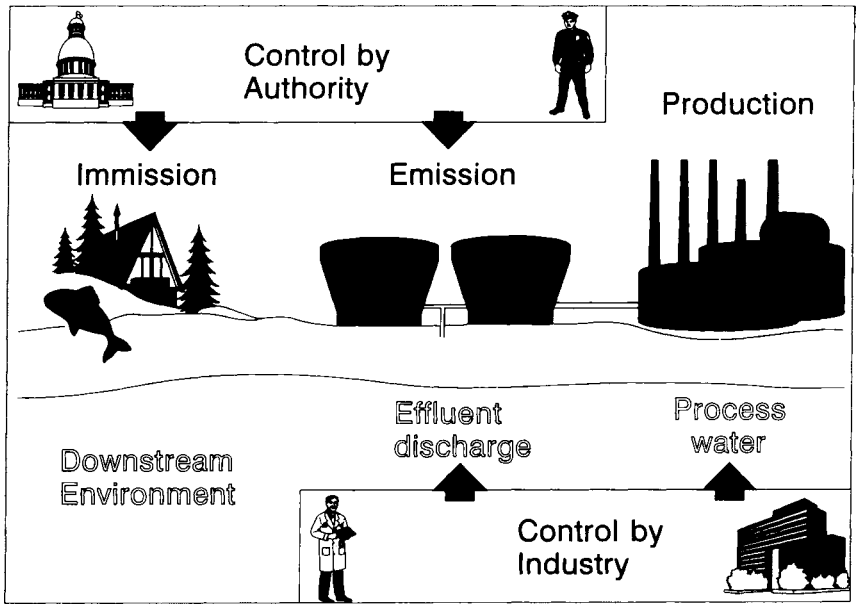

Figure 14.3 *Scenarios for a toxicity assessment by authority and industry*

Three situations with different applications for biotests must be distinguished (Figure 14.3):

14.6.1 Emission control

With effluent discharge the wastewater from a local source enters the environment. This 'end of the pipe' is the crucial point where emission limit values have to be set to avoid damage for the environment. These emission limit values should be controlled frequently by the local industry itself and checked randomly by the regional authority. As emission limit values may have severe economic consequences, the methods used to define ELVs must be standardized and reliable. In Germany, the fish and daphnia test are already used for that purpose, whereas the algae and the luminescent bacteria tests are still on trial. If the test results exceed emission limit values, it will have financial consequences for the polluter and cause prosecution for the responsible managers.

14.6.2 Immission control

If the recipient water is a river, the relevant point for the ecosystem is the place where the effluents have completely mixed with the river water. As that place is usually several kms away from the point of discharge, the regional authority is the competent institution for water control in that area. Downstream from that zone of complete mixture, all organisms in the recipient water are exposed to the effluent constituents. Adequate testing is required for the detection of harmful effects from immissions. In the proposal for the EC directive on the ecological quality of water a kind of inventory of organisms in the recipient surface water is encouraged. Such examinations have a tradition of more than 80 years in central European regions. In Germany, the methods of the so-called 'saprobic system'[10] have been standardized in DIN 38410 part 1 to 7.[11] Besides a classification of water quality, the disappearance of species can be detected by this method. The results of examination depend on river water flow and change slightly with the seasons; therefore, it is applicable only as a long term control procedure. For short term immission control biotest systems may be used which permanently or frequently expose the test organisms to water from the recipient river, eg, the fish monitor developed by the Water Research centre in Medmenham.[12] Currently, such automated test systems are installed and tried out at the Rhein, Main, and Moselle rivers in Germany.[13] Automated test systems even respond to the different background situation of the rivers;[14] this is an indication that they can better reflect the ecosystem than any laboratory test. Several deteriorations of the ecosystem, eg, by accidental emissions of mineral oil, could successfully be detected by automated tests. The development of automated test systems similar to the test systems used for emission control, but with a sensitivity high enough for immission control, would enable a correlation between environmental quality

objectives based on automated systems and emission limit values based on the standardized tests for wastewater.

14.6.3 Process water control

After a setting of environmental quality objectives and the definition of emission limit values a situation might occur where industry has to find out which one of several processes at a work is responsible for emission problems. Sometimes, especially in complex effluents, it is not possible to correlate toxicity with one of the analyzed chemical substances. Principally, standardized biotests can also be used for the examination of single process waters. However, it makes no sense to test untreated process water if the wastewater is treated before effluent discharge. The amount of toxic substances present is often dramatically reduced during the treatment, eg, in a biological wastewater treatment plant. To simulate the composition of a process water after biological treatment, a modified Zahn-Wellens-Test (modified OECD 302 B procedure) is used. This relatively simple static procedure is carried out under conditions resembling the COD elimination rate of the biological treatment plant. For our treatment plant near Frankfurt, this test will take seven days at a realistic sludge/wastewater ratio. Table 14.2 shows that data from the seven day-test and the real COD elimination are almost identical. The calculation of total COD elimination is an addition of all the process waters, considering their contribution to the total wastewater volume. It should be noted, however, that the calculation of a biotest result is not additive, because the effect induced by a process water is normally caused by another biochemical reaction mechanism than the same effect of other process waters. Synergistic effects are very rare; no synergistic effect could be detected by testing 70 process waters, ie, ~1000 substances, during our work.

Table 14.2 Simulation of the elimination in a wastewater treatment plant by analyses of the single process waters. A comparison between the simulated treatment (7-day test) of process waters and the real wastewater treatment plant of Hoechst near Frankfurt.

Process No.	COD before simulated treatment mg l^{-1}	COD after simulated treatment mg l^{-1}
1	16716	4477
2	960	149
3	9133	2613
4	3758	1193
5	1226	360
6	6624	658
7	2149	274
8	20480	1647

Process No.	COD before simulated treatment mg l^{-1}	COD after simulated treatment mg l^{-1}
9	34211	4951
10	13564	1265
11	5465	512
12	2148	163
13	454	226
14	6636	1599
15	1708	302
16	7060	422
17	7860	975
18	1869	163
19	31782	9898
20	409	72
21	797	669
22	3452	894
23	44992	3530
24	5427	661
25	1325	149
26	9132	835
27	13646	2529
28	1088	65
29	2920	117
30	7642	422
31	5394	135
32	2136	571
33	878	35
34	7594	488
35	10724	362
36	10523	353
37	36121	1251
38	1841	484
39	1602	75
40	624	71
41	176	176
42	1506	87
43	3382	210
44	629	262
45	73	45
46	21837	191
47	13525	427
48	295	91
49	3583	99
50	3094	133
51	492	83
52	258	135
53	1217	329
54	1596	98
55	962	35

Process No.	COD before simulated treatment mg l^{-1}	COD after simulated treatment mg l^{-1}
56	74216	13701
57	338	67
58	2654	14
59	638	152
60	270	6
61	629	3
62	643	2
63	68	8
Total	**474351**	**61969**

The elimination rate calculated from the simulation treatment is 86.9%. The corresponding rate from the wastewater treatment plant is 88.8%.

14.7 CONCLUDING REMARKS

Currently, standardized biotests provide an efficient means to define emission limit values for complex effluents. If biotests for immission control can be adequately chosen, it will be possible to correlate water quality objectives with emission limit values in the future. Chemical analyses or a biotesting of process water may help industry to elucidate emission problems.

For regulatory purposes it is necessary that emission limit values based on biotests must adequately consider the reproducibility of the methods.

14.8 ACKNOWLEDGEMENT

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15 Biodegradation Testing in the Regulatory Environment

Brian Alexander

15.1 INTRODUCTION

In order to assess properly the environmental risks of a chemical it is necessary to have information which may, at least, enable an estimation of the approximate concentrations in the environment. This estimate is usually based on the predicted use and disposal patterns of the chemical, its physical-chemical properties and its environmental fate and the properties of the environment within which the chemical is to be used. This information on the chemical is required by national regulatory authorities for registration and notification procedures in which the authority receiving the information can evaluate the potential of the chemical to cause a hazard to man or the environment. These predictions are refined once relevant tests are performed to determine the environmental fate of the chemical.

The continued increase in use of chemicals such as human and veterinary pharmaceuticals, polymers, surfactants and other industrial chemicals in today's society means that there is consequently an increase in the environmental exposure to such chemicals. The persistence of the chemical in the environment depends on its chemical and physicochemical stability and its resistance to degradation. This stage in the chemicals' life-cycle is very important. If a chemical cannot be broken down to non-toxic products in the environment it can affect ecosystems adversely, causing problems in the health of humans and animals, phytotoxicity in crops, effects on soil organisms, etc.

Biodegradation is a significant loss mechanism in soil and aquatic systems and plays an essential role in wastewater treatment. The definition of biodegradation can be described as the molecular degradation of a material resulting from the integrated action of microorganisms. Complex organic molecules are broken down into simpler molecules eg, (CO_2+H_2O) by microorganisms in the presence or absence of oxygen.

Biodegradation can be divided into at least two types; primary and ultimate biodegradation:

(i) Primary biodegradation is the result of any biologically induced structural transformation in the parent molecule that changes its molecular integrity, eg, as demonstrated by loss of color, surface activity, structural integrity; and

(ii) Ultimate biodegradation is the biologically mediated conversion of organic compound to products associated with normal metabolic processes. Biodegradation can occur in the absence of oxygen (anaerobic biodegradation) and will additionally produce methane. (See also Chapters by Evans and Moore, Handley and Knight.)

Almost all the reactions involved in biodegradation can be classified as oxidative, reductive, hydrolytic or conjugative (methylation, acetylation). Biodegradability is an important property of a chemical which plays a part in its fate in the environment.

Biodegradation tests are usually conducted in accordance with various guidelines which form the strategy of environmental fate testing. This chapter will deal with biodegradation testing in the aquatic environment with reference to the strategies and the type of tests performed.

15.2 GUIDELINES FOR BIODEGRADATION TESTING

The most commonly used guidelines for biodegradation testing internationally are those of the Organization for Economic Cooperation and Development (OECD). European Community (EC) and the majority of the United States Environmental Protection Agency (USEPA) guidelines are derived from those of the OECD, whilst the Japanese Ministry of International Trade and Industry (MITI) guidelines have been adopted (with slight modifications) as OECD guidelines. An OECD guideline for testing biodegradability in seawater also exists, and is the preferred method of the Paris Commission (PARCOM) guideline,[1] 'Notification Scheme for the Selection of Muds and Chemicals to be used Offshore', for initial testing of this parameter. (For details see Chapter by Evans and Moore, Handley and Knight.)

The first legislation requiring biodegradation testing was introduced for surfactants used in detergents. In the 1960s Germany introduced a regulatory limit to the biodegradability of anionic surfactants. This was followed on by the OECD in 1971[2] which recommended a screening test (a die-away static test) and a confirmatory test (a continuous activated sludge test). The EC then followed with the EC Directive 73/404/EEC[3] complemented by specific directives on anionic and non-ionic surfactants.[4–6] Guidelines for safety assessment of industrial chemicals were published by the OECD in 1981.[7] The ready biodegradability and Zahn-Wellens/Swiss Federal Laboratories for Materials Testing and Research (EMPA) Test guidelines were updated and a Biodegradability in Seawater Test guideline added in 1993.[8] (For other legislation see Chapters by Handley and Knight, Evans and Moore, and references 9–13.)

15.3 STRATEGIES INVOLVED IN AEROBIC BIODEGRADATION TESTING

Most biodegradation guidelines have a decision strategy for the testing of a chemical. These guidelines are operated by means of a positive/negative result in a flowchart format. The strategy is designed to reduce the uncertainty that a chemical may or may not degrade by biotic means in the aquatic environment.

The sequence of testing for biodegradability for Annexes VIIA, VIIB, and VIII in Directive 92/32/EEC (Seventh Amendment)[11] generally follows:

(i) Ready Biodegradability at the 'Base Set' level (Annex VIIA);
(ii) Inherent Biodegradability at Level I (Annex VIII); and
(iii) Biodegradability in special environments at Level II (Annex VIII).

At the 'Base Set' level, ready biodegradability tests are static tests in which a small amount of test material and a low biomass of inoculum are incubated. The pass level for a test material ie, a readily biodegradable material, are 70% removal of DOC and 60% of the theoretical oxygen demand (ThOD) and CO_2 production ($ThCO_2$) for respirometric methods. These pass levels have to be reached in a ten day window within the 28 d period of the test. The ThOD and $ThCO_2$ are derived from the structure of the test material and the formulae to calculate them are given in both the OECD and EC guidelines. Test materials which reach the pass levels after day 28 are not readily biodegradable. The 10 d window does not apply to the MITI Test (I). Indeed, the concept of the 10 d window for all ready biodegradability tests has recently been questioned.[13] (See also Chapter by Evans and Moore.)

In general, when low (<20%) biodegradability results are obtained at the 'Base Set' level inherent biodegradability tests are performed. These tests can be either static or semicontinuous. A small amount of test material and a large biomass of inoculum are incubated under aerobic conditions in a suitable mineral nutrient medium.

If poor biodegradability (<20%) is obtained at Level I then special measures are necessary to develop a microbial population able to degrade the test material. In such cases the test material and inoculum are incubated in a model sewage plant fed continuously with an artificial sewage containing the test material (Sewage Simulation Tests).

Biodegradation testing strategy is described in the EC document XI/841/86 final[14] and is currently being updated in context of the EC Directive 92/32/EEC.[11] In certain cases the normal sequence of testing of a test material is not followed (Figures 15.1–15.3), such materials would be classified as:

(i) Materials of Concern:
- Materials classified as 'dangerous to the environment';
- Materials having the potential to bioaccumulate (Log P_{ow}>3.0);
- Materials having potential to cause adverse human health effects;
- Surface active materials;

(ii) Materials defined as Special Cases;
- Materials that are; volatile, react with water, form unstable suspensions or dispersions, poorly water soluble, photodegrade, adsorb to solids; or

(iii) Materials that do not come under these headings are classified as Materials of No Concern and follow the normal testing strategy.

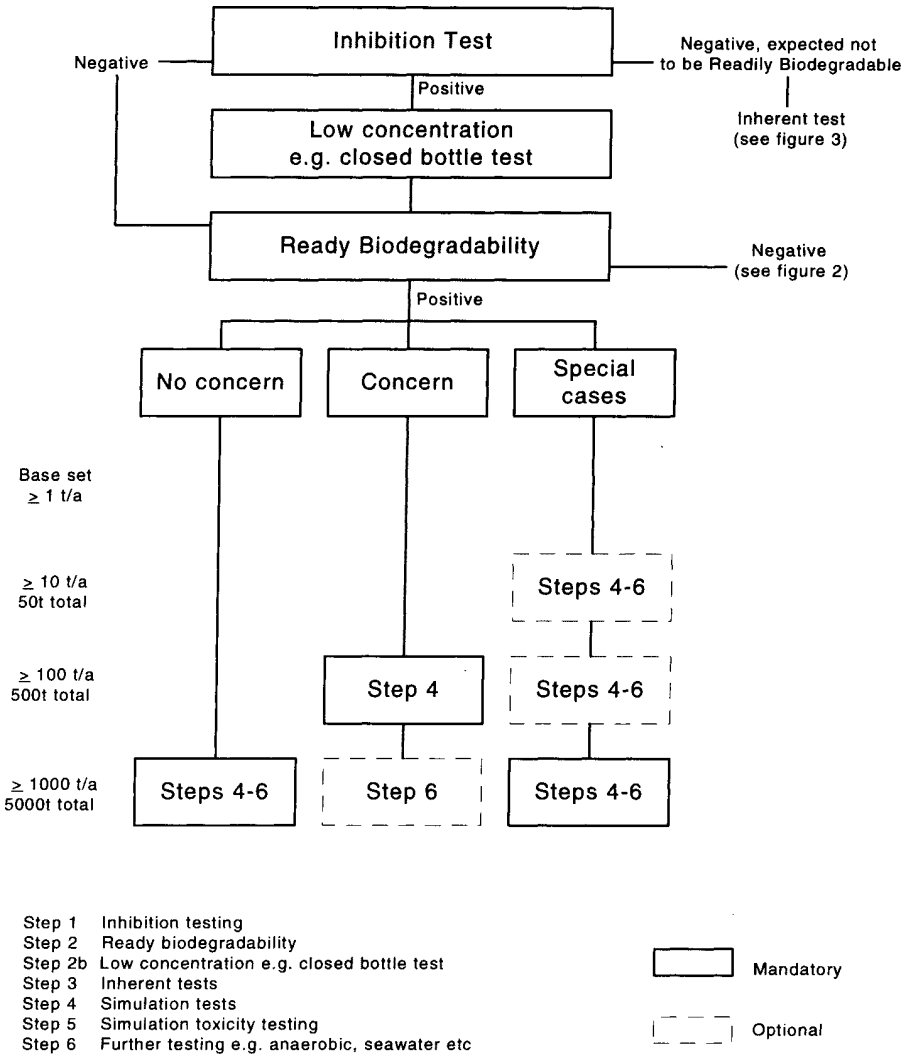

Figure 15.1 *Testing strategy for a readily biodegradable test material*

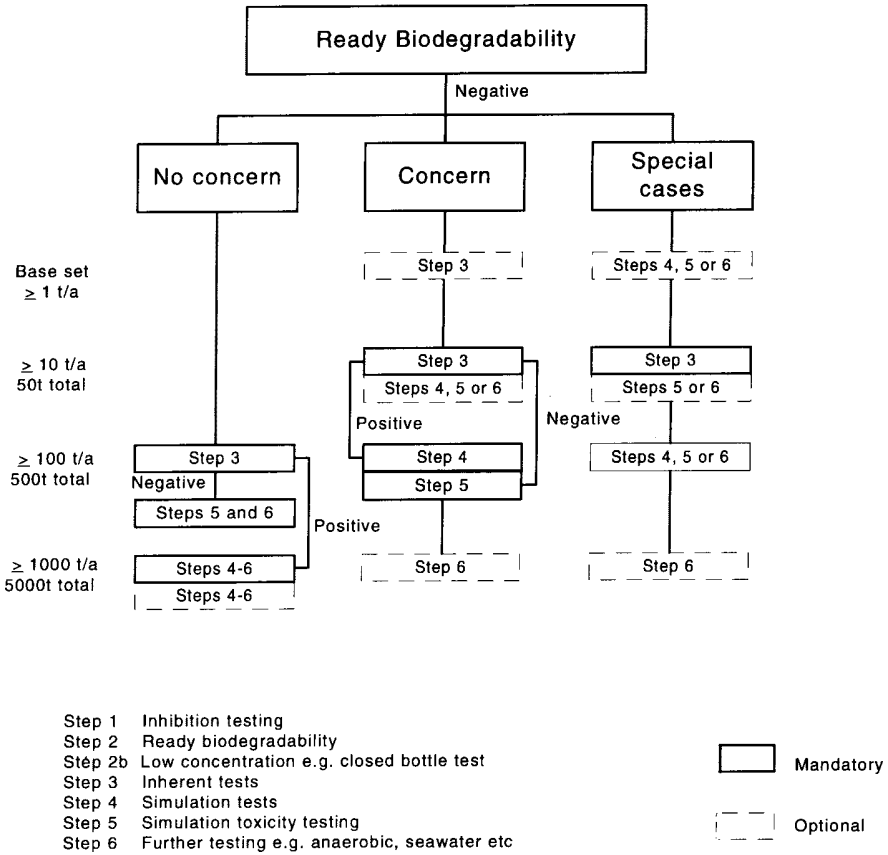

Figure 15.2 *Testing strategy for a non-readily biodegradable test material*

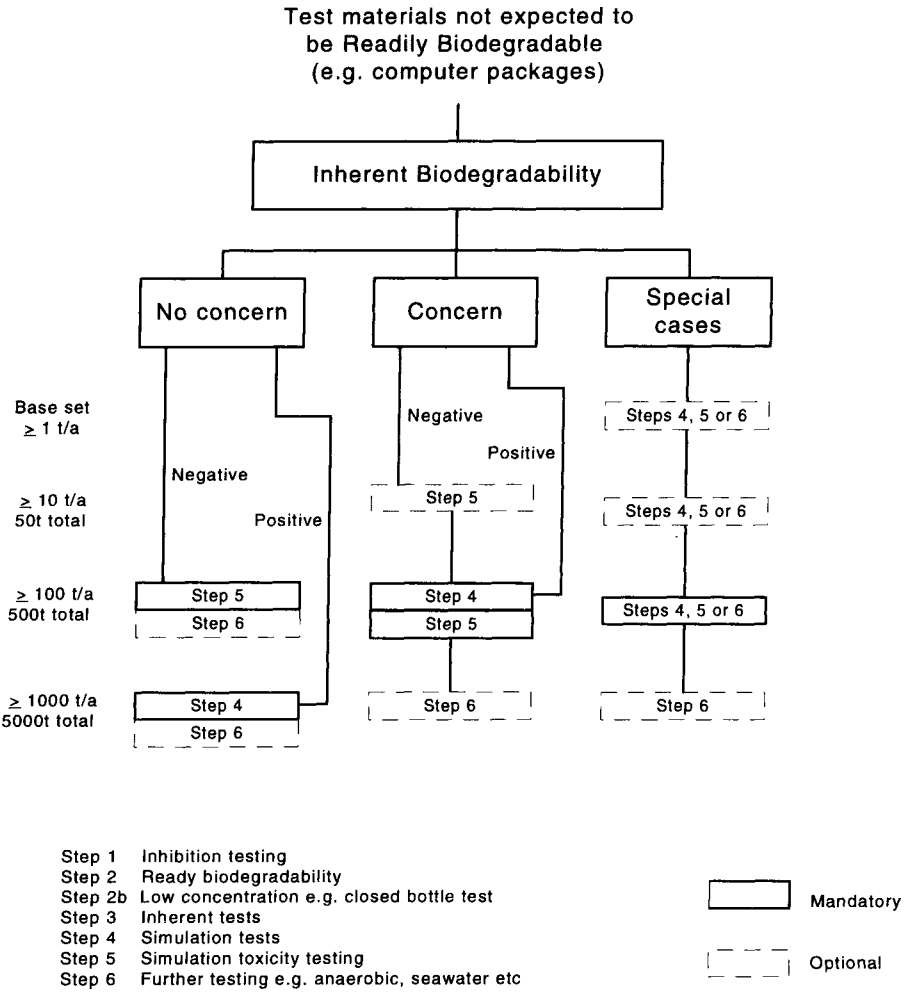

Figure 15.3 *Testing strategy for inherently biodegradable test material*

15.4 BIODEGRADATION TESTS

15.4.1 Toxicity tests

In all cases of biodegradation testing it is important, prior to performing the test, that the test material is checked to ensure that it is not toxic to the bacteria at the concentration suggested in the guideline. The OECD ready biodegradability test guidelines[8] suggest that a toxicity control is performed in parallel with the main test. Ideally, the inhibitory potential of the test material may be assessed from the structure eg, chlorobenzene derivatives may inhibit aerobic degradation, ester bonds may hydrolyse easily, polyaromatic structures are more difficult to biodegrade than simple benzene derivatives. However if the toxicity of the material is not known then specific toxicity tests should be performed prior to the ready biodegradability test to avoid the situation outlined above. (See also Chapter by Evans and Moore.)

The Activated Sludge Respiration Inhibition Test (OECD 209) and the BOD_5 Inhibition Test described by the HMSO[15] are the most commonly used tests for estimating the inhibitory effects of a chemical prior to biodegradation testing.

The Activated Sludge Test measures the inhibitory effect of the test material on oxygen uptake while it is degrading a standard substrate at high concentrations. This test is suitable for tests which have a high inoculum.

The principle of the BOD_5 Inhibition Test is the same as the toxicity control test run in parallel to the main ready biodegradation test described above. The inhibitory effects of the test material are measured by O_2 uptake.

Reynolds et al[16] compared different types of inhibition tests and concluded that the Microtox® and the Nitrification Inhibition Tests were too sensitive as a toxicity screen. The most applicable tests were the Activated Sludge and the BOD_5 Inhibition Tests. (See also Chapter by Fearnside and Booker.)

It is recommended that 1/10 of the EC_{50} (ie, the concentration of the test material which inhibits the microbial function by 50%) determined in the toxicity test, should be the concentration used in the biodegradation test. This aims to ensure that there would be no inhibitory effect of the test material to the bacteria. If an EC_{50} of >300 mg l^{-1} was determined then a concentration of 30 mg l^{-1} would be used in the main test. An EC_{50} value of 20–300 mg l^{-1} would require more detailed testing to determine the no-effect level. The Closed Bottle Test can be performed if the EC_{50} is in the range 20–300 mg l^{-1} since this test can be used at low concentrations down to 2 mg l^{-1}. Problems may be encountered if EC_{50} values of <20 mg l^{-1} are determined. The Closed Bottle Test may be used or alternatively the inoculum may be adapted to the test material in order to use higher concentrations of the test material. In such cases the inoculum and the test material are aerated together for approximately seven days with the continuous addition of inoculum. It is worth noting that when using acclimatised inocula in ready biodegradation tests the specific criterion of the ready test is lost. Another alternative in cases where the EC_{50} is <20 mg l^{-1} is to use radiolabeled ^{14}C techniques which can allow low concentrations of the test material to be used but limits the tests to those which monitor carbon.

15.4.2 Ready biodegradation tests

Following the EC Directive,[14] the next step after the toxicity test in the biodegradation strategy is to perform one of the ready biodegradability tests. This is to be performed at 1/10 of the concentration determined in the toxicity test. The type of test performed is determined by the physical characteristics of the material itself and the result of the toxicity test. Table 15.1 lists the criteria used by the EC for selecting the most appropriate test for the test material. For example, the test recommended for a poorly-soluble non-volatile material with a determined EC_{50} of 20 mg l^{-1} would be the Closed Bottle Test since 1/10 of the EC_{50} would require a concentration of 2 mg l^{-1} to be used in the test. If the EC_{50} had been determined as 300 mg l^{-1}, then the recommended tests would be a choice of either the Closed Bottle Test, Modified Sturm Test or Manometric Respirometry.

Table 15.1 *Selection criteria for the appropriate ready biodegradation test for the test material*

Test	Analysis method	Poorly soluble	Volatile	Adsorbing
DOC-die away (301A)	DOC	-	-	+/-
CO_2 evolution (301B)	Respirometry CO_2	+	-	+
MITI (I) (301C)	respirometry O_2	+	+/-	+
Closed Bottle (301D)	O_2	+/-	+	+
Modified OECD (301E)	DOC	-	-	+/-
Manometric respirometry (301F)	O_2 Consumption	+	+/-	+

In ready biodegradability tests the test material is exposed to a low biomass of inoculum containing a variety of microorganisms which have not been specifically adapted. The test material is the source of carbon in an inorganic nutrient medium. Biodegradation is measured by oxygen consumption, carbon dioxide production or Dissolved Organic Carbon (DOC) disappearance. These methods measure ultimate biodegradation.

Table 15.1 also lists the different types of ready tests that can be used. Ready biodegradability tests are stringent but simple and cheap. A positive result with this type of test would indicate that the test material is unlikely to persist (under aerobic aqueous conditions) in most environmental situations. The ready biodegradation tests are not definitive tests and are not expected to have a higher biodegradation potential than sewage treatment systems.[17] Therefore, it is common to find that the majority of test

materials examined by these methods **do not** fit into the category as being readily biodegradable as defined in the tests. This does not mean that the test material will not degrade but indicates that further work is required.

15.4.3 Inherent biodegradation tests

If the result from the ready test show that the percentage biodegradation is between 20–50%, then this would indicate that whilst the material was not readily biodegradable, it may potentially be inherently biodegradable. Thus the next step would be to test the material for inherent biodegradation. If after 28 d degradation has reached 20%, the material can be considered to be inherently biodegradable. Conversely, a negative result would indicate that the material would probably persist in the environment. (See also Chapters by Evans and Moore, Handley and Knight, Fearnside and Booker and Hiley.)

In the Modified SCAS Test the activated sludge and test material (20 mg l^{-1}) are mixed daily and the supernatant liquor is removed and analyzed for DOC. The supernatant is replaced by settled domestic sewage and the test respiked with test material. Since the test provides adaption of the microorganisms and uses a high level of biomass with long exposure time, it produces biodegradation results that can be taken as the inherent biodegradability of the material. This test is thought to be the most reliable and convenient to test for inherent biodegradation.

The Zahn Wellens/EMPA test is a batch activated sludge test using a high test substance concentration (50–400 mg DOC l^{-1}), unlike the modified SCAS test there is no removal and replacement of sludge or test material. Hence, it determines the removal of DOC; however, it is susceptible to microorganism toxicity.

The Modified MITI (II) Test is usually performed in cases where the ready Modified MITI Test (I) (OECD 301 C) has shown the test material to indicate low biodegradation. Inoculum from at least 10 different sites is required for this test, from areas where a variety of chemicals may be considered to be consumed and discarded. Unlike the other inherent tests (the Modified SCAS and the Zahn-Wellens/EMPA require at least 20 mg and 50 mg DOC l^{-1} respectively) the Modified MITI (II) Test can be performed using poorly water soluble test materials. The analysis in this test is performed by oxygen consumption using a BOD meter. Test material is mixed with inoculum in the testing vessels and the BOD is measured continuously. Biodegradation is calculated from the BOD and DOC or specific chemical analysis.

Generally, if a negative result is obtained with the Modified SCAS Test then there is no need to perform a Simulation Test but with the Zahn-Wellens/EMPA and Modified MITI (II) Tests a negative result indicates that a Simulation Test should be carried out.

A simple method using the Closed Bottle Test for inherent biodegradability has been proposed by Ginkel and Stroo[18] in which the test medium is not removed from the BOD bottles and the test prolonged for up to 200 d.

In the inherent tests problems may occur if the material is sorptive since it may be difficult to distinguish between biodegradation or adsorption on the particulate matter or vessels of the inoculum, both of which result in the removal of the DOC.

15.4.4 Simulation testing

The aim of these tests is to provide information about rates of biodegradation in specific environments under environmentally realistic conditions. The test material is exposed to a laboratory scale activated sludge based on an aerobic treatment stage of a wastewater treatment plant or other environmental situation, eg, river. Generally simulation tests measure bioelimination of a test material. Adsorption, if it occurs, is usually most marked at the start of the test and an equilibrium is reached. Biodegradation is shown as a steady upward trend of removal of test material to a constant plateau at the steady state. The extent of adsorption can be estimated by determination of the adsorption coefficient K_{oc}. The simulation tests for chemicals which are most frequently conducted are the Aerobic Sewage Treatment: Coupled Units test (OECD 303 A) and the Porous Pot method described in the HMSO, 1981.[19]

The 'Coupled Units Test' was adapted from the OECD Confirmatory Test used for surfactants by Fischer et al[20] based on the original design of Husmann[21] and Husmann et al[22] The unit of the system is called the Husmann unit which is made from acrylic or glass and consists of a cylindrical aeration chamber of 3 l capacity with a conical base and an outlet passing into the conical base of the settlement chamber. Near the top of the settlement chamber an outlet is fitted to maintain the 3 l capacity. The test conditions are based on those in real sewage treatment plants but do not simulate exactly the process in a sewage treatment plant since the airlift pump which returns the settled sludge at a high rate (>12:1 of sewage flow) does not allow for anaerobic conditions to develop during settlement. The method is performed with two small activated sludge plants running in parallel. One plant is inoculated with test material and synthetic sewage and the other with synthetic sewage only (control plant).

The DOC concentrations are measured in both effluents, the difference in the values is due to non- or partially degraded test material. The tolerance limits of the mean are calculated by means of a computer programme.

Unlike the EEC units the Porous Pot method, developed by Painter and King,[23] does not have a settlement chamber and so no predictions on settleability of the sludge can be made. The porous pots are 14 cm cylinders in diameter constructed from porous polythene. The advantage of this system over the 'Coupled Units' method is that the porous pots are cheap to construct and easier to place in a waterbath for temperature control. The effluents produced with the Porous Pot method have a lower concentration of suspended solids. As with the 'Coupled Units', the Porous Pot method has a control system running in parallel.

15.5 BIODEGRADABILITY IN SEAWATER

In the case of the PARCOM guidelines[1] for offshore chemicals the biodegradation testing strategy begins with either the Closed Bottle Test in Seawater or the Shake Flask Method in Seawater (OECD 306). The method selected depends upon the solubility of the test material. If the solubility of the test material or formulation is <5 mg C l^{-1} then the Closed Bottle would be the method of choice. These methods are based on ready biodegradation tests but are not actual tests for ready biodegradability since no inoculum is added in addition to the microorganisms already present in actual seawater which is used as the diluent. The tests can be argued to not simulate the marine environment since nutrients are added and the concentration of the test material or formulation is much higher than would normally be present in the sea. For these reasons the term 'Biodegradability in Seawater' is used.

If a test material or formulation is determined to be biodegradable in seawater no further testing is necessary, otherwise one of the tests for inherent biodegradability described above is required.

15.6 ANAEROBIC BIODEGRADABILITY

Anaerobic biodegradation is an important aspect of the overall biodegradation picture since chemicals that are resistant to this process may accumulate in anaerobic environments such as marine sediments and anaerobic muds of freshwater lakes, streams and marshes. It is widely used as a wastewater treatment process for the conversion of concentrated organic wastes to methane and carbon dioxide. It is therefore important to determine whether or not the chemicals are likely to degrade under these conditions.

Some chemicals may be toxic to the microorganisms in the anaerobic digester and interfere with the normal treatment of other wastes. It is therefore desirable to examine the effects chemicals have on the anaerobic process.

Anaerobic testing is not part of the EC 'Base Set' but is indicated in the EC testing strategy when one or more of the following properties of the test material are found:

(i) Potential to bioaccumulate (Log P_{ow}>3.0);
(ii) High adsorption coefficient; or
(iii) Low water solubility.

Anaerobic testing is required for the PARCOM guidelines when an offshore chemical or formulation has one or more of the following properties:

(i) Organic carbon adsorption coefficient (K_{oc}) of >1000;
(ii) The test material is a mud; or
(iii) The material or formulation is likely to be deposited in the sediment.

To date, there are no internationally adopted methods for anaerobic testing but this is currently being addressed.

As with the EC aerobic biodegradation strategy the first step would be an anaerobic inhibition test if the test material was suspected of being toxic to the microorganisms. One of the anaerobic inhibition methods, SCA (Standing Committee of Analysts) 1986, is a modification of the HMSO method.[24] Primary digesting sludge is incubated with different concentrations of test material. The amount of gas (methane and carbon dioxide) produced is measured by an increase in the pressure in the test bottles. This is converted to volume by a calibration graph where the EC_{50} can be calculated and an estimate of the no effect concentration determined.

Anaerobic biodegradation was recently the subject of a ring-test and an ISO Committee Document (ISO CD 11734).[25] This method based on the ECETOC Technical Report Number 28[26] and Birch et al[27] uses dilute sludge (to 10% v/v) in order to reduce the amount of gas produced by the sludge alone (control vessel). This improves the precision with which the amount of gas produced from the test material can be determined.

15.7 COMPUTER PACKAGES TO PREDICT BIODEGRADATION

It is worth noting that computer packages are now available that can estimate the biodegradability of a material based on quantitative and qualitative biodegradation data as well as a number of structural and/or physical/chemical properties. Investigators using qualitative data include Geating[28] who used discriminant function analysis on a large number of chemicals, and Niemi et al[29] who used standard 5 d biochemical oxygen demand (BOD_5) data from a large number of chemicals. These were divided into degradable and persistent chemicals by molecular connectivity indexes and physicochemical properties.

Correlation between quantitative biodegradation rates and structural and physical/chemical properties has been developed by a number of investigators, including Dearden and Nicholson,[30] Banerjee et al,[31] Paris et al,[32–34] Mudder,[35] Pitter,[36] Boethling,[37] Cantier et al[38] and Desai et al[39] A comprehensive discussion of these studies is given by Howard et al.[40]

These qualitative structural-biodegradability relationship (QSBR) studies may indicate that the material is unlikely to be readily biodegradable and thus may eliminate some unnecessary testing and this would be useful in terms of saving time and money, though the actual testing is likely to be required if ready or inherent biodegradation is claimed. This is likely to be particularly true until the computer packages are validated.

15.8 CONCLUSION

As long as chemicals are being introduced into the environment there will be a need to predict their environmental fate. At the present this has to be performed by relevant biodegradation tests in accordance with particular guidelines. These guidelines vary depending upon the material, its use and its likely environmental exposure. Ideally, they should predict the persistence or otherwise of the test material in a particular environment and have decision strategies that avoid unnecessary testing. Overall, biodegradation testing is an essential part of the environmental fate testing process.

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16 Some Observations on Biodegradabiiity Testing of Chemicals

Mike Evans and Keith Moore

16.1 INTRODUCTION

Prediction of the environmental fate of organic chemicals requires a realistic evaluation of their susceptibility to mineralisation, that is their conversion to biomass, carbon dioxide, water and various inorganic compounds. Whilst physical and chemical processes play a role in the degradation of some chemical moieties, they are rarely capable of complete destruction of the compound. Mineralisation is usually accomplished as a consequence of microbial activity. Thus, tests of degradability are designed to measure susceptibility to biological attack, or biodegradation.

Biodegradation is the biological transformation of a chemical into a different form; no extent is implied. Furthermore, biodegradation need not have a benign outcome. An innocuous compound could be converted to a toxic one, a readily metabolisable substrate could be rendered recalcitrant, or its toxicity could be altered so that it acts against a different organism. The terms primary, partial, or ultimate are often used to quantify the extent of biodegradability. Primary biodegradation is used to denote a single transformation, ultimate biodegradation denotes the complete mineralisation of the substrate, and partial biodegradation indicates any stage between these two extremes. The term recalcitrance has been defined as the inherent resistance of the compound to any degree of biodegradation, and persistence is commonly used to indicate a failure to biodegrade under specified test conditions.[1]

The ability to degrade xenobiotic compounds at all is perhaps rather surprising given the specificity of the enzymes employed by biological systems. In order for biodegradation to occur, the degrading organism must be able to utilize the substrate for energy and/or as a carbon source for growth. The ability of microorganisms to degrade xenobiotic compounds may depend on a close analogy to a naturally occurring substrate. If the novel compound is sufficiently similar to the normal substrate for a given enzyme, it may well be recognised by the enzyme, and undergo a programmed reaction. Since most enzymes act as a catalyst in a series of controlled single step reactions, or metabolic pathway, it is possible that the product of the first enzyme catalyzed reaction may be an analogue of the second substrate in the series, and so on. Thus, after several transformations the foreign nature of the molecule may be lost and the intermediate formed undergo the normal metabolic processes. This ability to metabolize foreign compounds by their relation to a recognised substrate has been called gratuitous metabolism.

A special case of gratuitous metabolism occurs when a molecule can be recognized and acted upon by an organism only in the presence of another substrate. This is termed co-metabolism.

A single microorganism may not have the capability to completely mineralize a xenobiotic substrate. In this case, a single transformation may take place, after which the altered molecule will be released. It may be found that a second species present in a mixed population will be able to degrade the product of the first, and thus many compounds are found to be biodegraded by the concerted activity of a mixed population.

Since the conditions under which biodegradation will occur may be very different for different compounds, and in differing circumstances, there is a great problem associated with the strict description of a new compound as being biodegradable or recalcitrant. Many recognized test methods have been published, which attempt to simulate specific conditions in a reproducible manner.

The need to measure the degradation of chemicals in the environment has been recognized for many years but the objectives of such measurements have changed over time. At the turn of the century, the five day biochemical oxygen demand test (BOD_5) was described as a measure of river water quality and used in the classification of rivers. That this test is a measure of biodegradation was not fully recognized until much later, although it is too stringent a test for most xenobiotic chemicals. When foam on rivers became a nuisance, resulting from surfactant use, tests were developed to differentiate surfactants which rapidly lost foaming properties from those which did not. The surfactant tests, simply measure surfactant activity as an indicator of the ability to foam and are only a measure of primary biodegradation. The surfactant tests were however the first truly internationally accepted biodegradation test methods following their publication by the OECD.[2]

Assessment of the environmental profile of novel compounds is now required for new product registrations in many countries (see also Chapters by Alexander, Handley and Knight), Governmental, inter-governmental, and international industrial trade associations are all concerned with the applicability, reliability, and standardisation of such tests in order to control the validity of the data produced.

Several authors have advocated a multi-tiered approach to biodegradability testing, based largely on the practicality of investigating what may be a many factored problem of persistence, and the resultant high costs.[3]

In the first tier of tests, an inoculum may be used which has not previously been in contact with the test substance. These are screening tests and only substances which are easily biodegraded as the sole carbonaceous substrate will pass these tests. Co-metabolism may not occur. Failure to biodegrade in these tests does not necessarily imply recalcitrance, so much as a failure of the test to provide conditions favoring biodegradation.[1] Indeed, true recalcitrance is very difficult to prove conclusively in any test. Whether the test shows primary or ultimate biodegradation will depend on the analyte chosen to monitor the utilisation of the test substrate. Respirometric parameters, such as oxygen uptake or carbon dioxide evolution, can be related to the theoretical values expected from knowledge of the empirical formula for oxidation of the test molecule, and can give an evaluation of complete mineralisation. Surfactant activity, residual color from dyestuffs, infra-red analysis of C–H stretch in the CH_2–CH_3 bond

used in an oil biodegradation test,[4] or parent compound analysis all demonstrate only primary biodegradation.

Tests in the second tier allow acclimatization of the inoculum to the test substrate, may involve the use of secondary growth substrates allowing materials requiring co-metabolic effects for biodegradation to be assessed, and will generally be of longer duration.

Third tier tests are generally semi-continuous or continuous simulations of specific situations, such as activated sludge or biological filter treatment plant. Here the emphasis is on optimising conditions favoring biodegradation by the establishment and enrichment of acclimatized, competent populations of microorganisms in order to assess whether a compound can be removed in biological treatment of wastewaters. An assessment of the rate and extent of biodegradation attainable may be made at this stage.

16.2 OECD TEST GUIDELINES

The OECD took a lead through its Environmental Directorate in establishing standard methods which form the basis of the regulatory tests currently required in many countries for product registration. In 1976 the OECD produced test guidelines for the biodegradability of surfactants. Later, the OECD[5] brought together a number of test methods for assessing the biodegradation of chemicals. These methods were amended and updated following a ring test in 1987.[6]

The problems with the concepts of biodegradation are that not only does the term have different meanings to different people but the data from current tests are now being used for different purposes other than those for which the tests were originally designed.

The OECD define three levels of tests, namely the **'Ready', 'Inherent'** and **'Simulation'** biodegradation tests. All the tests, except for the MITI tests normally, use an inoculum derived from domestic sewage biological treatment and incubation is at 22+/-2°C. In the **Ready** tests the inoculum may be pre-conditioned to the experimental conditions but not pre-adapted to the test substance. Abiotic controls are optional, as is the need to assess toxicity of the test substance to the inocula.

There are six **Ready** tests. Three of these OECD tests, 301C (MITI I), 301D (Closed bottle) and 301F (Manometric respirometry), measure oxygen uptake, two, 301A (DOC Die-away) and 301E (Modified OECD screening), measure loss of dissolved organic carbon (DOC) and the sixth test, 301B (Modified Sturm) measures carbon dioxide evolution.

16.2.1 Interpretation of the data from OECD tests

The OECD screening tests of **Ready** biodegradability vary widely in their applicability to differing substrate properties, and can give vastly different results for any one compound. The need to standardize methodology is widely recognized and

would be expected to improve the comparability of test results produced by different methods and laboratories.[7,8] Many of the differences between methods derive from historical or traditional factors. The scientific justification for differences between test conditions is frequently unclear. By definition, a standard method must display uniformity of results and a great deal of effort has been expended to this end, with international inter-laboratory ring-tests being used as an indicator of compliance with this aim.

The OECD test guidelines[6] set limits for the requirements of a test chemical passing their tests of **Ready** biodegradability. In the case of tests measuring DOC as analyte, 70% removal must be seen. Where a specific analysis for the test chemical moiety is followed, 80% removal must be seen. The accepted pass level for tests using respirometric parameters is set at 60% of theoretical carbon dioxide evolution ($ThCO_2$) or. oxygen demand (ThOD). In all cases, this is to be achieved within 10 days of the initiation of biodegradation. Initiation of biodegradation is taken to have occurred when 10% removal of DOC or of the measured direct parameter is seen, or 10% of ThOD or $ThCO_2$ has been reached. The overall time allowed for the test is 28 days.[6]

These somewhat arbitrary acceptance levels were established in the original OECD report[9] on test guidelines and based on practical experience. There is some weight of evidence, however, that they may be overly stringent. On examining the data generated by an OECD ring test[10] Kuenemann et al[11] found that over 40% of substrate DOC can be used in new cell growth, so that when carbon dioxide evolution is measured, <60% $ThCO_2$ pass level is attained despite near complete DOC removal. The stipulation of test duration being 28 days and the inclusion of a 10 day 'window' for biodegradation to occur appear particularly subjective,[12] being set with the aim of maintaining test stringency only. The present testing regime allows up to 18 days acclimatization time for the inoculum. It is considered that removal of test substrate taking longer than 10 days indicates that the substance will be difficult to treat in practice. This view is questioned by Painter and King.[13]

The OECD guidelines were all designed to provide data on the potential biodegradability of chemicals when released into an aerobic aquatic environment and by and large they all provide such information. They do not provide information on the likelihood of biodegradation in other environments. Complex chemicals that are difficult to degrade in the OECD tests are sometimes degraded by acidophilic and/or surface colonising microbes in soil.

The OECD tests were not designed to measure rate or extent of biodegradation in the natural environment nor to label any chemical as recalcitrant. The sub-division into **Ready** and **Inherent** biodegradability indicates whether or not a prolonged lag period may be expected before degradation occurs, but does not provide any insight into the likely length of a lag phase in the environment.

16.2.2 Inocula

The source, quantity and pre-treatment of the inoculum used is probably the one factor having the greatest influence on assay performance.

The OECD protocols for **Ready** biodegradability testing stipulate conditions to be met. The inoculum must be from a 'natural' source. This includes sewage, activated sludge, soil, mud, river water and lake water.

Where the source is a wastewater treatment plant, the sewage treated must be predominantly of domestic origin. This is curious since domestic sewage contains a range of concentrations of a wide diversity of products of the chemical industry. Trade wastes may contain higher concentrations of a more limited range of chemicals and chemical industry wastewaters contain a restricted number of products together with a variety of chemical intermediates. Therefore sludge from purely domestic sewage origin can be expected to be acclimatized to a range of xenobiotic chemicals.

Cell density is approximately 10^5 l^{-1} in the closed bottle test and modified screening test or 30 mg suspended solids l^{-1}, equivalent to about 10^8 cells l^{-1} in the manometric respirometry, modified Sturm and DOC die-away tests. It is assumed that any size of inoculum will eventually remove a biodegradable substance, as long as the test chemical is below a toxic threshold, at a rate predictable by calculation of die-away using Monod-type bacterial kinetics.[13] Conversely, inoculum concentrations above about 100 mg suspended solids l^{-1} can give rise to significant errors due to sorption and desorption effects in the solid mass.[14]

Pre-treatment of the inoculum may include acclimatization to the specific test conditions, but should not include exposure to the test chemical. Such pre-exposure favors the growth of those species which are capable of utilising the test compound and it is then assumed that the test itself will be performed with an atypical culture and cannot be accepted as a true test of **Ready** biodegradability.

The inoculum used in the Japanese MITI tests is slightly different in that several sources, including industrial wastewater treatment works, rivers, lakes and sea are sampled. These are blended and the supernatant, after settling, grown on a synthetic sewage to provide an activated sludge for use as inoculum. It is doubtful whether this protracted process truly yields a more acceptable or more consistent inoculum as the microbial population diversity of the original mixed sample may be lost during the subsequent growth on synthetic sewage.[15]

16.2.3 Incubation conditions

Incubation temperature and time are purely arbitrary. Ambient temperatures are often lower than 22°C but the rate of microbial activity decreases at lower temperatures which would necessitate longer incubation times. Some customers for biodegradation tests already consider 28 days to be too long.

16.2.4 Analyte

It is generally assumed that oxygen uptake is an indirect measure of mineralisation. However some oxygen consumption can be attributed to partial oxidation of organic molecules, or primary degradation. When test materials are rich in nitrogen, ammonification may occur followed by nitrification which has a high oxygen demand. In the five day BOD test it is common to use allylthiourea as a nitrification inhibitor but it is not suitable for the 28 day test since it is itself biodegradable.

DOC removal indicates bioelimination but fails to distinguish between sorption and mineralisation. Carbon dioxide evolution is a direct measure of mineralisation, although if nitrification occurs, a small proportion of the released CO_2 may be utilized for the autotrophic growth.

More frequent measurement of oxygen uptake or CO_2 evolution than called for in the OECD guidelines will give kinetic rate constants but only for the conditions of the test. Extrapolation to the natural environment needs to take account of other factors including temperature, pH values and the presence of other substrates.

The accumulated data from testing of chemicals over the past few years only provide the percentage biodegradation in one of the OECD tests. They cannot be used to extrapolate to truly meaningful kinetic rate constants.

16.2.5 CO_2 evolution test methods

The method described by Sturm[16] which is the basis of OECD 301B is the only current test protocol making a direct measure of a product of biodegradation. It measures the extent of biodegradation of a test compound by monitoring the carbon dioxide produced by the microbial population as a result of their metabolising the substrate. Knowledge of the organic carbon content of the substance allows an estimation of the expected carbon dioxide output from its complete oxidation. This is not seen in practice because of the incorporation of some of the substrate carbon into new cells.

The method originally described by Sturm suffers from several practical drawbacks.[17] It involves the use of a steady carbon dioxide-free air supply to drive carbon dioxide from the test medium to bubble impingers containing barium hydroxide as a carbon dioxide absorbent. The alkali is then titrated against a standard acid to estimate the barium hydroxide consumed and thus the carbon dioxide production is quantified. This entails the use of bulky and complex apparatus. The continuous gas stream passing through the test vessel may not completely purge the produced carbon dioxide from the reaction vessel, leading to false low results. Birch[18] reported finding a higher rate of DOC removal than carbon dioxide collection, with a sufficient differential for some chemicals to pass the criteria for DOC removal, but to fail the 10 day 'window' requirement when ThCO_2 is measured. This finding was confirmed by Weytjens et al,[19] who followed dissolved inorganic carbon (DIG) in the medium concurrently with the carbon dioxide trapped by the impingers. It was

found that the conditions of the test do not allow the rate of collection of carbon dioxide in the alkaline traps to reflect the true rate of production by the microorganisms present. The continuous purging also renders the method unsuitable for the estimation of biodegradability of volatile compounds. Ingress of atmospheric carbon dioxide into the alkali absorbent, which is remote from the test vessel may also be manifest. Unlike the die-away tests, it is, however, suitable for both soluble and poorly soluble non-volatile compounds.

Improvements to the original method of Sturm include a significant reduction in the test vessel volume by Gledhill[17] and the addition by Oudot[20] of a U-tube containing carbon dioxide absorbent to the exhaust from the test apparatus to prevent the ingress of atmospheric carbon dioxide.

Several modifications of the original method have since been developed utilising a closed bottle from which samples are drawn in order to assay the carbon dioxide as gaseous or dissolved inorganic carbon.[21,22] The use of a closed system implies that the method will accommodate volatile samples. There is a considerable saving in space and equipment and thus the opportunity for greater replicate numbers, conferring improved precision.

The method of Birch and Fletcher[22] makes very few changes to the original test conditions of the Sturm test, except in that the test vessel is much more compact (125ml nominal capacity sealable bottles are employed, as opposed to the original 5l flasks) and in the manner of assaying the produced carbon dioxide. Here the gaseous carbon dioxide and the dissolved carbon dioxide, as dissolved inorganic carbon (DIG) is measured by withdrawing samples via the septum sealed closure. Analysis was performed by use of a non-dispersive infra-red analyzer. Results for a range of compounds were found to be comparable with those obtained by the standard technique.

