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ENVIRONMENTAL CHEMISTRY

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Effect-Directed Analysis of Complex Environmental Contamination

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The Handbook of Environmental Chemistry
ISSN 1867-979X e-ISSN 1616-864X
ISBN 978-3-642-18383-6 e-ISBN 978-3-642-18384-3
DOI 10.1007/978-3-642-18384-3
Springer Heidelberg Dordrecht London New York

Library of Congress Control Number: 2011926782

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Cover design: SPi Publisher Services

Printed on acid-free paper

Springer is part of Springer Science+Business Media (www.springer.com)

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Aims and Scope

Since 1980, *The Handbook of Environmental Chemistry* has provided sound and solid knowledge about environmental topics from a chemical perspective. Presenting a wide spectrum of viewpoints and approaches, the series now covers topics such as local and global changes of natural environment and climate; anthropogenic impact on the environment; water, air and soil pollution; remediation and waste characterization; environmental contaminants; biogeochemistry; geo-ecology; chemical reactions and processes; chemical and biological transformations as well as physical transport of chemicals in the environment; or environmental modeling. A particular focus of the series lies on methodological advances in environmental analytical chemistry.

Series Preface

With remarkable vision, Prof. Otto Hutzinger initiated *The Handbook of Environmental Chemistry* in 1980 and became the founding Editor-in-Chief. At that time, environmental chemistry was an emerging field, aiming at a complete description of the Earth's environment, encompassing the physical, chemical, biological, and geological transformations of chemical substances occurring on a local as well as a global scale. Environmental chemistry was intended to provide an account of the impact of man's activities on the natural environment by describing observed changes.

While a considerable amount of knowledge has been accumulated over the last three decades, as reflected in the more than 70 volumes of *The Handbook of Environmental Chemistry,* there are still many scientific and policy challenges ahead due to the complexity and interdisciplinary nature of the field. The series will therefore continue to provide compilations of current knowledge. Contributions are written by leading experts with practical experience in their fields. *The Handbook of Environmental Chemistry* grows with the increases in our scientific understanding, and provides a valuable source not only for scientists but also for environmental managers and decision-makers. Today, the series covers a broad range of environmental topics from a chemical perspective, including methodological advances in environmental analytical chemistry.

In recent years, there has been a growing tendency to include subject matter of societal relevance in the broad view of environmental chemistry. Topics include life cycle analysis, environmental management, sustainable development, and socio-economic, legal and even political problems, among others. While these topics are of great importance for the development and acceptance of *The Handbook of Environmental Chemistry,* the publisher and Editors-in-Chief have decided to keep the handbook essentially a source of information on "hard sciences" with a particular emphasis on chemistry, but also covering biology, geology, hydrology and engineering as applied to environmental sciences.

The volumes of the series are written at an advanced level, addressing the needs of both researchers and graduate students, as well as of people outside the field of "pure" chemistry, including those in industry, business, government, research establishments, and public interest groups. It would be very satisfying to see these volumes used as a basis for graduate courses in environmental chemistry. With its high standards of scientific quality and clarity, *The Handbook of*

Environmental Chemistry provides a solid basis from which scientists can share their knowledge on the different aspects of environmental problems, presenting a wide spectrum of viewpoints and approaches.

The Handbook of Environmental Chemistry is available both in print and online via www.springerlink.com/content/110354/. Articles are published online as soon as they have been approved for publication. Authors, Volume Editors and Editors-in-Chief are rewarded by the broad acceptance of *The Handbook of Environmental Chemistry* by the scientific community, from whom suggestions for new topics to the Editors-in-Chief are always very welcome.

Damià Barceló
Andrey G. Kostianoy
Editors-in-Chief

Volume Preface

Economic and social development of human societies comes along with the production, use, and emission of constantly increasing numbers of chemicals. At present, about 100,000 chemicals are in daily use while more than 50 Mio chemicals are known and registered in the Chemical Abstracts System (CAS). Comprehensive chemical monitoring of all these chemicals together with the enormous number of by- and transformation products is impossible and because of the limited availability of effect data not very helpful. Thus, effect-directed analysis (EDA) and related approaches were designed to direct chemical analysis toward those chemicals that actually cause hazards mostly indicated by laboratory in vitro and in vivo bioassays. The basic assumption behind EDA is that although ecosystems and humans are exposed to complex mixtures of compounds, only few toxicants dominate adverse effects. The identification of these toxicants is the key to efficient mitigation of toxic risks.

EDA is based on biotesting of environmental mixtures in combination with a sequential reduction of mixture complexity by fractionation. The procedure is directed by the biotests aiming to remove compounds without significant contribution to sample toxicity and isolating and identifying predominant toxicants. This book aims to give an overview on concepts and methodologies that are or may be applied in EDA to identify toxicants in complex environmental mixtures and to establish cause–effect relationships between chemical contamination and measurable effects.

Toxicity identification evaluation (TIE) based on whole effluent testing (WET) is a scientific concept and a regulatory approach developed by the US Environmental Protection Agency (EPA) to characterize and identify chemicals in toxic effluents causing effects to aquatic organism. Historical backgrounds, concepts, and procedures of TIE that inspired a lot of development in EDA all over the world are presented in Chapter "Early Evolution of the Toxicity Identification Evaluation Process: Contributions from the United States Environmental Protection Agency Effluent Testing Program." New developments in TIEs of whole sediments as discussed in Chapter "Recent Developments in Whole Sediment Toxicity Identification Evaluations: Innovations in Manipulations and Endpoints" include innovative manipulation procedures and the integration of genomic endpoints. A key issue

of EDA and TIE of contaminated sediments is bioavailability in order to avoid a bias toward toxic but nonavailable compounds. Concepts and approaches to address this issue are presented in Chapter "Considerations for Incorporating Bioavailability in Effect-Directed Analysis and Toxicity Identification Evaluation."