The method of Struijs and Stoltenkamp[21] is essentially similar to that of Birch and Fletcher, except that the contents of the test bottle are acidified prior to analysis. At a pH less than 3.0 the partition of the inorganic carbon (IC) between the liquid and headspace gas was claimed to be consistent, with equal concentration in each phase. Only one phase therefore need be measured and the total IC extrapolated from knowledge of the relative volumes of each phase. Whilst there can be no doubt that all the produced carbon dioxide must be measured by the method of Birch and Fletcher, there is some evidence to suggest that the release of DIG to the headspace gas after acidification may not be entirely efficient, and could, lead to low assays being reported.[23]

A further modification has been suggested by Peterson[24] in which the medium is treated with alkali in order to disturb the gaseous partition in favour of the resorption of carbon dioxide to the medium. This implies that only the DIG need be estimated, the contribution of the gas phase IC being negligible.

Pallett[23] compared the three methods of measuring CO_2 evolution in the closed bottle modification of the Sturm test. Pallett measured the biodegradability of sodium acetate and Enerpar M2632 (a slowly degradable white oil used as one of the reference oils in the CEC test[4]) in 3 sets of bottles. Acid was injected into one set of bottles and alkali into the second set. CO_2 was then measured in both the liquid and gas phases of all the bottles. All the CO_2 was adsorbed into the liquid by alkination but the partition

between the liquid and gas after acidification was not always unity. However all three measurements gave similar results for biodegradability (Figure 16.1).

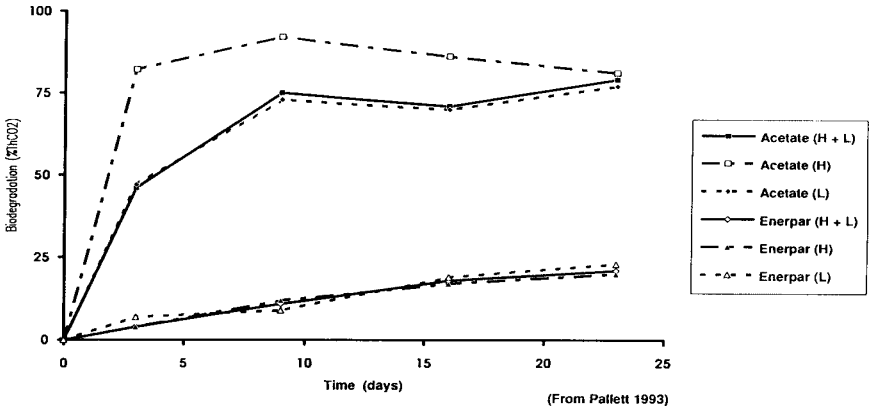

Figure 16.1 *Biodegradation of sodium acetate and enerpar. Carbon dioxide measured in gas and liquid (H+L); gas after acid addition (H); liquid after alkali addition (L)*

Moore[25] studied the effects of inoculum and substrate concentrations on the assay performance in the closed bottle CO_2 evolution test. Tests were done with initial inoculum concentrations between 10^2 and 10^6 cell forming units (cfu) ml^{-1} and test substance concentrations of 10, 20 and 30 mg C l^{-1}. The percentage biodegradation values were similar in each case for a number of chemicals as illustrated in Figures 16.2 to 16.5. With the higher inoculum concentration and test substance concentrations of 10 mg C l^{-1} there was no significant increase in cell numbers throughout the test period. With lower initial cell numbers or higher substrate concentrations cell numbers increased during the test. The evolved CO_2 was slightly lower and comparable to that calculated to have been incorporated into new cells.

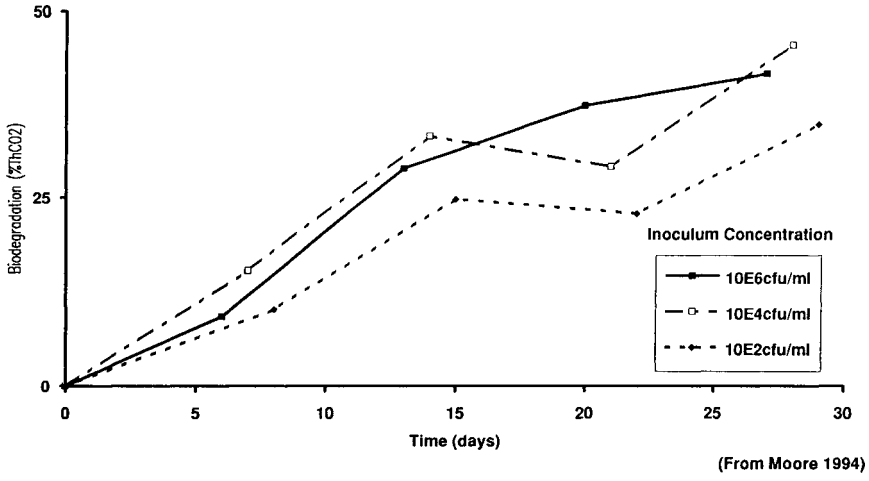

Figure 16.2 *Biodegradation of DITA at various inoculum concentrations*

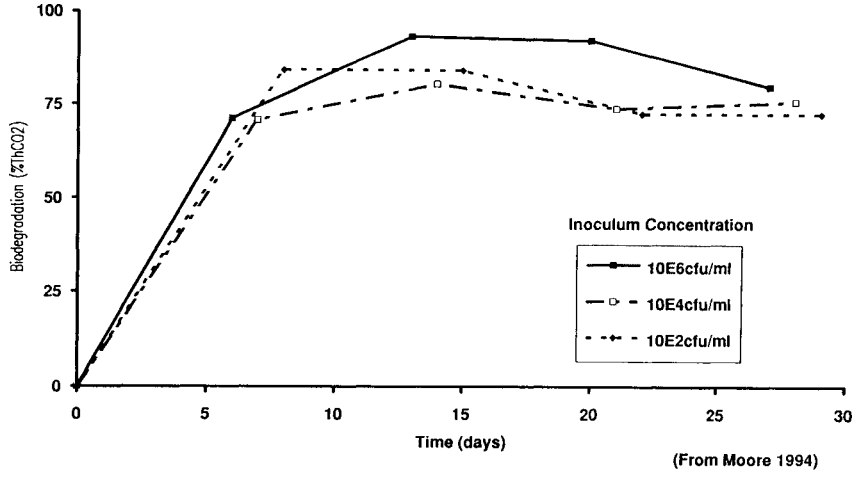

Figure 16.3 *Biodegradation of sodium acetate at various inoculum concentrations*

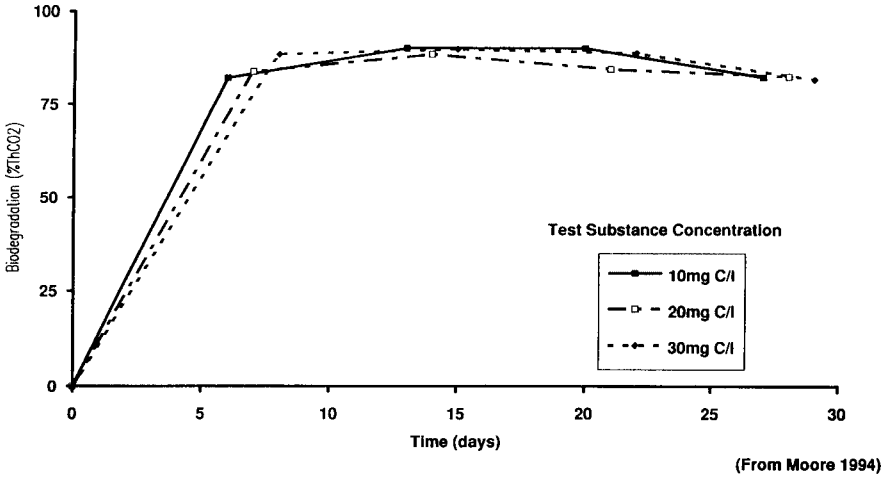

Figure 16.4 *Biodegradation of sodium acetate at various test substance concentrations*

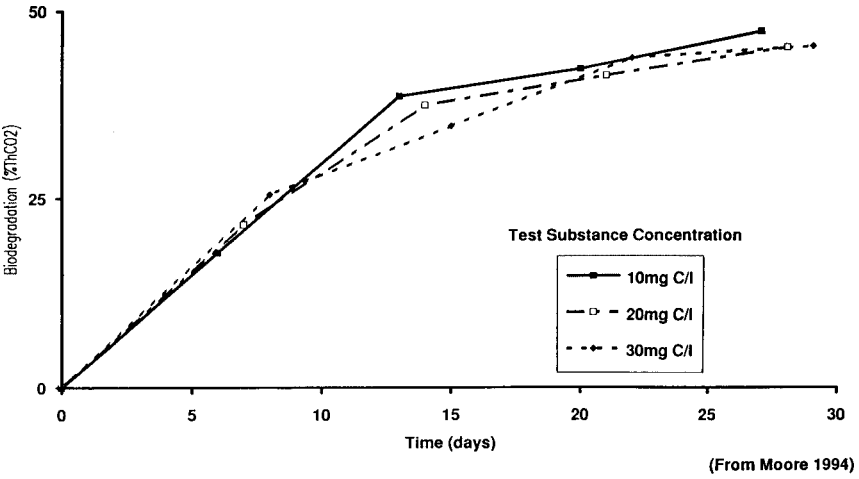

Figure 16.5 *Biodegradation of DITA at various test substance concentrations*

16.3 CONCLUSIONS

Whilst the **Ready** biodegradation test methods described by the OECD meet their original objectives, they do not all confirm ultimate biodegradation. Current requirements are to predict the environmental fate of chemicals, in particular the extent and rate of biodegradation. CO_2 evolution tests provide direct evidence of ultimate biodegradation. The modified Sturm test (301B) has a number of drawbacks that are overcome by a recent closed bottle modification of the OECD test. However even frequent measurements of evolved CO_2 in multi-replicate bottles do not provide adequate data on biodegradation rates to be expected in the natural environment.

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SECTION 5: CASE STUDIES

17 The Austrian Water Quality Monitoring System and its Integration into the Water Management Concept

Wilhelm R.Vogel

17.1 INTRODUCTION

Austria—situated in Central Europe—has different landscape types: high alpine areas, large valley landscapes with intensively agricultural valley floors and the north-eastern parts of the country which are strongly influenced by the pannonian climate, thus exhibiting a nearly steppe-like character. Precipitation varies according to altitude, on average amounting to about 1200 mm. Whereas precipitation in higher regions often exceeds 2000 mm, it remains ~500 mm in the areas influenced by the pannonian climate. Beside all other aspects the quantity of precipitation alone influences water quality.

In the mountainous areas water quality is influenced by extensive agricultural activity and those industrial branches which on account of their location close to resources have always been situated in these areas, eg, the pulp and paper industry and the metallurgical industry. During the last decades tourism has become an important factor. Lower areas are characterized by extensive urban development combined with high traffic volumes and various branches of industry and commerce in addition to intensive agriculture especially in the eastern parts of the country.

The first step in the remediation of lakes suffering from excessive nutrient input was initiated in the late 1960s. The lakes were chosen for economic reasons since they represent an important factor for tourism. With a few exceptions remediation was carried out by ring channelization.

Until the late 1980s, the pulp industry was one of the main causes of contamination of receiving waters. The location of the pulp mills could be identified easily on the water quality maps established since the 1960s on the basis of the saprobiological system on account of the red colour indicating poor water quality. By 1991 emissions from the pulp and paper industry were reduced by >90% by corresponding measures such as severe emission standards and an improved state funding system. A second significant cause of contamination is municipal wastewater. In 1968, ≯3% of the population were connected to biological wastewater treatment plants, which only reached 50% by 1981. Subsequently, this number has risen to ~70%. On account of the specific settlement structure in Austria (scattered villages and individual farmhouses) 88% would appear to be a reasonable goal.

With the remediation of the lakes and the disappearance of scum from the river surfaces the more evident signs of contamination have disappeared. Further remediation measures are still required not only in the field of easily degradable

substances (where significant progress has been made) but also with regard to persistent toxic substances. However, localization of problematic areas has become increasingly difficult, effective remediation measures (and thus efficient use of resources) depend on detailed information on temporal and spatial pollution distribution. Similar principles apply to the development of remediation strategies for groundwater. Groundwater is known to be contaminated by nitrate input from agricultural activities in addition to leakage from waste disposal sites. In the sensitive karst areas tourism represents an additional stress factor. In a country in which almost all drinking water is derived from groundwater sources (including springs) these problems must be taken seriously.

Chemical investigation of waters commenced in the 1970s. But the databases was totally insufficient to meet the requirements of a modern water protection system. In order to obtain current, accurate and comparable information on the contamination of surface and ground waters the establishment of a nationwide monitoring system started in 1991. By now, this programme forms an integral part of the Austrian water protection concept. This monitoring system and its role within the water protection concept will be explained in greater detail.

17.2 PRINCIPLES OF THE AUSTRIAN WATER PROTECTION CONCEPT

The main features of water protection are prescribed in the Federal Water Act.[1] With reference to water protection the Federal Water Act stipulates that all water bodies including groundwater have to be kept clean so as not to jeopardize the health of man and animals, and to ensure that ground and spring water can be used as drinking water. The natural state of water bodies regarding physical, chemical and biological aspects has to be maintained. The protection covers the conservation of waters in their natural state including the adjoining terrestrial areas necessary for maintaining the ecological functional integrity.

This formulation defines certain objectives,[2] the achievement of which depends on a variety of further regulations whose interaction are explained.

17.2.1 **Emission standards** stipulated in ordinances shall limit pollutant input into surface waters. This ordinance sets limit values according to the 'best available means' which have to be met regardless of type and size of the water body. One general and about 60 specific ordinances, of which 20 have been promulgated to date, define the maximum admissible emission levels for receiving water bodies and also sewage treatment plants. These emission standards include physical and chemical parameters in addition to toxicity to fish and (up to now in one ordinance) for luminescent bacteria.

17.2.2 **Limit values for the contamination of larger rivers** distinguishing between mountainous and lowland rivers are stipulated in a draft ordinance. These limit values were set with a view to meeting the above mentioned qualitative objectives and to reaching at least water quality class II according to the saprobiological system (Kolkwitz-Harsson-Liebmann system).[3] Furthermore, this ordinance defines the requirements for an investigation of these water bodies (investigation period, etc.). In case of an exceedance of the limit values the provincial governor has to designate the areas concerned as remediation areas and stipulate appropriate remediation concepts. These measures may *inter alia* include the setting of more severe emission standards. Currently, there are no similar regulations for smaller rivers or lakes.

17.2.3 **Threshold values for groundwater pollution** are already in force.[4] These threshold values were defined with regard to drinking water standards (see above). In general the threshold value is set at 60% of the drinking water limit value. If in a specific groundwater area the groundwater threshold value is exceeded for one parameter at measuring sites in >25% of the investigations, this area has to be designated a remediation area and recovery concepts have to be designed accordingly. In this case remediation measures might consist of the remediation of contaminated sites or by restrictions on agricultural practices (reduction of the amount of fertilizers or pesticides applied).

The regulating mechanism described above requires detailed information on the *status quo* of water quality of ground and surface waters. This information will be provided by the Austrian Water Quality Monitoring System.

17.3 THE AUSTRIAN WATER QUALITY MONITORING SYSTEM (AWQMS)

According to Meybeck et al[5] 'monitoring is a long-term, standardized measurement, observation, evaluation, and reporting technique for the aquatic environment in order to define its status and trends'. 'It is purpose orientated; it tells how something(s) is/are changed; thus it is dynamic in philosophy…'

The only nationwide system meeting this standard in the field of the aquatic environment has been monitoring based on the saprobiological system. Investigations have been earned out since 1961 and water quality maps have been published in intervals over some years. The information provided by these maps have proved to be very helpful, but they lack concentration data totally. Many substances although known to be harmful to the environment cannot be detected. Investigations for specific substances have often been carried out but only on the local or regional levels with different sampling methods, different analyzing methods and differing sampling sites. This is why the data so obtained have rarely been comparable.

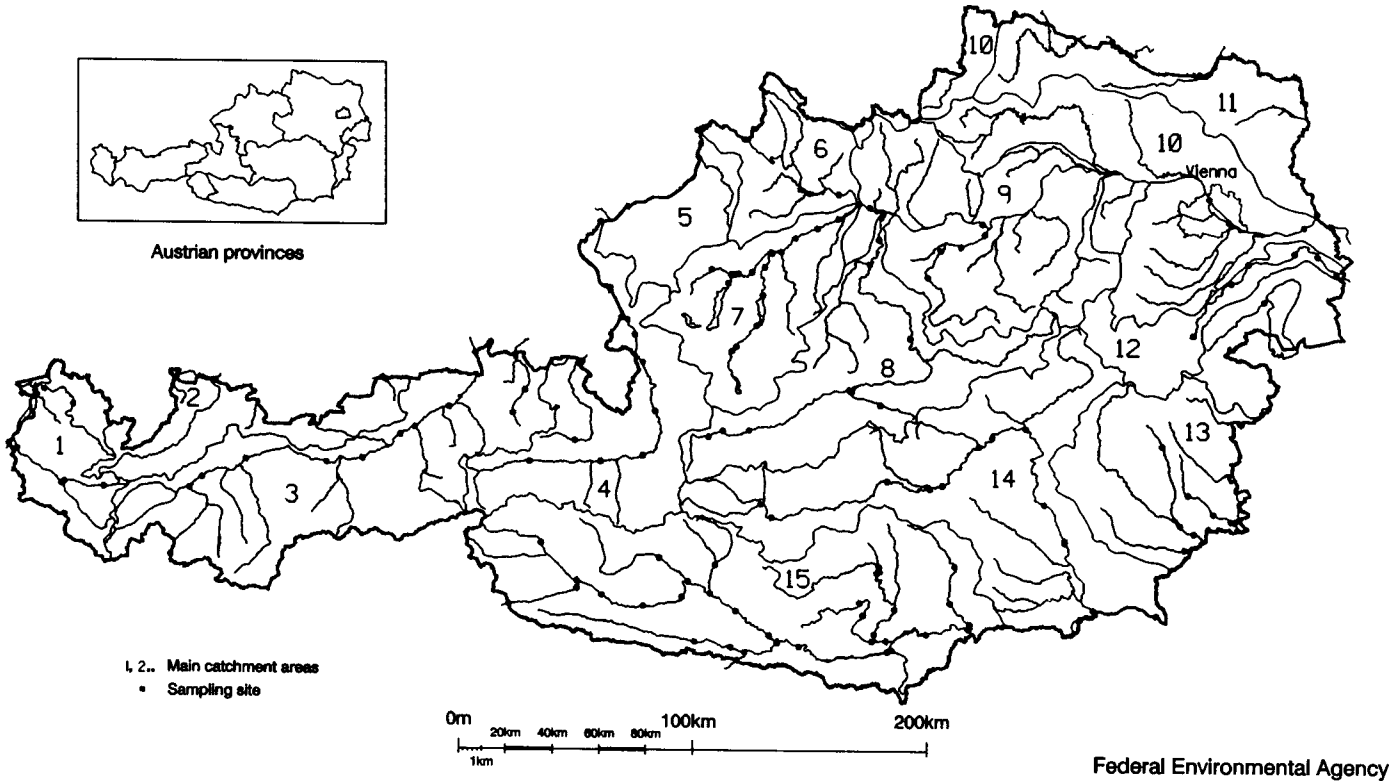

Figure 17.1 *River monitoring sites (1993)*

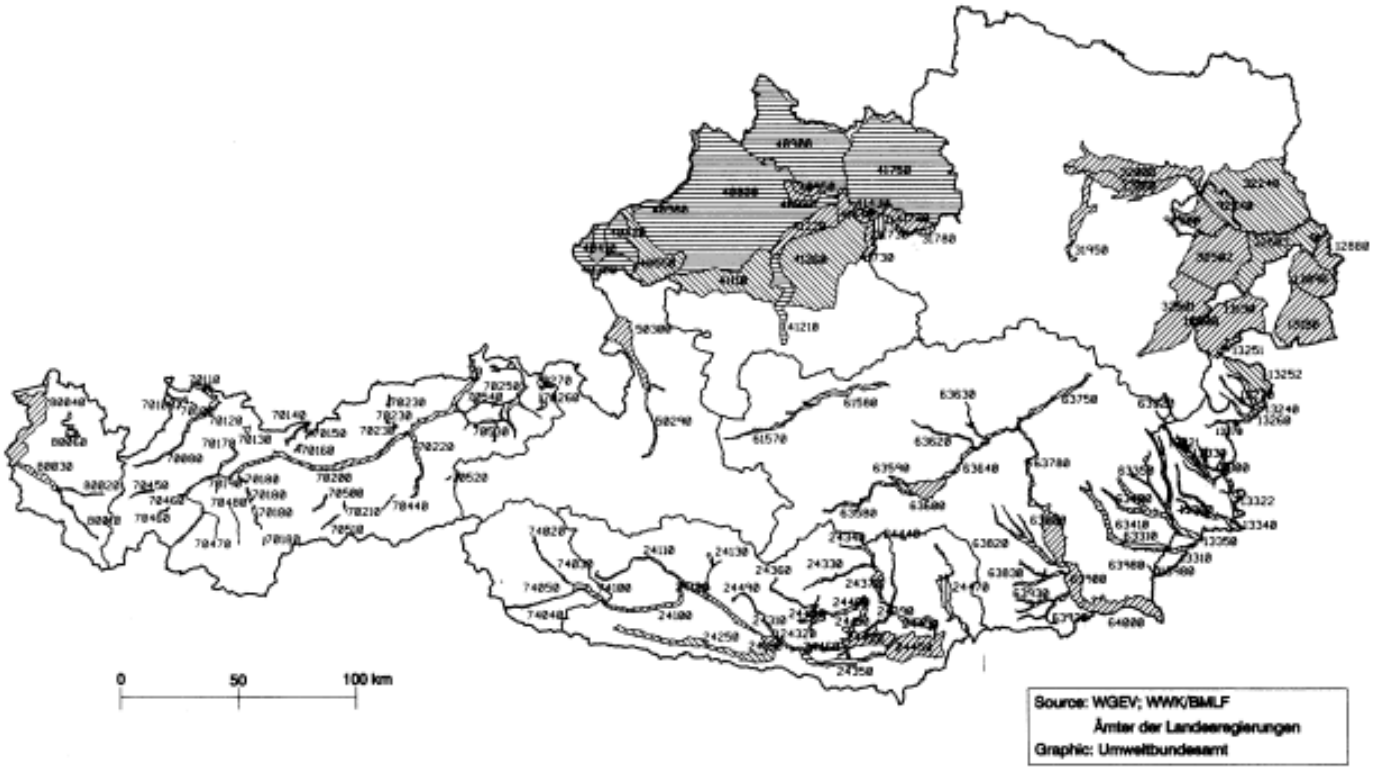

Figure 17.2 *Groundwater areas in porous media (1993)*

17.3.1 The River monitoring network

The installation of the river monitoring network commenced in 1991 with 85 sampling sites. The first report[7] comprised 150 sites which are indicated on the map given in Figure 17.1; in 1994, the network covers some 250 sites. The investigational programme includes chemical analysis of water samples (bimonthly) and of sediments (annually). Analyses of biota (saprobiological index) are also carried out annually. The investigations are carried out in six-year cycles, the first two years being regarded as first investigation, the following four years as follow-up investigation, followed by the first investigations of the next cycle.

At each site some 50 parameters are measured. The parameters are grouped in three sets, the first includes those parameters which allow a general characterization of a river and a first assessment of the water quality including nutrients (nitrate, phosphate) and organic matter such as BOD, TOC and DOC.

Table 17.1 *List of parameters for groundwater investigations*

Set 1	Set 2	Set 3
Depth	Arsenic	Aluminium
Discharge	Lead	Copper
Colour	Cadmium	Nickel
Turbidity	Chromium	Zinc
Odour	Iron	Hydrocarbons
Temperature	Manganese	Fluoride
pH	Mercury	Cyanide
Conductivity	AOX	Dichloromethane
Dissolved oxygen	Trichloroethene	1,1,2-Trichloroethane
Total hardness	Tetrachloroethene	substances listed in the Austrian foodstuffs code
Carbonate hardness	1,1,1-Trichloroethane	Phenol-index
Hydrogen carbonate	Trichloromethane	Benzene
Calcium	Tetrachloroethane	Toluol
Magnesium	1,1-Dichloroethene	Xylol
Sodium	1,2-Dichloroethane	
Potassium	PAHs	
Nitrate	PCBs	
Nitrite	Pesticides	
Ammonium		
Chloride		
Sulfate		
Orthophosphate		
Boron		
DOC		

Table 17.2 *List of parameters for receiving water investigations*

Set 1	**Set 2**	**Set 3**
Water:	**Water:**	**Water:**
Velocity	Total hardness	Aluminium
Water temperature	Carbonate hardness	Arsenic
Air temperature	Calcium	Lead
pH	Magnesium	Cadmium
Colour	Potassium	Chromium
Turbidity	Sodium	Iron
Smell	Chloride	Copper
Filterable substances	Sulphate	Manganese
Conductivity	AOX	Nickel
Dissolved oxygen	Hydrocarbons	Mercury
Oxygen saturation	Phenol index	Zinc
BOD_5		COB
TOC	**Sediment:**	BOD_5
DOC		Boron
Orthophosphate	sampling procedure	Cyanide
Total phosphorus	Ignition loss	Fluoride
Ammonium	Arsenic	Dichloromethane
Ammonia	Lead	1,2-Dichloroethane
Nitrate	Cadmium	1,1-Dichloroethene
Nitrite	Chromium	Parameters listed in the Austrian foodstuffs code
Biological quality	Copper	Benzene
	Manganese	Toluol
	Nickel	Xylol
	Mercury	PAH
	Zinc	PCB
	EOX	Tensides
		Pesticides
		Sediment:
		EOX
		POX
		Aerobic and facultatively anaerobic
		Heterotrophic germs
		Fecal coliforms
		Ecotoxicological parameters: bacteria, algae, and *Daphnia*

Parameters in set 2 have to be analyzed in the course of the first investigations of each cycle, later only if they are suspected to occur, or if there is some evidence from the first results. Set 3 is an open ended list of parameters which—although of high ecotoxicological relevance as, eg, pesticides or some organochlorines—are not measured routinely. Due to the fact that these analysis demands very sophisticated and expensive procedures they can only be taken into consideration in special programmes, which may or may not to be restricted to certain regions. In 1993, a programme for tensides was undertaken. Another programme under consideration is to investigate new pesticides following certain times of application. Generally, the investigation scheme is a flexible one, additional parameters can be chosen at any time.[8,9]

17.3.2 Groundwater monitoring

Due to the fact that drinking water is derived to ~98% from ground water sources, the groundwater monitoring system is much more elaborated. Out of ~2000 monitoring sites ~1600 are situated in porous media (see Figure 17.2), 400 in karst and crevice groundwater areas (including springs). Similarly, surface waters monitoring is carried out in six-year cycles, the first year of each cycle being considered as first investigation. The investigations are carried out quarterly. As with surface waters, parameters are grouped in three sets. Provided that no contamination is detected and not to be expected a reduced investigation frequency can be agreed upon from every third year.[8,9]

17.3.3 Organisational aspects

The Federal Water Act[1] and the Federal Act on Hydrography[9] were amended in 1990 to provide a legal, administrative, and financial basis for a new countrywide water monitoring system. The enforcement and administration of the monitoring system (including field sampling operations, laboratory analyses, data transfer and management, reporting) is carried out in close cooperation by the Department for Federal Water Management Register (FWMR) of the Federal Ministry of Agriculture and Forestry, The Federal Environmental Agency (FEA) and the provincial authorities. Costs were met by Federal (60%) and Provincial (33%) authorities,[7,10]

17.3.4 Standardised sampling methods and analytical methods

As mentioned previously, data obtained in the past have rarely been comparable. Therefore, one of the first tasks was to establish standard procedures in the field of sampling (including preparation of the transport bottles and stabilisation), transport, storage, chemical analysis, data collection and processing etc. Relevant procedures are described in detail in manuals[11] and round robin tests were also organized.[12] Sampling and chemical analysis

are carried out by private laboratories selected every year by official calls for tender. Training programmes for the laboratories involved are organized regularly. Laboratories may be subjected to controls at any time (eg, check of correct application of the methods, adequate sample storage, correct keeping of laboratory records). Double samples are infiltrated occasionally.

17.3.5 Data flow

The software, including PC-software, has been developed by the Federal Environmental Agency.[7,13] The PC-software including data input masks is made available to the laboratories. The data discs are sent to the provincial authorities, where the first data check is carried out (in some cases data input is still managed at the level of the Provincial authorities) and the data carriers are sent to the FWMR and to the FEA subsequently.

17.3.6 Publishing of data

Investigation results are published in annual reports, the first one of which was published in December 1993.[7] Apart from this report a shortened version of the results of the AWQMS is published in the biennial environmental control report to the parliament and in the water protection report published every three years. Individual aspects are covered by publications of the FMWR/FEA and in scientific journals (eg, references 14–17). According to the Environmental Information Act water quality data are made available to all interested persons. Administrative authorities and private companies increasingly turn to the FEA or the FMWR for such information.

17.4 RESULTS OF WATER QUALITY MONITORING

Data of the first investigation period December 1991 to December 1992 were published in an aggregated form in the annual report 1993.[7] The following results are presented briefly.

17.4.1 Ground water monitoring

The ordinance on nitrate in drinking water[18] stipulates that the nitrate concentration (NO_3^-) must not exceed 50 mg l^{-1} (from 1.7.94), or 30 mg l^{-1} (from 1.7.99), respectively. The ordinance on groundwater threshold values[3] sets the following thresholds: 45 mg l^{-1} until 30.6.1997, and 30 mg l^{-1} until 30.6.1999, then 18 mg l^{-1} (the latter two corresponding to 60% of the respective drinking water limits).

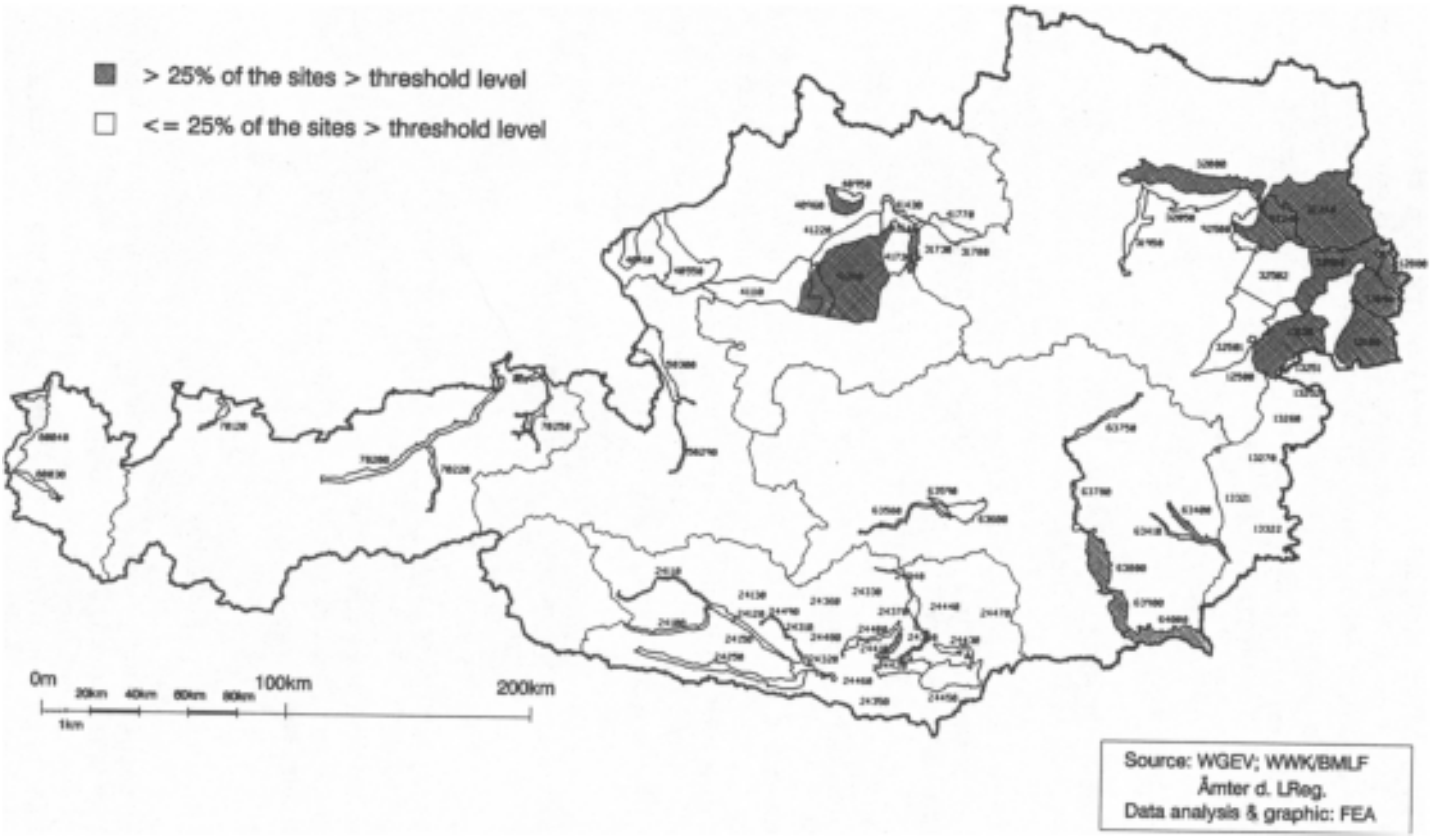

Figure 17.3 *Results of groundwater monitoring: nitrate*

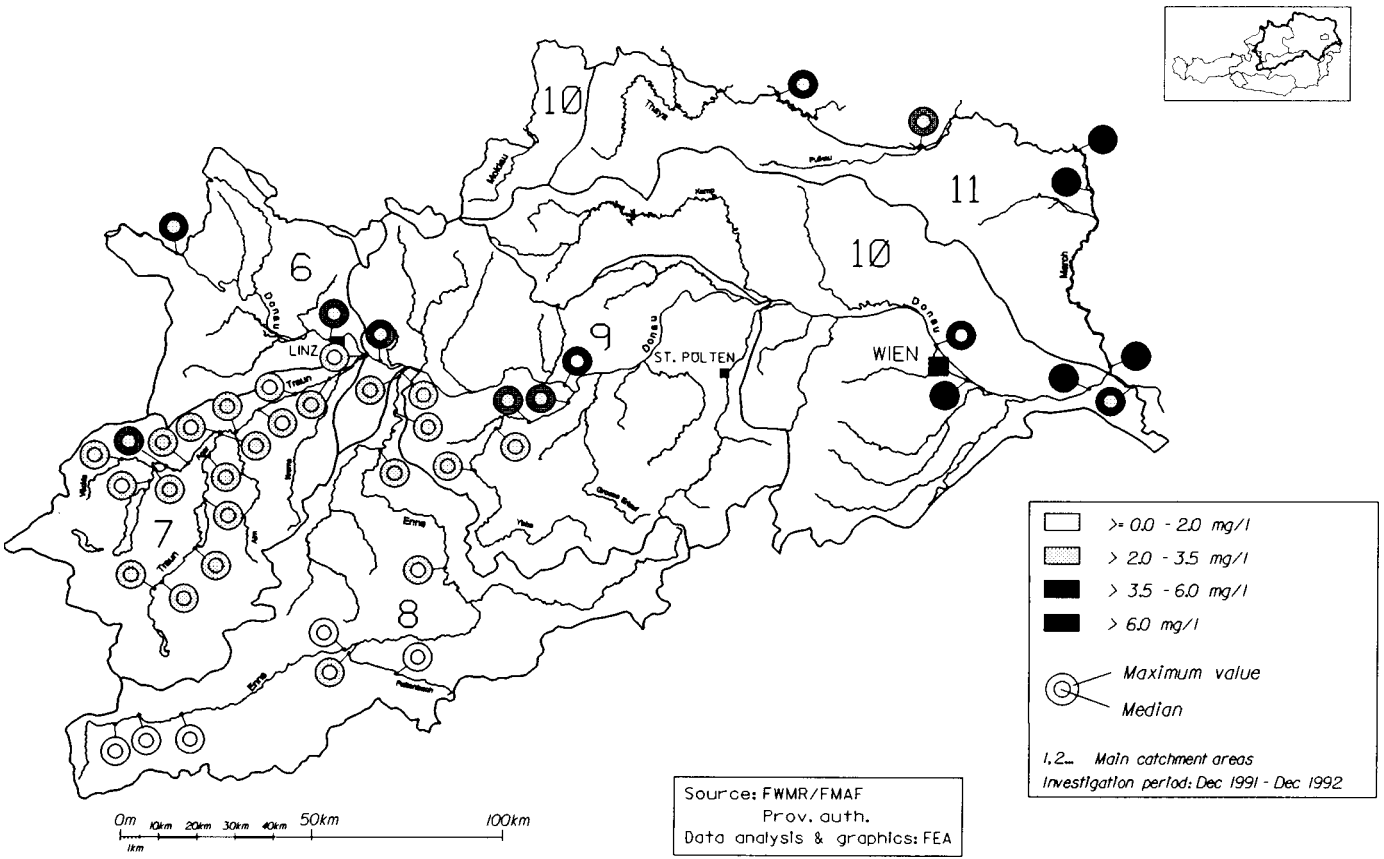

Figure 17.4 *Results of river monitoring: BOD in the north-eastern catchments*

Figure 17.5 *Results of river monitoring: AOX in the southern catchments*

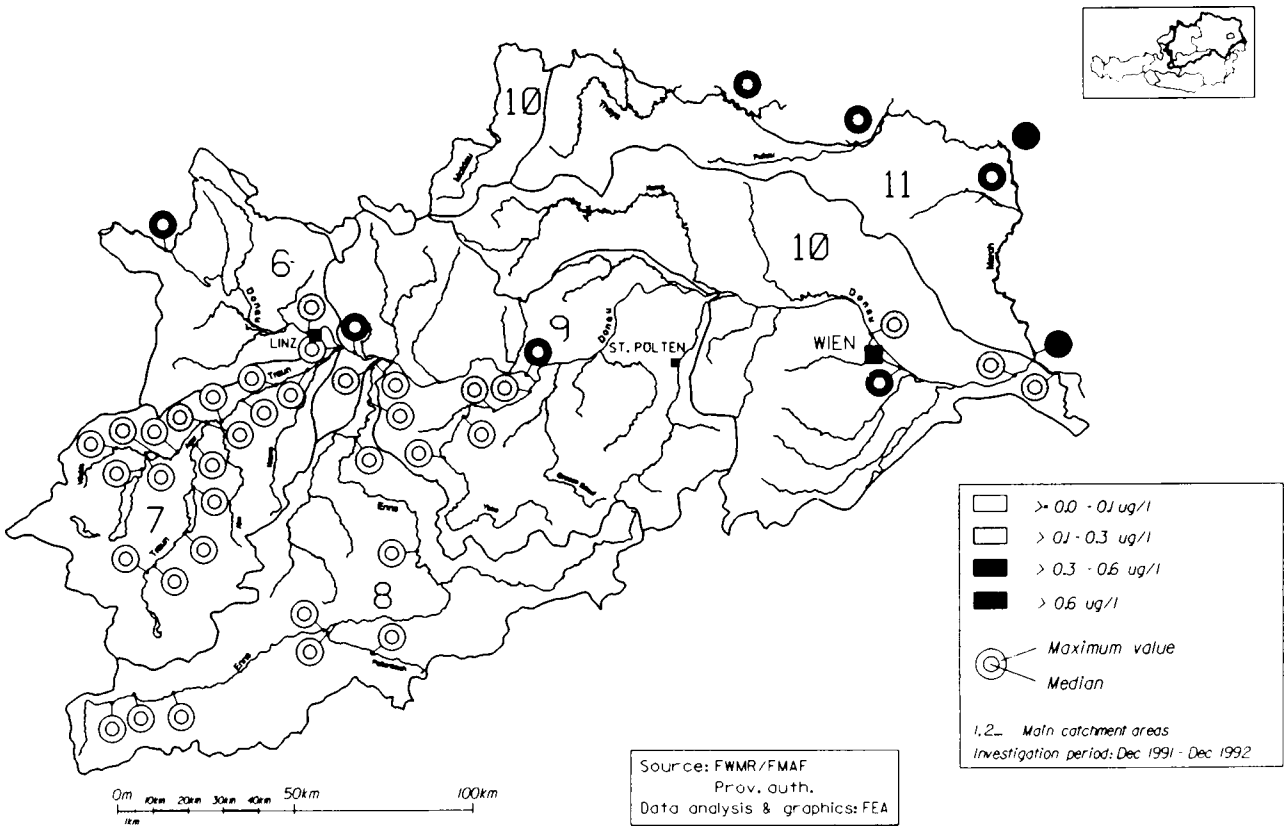

Figure 17.6 *Results of river monitoring: atrazine in the north-eastern catchments*

Nitrate proved to be a major problem in areas with intensive agricultural activity. From 944 samples analyzed in autumn 1992, 222 exceeded the threshold value of 45 mg l^{-1}, in 33 of these samples concentrations >100 mg l^{-1} could be detected. In nine groundwater areas from a total of 48 at more than 25% of the sampling sites the threshold value has been exceeded three times during three investigations. At least nine groundwater areas are expected to become sanitation areas after two years of investigation (see Figure 17.3). Nitrate contamination of groundwater is caused by agricultural activities. Thus, reducing the amount of fertilizers used appears to be a possible strategy for recovery.

Pesticide contamination could also be established. While most of the pesticides could only be detected in a very few cases, atrazine could be detected in 270/943 samples in the third sampling series (detection limit: 0.1 μg l^{-1}). Desethylatrazine, a metabolite of atrazine (and of simazine) was detected in 286 samples, desisopropylatrazine in 9 samples. Since July 1 1994, pesticide concentrations in drinking water must not exceed 0.1 μg l^{-1} (=threshold level). With regard to atrazine it is assumed that at least nine groundwater areas will have to be designated remediation areas. Since the application of atrazine is forbidden in Austria since 1994 no further measures will need to be taken.

Exceedances of the following parameters according to the threshold value ordinance (at least three sampling series, 25% of the measuring sites of a given area) were recorded in one or more areas: potassium, boron, ammonium, nitrite, chloride, and tetrachloroethene.

17.4.2 River monitoring

The results of the BOD investigation indicate severe pollution in the rivers of the northern and eastern parts of Austria. The results for these areas are shown in the map (Figure 17.4). Absorbable halogens (AOX) have been detected in most river systems (Figure 17.5). Higher levels are probably caused by pulp and paper mills many of which are located in the catchment areas of the Traun and Mur rivers. The highest concentrations (maximum value 2300 μg l^{-1}) were measured in the Pöls river, a tributary to the Mur river, resulting from a pulp mill using chlorine or chlorine derivatives for bleaching. Earlier investigations indicated concentrations of up to 5000 μg l^{-1} in this river.[19] In addition to the high concentrations originating from industrial effluents, in nearly all rivers throughout the country, low AOX concentrations could be detected in a range of up to 10 μg l^{-1}, which are likely to be caused by domestic effluents contaminated by chemicals used in the home or from chlorine bleached toilet paper (or toilet paper made from originally chlorine bleached waste paper). Unfortunately, AOX elimination in biological wastewater treatment plants is very ineffective.

Similarly to groundwater, atrazine could be detected in some rivers draining areas with intensive agriculture (Figure 17.6). The highest levels of atrazine measured during the investigation period amounted to 3 μg l^{-1}.

17.5 DISCUSSION

The main features of the water quality monitoring system are:

(i) The monitoring system at any time provides current and detailed information on river and ground water quality;
(ii) Changes in water quality are indicated quickly—trends can be estimated;
(iii) Main areas of water pollution can be detected and remediation measures carried out effectively; and
(iv) The success of remediation measures can be supervised by monitoring their effect on the environment.

Hence, the monitoring system provides basic information necessary for extensive water protection; corresponding legal regulations allow a quick implementation of the results. Experience will show the effectiveness of this system, which is not static, but depends on permanent adaptation to changing requirements for its function.

Most changes will originate from new scientific findings especially in the field of ecotoxicology, where there is a great need for further research. However, many aspects, for example, the toxicology of interacting substances can never be fully predictable due to their great number. This is another reason to handle xenobiotics very carefully and to adopt rather strict legal regulations.

Furthermore, the system has to be adapted with regard to the political situation, for example Austria's accession to the European Union in 1995. The amendment to the Federal Water Act of 1990 has already taken community law into consideration. Due to this fact no major problems are to be expected. The implementation of substance related community directives could nevertheless cause a lot of work in case of the Austrian emission standards as for each new substance in the extreme case all 60 ordinances may have to be amended, but environment related legislation has to be adapted continuously to changing requirements. The revision of the water law has become a permanent task.[19]

With increasing water quality, the structural aspect of rivers and lakes becomes increasingly important. In many cases it is no longer water pollution but inadequate structure which impedes the development of natural and site-specific biocenoses. The existing monitoring systems cover this aspect insufficiently.

Sediment investigations have proved to be a suitable tool in water pollution assessment (eg, references 21–24). The number of parameters used in the water quality assessment for sediments appears to be insufficient at first glance as it covers only a few organic pollutants (mostly summation parameters). There is a need for special investigations according to the open ended list of parameter set three. An enlargement of the parameter sets one and two (requiring regular investigations) involves exorbitant costs and can thus not be put into practice. In terms of analytical quality problems arose mainly with sediment investigations.

In Austria, karst groundwater resources are of great importance. Almost half of the drinking water originates from those sources which are characterized by low contamination and high vulnerability. Threshold values stipulated in an ordinance[4] which were set primarily for groundwater in porous media have proved to be only partly suitable for karst waters. The adoption of different values adapted especially to the sensitive alpine ecosystems would be necessary.

17.6 ACKNOWLEDGEMENTS

The author gratefully thanks Mrs. Ulli Stärk for her linguistic corrections.

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18 Bromate Survey on European Water Utilities

Bernard Legube

18.1 INTRODUCTION

Ozonization of drinking water is a process commonly used in France and Europe. Ozone is an excellent agent for the disinfection, oxidation of metals and organics, removal of taste and odors, removal of trihalomethane (THM) precursors to increase the biodegradability of organics prior to granulated activated carbon (GAC) filtration, etc. Though ozonization does not directly produce toxic organic by-products, some traces of bromide in water can lead to the formation of both hypobromous acid and hypobromide ion (bromine). These brominated entities can react subsequently either with organic substances or with ozone to form organobrominated compounds and bromate respectively.

There is no European directive concerning bromate, but recent WHO guidelines[1] recommended 25 ppb ($\mu g\ l^{-1}$). The formation of some tens of ppb of bromate was recently observed during ozonation of water containing bromide, in laboratory or pilot-scale experiments.[2–5]

The purposes of this chapter are:

(i) To present a short synopsis relating to the origins of bromate in water, its toxicology and its analysis;
(ii) To illustrate the main results obtained from some French, English, and Spanish water utilities; and
(iii) To take stock of methods and processes which allow the inhibition of bromate formation or its removal.

18.2 ORIGIN OF BROMATE IN WATERS AND MECHANISMS OF FORMATION

18.2.1 Bromate in natural waters

The presence of bromate in surface waters is very small (or accidental). It can be present in treated waters. The precursor of bromate formation is bromide. Bromide levels in natural soft waters, on an average, are between 100 and 200 $\mu g\ l^{-1}$. Masschelein and Denis[6] state that the highest concentrations, in soft waters, were ~2000 and 3000 $\mu g\ l^{-1}$, the lowest being a few ppb. According to the same authors, bromide and chloride are associated in nature and the ratio of their concentrations is constant in natural waters, an average of 2.5 μg Br per mg Cl^-.

18.2.2 Bromate and ozonation

Molecular ozone (O_3) can react with bromide to produce hypobromous acid (HOBr) and hypobromite ion (BrO^-). Only hypobromite can react subsequently with either one mole of ozone to give bromide, or with two moles of ozone to form bromate (Figure 18.1).

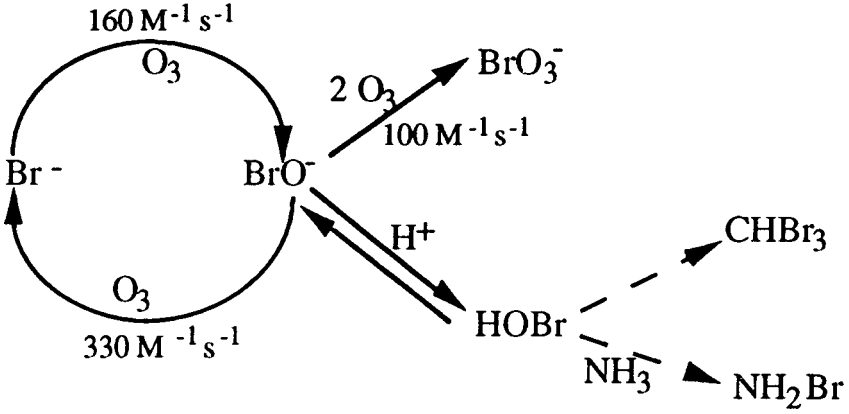

Figure 18.1 *Principle mechanisms for the formation of bromate by ozonation of bromide in pure water (modified from Haag and Hoigné)*[7]

From values of the rate constants for each reaction (as shown in Figure 18.1), a simple calculation shows that the first step of the ozonation of a hypobromite solution in pure water, would lead to 77% of bromide ion and 23% of bromate ion. However, subsequent unlimited ozonation can yield only one product, bromate ion, which does not react with ozone.

Fortunately, the reactions of ozone on bromide and hypobromite ions are relatively slow. Hence, ozonation of waters during a brief period of time (limited to a few minutes) will lead to a low oxidation yield of bromide in bromate. Furthermore, pH will be preponderant, because only hypobromite is the precursor of bromate formation. Moreover, the action of ozone on organic matter is fast ($k \sim 10^3$ M^{-1} s^{-1}), as well as the reaction of bromine on ammonia ($k \sim 10^7$ M^{-1} s^{-1}). It is obvious that bromate formation by limiting the ozone dose will depend also on organic matter and ammonia concentrations in water.

18.2.3 Bromate and hydroxyl ion

From Von Gunten and Hoigné[8], bromate formation by reaction of hydroxyl ion on bromide, is possible only in the presence of hypobromite. Moreover, hypobromite ion (and hypobromous acid) cannot be formed by reaction of hydroxyl ion on bromide, from the same authors study. In other words, hydroxyl ions alone are not precursors of bromate formation.

$$2\ Br^- + OH^\circ \rightarrow Br_2^\circ \qquad Br_2^\circ + BrO^- \rightarrow BrO^\circ \qquad BrO^\circ + O_3 \rightarrow BrO_3^\circ$$

18.2.4 Bromate and peroxone

The peroxone system (O_3+H_2O_2) is used in drinking water treatment in order to form hydroxyl radicals which are powerful oxidants, capable of oxidizing pesticides (eg, atrazine).

$$2\ O_3 + H_2O_2 \rightarrow 2\ OH^\circ + 3\ O_2 \text{ (very fast reaction, } k \sim 10^6\ M^{-1}\ s^{-1}$$

There are conflicting data available on the effect of peroxone on bromate formation. Indeed, some authors found that hydrogen peroxide injection promotes bromate formation as compared to ozonation when applied alone at the same dose,[9] while others showed the converse.[10] In fact, the effect of hydrogen peroxide addition probably depends on application conditions, particularly at the place of injection of H_2O_2 in ozonation reactor.

18.2.5 Bromate and chlorination

Chlorine reacts quickly with bromide to produce bromine. In the presence of an excess of chlorine (ie, in concentrated solutions), some secondary reactions with hypobromous acid can lead to the formation of bromate. The available data are sparse and it seems that the presence of bromate in water after chlorination is due to the presence of bromate in sodium hypochlorite used in chlorination step.[9] Bromate could be an impurity in sodium hypochlorite.

18.3 HEALTH EFFECTS AND GENOTOXICITY STUDIES

18.3.1 Health effects

Laboratory studies have been carried out on the health effects of potassium bromate in animals. In 1986, Kurokawa et al[11] studied the carcinogenic potential of potassium bromate in rats. Groups of 4–6 week-old rats (F344) were supplied with drinking water (0, 250, or 400 mg l^{-1} BrO_3^-). After 110 weeks the incidence of renal tumors in

the three groups (0, 250, and 400 mg l^{-1} BrO_3, or 0, 12.5, and ~26–27 mg kg^{-1} d^{-1}) was 6%, 60%, and 88% in males, and 0%, 56%, and 80% in females ($P < 0.001$). The incidence of peritoneal mesotheliomas in males was 11%, 33% ($P < 0.05$), and 59% ($P < 0.001$), at the three doses, respectively.

The same authors[11] exposed male rats with increasing doses of bromate in water (0, 15, 30, 60, 125, 250, or 500 mg l-1) for 104 weeks. The incidence of renal cell tumors was statistically significant (45%, $P < 0.001$) from bromate concentrations in water of 250 mg l^{-1}. Incidences of dysplastic foci were noted (50%, $P < 0.001$) from 125 mg l^{-1}. Based on the dose response data, the concentration corresponding to an excess cancer risk of 10^{-6} was calculated (using the Probit model) to be 0.95 mg l^{-1}.

Similar experiments with mice and hamsters have shown that these species are less sensitive to the renal effect of potassium bromate.[12]

The mechanisms of carcinogenicity might occur by causing oxidation of lipids in cell membranes to produce active oxygen species which can, in turn, produce damage to DNA.[13]

Richardson[14] proposed that 4 μg kg^{-1} body weight day^{-1} could be used for oppressing any risk arising from human bromate ingestion. Applying the IARC recommendation for drinking water, a worst case concentration of 14 μg p^{-1} BrO^-_3 would appear to be an advisable criteria by this author.

Some attempts have been made[15] to evaluate the potential impact of the increased potassium ion administration during the long periods, radioactivity, and radiolytic effects on specific sites of stressed kidneys does not appear to be negligible.

18.3.2 Genotoxicity

Studies on the ability of potassium bromate to cause mutations in bacterial test systems have given negative results, or only weak activity.[13] As for the Ames test, potassium bromate at 3 mg $plate^{-1}$ indicated a positive effect with *Salmonella typhimurium* TA 100 (+S9),[14] while sodium bromate at 5 mg $plate^{-1}$ gives negative results.[15] Some experiments in progress at Poitiers (using SOS chromotest and Ames fluctuation test) indicated that ozonation (at pH7 with 2 mg of ozone applied mg^{-1} of dissolved organic carbon (DOC) of solutions of aquatic humic substances (50 mg l^{-1} COD) containing bromide (1 mg l^{-1}) does not lead to genotoxicity for the tested concentrations (5–40 μgC ml^{-1}).

18.4 REGULATIONS

Referring mainly to the Kurokawa studies,[11] IARC has been able to classify bromate in Group 2B. Experts from WHO, FAO, and JECFA recommended absence of bromate in food such as bread (when bromate is added to flour at some 10 ppm). Based on a linearized multistage model for a consumption of 2 1 d^{-1} by a 70-kg adult, the bromate concentrations in water associated with an excess lifetime cancer risk of 10^{-4}, 10^{-5}, and 10^{-6} are respectively 30, 3, and 0.3 μg l^{-1}.[1]

Taking into account the analytical feasibility (in 1992–93) WHO recommended a provisional guideline value of 25 $\mu g\ l^{-1}$ for drinking water.[1] The discussion of a regulatory standard for bromate in drinking water (for US EPA and European Commission) is actually governed by three main factors, toxicity of bromate, detection limit in natural water, and disinfection safety criteria.[8]

18.5 ANALYSIS OF BROMATE

Recent analytical methods for bromate include pulsed polarography (detection limit (dl) 150 ppb), capillary electrophoresis (dl:between 10 and 100 ppb depending on the origin of the water sample), and ionic chromatography with sample preparation (dl:between 1 and 10 ppb).

The Water Research centre (WRc), KIWA, the French water companies (C.G. Eaux, Lyonnaise des Eaux, SAUR), the Spanish society SGAB, and the research laboratory of the University of Poitiers, set up a similar method using Dionex ion chromatograph. This method, roughly presented in Figure 18.2, has been the object of tests of reproducibility and accuracy between the different laboratories of the above mentioned organizations. The detailed description of the method and the results obtained from the interlaboratory tests were previously presented.[16] The total time of analysis is ~1 h, the detection limit is ~2 ppb, and the reproducibility decreases as bromate concentration decreases. It can be noted that the standard deviation (between 7 laboratories) of ~5, 10, and 20% corresponding to 50, 25, and 10 $\mu g\ l^{-1}$ bromate in natural waters.

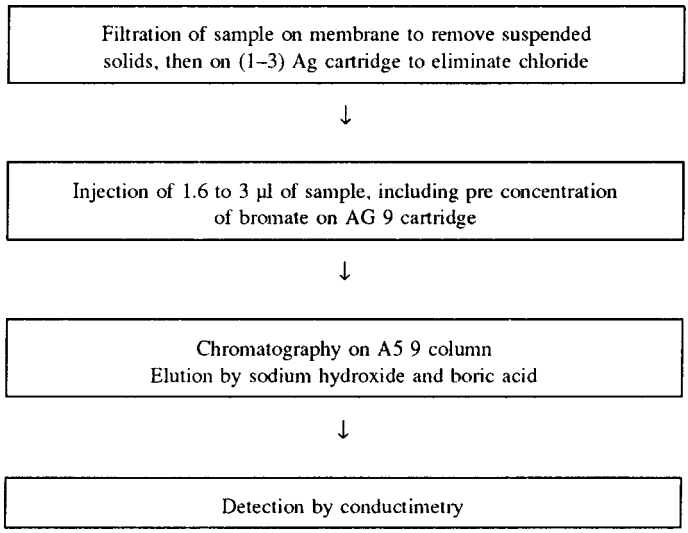

Figure 18.2 *Summarized analytical method for bromate in natural water* (for more details, see reference[16])

18.6 BROMATE FORMATION IN DRINKING WATER UTILITIES

18.6.1 Water utilities studied

Thirty-six water utilities have ben considered, at this time: 14 in the group 'Compagnie Générale des Eaux', 12 in the group 'Lyonnaise des Eaux-Dumez', 7 in the French society SAUR, 2 in the French society SAGEP, and 1 in the Spanish society SGAB. Samples of water have been subjected to different stages within the plants, mainly in upstream and downstream of each ozonation step, after GAC filtration, and before distribution. Some parameters were analysed (such as temperature, pH, alkalinity, total organic carbon, ammonia, bromide, bromate, bromoform, dissolved ozone), and oxidation conditions have been evaluated (oxidant doses, ozonation contact time). Figure 18.3 presents the main treatment processes involved in these utilities.

Utility 1 (river-F)
COAGULATION—DECANTATION—SANDFILTRATION—OZONATION—DISINFECTION (Cl_2)

Utility 2 (reservoir-F)
PREOXIDATION (ClO_2)—COAGULATION—DECANTATION—SAND FILTRATION—OZONATION—GAC FILTRATION—DISINFECTION (Cl_2)

Utility 3 (river-F)
COAGULATION—DECANTATION—SAND FILTRATION—OZONATION—GAC FILTRATION—DISINFECTION (Cl_2)

Utility 4 (river-UK)
PREOZONATION—COAGULATION—DECANTATION—OZONATION—GAC FILTRATION—DISINFECTION (Cl_2)

Utility 5 (river-UK)
PREOXIDATION (Cl_2)—COAGULATION—DECANTATION—OZONATION—GAC FILTRATION—DISINFECTION (Cl_2)

Utility 6 (groundwater-F)
NITRIFICATION—SAND FILTRATION—OZONATION—DISINFECTION (Cl_2)

Utility 7 (groundwater-F)
NITRIFICATION—OZONATION (+ H_2O_2)—GAC FILTRATION—DISINFECTION (Cl_2)

Utility 8 (groundwater-F)
NITRIFICATION—OZONATION (+ H_2O_2)—GAC FILTRATION—DISINFECTION (Cl_2)

Figure 18.3 *Main treatment steps in the utilities studied*

Utility 9 (groundwater-F)
OZONATION (+H_2O_2)

Utility 10 (river-F)
PEROXIDATION (Cl_2)—COAGULATION—DECANTATION—SAND FILTRATION—OZONATION—GAC FILTRATION—DISINFECTION

Utility 11 (river-F)
PEROXIDATION (Cl_2)—COAGULATION—DECANTATION—SAND FILTRATION—OZONATION—GAC FILTRATION—DISINFECTION

Utility 12 (river-F)
PEROXIDATION (Cl_2)—COAGULATION—DECANTATION—SAND FILTRATION—OZONATION—GAC FILTRATION—DISINFECTION

Utility 13 (groundwater-F)
AIR OXIDATION—CHEMICAL SOFTENING—COAGULATION—DECANTATION—SAND FILTRATION—OZONATION—DISINFECTION (Cl_2)

Utility 14 (reservoir-F)
PEROXIDATION (ClO_2)—COAGULATION—DECANTATION—OZONATION—SAND/ANTHRACITE FILTRATION—DISINFECTION (ClO_2)

Utility 15 (reservoir-F)
PEROXIDATION (Cl_2)—COAGULATION—DECANTATION—OXIDATION (Cl_2)—SAND FILTRATION—OZONATION—DISINFECTION (Cl_2)

Utility 16 (reservoir-F)
PEROXIDATION (ClO_2)—COAGULATION—DECANTATION—SAND FILTRATION—OZONATION—GAC FILTRATION—DISINFECTION (ClO_2)

Utility 17 (river-F)
COAGULATION—DECANTATION—SAND FILTRATION—OZONATION (+H_2O_2)—GAC FILTRATION—DISINFECTION (ClO_2)

Utility 18 (river-F)
PREOZONATION—COAGULATION—DECANTATION—SAND FILTRATION—OZONATION—DISINFECTION (Cl_2)

Utility 19 (river-F)
PREOZONATION—COAGULATION—DECANTATION—SAND FILTRATION—OZONATION—GAC FILTRATION—DISINFECTION (ClO_2)

Figure 18.3 *Main treatment steps in the utilities studied*

Utility 20 (river-F)
PREOZONATION—COAGULATION—DECANTATION—SLOW SAND FILTRATION—OZONATION—GAC FILTRATION—DISINFECTION (Cl_2)

Utility 21 (river-F)
STORAGE (2 days)—PREOZONATION—COAGULATION—DECANTATION—SAND FILTRATION—OZONATION—DISINFECTION (Cl_2)

Utility 22 (river-SP)
PREOZONATION (Cl_2)—COAGULATION—DECANTATION—SAND FILTRATION—OZONATION—GAC FILTRATION—DISINFECTION (Cl_2)

Utility 23 (reservoir-F)
COAGULATION—DECANTATION—OZONATION—SAND FILTRATION—OZONATION—DISINFECTION (Cl_2)

Utility 24 (groundwater-UK)
PREOXIDATION (Cl_2)—SAND FILTRATION—OZONATION—DISINFECTION

Utility 25 (river-F)
PREOXIDATION (ClO_2)—COAGULATION—DECANTATION—SAND FILTRATION—OZONATION—GAC FILTRATION—DISINFECTION (ClO_2)

Utility 26 (river-F)
PREOZONATION—COAGULATION—DECANTATION—SAND FILTRATION—OZONATION—GAC FILTRATION—DISINFECTION (Cl_2)

Utility 27 (river-F)
PREOXIDATION (Cl_2)—COAGULATION—DECANTATION—SAND FILTRATION—GAC FILTRATION—OZONATION—DISINFECTION (Cl_2)

Utility 28 (river-F)
PREOZONATION—COAGULATION—DECANTATION—SAND FILTRATION—OZONATION—DISINFECTION (ClO_2)

Utility 29 (reservoir-F)
COAGULATION—OZOFLOTATION—COAGULATION—DECANTATION—SAND FILTRATION—OZONATION—DISINFECTION (ClO_2)

Utility 30 (reservoir-F)

COAGULATION—DECANTATION—OZOFLOTATION—SAND FILTRATION—OZONATION—GAC FILTRATION—DISINFECTION (Cl_2)

Figure 18.3 *Main treatment steps in the utilities studied*

Utility 31 (river-F)
PREOZONATION—COAGULATION—DECANTATION—SAND FILTRATION—OZONATION—GAC FILTRATION—DISINFECTION (Cl_2)

Utility 32 (groundwater-F)
NITRIFICATION—OZONATION (+H_2O_2)—GAC FILTRATION—DISINFECTION (Cl_2)

Utility 33 (groundwater-F)
NITRIFICATION—SAND FILTRATION—CHEMICAL SOFTENING—GAC/SAND FILTRATION—OZONATION—DISINFECTION (Cl_2)

Utility 34 (river-F)
PREOZONATION—COAGULATION—DECANTATION—SAND FILTRATION—OZONATION (+ H_2O_2)—GAC FILTRATION—DISINFECTION (ClO_2)

Utility 35 (river-F)
PREOXIDATION (Cl_2)—COAGULATION—SAND FILTRATION—OZONATION—DISINFECTION (Cl_2)

Utility 36 (river-F)
PREOXIDATION (ClO_2)—COAGULATION—DECANTATION—SAND FILTRATION—OZONATION (+H_2O_2)—DISINFECTION (ClO_2)

Figure 18.3 *Main treatment steps in the utilities studied*

18.6.2 Effect of the ozonation steps

Preozonation of nine studied raw water (utilities 4, 17–21, 26, 28, 31, and 33) did not produce bromate. Two theoretical interpretations can be given:

(i) Ratios O_3 applied/TOC are always very low in the preozonation step and ozone is consumed firstly by organic matter;
(ii) Even if secondary formation of hypobromite occurs (reaction of ozone on bromide), hypobromite reacts very quickly with ammonia, and its reaction with ozone cannot take place.

As for interozonation step. Table 18.1 presents the results obtained from the most important samples, that is to say:

(i) Before the interozonation step or peroxonation step: temperature (parameter A Table 18.1, in °C), pH (B), alkalinity (C in ppm $CaCO_3$), ammonia (D in ppm N), total organic carbon (E in ppm C), bromide (F in ppb), bromate (G in ppb);
(ii) After interozonation step (or interperoxonation): bromide (F' in ppb), bromate (G' in ppb), dissolved residual ozone (I in ppm), bromoform (L in ppb); and
(iii) The principal characteristics of preoxidation and ozonation steps: dose of oxidant applied in preoxidation (H in ppm), dose of ozone in interozonation (H' in ppm), dose of hydrogen peroxide in interperozonation (I in ppm), contact time for interozonation (K in min).

Table 18.1 *Analyses of water sampled before and after interozonation*

Utilities Origin–Country Date	A	B	C	D	E	F F	G G'	H H'	I	J	K	L
Utility 1 River–F 31/08/93	18.6	7.6	165	<ld	2.5	**45** 40	<ld **11**	0 2.5	0	0.45	32	<ld
Utility 2 Reservoir–F 02/09/93	20	6.2	35	430	6	**155** 150	<ld **<ld**	0 **2**	0.9	0.3	nd	<ld
Utility 3 River–F 16/09/93	nd	6.2	110	50	3.4	nd nd	nd **2**	1 2	0	nd	6	nd
Utility 4 River–UK 25/05/93	nd	7.7	150	nd	4-4.3	87 84	<ld **12**	1.6 4	0	0.32	12	nd
Utility 6 Gr.water–F 28/07/93	15.9	7.8	253	<100	1.5	**107** 102	<ld **<ld**	0 0.9	0	nd	10	<2
Utility 7 Gr.water–F 19/07/93	14.2	7.7	246	<ld	2.1	**60** 57	<ld **3**	0 0.6–0.8	nd	0.2	8	<2
Utility 8 Gr.water–F 19/07/93	17.1	7.5	193	<100	<0.5	**48** 48	<ld **2**	0 0.6–0.8	nd	0.2	3	<2
Utility 9 Gr.water–F 19.07.93	21.7	nd	nd	nd	nd	**55** 45	<ld **8**	0 1.2–1.5	0.4–0.5	0.4	8	<2
Utility 10 River–F 07/09/93	nd	7.5	159	<ld	1.8	**20** 22	<ld **<ld**	nd nd	0	nd	nd	nd

Utilities Origin–Country Date	A	B	C	D	E	F F	G G'	H H'	I	J	K	L
Utility 11 River–F 07/09/93	19	7.5	151	<ld	1.3	**25** 30	<ld **5.5**	nd nd	0	0.4	20	<2
Utility 12 River–F 07/09/93	19	7.5	151	<ld	2.6	**30** 35	<ld **3**	nd nd	0	0.4	20	<2
Utility 13 Gr.water–F 10/08/93	16	7.7	219	nd	1.6	**207** 206	<ld **<ld**	0 0.32	0	0.08	6	5
Utility 14 Reservoir–F 13/08/93	16	5.7	14	600	3.1	**12** nd	<ld **<ld**	1.9 1.05	0	0.1	6	<ld
Utility 15 Reservoir–F 01/09/93	18.9	7.7	112	100	6.8	**44** 62	4.7 **5.8**	4.3–5.3 1.7	0	0.06	5–6	<ld
Utility 16 Reservoir–F 02/09/93	17.8	6.5	19	150	2.4	**51** 47	<ld **traces**	1 0.8	0	0	14	<ld
Utility 17 River–F 10/08/93	21	8.2	60	50	1.9	**200** 202	<ld **2.5**	0 1.6	0.4	0	13	<ld
Utility 18 River–F 09/08/93	21.1	8.2	93	100	2.5	**97** 94	<ld **4.9**	0.1 1.7	0	1.0	7	<ld
Utility 19 River–F 16/08/93	26.8	7.5	107	30	1.6	**45** 42	<ld **16**	0.8 1.5	0	0.4	10	<ld
Utility 20 River–F 31/08/93	20.4	7.9	175	<ld	1.8	**34** 30	<ld **5.5**	0.75 0.9	0	0.4	8	<1
Utility 21 River–F 31/08/93	19.7	7.5	160	<ld	2.0	**28** <5	<ld **3**	0.75 2.1	0	0.4	13	<1

Utilities Origin–Country Date	A	B	C	D	E	F F	G G'	H H'	I	J	K	L
Utility 22§												
River–SP	6–16	6.9–7.4	163–175	<20-500	4.5–5.0	**300** 300	<ld–2 **3–5**	12–28 2–3.5	0	0.1–0.8	9	50–70
10/12/93												
Utility 23												
Reservoir–F	17	8.6	76.5*	80	3.7	**155** 120	<ld **<ld**	0 1.2	0	0	nd	<ld
31/08/93						**35*** 20*	<ld **4***	0.7*	0*	0*	nd*	<ld*
Utility 25												
River–F	18.4	7.3	86	<ld	1.4	**<20** <20	<ld **<ld**	0.8 1.5	0	0.61	8	nd
15/09/93												
Utility 26												
River–F	12.5	7.3	107	<ld	2.4	**36** 40	<ld **3.1**	nd 1.9	0	0.43	14	nd
08/11/93												
Utility 27												
River–F	19	7.5	81	50	2.3	**50** 61	<ld **5**	0 nd	0	0.28	16	nd
20/09/93												
Utility 28												
River–F	11.5	6	6.5	100	1.5	**112** 92	<ld **<ld**	0 0.87	0	0.48	7	<ld
03/11/93												
Utility 29												
Reservoir–F	16.9	7.8	270	20	1.7	**29** 32	<ld **8**	0 0.92	0	0.29	4	<ld
13/10/93												
Utility 30												
Reservoir–F	15	7.3**	104**	150**	8.5**	**120****	<ld	0.7**	0	nd	11**	<ld
27/09/93		7.2	90	30	5.6	120 120	<ld** **2.5**	1.3			3.5	
Utility 31												
River–F	13.7	7.8	184	<20	2.9	**28** 36	<ld **2**	0 4	0	0.4	4	<ld
13/10/93												
Utility 32												
Gr.water–F	13.3	7.3	250	600	2.4	**109** 112	<ld **<ld**	0 0.7	0	0.2	nd	<5
17/01/94												
Utility 33												
Gr.water–F	15	7.9	186	<100	1.7	**144** 144	<ld **4**	0 0.1	0	0.09	nd	<5
18/01/94												

Utilities Origin–Country Date	A	B	C	D	E	F F	G G'	H H'	I	J	K	L
Utility 34 River–F 13/09/93	17.5	7.5	154	<100	2.2	**60** 45	<ld **7**	nd 1.5	0.5	nd	25	<5
Utility 35 River–F 29/03/94	11.2	7.1	92	<100	1.5	**<20** <20	<ld **2**	nd nd	0	0.39	4	<5
Utility 36 River–F 23/03/94	11.3	7.4	61	200	1.5	**69** 60	<ld **10**	nd 3	0.6	0.3	8	<5

A: temperature (°C)
B: pH before interozonation
C: alkalinity (ppm ($CaCO_3$)
D: ammonia (ppb) before interozonation
E: TOC (ppm) before interozonation
F: bromide (ppb) before interozonation
F': bromide (ppb) after interozonation
nd: not determined, ld: limit of detection

G: bromate (ppb) before interozonation
G': bromate (ppb) after interozonation
H: oxidant dose (ppm) in preoxidation
H': ozone dose (ppm) in interozonation
I: H_2O_2 dose (ppm) in interozonation
J: dissolved residual O_3 (ppm) after interozonation
K: contact time in interozonation (min)
L: bromoform after interozonation (ppb)

Whilst awaiting the second campaign of sampling to attempt to show relationships between some of the parameters, the following comments and remarks can be mentioned.

(i) Concentrations of bromide before interozonation (parameter F, Table 18.1) are relatively low, globally between 20 and 200 $\mu g\ l^{-1}$, except for water utility 22 where the bromide concentration in raw water is very high;

(ii) These bromide concentrations appear to have no effect on bromate formation in interozonation step (G', Table 18.1). Indeed, ozonation of some waters 'highly' concentrated in bromide produces little bromate (water utilities 2, 6, 13, 17, 23, 28, 30, 32, and 33), while ozonation of other raw waters, less concentrated in bromide, leads to 'high' bromate concentrations (water utilities 1, 4, 9, 11, 19, and 29);

(iii) As pH of water to ozonate (B, Table 18.1) is slightly acidic (<6.5), bromate formation is never significant (water utilities 2, 3, 14, 16, and 28);

(iv) The presence of ammonia (D, Table 18.1) at some tenth of ppm, appears to have an inhibiting effect on bromate formation (water utilities 2, 14, and 32);

(v) It is difficult to conclude on the TOC effect (E, Table 18.1) as some high TOC-containing waters are precursors of bromate formation (water utilities 4 and 15) while others are not precursors (water utilities 22 and 30). An attempt was made to deduce relationships between bromate formation and the relative ozone dose (ozone applied/TOC), or the Ct product (dissolved residual ozone x ozonation contact

time). These relationships are not obvious, even when utilities where peroxonation is applied (I>O, Table 18.1) are excluded, and where preoxidation step is included (H>O, Table 18.1). However, as relative ozone dose applied (ozone dose/TOC) is >0.7 mg O_3/mg TOC, the bromate formation is often greater than detection limit of 2 ppb (water utilities 1, 12, 18, 19, 21, and 26), while note can be taken of the absence of bromate when relative ozone dose is <0.7 (water utilities 6, 13, 23, and 30). However, there is some exceptions, such as water utilities, 20, 22, 25, 29, and 31;

(vi) Injection of hydrogen peroxide in interozonation step (I>O, Table 18.1) have no apparent effect on bromate formation. Sometimes the bromate production is enhanced (water utilities 9 and 34), sometimes the reverse effect is observed (water utilities 7, 8, and 17); and

(vii) Bromoform formation (L, Table 18.1) by ozonation has never been observed, except for water utility 22, where the high dose of chlorine applied in preoxidation probably lead to brominated compounds.

18.6.3 Effects of GAC filtration and final chlorination

The results are shown in Table 18.2, for samples of interozonated waters (G', Table 18.1), GAC filtered waters and disinfected waters.

The action of GAC filtration appears to be very aleatory, since it is sometimes positive (water utilities 3, 4, 20, 22, and 26), sometimes zero or negative (water utilities 7, 8, 12, 17, 20, 30, 31, and 36). In fact, the differences are very small and it is difficult and incautious to make conclusions.

The chlorine disinfection leads occasionally to the appearance of bromate in final waters (water utilities 1, 2, and 35), as already mentioned above. It is noted that water utility 15 (table 18.1) bromate is present in water before interozonation, due to the applied prechlorination (not shown in this chapter).

Chlorine dioxide disinfection is not a precursor of bromate formation for the cases studied (water utilities 14, 16, 17, 19, 25, 28, and 34).

Table 18.2 *Bromate levels in GAC filtered and disinfected waters*

Utilities	Origin–Country Date	Bromate (ppb) Interozonated waters	Bromate (ppb) GAC filtered waters	Bromate (ppb) Disinfected waters
Utility 1	River–F 31/09/93	11	No GAC	18
Utility 2	Reservoir–F 02/09/93	<ld	<ld	4.2
Utility 3	River–F 16/09/93	2	<ld	nd

Utilities	Origin–Country Date	Bromate (ppb) Interozonated waters	Bromate (ppb) GAC filtered waters	Bromate (ppb) Disinfected waters
Utility 4	River–UK 25/05/93	12	6–11	7
Utility 5	River–UK	no ozone	<ld	<ld
Utility 6	Groundwater–F 28/07/93	<ld	No GAC	<ld
Utility 7	Groundwater–F 19/07/93	3	3	3
Utility 8	Groundwater–F 19/07/93	2	2	2
Utility 9	Groundwater–F 19/07/93	8	no GAC	*
Utility 10	River–F 07/09/93	<ld	<ld	nd
Utility 11	River–F 07/09/93	5.5	no GAC	nd
Utility 12	River–F 07/09/93	3	4	nd
Utility 13	Groundwater–F 10/08/93	<ld	no GAC	<ld
Utility 14	Reservoir–F 13/08/93	<ld	no GAC	<ld
Utility 15	Reservoir–F 01/09/93	5.8	no GAC	3
Utility 16	Reservoir–F 02/09/93	traces	<ld	<ld
Utility 17	River–F 10/08/93	2.5	2.5	2.5
Utility 18	River–F 09/08/93	4.9	no GAC	4.5
Utility 19	River–F 16/08/93	16	nd	*
Utility 20	River–F 31/08/93	5.5	4	3
Utility 21	River–F 31/08/93	3	no GAC	3
Utility 22§	River–SP 10–12/93	3–5	<ld–3	<ld–2
Utility 23	Reservoir–F 31/08/93	4	nd	5
Utility 24	Groundwater–UK 10/06/93	no ozone	no GAC	2
Utility 25	River–F 15/09/93	<ld	<ld	<ld
Utility 26	River–F 08/11/93			

Utilities	Origin–Country Date	Bromate (ppb) Interozonated waters	Bromate (ppb) GAC filtered waters	Bromate (ppb) Disinfected waters
Utility 27	River–F 20/09/93	5	no GAC	5
Utility 28	River–F 03/11/93	<ld	no GAC	<ld
Utility 29	Reservoir–F 13/10/93	8	no GAC	*
Utility 30	Reservoir–F 27/09/93	2.5	3	3
Utility 31	River–F 13/10/93	2	4	4
Utility 32	Groundwater–F 17/01/94	<ld	<ld	<ld
Utility 33	Groundwater–F 18/01/94	4	no GAC	2
Utility 34	River–F 13/09/93	7	7	4
Utility 35	River–F 29/03/94	2	no GAC	8
Utility 36	River–F 23/03/94	10	13	nd

ld: limit of detection: not determined, * water mixed with other water before disinfection
§: five sampling periods between October and December 1993 (utility 22)

18.7 MEANS TO PREVENT BROMATE FORMATION AND TO REMOVE BROMATE

Some study programs on the problems regarding bromate are undertaken currently by several research teams. The purpose of this section is to give a brief synopsis regarding the state of the art.

If future European guidelines are to be between 10 and 25 ppb, the principal actions to undertake against bromate should be more preventive than curative. To substitute chlorine for ozone is not a solution, it can lead to the replacement potential of the formation of bromate by an assured formation of toxic organohalides and tastes, for the same disinfection efficiency. Moreover, suppression of ozone negates the virulicide effect of ozonation and advantages of the coupled ozone-GAC filtration process, such as a decrease in DOC, THM precursors destruction and pesticides removal. To remove bromide in raw water is an utopia. So, it remains to modify ozonation conditions or to remove bromate following its formation (table 18.3).

Table 18.3 *The present means available to decrease bromate concentration in drinking waters*

Processes	Other advantages	Main drawbacks
pH decrease prior to ozonation	Improved stability of ozone for disinfection Improved removal of organic matter if acidification is prior to clarification	Inhibition of manganese oxidation by ozone Inhibition of pesticides removal by ozone
Decrease applied ozone dose	none	Decrease all ozonation effects (disinfection, toxic oxidation, removal of THM precursors, contribution to organics removal with biological activated carbon)
Decrease ozonation contact time	none	*idem*
Addition of ammonia	none	Necessity to remove excess of ammonia (biological nitrification of chlorination)
Filtration on fresh GAC	Classical advantages of GAC filters	Low removal yields and very short period
Ferrous iron reduction	none	Slow reaction Necessity to oxidize iron (II) and to clarify prior to final disinfection
UV radiation	Disinfection	Retention time and cost

18.7.1 pH decrease

Some recent works[9] have shown that a pH decrease prior to ozonation, at 6.5, leads to inhibition of bromate formation due to the value of the equilibrium constant of hypobromous acid/hypobromite ion (2.06×10^{-9} at 25°C), and the fact that only hypobromite ion can react with ozone to give bromate. To decrease pH in drinking water treatment is often possible, the preferred place of acidification is in the coagulation mixing prior to the clarification processes. Note that the efficiency of pesticide and manganese removals by ozonation are reduced when ozonation pH decreases.

18.7.2 Ozone dose decrease

Taking into account the low values of the rate constants mentioned in Figure 18.1 as compared to elevated rate of ozone reaction with organic matter (10-fold greater), it is obvious that ozone will always be consumed firstly by organic matter, and secondly by bromide and hypobromite ion. In other words, for a given natural water, the lower the ozone dose is, the less residual dissolved ozone will be in contact with bromide, and the less bromate formation will occur. Some authors have already shown this important effect of ozone dose. A calculation from our research data on the ozone consumption of principal groups of aquatic organic matter (humic substances and amino acids) has shown that ozone doses <0.5 to 0.7 mg O_3/mg TOC could not lead to bromate formation.[17] This value is partially verified from data in Table 18.1. However, decreasing the ozone dose in the interozonation stage on water utilities will cause certain drawbacks (see Table 18.3).

18.7.3 Ammonia addition

Figure 18.1 shows the role of ammonia. Indeed, the reaction between ammonia and bromine is so fast that hypobromite ion cannot react with ozone if ammoniacal nitrogen concentration is high enough (probably some tenths of mg l^{-1}).

18.7.4 Hydrogen peroxide injection

As mentioned above, there are some ambiguities concerning the effect of H_2O_2 injection during the ozonation stage. The place of injection port of H_2O_2 could play an important role.

18.7.5 Direct reduction

Bromate reduction with sodium sulfide is a very slow reaction (~30 M^{-1} s^{-1}). Ferrous iron can reduce bromate. However, only soluble iron appears to be efficient and the reaction is too slow to apply to this process during drinking water treatment.

18.7.6 Activated carbon effect

From laboratory scale-studies, it appears that only fresh activated carbon can reduce bromate (in bromide). This has also been observed in water utilities. For the water utility 4 (table 18.2), the first GAC filter was just regenerated before the sampling campaign, while the second filter had been regenerated 13 months before. A bromate reduction (from 12 to 6 ppb) was only detected on the first filter (table 18.2).

18.7.7 UV radiation

UV radiation (at 254 nm) appears to have a positive effect on bromate reduction. However, retention times and lamp power required to remove bromate in drinking water are not known adequately to estimate correctly the efficiency of such a process.

18.8 CONCLUSIONS

The main conclusions which can be drawn from this chapter are:

(i) Bromates are rarely present in natural waters. Their appearance in drinking water is mainly due to the reaction of ozone with bromide and sometimes to the bromate contamination of sodium hypochlorite;

(ii) Referring mainly to carcinogenicity studies on rats, bromate was classified in group 2B by the International Agency for Research on Cancer (IARC);

(iii) The bromate concentration in water, associated with an excess lifetime cancer risk of 10^{-5}, is 3 $\mu g\ l^{-1}$. But, taking into account the analytical feasibility, WHO recommended a provisional guideline value of 25 $\mu g\ l^{-1}$ for drinking water;

(iv) Bromates may be analyzed by ionic chromatography. The analysis is not easy, long and expensive. The standard deviation (between seven laboratories) was found to be about 5, 10, and 20% for respectively 50, 25, and 10 $\mu g\ l^{-1}$ in treated natural waters;

(v) Except for some cases of water utilities, the studied drinking waters distributed by 36 water utilities presented bromate levels $<10\ \mu g\ l^{-1}$ formed mainly in the interozonation stage and sometimes caused by disinfection by sodium hypochlorite. Note that the results presented were issued from only one sample regime carried out at the end of summer 1993 for certain utilities, and during following autumn and winter for others. Second samples taken in the spring and summer periods could show higher concentrations due to the increase of ozone dose applied against pesticides; and

(vi) Among the means available to reduce bromate formation, decrease of pH of water to ozonate is probably the process which presents less drawbacks. To decrease ozone dose applied to ozonation contact time is another possible mean. The processes leading to bromate removal are not currently or adequately efficient.

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19 Pesticide Residues in Foodstuffs in Pakistan: Organochlorine, Organophosphorus and Pyrethroid Insecticides in Fruits and Vegetables

Syed Zafar Masud and Nusrat Hasan

19.1 INTRODUCTION

Fruits and vegetables are grown in abundance in Pakistan. The country, besides meeting its own requirements, is also exporting modest quantities of fruits and vegetables worldwide and to the Gulf States in particular. These crops receive insecticidal treatment for the control of different pests. While the application of pesticides is necessary to increase crop production, its increasing use poses a serious threat to public health.

In advanced countries of the world, regular and long-term pesticide monitoring programmes are undertaken with a view to examining pesticide residue levels in commodities destined for consumption by humans or livestock. Egan and Weston[1] discussed the role of surveys in monitoring pesticide residues in specific foods with particular reference to food surveys in the United Kingdom. A survey of foodstuffs in the United States[2] showed the presence of organochlorine and organophosphorus pesticides in most of the samples analyzed.

Chlorinated pesticides have been found by Parveen and Masud[3–5] in samples of milk, feed, and cattle drinking water drawn from Karachi Cattle Colony. Of the samples analyzed, 37, 36, and 13% samples, respectively, were found to be contaminated with BHC isomers, *p,p'*-DDT, *p,p'*-DDE, heptachlor epoxide, aldrin, and dieldrin. The most frequently occurring pesticide was γ-BHC. A survey of fruits and vegetables from the wholesale market of Karachi by Masud and Hasan[6] showed the presence of organochlorine, organophosphorus, and pyrethroid pesticides in a large number of the samples analyzed. Out of a total of 250 samples screened, 93 contained pesticide residues including 45 samples which contained residues above the maximum residue limits (MRLs) proposed by FAO/WHO.[7]

Since sufficient data on pesticide residue levels in food commodities is not available in Pakistan, this work forms part of our investigations being carried out to determine the level of contamination of foodstuffs with persistent organochlorine, organophosphorus, and pyrethroid pesticides. This chapter presents results of a four-year pesticide monitoring programme earned out on 550 samples of fruits and vegetables procured from different places in Pakistan. The work was earned out in three phases:

(i) From Karachi (Sindh) wholesale market during July 1988 and June 1990;
(ii) Main selling points and growers' fields of North West Frontier province and Islamabad during December 1990 and June 1991; and

(iii) Growers' fields and main selling points of Quetta/Pishin districts of Baluchistan province during September 1992.

The data indicate if pesticide residues were present in these commodities and if these residues were in violation of MRLs permitted by FAO/WHO.[7]

19.2 PROCEDURES

19.2.1 Sampling

Samples of fresh fruits and vegetables were procured from growers' fields and main selling points at different places. Generally, each composite sample weighed about 0.5 kg—all were random samples and drawn according to the prescribed procedure.[8]

19.2.2 Sample preparation and extraction

Fruits and vegetables were sliced into small pieces. Sub-samples were then taken for analysis. For extraction, the method of Johansson[9] modified by Masud[10] was followed. According to this procedure, the sample (30 g) was finely homogenized in a high-speed blender using a mixture of 75 ml toluene:*n*-hexane (3:1). The homogenate was shaken (electric shaker) for 3 h and then left in a deep freezer at -20°C overnight. After 24 h the clear solution was decanted quickly and the extract was concentrated to ~2 ml in a rotary vacuum evaporator at 60°C.

19.2.3 Clean-up

The method of Masud[10] has been adapted after slight modification. The modification is the addition of 0.5 g activated charcoal (Merck Art No. 2183) to Florisil at the column clean-up stage.

According to this procedure, Florisil 60/100 mesh (BDH or Merck), activated at 650°C for 3 h, was used for clean-up. A slurry of 10 g Florisil and 0.5 g activated charcoal in redistilled petroleum ether was prepared. It was then transferred to a 1.5 cm (i.d.)×60 cm chromatographic column containing a loose plug of cotton wool. After the entire quantity of Florisil had been poured into the column, an approximately 4 cm layer of anhydrous sodium sulfate was placed on top of the settled bed. The column bed should be kept covered with solvent to prevent dryness and subsequent 'channeling'. The column was first washed with 50 ml elution solvent toluene:acetone (99:1 v/v). The sample extract (2 ml) was then transferred quantitatively to the column and eluted with elution solvent. In each case, 100 ml eluate was collected. Solvent flow rate through the column was adjusted to 1 ml 1.5 min^{-1}. The eluate was evaporated to almost dryness and dissolved in 5 ml *n*-hexane in a volumetric flask for GLC determination.

19.2.4 Gas chromatographic determination

Pye-Unicam Series 204 gas-liquid chromatograph, equipped with ^{63}Ni electron capture and flame ionization detectors, was employed with the following operating parameters.

19.2.4.1 *Organophosphorus pesticides*

Glass column 1 m long×4 mm i.d. packed with 3% OV 101 on 100–120 mesh chrom WAW.DMCS treated. Temperatures: injector 250°C, column oven 180°C, detector (FID) 250°C. Attenuation 64, range 1, nitrogen carrier gas 30 ml min^{-1}. Hydrogen 28 ml min^{-1} and air 300 ml min^{-1}. The detector was linear in the range of 0.01–2.0 ng for detected pesticides.

19.2.4.2 *Synthetic pyrethroids*

Similar to Organophosphorus pesticides except for the column oven temperature, which was 200°C. The detector was linear in the range 0.21–2.0 ng.

19.2.4.3 *Organochlorine pesticides*

Glass column 1 m long×4 mm i.d. packed with a mixture of 1.5% SP 2250 and 1.95% SP 2401 on 100–200 mesh Supelcoport. Temperatures: injector 250°C, column oven 180°C, detector (ECD) 250°C, current 8×10^{-10}A, attenuation 64, nitrogen (carrier) 30 ml min^{-1}. The detector was linear in the range 0.01–1.0 ng for detected pesticides.

Servoscribe strip chart recorder (Kelvin Electronics Co. Ltd, England) with a speed of 120 mm h^{-1} was employed with the gas chromatograph.

Prior to use, each packed column was conditioned for 24 h under a slow stream of nitrogen at temperatures 50°C higher than their working temperatures.

Each cleaned-up sample extract was gas chromatographed thrice together with the relevant insecticide standard (analytical grade supplied by the manufacturers) in *n*-hexane using 0.1–0.2 μl injection. The amount of insecticide in each sample extract was calculated by comparing its peak height with that of the standard. Peak height was calculated by measuring the vertical distance from the peak apex to a line forming the base line of the peak. A typical gas chromatogram of cleaned-up extract of coriander is presented in Figure 19.1.

19.2.5 Thin layer chromatography

Extracts of samples found to contain pesticides were also screened for further confirmation of identity by thin layer chromatography on 20/20 cm silica gel coated glass plates (Merck Art No. 5715) using toluene:acetone (10:2) as a mobile phase. The plates were evaluated using a mixture of 10% zinc chloride and 20% diphenylamine in acetone as chromogenic reagents.[11]

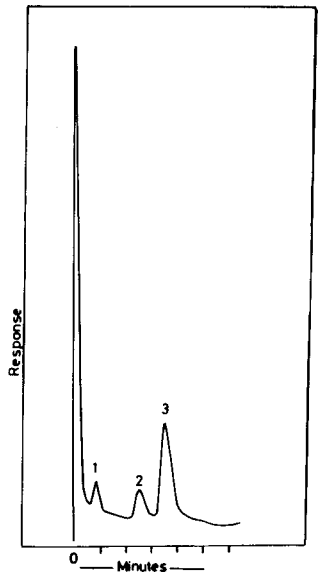

Figure 19.1 *Gas chromatogram of pesticides detected in coriander. 1. Cypermethrin. 2. γ-BHC. 3. Malathion.*

19.3 RESULTS AND DISCUSSION

Pesticide monitoring studies on such a large scale involving 550 samples and a duration of nearly 4 years have been carried out for the first time in Pakistan. As indicated in section 19.1, the treatment history of the produce from the main selling points was unknown; surveillance was, therefore, designed to check for as many pesticides as possible. Since treatment history in respect of samples from growers' fields was known, monitoring studies were confined mostly to pesticides used in-the area. Multiresidue procedures were applied to determine organochlorine, organophosphorus, and pyrethroid pesticides in samples of fruits and vegetables.

For clean-up of sample extracts, the method of Masud[10] was slightly modified. The addition of 0.5 g activated charcoal to a slurry of Florisil for the column clean-up of extracts assisted in the removal of chlorophyll and other plant colouring materials from sample extracts thus prolonging the life of gas chromatographic stationary phases in the GC column and giving results free from interference. Gas chromatography of the extracts showed the efficiency of extraction and clean-up methods. No interfering peak was observed in any of the samples.

Pesticide monitoring studies indicated the level of its residues present in or on food commodities and thus provide the adoption of precautionary measures. Since no MRL has so far been fixed for any pesticide on fruits and vegetables and other food commodities in Pakistan, it would be desirable to compare our results with internationally accepted Codex Limits.[7]

Out of a total of 550 samples of fruits and vegetables analyzed, 214 samples contained different organochlorine, organophosphate, and pyrethroid pesticides. Results are presented in Table 19.1 and are compared with MRLs of detected compounds as far as possible. Area-wise results are summarized in Table 19.2. For some of the compounds, no MRL was available in the literature. This means that either the MRL has not so far been prescribed for such compounds, or they are not approved for use on fruits and vegetables. It is evident from Table 19.1 that in 79 out of 214 contaminated samples, the quantities of detected compounds exceeded the maximum residue limits;[7] hence, these samples could pose pesticide hazards to the consumer.

Table 19.1 *Summary of vegetable and fruit samples analyzed, number of contaminated samples, pesticides detected with quantities, and proposed maximum residue limits.*

Commodity	No. of Samples	No. of Contaminated Samples	Pesticides Detected	No. of Samples	Quantity/ Range (mg kg^{-1})	MRL
VEGETABLES						
Luffa (turi)	11	1	Lindane	1	4.21	0.5
			Cypermethrin	1	1.63	NA
			Methamidophos	1	2.11	NA
			Methyl parathion	1	1.71	NA
Pumpkin	12	4	*p,p'*-DDT	1	Traces	1.0
			Lindane	2	0.03–0.12	1.0
			Cypermethrin	2	Traces–0.11	NA
Cauliflower	14	10	*p,p'*-DDT	5	0.8–10.3	1.0
			o,p-DDT	3	Traces–0.3	1.0
			Lindane	3	0.12–0.8	0.1
			Methyl parathion	1	2.5	0.2
			Thiometon	1	1.0	0.5
			Malathion	1	2.5	0.5
			Methamidophos	1	2.60	1.0
			Menvalerate	1	0.10	0.10
			Cypermethrin	3	0.1–0.8	NA
Spinach	16	13	*p,p'*-DDT	4	0.04–1.0	1.0
			p,p'-DDE	1	Traces	1.0
			Lindane	5	Traces–1.5	2.0
			Malathion	9	1.7–5.0	8.0
			Cypermethrin	1	Traces	NA
			Methyl parathion	1	0.15	2.0

Commodity	No. of Samples	No. of Contaminated Samples	Pesticides Detected	No. of Samples	Quantity/ Range (mg kg^{-1})	MRL
White gourd (tinda)	16	4	Lindane	2	0.2–1.5	NA
			Cypermethrin	3	0.11–0.15	NA
			Fenvalerate	1	0.16	NA
Coriander	10	9	*p,p'*-DDT	4	Traces–1.0	1.0
			o,p-DDT	3	Traces–0.04	1.0
			Lindane	5	0.2–3.2	2.0
			Methyl parathion	1	1.5	0.2
			Thiometon	1	0.4	0.5
			Malathion	4	2.5–7.5	3.0
			Methamidophos	1	2.6	1.0
			Fenvalerate	1	Traces	0.1
			Cypermethrin	2	0.17–0.3	0.5
Cucumber	16	5	*p,p'*-DDT	1	0.3	1.0
			Lindane	3	2.09–2.10	NA
			Malathion	3	2.50–4.0	NA
			Cypermethrin	2	0.16–1.80	NA
			Deltamethrin	1	0.13	NA
Bitter gourd	12	9	Lindane	5	0.02–0.15	1.0
			Malathion	4	1.30–3.47	8.0
			Methyl parathion	1	4.0	0.2
			Methamidophos	1	Traces	1.0
			Cypermethrin	1	1.50	NA
			Triazophos	1	2.11	NA
			Deltamethrin	2	1.12–1.70	NA
Fenugreek	8	4	*p,p'*-DDT	3	Traces	1.0
			Methyl parathion	1	0.5	0.2
			Malathion	1	1.8	8.0
Lettuce	8	5	*p,p'*-DDT	2	Traces–1.8	1.0
			Lindane	2	7.5	2.0
			Malathion	1	1.2	8.0
			Methamidophos	1	0.09	1.0
			Cypermethrin	1	1.0	2.0
Aubergine (brinjal)	26	14	*p,p'*-DDT	4	0.09–4.0	1.0
			p,p'-DDE	2	Traces	1.0
			o,p-DDT	2	Traces	1.0
			Lindane	5	Traces–0.5	0.5
			Malathion	4	2.5–10.5	8.0

Commodity	No. of Samples	No. of Contaminated Samples	Pesticides Detected	No. of Samples	Quantity/ Range (mg kg^{-1})	MRL
Onion	18	4	*p,p'*-DDT	1	0.04	1.0
			Methamidophos	2	0.17–4.61	0.5
			Malathion	1	8.52	0.5
			Methyl parathion	3	1.09–3.15	NA
			Cypermethrin	2	1.23–1.8	0.1
			Fenvalerate	1	2.21	0.05
Turnip	20	12	*p,p'*-DDT	5	Traces–6.2	1.0
			o,p-DDT	2	0.2–0.8	1.0
			p,p'-DDE	1	2.1	1.0
			Lindane	5	0.03–3.0	1.0
			Malathion	6	Traces–2.6	3.0
			Fenvalerate	5	Traces–2.0	0.1
			Cypermethrin	10	0.05–3.0	0.1
Sweet potato	1	—	—	—	—	—
Tomato	30	15	*p,p'*-DDT	1	0.15	1.0
			Lindane	5	0.06–2.18	2.0
			Malathion	7	0.21–10.0	3.0
			Cypermethrin	2	Traces–0.15	0.5
			Fenvalerate	4	0.13–2.25	1.0
Lady's finger	28	23	*p,p'*-DDT	8	0.51–8.10	1.0
			p,p'-DDE	1	Traces	1.0
			Lindane	7	0.02–4.30	1.0
			Malathion	12	0.17–10.31	8.0
			Methamidophos	6	0.21–1.83	1.0
			Methyl parathion	4	1.83–2.83	NA
			Cypermethrin	7	0.3–3.43	0.1
			Fenvalerate	3	0.1–1.7	NA
Cabbage	8	5	*p,p'*-DDT	3	0.18–8.6	1.0
			Lindane	4	0.1–0.15	0.5
			Malathion	2	Traces–5.11	8.0
			Methamidophos	1	3.1	1.0
			Cypermethrin	3	1.1–3.3	NA
Potato	13	1	*p,p'*-DDT	1	Traces	1.0
			Lindane	1	Traces	0.05
Green pepper	9	—	—	—	—	—
Capsicum	8	1	Lindane	1	1.31	NA
			Fenvalerate	1	3.23	NA

Commodity	No. of Samples	No. of Contaminated Samples	Pesticides Detected	No. of Samples	Quantity/ Range (mg kg^{-1})	MRL
Mint	9	5	*p,p'*-DDT	2	Traces	1.0
			Lindane	3	Traces–1.40	2.0
			Malathion	2	3.0–5.5	8.0
			Cypermethrin	2	0.14–2.5	NA
Dill (soya)	5	5	*p,p'*-DDT	1	0.04	1.0
			o,p-DDT	2	0.07–0.5	1.0
			Malathion	3	0.3–2.1	8.0
			Methamidophos	1	Traces	1.0
			Cypermethrin	1	1.2	NA
Beet sugar	9	4	Lindane	1	0.19	NA
			Malathion	2	1.5–4.3	0.5
			Cypermethrin	1	0.10	NA
Carrot	10	6	*p,p'*-DDT	3	2.3–3.2	1.0
			Lindane	2	0.3–3.38	NA
			Malathion	2	1.2–1.5	0.5
			Fenvalerate	1	0.20	0.5
			Cypermethrin	3	0.04–3.0	0.1
Radish	22	6	*p,p'*-DDT	3	0.08–1.4	0.1
			Lindane	2	0.12–0.65	2.0
			Malathion	2	1.5–2.0	0.5
			Fenvalerate	1	0.05	1.0
Garlic	10	—	—	—	—	—
Peas	5	—	—	—	—	—
French beans	3	—	—	—	—	—
Mustard	1	—	—	—	—	—
Ginger	6	1	*p,p'*-DDT	1	Traces	1.0
			o,p-DDE	1	Traces	1.0
			p,p'-DDE	1	Traces	1.0
Arum	9	1	Lindane	1	0.12	NA
Gourd	2	—	—	—	—	—
Cow pea	2	—	—	—	—	—

Commodity	No. of Samples	No. of Contaminated Samples	Pesticides Detected	No. of Samples	Quantity/ Range (mg kg^{-1})	MRL
Lambs quarter (bathwa)	4	2	*p,p'*-DDT	2	Traces–0.05	1.0
			Lindane	2	Traces	2.0
			Cypermethrin	1	Traces	2.0
Radish seeds (mongray)	2	1	*p,p'*-DDT	1	0.05	1.0
			Fenvalerate	1	0.05	0.05
Parsley	1	—	—	—	—	—
Long cucumber	7	1	Fenvalerate	1	0.12	NA
Green peas	1	1	Cypermethrin	1	1.1	0.05
Lemon	6	—	—	—	—	—
Cluster bean	1	—	—	—	—	—
FRUITS						
Mango	16	12	*p,p'*-DDT	5	0.07–1.5	1.0
			o,p-DDT	2	0.07–0.8	1.0
			p,p'-DDE	3	Traces	1.0
			Lindane	2	Traces–0.11	0.5
			Malathion	2	3.4–4.1	8.0
			Methamidophos	7	Traces–2.3	1.0
			Cypermethrin	1	1.10	NA
			Fenvalerate	1	0.13	NA
Peach	8	5	*p,p'*-DDT	2	Traces–0.85	1.0
			o,p-DDT	1	Traces	1.0
			p,p'-DDE	1	Traces	1.0
			Lindane	3	0.24–0.4	0.5
			Methamidophos	2	Traces–3.4	1.0
Apple	41	16	*p,p'*-DDT	1	Traces	1.0
			Lindane	1	0.83	0.5
			Azinphos methyl	9	0.02–0.19	NA
			Methidathion	9	Traces–0.08	NA
Parsimon (amlok)	2	—	—	—	—	—
Banana	2	—	—	—	—	—
Pomegranate	4	—	—	—	—	—

Commodity	No. of Samples	No. of Contaminated Samples	Pesticides Detected	No. of Samples	Quantity/ Range (mg kg^{-1})	MRL
Grapes	3	—	—	—	—	—
Musk melon	8	—	—	—	—	—
Orange	15	—	—	—	—	—
Grapefruit	1	—	—	—	—	—
Papaya	5	—	—	—	—	—
Guava	8	3	*p,p'*-DDT	2	Traces–2.0	1.0
			o,p-DDT	1	Traces	1.0
			Lindane	2	0.03–0.04	0.5
			Methamidophos	1	2.65	1.0
			Cypermethrin	1	4.0	1.0
Sepota	2	—	—	—	—	—
Ziziphus	2	—	—	—	—	—
Cherry	2	—	—	—	—	—
Plum	15	2	Triazophos	1	1.4	NA
			Fenvalerate	2	1.31–1.90	NA
Apricot	7	2	*p,p'*-DDT	1	Traces	1.0
			Lindane	1	0.17	NA
			Malathion	1	Traces	6.0
			Methamidophos	1	0.7	1.0
Pear	4	1	Lindane	1	Traces	0.5
Dates	2	1	*p,p'*-DDT	1	Traces	1.0
			Cypermethrin	1	1.4	1.0
Water melon	2	—	—	—	—	—
Sugar cane	1	—	—	—	—	—
Cheeko	1	—	—	—	—	—
Total	**550**	**214**				

NA = Not available

Table 19.2 *Area-wise comparative picture of pesticide residue levels in fruits and vegetables.*

Area/Province	No. of Samples Analyzed	No. of Samples Found Contaminated	No. of Samples Above MRL
Karachi (Sindh)	250	93	45
NWFP	154	54	22
Islamabad	96	48	11
Quetta/Pishin (Baluchistan)	50	19	1

19.4 CONCLUSION

In view of the above findings it has become essential to educate the farming community regarding the proper use and the dangers involved in the misuse of pesticides. Pesticides pay dividends only when used in accordance with 'good agricultural practice' and become a curse if misused.

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20 The Effects of Industrial Wastes on Nitrification Processes Found Within Wastewater Treatment Works

David Fearnside and Helen Booker

20.1 INTRODUCTION

There is increasing pressure from the regulators to reduce the amount of ammonia discharged to the freshwater environment. A major source of this material is the wastewater treatment works (WWTWs). Current regulations permit certain works to discharge relatively high levels of ammonia, perfectly legally, before consent is breached. The regulators are reviewing consents on certain environmental discharges to more acceptable levels with, in some cases, ≥ 1 mg l^{-1} being the target concentration. Some works could fail consent if some form of treatment is not introduced to remove ammonia.

WWTWs are being designed specifically to remove ammonia from sewage effluents via the nitrification process. Some plants recently commissioned exhibit a significant shortfall in performance in terms of their ability to nitrify. The cause could be any one of the following:

(i) Poor operational practice;
(ii) Poor design;
(iii) Organic overload; or
(iv) Toxicity/inhibition.

Regarding (iv), the Water Industries Act 1991[1] states that:

> *'…no person shall throw, empty or turn, or suffer or permit to be thrown or emptied or to pass, into any public sewer, or into any drain or sewer communicating with a public sewer—*
>
> *(a) any matter likely to injure the sewer or drain, to interfere with the free flow of its contents or to affect prejudicially the treatment and disposal of its contents; or…'*
>
> If toxicity/inhibition is interpreted as 'prejudicially affecting treatment and disposal' then clearly no effluent entering the sewer should exhibit any degree of inhibition to the various biological treatment processes. If toxicity/inhibition is present, then the discharger is breaking the law'.

This chapter describes Yorkshire Water's approach to the problem, the test used to identify and quantify inhibition to nitrification, how analysis is interpreted in terms of effect to the processes and outlines some case studies.

20.2 NITRIFICATION INHIBITION TEST METHOD

20.2.1 Principle

In sewage treatment the removal of ammonia is earned out by biological processes in percolating filter beds, activated sludge plants etc. The process involves the oxidation of nitrogen (N) in NH_3 by *Nitrosomonas* bacteria to nitrite and then to nitrate by *Nitrobacter* bacteria. The biochemical processes involved in this transformation are sensitive to toxic substances which will have an inhibitory effect on the oxidation of NH_3. The nitrification inhibition bioassay monitors the ability of nitrifying bacteria to remove NH_3 and/or produce NO_3^- in the presence of the test sample.

The procedure is based on the Department of the Environment Standing Committee of Analysts 'Blue Book' method[2] and the Nitrotox Toxicity Test,[3] which uses a pure culture of the two species of bacteria responsible for the nitrification process. These have been combined to produce the Yorkshire Water Standard Operating Procedure for Nitrification Inhibition.[4]

20.2.2 Outline of procedure

Pure cultures of the bacteria are purchased commercially. Different batches of bacteria can have different levels of activity. Before a test is performed the activity of the bacteria must be established. This is undertaken by screening different concentrations of bacteria as blank determinations as described in the procedure below, in order to determine the concentration which provides 80% removal of ammonia over a 4 h contact period.

The ammonia and total oxidized nitrogen (TON) (as N) concentration of the test sample is determined. Dilutions of the test sample are then prepared as necessary with distilled water. 100 ml of test sample (or dilution of) and a non inhibitory control are each placed in 100 ml cylinders. The solutions are buffered to pH 8.2 using potassium hydrogen carbonate and the ammonia concentration adjusted to 60 mg l^{-1} with ammonium chloride. The appropriate quantity of bacteria, as determined previously, is added to the cylinder and the ammonia and TON concentration in each cylinder determined. The mixture is then aerated (to ensure the bacteria are in constant contact with the whole sample) for a period of 4 h. After 4 h aeration is terminated, the bacteria are allowed to settle for a few minutes and the final ammonia and TON determined.

20.2.3 Expression of results

The degree of inhibition is calculated from nitrification rate of the test sample compared to the nitrification rate of a non-inhibitory control and is expressed as percent inhibition. The effective concentration that kills 50% of the test organisms (EC_{50}) is calculated in the normal manner.

20.3 INTERPRETATION OF RESULTS

Although a particular effluent when analysed by the above protocol may produce a measurable amount of inhibition it does not mean that the effluent will affect biological treatment by the time it reaches the treatment works. If the effluent under scrutiny is only being discharged in very small quantities, say 2 or 3 m^3 d^{-1} into WWTWs treating 100,000 m^3 then the dilution effect will be so great that inhibition effects may be negligible. However, although dilution may be available within sewerage systems, if there are 30 other traders discharging effluent in similar quantities and of similar toxicity, additive effects will come into play. Hence some caution should be exercised when assuming toxicity will be diluted out within sewerage systems.

Considerable research has been undertaken to determine the amount of dilution required before a particular chemical or industrial effluent is guaranteed not to affect biological treatment processes or the environment to which it is being discharged.[5] Many individual chemicals have been tested for inhibition to the nitrification process,[6–11] but few mixed effluents. This 'guaranteed no effect' dilution is calculated by multiplying the EC_{50} (times dilution), of the organism or organisms being tested, by 100. In order to relate this theoretical 'guaranteed no effect dilution' to actual dilution available we have developed the concept of the TR (toxicity ratio).

A toxicity ratio is a means of expressing whether the measured amount of inhibition in an effluent discharged to a sewage treatment works will, by virtue of dilution, actually have an effect upon biological treatment processes at a works.

The ratio is calculated by dividing the 'guaranteed no effect dilution' of the effluent by the actual dilution of the effluent to the volume of sewage available within the system, ie.

$$TR = EC_{50} \times 100/(V_S/V_E)$$

where: V_S = Volume of sewage available within the system.
V_E = Volume of effluent discharged to the system.

Based on the above equation, if an effluent has a TR $\geq$ 1 it will be guaranteed not to effect to treatment processes. If the TR is in the range 1–10 then there is a possibility of an effect, producing perhaps some shortfall in treatment performance. If the TR>10 then the treatment process is likely to be affected severely.

This has proved to be a very effective method of prioritizing and targeting problem effluents.

20.4 OVERVIEW OF NITRIFICATION INHIBITION PROBLEMS

Until relatively recently there has been little obligation for WWTWs to remove ammonia as it has not been included in many consent conditions. It is only recently that the regulators have begun to review and tighten ammonia consents. Where sites have been identified for consent review, some form of adaption or extension of existing treatment

processes has been required in order to remove or reduce ammonia to acceptable levels for discharge.

This usually involves substantial amounts of capital investment (see Chapter by Hiley). Yorkshire Water has made this investment at many of its works throughout the region. Unfortunately at certain locations the purpose built nitrification process has not operated to its designed capability or at worst failed to function at all. The reason for failure has varied from poor design, poor operation, mechanical failure and toxicity/inhibition. One area suspected as a problem was that effluents received via the sewer were toxic and to varying degrees, inhibiting biological processes. Until recently there was no facility to evaluate, identify and quantify this type of problem.

In 1990 Yorkshire Water set up its Ecotoxicity Laboratory which is specifically designed to identify and quantify toxic effects on biological treatment processes and the environment.

Part of the section's remit was to develop and implement screening programs to detect and quantify inhibition to the nitrification process. The primary targets being WWTWs where ammonia consent may be considered for review within the next few years. WWTWs (~110) in the Yorkshire region have been identified as receiving significant quantities of industrial effluent. To date 41 have been screened (Figure 20.1; of these 23 have been identified as definitely having inhibition problems, ie, they are receiving persistently toxic effluent, which, on average produces inhibition levels >10% and occasionally levels rise to >50%. Eight works have possible inhibition problems, that is they receive on average sewage effluent with inhibition levels >5% but <10% and occasionally inhibition is >20%. The remaining 10 works receive sewage effluent which is non inhibitory, domestic in nature and should be fully treatable.

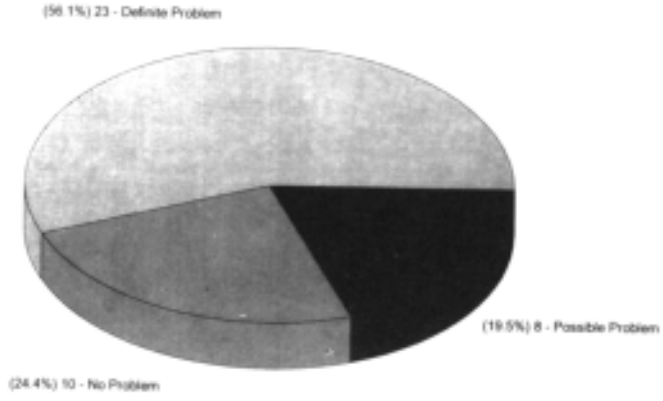

Figure 20.1 *Summary of Yorkshire Water's 41 WWTWs so far screened for nitrification inhibition*

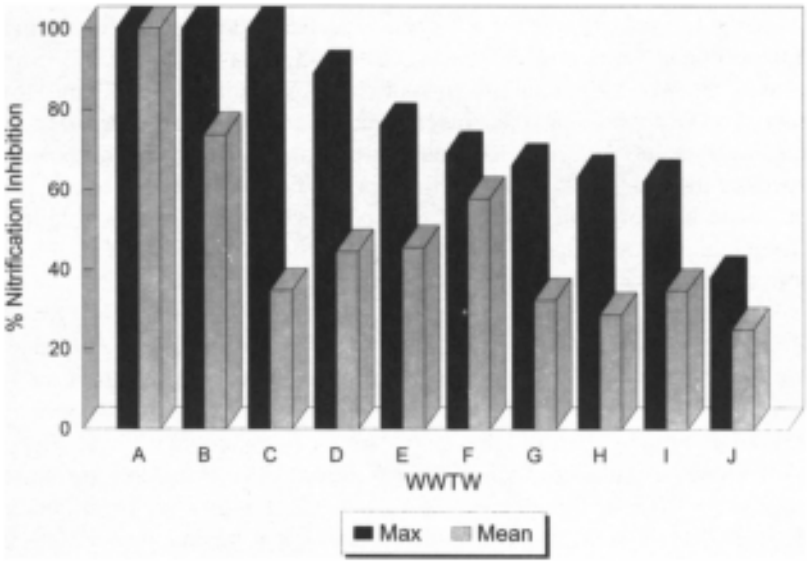

Figure 20.2 *The level of nitrification inhibition encountered at 10 of Yorkshire Water's more severely affected WWTWs*

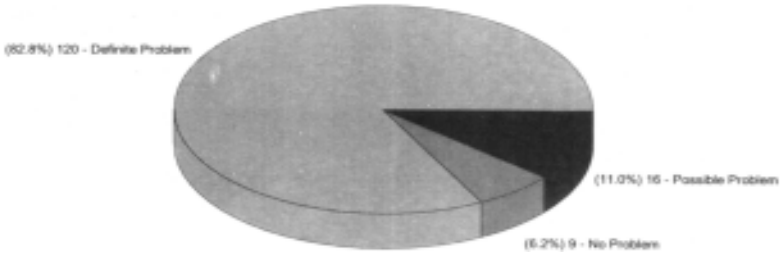

Figure 20.3 *Summary of trade effluent discharges contributing to nitrification problems detected at the 41 WWTWs.*

Different WWTWs are subjected to differing degrees of inhibition. Figure 20.2 demonstrates the degree to which nitrification inhibition effects 10 of the more severely affected works in the Yorkshire region. WWTWs *A* and *B* are continually receiving very high levels of inhibition; whereas works *C* on average inhibition is relatively low but intermittently receives high doses of inhibition.

The next stage was to identity the source of the problem (Figure 20.3). Potentially there are over 360 trade effluents discharging significant quantities of industrial effluent to Yorkshire Water's WWTWs. To date 145 traders which are directly associated with 31 WWTWs which have some form of inhibition problem, have been screened. Over 93% of the traders screened have been identified as contributing significant levels of toxicity, preventing nitrification processes performing their task.

20.5 CASE STUDIES

The following case studies are but two examples of the many ongoing investigations into toxicity/treatability problems identified by Yorkshire Water's Ecotoxicity Department.

Nitrification inhibition is not the only area of toxicity/inhibition under investigation. Others include the shortfall in performance of carbonaceous treatment processes (those designed to remove polluting material normally quantified as BOD), the fate of toxic effluents (the ability of biological treatment processes to breakdown the chemical components toxic effluents) and many others.

20.5.1 Wastewater treatment works *A*

In the early 1990s WWTWs *A* was redesigned in order to deal with increasing organic load and to reduce the level of ammonia in its final effluent discharge. An ammonia level of 1 mg l^{-1} was consented by April 1994. A capital scheme costing GB£5 million was required in order to achieve the new performance objectives.

Soon after commissioning it was realised that the plant would not nitrify. Operational and design problems were investigated and eliminated, the only source of the problem was thought to be toxic sewage effluent. A program of screening selected sample points was initiated.

The mixed crude sewage entering the works via two major sewerage systems (Figure 20.4) was screened first. Nitrification inhibition analysis indicated the works was being subjected to very high levels of inhibition. The two receiving sewers were then individually screened. Both were found to contain toxic material which would inhibit the nitrification process. Unusually there was a significant 'additive' effect when the two sewers mixed. In theory, the resultant inhibition after mixing should have been no greater than the average of the sum, as the volumes of sewage within the sewers are very similar. It is believed that some form of chemical interaction resulted in the mixed sewage being more toxic.

The next stage was to identify and screen the trade discharges that were suspected of contributing to the toxicity measured (Figure 20.5). Six major companies were identified for screening. The types of discharges ranged from waste from metal processing, chemical manufacturing, waste recovery (oils etc.), metal plating and a dairy and creamery. Inhibition analysis identified three companies with measurable inhibition values. When the analysis was converted to toxicity ratios only two were confirmed as the major

source of the problem. Both effluents were from metal plating processes. Each were approached by our Trade Effluent Control Department in order to establish whether, with cooperation, the toxicity within their discharge could be identified and eliminated.

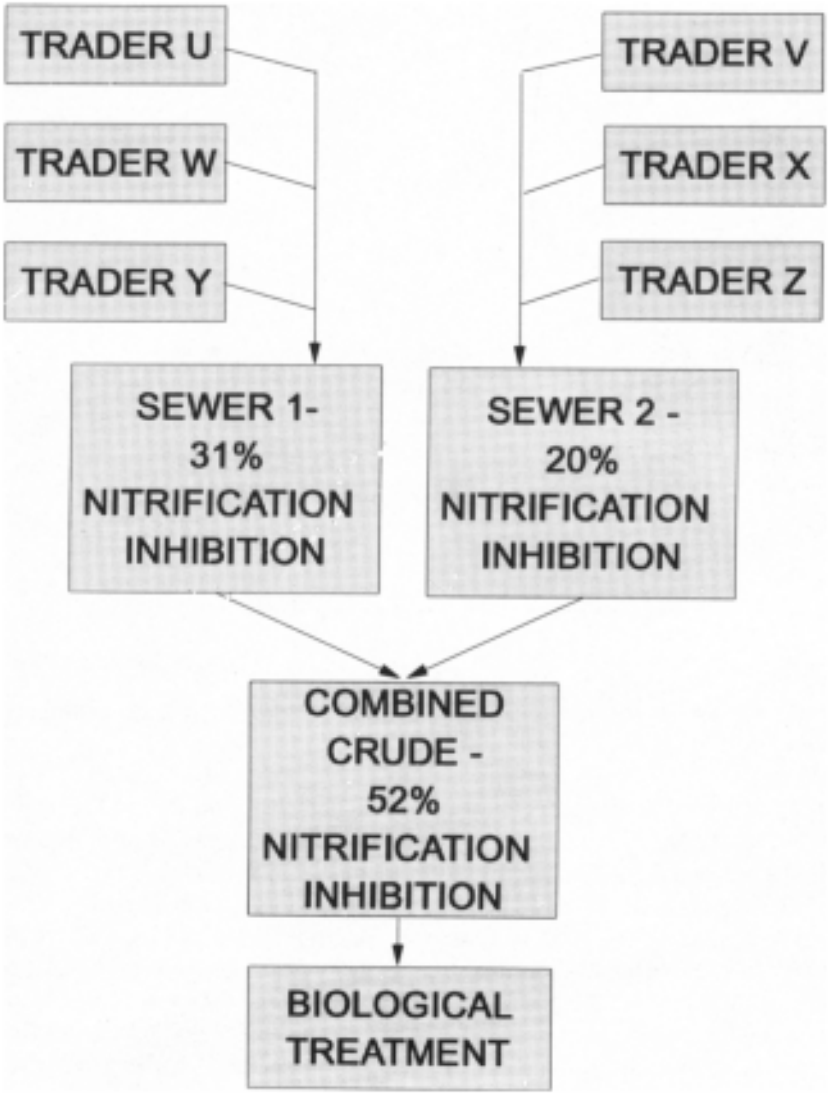

Figure 20.4 *Levels of nitrification inhibition measured at WWTWs A*

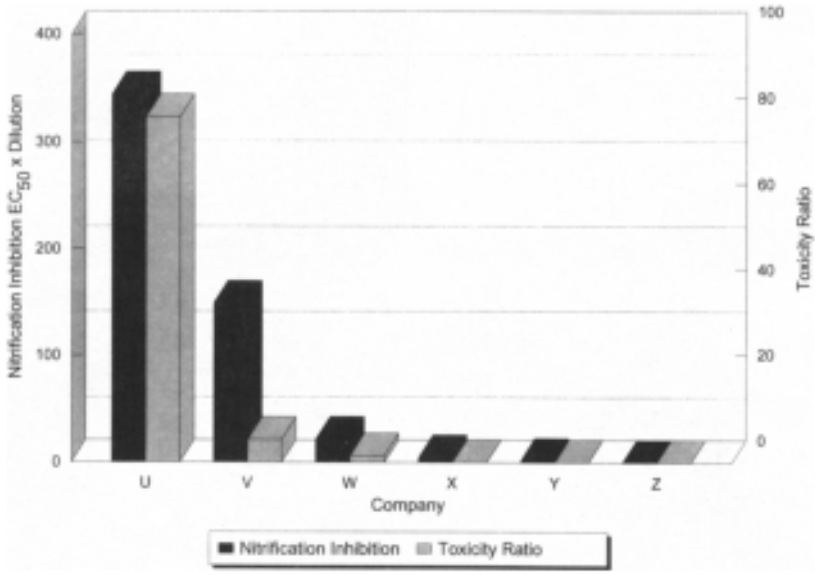

Figure 20.5 *Levels of nitrification inhibition measured on the companies discharging to WWTWs A and their potential effect expressed as a toxicity ratio*

Analysis of their internal process effluents (Figures 20.6 and 20.7) identified a discharge from the 'waste spill dump' at company *U* and the 'cyanide rinse' process effluent at company *V,* as the source of problem. Company *U* was able to very quickly alter the chemical process used, resulting in a drastic reduction in toxicity. Company *V* has not yet implemented any changes to processes which will reduce the toxicity of their effluent (Figure 20.8). Company *W.* though not thought to be a significant contributor to the problem, has also reduced the toxicity of its effluent.

Since going through the process of 'toxicity reduction evaluation' (TRE) there has been a measurable improvement at WWTWs *A.* On the whole the sewage received is now not significantly less treatable than domestic sewage, only occasionally does the works receive a 'shock toxic discharge' resulting in measurable levels of nitrification inhibition at the works. This was the situation in early 1994.

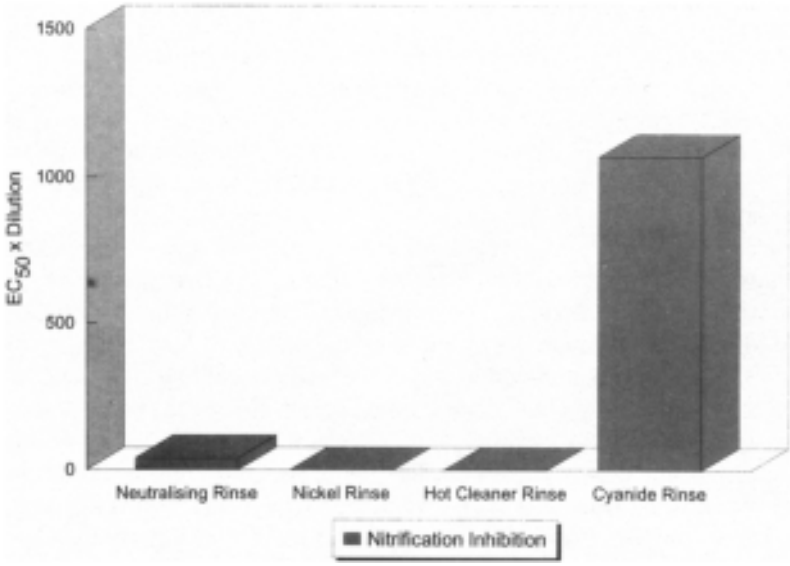

Figure 20.6 *Internal screen of company W's various waste streams from its operational processes*

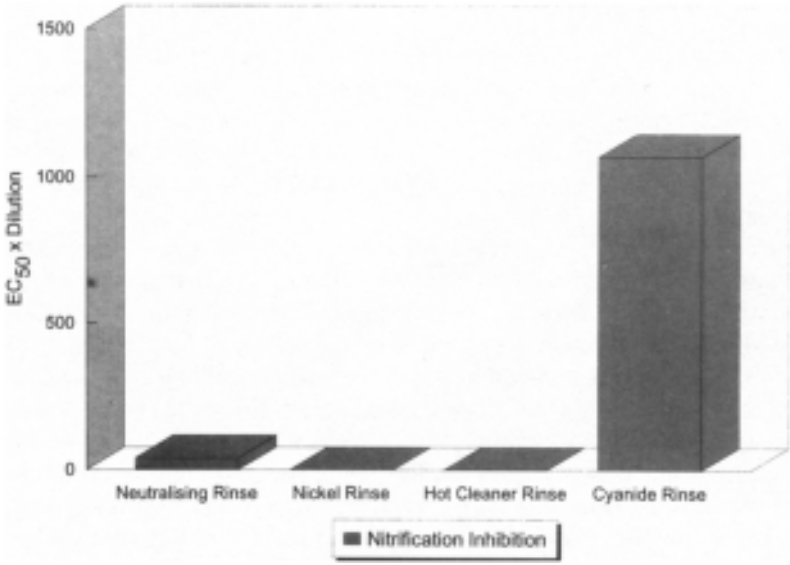

Figure 20.7 *Internal screen of company V's various waste streams from its operational processes*

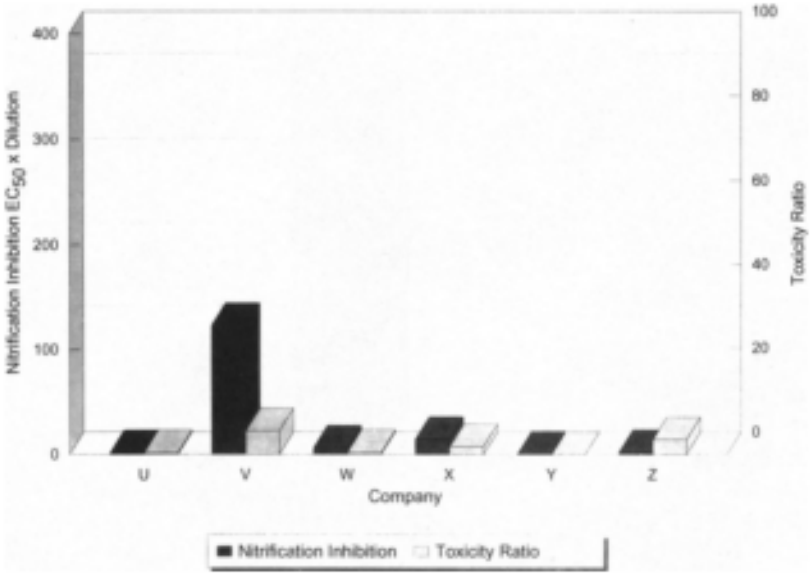

Figure 20.8 *Nitrification inhibition of companies discharging to WWTWs A after toxicity reduction evaluation*

20.5.2 Wastewater treatment works *B*

WWTWs *B* is another works which in the near future will be required to reduce the amount of ammonia it discharges into the environment. In 1992 a purpose built nitrification process costing GB£2 million was installed. After commissioning in early 1993 it was soon obvious the plant was not performing to design specification. At any one time the plant would only remove at most ~25% of the ammonia it was designed to remove.

In 1991–2 some investigational work was undertaken to determine whether toxicity to nitrification would be a problem. Figure 20.9 shows that some of the time the sewage is non-inhibitory (domestic in nature), but at other times the works is subjected to quite severe levels inhibition to nitrification via 'shock toxic loads'. It was assumed at the time that providing the shock loadings could be removed, the introduction of the new nitrification process would be a success, hence approval for capital went ahead.

The evaluation of the trade effluents discharging to the sewer revealed only one source of severe toxicity (Figure 20.10), company *R*. The nature of this discharge is effluent from processing imported chemical wastes of varying composition. Company *R* has, as yet, failed to make any improvement in the quality of the effluent it discharges. If there is no improvement in effluent quality, it is unlikely the works will achieve full nitrification and meet future consent requirements.

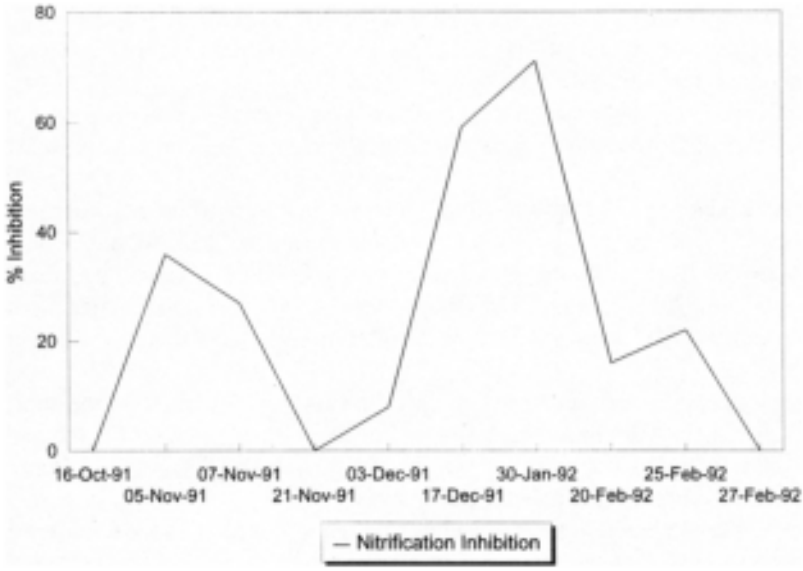

Figure 20.9 *Fluctuation of nitrification inhibition at WWTWs B*

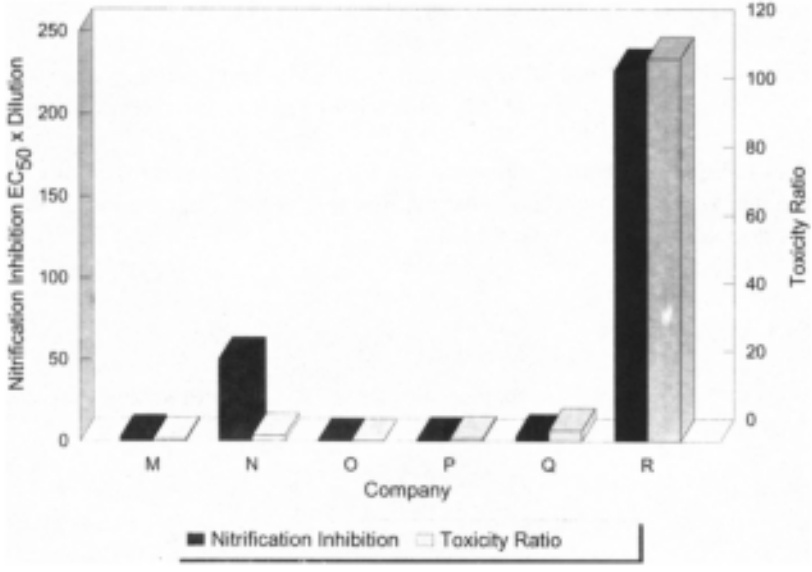

Figure 20.10 *Nitrification inhibition of companies discharging to WWTWs B and their potential effect expressed as a toxicity ratio.*

20.6 CONCLUSIONS

It is generally accepted that nitrifying bacteria are more sensitive to toxic chemicals than bacteria responsible for carbonaceous treatment. Therefore, it is essential to remove these chemicals which may prejudicially affect the nitrification process prior to installing such treatment processes.

The problems around under-performance of nitrifying processes are numerous, but there is no doubt that in Yorkshire toxic industrial effluents are compounding the problems.

Extending or adapting existing WWTWs is not practical from a cost point of view if toxic chemicals are present and cannot be eliminated or at least reduced to acceptable levels. Even with the elimination of background toxicity, treatment processes are still likely to be at risk from shock toxic loads. No matter what capacity these newly build plants may have, if inhibition is present, they will never work.

In order for plants to perform to design specification, toxicity must, in the short term, be reduced, and in the long term, removed at their source. This could potentially be a method for controlling wastewater quality.

There are several ways to achieve this:-

(i) Discharges could be controlled by subjecting them to toxicity based consents, in the same way as chemicals are controlled;
(ii) Schemes to encourage toxic waste minimization should be promoted;
(iii) If biological or chemical treatment is thought to be the answer these processes must be placed at the source of the problem;
(iv) Best effort should be made to ensure treatment processes are fully optimised; or
(v) On-line toxicity monitoring should be introduced to protect treatment processes and WWTWs. Policing the problem via normal trade effluent control will not be enough.

Improving the quality of toxic industrial discharges will improve the ability of treatment plants to remove polluting material, this will in turn improve the quality of effluents discharged to the environment.

20.7 DISCLAIMER

The opinions expressed in this chapter are those of the authors and do not necessarily represent those of Yorkshire Water plc.

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21 Environmental Audit of a Former Soviet Port

Chris Hoggart

21.1 INTRODUCTION

21.1.1 Environmental auditing

Environmental auditing is a method of gathering environmental information which encompasses a number of different techniques such as interview, inspection, document and data gathering, sampling, analysis and measurement. Environmental auditing can be used to gather evidence concerning current or past pollution and, in this way, differs from environmental assessment which attempts to characterize the likely environmental impacts caused by some future activity.

Normally, environmental auditing is applied to an operational site, such as a working factory, or a piece of land suspected of some past contaminative use. Auditing techniques are then used to characterize the pollutants which have been, or are being, discharged. This is normally followed by in-depth and targeted investigations aimed at identifying precisely the contaminants of concern. This process results in a more cost-effective study as the expensive, chemical and ecotoxicological analysis steps can be reduced in scope to target only those pollutants suspected as a result of the audit.

21.1.2 Auditing Klaipeda port

In the case of the present study, auditing techniques were used to attempt to characterize the likely contaminants which had been discharged to Klaipeda harbor over a number of years. It was hoped that this would considerably reduce the chemical and ecotoxicological investigations required and thus result in a better targeted and, therefore, a more productive and economical study.

21.2 KLAIPEDA PORT

21.2.1 Location and strategic importance

As the title of this chapter suggests, Klaipeda is a former Soviet port on the Baltic Sea. It is the principal port of Lithuania and is important strategically because, unlike other, more northerly, Baltic ports, it is ice free throughout the year. This, combined with its

good road and rail connections to Vilnius (Lithuania's capital city), Byelorussia, the Ukraine and north-west Russia, gives it great potential for attracting future trade and thereby promoting Lithuania's economy. Therefore, it is important that the port can continue to operate and expand if this becomes necessary.

21.2.2 Environmental constraints on port operations

There are two major environmental constraints which will affect the future operation of the port. These difficulties must be overcome if the port is to operate and expand in a sustainable fashion.

The first potential problem is conserving the area of land which lies opposite the port known as the Kursiu Spit. This sand peninsula, which formed over 5000 years ago, now comprises a series of habitats ranging from and dunes on its western edge adjacent to the Baltic Sea, through pine and deciduous forests in the centre to wetlands and reed beds on the eastern extremity opposite the working port. Given its singular nature, and the fact that it is home to many rare, vulnerable and endangered species of plants and animals in Lithuania, it is important that this area is conserved. Parts of the peninsula are designated as a national nature reserve and are thus subject to legal control, nevertheless the existence of a major port so close to the peninsula means that this unique region is always under a potential threat.

A further problem arises from the legacy of pollution which has been left from past and current uses of the port. This has resulted in sediments within the port becoming contaminated with a variety of pollutants to such a degree that dredging and dumping of these sediments at sea is forbidden by international treaties. Clearly, in order that the port can function, it is necessary to carry out regular maintenance dredging and this is being inhibited by the contamination present in the sediments. Further, port expansion is being prevented by the inability of the harbor authorities to dredge areas where construction activity is due to take place. Thus, an environmental problem, serious in itself, is also becoming an economic problem, as Klaipeda port is essential to Lithuania's current and future trade.

21.2.3 Port pollution

Pollution within Klaipeda port would have been contributed by the many different industries and operations which have been earned out there over the years including:

(i) An oil export terminal where Russian 'muzut' oil is offloaded from rail tankers i nto storage and thence onto ships;
(ii) A commercial harbor handling 3.8 million tonnes of cargo including fertilizers, bulkgrain, steel, scrap and building materials;
(iii) A paper mill and board factory;
(iv) A fishing harbor;

(v) An international ferry terminal;
(vi) Ship building and ship repair yards; and
(vii) A fish cannery.
In addition, the waters of Klaipeda port receive contaminated runoff from surrounding areas, untreated sewage from the town's sewerage system and polluted freshwater inputs from rivers and the adjacent Kursiu lagoon.

21.3 ENVIRONMENTAL CONDITIONS

21.3.1 Aquatic ecology

The contaminated sediments in the port were known to be affecting the marine ecology of the area. It was known prior to the audit, for example, that the aquatic ecology had, in places, been badly affected by contaminants within the sediment. This can be illustrated by reference to biomass and species diversity data gathered from sediment samples taken within and around the port area as follows:

	Unpolluted areas	**Polluted areas**
Biomass	50 gm^{-2}	0.1 gm^{-2}
Species diversity	≥30 species	<10 species

21.3.2 Terrestrial ecology

Documented data on the terrestrial ecology of the area was available, due to surveys performed during the recent production of a Lithuanian Red Data Book on rare and endangered species. Data extracted from this document gave the following information on the terrestrial ecology in and adjacent to the port:
(i) Twelve rare species in the area including the butterfly, *Cucullia balsamitae;*
(ii) Nine vulnerable species in the area including the beetle, *Calosoma sycophanta;*
(iii) Five endangered species in the area including the flowering plant, *Erica tetralix;* and
(iv) Two indeterminate species in the area.
Thus, it can be seen that the study area is ecologically valuable and will, therefore, require careful conservation and management if it is to co-exist with a large working port.

21.3.3 Water pollution

Limited quality data were available for the water within the port. In terms of organic, oxygen-consuming material, averaged data were obtained for a particular sampling point inside the port's working area. This data is shown in Table 21.1 together with comparable limits derived from the United Kingdom water quality classification system.

Table 21.1 *Quality data for water within the port and relevant United Kingdom limits*

	Concentration (mg l^{-1})	United Kingdom 'Fair' waters (mg l^{-1})
BOD	109	5
Ammoniacal nitrogen	25.1	2.33

In addition, some average data were available giving the extent of inorganic contamination within the port (Table 21.2).

Table 21.2 *Comparative data from Klaipeda Port and relevant limits for the protection of marine life as originally set by the Water Research centre (WRc)*

	Concentration (mg l^{-1})	WRc limits for the protection of marine life (mg l^{-1})
Nickel	50	30
Copper	33	5
Chromium	31	15
Zinc	380	40

It is likely, given the other known discharges to the port, that other pollutants are also present.

21.3.4 Sediment pollution

The limited amount of previously documented data available on sediments within the port area indicated that serious pollution had occurred in the past. It was known, for example, that some chemical analysis had been carried out on the sediments in order to decide whether or not they could be disposed of to sea, although the precise analytical results were not available during the audit. All that was obtainable for inspection were plans describing in subjective terms the extent of pollution in the port sediments (see Figure 21.1). However, given that it was known that the analysis results precluded disposal to sea of sediments in certain areas, it could be assumed that some significant contamination had been found.

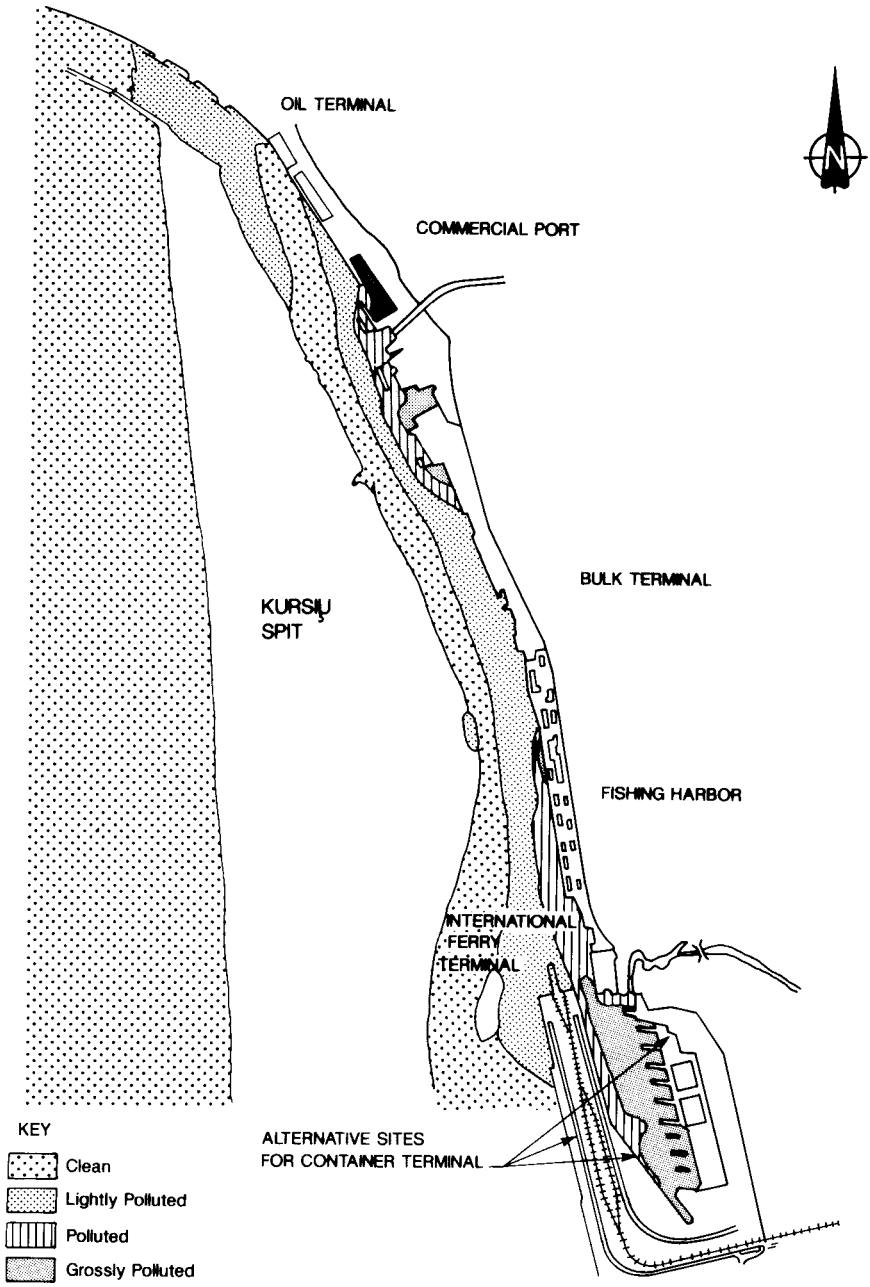


Figure 21.1 *Classification of sediment according to pollution*

21.4 ENVIRONMENTAL AUDITING

The environmental audit of Klaipeda port was carried out using a variety of techniques including:

(i) Skilled-eye inspection of the industries and facilities within the port;
(ii) Gathering of existing data, current and historical records and official documentation; and
(iii) Interviews with selected personnel.

The interviews held during the audit were particularly wide-ranging and included, in addition to staff employed at the various organizations within the shipyard, representatives from the National Institute of Ecology, the Environmental Protection Department, the Sea Port Authority and local authorities. In addition, much useful information was gained by attending, and interviewing delegates to, the First Lithuanian Conference on the future of the Kursiu Spit which was held during the audit.

Due to time and budget constraints it was not possible to carry out any sampling or perform any additional chemical or biological analysis. Such information had to be inferred from the results obtained from the audit with a view to carrying out future confirmatory testing.

21.5 RESULTS

A number of significant polluting and potentially polluting effluents and processes were discovered during the audit. These can be summarized as follows:

(i) The oil terminal was contaminating land and port waters due to past hydrocarbon spillages. Other oil products were also being discharged from the fishing harbor and the shipyards;
(ii) Loose particulate goods spilled during loading, unloading and storage were often washed into the port and were thus contaminating water and sediments;
(iii) The paper mill and the fish cannery sewage works had been discharging gross solids and organic material thus polluting port waters and contributing to sediment contamination;
(iv) The outlet of untreated sewage from the town's sewerage system was polluting the waters of the port with both sewage-derived organic matter and, largely inorganic, trade effluents including discharges from a battery factory and a pulp mill. The pollution caused by this discharge was exacerbated by large ferries turning within the port and thereby resuspending solid material;
(v) Ballast water, taken on board in foreign locations and subsequently discharged to the port from fishing fleet oil tankers, was suspected of introducing alien species to the area;
(vi) Heavy metals and anti-fouling paints were likely to have been discharged to port waters from the shipyards; and

(vii) Waste sludge and other material were being inadequately burnt in an outdated i ncinerator within one of the shipyards.

Given the above results, and other documented and hearsay evidence on the actual chemical species used by the various operators within the port, it was possible to further characterize the chemical contaminants which were likely to have been discharged to the harbor area over the preceding years. The resulting data were used to plan further surveys and studies aimed at providing enough information to allow environmental remediation of the port to proceed.

21.6 CONCLUSIONS

21.6.1 Audit findings

The environmental audit of Klaipeda port identified successfully many pollutants which were likely to have been discharged to the waters of the port over time. The following is a summary of the information gained:

(i) Resins, terpenes, pentose, methanol, acetic acid, formic acid and cellulose from the wood pulp factory via the town's s sewerage system;
(ii) Heavy metals particularly zinc, copper, nickel, cadmium, manganese, lead and mercury from the battery factory via the town's sewerage system;
(iii) Fuel oil, petroleum, phenols, polycyclic aromatic hydrocarbons (PAHs) and various other organic chemical species from assorted oil handling and storage facilities throughout the port;
(iv) Urea, phosphates and nitrate compounds from loading, unloading and storage facilities within the port; and
(v) Detergents, cyanides, a range of heavy metals, polychlorinated biphenyls (PCBs), tributyl tin compounds and a number of organic solvents rom the ship building and repair yards.

21.6.2 Further studies

Following the audit of a number of further studies were recommended. These were to be carefully targeted studies looking for the pollutants, and the known effects of these pollutants, which had been found during the audit to be those most likely to be contaminating the port waters and sediments. Studies recommended included:

(i) Carefully targeted chemical analyses;
(ii) Leaching tests and ecotoxicological studies on sediments within the port to determine pollutant availability and likely ecological effects;
(iii) Further ecological studies to relate known pollutant discharges to observed effects; and
(iv) Pilot clean-up and remediation studies with associated chemical and ecotoxicological testing.

22 Biological and Environmental Properties of Nitro-, Nitramine- and Nitrate Compounds: Explosives and Drugs

Application of QSPR and QSAR Studies in Environmental Toxicology Assessment

Slobodan Rendić, Blaženka Jurišić and Marica Medić-Šarić

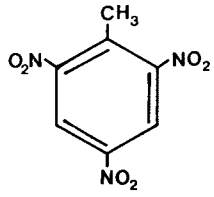
22.1 INTRODUCTION

Structure-property and structure-activity relationship studies (SPR and SAR, respectively) are important tools in pharmacology, toxicology, environmental research and medicinal chemistry for predicting effects of chemicals on the biological environment. When such effects pertain to additional processes such as metabolism and distribution then these studies are even more important for the deduction, interpretation and generalization of the compounds' behavior in the environment, since they greatly depend on the structure and physicochemical properties of the chemical(s) in question.[1] The QSPR and QSAR studies might lead to a better understanding of the reactions producing toxic metabolite(s) and contribute to the elucidation of the mechanisms of toxicity of these chemicals. Therefore such studies can be used effectively in environmental toxicology assessments.

22.2 BIOLOGICAL AND ENVIRONMENTAL EFFECTS OF EXPLOSIVES AND RELATED DRUGS

22.2.1 Nitroaromatic compounds

Trinitrotoluene (TNT), 1 (Figure 22.1, Table 22.1) is the representative compound most commonly used as an explosive. Following acute or prolonged exposure TNT causes liver injury, marked changes in the hematopoietic system producing anaemia, hepatomegalia and cataract in humans.[2,3] The compound has been reported to be harmful to rats, mice, dogs, fish, and marine life (oysters, green algae) as well as for many fungi, yeasts, actinomycetes, and other microorganisms in soil contaminated with munitions and was mutagenic by the Ames test.[3–5] Similar effects were observed also following high doses of other nitroaromatics such are dinitrotoluene (DNT), dinitrobenzene (DNB) and picric acid.[6]



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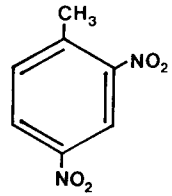

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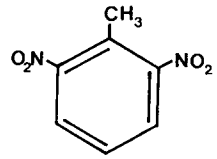

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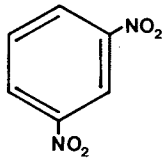

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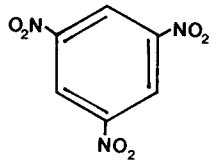

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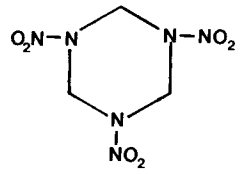

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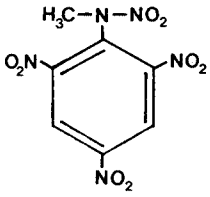

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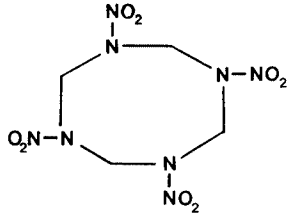

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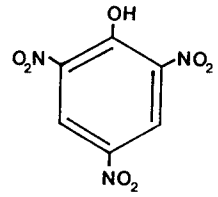

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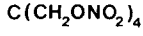

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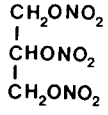

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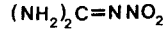

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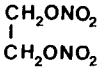

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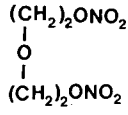

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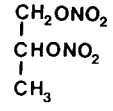

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Figure 22.1 *Structural formulae of explosives and propellants*

Table 22.1 *Full names, abbreviations and Chemical Abstract Service Registry Numbers (CAS RN) of explosives and propellants*[1]

Company No.	Full Name	Abbreviation	CAS RN
1	Trinitrotoluene	TNT	118-96-7
2	2,4-Dinitrotoluene	2,4-DNT	121-14-2
3	2,6-Dinitrotoluene	2,6-DNT	606-20-2
4	1,3-Dinitrobenzene	DNB	99-65-0
5	1,3,5-Trinitrobenzene	TNB	99-35-4
6	Hexahydro-1,3,5-trinitro-1,3,5-triazine	RDX	121-82-4
7	Octahydro-1,3,5,7-tetranitro 1,3,5,7-tetrazocine	HMX	2691-41-0
8	N-2,4,6-Tetranitro-N-methylaniline	TETRYL	479-45-8
9	Picric acid	PA	88-89-1
10	Pentaerythritol tetranitrate	PETN	78-11-5
11	Nitroglycerin	NG	55-63-0
12	Nitroguanidine	NQ	556-88-7
13	Ethylene glycol, dinitrate	EGDN	628-96-6
14	Diethylene glycol, dinitrate	DEGDN	693-21-0
15	Propylene glycol, dinitrate	PGDN	6423-43-4

22.2.1.1 *Application and environmental characteristics*

Nitroaromatic compounds are used in industry producing:

- Munitions chemicals (explosives and propellants);
- Pharmaceuticals;
- Dyestuffs;
- Pigments;
- Textiles;
- Plastics; and
- Fuel additives etc.

Pollution can be related to:

- Exploded and unexploded munitions in peace- and wartime;
- Industrial spillages containing end products of synthesis, intermediates and impurities such as nitro derivatives, sulfonated dinitro compounds, etc.;
- Disposal procedures;

- Clean-up procedures; and
- Wastewater discharge.

Environmental pollution by compounds, **1–5** (Table 22.1) was previously detected in:
- Surface waters;
- Sediments;
- Soils; and
- Groundwaters near to production, decontamination and military facilities

22.2.1.2 *Fate in the environment*

22.2.1.2.1 Biological degradation of nitroaromatic compounds in the environment was reported to occur slowly or not at all.[7]

In addition to the reduction of the nitro group resulting in formation of different, often toxic, intermediates,[6] explosives could be utilized by microorganisms as a carbon and nitrogen source.[8] The rate of biological degradation with formation of active toxic species depends on structural as well as on electronic properties of the compounds.[9–11] Organisms in the environments eg, bacteria, algae, vascular plants, invertebrates and fish, were found to be affected by the presence of nitroaromatic compounds in munitions' plant sediments and effluents.[7]

22.2.1.2.2 *Chemical degradation*. For toxicological properties, photochemical transformation of munitions chemicals is often more important than microbiological, due to the effect of sunlight, particularly when in admixture.[12] The principal photochemical, sunlight promoted trinitroglycerol products are 2, 4, 6-trinitrobenzaldehyde (converted further through 2-nitroso-4, 6-dinitro-benzoic acid to the azoxydicarboxylic acid) and 1, 3, 5-trinitrobenzene. The latter product is photostable and undergo further biotransformation via reduction of the nitro group to 3, 5-dinitroaniline. The photolysis under laboratory conditions yielded a number of compounds in complex mixtures and the primary product was the same as found in sunlight promoted photolysis. Other nitroaromatic compounds are transformed to structurally similar products.[6]

22.2.1.3 *Metabolic studies and toxicity*

In mammalian systems the principal metabolites of 2, 4, 6-trinitrotoluene are the 2-and 4-amino-dinitro derivatives but additional metabolites such as diimino-mononitro compounds have also been found. These metabolites were found in urine of rats, rabbit blood, and in munitions workers' urine.[6,13] The toxicity of the aromatic nitrates has to be associated with their reductive as well as their oxidative metabolism[14] (Figure 22.2). The following metabolites were found to be covalently and dose dependently bound to plasma, hepatic and renal proteins: 2-amino-4, 6-DNT and 4-amino-2, 6-DNT; as

toxic intermediates the oxidated forms of the corresponding hydroxylamines are proposed.[2]

The most carcinogenic compound was reported to be 2, 6-dinitro toluene, while 2, 4-dinitrotoluene is a pure promotor and less carcinogenic.[6,15]

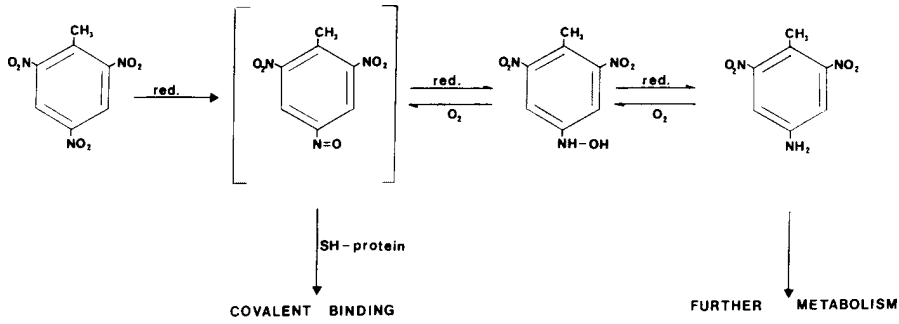

Figure 22.2 *Metabolism and proposed mechanisms of bioactivation and covalent binding of 2, 4, 6-trinitrotoluene (TNT)*

Model systems used for toxicity testing include:

22.2.1.3.1 For *in vivo* testing, the compounds are administered to different experimental animals, followed by measurements of covalent binding and structure elucidation of the compounds bound to tissue proteins.[2,15] Mammals used as models for *in vivo* toxicological studies are mink, monkeys, cows, pigs, humans (for skin sensitization studies). Other organisms used are fish (bluegill, Japanese medaka, fathead, zebra fish), micro-crustaceans *(Daphnia magna, Ceriodaphnia),* amphibian *(Xenopus),* birds (chickens, Japanese quail, bobwhite quail, Mallard ducks), and domestic plants.[6,16]

The following toxicological studies are usually performed: acute LD_{50}; sub-chronic; sub-acute; chronic; teratogenicity; mutagenicity; carcinogenicity; reproduction; and toxicokinetics/metabolism.

22.2.1.3.2 For *in vitro* studies radioactive labeled compounds were incubated with different mammalian tissue fractions in the presence of enzyme activating systems.[2,4] The following literature references describe the application of model studies (ie, using plants, bacteria and others) in addition to chemical methods:

(i) Systems using fathead minnows, water fleas, bluegills, freshwater micro-crustacean *Ceriodaphnia dubia;*[17]
(ii) Neutral red uptake assay;[7] and
(iii) Bacterial *in vitro* models: *Nitrosomonas* species,[18] *Salmonella* species,[19] *Photobacterium phosphoreum,*[12] etc.

22.2.2 Nitric acid esters

Nitroglycerin, **11** (Figure 22.1, Table 22.1) synthesized by A.Sobrero in 1846 is a representative of nitric acid esters of the lower alcohols ie, glycerol, diethylene glycol, ethylene glycol, propylene glycol, and the higher alcohol pentaerthrytol. The esters of nitric acids (compounds **11, 13, 14, 15**) are used as:

- Explosives (for instance dynamite prepared by mixing nitroglycerin with charcoal and diatomaceous earths (A.Nobel); and
- Drugs for treatment of angina pectoris possessing vasodilatating properties (in highly diluted form).

Pollution by nitric acid esters can occur by the same procedures as described for nitroaromatic compounds.

Environmental pollution was detected previously in washwaters from nitroglycerin producing plants.

22.2.2.1 *Fate in the environment*

22.2.2.1.1 Biological degradation. Nitroglycerin and other aliphatic nitric acid esters undergo aerobic biodegradation readily via successive removal of nitrate groups to isomeric derivatives.[6]

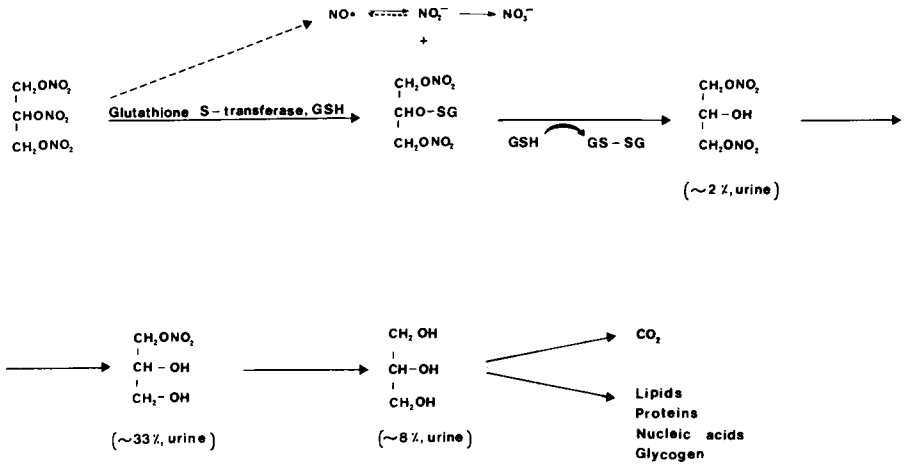

Figure 22.3 *Metabolism of glycerol trinitrate*

Microbial metabolism and the fate of nitric acid esters has been recently reviewed.[20] Glycerol-1, 2, 3-trinitrate can be metabolized to the corresponding mono-and dinitro-derivatives, and to glycerol by both mixed bacterial cultures and a number of fungi. Formation of isomeric mono- and dinitro-derivatives from nitroglycerin was accompanied by production of nitrite, nitrate and nitroxide (Figure 22.3). The latter was found to be reactive and bound to reduced heme and to non-heme Fe-S proteins as well as to proteins with cytochrome P-450 like activity. GSH S-transferase as a nitrite liberating enzyme was suggested for fungi. Other nitrate esters including pentaerythritol tetranitrate also undergoes microbial metabolism by cleaving the nitrate ester group. Ethylene glycol dinitrate could be used by bacteria as a sole source of nitrogen.

22.2.2.1.2 Chemical degradation. Nitrate esters are not significantly photolabile, but they are readily hydrolyzed depending on the pH value. Trinitroglycerin is hydrolyzed more slowly than mono- or dinitro derivatives, and the products are complex mixtures of nitrate, nitrite and oxalate.[6]

22.2.2.1.3 Toxic effects of nitric acid esters. Toxicological studies (LD_{50} determinations) indicated higher toxicity of nitroglycerin compared to other esters.[6] Animals exposed to nitroglycerin, for instance, suffered from convulsions and died.[21] (21). Administration of the organic nitrates increases dopamine release from the striatum in rats. These experiments indicated that the central nervous system might be implicated in the acute phase of exposure to organic nitrates.[22]

22.2.2.2 *Metabolic studies and toxicity*

Formation of nitric oxide radical (nitroxide, NO•) as an inorganic free radical gas during biotransformation reactions was associated with toxic as well as with therapeutic effect of nitroglycerin and other esters of nitric acid.[23–25] The nitric oxide radical is also synthesized endogenously from *L*-arginine by family of enzymes known as NO-syntheses.[26] Different mechanisms were proposed for the formation of the free radical species from trinitroglycerin (ie, sulfhydryl dependent and heme-dependent mechanism possibly via cytochrome P-450 enzymes).[1] The formation of 3-nitroso-1, 2-glyceryl dinitrate as a putative intermediate has been proposed by Buckell et al.[27] Posedas del Rio et al[28] have reported that the biotransformation of the nitrate esters following oral administration commences at the absorption site (ie, small intestine) followed by reactions in other human tissues. Inorganic nitrate and nitrite were identified as metabolic products in blood following administration of nitroglycerin and other esters (pentaerythritol tetranitrate, propylene glycol dinitrate and ethylene glycol dinitrate).[1] The following toxic reactions were attributed to administration of the nitroglycerin as a consequence of increased concentration of nitric oxide: interference with DNA replication and with mitochondrial respiration enzymes, inhibition of metabolic pathways (Krebs cycle), oxidation of low density lipoproteins resulting in cytolysis and cytostasis.[29–31] The formation of nitric oxide was confirmed by measuring the concentrations of NO in exhaled air in anaesthetized rabbits following infusion of trinitroglycerol.[32] It has been suggested

that nitric oxide in the presence of superoxide radical ($.O_2^-$) might produce the highly toxic peroxynitrite ion ($ONOO^-$) causing neuronal death, however, this mechanism could not be confirmed.[33]

22.2.3 Nitramine compounds

Representative of nitramine compounds used as explosive are 1, 3, 5-trinitro-1, 3, 5-triazine (RDX) and octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX) (Figure 22.1, Table 22.1).

RDX and HMX are used as explosives in a variety of munitions applications.

Pollution can be related to the environment in a similar manner to that described for nitroaromatic compounds.

Environmental pollution has been detected at munitions production plants in: aquatic environments; soils; and sediments.

22.2.3.1 *Fate in the environment*

22.2.3.1.1 Biological degradation. Nitramine compounds in the environment (eg, aquatic or soil) are biologically stable and migrate very slowly.

Nitramines are strongly resistant to biodegradation. Aerobic microorganisms in surface waters do not affect these compounds and anaerobic processes are of limited significance. However, under anaerobic conditions biodegradation of RDX produced a number of metabolites resulting from the reduction of the NO_2 groups.[34] Such products were reported to possess mutagenic and carcinogenic properties.[6]

22.2.3.1.2 Chemical degradation. In an aqueous solutions photolysis by sunlight of RDX and HMX is slow. Rates obtained are in days and RDX reacted 2–3 times faster than HMX. The ultimate products identified are nitrates, nitrite, ammonia, formaldehyde and nitroso compounds.[6]

22.2.3.2 *Toxic effects and metabolism of nitramine compounds*

Because of its toxicity in rats, RDX was used as a rat poison. The compound was not mutagenic but it showed detrimental reproductive effects when administered to rats. Toxic reactions observed in humans following exposure at munitions manufacturing plants,[35] occupationally related case of human toxicity of RDX have been reported in USA.[34] Toxic reactions might result from reductive metabolism with formation of reactive nitroso- and hydroxylamines.

RDX has been reported to cause adverse effects on central nervous system in mammals, producing convulsions and/or unconsciousness either by inhalation or by ingestion. RDX was found to be acutely toxic both to water invertebrates when tested at its solubility limits and to reduce reproductive success.[36]

Nitrate, nitrite, and nitrogen dioxide radical formed by photolytic reactions (Figure 22.4) from nitramines could react with environmental pollutants such are polycyclic aromatic hydrocarbons producing carcinogenic nitroarenes. These compounds are potent inducers of the cytochrome P-450 1A1 family of enzymes which activate polycyclic aromatic hydrocarbons to carcinogenic dihydrodiol

epoxides. The nitrogen dioxide radical formed by photolytic decomposition of nitramine RDX has been measured by a spin trapping method. It was proposed that the radical is formed by the reaction between superoxide anion and nitroxide via an intermediate peroxynitrite.[37]

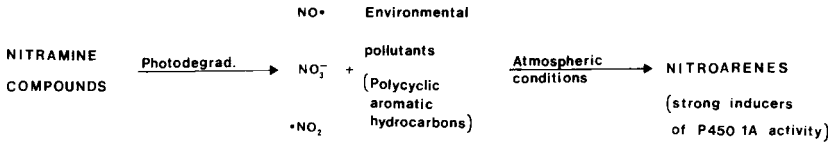

Figure 22.4 *Toxic effects of nitramine compounds*

Model systems and organisms used for toxicity testing are similar to those described for nitro aromatic compounds.

22.2.4 Nitroguanidine

Nitroguanidine (Figure 22.1, Table 22.1) is manufactured for use as a component of military propellants and munitions.[6,38]

Other pollution related characteristics are similar to those described for nitroaromatics.

22.2.4.1 *Fate in the environment*

22.2.4.1.1 Biological degradation:

(i) Nitroguanidine is not susceptible to aerobic biodegradation and, hence, because of its water solubility, it is a potential environmental contaminant; and

(ii) The compound could be reduced anaerobically into nitrosoguanidine and its degradation products cyanamide, cyanoguanidine, melamine and guanidine.[6]

22.2.4.1.2 Chemical degradation. Nitroguanidine can be readily photolyzed by sunlight with the rate which corresponds to that for RDX. The products are primarily guanidine, urea, and nitrite, with nitrosoguanidine as an intermediate.[6]

22.2.4.2 *Toxicity and metabolism of nitroguanidine*

Nitroguanidine is not metabolized in rodents and, therefore, is a low toxicity compound to these species. Nitroguanidine is also essentially non-toxic to aquatic organisms such are fathead minnows, water fleas, and rainbow trout. The mixture of photolysis products was, however, about 100 times more toxic.[6,38]

22.3 APPLICATION OF QSPR AND QSAR STUDIES IN ASSESSMENT OF BIOLOGICAL ACTIVITY

A number of papers have been published on QSPR and QSAR of explosives and related compounds. It is reported[1] that physicochemical parameters such are molecular weight, density, diffusion coefficient, heat of fusion, and vapor pressure may be correlated with selected molecular descriptors (the Wiener Index, the First-Order Connectivity Index, the Fifth-Order Connectivity Index, the Sixth-Order Valence-Connectivity Index, Balaban Index, and the number of non-hydrogen atoms in the molecule). Correlation studies were proposed for determination and prediction of the behavior of explosives as pollutants in the environment in relation to their safety in production.[39] QSAR of mono- and dinitrobenzene derivatives was investigated by using toxicity data from *in vitro* model studies.[40] Mono- and dinitro-derivatives producing different acute and semichronic toxic response were statistically evaluated and the QSAR data discussed in regard of uses of Hammett constants and log P_{ow} as parameters.[40] (See also chapter by Dearden and Cronin.)

It is proposed to use quantitative studies (QSPR and QSAR, respectively) to correlate structural characteristics with physicochemical properties, lipophilicity (log P_{ow}) with chemical and biological effects and environmental fate. The results from own studies and from studies obtained from literature sources using diverse assay systems will be used

22.4 RECOMMENDATIONS

For toxicology assessment of chemicals used as drugs and explosives it is suggested that studies on structural parameters in correlation with the following are undertaken:

(i) The rate of biological degradation (metabolism) of the compounds in the environment as a factor for determining toxicity by conversion to more potent biologically active metabolites;
(ii) The rate of chemical transformation (phototransformation, thermal composition, interaction with other chemicals in the admixture) and toxicity of the products and intermediates formed;
(iii) Biological properties (toxicity) induced by other compounds present in mixtures; and
(iv) Toxicity which results from industrial operating conditions and from chemical and biological purification methods.[18]

Such studies will enhance the scientific undertaking and development of environmental toxicology assessment especially in those areas devastated by warfare (eg, Oštarije, Delnice and elsewhere in Croatia).[41,42]

22.5 ACKNOWLEDGEMENTS

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Permission to reproduce Figure 22.1 and Table 22.1 from *Drug Metabolism Reviews* 1994, **26,** 717–738 from Marcel Dekker Inc., New York is acknowledged with thanks.

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23 The Idrija Mercury Mine, Slovenia, A Semi-millennium of Continuous Operation: an Ecological Impact

Ladislav A.Palinkaš, Simon Pirc, Slobodan F.Miko, Goran Durn, Ksenija Namjesnik and Sanja Kapelj

23.1 INTRODUCTION

Serious ecological disasters in the 1950s in Japan and Sweden brought mercury into the limelight of public interest. The semi-millennium of mining and processing of mercury ore at one of the largest natural mercury accumulations of the world—the Idrija mercury ore deposit—did not attract attention; which was probably due to the lack of an accidental event, at least in recent history. Weathering of the ore deposit with its load of 500,000 tonnes of mercury in the form of cinnabar and native metal has been a low intensity process in comparison with the anthropogenic intervention. This implied large mass transport and energy transfer inducing significant chemical disequilibria and intensive dispersion of mercury as a pollutant. The primary task of the survey outlined in this chapter was to complete an overall picture of the pollution within different environmental compartments. The cessation of mining was a decision of the Slovenian government as a requirement for environmental protection did not quieten public anxiety. Thousands of tonnes of mercury has been spilled or emitted in some other manner to the environment at Idrija. This survey is an attempt to illustrate a more realistic and complete assessment of the size of the contamination, its character and fate, which may assist in any future health adverse effects and a requirement for remediation.

23.2 LOCATION, CLIMATE, GEOLOGY

The town and the mine of Idrija is situated 50 km west of Ljubljana in the region named Cerkno-Škofja Loka Cliffs, a part of the Alpine promontory, transitional zone between the Alps, Karst and Adriatic.[1] The town is 310 m above sea level in the valley of the Idrijca river, a left-hand tributary of the Soca river within the drainage areas of the Adriatic (Figure 23.1). The valley of the Idrijca river, wider in the clastics and narrows to almost a gorge in limestone rocks and is surrounded by moderately high mountains (700–1100 m). At the highest elevations, steep cliff walls of the gorge flatten gradually into a highlands with unexpressive cupola-like peaks which are remnants after the peneplanization of the limestones.

The climate in such a diversified relief with deep valleys between the Inner Slovenia, Alps and the Adriatic, has a complex temperate character.

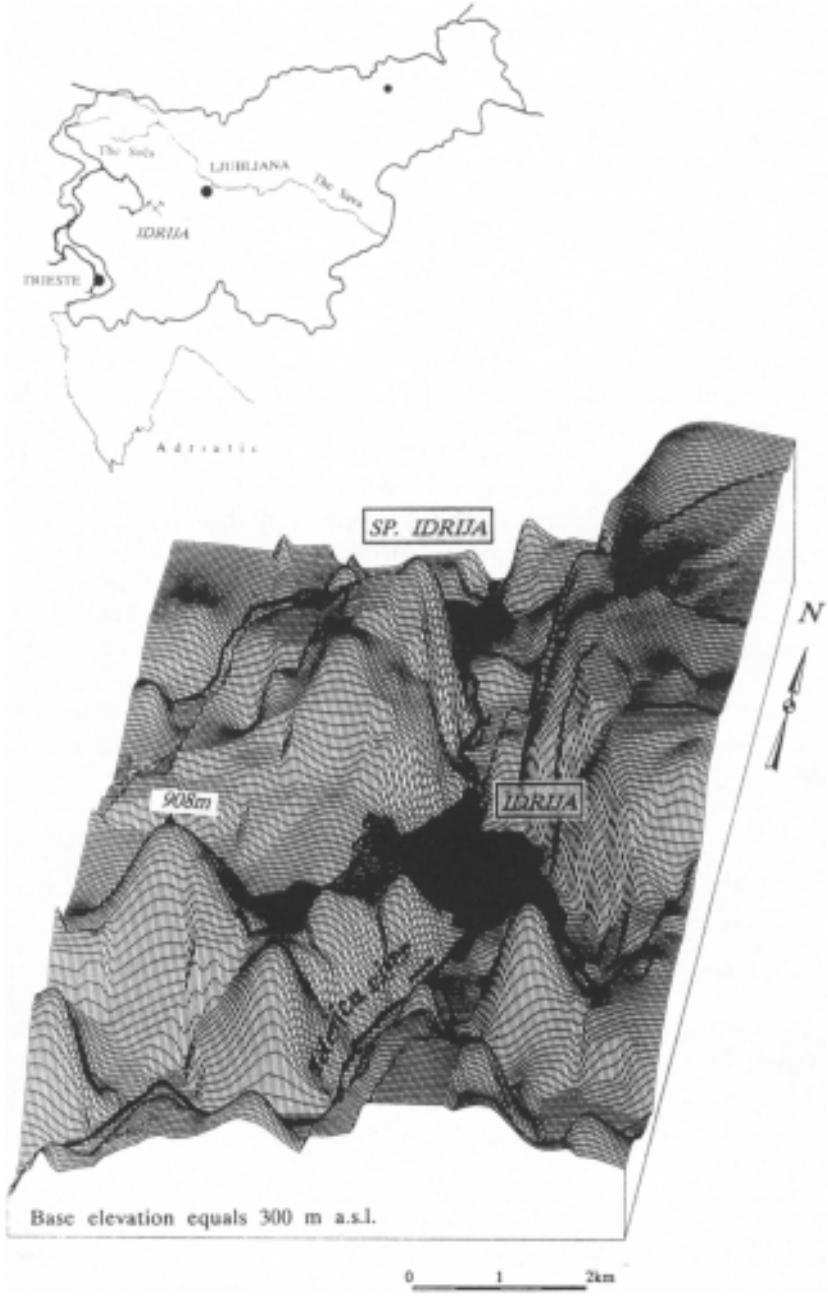


Figure 23.1 *Geographical position and local topography of Idrija and surroundings*

However, four well-defined seasons are recognized. Summers are not unbearably hot and bitter cold winters are rare. Antagonism among continental, altimontane, submontane and sub-mediterranean climates results in high mean annual precipitation, 2000–3000 mm y^{-1}, which is irregular throughout the year. Wet air masses from the sea contact.cold air causing ~80–100 foggy days. The average temperature in July is 19–20 °C but in January -2 °C is not representative of the whole region since a temperature inversion in the foggy valleys may reach 15 °C. The wind directions at higher altitudes coincide well with general trends in Western Slovenia forming a regular wind-rose but draught occurs in the valleys, dependent on the valley shape. Calm periods also occur. The area is forested and sparsely populated. The town of Idrija has ~10,000 inhabitants.

The geology of the region has developed through the Mesozoic and Tertiary evolution of the northernmost Dinarides. It is exceptionally well investigated due to the semi-millennium mining operation and applied geological studies by a number of geologists: Lipold,[2] Kossmat,[3] Kropač,[4] Berce,[5] Mlakar,[6,7], Drovenik,[8] Placer,[9,10] Čar,[11–14] Čade•,[15] et al. Currently >80 geological papers have been published.[13]

The Idrija ore deposit was formed by a volcano-sedimentary process within the Middle Triassic trough[16] deepened by extensional tectonics during the rifting stage of the Tethyan ocean opening. Middle Triassic tectonics led to the upwelling of hydrothermal solutions which expelled their deposits onto the sea bed through a thick layer of Upper Palaeozoic, Permian, Scythian and Anisian clastics and carbonate rocks (Figure 23.2). They mineralized fissures, pores, faults, planes, breccia zones, replaced metasomatic carbonates and cement in clastics, assigning the clear epigenetic character to the cinnabar ore bodies whilst percolating towards the sea bed. A reducing environment in the carboniferous carbonaceous schists favored deposition of the native mercury from the ore-forming solutions. The thickness of the ore bearing sequence is estimated to be 800 m.[16] Thermal springs, deposited their mercury load onto the sea bed forming synsedimentary ore beds and lenses in the black Sconca shales and tuffs, which is a member of the Langobardian sedimentary sequence of the Ladinian age. Currently, the Idrija ore deposit is incorporated into a complex geological structure which was formed during the paroxysm of the Alpine orogenesis. The ore deposit was uplifted and cut into blocks by napping and thrusting and pushed several kilometers from its place of origin.[5,7] In the final phase of the Alpine orogenesis the ore bodies were disintegrated and moved along the faults. A part of the deposit was displaced for about 2.5 km south-east along the Idrija fault,[17] cinnabar milonites and cinnabar ore can be found in these faults.

The mineral paragenesis of the deposit is almost monometallic and consists of cinnabar, metacinnabar, native mercury, sporadic iron sulfides and gangue minerals calcite and quartz. Its volcano-sedimentary origin implies that epigenetic ores such as veins, veinlets, disseminations, stockworks, ore breccias, etc. and syngenetic, conformable ore beds and lenses were formed. These varieties of the ore textures furnishes a large span of ore grades from several tens to <1% of mercury.

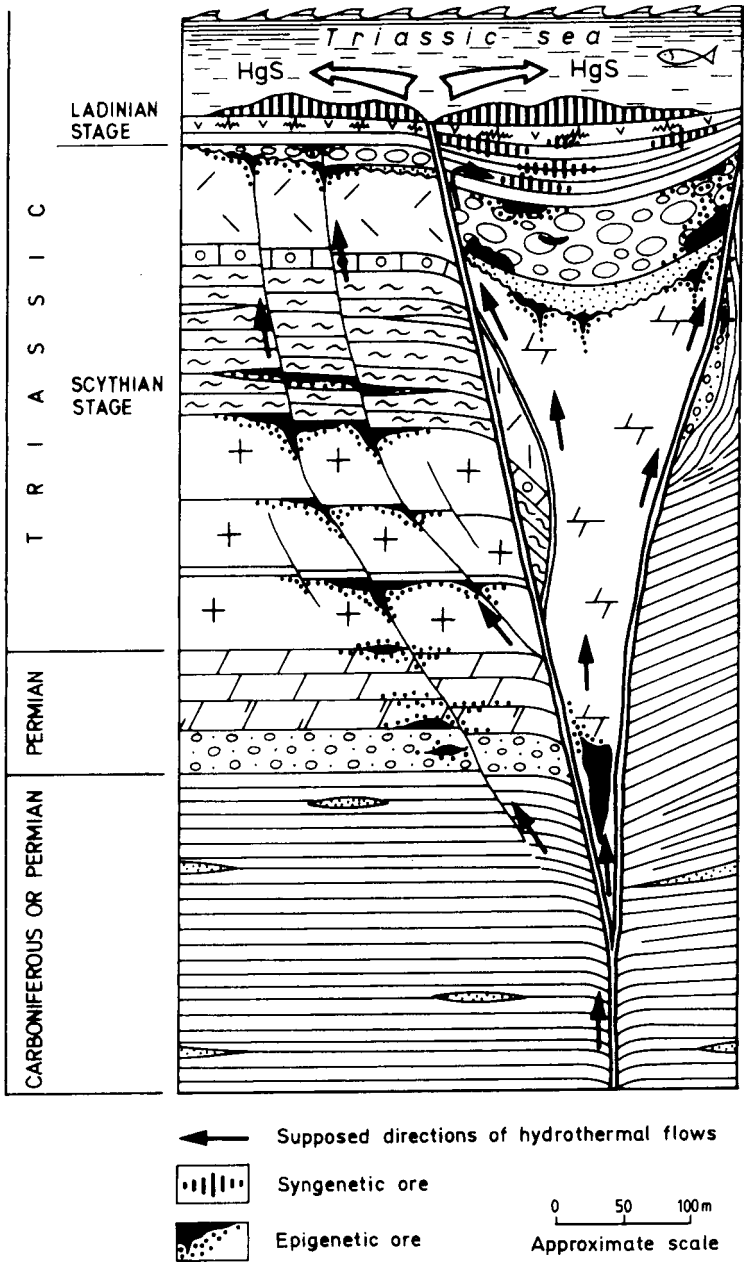


Figure 23.2 *Schematic ore forming processes in the Middle Triassic time. Hydrothermal water upwelling to the bottom of the Tethyan ocean, mineralized surrounding rock conduits and loaded its mercury content around the underwater springs in the Middle Triassic time. Probably, it was the largest natural pollution in the Earth's history*

23.3 PAST MINING ACTIVITIES

The real pollution hazard in the valley of the Idrijca river commenced in 1490 when a local cooper found a droplet of native mercury in the carboniferous shale. Until 1508, only native mercury from the shale was being recovered when rich cinnabar veins were discovered which initiated intensive production and paved the way to a large-scale ecological contamination. A concise outline of the Idrija mercury mining through the centuries is given by Mlakar.[18]

An increased requirement for mercury commenced with the discovery of the Americas and exploitation of their gold and silver mines. It was used largely for recovery of precious metals by amalgamation. Such world needs had been covered by three mines: Almaden (Spain), Idrija and Huancavelica (Peru). Between 1500 and 1599 the Idrija participation to the world production was 37% but its share decreased subsequently and today it is ≯6%. A similar trend may be observed with the ore grade, from 18% in early mining days to 0.1–0.2% in the 1970s an average for the total excavated ore was 1.7%. As expected, the recovery has been increasing steadily, following advances in technology, from 50% in the pioneering days to 90% in the 1970s, averaging ~73%. Up until now the entire world production was 800,000 tonnes of mercury with 13% from Idrija. Almaden (280,000 tonnes) and Idrija (105,000 tonnes) together have produced nearly 50% of the total world output. The Idrija ore deposits are still ~10% of the world mercury reserves.[19] For comparison purposes the total quantity of the recovered mercury from Idrija could have filled two Olympic swimming pools (25 m×50 in×3.1 m) with a value estimated at US$ 1 billion.[18] Assuming an average recovery of 73%, the total production amounts to 144,000 tonnes. According to experts, with an ore content of 0.1–0.2% mercury, Idrija is one of the poorer ore deposit producers.[20] Unfavorable market conditions, in addition to the raised public awareness on the environmental protection, forced the Slovenian Assembly to promulgate a special law regulation on a gradual closing of the Idrija mine until the year 2003. Monitoring of eventual accidental effects will be furnished until 2006.

23.4 CASE HISTORY: RECOGNITION OF THE POLLUTION

The Idrija ore deposit, with its huge metal load exceeding a few 100,000 tonnes of mercury is situated below a surface of 350,000 m^2 and is a worldwide phenomenon.[18] It is surpassed only by Almaden with the total metal quantity. The birth of such a metal accumulation by virtue of mercuriferous thermal springs on the bed of the Middle Triassic sea is a unique event and must have been the largest natural environmental pollution in the earth's history. However, natural mercury pollution by volcanic hydrothermal activity does not mean necessarily poisoning of the biota. Strong bonding between mercury and sulfur in cinnabar, precipitated within euxinic sedimentary or hypogene environment, rich in hydrogen sulfide, assures low concentrations of possible mercurials. Up until now, recent natural disasters by hydrothermal mercury poisoning have not been reported.

Weathering of the mercury-bearing ore deposits might be a facet of the natural pollution hazard. Cinnabar, as a chemically resistant mineral, is concentrated in the heavy mineral fraction of stream sediments and can be washed from deposits. Its poor mechanical durability results in dispersion through disintegration within a drainage system. Additionally, weathering cycles of other mercury-containing minerals which includes ionic chemical species might be a threat to the environment but no tragic episodes have been recorded.

A major part of the ore reserves in Idrija is liquid, metallic mercury disseminated within the black carbonaceous schists of the Carboniferous age. Weathering of the rocks, hosting liquid mercury droplets and which crop out on a large surface of the rocks, contributes to the pollution of the area through degassing, oxidation of mercury and its transport via aquatic media and possible methylation in organic rich soils. Natural discharges are usually of low intensity controlled by the rate of weathering. In contrast, anthropogenic release of mercury into the environment might be considerable due to the large mass transport and energy transfer, which induce a significant chemical disequilibria and consequently high mobility of possible toxicants.

Termination of the mine operation does not mean an end of the public anxiety. A semi-millennium of production spilled 39,000 tonnes of mercury due to poor recovery (73%) through smelter stacks and flues by gaseous and particulate matter in the atmosphere. The emissions have contaminated soils in a wider area by dry and wet fallout. Even in 1977, when the smelter was still in full operation, 20 kg Hg day^{-1} was being emitted into the atmosphere.[21] Effluent from flotation, tailings, mining spills, dumps, etc. affected stream and flood sediments in the Idrijca river. In the past, the existence and temporary activities of small smelters in the Idrija countryside, in addition to negligent, cattle-driven transportation of mercury in impractical containers, contributed to the dispersion of the pollutant.

However, long-term mining and processing of the ores in the surrounding area of Idrija does not allow quantitative discrimination of the natural and man-made pollution but some attempts have been undertaken during this research. The vertical distribution of mercury at some selected soil profiles suggests one source of pollution—anthropogenic.

Toxicological effects of mercury had been already recognized by ancient observers and the danger of poisoning by mercury had been demonstrated as early as 1493.[22] The life expectancy of native miners in Mexico and South America in the 16th century, who used mercury in the manufacture of silver by amalgamation, was about six months.[23] Paracelsus described a therapy for mercurial diseases of miners in 1567.[22] The affection of the huge natural mercury accumulation in Idrija by manmade intervention and its immediate influence on the environment had been observed by early poisoning of the Idrijan miners. Several disastrous underground fires in the Idrija mine in 1766, 1803 and 1846, killed and impaired the health of thousands of miners mostly through mercury vapor poisoning.[24,25] It is no wonder that humans were the first subjects to be studied. The monitoring of mercury retention in the human body and various organs has been the primary task for many years. Kosta et al[26] measured 101 $\mu g\ g^{-1}$ mercury in certain human organs from underground miners and 14.4 $\mu g\ g^{-1}$ mercury by some individuals inhabiting Idrija who did not participate in mercury processing.

The serious ecological disasters that occurred in the 1950s in Japan and Sweden initiated a public interest in mercury but the Idrija area did not attract very much attention, probably due to the very specific and local geochemical conditions which did not lead to an accidental situation. A certain number of scientific papers dealing with contamination of almost all environmental compartments: biota, stream waters, stream sediments, soils, air and potable water, offer inconsistent information but did report, however, an extraordinary pollution.[26–32]

Kavčič[33] studied contamination of air by 24 h sampling at seven sites within the Idrija town limits and obtained values between 0.5–30 μg m^{-3} Hg. Dermelj[34] reported mercury values in potable waters (0.1–0.3 μg l^{-1} Hg), in the Idrijca river (0.1–0.2 μg l^{-1} Hg) within the less polluted area, and 4–70 μ l^{-1} Hg in the vicinity of the mine and smelter. Extremes of 100 μg l^{-1} Hg was found in an effluent from the mine and smelter. Grašic[35] reported high valuations of mercury concentrations in the air with a maximum of 8500 ng m^{-3} Hg near to the smelter stacks and 570 ng m^{-3} Hg downtown, depending on the influence of wind, temperature and local morphological conditions. Kobal et al[30] recorded a mercury content in the air of 10–490 n gm^{-3} downtown and in potable waters in the range of 0.1–1.2 μg l^{-1} (detection limit 0.1 μg l^{-1} Hg). Kosta et al[26] determined values of 5800 ng m^{-3} Hg in the air close to the smelter and 580 ng m^{-3} Hg downtown. Hess[29] inspected a number of stream and flood sediments, soils (1.84–885 mg kg^{-1} Hg) and two water samples (3.1 and 0.35 μg l^{-1} Hg respectively). Flood and stream sediments of the Idrijca river along the valley a few km up and downstream were inspected systematically by Gosar.[31] The upstream mercury values are low (2 mg kg^{-1}), between the towns of Idrija and Spodnja Idrija up to 100–760 mg kg^{-1} Hg were found and downstream towards the confluence with the Soca river reduced gradually to 5–100 mg kg^{-1} Hg. Flood sediments are several times those relative to the stream sediments in the downstream sampling section (3–993 mg kg^{-1} Hg). Approximately 10% of the total mercury in stream sediments is in the form of cinnabar as determined by the heavy mineral fraction, although clay-sized particles have not been examined and could change these percentages significantly (author's comments). The Müller index of geoaccumulation in the sediments (Igeo)[36] calculated from the background value of 2 mg kg^{-1} Hg, places the Idrijca river into the group of exceptionally polluted rivers.

23.5 CASE STUDY: POLLUTION OF SOIL, SOIL GAS, WATER, STREAM SEDIMENTS AND AIR

23.5.1 Materials and methods

Inconsistency of the earlier analytical data result from diversified sources. Use of different analytical techniques, choice of sampling sites, seasonal or even diurnal variation of climate parameters (temperature, barometric pressure, humidity. precipitation) soil moisture, mining intensity, etc. may affect significantly the overall picture of the pollution. The content of mercury in air, soil gas and stream waters are influenced by weather conditions. For these reasons the sampling campaign was

undertaken in only two days (24 and 25 May 1990) in order to obtain comparable results within all environmental compartments or with those of possible subsequent monitoring. The mining production during that time was in significant decline as part of the planned closure procedure.

23.5.1.1 Soils and soil air

A total of 100 soil samples were collected at 56 sampling sites. Fifty-two soil samples were collected as single samples (Figure 23.3). A sampling grid was spaced irregularly along the Idrijca valley and the slopes of the surrounding hills. To discriminate between the natural from man-made pollution, an additional four vertical soil profiles were selected and 48 soil samples obtained. They were dried and sieved to -80 mesh size prior to digestion and cold vapor atomic absorption spectrometry (CVAAS) analysis for mercury.

At the same sampling sites soil air was analyzed for mercury-vapor content after exhaustion by vacuum soil pump and adsorption on gold wire in the collector of the field mercury atomic absorber. The soil types of the region are indicated only as a broad overview. According to Stritar,[37] on hard carbonate country rocks rendzinas, brown rendzinas, decarbonate soil, leached decarbonate soil and leached soil occur. Rendzinas (pararendzinas), brown carbonate soils and brown saturated soils are developed on marly rocks. On siliceous clastic rocks rankers, brown acid soils, podzolic brown soils and pseudogleyic leached soils occur. Alluvial or fluvial soils on gravel and sand occur on the alluvium in valleys. Also regosols are developed on mine tailings. In parks and landfill areas of the town of Idrija, anthropogenic soils were found.

Soil analysis: Soil and stream sediment samples were digested with a mixture of concentrated nitric and hydrochloric acids (1:3). The mercury analyses were performed by cold vapor atomic absorption (CVAAS) on a Pye Unicam model SP9 atomic absorber spectrophotometer. The soil sample solution was transferred to a mercury reduction vessel containing 10% stannous chloride and 20% hydroxylamine hydrochloride solutions in distilled water. The evolved mercury vapors were passed through a silica-glass cuvette on which the mercury concentration was measured. A mean analytical sensitivity of 0.05 mg kg^{-1} Hg was achieved. Stream sediments were analyzed additionally for lead.

Soil air analysis: Mercury analysis of soil air was performed with a field mercury atomic absorption spectrometer (AGP-1 (Analizator Gaza Pochve)). The soil gas is pumped out of the soil at a depth of ~30 cm from a conical metal probe into the instrument where mercury is then amalgamated onto a gold wire, usually one 1 of soil air is passed through the gold wire trap for mercury which is afterwards electrothermally heated and the adsorbed mercury released and measured. The instrument is calibrated with the use of a synthetic mercuric sulfide quartz standards from which mercury is

evaporated in a specially designed electrothermic furnace.The mean analytical sensitivity of 50×10^{-6} mg m^{-3} of gas was achieved which is equivalent to a sensitivity of 5 ng g^{-1} Hg in a solid sample (soil, rock, etc.).

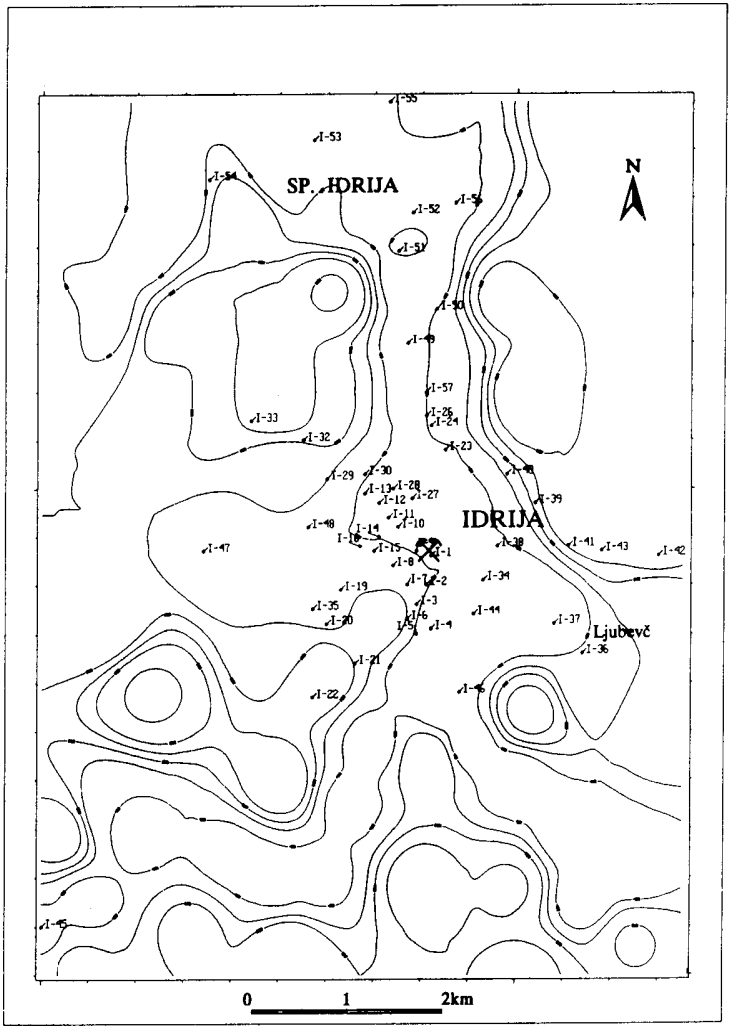


Figure 23.3 *Soil sampling map*

23.5.1.2 Stream sediments and water

Stream sediments were sampled along the Idrijca valley 7 km upstream and 18 km downstream and in the Idrija town itself. In total, ten samples were taken, dried and sieved to -80 mesh size, together with 100 soil samples, prior to digestion and CVAAS analysis for mercury.

Water samples were collected at five springs used as part of the municipal water supply system. Four of them are in the town or its immediate vicinity and one is the spring of the Idrijca river. The Idrijca river and its tributaries were sampled randomly at 16 sites along the valley between the points; 7 km upstream, 18 km downstream and in the mining area within the town (Figure 23.4 and Table 23.1).

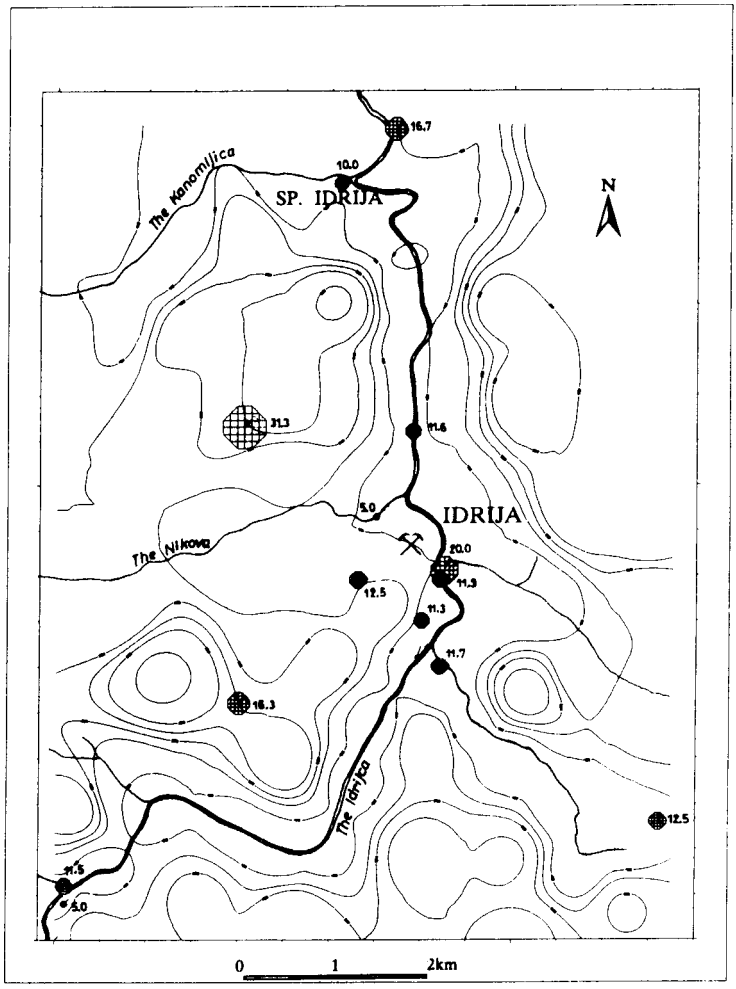


Figure 23.4 *Map of mercury in stream and springwaters, ng l^{-1} Hg*

Table 23.1 *Mercury content in springs and stream waters*

Sample No.	Locality	pH	T °C	HCO^-_3 mg l^{-1}	Hg ng l^{-1}	Comment
1	Spring–Černika	8.3	9	185	11	mining area
2	Spring–Češnjice	7.80	9	271	31	mining area
3	Spring–Lačna voda	8.10	7	220	16	
4	Spring–pri Kosmaču	7.90	10	269	13	mining area
5	Spring–Divji Potok	7.65	7	208	10	
6	Idrijca river	7.65	11	188	12	7 km upstream
7	Belca river tributary of Idrijca	7.60	11	209	5	7 km upstream
8	Idrijca river	7.58	11		3	1 km upstream
9	Zala river	7.60	14	231	12	upstream, close to mining area
10	Idrijca river	7.60	11		11	upstream, close to mining area
11	Ljubevšca river tributary of Idrijca	7.55	12.5		20	mining area
12	Mining water	7.50	11	200	11	mining area
13	Idrijca river	7.50	12.5		5	mining area
14	Mining water	7.40	12		21	mining area
15	Idrijca river	7.50	11.8	215	12	0.5 km downstream
16	Idrijca river	7.62	11.8	209	17	2.5 km downstream Spodnja Idrija village
17	Idrijca river Reka village, downstream	7.50	12.8	208	12	10 km downstream Reka village
18	Idrijca river	7.50	12	206	4	18 km downstream Otalež village
19	Kanomljica river tributary of Idrijca	7.70	12.2	211	10	3 km downstream
20	Nikova river tributary of Idrijca	7.60	9.5	248	5	mining area
21	Zala river	7.70	9.5	242	13	5 km upstream close to traffic

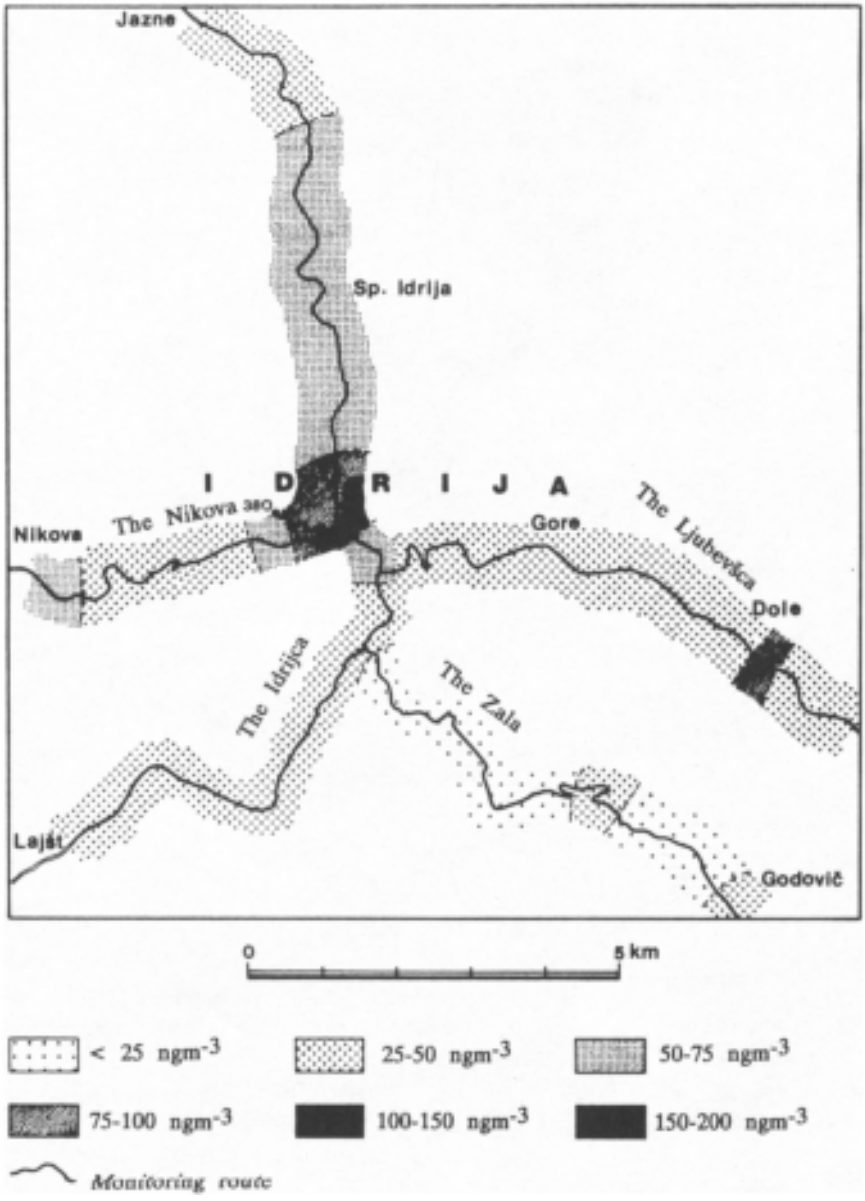


Figure 23.5 *Schematic map of mercury in air, ng m^{-3} Hg*

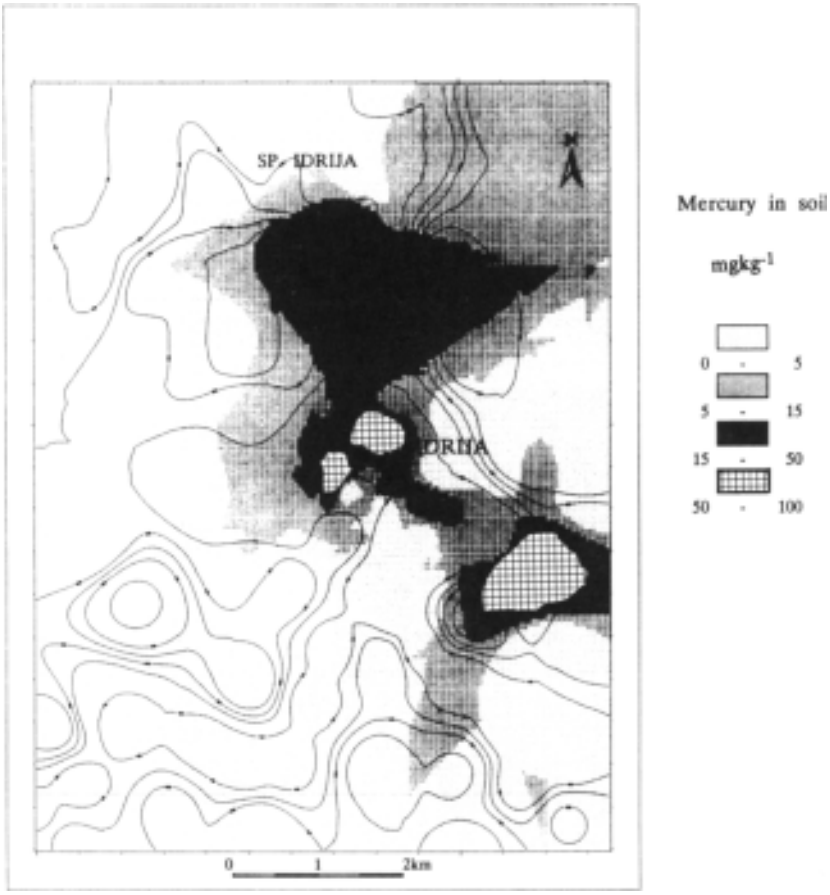


Figure 23.6 *Map of mercury in soil, mg kg^{-1} Hg*

Water analysis: Water samples were collected in one 1 glass bottles which were washed with nitric acid prior to sampling. After sampling, 1 ml of concentrated nitric acid was added to the samples. CVAAS was applied for mercury analysis using a Pye Unicam model SP9. The analytical work was performed 48 h after sampling. An aliquot of 400 ml of water sample was placed into the mercury reducing vessel containing 10% stannous chloride solution. The vessel was subsequently purged to release the mercury vapor to pass over a gold wire in a silica glass tube which acted as an amalgamation trap. The gold wire was electrothermally heated to ~800 °C and the deposited mercury was liberated and allowed to pass through a silica glass tube where the mercury content was measured. Calibration was performed with the addition of standard solutions and blanks of all reagents were determined and subtracted. The results are means of two measurements performed on each sample. Mean analytical sensitivity of 1 ng l^{-1} was achieved.

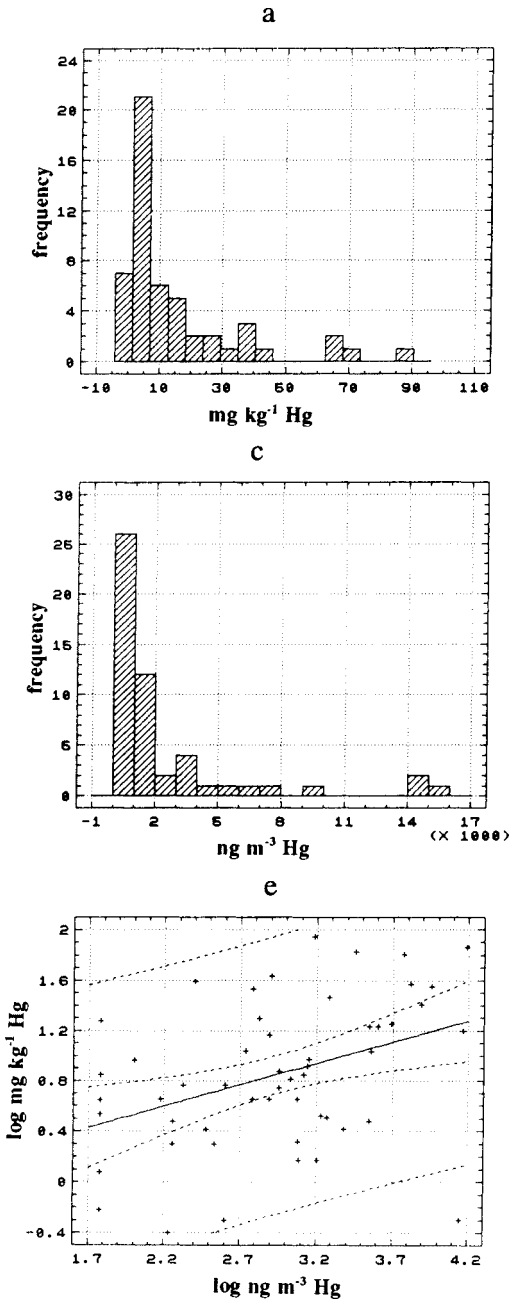
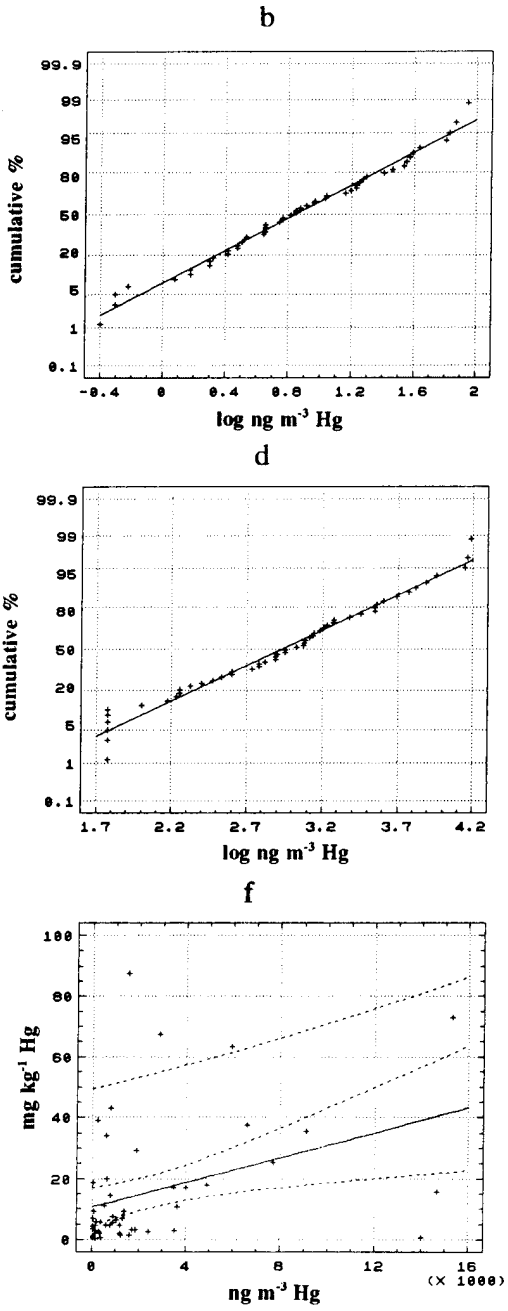


Figure 23.7 *Frequency distribution diagram and normal probability plot of mercury in soil (mg kg^{-1}) and mercury in soil air (ng m^{-3})*



Regression analysis of mercury in soil and mercury in soil air (correlation coefficient, r=0.39)

23.5.1.3 Air

Measurements of mercury levels in air were undertaken on 5 May 1990 between 11–15 h in the valley of the Idrijca river and its tributaries, the Nikova, Zala and Ljubovšca, through the center of the town and towards the village of Spodnja Idrija and Jazne (Figure 23.5). It was a quiet, sunny day with a northerly breeze. The survey was performed by a portable (car) mercury atomic absorber based on the Zeeman-background corrector, RGA-II (constructed by the Institute of the Earth Crust, Sankt Peterburg, Russia). Detection limit is 10 ng m^{-3} and precision ±30% in the range 10–100 ng m^{-3} and ±10% in the range 100–10,000 ng m^{-3} Hg.[38,39] The 72 km long monitoring route comprised 84 measuring points at an average distance of 1 and 0.5 km in the town of Idrija. The results were presented in the form of a schematic map (Figure 23.5).[40]

23.6 RESULTS AND DISCUSSION

The irregular, random, sampling grid is not based on the analysis of variance and does not assure a stabile geochemical map of the Idrija surroundings but gives a definitive extension and intensity of the mercury pollution and a rough pattern of the anthropogenic geochemical anomaly (Figures 23.3 and 23.6). Basic statistics gives some more information (Figure 23.7, Table 23.2). The frequency distribution diagram and the normal probability diagram suggest log-normal distribution of mercury in the soil. The maximum values (87.6 mg kg^{-1} Hg) were determined in the anthropogenic soils and regosols in the mining area and the vicinity of the smelter, In order to evaluate a natural state of the anthropogenic pollution, four vertical profiles, drilled in the soil over the ore-bearing and barren country rocks, were examined (sampling location 14, 15, 22, 36, Figure 23.3).

The distribution of mercury along the profiles (Figure 23.8) suggests a predominant influence of the fallout on the accumulation of the pollutant. Natural processes such as degassing of mineralization, diffusion of natural leachates, weathering products, percolation of ascending, capillary waters contribute little or nothing to the distribution along the profile. In the case of the ore-bearing bed rocks (the Carboniferous shales) mercury is augmented slightly at the contact regolith—parent bed rock, but the solum is strongly enriched by the descending, man-made pollutant (profiles at the locations 14, 15). At the location 36 (profile 36, Ljubevc) the bed rock—the Lower Triassic clastics, although underlain at some depth with mercury mineralization, contributes very little to the pollution in comparison to the mining and smelter. The origin of the pollution is explained convincingly at the location 22 (profile 22, Figure 23.8) where underlying ore-barren Cretaceous limestones and a modest distance from Idrija reduces pollution almost to the regional background.

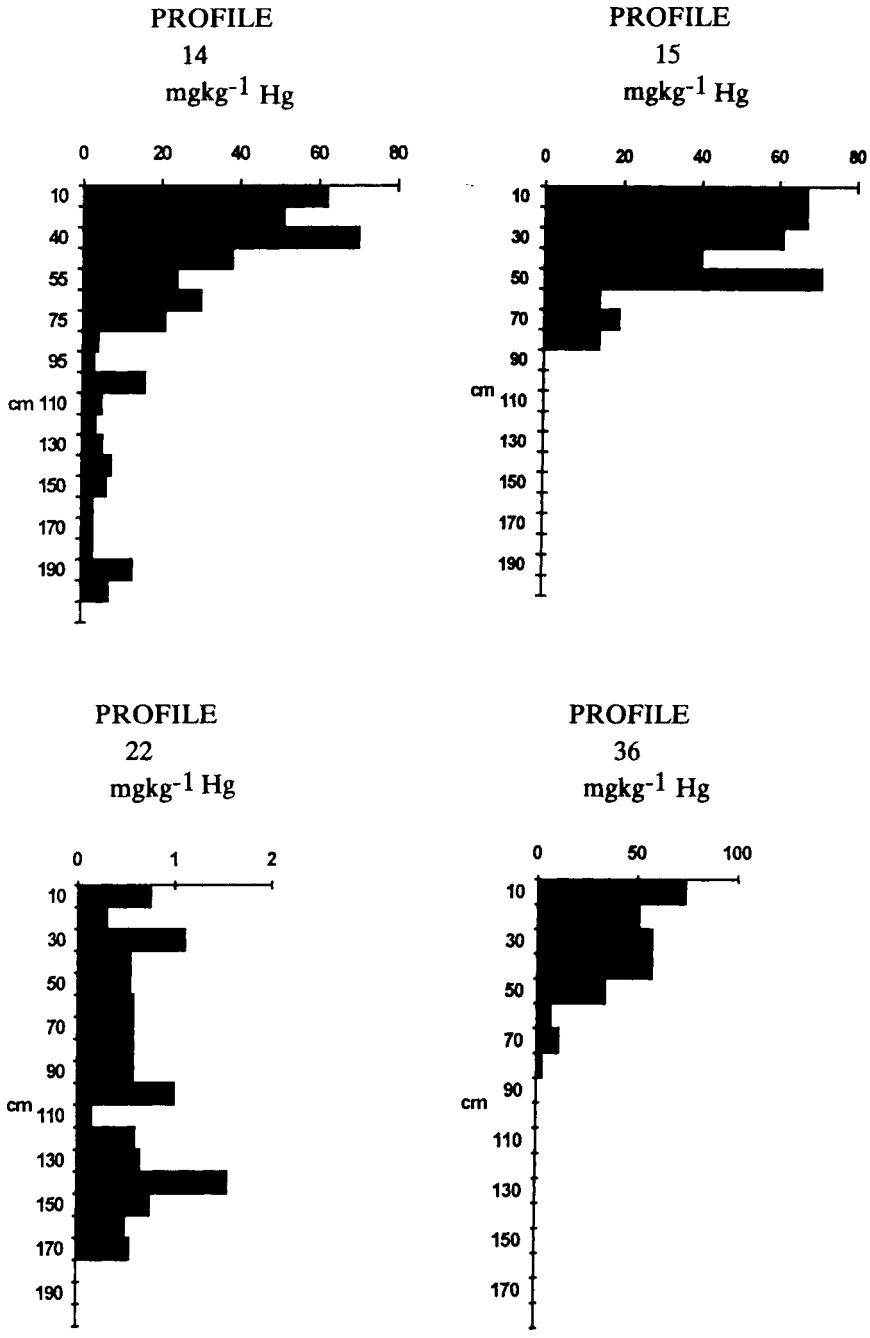


Figure 23.8 *Mercury content along the soil profiles at the locations 14, 15, 22 and 36, in mg kg^{-1} Hg*

Table 23.2 *Statistics, mercury in soil, mg kg^{-1}*

Original number of samples	52
Minimum sample value	0.4
Maximum value	87.6
Mean	15.4
Standard deviation	20.2
Standard error of mean	2.8
Median	44
Mode	4.5
Geometric mean	7.0
Geometric standard deviation	3.8
Skewness	1.95
Kurtosis	6.19

Table 23.3 *Statistics, mercury in soil air, ng m^{-3}*

Original number of samples	52
Minimum sample value	60
Maximum value	15,300
Mean	2403
Standard deviation	3673
Standard error of mean	509
Median	7680
Mode	60
Geometric mean	866
Geometric standard deviation	4.77
Skewness	2.32
Kurtosis	7.76

The pollution at some places may probably reach a much higher level (885 ppm[29]) but the number of 52 soil samples gives a confident average, which appears to be a good representative of the endangered area (15 ppm). It is sufficiently high above the global average in soil (50–100 μg kg^{-1},[41] and maximum allowable concentration (MAC) about 2 mg kg^{-1},[29,42] to alert public awareness.

The fate of the contamination is almost unpredictable. The area is covered by numerous soil types which differ in mineralogy, organic chemicals, pH, redox potential, groundwater regime, etc. and create variable mercury cycles. Degassing of the native mercury is one of the possible pathways and measurements of mercury levels in ambient air of the soil

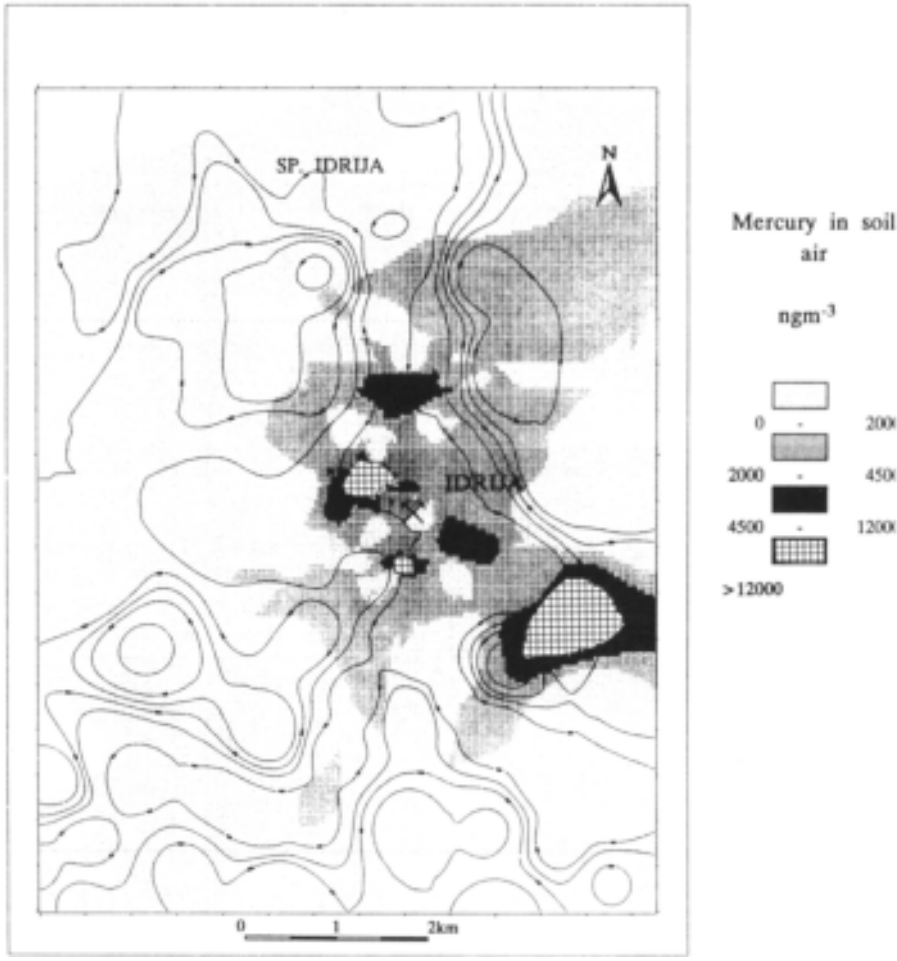


Figure 23.9 *Map of mercury in soil air, ng m^{-3} Hg*

pore space may be indicative. Mercury vapor in the soil air develops through reduction of different mercurials into native mercury.

Mercury is frequently used as a pathfinder element in the exploration of blind sulfide ore deposits,[43–45] it has been analyzed in all compartments including soil air.[43,46] Measurements in the Idrija region were undertaken at the same 52 sites as used for the soil sampling. The statistics are shown in Table 23.3. Normal probability plot and frequency diagram suggests log-normal distribution of the values (Figure 23.6b and 23.6d). There is a wide span of values from 60 to 15300 ng m^{-3}, with the mean 2403 ng m^{-3}. The highest value, obtained in regosol over an old dump in the Idrija downtown park enriched 110,000 ng m^{-3} and was not included in the statistics. Low background values in the unmineralized areas elsewhere (6–10 ng m^{-3})[46] have enabled recognition of the anomalous values above blind ore bodies seated deeply several 100 m distance

(10–17,200 ng m^{-3}).[46,47] The values in the Idrija region are in the same order of magnitude but it is impossible to discriminate anthropogenic from natural contributions. Geochemical map patterns of the mercury in soil and mercury in soil air coincide well (Figure 23.6 and Figure 23.9). Linear regression analysis (Figure 23.7) and corresponding correlation coefficient r=0.39 place constraint on some other variables which affect the mercury in soil air beside mercury accumulation in soil. The phenomenon, already observed within an area of the old mining sites in Croatia,[48] has ben referred to the inherent geochemical and physical soil conditions.

Stream sediments are the most contaminated media in the Idrija region. The highest value of 2857 mg kg^{-1} Hg, obtained 0.5 km downstream from the smelter is in contrast to 2 mg kg^{-1}, 7 km upstream, this reflects the semi-millennium uncontrolled accumulation of the pollutant (Table 23.4). The large increase in mercury levels in the active stream sediments within the Idrija town, and the steady decrease towards the Soca river[31] and the Adriatic[49] has the standard decay pattern of a geochemical anomaly in sediments, regardless of the anomaly that occurs as residual detrital grains or as precipitates of hydromorphic origin.[50] These results compares to the other contaminated rivers flowing through mining areas eg, Madeira, Brazil (157 mg kg^{-1}),[51] the stream close to Monte Amiata, Italy (288 mg kg^{-1}),[52] and the Laba river in the former East Germany (157 mg kg^{-1}.[53]

Sample No.	Locality	Hg mg kg^{-1}	Pb mg kg^{-1}	Comment
1	Idrijca river	2	70	7 km upstream
2	Belca river	2	92	tributary 7 km upstream
3	Zala river	3	135	tributary 1 km upstream
4	Idrijca river	5	111	1 km upstream
5	Idrijca river	34	113	upstream mining area
6	Ljubevšca river	445	192	tributary mining area
7	Idrijca river	97	157	mining area
8	Idrijca river	2857	242	downstream smelter
9	Idrijca river	1000	188	0.5 km

Extremely low concentrations of mercury in the Idrijca river water and its tributaries (3–20 ng l^{-1}) implies that cinnabar is the predominant chemical form in the sediments due to its chemical resistivity. Coarse-grained clastics in the river bed, with a low content of organic chemicals, paucity of viable biota, high energy, turbulent and

well-aerated water do not favor efficient methylation and concentration of alkylmercurials, at least not in the upper reaches of the Idrijca river. Low concentration in the waters may be also attributed to very low content of suspended particulates, and very high pH, characteristics of the karstic waters (pH 7.50–8.30, Table 23.1). It prevents the release of other mercurials adsorbed on the stream sediments and suspended matter. It should be noticed that in the area of acid mining effluent a slight decrease of pH may be observed (Table 23.1).

This should not be the case within the flood and stream sediments in quieter and flatter reaches of the Idrijca and the Soča rivers downstream. Erosion of the contaminated flood sediments, scouring of the bed load and direct contribution from runoffs, sheetwashes and soil creeps will form a long duration supply of the pollutant, even after the cessation of the mining activity. Already contaminated sediments of the rivers will continue to yield highly toxic methyl mercury into the water for many future years.

An extremely low content of mercury in the stream waters and springs in the Idrija surrounding (3–31 ng l^{-1}, Table 23.1) approximates to the waters in the unpolluted areas and differs greatly from other mining areas (1–1000 ng l^{-1}).[41] In the Nikitovka cinnabar mine, Ukraine, the ore bodies are situated in the host rocks with low buffer capacity (pH 2–6.5) and water acquires 1–10 μg l^{-1},[54] but quickly reduces to <1 μg l^{-1} in the neighboring main stream.

Due to an abundance of carbonate rocks in the surroundings of Idrija groundwaters there is a considerable degree dominated by karst hydrology. The spring catchment of Češnjice has a slight augmentation of mercury levels (Table 23.1) due to the proximity of the Idrija fault, along which tectonically crushed, mineralized Carboniferous shales occur. Mercury contamination might be expected also in the Černike catchment. In the Lačne vode catchment a small amount of native mercury has been detected periodically. According to geological interpretation, the Carnian sandstone is overlain by the Anisian dolomite, which is a part of the ore deposit structure. In the spring, Divji Potok and 'pri Kosmaču', resurgence of high mercury contamination is not possible, following the present state of understanding of the regional hydrogeology (J. Čar, 1994, personal communication).

Water quality in Idrija is protected naturally by specific conditions in the karst hydrology. High pH of well-buffered waters, controls low level of retrievable 'hydrogenous' fraction of ions by: promoting hydrolysis of metals with high ionic potential, preventing proton displacement from organic chemicals (humic and fulvic acids), clays and obstructing partial dissolution of sparingly soluble compounds, such as cinnabar.

The air pollution map does not illustrate an overall picture, and may differ significantly depending on weather conditions. However, it confirms the mining and smelter zone of Idrija to be a regional point-source with values exceeding 380 ng m^{-3} Hg, in the center of the town. Mercury levels decrease steadily toward acceptable 'background' values at a few km distance (<25 ng m^{-3}). The closure of the mine and the smelter will be a satisfactory measure to commence remediation.

23.7 CONCLUSIONS

A semi-millennium of mining and processing of the ore in Idrija induced severe contamination of all natural compartments: soils, soil air, air and stream sediments with the exception of spring- and stream-waters. Long-term human intervention into the huge natural accumulation of mercury, the ore deposits and its wide-spread dispersion into the environment prevents quantitative discrimination of the natural and man-made contribution. A study of the soil profiles over the barren and orebearing bed rocks suggests man's activity as the predominant source of the pollution.

Mercury levels in soils and stream sediments exceeds MAC values greatly and will be a matter of concern for many years. Whilst the air pollution will terminate with the closure of the operation and no remediation is required, the water media is not endangered due to the protective nature of well-buffered karstic waters which controls the release of mercury pollutants from the sediments by their high alkalinity and pH. The downstream reaches of the Idrijca and the Soca rivers, with finer-grained contaminated clastics will face a less optimistic future. Large sedimentary loads from Idrija will be a long duration supply of cinnabar detritus, a potential source of all inorganic and organic mercurials. A lack of extensive lowland marshy or agricultural area and generous contribution of unpolluted, high pH, karstic waters from the clean surroundings, which dilute and prevent the release of deleterious mercury species are the safeguard barrier to the development of 'Itai-itai' or 'Minamata' disastrous scenario.[55] Some local adverse effects to gardening and fruit planting are unavoidable and monitoring of mercury retention in humans and biota will be necessary. Planned and controlled closure of the mine by a governmental decision, primarily for environmental protection, is a result of the raised public awareness and guarantees a more satisfactory future.

These studies include the contribution of both ecotoxicology and occupational working activity to environmental toxicology.

23.8 ACKNOWLEDGEMENTS

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SECTION 6: RISK ASSESSMENT, SAFETY AND ECONOMICS

24 EC Environmental Risk Assessment of New and Existing Chemicals

John W.Handley and Derek J.Knight

24.1 INTRODUCTION

The intrinsic chemical, health and environmental hazards of chemical substances and preparations have to be communicated to the user, to enable the material to be used and stored safely. This is achieved by standardized classification and labeling of 'dangerous' chemicals, and by providing safety data sheets. This information is used to ensure chemical workers are operating under safe conditions, with appropriate engineering controls to minimize chemical release and using adequate protective equipment to minimize exposure to released chemicals, and that appropriate environmental protection measures are taken.

The information used to classify a chemical substance as 'dangerous', either to health or the environment, can be used for hazard assessment, which can be combined with chemical exposure data to produce a risk assessment. Further information on toxicity or exposure may be needed to refine the risk assessment, before any necessary management action is taken to ban or restrict the use of the chemical. Risk management measures for chemicals, which may be taken after a risk/benefit evaluation, can be in the form of recommendations for safe use, labeling or occupational exposure limits. Most developed countries also have legal provisions for banning chemicals, or restricting their use to safe conditions.

Chemical hazards can be categorized into physico-chemical, toxicological and environmental,

(i) Physico-chemical hazards, such as explosivity, oxidizing properties and flammability, are caused by the intrinsic physical or chemical properties of the substance;
(ii) Toxicological hazards arise from a chemical causing harmful effects to living organisms, which in practice normally means death, injury or adverse effects in man when ingested, inhaled or absorbed through the skin. Toxic effects may be acute or chronic, local or systemic and reversible or irreversible. They include corrosivity and irritancy to skin, eyes, and the respiratory tract. Specific toxic effects are skin and respiratory sensitization, carcinogenicity, mutagenicity, and reproduction effects; and
(iii) Environmental hazards relate to the potential of a chemical to damage one or more environmental compartments, ie, the air, soil or water, including groundwater.

Finally, there may be hazards to human health by exposure to the chemical via the environment.

Chemical control in the European Community (EC), commonly referred to as the European Union (EU), is based on a network of legislation for hazard communication and safety assessment. This EC legislation is brought into force in individual Member States by national laws, regulations, and administrative procedures and hence although chemical control is fundamentally harmonized, there can be minor differences between countries. The apparently complicated legal framework governing chemicals perhaps reflects how control measures have developed in the EC. Hence, in order to understand the comparatively recent development of form EC risk assessment of chemicals, it is necessary to understand the supporting control measures, which are summarized in section 24.2.

Austria, Finland, Iceland, Liechtenstein, Norway, and Sweden, but not Switzerland, are part of the agreement between the European Free Trade Association (EFTA) and the EC to form the European Economic Area (EEA). This agreement requires these countries to harmonize their environment legislation with that of the EC, by a scheduled deadline of 1 January 1995. Hence, the measures for EC chemical control will apply to a wider European market.

24.2 SUMMARY OF EC CHEMICAL CONTROL MEASURES

24.2.1 Hazard communication

All 'dangerous' substances have to be classified, packaged and labeled according to the requirements of Council Directive 67/548/EEC,[1] and it is the responsibility of suppliers to ensure that these requirements are met. Substances officially classified as dangerous are listed in Annex 1 of the Council Directive 67/548/EEC. The criteria to enable substances to be classified and labeled are given in Annex VI of Council Directive 67/548/EEC, as Commission Directive 93/21/EEC.[2] Substances are classified for labeling by evaluation of their physical, toxicological and ecotoxicological properties, and there are 15 'dangerous' classifications: explosive, oxidising, flammable, highly flammable, extremely flammable, harmful, toxic, very toxic, irritant, corrosive, sensitizing, carcinogenic, mutagenic, toxic for reproduction, and dangerous for the environment. Labeling consists of appropriate hazard symbols and information on risks in the form of standard Risk Phrases and safety advice as Safety Phrases.

The EC scheme for classification, packaging, and labeling of dangerous 'preparations' (ie, formulated products consisting of a mixture of 'substances') is specified in Council Directive 88/379/EEC.[3] The physico-chemical properties of the preparation are determined using the standard EC test methods. General health hazards of preparations can be assessed either by studies using EC methods or by evaluation from the dangerous components using the procedure given in Council Directive 88/379/EEC. The required concentration limits for individual dangerous substances already classified by the CEC are given in Annex 1 of Council Directive 67/548/EEC, and for others the general

limits for individual 'dangerous' properties from Annex 1 of Council Directive 88/379/EEC are used. Preparations containing substances which are carcinogenic, mutagenic or toxic for reproduction are classified only by the calculation method, and studies on the preparation are not appropriate. Classification and labeling as 'dangerous for the environment' does not yet apply to chemical preparations, although criteria are being developed as part of a First Amendment to Council Directive 88/379/EEC. Industrial users of dangerous chemical substances or preparations must be supplied with a free safety data sheet (SDS). The SDS scheme was implemented by Commission Directive 91/155/EEC[4] for preparations, which has been amended by Commission Directive 93/112/EEC[5] to implement the equivalent requirement for substances as from 1 January 1995. SDSs have to contain the specified headings, although the sequence in which the information is given is permitted to vary. A guide to what information to include under each heading is given in the Directive as the Annex.

24.2.2 Notification of new chemical substances

Council Directive 92/32/EEC[6] (which is the 'Seventh Amendment' of Council Directive 67/548/EEC) requires pre-marketing notification of new chemical substances, and classification, packaging and labeling according to the degree of hazard. Thus sufficient information on new substances is available to enable the hazard to be assessed and any necessary control measures to be taken. Chemicals controlled under separate EC legislation are exempt from notification, as are 'existing' chemical substances, which are defined as those listed in the European Inventory of Existing Commercial Chemical Substances (EINECS)[7] because they were reported as supplied within the EC from 1 January 1971 to 18 September 1981.

Full notification is required 60 days before a substance is to be supplied to the EC at an amount of 1 t yr^{-1} (or 5 t cumulative). The information required for the notification (the 'Base Set') is specified in Annex VIIA of the Directive. It consists of the identity of the substance, commercial information, recommendations for safe handling and use, physico-chemical properties, animal toxicology, mutagenicity studies, ecotoxicology, recommendations for disposal, the proposed classification and labeling, and a draft SDS for 'dangerous' substances. Reduced notification applies at supply below 1 t yr^{-1} (or 5 t cumulative), with Annex VIIB or Annex VIIC data for supply at above or below 100 kg yr^{-1} (or 500 kg cumulative) respectively.

Further information must be submitted when the amount of substance supplied to the EC reaches the Level 1 and Level 2 trigger points. The possible studies are given in Annex VIII of the Directive, although those required will depend on the particular substance. Level 1 testing may be required at 10 t yr^{-1} (or 50 t cumulative), but will definitely be required (if not already done) at 100 t yr^{-1} (or 500 t cumulative). Level 2 testing will be required at 1000 t yr^{-1} (or 5000 t cumulative).

The EC study methods are given in Annex V of the Directive. The Base Set tests are published as Commission Directive 92/69/EEC,[8] and some of the methods for Level 1 studies are contained in Commission Directive 87/302/EEC.[9] The EC methods are

derived from the OECD Guidelines.[10] The Annex V methods must be followed unless deviation is scientifically justified. Omission of a test for technical reasons must also be justified. Tests must be performed in compliance with GLP.

A Competent Authority will not accept a notification until all the required information has been provided and is to the necessary standard. Risk assessment has to be undertaken in accordance with the general principles of Commission Directive 93/67/EEC,[11] which involves hazard identification and dose (or concentration)/ response (effect) assessment and comparison with an exposure assessment to produce a risk characterization for both human health and environmental effects, and subsequent combination in an overall integration of conclusions. The Competent Authority will carry out the detailed assessment, but notifiers are encouraged to include a preliminary risk assessment with the notification. It may be necessary to refine the risk assessment by improving the exposure assessment and requesting additional studies to evaluate the hazard further. If necessary, risk management measures can be taken using existing EC provisions.

24.2.3 Evaluation of existing chemicals

The EC Council Regulation Number 793/93[12] on the evaluation and control of the risks of existing substances applies to all EC manufacturers or importers of existing EINECS-listed chemical substances. They must report to the Commission of the European Community (CEC) Joint Research Centre (JRC) the available Annex III data by 4 June 1994 for substances listed in Annex 1 which they supply at >1000 t yr^{-1}. Data are to be reported on non-Annex 1 substances supplied at >1000 t yr-1 by 4 June 1995, and the more limited data specified in Annex IV on all substances supplied at 10 to 1000 t yr^{-1} between 4 June 1996 and 4 June 1998. However, there is an Annex II which lists high-volume substances which are obviously non-hazardous to health or to the environment and which are hence not reportable unless separately requested by the CEC. Data must be reported in summarized format using Harmonized Electronic Data Set (HEDSET) computer diskettes. The JRC will collate all the HEDSET data into an EC database called EUCLID.

In practice out of the many reported chemicals, only a limited number can be subjected to full risk assessment each year. Therefore, reported chemicals are to be selected for full assessment in a priority setting scheme using criteria which are currently being finalized. Lists of priority chemicals will be published annually in the *Official Journal of the European Community (OJ)*. The first list of 42 chemicals selected on an *ad hoc* basis from Member States individual nominations was published in the *OJ* as Commission Regulation No. 1179/94.[13] The principles for risk assessment are set out in Commission Regulation No. 1488/94,[14] and the CEC will publish detailed guidelines on how to conduct the assessment, which can be updated and revised as necessary. This risk assessment scheme will correspond closely with that for notified new chemicals required under Council Directive 92/32/EEC.

A rapporteur Competent Authority will be selected to conduct the risk assessment on each priority chemical. The HEDSET summary data for the selected priority chemicals must be supplemented with full reports and other existing data within 6 months of listing. A full EC 'Seventh Amendment' Base Set must be made available for risk assessment, and any necessary new studies to fill 'data gaps' have to be provided within 12 months of listing, unless the notifier applies for an extension of the time limits or derogations for data requirements on the grounds that the test is technically impossible or it is unnecessary for risk assessment. The rapporteur then evaluates this data package, and decides on whether any non-GLP or non-standard studies are adequate. The rapporteur may request repeat testing or additional GLP-compliant studies if these are necessary for risk assessment, but this has to be approved at EC level first. The manufacturers and importers who originally reported the substance are jointly responsible for providing these new studies, but data sharing by consortia formation is encouraged to prevent repeat animal testing. The rapporteur then produces a draft risk assessment regarding human health and the environment, and proposes any necessary risk limitation strategies. Such measures are for adoption at EC level using existing provisions, and can be undertaken only after a risk/benefit evaluation on the chemical.

24.3 FRAMEWORK FOR EC RISK ASSESSMENT OF NEW AND EXISTING CHEMICALS

The general principles for risk assessment of notified substances of Commission Directive 93/67/EEC[11] do not include extensive technical details for conducting hazard identification, exposure assessment and risk characterization in relation to human health and the environment. Hence, the 'Risk Assessment of Notified New Substances Technical Guidance Document',[15] which advises Competent Authorities and notifiers on risk assessment and additional testing strategies, has been developed by the CEC, with assistance from the Competent Authorities, to provide supplementary technical detail. The guidance is not legally binding, and other methods or approaches may be used in the risk assessment process if they are considered more appropriate, and can be justified, providing they are compatible with the general principles of Commission Directive 93/67/EEC. The technical procedures relevant to the different aspects of risk assessment in this CEC technical guidance are subject to continuous refinement and development, and hence the manual will be updated as necessary. Corresponding technical guidance for risk assessment of existing EINECS-listed chemical substances will be developed by the CEC, and in due course the two manuals are to be combined into a single document. The intention is to harmonize as far as possible how risk characterization is conducted by the different assessor authorities.

Risk assessment of both new and existing substances comprises the following steps for human health (toxicology and physico-chemical properties) and the environment:

(i) Assessment of effects, comprising:

- Hazard identification (identification of the intrinsic hazardous properties of the substance)
- Elucidation of the dose (concentration)—response (effects) characteristics, when appropriate;

(ii) Exposure assessment for the human populations (ie, workers, consumers and man exposed indirectly via the environment), and for the different environment compartments (water, soil, air) likely to be exposed to the substance; and

(iii) Comparison of information on hazardous properties and effective dose levels/concentrations with exposure levels in order to characterize the degree of risk posed by the substance to human health or to the environment.

In conducting an exposure assessment, the assessor takes into account those human populations or environmental spheres for which exposure to the substance is known or reasonably foreseeable in the light of available information on the substance, with particular regard to manufacture, transport, storage, formulation into a preparation or other processing, use and disposal or recovery. Also, for certain particular effects such as ozone-depleting potential for which the above steps are impracticable, risk assessment is conducted on a case-by-case basis. Finally, if an existing substance appears on a priority list again after already being assessed, the first risk assessment is taken into account.

Assessors will reach a conclusion following risk characterization of each of the hazardous properties identified and/or those for which there are other reasonable grounds for concern for each appropriate human population and/or environmental compartment. For notified new substances, the administrative risk assessment conclusions are set out in paragraph 4 of Article 3 of Commission Directive 93/67/EEC as follows:

(i) The substance is of no immediate concern and need not be considered again until further information is made available in accordance with Article 7(2), 8 (3), 8 (4), or 14 (1) of Council Directive 92/32/EEC;

(ii) The substance is of concern and the Competent Authority shall decide what further information is required for revision of the assessment, but shall defer a request for that information until the quantity placed on the market reaches the next tonnage threshold as indicated in Article 7 (2), 8 (3), or 8 (4) of Council Directive 92/32/EEC;

(iii) The substance is of concern and further information should be requested immediately; or

(iv) The substance is of concern and the Competent Authority should immediately make recommendations for risk reduction.

Since a risk characterization may have been carried out for more than one potential adverse human health effect or human population, regarding toxicity and/or physico-chemical properties, or in different environmental compartments, it is necessary for the assessor to produce integrated conclusions for each category. Finally,

an overall integration of conclusions in relation to the totality of the risks identified in the risk assessment is made, with any proposals for additional testing or risk reduction measures, in the risk assessment report by the assessor for the CEC (in the format specified in Annex V of both Commission Directive 93/67/EEC for new substances and Commission Regulation No. 1488/94[14] for existing substances).

For new substances, notifiers are encouraged to submit a preliminary risk assessment with the notification, both to save Competent Authority resources and because the notifier may be able to produce a more realistic exposure assessment, and hence the 'default' values for parameters in the various models need not be used. The assessor Competent Authority will permit comments from the notifier before the integrated risk characterization summary is sent to the CEC for circulation to the other Competent Authorities. This feedback is important to ensure that a 'realistic worst case' assessment has been made for each stage of the lifecycle of the substance in the EC, especially if the assessor had to prepare the risk assessment from scratch without initial input from the notifier. Hence it is essential to include detailed use data in the notification summary, to enable human and environmental exposure to be estimated. A standard format for risk assessments is being developed at EC level.

Any risk reduction recommendations resulting from risk assessment of existing and notified new chemical substances will be measures for adoption at EC level using existing provisions, and for existing chemicals those risk reduction measures already applied are taken into account. Recommendations may be modifications to classification, packaging or labeling, the SDS or the recommended precautions or emergency measures. Alternatively, the relevant control authorities may be advised to consider appropriate measures for the protection of man and/or the environment from the risks identified, by means, for example, of occupational exposure or environmental discharge limits, or, very occasionally, by restrictions on marketing and use under the provisions of Council Directive 76/769/EEC (as amended).[16]

24.4 ENVIRONMENTAL RISK ASSESSMENT

24.4.1 Principles

Existing chemicals appearing on the priority lists are subject to full risk characterization, and at the first stage of hazard identification the effects and/or properties of concern are identified and the environmental classification is reviewed in terms of all the available data. Full risk characterization is required for notified new substances which are provisionally classified as 'dangerous for the environment', or if there are the following other reasonable grounds for concern in relation to environmental effects for substances not so classified on the basis of full notification data or if there are insufficient data for definitive classification following reduced notification:

(i) Indications of bioaccumulation potential;
(ii) The shape of the toxicity/time curve in ecotoxicity testing;

(iii) Indications of other adverse effects on the basis of toxicity studies, eg, classification as a mutagen, toxic or very toxic or harmful with the risk phrase R 40 ('Possible risk of irreversible effects') or R 48 ('Danger of serious damage to health by prolonged exposure'); or
(iv) Data on structurally analogous substances.

The objective is to predict the concentration of the substance below which adverse effects in the environmental compartment of concern are not expected to occur, ie, the predicted no effect concentration (PNEC). However, in some cases, it may not be possible to establish a PNEC, and a qualitative estimation of the dose (concentration)—response (effect) relation would have to be made instead. The PNEC may be calculated by applying an assessment factor to the values resulting from acute and/or long-term studies on aquatic organisms (ie, EC_{50} etc. and NOEC etc., respectively). The assessment factor is an expression of the degree of uncertainty in extrapolation from test data on a limited number of species to the real environment, and thus in general, the more extensive the data and the longer the duration of the tests, the smaller is the degree of uncertainty, and the size of the assessment factor.

The objective of the exposure assessment is to predict the concentration of the substance which is likely to be found in the environment, ie, the predicted environmental concentration (PEC). However, in some cases, it may not be possible to establish a PEC and a qualitative estimation of exposure would have to be made. A PEC or qualitative estimation of exposure is only determined for the environmental compartments to which emissions, discharges, disposal or distributions are known, or are reasonably foreseeable. It takes account of the following factors:

(i) Adequately measured exposure data;
(ii) The quantity of the substance;
(iii) The form in which the substance is produced, imported and used (eg, substance itself or as component of a preparation);
(iv) Use pattern and degree of containment;
(v) Process data;
(vi) Physico-chemical properties of the substance, in particular melting point, boiling point, vapour pressure, surface tension, water solubility, and P_{ow};
(vii) Breakdown products and/or transformation products (for existing chemicals);
(viii) Likely pathways to environmental compartments and potential for absorption/desorption, and degradation; and
(ix) Frequency and duration of exposure.

For new substances to be placed on the market in quantities at or below 10 t yr^{-1} (or 50 t cumulative), the PEC or qualitative estimation of exposure is usually determined for the generic local environment in which release of the substance may occur. Also, for existing substances with adequately-measured, representative exposure data, special consideration is given to these when conducting the exposure assessment. Where calculation methods are used for the estimation of exposure concentrations, adequate models have to be applied, and if appropriate, on a case-by-case basis, relevant monitoring data from substances with analogous use and exposure patterns or analogous properties may also be considered.

For any given environmental compartment, the risk characterization as far as possible entails comparison of the PEC with the PNEC, in the PEC/PNEC ratio. For notified new substances, if this ratio is ≤1, the conclusion at Article 3 (4) (i) of Commission Directive 93/67/EEC applies, and the substance is of no immediate concern. If the ratio is >1, the assessor decides on the basis of its value and the factors giving reasonable grounds for concern (numbered as (i) to (iv) above), which other administrative conclusions applies. Similarly, for existing chemicals, if PEC/PNEC ≤1, no further information and/or testing and no new risk reduction measures are required, whereas if the ratio is >1, the assessor may recommend such measures as appropriate. If it has not been possible to derive a PEC/PNEC ratio, the risk characterization consists of a qualitative evaluation of the likelihood that an effect is occurring under the current conditions of exposure or will occur under the expected conditions of exposure.

The Base Set testing package for the notification of a new chemical substance consists of the following data relevant to the aquatic environment:

- Biodegradation (biotic and abiotic)
- Acute toxicity to fish, *Daphnia* and algae.

Little data of relevant to the atmospheric and terrestrial compartments is generated at this stage or for that matter at the Level 1 and 2 stages of notification. Where consideration of these compartments is relevant to the environmental risk assessment then further testing and revision of the PEC/PNEC ratio should be carried out on a case-by-case basis.

The decision to proceed with further testing of new substances should be made on a case-by-case basis with the objective to revise either the PEC and/or the PNEC. Resources should be geared to which of the PEC or PNEC would be more sensitive to revision from the result of additional testing as well as the need to reduce the amount of testing with vertebrate animals.

As a notified new substance reaches each tonnage trigger point or, after any revision of the PEC/PNEC ratio care should be taken as to the need for further testing. At higher tonnage levels the PEC may be revised on the basis of local or regional models dependant on the nature of the substance and its use pattern.

A complete discussion on the method of risk assessment would, inevitably take some time and is outside the scope of this chapter, and hence, consideration will be given to the testing methods used for hazard identification for notified new substances.

24.4.2 Environmental exposure assessment

24.4.2.1 *General considerations*

Environmental exposure assessment involves an estimation of emission rates and subsequent spreading from each identified emission source of the substance in the various stages of its life-cycle to the different environmental compartments. For a first

assessment, the initial PEC for each compartment of concern is estimated, using reasonable worst-case assumptions, from the key parameter of discharge rate, after taking account of elimination by treatment on- or off-site and/or dilution in the receiving compartment. Subsequent revisions also consider the longer-term situation likely some time after discharge, by estimation of the local PEC, which takes into account further environmental fate processes such as degradation, volatilization and adsorption, and/or the regional PEC, which takes into account the further distribution and fate of the released chemical. Exposure from accidental release and improper use are not considered. A PEC can normally only be estimated from point-source emissions, and exposure assessment for diffuse emission sources simply consists of identifying the environmental compartments likely to be polluted, so if both types of emission occur, normally only that from point-sources is considered.

Ten emission scenario documents are currently available in the Technical Guide[15] for the main use categories which account for ~85% of notified new substances. These enable a reasonable worst-case initial PEC to be calculated for common processes and production techniques for the aquatic environment, by using substance-specific data and/or 'default' values from the exposure models. For other use categories, the generic emission scenario equation is used. Finally, in certain cases it can be justified to calculate a site-specific initial PEC, instead of a reasonable worst-case scenario intended to be applicable throughout the EC.

24.4.2.2 *Aquatic environmental compartment exposure assessment*

The present risk assessment models in use assume that all chemical substances entering the aquatic compartment firstly pass through a wastewater treatment plant prior to discharge to the environment. This is considered to be a reasonable assumption on the basis of the current levels of progress within the Member States of the EC. However, for some substances it may be necessary to determine a PEC based on the assumption that no form of treatment process occurs before discharge.

The degree of removal of a substance in wastewater treatment plants is determined by a number of factors:

- Volatility
- Adsorption onto sludge
- Sedimentation of insoluble materials
- Biodegradation.

The degree of removal may then be estimated on the basis of Henry's constant, log P_{ow} and biodegradation (ready) test performed at the Base Set.

A substance's biodegradability may be defined at two different levels:

(i) Primary biodegradation: the loss of a specific function, eg, loss of surface activity or loss of color; or

(ii) Ultimate biodegradation: the complete mineralization of a substance to carbon dioxide and water.

Primary biodegradation may involve the modification of a chemical group only, and hence the level of degradation achieved may be in excess of that determined in an ultimate biodegradation test. In practical terms it is likely that the only results available will be those of an ultimate biodegradation test performed at the Base Set.

The ready biodegradation tests employed at the Base Set are described in OECD Guidelines 301 A-F[10] and are as follows:

301A DOC Die-Away	301B CO_2 Evolution (Sturm test)
301C MITI (I) test	301D Closed Bottle test
301E Modified OECD Screening	301F Manometric Respirometry

All these tests are seen as harsh, stringent tests and do not favour biodegradation as the ratio of test substance to inoculum is high and the amount and diversity of the inoculum may not be sufficient for complete mineralization of the substance.

In order to select the most appropriate methods information on the chemicals water solubility, vapour pressure and adsorption characteristics is useful. The chemical formula or structure, together with information on the purity and impurities, are required in order to interpret the results obtained.

Degradation may be followed by determining one or more of the following parameters:

(i) Loss of dissolved organic carbon	:	DOC Die Away OECD Screening test;
(ii) Oxygen consumption	:	Closed Bottle test MITI test ManometricRespirometer;or
(iii) Carbon dioxide evolution	:	CO_2 Evolution test

Additionally, compound specific analysis for the parent substance and its metabolites may be incorporated, eg, MITI test.

The pass levels for ready biodegradability tests are defined as 70% for removal of DOC and 60% if the consumption of oxygen or evolution of carbon dioxide is studied. These pass levels have to be in a 10 d 'window' within the 28-d study period. The concept of the 10 d 'window' does not apply in the MITI test. Those substances attaining the pass level after 28-d but failing to comply with the 10 d 'window' are determined not to be ready biodegradable.

For substances found not to be ready biodegradable, it is necessary to determine any inhibitory effects on the inoculum at the concentration employed in the ready biodegradation test. This is done by either conducting an Activated Sludge Respiration Inhibition test (OECD Guideline No. 209), or by incorporation of a toxicity control in the test. If inhibition of microbial activity is observed the test should, if possible, be repeated at a lower non-inhibitory concentration.

The results of a hydrolysis study (EC Test Method C.7)[8] may be used in the estimation of the PEC. The results of the ready biodegradation test will show whether the hydrolysis product(s) is biodegradable or not. For substances with a half life of less than 12 h any environmental effects are likely to be the result of the hydrolysis product(s) rather than the parent substance.

Information on volatility (Henry's constant) and from any adsorption/desorption studies (not currently a Base Set test) can be used in wastewater treatment models to determine elimination of the substance.

Inherent biodegradability data may be used to revise a PEC. The data may be obtained from further testing, or if a chemical has achieved the pass levels for biodegradability but has not met the 10 day window validation criteria, then it may be considered to be inherently biodegradable. Also substances which have shown a significant amount of degradation in a 'ready' test but have failed to achieve any of the preset pass levels may also be regarded as inherently biodegradable after review of the data and degradation curves.

The OECD Guidelines for Testing of Chemicals currently lists three inherent biodegradation test methods:

302A SCAS test (Semi-Continuous Activated Sludge);
302B Zahn Wellens/EMPA test; and
302C MITI (II) test.

The test methods are designed to be less stringent than the 'ready' tests and have a lower ratio of test substance to inoculum. The duration of the studies is defined as 28-d, but they may be continued for longer periods and there is no 10 day window validation criteria.

Degradation may be followed by oxygen consumption (MITI test) or by reduction in DOC, or loss of parent material (SCAS and Zahn Wellens test). For poorly soluble substances the choice of test is limited, in practical terms, to the MITI (II) test.

Those substances showing results of $\leq$20% degradation after 28-d can be considered to be 'partially degraded', while a result of between 20 and 70% suggests that stable breakdown products may be formed, which it may be necessary to identify for a full risk characterization. Degradation values in excess of 70% indicates inherent biodegradability.

If, at further tonnage trigger points additional biodegradation data are required, then simulation tests may be performed. This involves a laboratory system designed to simulate either environmental compartments, eg, river or sediment, or more commonly, the activated sludge treatment stage of a wastewater treatment plant.

The only current OECD Guidelines for testing are those for the wastewater treatment plant, eg. Coupled Units and Porous Pot tests. Degradation is followed by the decrease in DOC or COD.

24.4.2.3 *Soil environmental compartment exposure assessment*

It is assumed that at Base Set tonnages any soil exposure will be by landfill and/or by application of contaminated sewage sludge from wastewater treatment plants to farmland. Other diffuse exposure sources and redistribution to the soil compartment can be considered at the 10 t yr^{-1} (or 50 t cumulative) supply level. The concentration of the chemical in sewage sludge can be calculated, and country-specific typical application rate data can be used to calculate the initial PEC in soil for arable and/or grass land.

This PEC can be refined by taking account of the reduced concentration of the substances in digested sludge from anaerobic biodegradation in certain wastewater treatment plants, degradation in soil and leaching from soil.

24.4.2.4 *Air exposure assessment*

It is assumed that at Base Set tonnages, concentrations in air are low because of dilution effects, but a first PEC calculation using an appropriate model may be necessary at 10 t yr^{-1} (or 50 t total), especially regarding potential degradation of air quality. Data on the long-term fate in the atmosphere may be needed to revise the PEC.

24.4.3 Environmental effects assessment

24.4.3.1 *Aquatic environmental effects assessment*

The ecotoxicity tests at the Base Set level are designed to identify short-term acute effects. The following tests are conducted:

(i) Acute toxicity to fish (OECD Guideline No. 203);
(ii) Acute toxicity to *Daphnia magna* (OECD Guideline No. 202 Part I); and
(iii) Algal growth inhibition (OECD Guideline No. 201).

Typically, rainbow trout is the species most commonly tested for fish toxicity, although any of the recognized species in the OECD/EC test guidelines may be used. Similarly, for the algal growth inhibition test, two species are listed with *Scenedesmus subspicatus* being the next most commonly used. The test methods allow limit tests to be performed at a concentration of 100 mg l^{-1} to demonstrate that the $L(E)C_{50}$ is greater than this concentration.

The testing of pure, stable water soluble substances is comparatively straightforward. However, many substances have physical or chemical properties which make testing difficult.

Provision is given for low solubility substances such that limit tests are performed at a concentration equal to the water solubility of the substance, or the maximum concentration forming a stable homogenous dispersion. For highly colored substances, where it can be demonstrated that the reduction in light intensity is responsible for the toxicity observed in an algal growth test, by a regrowth experiment, then this data may be omitted from the classification.

For 'difficult substances', it is not always possible to meet the validation criteria for maintaining exposure concentrations at $\geq$80% of the initial concentration. Here, advice is available from the Competent Authorities, eg, the United Kingdom Department of the Environment Advisory Document on Dealing with 'Difficult' Substances.[17]

For the purposes of the risk assessment, a PNEC is calculated by extrapolation from laboratory test data (acute or chronic) using an assessment factor. Assessment factors vary according to the level of confidence with which the PNEC is calculated. Lower

factors are, therefore, applied to No Effect Concentrations (NOEC) derived from a long-term study than to a short-term $L(E)C_{50}$.

When calculating PNEC, data from the most sensitive species tested is used as it is generally accepted that ecosystem function is protected by protecting the most sensitive species. Thus, when additional tests are conducted to revise the effect's assessment, species sensitivity should be taken into account, ie, the species with the lowest $L(E)C_{50}$ value.

If additional testing is required this should be directed to revising the PEC or PNEC and thus lead to a recalculation of the PEC/PNEC ratio. Long-term testing will normally result in the use of a lower assessment factor and hence lead to a revision of the PNEC.

In some circumstances, additional testing may not be justified, for example, if the PEC is higher than the acute toxicity values, the substance will already be a candidate for risk reduction and further testing would not change this decision. If the results of a long-term study on one species indicate the need for risk reduction, it is doubtful whether a study on a second species would modify this.

Although, in general, the most sensitive species from the acute toxicity tests should be used for long-term studies, the potential of the test substance to bioaccumulate must be considered. Substances which bioaccumulate may lead to a long-term or delayed effect and this should be considered for long-term fish and *Daphnia* studies as development in the early stages of the life cycles may be affected by an accumulation of the test substance.

Further testing is not required when no short-term toxicity ($L(E)C_{50}$ > limit value) has been demonstrated for a species or when the PEC/PNEC<1 when the PNEC has been calculated based on the toxicity data of that species. This does not apply to low water soluble (<1 mg l^{-1}) substances where no acute toxicity has been demonstrated as steady state between the water and test organism was not achieved.

The following tests are available for the investigation of the long-term effects of a substance:

(i) Full Early-Life Stage (FELS) test (OECD Guideline No. 210);
(ii) Egg and Sac-Fry Stage test;
(iii) 28-d Juvenile Fish Growth; and
(iv) *Daphnia* Reproduction test (OECD Guideline No. 202 Part II).

Assessment factors of 50 or 10 may be applied to the NOEC values from the studies. The Prolonged Fish Toxicity test (OECD Guideline No. 204) is not recommended as this is merely a 'prolonged' acute toxicity test. The algal growth study performed at the Base Set stage can also provide a NOEC to which factors of 50 or 10 may be applied as it is a multigeneration study.

Mesocosm and field studies, may be considered, however, it is unlikely that this option would be considered for low-tonnage chemicals.

The Competent Authorities have proposed that the following assessment factors are used:

(i) A factor of 1000 applied to the lowest $L(E)C_{50}$ from the Base Set toxicity data. This factor is designed to be a conservative and protective measure to ensure that all substances with the potential to cause adverse environment effects are identified. It assumes that the following uncertainties arising from the

extrapolation of single-species laboratory data to multi-species ecosystems are taken into account:
- Inter-species variations
- Extrapolation of acute to chronic toxicity and then laboratory to field situations;

(ii) A factor of 50 applied to the lowest NOEC value from long-term toxicity data on two species from different taxonomic groups;
(iii) A factor of 10 applied to the lowest NOEC from long-term toxicity data from fish, *Daphnia* and algae, ie, three species from three different taxonomic groups. It may be possible to show that the most sensitive species has been tested by demonstrating that the NOEC from a second species in a different taxonomic group is higher than the data available, and so apply the factor of 10 when only two species have been tested; or
(iv) Assessment factors for mesocosm and/or field data would be reviewed on a case-by-case basis.

24.4.3.2 *Terrestrial environmental effects assessment*

No data on toxicity to soil organisms is required as part of the Base Set. However, it can be possible to obtain an indication of potential adverse effects on the terrestrial environment by comparing the concentration of the substance in soil pore water, calculated from the initial PEC for soil, with the PNEC for aquatic organisms. Relatively short-duration toxicity tests in plants and earthworms are required at Level 1, and pertinent supplementary data on accumulation, degradation and mobility are to be provided at Level 2.

24.4.3.3 *Preliminary evaluation of atmospheric effects*

In the absence of a PNEC for air exposure to environmental species, risk characterization by estimation of a PEC/PNEC ratio is not applicable, and the alternative approach is followed of identifying chemicals which have a potential to harm the atmosphere, according to defined selection criteria, in any of the following ways:

(i) By degrading air quality (eg, visibility, effects on human health, etc.) (DAQ);
(ii) By tropospheric ozone building (TOB);
(iii) By acidification;
(iv) By contributing to eutrophication processes, ie, the progressive enrichment of an 'environment' by certain chemical elements or compounds to the ultimate detriment of that 'environment';
(v) By ozone layer depletion (ODP); or

(vi) By warming the atmosphere, the greenhouse warming potential (GWP).

24.4.3.4 *Evaluation of bioaccumulation and secondary poisoning*

Chemicals can be accumulated in organisms either by direct uptake or through the food chain. In the aquatic environment, bioaccumulation (or bioconcentration) is related to the uptake of a substance from water via the gills or skin or by ingestion. The potential of a substance to bioaccumulate cannot be related directly to its toxicity, but this potential does give cause for concern, especially if the substance does not biodegrade.

One way to assess the potential for bioaccumulation in aquatic species is to measure the Bioconcentration Factor (BCF). The static bioconcentration factor is the ratio between the concentration in the organism and the concentration in water in a steady state (ie, equilibrium) situation. When uptake and depuration kinetics are measured, the dynamic bioconcentration factor can be calculated from the quotient of the uptake and depuration velocity constants. A comprehensive assessment of the potentials for bioaccumulation and biomagnification cannot be based on BCFs alone because information is required on the kinetics of uptake and depuration, metabolism of the chemical, and the occurrence of bound residues.

Biomagnification is the uptake of a substance via the food chain involving different pathways and different trophic levels. A specific effect of biomagnification can be secondary poisoning, which is concerned with toxic effects in the higher members of a food chain, either living in the aquatic or terrestrial environment, resulting from ingestion of organisms at the different trophic levels that contain accumulated substances.

At the Base Set level, no specific information on bioaccumulation potential is available, although an assessment can be made on whether or not there are indications for a potential for bioaccumulation. This estimation is used as a first step in the testing strategy for bioaccumulation and secondary poisoning, and is also relevant for deciding if there are other reasonable grounds for concern in risk characterizations (see section 24.4.1), and to select the assessment factor and longterm toxicity testing for aquatic organisms (see section 24.4.3.1). The criteria for deciding if a substance has indications of bioaccumulation potential are from the P_{ow} (ie, if log $P_{ow} \geq 3$, but not if log $P_{ow} > 6$, the molecular weight is >700, or if the substance ionizes in water or is surface-active), adsorption (ie, log $K_p > 3$ is indicative of bioaccumulation potential), surface tension (ie, ≤ 50 mN m^{-1} at an aqueous concentration of ≤ 1 g l^{-1}, even if log $P_{ow} < 3$), structure activity relationships and structural functions, unless there are mitigating properties (ie, hydrolysis with $t½ < 12$ h, or ready biodegradation).

A strategy for the assessment of the potential for secondary poisoning has been developed to support the decision when to request a bioaccumulation test. This strategy takes account of the PEC in water, the resulting concentration in food of higher organisms and the mammalian toxicity of the chemical (as an indication of possible effects on birds and mammals in the environment). Assuming indirect exposure is possible, there are indications of bioaccumulation potential as discussed above, and the substance is classified as 'very toxic', 'toxic', or 'harmful' with R 40 ('Possible risk of irreversible effects'), or R 48 ('Danger of serious damage to health by prolonged exposure'), a

provisional assessment of secondary poisoning is performed. At this stage a simple estimation is made of whether the PEC in water can lead to concentrations in fish that may lead to deleterious effects in higher organisms that eat fish by using a BCF estimated from the known P_{ow}. The value of the oral PEC/PNEC ratio determines if a bioaccumulation test is required, and at what stage, in order to refine the risk characterization by using a measured BCF in estimating an improved oral PNEC. If this refined oral PEC/PNEC is still >1, there is potential for secondary poisoning of fish eaters and more-specific tests may thus be required. The results of the bioaccumulation test can be used to decide if the substance is of concern in relation to biomagnification potential.

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25 Private-Public Sector Cooperation to Improve Pesticide Safety Standards in Developing Countries

William Wyn Ellis

25.1 INTRODUCTION

In 1990, GIFAP (the International Group of National Associations of Agrochemical Manufacturers) highlighted industry's role in ensuring good stewardship of pesticides. In a major industry initiative, the major international agchem companies invested over US$ 4 million to start the pilot Safe Use Projects (SUPs) in Kenya, Guatemala and Thailand. In setting up these projects, industry accepted a major challenge addressing a complex matrix of issues, many of them socio-economic rather than purely technical. These required different approaches and a much wider and more open industry perspective than had prevailed previously.

This chapter draws on the author's experiences of the three-year pilot phase of the SUP in Thailand. It exemplifies how, by liaising with a variety of organizations, such an initiative can generate a sustainable momentum that can lead to significant policy changes. In the longer-term farmers and consumers benefit through focusing government resources on safety education, training and enforcing relevant legislation. The ecotoxicological burden is minimized through more efficient application practices and by using integrated pest management to reduce the overall need for agrochemicals. The project's progress is evaluated and the need for collaboration between all relevant agencies is highlighted.

25.2 SAFE USE PROJECT: THAILAND—BACKGROUND

Prior to the start of the SUP in Thailand, some excellent safety-related efforts were already under way, earned out by individual companies, government, NGOs, the Thai Pesticide Association (TPA), and by international agencies. The SUP aimed to complement these programmes, for example by providing 'standard' training and resource materials, guidelines and technical advice.

To maximize awareness within target groups, the project built on such existing initiatives. The SUP worked with government and non-government agencies in addition to the agrochemical companies themselves. This collaboration reflected industry's belief in the importance of delivering a common, consistent message to target groups, to maximize the project impact.

In Thailand the SUP's objectives were as follows:

(i) To raise awareness and compliance in the safe handling and storage of pesticides within the industry, the medical profession and end-users;
(ii) To reduce the incidence of pesticide poisoning;
(iii) To protect the environment;
(iv) To help relevant government agencies with resources, expertise and training.

The basic set of 'benchmark' standards was the FAO International Code of Conduct on the Distribution and Use of Agrochemicals,[1] and the GIFAP Guidelines referred to therein.[2] Since improving compliance with the Code was a fundamental project objective, the SUP targeted the whole distribution chain, from importer/ formulator, through to the end-user, ie, a 'cradle to grave' approach. Additionally, the medical profession, teachers and school students were also targeted.

An independent baseline survey[3] conducted at the start of the project helped establish current standards. This subsequently allowed easy evaluation of project impact through an interim audit. Although such audits provided important information that helped fine-tune priorities, subsequent experience shows such quantitative surveys are less useful in evaluating total project progress, at least in the short term. This is further discussed below.

25.3 STATUS

The SUP has now been in progress in the pilot countries for over three years. During this period the project set up working links with a wide range of organizations. These included government departments, universities, hospitals, international agencies, NGOs (Non-Government Organizations) and private sector companies. This allowed the project to make use of the 'multiplier effect' to reach target groups.

A key long-term objective from the start was to stimulate concerned agencies of all kinds to:

(i) Give high priority to pesticide safety in their own ongoing activities;
(ii) Allocate adequate funding from within their own budgets; and
(iii) Work with other agencies where appropriate, to combine all the expertise, opinions and resources for maximum impact.

The SUPs in all three pilot countries have used the following means to achieve these objectives:

(i) Generate local language training resource materials for use by various categories of trainers, from government, NGOs and the industry itself;
(ii) Provide basic training so that government officers have the technical competence, communication skills and motivation to conduct further training with confidence and credibility;
(iii) Provide 'seed money' to start small-scale activities to prove what can be done and so further stimulate interest at senior levels;
(iv) Lobby for changes in government policies and regulations; and

(v) Act as a focal point for pesticide safety-related information so that other organizations can be introduced to useful contacts, training materials, resources and potential partners, eg, in research and training.

Table 25.1 shows how the SUP has succeeded in reaching large numbers of people in its efforts to raise safety awareness.

Table 25.1 *Quantitative objectives and achievements 1991–94*

Programme	**3-Year Quantitative objective**	**Achieved June 94**
Trainer Training	130 trained trainers	1630
Retailer Training	600 trained retailers	925
Farmer Training	(Reading) 530,000 farmers	423,400
Education	144 schools in pilot areas	253 schools
	100,000 students	65,000 students
Medical	Train 800 doctors/paramedics Reach 600 hospitals	1235 doctors + nurses 485 hospitals
Protective Clothing (PC)	Distribute 50,000 pieces	18,500 PC for tree crops 17,700 back shields 325,000 face masks 24,800 face shields 12,564 pairs gloves
Industry Standards	4 full GIFAP Accreditation 10 Improvement awards	5 applied for Full Accreditation 1 Improvement Award

Clearly, judging the success of the SUP by quantitative criteria alone, shows only part of the overall picture. Nevertheless, the project could not have reached these targets without a high level of cooperation between participating agencies.

Independent audits show an increased level of awareness of key safety issues in target groups (Figure 25.1). However, given the socio-economic and other constraints operating on the rural population, the timescale for generating sustainable behavioral change in farmers can be long.

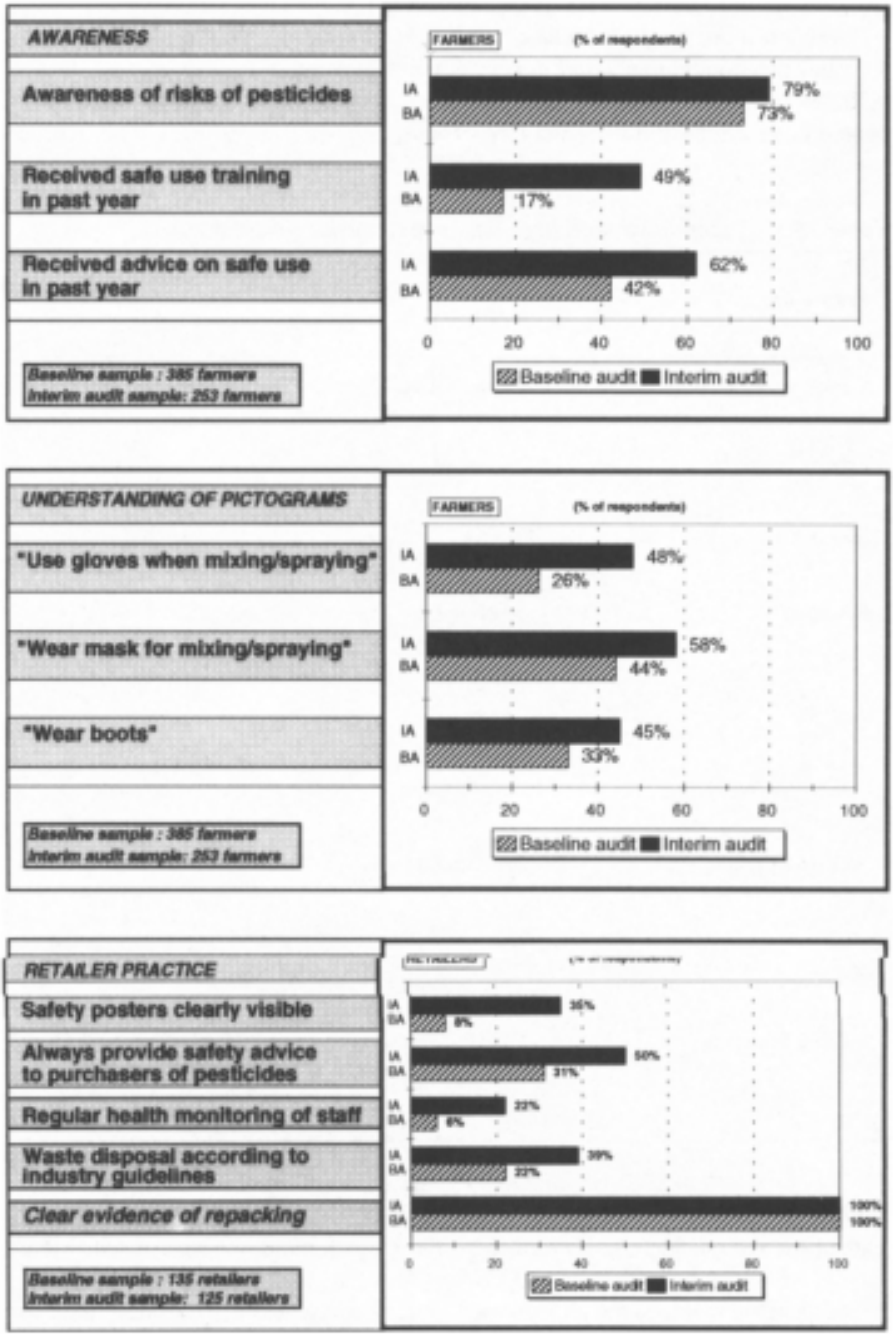

Figure 25.1 *Interim audit summary—September 1993. Progress in pilot areas (Nakhon Pathom/ Sukhothai provinces)*

25.4 EVALUATION

SUP Thailand commissioned independent audits and surveys at various levels to evaluate the effectiveness of the activities shown in Table 25.1.[4] Although the results indicate greater awareness and improvements in some aspects of practice, at this relatively early stage in the SUP's implementation, more widespread improvements in farmer practice cannot be expected. (Extension experts generally acknowledge that to achieve a truly sustainable change in attitudes and behaviour, a timeframe of 10 years, rather than three, is probably more realistic). KAP ('Knowledge-Attitude-Practice')—type surveys can effectively track awareness levels (quick to change) but a longer time frame is required to detect meaningful changes in farmer practice.

Although SUP will continue where necessary to work with existing partners, SUP does not need or aim to be a 'working partner' in future initiatives. Indeed, the main purpose is to stimulate and facilitate **independent** SU initiatives at all levels and to foster broad-based cooperation. The extent to which this is happening is one measure of the **sustainability** of current efforts beyond the pilot phase.

Having reached the end of the three-year pilot phase, it is therefore important to assess the project from this perspective.

25.5 SO HOW SUSTAINABLE IS THE SUP IN THAILAND?

The SUP's six key programmes, highlighting factors that contribute to longer-term sustainability, are detailed below together with the principal cooperating agencies.

25.5.1 Training of trainers, retailers and farmers

In 1993, Thailand's Department of Agricultural Extension (DOAE) changed radically its policy to set priorities for safe use training for all levels of extension workers, retailers and farmers. The Thai Cabinet has backed this policy, approving a training budget of US$ 200,000 y^{-1}.

To 'kick-start' this initiative, SUP provided training materials, a five-day course curriculum and a course management/organization structure. This programme is now already implemented almost entirely by DOAE with minimal need for external support. So far, the SUP has trained 1,630 trainers, 925 retailers and over 450,000 farmers. Extension officers nationwide are using the GIFAP Fanner Training Modules translated into the Thai language.

Cooperation with aid-financed development projects has helped broaden the scope of the SUP. In Mae Hong Son Province, northern Thailand, the Deutsche Gesellschaft für Technische Zusammenarbeit (GTZ)-funded Thai-German Highland Development Project (TGHDP) works with SUP trainers in remote border areas; TGHDP recently integrated safe use training in its four-year Master Plan for Thailand.

In upgrading standards within the distribution chain, lack of enforcement of existing legislation seriously impedes progress, especially with regard to retailer licensing. Whilst recognizing this, the Department of Agriculture (DOA) recently announced that newly-drafted legislation[5] will oblige all retailers to undergo training/ accreditation before receiving their sales licence; DOA plans to start the training on a national scale by the end of 1995. The SUP's retailer training course will form the basis of the course curriculum.

Based on current progress, DOAE is now proposing a major national safe use training project, for submission to donor aid agencies. The main aims will be to reduce use rates of pesticides to protect users and the environment from contamination and to ensure quality produce. This last is increasingly important as international food residue standards for export become more strictly enforced.

At a regional level, there are several encouraging developments. For example, a joint proposal was submitted by the UN's Economic and Social Commission on Asia and the Pacific (ESCAP) and GIFAP, to the EU and other donors, for a regional program on pesticide policy formulation. This included pesticide safe use training as an integral element. Secondly, FAO's Regional Office for Asia-Pacific will jointly organize with GIFAP an Expert Consultation on Safe Use of Pesticides in late 1995. The latter will certainly help to inform governments within the Asia-Pacific region and hopefully generate the wider participation and funding necessary to address this major issue.

25.5.2 Schools programme

SUP also supports NGOs working at field level in Thailand. CARE International, a leading NGO active in Thailand for 16 years, has a clearly-defined pesticide policy which insists on safe use training and personal protection in all CARE projects. CARE now includes pesticide safety as a topic in its schools' newsletter, *Children's Health and Environment*.

25.5.3 Medical training

The SUP has arranged a training programme for the medical profession with the cooperation of the Ministry of Public Health (MoPH), Food and Drug Administration and the Toxicological Society of Thailand. The programme is training approximately 1300 doctors, paramedics and nurses in first aid, diagnosis and treatment of pesticide poisoning. A medical text book[6] and a cross-referenced trade/common name index of registered pesticides[7] have also been distributed nationwide to provide updated information to hospital emergency rooms.

WHO has expressed an interest in the programme. Its International Programme on Chemical Safety (IPCS) intends to establish Thailand as a regional (Asia-wide) centre of expertise for training doctors, nurses and technicians. IPCS has produced a modular

trainer training manual and discussions are now under way to upgrade the existing programme and build on the cooperation already established.

To supplement this training course, doctors are kept in touch with developments by a Medical Newsletter,[8] set up for doctors nationwide. This is distributed by Ramathibhodi Hospital's National Poison Information Centre in Bangkok.

The ready availability of such reference materials and newsletters should have a lasting effect. The MOPH and major hospitals are making good progress in developing databases at the Poison Information Centre. Ultimately, all hospitals will have on-line access to these databases. GIFAP Thailand already has on-line access to the CD-ROM medical databases at Ramathibhodi Hospital Poison Information Centre. In fact, on-line networking between provincial poison control centres throughout Thailand is likely to become a reality sooner rather than later.[9]

25.5.4 Protective clothing

The promotion of cost-effective items of protective clothing has been a key priority. In 1988–1989, GIFAP conducted a detailed study of protective clothing in Thailand.[10] A project task force initiated a follow-up study to establish which items were most needed by 'high-risk' growers. (Surveys identified citrus/fruit trees, tall field crops such as tobacco and cotton and rice, as high-risk crops). Face and body protection equipment available in the market resulting in a major requirement for improvement. Most types evaluated were either too expensive or did not provide an acceptable combination of durability, comfort and protection.

A project task force compared available and new materials and designs of mask and body covering. These were field- and market-tested in cotton and fruit tree areas. Protective clothing is now distributed to retailers (at cost price) via companies' own distribution channels, via radio programmes, the Royal Projects, and by other government programmes.

The SUP supports this marketing effort with promotional materials and a video. The strategy is an attempt to encourage farmers to see the benefits of protective clothing and be prepared to pay for them. Generally, this aim is better served by marketing at nominal cost rather than giving away the items. However, the programme does need to retain some flexibility to allow companies to offer free protective clothing as part of their promotional campaigns.

As of June 1994, approximately 300,000 items had been distributed. The Ministry of Public Health is now about to start a nationwide campaign to promote use of protective clothing and has requested SUP support to set up this programme.

25.5.5 Industry standards

At the industry level, the project provides assistance to manufacturers, formulators, repackers and distributors to enable them to meet the standards laid out in the FAO Code. The Code, together with the relevant national rules and regulations and GIFAP

Guidelines,[11-13] were used to develop a checklist[14] providing minimum acceptable and target standards for the premises of TPA member companies. This applies to structure and location, storage, packaging and labeling, employee safety, transportation and disposal.

Three expert advisory groups have conducted surveys of a number of plants. These groups now use the checklist to advise companies of their obligations under the Code and national law. Companies can now easily ascertain what cost-effective measures are available to meet these basic standards. Such advice is obviously tailored to the company's individual circumstances. The scheme has met with some success and companies, large and small, are now much more ready to open their doors and share safety technology with other companies.

A key objective is to establish appropriate disposal facilities for pesticide waste and bulk packaging. Private sector investment is likely to solve this problem, at least for one major industrial estate. Japanese investors are currently planning a US$32 million total disposal facility (incinerator, scrubber, water and ash treatment units). This will serve the entire estate, with completion by end 1995. At least 10 major formulation/repacking plants at this location (40 km east of Bangkok) will benefit.

25.6 MODEL FARM

Working with the DOA's Pesticide Application Research Team and other Divisions of the DOA, this project addresses the specific issue of boat spraying of citrus just north of Bangkok. It aims to reduce pesticide use to what is strictly necessary, by bringing together elements of the SUP such as training, protective clothing, and (especially) improved application technology.

Traditional boat sprayers use three operators and two spray lances. Coverage is poor and slow and operator contamination can be serious. The new design (adapted from an innovation by a citrus farmer) provides improved canopy penetration and droplet size distribution and greatly reduces spray volume and product usage.

Preliminary comparisons of traditional vs. improved designs are given in Table 25.2. The study is still ongoing, with supplementary work being conducted by the British Natural Resources Institute, Pest Management Division, Silwood Park, Ascot, Monitoring of residues in soil, water, spray operators and produce is being conducted in cooperation with the DOA's Hazardous Substances Division.

By adopting these practices (which are consistent with generally accepted GAP), it is anticipated that farmers can raise their gross margins considerably. Users, consumers and the environment will also benefit from more judicious use of pesticides. (The GTZ's Thai-German Plant Protection Project provides recommendations for selection of appropriate products and IPM practices).

There are indications that farmers are voting with their feet, since the new sprayers have already proved popular and cost-effective. Traditional lance-hose boat sprayers are now hard to find in the main citrus areas.

Table 25.2 *Comparison of improved air blast vs traditional boat sprayer, Rangsit, Thailand*

Sprayer	Traditional Boat Sprayer	Improved Air-blast
System	Pump + 2 hoses, Open spray tank	Mounted adjustable Silvan nozzles Self-propelled, closed spray tank
Spray volume (l ha^{-1})	3,125–3,750 l ha^{-1}	625–1,250 l ha^{-1}
Man h ha^{-1}	6.25	0.31
Spray reaching ground (%)	26	6
Coverage (ha h^{-1})	0.48	3.2
Operators	3	1

25.7 CONCLUSIONS

Since its inception. The SUP in Thailand has grown from an industry initiative into a broader movement. Now many different organizations are involved. It appears that there is sufficient momentum, especially within government, to ensure higher priority for enforcing policies related to Code implementation.

Growing public awareness of environmental issues in Thailand has certainly stimulated this process. The 7th National five-year Development Plan[15] reflects this concern in a strong environmental policy. Together with the formation of the US$20 million Environment Fund, this will do much to maintain continuing interest in (and funding for) such programmes.

From the experiences of the three-year pilot phase of the SUP, several lessons may be drawn:

- Pesticide safety needs to be addressed by all concerned agencies in a joint effort;
- A rural development perspective must be adopted in improving pesticide safety;
- Integrated Pest Management (IPM) training programmes must include precautionary advice for proper handling, use and disposal of pesticides, wherever these are necessary; and
- Safety training activities must be supported by a 'social marketing' approach, linking different programmes and reinforcing key messages using mass media.

The agrochemical industry is committed to minimizing the environmental impact of pesticides. There are strong trends within the major research-based international companies towards developing new molecules/products with low use rates, high

target-specificity, very low non-target toxicity, and with favorable ecotoxicological profiles. Such trends will certainly enhance the intrinsic safety of pesticides, but GIFAP believes that progress in R&D must nevertheless be complemented by large-scale training and education programs. Taken together, these will contribute towards improved user and environmental safety in developing countries.

25.8 THE FUTURE?

There must be a collective responsibility to protect users and the environment must be a collective one. Industry, government, NGOs and international agencies have to work together if such common goals are to be reached. The SUP has demonstrated on a small scale the way in which such cooperation can work effectively. Accordingly, with the experience of the past three years, GIFAP is extending its support to other countries outside the pilot country, Thailand.

The agrochemical industry is committed to raising standards of manufacturing, transport, handling, usage and disposal. Therefore, GIFAP is supporting the efforts of National Agrochemical Associations in Asia to accomplish these aims in cooperation with government agencies. There is no doubt that attaining this ambitious goal will require higher prioritization of pesticide safety and related ecotoxicological issues in development programmes funded by governments and the international community. GIFAP strongly believes that through such cooperation the Safe Use Project can make a worthwhile contribution to worker safety, environmental protection and, of course, consumer safety.

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26 The Role of the National Chemical Emergency Centre

John White

26.1 INTRODUCTION

The National Chemical Emergency Centre (NCEC) was established in 1973 by the Department of the Environment and the Home Office and forms part of the National Environmental Technology Centre, itself part of AEA Technology. This chapter is based on previous work by the author[1,2] and by the Founder of the NCEC[3,4] and describes the origin, development and functions of the NCEC.

The basic purpose of the Centre is the identification of hazards and the remedial actions needed to counter them in the event of an uncontrolled chemical 'release'; that is to say, in case of a chemical emergency. It achieves that purpose both by giving advice (often to the emergency services) by telephone and by providing information often in the form of computerized databases to numerous customers worldwide.

The NCEC's principal function has always been the provision of chemical information to the emergency services (in particular the Police and the Fire Service) when normal means of obtaining such information fail them. In this role, in conjunction with the Chemical Industries Association, it plays a key role in the national CHEMSAFE scheme. (CHEMSAFE is the CHEMical Industry Scheme for Assistance in Freight Emergencies.)

26.2 CHEMSAFE

Before 1973 there were no formal national arrangements for dealing with chemical transportation accidents. The CHEMSAFE scheme[5] came into operation in January 1974, coordinating existing emergency response arrangements provided by some chemical manufacturers and at the same time encouraging all companies responsible for causing the conveyance of hazardous substances to establish adequate emergency procedures and labelling standards. Many of those voluntary principles have since been incorporated into legislation.

The primary objective of the CHEMSAFE scheme is to provide prompt technical advice and assistance to the emergency services dealing with incidents involving chemicals in transit, so that any adverse effects are minimized. CHEMSAFE relates to all chemical products, not just dangerous substances; thus the scheme is additional to regulatory/legislative requirements.

Furthermore, the sound reasons for which CHEMSAFE was set up have over recent years become all the more important in view of the greater concern for the environment and in the light of the 'Responsible Care' programme.

The companies who participate in CHEMSAFE are all committed to providing help, but there are three different levels of participation:

(i) Level A is a commitment to providing a specialist advice telephone number on product labels and documentation, from which advice is obtainable;
(ii) Level B includes, in addition to Level A, the provision of assistance at the scene of an incident where a participating company's own products are involved; and
(iii) Level C involves, in addition to Level B, the provision of assistance at the scene of an incident that involves products not belonging to the participating company.

All three levels of participation also involve a commitment to supplying product information to NCEC. These companies participating in CHEMSAFE provide the mainstay of the scheme and the effective operation of CHEMSAFE relies very much on their participation.

In addition to the company involvement, however, there is an aspect of the scheme which relates more directly to NCEC—the Chemsafe 'longstop' procedure. This is essentially a 'system of last resort' whereby the Fire Brigades or the Police, when dealing with an incident and having tried without success to obtain information by normal means, can call the NCEC for advice and help at any time.

In spite of the emergency arrangements being established within the chemical industry, it was anticipated that there would be some transport accidents involving chemicals where important information could not be found. This might be, for example, where the manufacturer could not readily be contacted, or where imported chemicals or inadequately-labelled trade name products could not be identified. It is in such cases that the emergency authorities would invoke the Chemsafe longstop procedure by telephoning the NCEC using an unlisted number given to them by the Home Office. This telephone line gives access to one of a team of technically qualified Duty Officers who is always available to give advice and assistance.

26.3 NCEC SOURCES OF INFORMATION

The NCEC holds information on both pure chemical substances and trade name products, much of which has been obtained from manufacturers, traders or importers, in collaboration with the Chemical Industries Association. Information sought from industry is based on meeting the needs of the emergency authorities. The Emergency Duty Officers have access to this information via computerized databases, but in the event of the information needed not being held on the Centre's computers there is also an extensive reference library which can be consulted. This contains numerous reference books on chemical hazards, nomenclature, toxicology, protective clothing, and the like, as well as references to legislation/regulation in the United Kingdom, Europe and Worldwide. There are also numerous trade name directories, including overseas directories to aid in the identification of imported products.

26.3.1 CHEMDATA

Over the first few years of NCEC's existence, a large 'free text' chemical database was built, mainly from information supplied by industry and, subsequently, Fire Brigades were given access to this via a telephone line and a remote terminal/printer. Although this arrangement proved useful to the Fire Brigades, it was expensive to operate and sometimes caused some difficulty (in that anyone interrogating the database was presented with a lot of information from which the facts required had to be selected and often interpreted). This need for interpretation could prove difficult for a non-scientifically qualified person, so a demand arose for a database which gave information in a simple and straightforward manner, and which was above all user friendly. This demand led to the production of CHEMDATA.[5]

Information from the Centre's computer network, supplemented by standard reference material, is used to provide a microcomputerized chemical hazard databank called CHEMDATA. This database uses a system of standard phrases which are selected on the basis of known properties of the chemical. Each data record consists of 18 datafields of which 8 contain code numbers which relate to the selected standard phrases. Decoding of these fields by reference to the phrases takes place during the data retrieval process so that the user sees normal text.

At the moment, more than 80,000 product names are on CHEMDATA; searches can be made by full or part name, by Substance Identification Number (UN) or by CAS registry number. A search can also be made for company information. CHEMDATA is designed to operate from a user's own hardware on a stand-alone basis, and in the case of many emergency authority users it forms part of an integrated Command and Control System. It is supplied by NCEC on a subscription basis, and is updated every six months when copies of the updated information are despatched to users on various magnetic media.

The system is used by the majority of the Fire Brigades in the UK and has been adopted as a standard system in Ireland. In fact, it is used in over twenty countries around the world, including Europe, Scandinavia, the Middle East, Far East, Africa, Australasia, and North America. Users include fire brigades, police authorities, airports and sea ports, health and environmental agencies, and industry.

As well as regular updates and the addition of product and company information there are also, from time to time, improvements in operating software, and in the scope of information provided. The most recent example of the latter is the addition of the IMDG Emergency Schedules (EmS)[6] to CHEMDATA. The next update of CHEMDATA will include four additional datafields giving information on, for example, EEC No. and Packing Group. In the near future there will also be a Microsoft Windows version of CHEMDATA.

The adoption of the coded standard phrases system for CHEMDATA led to a dramatic saving in data storage space compared to the old free-text database. This was initially a very important step because it enabled a comparatively large database to be accommodated on microcomputer equipment. It meant that the database could be operated on a 'stand-alone' basis, each user having access to his own copy of the

Table 26.1 CHEMDATA readout

CHEMDATA – (c)UKAEA 1987–1994 Database Version:9.0/0494/GB
[DOC #] 08442 [E.A.C.] 2XE APP CODE A (SEEK SPECIALIST ADVICE) & C

[*product name*]
TOLUENE DIISOCYANATE

[UN No.]	[UN HAZARD]	[ADR/RID]	[TREMCARD]
2078	6.1	60	173/61GO6b
[IMDG]	[NFPA]		
6269	3-1-1		

[*protection*]

- FULL CHEMICAL PROTECTION SUIT WITH POSITIVE PRESSURE BREATHING APPARATUS BECAUSE OF HIGHLY TOXIC PROPERTIES OF GASES OR VAPOUR
- SUBSTANCE ATTACKS PROTECTIVE CLOTHING AND EXPOSURE TO HIGH CONCENTRATIONS SHOULD BE LIMITED TO DURATION OF ONE BA CYLINDER

[*hazards*]

- TOXIC
- IRRITANT
- COMBUSTIBLE (F.PT > 100°C)
- GIVES OFF TOXIC OR IRRITANT FUMES IN A FIRE — KEEP UPWIND
- DECOMPOSES IN WATER

[*form*]

LIQUID, COLOURLESS, IMMISCIBLE WITH WATER, HEAVIER THAN WATER

[*precautions*]

- AVOID ANY PERSONAL CONTACT
- KEEP CONTAINER(S) COOL IF INVOLVED IN A FIRE
- PREVENT SUBSTANCE ENTERING WATERCOURSES AND SEWERS
- ABSORB SPILLAGE IN EARTH OR SAND

[*fire*]

- EXTINGUISH WITH FOG (SPRAY)

CHEMDATA – (c)UKAEA 1987–1994 Database Version:9.0/0494/GB
[DOC #] 08442 [E.A.C.] 2XE APP CODE A (SEEK SPECIALIST ADVICE) & C

. . . . . .

[product name]
TOLUENE DIISOCYANATE

. . . . . .

[decontamination]

- WASH WITH COPIOUS AMOUNTS OF WATER AND DETERGENT

. . . . . .

[FIRST AID]

- If substance has got into the eyes, immediately wash out with plenty of water for at least 15 min.
- Remove contaminated clothing immediately and wash affected skin with soap and water.
- Apply artificial respiration only if patient is not breathing or under medical supervision.
- Seek medical treatment when anyone has symptoms apparently due to inhalation, swallowing, contact with skin or eyes.
- Persons who have been in contact with the substance or who have inhaled the vapour or fumes produced in a fire may not show immediate symptoms and should be taken to a doctor. Patient should be kept under medical observation for at least 48 h.

CHEMDATA – (c)UKAEA 1987–1994 Database Version:9.0/0494/GB
[DOC #] 08442 [E.A.C.] 2XE APP CODE A (SEEK SPECIALIST ADVICE) & C

. . . . . .

[product name]
TOLUENE DIISOCYANATE

. . . . . .

[ADR/RID]

- 60 TOXIC OR SLIGHTLY TOXIC SUBSTANCE

. . . . . .

[E.A.C.]

- 2: FOG — Use water fog for fire situations
  NOTE: In the absence of fog equipment a fine spray may be used.
- X: Wear full body protective clothing with BA.
  Prevent, by any means available, spillage from entering drains or watercourse.
- E: Consider evacuation, but it is usually safer to shelter in buildings with all doors, and windows closed.
  Consult Control, Police and product expert.

. . . . . .

[TREMCARD SINGLE SUBSTANCE TITLE]

- TOLUENE DIISOCYANATE (TDI)

. . . . . .

[NFPA]

HEALTH – 3: Materials which on short exposure could cause serious injury (temporary or residual) even though prompt medical treatment were given.
FIRE – 1: Materials that must be pre-heated before ignition can occur.
REACTIVITY

- 1: Materials which in themselves are normally stable, but which can become unstable at elevated temperatures and pressures or which may react with water with some release of energy but not violently.

database in his own Control Room, hence avoiding the complicated accessing arrangements of the old database. In more recent years, the saving of space has had less importance because of the rapid increases in hard disk capacity of PCs. However, another advantage of using standard phrases remains in place to this day. It is the ease with which the system can be translated into other languages. At the moment CHEMDATA is available in Dutch, French, German, Spanish, and Polish, as well as English.

CHEMDATA is a registered trade mark of the United Kingdom's Atomic Energy Authority. A sample CHEMDATA readout is shown in Table 26.1.

26.3.2 Transport Emergency Cards (TREMCARDS)

When the emergency authorities are called to the scene of an incident involving chemical substances, they need to know if the substance involved is hazardous, what precautions need to be taken in handling it, and whether it can be dispersed or should be contained. Whilst some information can be obtained from the vehicle or package labelling, more detailed information in cases of emergency can be provided by Transport Emergency Cards. These cards, which are normally carried in the vehicle cab, provide the 'instructions in writing' required both by European Agreement covering the transport of dangerous goods (ADR/RID)[7,8] and by United Kingdom legislation.[9-11] Most cards in use in the United Kingdom are those prepared by the European Chemical Industry Council (CEFIC) in an established standard format using a system of standard phrases agreed internationally by the member Federations of CEFIC.

TREMCARDS were originally prepared to cover single substances but later a series of 'Group Text' cards have been produced, each designed to cover a group of substances possessing a specific range of properties within ADR hazard classes. More recently still, a series of mixed-load cards have been evolved, designed to simplify the carrying of 'instructions in writing' when conveying several different chemical substances of the same hazard class, in packages.

The NCEC in close collaboration with CEFIC produces a computerized system which generates all current TREMCARDS in eleven European languages. The system operates on the same principles as CHEMDATA, using coded standard phrases which are decoded during data retrieval, printing normal text for the user in the language which he chooses. In order to ensure authenticity, translations of the extensive library of standard phrases have been prepared by the member Federations of CEFIC, the recognized voice of the European Chemical Industry. The most recent improvement to this computerized system has been the addition of seven more languages to its mixed-load card phrase file, thus extending the use of those cards to Eastern Europe and Russia. In the next few months the group text phrase library will be similarly enhanced, with the same result.

26.4 HEALTH AND SAFETY

The production of CHEMDATA and TREMCARD led to the development of other databases by NCEC, working on the same user-friendly principles—a diversification into the allied field of Health and Safety occurred. These newer databases will be described only briefly, because they work for the most part on the same basic principles as CHEMDATA, but with some variations in practice dependent upon the use to which they are put.

In addition to these databases, a recent innovation in NCEC's software production has been the creation of an expert system to classify and to advise on labelling requirements under the CHIP Regulations (see section 24.4.2).

26.4.1 COSHH (Control of Substances Hazardous to Health Regulations)[12]

The introduction of the United Kingdom's COSHH Regulations enabled the NCEC to diversify its activities, extending its sphere of interest from chemical emergency response into chemical Health and Safety. The Regulations created a legal requirement to assess the hazards in the workplace of chemical substances which are toxic, harmful, corrosive or irritant.

In order to provide the rapid identification of chemicals possessing such properties, coupled with essential data to assist the hazard assessment, the NCEC developed a computerized chemical information system called HAZDATA. Searchable by name, identification numbers, classification or location, the database contains information on over 2700 chemicals, providing details of the hazards, risks, and toxicity of a substance, as well as safety requirements, occupational exposure limits, and recommendations on ventilation, monitoring and health surveillance.

In view of the need to cater for a wide range of potential users, each of whom would generally have requirements for narrow groups of product data, HAZDATA also allows users to enter their own information on products of special importance to them (eg, a range of 'trade name' products). Thus the database can be customized by the user to include supplementary information and user-defined names.

26.4.2 CHIP (The Chemicals (Hazard Information and Packaging) Regulations)[13]

The European Community Preparations Directive and its United Kingdom's counterpart, CHIP, require that every hazardous chemical substance supplied to a customer must be accompanied by a safety data sheet in a specified format. Drawing on its experience in the development and production of databases for emergency response, and for meeting the requirements of the COSHH Regulations, the NCEC has produced a complete Safety Data Sheet system called DATASHEET +. This computerized system enables a chemical manufacturer/transporter/distributor to generate safety data sheets, in the required format, by selecting phrases from an extensive phrase file, and storing the resulting sheet in the computer for subsequent retrieval. Moreover, once a Safety Data Sheet is compiled it can be automatically translated by the system into any of ten

European languages. The system will also hold details of all of the user's customers supplied with each Safety Data Sheet, enabling them to be identified quickly and updated when revisions to a Safety Data Sheet are made.

26.4.3 LABEL ADVISER

The CHIP Regulations also require that an intricate classification process be undertaken in order to generate an official classification and hence a label for a hazardous substance. NCEC's 'LABEL ADVISER' is an expert system which carries out this process by presenting a series of questions to the user through a Microsoft Windows interface. The system takes the user through the whole classification process and devises an accurate label in a fraction of the time taken to do this manually.

26.5 CHEMICAL EMERGENCY AGENCY SERVICE (CEAS)[2]

It is a basic principle of CHEMSAFE that companies responsible for the transport of hazardous chemicals should have an adequate emergency response capability, and the simplest form of this (already referred to as Level A) is to provide a specialist advice telephone number. Some companies, however, especially chemical distributors and importers, are not able to provide a specialist advice telephone themselves. In view of this, and the legislative requirements of packaging and conveying dangerous substances which call for the display of a source of specialist advice under certain circumstances, the NCEC set up an Agency service to assist companies which were unable to comply with these requirements themselves.

The CEAS is provided on a commercial basis and an annual subscription is paid by each member company, the level of charge being dependent upon the number of products being transported. The Service works in a simple but very effective way; companies provide NCEC with details of their products, in an agreed format, and are then allowed to display a special NCEC telephone number on any package, TREMCARD or hazard warning panel relating to those products.

The information required on each chemical product is obtained by means of a questionnaire (The Chemical Product Information Form) which, when received at NCEC, is first scrutinized to establish its validity. The accuracy of the information given is checked, as is the consistency and agreement between the various sections of the form. Any amendments necessary are discussed and agreed with the company before being made. Sometimes, a company is unable to provide all the data on all of the products it wishes to register. During the validation process, help can sometimes be given by NCEC staff, eg, suggestions for additional information.

When it is deemed that the information provided for a product is satisfactory, the substance is accepted onto the CEAS as a 'specified material' and the company can then display the special CEAS emergency telephone number on vehicles or packages containing that product. Thus the emergency authorities can obtain the

necessary specialist advice for such products, at any time, by contacting the NCEC directly.

26.6 PUBLICATIONS

26.6.1 Hazchem List

The NCEC publishes, in collaboration with the Home Office, the well-known 'Hazchem List',[14] which has become an internationally recognized standard reference source of dangerous chemicals and emergency action codes. The list is updated from time to time in order to incorporate revisions to the United Nations List of Dangerous Goods,[15] and also to allow for any other changes, eg, those made by the Home Office Hazchem Technical Sub-committee. The Hazchem List is in two sections:

(i) An alphabetical list of chemical products with their Substance Identification Numbers (SINs); and
(ii) A numerical list of SINs identifying Home Office Emergency Action Codes and other references vital to emergency response.

It will be of particular interest to the readers to know that the latest version of the Hazchem List (No. 8) shows changes from its predecessor which will further protect the environment and reduce pollution. These changes are to some of the Emergency Action Codes. These codes are a key feature of the Emergency Services' initial reaction to a chemical emergency and give advice in a cryptic way on certain vital matters. One such vital matter, of especial interest to us, is the indication whether a spillage can be dispersed into the environment or whether it should be by some means contained, thus avoiding any pollution.

For example, an Emergency Action Code of 2R means, amongst other things, that the substance to which the code applies and which has presumably been spilt can be washed away to a drain with a large quantity of water. However, particular care should be taken to avoid unnecessary pollution of watercourses. In contrast an Emergency Action Code of 2X means, amongst other things, that a spillage of the substance to which the code applies should be contained. That is to say that the spillage must be prevented by any means available at the scene of the incident, from entering drains or watercourses.

There are many examples of changes in the latest Hazchem List where Emergency Action Codes have been altered from 'dilute and disperse' in the previous list, to 'contain' in the latest one. An example of the change from 2R to 2X is UN 1760 (Corrosive liquid, mixture, not otherwise specified, miscible with water). Whereas spillages of corrosive liquids which react with water, or which are immiscible with water have always been contained, those of corrosive liquids which are miscible with water were previously diluted/dispersed. Another example of this type of change is in the Emergency Action Code for ethanol or ethanol solutions (with more than 70% ethanol). In Hazchem List 7 the E.A.C. was 2(S)E (ie, dilute), whereas now, in Hazchem List 8 it is 2(Y)E (ie, contain).

There are many examples of this type of change which are a clear manifestation of the continuing trend towards a greater concern for the environment even in case of emergency.

26.6.2 Pocket Hazchem

The NCEC has also developed a computerized version of the Hazchem List which operates on a robust miniature hand-held computer (the PSION personal organizer). This is described as the 'Pocket Hazchem' or Personal Emergency Response System. Like the Hazchem List itself, the computerized version contains details on all current UN and UK Substance Identification Numbers, giving Home Office Emergency Action Codes, Home Office additional advice on personal protection, CEFIC TREMCARD reference numbers, and ADR Hazard Identification numbers.

26.6.3 CEFIC TREMCARDS

The NCEC also publishes, in collaboration with CEFIC and the UK Chemical Industries Association, a reference edition (in English only) of CEFIC TREMCARDS (Single Substance Cards, Group Text, and Mixed-Load cards).[16]

26.6.4 Guide

Also available is a guide to selecting the appropriate TREMCARD by substance name, SI/UN number, or ADR Class and Item number.[17]

26.7 CONSULTANCIES

Many years of experience in the fields of work already described enables NCEC to provide a consultancy service, offering for example the design, development, and preparation of specialized databases suited to a particular customer's needs. Examples of this type of service are the preparation of a health and safety database for a major international airline and the preparation of a cross reference system between ICAO[18] and ADR for another major international airline! Another example of this type of consultancy work led to the development by NCEC of the expert system to classify and advise on labelling requirements for chemical products under the CHIP Regulations.

Consultancy is also available for the development of national emergency response schemes, including the setting up of national/regional centres.

26.8 CONCLUSIONS

Since the CHEMSAFE scheme was established in 1974, arrangements for dealing with chemical transport accidents safely and effectively have been considerably improved in the United Kingdom. A United Kingdom national advice centre, which can give assistance at any time, has proven over many years to be an essential part of the CHEMSAFE scheme.

During the last twenty years there has also been an increasing awareness of and concern for the environmental effects of chemical emergency response and this has been especially marked over the last few years. A clear manifestation of this greater concern for the environment is the recent amendment to many Emergency Action Codes referred to in section 26.6.1. These amended codes mean that many spillages which could previously have been dispersed to the environment are now contained. However, these Emergency Action Codes are only applied to substances which are dangerous in the normally accepted sense, ie, those which can be assigned to one or other of the UN hazard classes. Other dangers to the environment exist from substances which may not be at all hazardous to us and these dangers too have become more widely recognized.

The versatile and wide-ranging chemical emergency services provided by the NCEC, which are probably unique, are coupled with an extensive expertise in software production. This has been made possible by the 'back-up' provided by AEA Technology and of the high degree of cooperation maintained with the Emergency Services, and with the Chemical Industries Association.

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27 The Economic Implications for Wastewater Treatment of Toxic or Inhibitory Sewage

Peter D.Hiley

27.1 INTRODUCTION

Wastewater treatment facilities belonging to English and Welsh water companies are only obliged by law[1] to treat wastes which will not prejudicially affect (eg, by being toxic or inhibitory) the treatment of the wastewater. They are designed, like any other industrial process, to accept certain qualities and quantities of input materials, energy etc.; delivering specified quantities of specified products. Measurement of the degree to which wastewater treatment may be affected by inhibitory wastes, and therefore its cost, has been difficult and contentious, especially since most wastes break down during treatment. Inhibition may have gone unnoticed because of oversized facilities. Biological waste treatment processes may acclimatize to inhibitory/toxic wastes and treat them but with variable consequences on performance.

An agreed form of measurement of the considerable extra costs due to toxicity is not possible currently. It would have to take account of acclimatization, degree, and speed of treatability as well as the difficulties of measuring wastewater treatment works performance. Controlling toxic waste inputs to almost the level at which effects are readily detected implies the acceptance of considerable damage in the longer-term which could amount to 25% of the total works cost. Currently, such practices are not accepted for environmental discharges.

Understanding the way that waste inputs affect biological processes in wastewater treatment is fundamental to their economic design and operation. Effective ongoing measurement of relevant factors is therefore vital. Wastewater toxicity should be controlled to a 'no-effect' standard based on laboratory tests of toxicity to an unacclimatized organisms, assuming no breakdown of toxicity in the wastewater treatment process. These are the most reproducible and precise tests currently available. The 'maximum potential inhibition' of the treatment process resulting from the discharge of the waste to the sewer may be calculated from these data. It suggests the maximum additional capital and revenue costs required in order to maintain the effluent quality and enables the prioritization of waste control actions.

For single substances discharged to the environment, no-effect discharge standards are set on the basis of laboratory toxicity tests and are monitored by chemical analysis. Standards can be set for wastewaters in the same way but monitoring can only be by toxicity tests, because wastewaters are mixtures, usually of unknown composition. Wastewaters and their associated toxic substances may react with one another and/or degrade in the sewer or treatment process to enhance or reduce the overall toxicity in an unpredictable way, so the toxicity of the wastewater should also be assessed and controlled. If water companies defined the chemicals they should not permit in wastes above given concentrations they would never succeed in defining them all and

would never be able to exert adequate control over the toxic effects of trade wastes. By defining the quality of wastes in terms of their effects on organisms one can be sure that a given level of toxic effect will not occur. The introduction of the National Rivers Authority (NRA) Toxicity Based Consents for discharges to watercourses will be the first time there has been a nationally agreed system of such standards in the United Kingdom.

From the results of a toxicity screening programme in Yorkshire, 55 works were identified as the worst affected, treating 70% of the company's wastewater. If there was a 5% improvement in the performance of these works it might save ~GB£3 million y^{-1} on capital. A result of control might be that waste dischargers become responsible for ensuring that toxic wastes are not discharged to the sewer. The environmental benefits of this would be a bonus today and make it easier for water companies to meet the new toxicity based consents in the future.

Waste dischargers may in future need to understand what is being controlled and why. The proposed tests are accessible to them, to help them discover the source of the problem and deal with it. More pretreatment is likely to result in weaker, less toxic wastewater. Better housekeeping may save the discharger by identifying wasteful practices. Some potential products may not be made because the wastes would not be treatable.

27.2 LEGAL ASPECTS

27.2.1 Obligations, present and future

The Water Industry Act 1991 (England and Wales)[1] states *'…no person shall… permit to be…emptied…into any public sewer…any matter likely…to affect prejudicially the treatment and disposal of its contents'* (alone or in admixture with wastewater). Therefore, there is no obligation to receive or treat toxic/inhibitory wastes, nor to pass on the costs of treating such wastes to the general water ratepayer. The term *'prejudicially affect the disposal of the contents'* should also refer to circumstances in which non-toxic wastes may combine or decompose to become toxic in the sewer or in the wastewater treatment process. If all wastes discharged to sewer were similar in toxicity and degradability to domestic wastewater, it is assumed they would comply with this Act. The Water Resources Act 1991[2] controls toxicity in effluents discharged to rivers by making it an offence to *'cause or knowingly permit any poisonous, noxious or polluting matter to enter …(watercourses)'*. Water companies may fail their consents as a result of toxic substances passing through the works, but unless they can demonstrate that the material originated down the sewer, they cannot avoid prosecution. The forthcoming Toxicity Based Consents, which are likely to be introduced by the end of 1995,[3] will set standards for the toxicity of effluent discharges. The US Water Quality Act[4] prohibits the discharge to watercourses of toxic substances above no-effect concentrations. European legislation controls the toxicity of certain discharges to estuaries.[5]

The substances or wastes requiring control under these laws can be different for wastewater and final effluents. Respiration inhibition of 35% in the crude sewage to works *X* (Water Industry Act) is broken down with no detectable inhibition in the final effluent except during toxic incidents. However, only 95% of the Microtox® toxicity in the crude sewage at this works is broken down, leaving readily detectable residues in the final effluent (Water Resources Act). Works *W* has a treatability factor of 0.6 due to the discharge of waste from a chemicals factory (Water Industry Act). At Works *Y* (Table 27.1) the removal of *Daphnia* toxicity was only 89% leading to obvious damage to the river life, ie, 'poisonous, noxious, etc.' (Water Resources Act).

Table 27.1 *Works Y removal of Daphnia toxicity compared to domestic wastewater*

Sample	*Daphnia* 24 h EC_{50} dilution
Works *Y* tank effluent	x 40
Works *Y* final effluent	x 6
Domestic tank effluent	x 1- x 5
Domestic final effluent	0

27.2.2 Ensuring cause and effect are correlated

Adverse effects observed in wastewater treatment works may be assumed wrongly to have been caused by a discharge of toxic waste, leading to a failed consent. It is vital to discover the types of toxicant present, their concentrations/toxicities, modes of action and frequency of discharge, with relation to potential target biological systems (different for filters and activated sludge) including the receiving watercourse and ensure that they could have caused the observed effects. The works may have been in poor condition before the discharge occurred. For example, either organic overload or toxicity in the wastewater can cause ponding, loss of fauna and loss of performance on percolating filters.

Recent work at sewage works *M* indicated inhibition of the microbiological processes, plus effects on the filter invertebrate grazers leading to excess film accumulations and loss of performance. A survey of the trade wastes in the wastewater (Table 27.2) identified the main companies causing the inhibition: one caused most of the respiration inhibition, one caused most of the nitrification inhibition and one caused most of the invertebrate toxicity. The last also accounted for the majority of the residual toxicity to river life in the final effluent, which was found by chemical analysis not to be due to known insecticides. (Toxicity ratio', a measure of potential for damage to wastewater treatment, is defined by Fearnside (see Chapter by Fearnside and Booker).

A degradable inhibitor may slow the decomposition processes in percolating filters so that the treatment of BOD takes place in the deeper zones where nitrifying bacteria

Table 27.2 *Toxicity of trade wastes discharged to works M, June 1994*

Trader	Toxicity ratio for respiration test	Toxicity ratio for nitrification test	EC_{50} to *Daphnia* 24 h test
A	0.1	0.8	1
B	1	0.9	6
C	0	1.6	23
D	0	5.7	**2122**
E	2.8	**10**	0.5
F	0	0.9	3
G	**5.5**	2	8
H	0	0.2	0.2
I	0	0.1	224
J	0	1.3	149

normally grow. The observed effect is loss of nitrification but toxicity tests would show no nitrification inhibition. Using a multiple test protocol,[6] respiration inhibition would be discovered.

At about 1980, at the medium sized works *N*, the concentrations of dieldrin, a very persistent and bioaccumulated pesticide, reduced as the less persistent eulan formulations replaced it for use in mothproofing, satisfying EC legislation to control its concentration in the Humber estuary. Some damage to the invertebrates on the percolating filters was noticed in 1981 (Figure 27.1). The less persistent and more effective permethrin formulations were introduced later in 1981, resulting in the death of most of the filter invertebrates and subsequent loss of filter performance. Toxicity tests using filter invertebrates confirmed that permethrin alone could have caused the damage to the filters and river life. The effluent from the company concerned was transferred to a much larger works which could handle it adequately, at a cost of GB£990,000 over two years.

The fauna or flora of the wastewater treatment works or receiving river may show serious damage as if from a pesticide, yet no identifiable ones can be found in the wastewater. Such damage may be caused by brief discharges of highly toxic pesticides, eg, at works *N* a spill of just 1 1 of undiluted permethrin at any time once a month would have been sufficient to maintain the damaged state of the wastewater treatment works. Short-term discharges of bactericides may affect only the bacteria causing a very short lived treatment failure followed by complete recovery in one to three days. Use of on-line biological tests may be the only way of tracing such discharges to source.

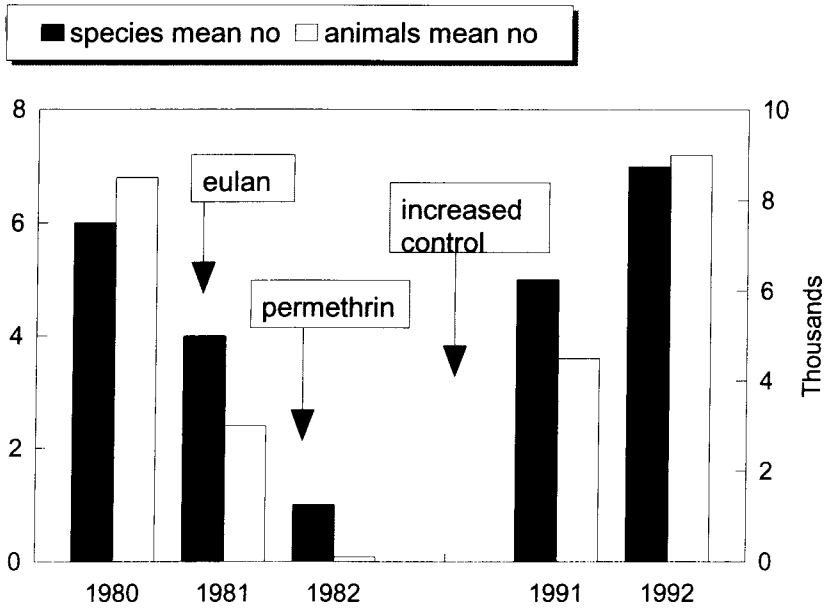

Figure 27.1 *Works N, effects of permethrin on filter fauna*

An additional investigation may be indicated when there is a mismatch in the data from different sources, eg, the finding of a significant reduction in the performance of works *X* was not confirmed by the normal 5 min or 3 h respiration tests. High concentrations of readily degradable matter masked the presence of a respiration inhibitor.[7] Substances which enhance respiration at the expense of the organisms, resulting ultimately in death but giving the impression of good health in a short-term test, were also found. In the first case running the test at several dilutions indicated the effect of the underlying toxicity and in the second case running the test for longer exposed the toxicant.

Toxicity to nitrification was suspected when, after apparently successful pilot trials, a full scale submerged aerated filter (SAF) failed to achieve its expected tertiary treatment performance. The SAF removed 7–11 mg l^{-1} of ammonia regardless of the concentration of the inflow rather than up to 30 mg l^{-1} as designed, except for toxic incidents when no ammonia was removed. The inflow ranged from 6 to 31 mg l^{-1} and the outflow normally from 1–20 mg l^{-1}. Tests of inhibition to nitrification never exceeded 30%, therefore could not account for the shortfall of nitrification capacity. The full scale unit was more lightly mixed and aerated than the pilot, leading to detectable short circuits which limited the effective amount of active biomass.

Good detective work on the part of the investigation team, which should include several disciplines covering the full understanding of the biology, chemistry and engineering of the processes, is essential to ensure successful control.

27.3 EFFECTS OF TOXIC WASTES ON WASTEWATER TREATMENT

27.3.1 Definitions

'Toxicity' is a measure of how poisonous something is and one of its effects is the 'inhibition' or slowing down of a treatment process.

'Treatability' is the extent to which a substance is broken down in wastewater treatment. A substance might be poorly treatable because it is toxic, or contains toxic components. Alternatively it may simply be difficult to biodegrade, in which case its presence in the wastewater will not affect the rate at which other substances degrade. The cost implications of toxicity are therefore higher than those of poor treatability because the whole biological process of wastewater treatment is affected.

'Performance' is the amount of treatment obtained from a unit of treatment plant.

27.3.2 Process inhibition

The biological stages of wastewater treatment works are a compression of the natural systems found in soils and rivers, having similar communities performing similar functions. Though they can degrade wastes in a wide range of conditions, their optimum performance is within a narrow range. The presence in the wastewater of toxicity, out of range pH and other conditions which are outside the optimum are therefore hazards to compliance with effluent consents, through their effects on performance.

Inhibition may affect one or more of the bacterial processes, depending upon the type of toxicant. A general inhibitor such as a low pH[7] or a broad spectrum antibiotic affects all bacterial processes so a reduction in effective works size is noticed, ie, biological removal of all matters is reduced. There are almost no organisms with performance optima outside the range pH 6–9. The effects of pH are more complex than this, though, including release of bound toxicants and alterations to the chemical state of the waste. A specific inhibitor may affect only one species of bacteria, for example the first in the two-species chain responsible for nitrification, leading to nitrite being found rather than nitrate in the final effluent. Inhibition or destruction of one of the ancillary biological processes may have direct and/or indirect effects. Damage to the protozoa in activated sludge can lead to a turbid effluent which lasts a day or so, while damage to the grazers in a percolating filter may have a delayed effect on the effluent when, after several months, film builds up to critical levels and clogs the filters, requiring many months for performance restoration.

27.3.3 Acclimatization

Acclimatization is a general term for the way that wastewater treatment biological systems adapt themselves to the presence of unusual, difficult to treat and toxic substances. The adaptations necessary vary with the type of organisms and the type of waste. The bacteria may 'find' an alternative metabolic route which enables them to use the new substance in a few hours. New varieties of the bacteria may have to develop, perhaps over a few days. New species of bacteria may have to arrive and establish themselves. Invertebrate grazers of percolating filters may become tolerant of pesticides after two or three years. A 'memory' of acclimatization may remain for two or three weeks because some of the organisms can exist on the normal wastewater with or without the waste to which they have become acclimatized. At works *B* in Yorkshire a compete change of community and associated loss of treatment capability takes place when the toxic waste from company *A* is introduced and when it is removed.

Treatment of the new, potentially toxic, waste does not take place efficiently until the acclimatization process is complete and the treatment of the normal waste may also be adversely affected for some time. Becoming acclimatized to one waste may reduce the performance of the works in respect of the other wastes being treated. Acclimatization may fail for reasons outside of the control of the water company and the trader discharging the waste to which the works is acclimatized. For instance, the treatment of thiocyanate is severely reduced if phenol, cyanide or ammonia are present. In some cases treatment is more efficient due to effects such as co-metabolism where bacteria are able to degrade difficult substances when in a plentiful supply of readily degradable material.

Because acclimatization can take place in so many ways, there is a tendency for dischargers of toxic wastes to argue from an example which is favorable to their case. The following hypothetical example (Fearnside personal communication) demonstrates how easily a satisfactory situation can turn into a disaster. In a simplified wastewater treatment works there is one toxic waste than can only be treated by two species of bacteria; it is toxic to all other organisms. These two species treat the waste and the rest of the wastewater at full performance. A second waste can be treated only by two different species of bacteria, so a mixture of both toxic wastes is totally untreatable.

In the case of variable effluents discharged to sewer the works may not get an opportunity to develop and retain acclimatization so it is irrelevant. Relying on acclimatization to deal with the usual level of inhibition in wastewater may give a false sense of security, since the works is likely to fail when a larger than normal load of toxic waste is discharged.

The definition of a suitable BOD seed, domestic wastewater treatment works final effluent, is given in the Standing Committee of Analysts (SCA) national standard[8] and regulation of effluent discharges to wastewater treatment works and watercourses is based on this, non-acclimatized, test.

Based on the above arguments it would only be possible to accept acclimatization in a wastewater treatment works to a trade waste if the discharge was unvarying in strength, toxicity and substances requiring acclimatization and the rest of the wastewater remained constant. The discharger would have to be responsible if acclimatization failed for reasons outside the control of the STW management including the results of other substances entering the sewer which interfere with acclimatization. The discharge would have to increase and decrease gradually ensuring that the STW does not fail final effluent consent at any stage. The costs of extra tests and any other special actions should be borne by the discharger.

27.4 MEASUREMENT OF INHIBITION

The presence of measurable effects in a wastewater treatment works would mean that the wastewater was clearly unsatisfactory. As with discharges to rivers and the sea, some damaging adverse effects begin subtly and are difficult to detect in the short-term. Koehler[9] states that 'true assessment of ecological impact is often limited by lack of knowledge of ecosystem functions' and this is certainly true of wastewater treatment. In order to prevent any effect, potential toxicants should be controlled to no-effect concentrations. The normal procedure in establishing no-effect environmental concentrations for national and international standards[10] is to take 1% of the measured no-effect concentration. This may be over stringent for wastewater treatment, especially activated sludge in which the ecosystem has a life of only a few weeks compared to percolating filters which take several years to stabilize and therefore may be more susceptible to long-term effects of low levels of toxicants.

In order to be of value in controlling inhibition, the tests used to establish and monitor the desired concentrations of wastes must have credibility in relation to their likely use and should not cost more to apply than the amount they are intended to save. The tests must give results fast enough to be used in incident management, ie, have a usual run time of minutes to a few hours. They should have nationally (preferably internationally) agreed protocols which run on commercially available apparatus so that they can be performed by any equipped laboratory wishing to check the results. Accepted variants of the tests should produce similar results, and the tests should be as reproducible as comparable testing procedures. The tests must relate to the actual treatment processes by using organisms which exist in, or represent those which exist in, wastewater treatment systems. The test conditions experienced by the organisms must be as close as possible to those in the full scale systems, given the needs for speed and accuracy. The results of the tests must be easy to understand and must interrelate well with one another.

27.4.1 Full scale plant

'toxic substances make the works grow smaller'. This is the net result of the presence of any toxic/inhibitory material in the wastewater received, but the extent of the effect is difficult to measure, especially at modest levels. The principal confusing factors are the variability of the incoming and outgoing wastewater, and the measurement of the effective size of the works. The performance in works known to be receiving only domestic character wastewater can be estimated by assessing the degree of wastewater treatment achieved. The uninhibited performance of works receiving trade wastes may be estimated during the holiday periods of the factories thought to be causing a problem. The performance can be expressed in terms of removal of BOD, COD, ammonia, etc. per m^3 media in the percolating filters, kg^{-1} of solids in the aeration stage, per unit area of overland flow reeds, etc. Mathematical models such as sewage treatment optimization model (STOM) and sewage treatment at risk (STAR) produce a treatability factor to quantify the loss in performance of a works affected by inhibition. Robinson[11] found a fair correlation between the results of toxicity tests on wastewater and the inhibition ('treatability factor') of the receiving works as measured on the STOM mathematical model, though the results of individual samples varied greatly from time to time. The STOM model requires data from many samples to arrive at a prediction and therefore cannot reflect the effect of short-term changes.

In cases where wastewater treatment works processes remained constant, changing only the toxicity would enable direct measurement of the resulting difference in performance.[12,13] Wastes usually contain several components which cannot readily be separated. Multivariate statistics may be used to attempt to separate the effects of removing a toxic waste and its associated BOD.

Crude sewage varies in quality and flow during the normal day, from day to day, week to weekend, with the weather and season to season. Within the works it is back-mixed in the various tanks during the settling and aeration/contact processes. It may take $\geq$12 h to pass through the works so the sample of the out-flowing final effluent represents wastewater which arrived 12 h previously mixed with a proportion of wastewater which arrived over the preceding and following hours. Comparing a sample of crude and final taken simultaneously will therefore give an imprecise and variable estimate of performance. The major works *K* (Figures 27.2 and 27.3) has a final effluent varying between 10 and 50 mg l^{-1} BOD, based on about 280 samples of final effluent taken over a two year period. The histograms show the normal distribution expected but with a long tail. This is almost certainly due to unauthorized trade discharges. The degree to which the mean might be taken to the left to keep all samples within the consent is a measure of the increase in works size required to cope with the intermittent toxicity. A small wetland works *AR* had a mean inflow BOD of 141 mg l^{-1} standard deviation 91.5 over 138 samples in four years, while the similar works *C* had a mean of 52 mg l^{-1} standard deviation 52 over 22 samples. Detecting even a 25% change in a works performance based on 5 or 6 samples is therefore difficult.

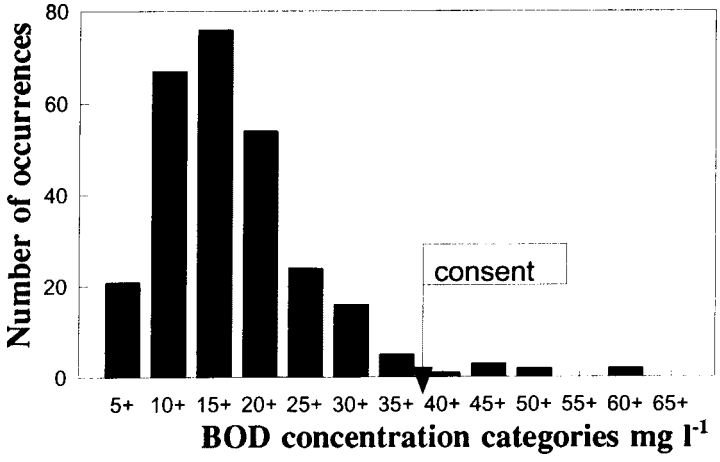

Figure 27.2 Works K, BOD frequency distribution in the final effluent, 1988–90, 271 samples

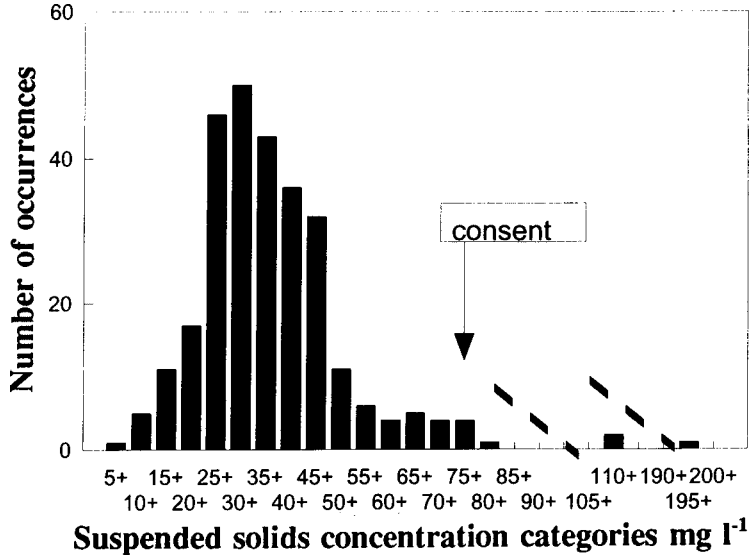

Figure 27.3 Works K, solids frequency distribution in the final effluent, 1988–90, 286 samples

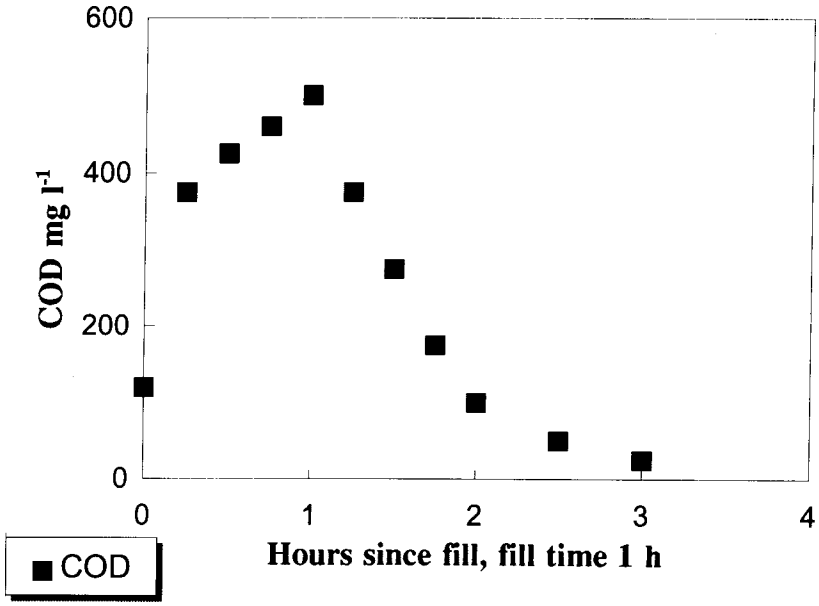

Figure 27.4 *COD removal in a fill and draw reactor*[14]

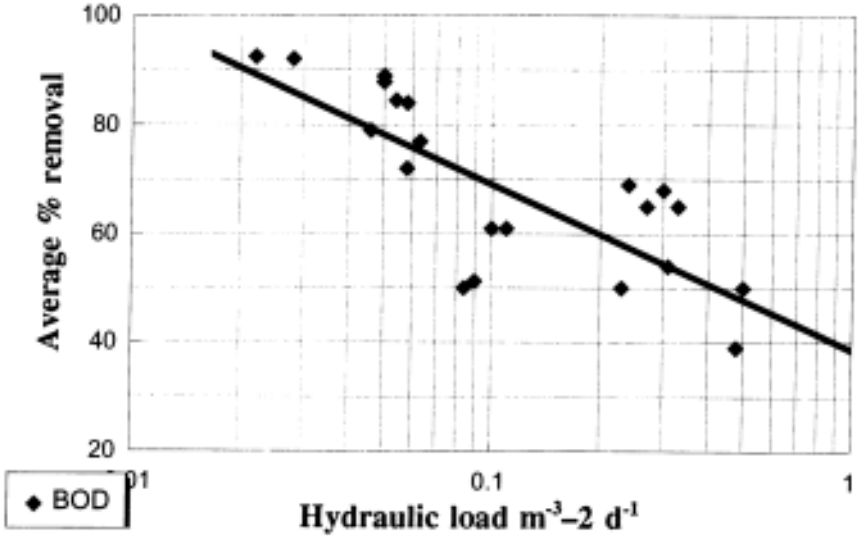

Figure 27.5 *BOD removal related to log of hydraulic load, overland flow wetlands*[15]

Because of the different needs and sizes of receiving watercourses, consent standards for final effluents vary. So the same process, with the same size of biological stage, may be loaded more or less to achieve the consent, with consequent effects on performance. Improvement in the accuracy of the estimates of inhibition should allow the construction and operation of works to tighter and therefore more economic limits.

Biological processes of wastewater treatment usually show a marked fall in removal of degradable matter once a certain concentration has been reached. Figure 27.4 shows the removal of COD in a fill and draw reactor of cycle time 8 h, in which the final COD of a few mg l^{-1} is almost reached after 2 h.[14] Figure 27.5 shows that the concentration of BOD in overland flow wetlands reduces in logarithmic fashion through the works.[15] Therefore the higher the standard of final effluent, the more difficult it is to detect by how much the incoming load could be increased with no significant effect on compliance. Works may have been designed larger than necessary to account for the effects of trade wastes, or to accommodate future increases in wastewater quantity.

Inhibitors may be completely degraded during their passage through the wastewater treatment works, but until they are completely degraded to non-toxic substances, inhibition will occur. The total amount of inhibition experienced by a works would be less if a substance was rapidly degradable, than if it degraded slowly. Figure 27.6 shows the results of toxicity removal test (a laboratory simulation) using Microtox on three wastewaters. One degrades almost immediately, one very slowly but completely and the third degrades rapidly but a small portion of hard toxicity is discharged to river (see Chapter by Fearnside and Booker). Figure 27.7[16] shows how toxicity to *Daphnia* remains fairly constant until degradation of the toxicant reaches a critical point. Acclimatization would perhaps reduce the degree of inhibition experienced by accelerating the breakdown of these substances. Some inhibitors are created during the breakdown of non-toxic substances, as in the case of cationic detergents.

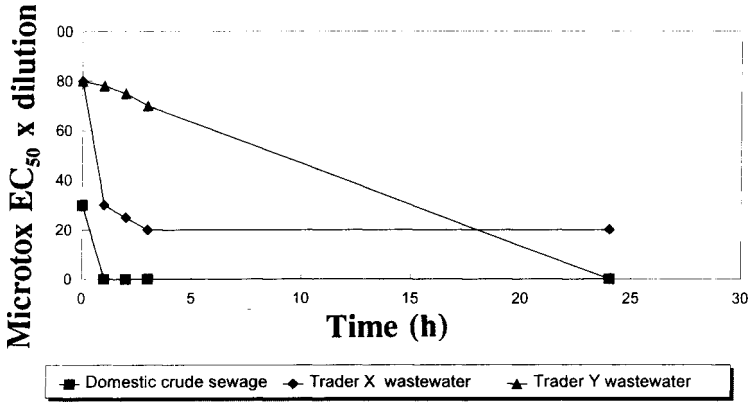

Figure 27.6 *Toxicity removal test using Microtox* (see Chapter by Fearnside and Booker)

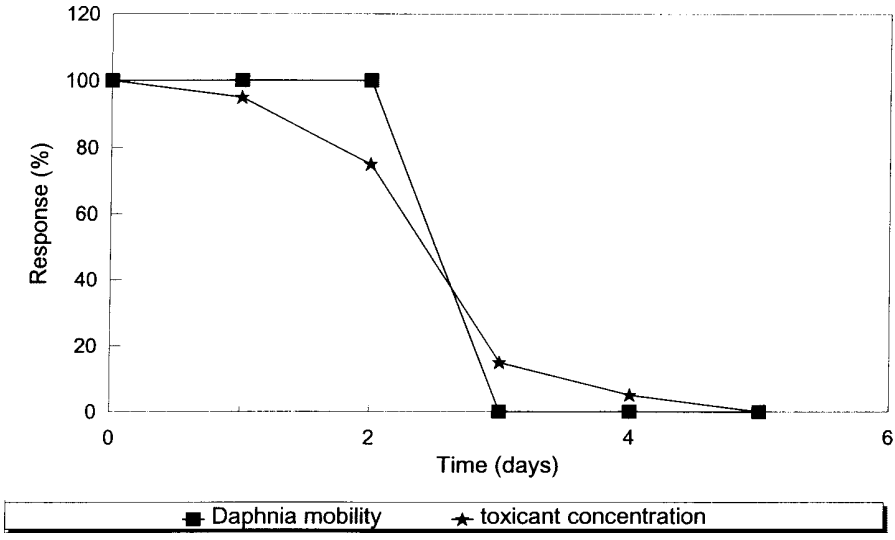

Figure 27.7 *Removal of Daphnia toxicant*[16]

27.4.2 Measurement using simulations

Simulations, such as porous pots and rolling tubes which run for many weeks in order to acclimatize, may be used to estimate performance. Depending on how they are operated they may give a relatively optimistic estimate. Feed variability may be low, aeration better and temperatures may be higher under laboratory conditions. Porous pots avoid solids loss due to bulking which, on the full scale plant, may make it very difficult to build up the desired quantity of sludge. The bulking sludge in the pots can be very efficient (as it would be on the full scale plant if it were possible to retain it in the process). Rolling tubes maintain the active surface area for biological growth in a well aerated condition throughout the year; whereas blockage occurs to a variable extent in the field.

Shorter-term simulations of acclimatized works would have to use the filter slime or activated sludge freshly harvested from the works. Because of the stratification of organisms in a percolating filter and the large amount of dead material often present in samples of filter slime, getting representative samples would be very difficult. The activity of such mixtures would perhaps be measured by ATP assessment, and their treatment of BOD, ammonia, etc. checked against the performance of similarly harvested materials from works treating only domestic wastewater.

Simulations may be valuable in individual cases but seem to be too variable and expensive to act as measures of inhibition across a wide variety of situations.

27.4.3 Measurement of inhibition by short-term toxicity test

Short-term tests (eg, 5 m to 3 h duration), which include measures of respiration and nitrification inhibition, can be standardized using domestic wastewater and OECD synthetic wastewater.[17] These tests generally have detection limits of ~10% inhibition, which would be suitable for the control of individual trade wastes. Additional tests may be appropriate to ensure the efficient operation of the wastewater treatment works and the production of an effluent which complies with the regulatory standards. The suite of tests allows the assessment of the potential degree of harm to crucial parts of the treatment process, and the risk of harm to the receiving watercourses.

The tests on unacclimatized organisms give results which are more reproducible and precise than those obtained when attempts are made to acclimatize the test organisms to the waste/sewage mixture. The harvesting of acclimatized filter slime for use in tests is particularly difficult (see section 27.4.2) These tests can also ensure the wastes potentially harmful to ancillary organisms and/or river life are excluded.

The application of the control of inhibition in this way is a recent development in the United Kingdom, so moves towards better understanding of the tests, the environment they present to the organisms, using more representative conditions and organisms, measuring changes in the organisms which relate to their success in wastewater treatment, understanding of the situations to which they are being applied, etc. are all important to the long-term success of the initiative.

27.5 COSTS

Plant optimization and improvements to process efficiency help water companies' wastewater treatment works to be maintained within their consents and minimize their operating costs. The cost consequences of not controlling toxicity in wastewater relate mainly to the extra capacity which has to be built in order to comply with the required effluent standards. The costs used below are approximate, based on 1993 data, and are intended only as a guide to the magnitude of cost savings involved against a water company's expenditure overall, if inhibition is controlled.

A toxicity survey of 105 works known to be receiving trade waste was made in the Yorkshire water company area. These tended to be the larger works, receiving the majority of the wastewater treated by the company. Thirty-one of the works had an average of 7% inhibition or more to the respiration test, but with a range of 0–89%. All works except one had zero respiration inhibition on at least one sample. A works will probably fail its consent at times of maximum inhibition, so this should be controlled. Fifty-five works had maximum respiration inhibition results of over 20%. They accounted for ~70% of the Yorkshire wastewater (population equivalent 7.5 million). If this measured toxicity caused an equal treatability factor, the saving on total replacement costs if these 55 were improved by 20% would be GB£235 million. Improving the treatability of the wastewater to these works by 5% would save GB£3 million on the annual capital cost of wastewater treatment. To make the

biological stage of works *K,* (Figure 27.2) 25% larger would cost ~GB£16 million. Four works originally studied by Robinson[11] (Table 27.3) have recently been found, using the STAR mathematical model, to have the potential for reduction in replacement costs of GB£16.7 million if the toxicity factors were all restored to one. Bearing in mind the difficulties of measuring performance, the effects of decomposition of toxics during treatment and other modifying factors already discussed, these figures should be seen as a theoretical ideal, with the practically achievable savings being less.

Table 27.3 *Treatability factors and replacement values updates on Robinson*[11]

Wastewater treatment works	Treatability factor	Replacement value (1993 data) GB£ M	Maximum saving on replacement value if toxicity factor = 1, GB£ M
W	0.62	6.8	2.6
X	0.85	66.0	9.9
Y	0.87	7.7	1.0
Z	0.93	45.7	3.2

With limited means of control, no matter the size of the works, there remains the risk of a toxic incident leading to consent failure, damage to river life and consequent costs. Given the problems of measurement of inhibition on a wastewater treatment works, accepting inhibitory wastes implies that there will be little effective control of the amount of inhibition received. The works will have to be built large enough to cope with the worst conditions. Ways of minimizing the environmental damage from incidents where a warning is given were discussed in Fearnside and Hiley.[6] The decision depends on the nature of the toxicants, eg, a degradable fish poison could be passed through the works safely at a rate which ensured it was treated; whereas a poorly degradable bactericide might severely inhibit the wastewater treatment works, allowing untreated wastewater to flow to the river for several weeks subsequently. If dischargers are accustomed to the control of toxicity, measures to minimize the occurrence of incidents and to deal with them effectively are much easier to implement. This has indirect value to the discharger through being demonstrably 'environmentally friendly'.

27.6 CONTROLLING TOXIC/INHIBITORY WASTES TO NO-EFFECT STANDARDS

The assessment of the toxicity of wastes to unacclimatized treatment processes and control to no-effect standards is based on assumptions including:

(i) Acclimatization is not sufficiently reliable to assure the required degree of compliance with effluent consent standards; and
(ii) Inhibitors do not lose their toxicity until the end of the biological stage.

Over a series of samples of each of the wastes discharged to a works, the maximum potential inhibition could be calculated from the results of relevant toxicity tests. This permits prioritization of control measures, towards effective achievement of compliance with effluent consent standards at the least cost. Ultimately, all sources of toxicity in wastewater should be controlled to have 'domestic wastewater characteristics', ie, to have no adverse effect on the wastewater treatment processes.

Laboratory tests of toxicity and treatability can rarely detect below 10% inhibition, but this would have large cost implications were it found in the wastewater. Controlling individual trade wastes with these tests brings the wastewater toxicity to a lower level but large volumes of trade waste may still cause significant potential inhibition in the wastewater. This approach represents the best control currently available.

If one assumes that the potential inhibition the waste causes a wastewater treatment works has the same effect as increasing the BOD load of the receiving wastewater treatment works by that percentage, the BOD equivalent of the toxicity can be calculated. The % inhibition data on an individual discharge into the sewer may be used to calculate the effect on the wastewater entering the wastewater treatment works as follows: A waste giving an EC_{50} to nitrification inhibition of 6.7% means that a 6.7% solution of the waste in the wastewater would cause 50% inhibition and a 6.7/50%=0.134% solution would cause 1% inhibition, ie, adding 1% to the effective BOD load on the works.

27.7 DISCLAIMER

The opinions expressed in this chapter are those of the author and not necessarily those of Yorkshire Water plc.

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SECTION 7: EPILOG

28 EPILOG

Mervyn Richardson

28.1 RATIONALE

Environmental toxicology assessment has to take into account hazards and their associated risks to both exposed persons and to all environmental media. This implies that in order to undertake such assessments it is of paramount importance to appreciate both the mammalian and effects to non-mammalian species.

Recent advances in information technology have ensured that ecotoxicological and toxicological data are now readily available to scientists. There are many standard text books, dictionaries and encyclopedias, learned journals, abstracting journals, of which the most widely known and extensive are Chemical Abstracts and BIOSIS. However, some care is necessary when using secondary sources such as dictionaries as they too often contain errors and recourse should be made to the original references. Abstracts are also available with other such sources in the increasing number of databanks and databases—many of the latter also now being available on CD-ROM. Whilst these may be available to the majority of scientists in the developed countries, regretably this is not the case in the developing countries.

One hour prior to writing this epilog a telephone call was received from a scientific colleague in a Central European emerging country requesting details on tributyl tin compounds that were to be used for painting the hulls of boats in marinas. Data sources such as those previously mentioned are not yet available to such colleagues. In Odessa in the Ukraine, the acquisition of Chemical Abstracts ceased in the late 1980s. In some countries in Asia telecommunications are too unreliable and far too expensive for scientists to facilitate their use. Far too many scarce resources, time and effort are being expended in repetitive research. Perhaps CD-ROMs and the INTERNET will overcome some of these problems.

Having established that a substance may be toxic (but at the same time remembering that toxic does not mean unacceptable), then that hazard needs to be integrated with a measurement to provide a risk assessment.

28.2 MEASUREMENT

Measurement of the extent of the toxic insult of a chemical, effluent stream, or any type of effluvia or emission in a working environment, or to the natural environment is a vital link in the stages in undertaking an *environmental toxicology assessment*.

During the last few decades analytical chemists and instrument manufacturers have developed increasingly sophisticated devices for the measurement of both inorganic and organic chemicals at ever decreasing concentrations in a variety of environmental media, body fluids, etc. Regretably, despite this sophistication, their success in measuring pesticide residues in water is poor. Of the 700 or so pesticides now in common usage it remains only possible to determine $\not>100$ in potable water samples at the 0.1 $\mu g\ l^{-1}$ concentration stipulated by the European Union Drinking Water Directive (80/778/EEC).

Whilst a number of pesticides involve reasonably complex molecules, other but simpler molecules are now of considerable concern. The chapter by Legube illustrates one of these, namely bromate. Whilst analytical chemical methods exist, they are inadequately sensitive. The concern over bromate in drinking water is not a matter of recent anxiety, as the problem was shown to be of potential concern by Thames Water Authority's Catchment Quality Control studies in the 1980s.

Within the British privatized water industry, far too many discrete measurements are being made. One such company, in its annual report, boasted as having made >1 million determinations! In the 1970s such results were neatly written in ledgers; in the 1990s, they are stored in more expensive ledgers—called computers. The concept of undertaking such colossal numbers of analyses is neither the best means of using of these facilities, nor the utilization of their customers' payments. The goal for the future has to be *environmental toxicology assessments*. One wonders, in the case of the water company who undertook the >1 million analyses, how many risk assessments were undertaken and their results published. It is conjectured that even less risk managements were undertaken to reduce such risks, ie, if these large numbers of determinants were justified from the projected hazards. It is foreseen that except perhaps for spillages and some other acute conditions, the days of such sophisticated chemical analytical techniques are numbered.

In the future, one will need to consider biological measurements. Some of the elegant techniques outlined in Sections 2 and 3 of this book illustrate the way forward. Techniques based on luminescent bacteria as outlined by Bulich and Bailey are extensions on methods which have been used rigorously for over a decade. The newer techniques using umu-C assays, tissue cultures and cytochrome P-450 as outlined by Hansen have considerable promise and certainly do not attract the problems which are so well known in using whole animals, especially fish, for continuous monitoring. However, one of the most original concepts is the use of cytochrome P-450 with DNA probes and fish hepatocytes, as illustrated by Gagné and Blaise in their chapter outlining their new research. This surely is a technique to be developed in the future.

Such generic techniques have enormous potential over discrete measurements, as aptly described by McHenery, Hiley, Fearnside and Booker, and others, the true chemical nature of effluents are rarely known. Even if they were known, it will be a brave scientist who would stipulate the many chemical reactions and interactions that may occur in wastewater systems, and even braver the biochemist who would predict the reactions induced by microorganisms in wastewater treatment works. The maize of

possible pathways that can occur in one species, *Vibrio fischeri,* is aptly described in the chapter by Bulich and Bailey.

Hence the way forward surely has to be generic measurements, especially those at the molecular level. Macroorganisms, such as algae and mosses, also have their place, as do structure-activity relationships (see Section 3).

In highly polluted areas, simple observations are often adequate for the initial audit (see Chapter by Hoggart)—are there any bees, birds, or butterflies in existence; does a river support ducks and fish; are trees suffering from phytotoxicity from acid rain or other causes?

28.3 ENVIRONMENTAL RISK

The estimation of the risk to human health from both xenobiotic and natural substances within our society has become increasingly critical. Toxic effects to both animals and other species are undertaken experimentally at high doses, usually for short periods. However, low-level chronic exposure effects largely remain unknown or could be biological, eg, induction of cytochrome enzymes—whilst for the short-term administration of drugs this is acceptable. It is not so for chronic exposure to environmental pollutants. Furthermore, ecotoxicological effects to individuals are often of less importance than are the effects to whole ecosystems and to populations. Reliance on extrapolation from animal studies can, and often has, led to a serious underestimation or overestimation of the risk.

Regulations and legislation based on overestimates can have serious and unnecessary economic consequences by keeping economically desirable products from being continued to be marketed and used to enact clean-up operations which have little real benefit. No action can equally be disastrous and many examples of both *laissez-faire* policies, often resulting from political dogma, are well documented in Central and Eastern Europe and other developing countries.

Properly conducted animal experiments and epidemiological studies provide a basis for the linkage between human health risks and environmental factors; from such associations, public health and environmental protection policies can be developed so as to minimize the risks to current and future populations.

28.4 BIOSENSORS

The development of biosensors, eg, biological or molecular markers that can illustrate a measurable effect in a biological media, tissues, cells or fluids, has to be one of the key developmental tasks for the end of the 20th century. Such markers will develop the concept of *toxic insult* without the precise chemical knowledge of the toxic agent. This in turn leads to a marker indicating a biologically effective dose, ie, the amount of absorbed chemical that has interacted with critical subcellular targets, measured in either a target or surrogate tissue. Hence, sequentially, exposure will lead to internal dose → biologically effective dose → biological response → altered structure/function

→ disease → prognosis; these stages will take into account susceptibility and an environmental lifestyle.

Through the development of biosensors or biomarkers and their associated molecular and toxicological techniques, an understanding of the intermediate steps between exposure and disease occurrence can improve the precision of the mode of exposure and outcome measurements.

If epidemiological techniques are also deployed, as they include large numbers of participants, the biological markers that are most desirable for use in human studies are those that may be measured with a minimum skill or equipment requirement in the smallest quantity of biological media and can be obtained with minimal invasive techniques. Additionally, these should be inexpensive, sensitive, as specific as possible, robust, peer reviewed and reliable. For such research and collaboration to succeed, researchers from many disciplines must learn the strength, weakness and, above all, the 'language' of other disciplines. Only by such undertakings can *environmental toxicology assessments* be successful.

28.5 ENVIRONMENTAL ASSESSMENT

Aquatic environmental assessment have reached a far more advanced stage than have those for soil, air and even human health. Such assessments are involved with ecosystems and the protection of their functions and hence are elevated towards populations. Currently, the target for the protection of an ecosystem function is as follows. If the most sensitive species has protection within a 95% confidence limit, taking into account two exposure routes:

- Direct environmental exposure from both point and diffuse sources; and
- Indirect exposures via food chains.

It will be necessary to consider separately the hazards to human health and the natural environment and subsequently integrate the results in one risk assessment. It is stressed that steps taken to reduce occupational exposure in some cases could increase environmental exposure. Conversely, replacement of an environmentally hazardous substance may have greater human toxicity. In order to obviate this, consideration needs to be given to a totally integrated approach.

28.6 ENVIRONMENTAL EXPOSURE ASSESSMENT

Environmental exposure assessment studies need to include both direct exposure of organisms from discharges and other related emissions and also indirect exposure through bioaccumulation in food chains. In determining direct environmental exposure the following criteria need to be appraised:

- Identification of the lifecycle of the substance;
- Identification of the target environmental compartment; and
- Estimation (or measurement wherever practical) of the environmental concentration.

The lifecycle of a substance will need to include all the stages from manufacture to ultimate disposal and the best means for a new substance is to include these steps during the research into its synthesis. The steps include:

- Manufacture;
- Transport/storage;
- Formulation (including production of preparations and articles);
- Use;
- Recovery/recycling; and
- Disposal.

All these steps require assessment which has to include potential releases of the substances to the environment. Not every chemical will need to be studied for all of these steps and the assessor will need to decide which are relevant.

For new substances the following are normal for estimating the points of release and target compartments:

- Production—exposure estimates;
- Proposed uses—exposure estimates, concentration in formulations and the proposed0 uses;
- Production (or imported quantity)—for every intended use; and
- Disposal—methods of destruction/disposal, including recycling.

The key criteria is the use category, eg, dyestuffs, lubricants, polymers, pesticides, domestic paints/inks, paper chemicals, photochemicals, plastic additives, intermediates, etc.

After identification of the lifecycle stages, the environmental compartment to which the release is most likely has to be determined. These include mobile compartments and air and water, however, the overriding consideration needs to be the ultimate fate of the substance following transport to, or through, other compartments. A substance's physico-chemical properties may result in absorption or adsorption on a sediment following release to water or on sludge during sewage treatment. Spreading of sludge on soil can result in terrestrial environmental contamination; a volatile substance present in the aquatic environment may be transported to the air, and a discharge to the atmosphere may lead to aquatic contamination via precipitation.

28.7 ASSESSMENT OF DATA

Toxicity test information may not be available for the key organisms in the ecosystems most at risk and cautious extrapolation will usually be inevitable. Toxicity tests may not relate to the naturally occurring conditions in regard to such parameters as temperature, water hardness, oxygen availability, trace metal content and the presence of other potentially toxic substances.

Assessment of environmental hazard and risk is not something that can be done simply by a set of rules: it is a continually developing skill which requires dedicated expertise and current awareness of the state of relevant knowledge.

Ecotoxicology, being a very new science and incorporating many disciplines, is extremely dynamic and hence requires constant vigilance to the importance of new initiatives. However, some generalizations can be made:

- An estimate must be made of the levels at which any potentially hazardous substance will appear in the environment and be taken in by living organisms including, ultimately, people. This is an estimate of probable exposure.
- An estimate must be made of the levels likely to be harmful to the organisms at risk. This is an estimate of dose-effect and dose-response relationships under expected conditions of exposure.
- The assessment should include whether environmental release and exposure may lead to human exposure.
- Initially, an estimation of the exposure can be made on the basis of simple transfer functions which relate between partitioning between air, water, soil, vegetation, animal.diet, lipid tissue and food products.
- It is recommended that assessments for air, water and soil concentrations should be undertaken on a regional basis and to use diets for the average in a region.
- Insights into the limitations and uncertainties of the transfer of substances between environmental media or quantitative structure activity relationships (QSARs) used in the indirect exposure assessment should be developed. Currently, such relationships have been deduced only for a limited range of classical hydrophobic substances and extrapolation to other substances need to be considered with extreme care.
- Better estimates of the variability in food habits and sourcing within and between regions should be compiled.
- Assessments could be further improved by incorporating concentration data for air, water, soil and food products, where available, even if these are generic measurements.

A comparison of these sets of data will indicate the likely safety margin if the chemical under consideration enters the environment following the mode of use or disposal assumed in the initial estimates. This safety margin should be very large before use and release of a chemical into the natural environment can be justified.

28.8 CONCLUDING COMMENTS

The 20th century is drawing to a close. Our planet Earth faces environmental crises unprecedented in human evolution. Entire ecosystems are threatened by human exploitation, exacerbated by increasing human activities and population. The disappearance of some ecosystems has the potential to create chaotic change on the scale that might even approach that of the asteroid impact which induced catastrophes seen in geological time.

There is a need to understand the forces that drive human self-destructive practices and this is why *environmental toxicology assessments,* leading to risk reduction, are imperative in the face of sustained human intervention. We must all strive towards sustainable development.

Contemporary human evolution is unique as it has been derived from non-genetic factors. Non-human life forms pass information genetically or by genetics coupled with learning. This too was the case until recently with human evolution. As with other advanced predators having social structures, human young learned information passed from the previous one or two generations. Human societal behavior and information technologies have reduced the significance of such limitations with the emergence of our complex spoken language, and hence initiated a divergent evolutionary recourse. A greater divergence occurred as the written word developed. Hence, humanity must now accept that further environmental damage which followed these developments cannot continue unabated. Control of the destiny of our planet, Earth, the only home we currently can inhabit has to be via non-genetic evolution and must accept that the consequences to nature of continuing irresponsibility.

Perhaps with the consensus and a sense of common purpose, coupled with pragmatic *environmental toxicology assessment* and management, the realities of survival in a complex but finite living system can be obtained for the future benefit of all countries, all peoples, and all organisms on which *Homo sapiens* depend.

28.9 ACKNOWLEDGEMENTS

The editor acknowledges with thanks the permission to reproduce sections 28.5–28.7 from the revised 'Environmental Toxicology and Ecotoxicology' section of the International Programme on Chemical Safety's Training Manual on The Fundamentals of Applied Toxicology, The Nature of Chemical Hazards, World Health Organization, Geneva, 1995.

Integration of environment and development concerns and greater attention to them will lead, to the fulfilment of basic needs, improved living standards for all, better protected and managed ecosystems and a safer, more prosperous future. No nation can achieve this on its own; together we can—in a global partnership for sustainable development.

Agenda 21, Chapter 1, Rio Conference, 1992.

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