While classical chemical analysis focuses on preselected target compounds, the driving force of EDA and the parameter directing chemical analysis are effects. Diagnostic mechanism-based in vitro assays not only represent relevant toxicological endpoints that may drive EDA, but also provide a valuable tool for characterizing underlying causes (Chapter "Diagnostic Tools for Effect-Directed Analysis of Mutagens, AhR Agonists, and Endocrine Disruptors"). These causes may be one, few, or many toxicants occurring in complex mixtures of natural and anthropogenic compounds present in the environment. To reduce the complexity of these mixtures by separation according to physicochemical properties is a crucial precondition for successful identification of the toxicants causing observed effects. In Chapter "Separation Techniques in Effect-Directed Analysis," separation techniques and underlying theoretical concepts are discussed that may help to fractionate, isolate, and identify toxicants in EDA. Affinity-based screening technologies including affinity chromatography are powerful tools used to exploit noncovalent binding of ligands to biological receptors for separation and characterization of specific toxicants and maybe of increasing relevance in EDA (Chapter "Simultaneous Screening and Chemical Characterization of Bioactive Compounds Using LC-MS-Based Technologies (Affinity Chromatography)"). When toxic fractions are isolated, these may still contain several compounds indicated by peaks in liquid or gas chromatograms. Many of them may be unknowns that require structural elucidation with advanced GC-MS and LC-MS tools as described in Chapter "Advanced GC–MS and LC–MS Tools for Structure Elucidation in Effect-Directed Analysis." However, highly sophisticated analytical tools need the support of powerful computer tools including databases, structure generation, and classifiers for candidate selection for successful and time- and cost-efficient structural elucidation (Chapter "Computer Tools for Structure Elucidation in Effect-Directed Analysis").

After discussing the major tools in EDA, this book wants to provide some prominent examples demonstrating the power and also the shortcomings of this approach. This starts with EDA of mutagens in ambient airborne particles, which has been an early focus of EDA and is still of great relevance particularly for human health (Chapter "Effect-Directed Analysis of Mutagens in Ambient Airborne Particles"). In Chapter "Effect-Directed Analysis of Endocrine Disruptors in Aquatic Ecosystems" on EDA of endocrine disruptors in aquatic ecosystems, examples are presented where EDA provided new insights into estrogens, androgens, progestagens, glucocorticoids, and thyroid hormone-like compounds in the environments. Pulp and paper mills are important sources of contamination of aquatic ecosystems in many countries emitting highly complex and hazardous effluents. EDA played a substantial role in characterizing and mitigating this problem as discussed in Chapter "Effects-Directed Studies of Pulp and Paper Mill Effluents." As a last example, Ah-receptor-mediated toxicants, mutagens, and endocrine disruptors in

sediments and biota are presented in Chapter "Effect-Directed Analysis of Ah-Receptor Mediated Toxicants, Mutagens, and Endocrine Disruptors in Sediments and Biota."

Since most EDA studies are based on in vitro and simple in vivo biotests, the relevance of EDA results for higher levels of biological organization in situ may be a matter of discussion. Tools that help to confirm the impact of identified toxicants on organisms, populations, and communities are in the focus of Chapter "Ecological Relevance of Key Toxicants in Aquatic Systems."

I want to thank all the contributors to this book and hope that the presented approaches, tools, and application examples help to support a new generation of monitoring and risk assessment focusing more on biological responses and the chemicals causing these responses than on preselected priority pollutants. The rapid increase in the number of chemicals produced, used, and emitted to the environment together with the many byproducts and transformation products cannot be addressed with target monitoring alone based on priority lists, which may need years to decades to be updated. Effect monitoring together with EDA approaches may help to increase environmental realism, to avoid that emerging toxicants are overlooked and to direct limited resources for monitoring and management to those chemicals providing major risks.

Leipzig, Germany Werner Brack
January 2011

Acknowledgments

This book was supported by the European Commission and provides an outcome of the Integrated Project MODELKEY (Contract No. 511237-GOCE) and of the Network of Reference Laboratories for Monitoring of Emerging Environmental Pollutants (NORMAN).

Contents

Early Evolution of the Toxicity Identification Evaluation Process: Contributions from the United States Environmental Protection Agency Effluent Testing Program 1
Gerald T. Ankley, James R. Hockett, Donald I. Mount, and David R. Mount

Recent Developments in Whole Sediment Toxicity Identification Evaluations: Innovations in Manipulations and Endpoints 19
Robert M. Burgess, Kay T. Ho, Adam D. Biales, and Werner Brack

Considerations for Incorporating Bioavailability in Effect-Directed Analysis and Toxicity Identification Evaluation 41
Werner Brack and Robert M. Burgess

Diagnostic Tools for Effect-Directed Analysis of Mutagens, AhR Agonists, and Endocrine Disruptors 69
Gisela Umbuzeiro, Miroslav Machala, and Jana Weiss

Separation Techniques in Effect-Directed Analysis 83
Werner Brack, Nadin Ulrich, and Mahmoud Bataineh

Simultaneous Screening and Chemical Characterization of Bioactive Compounds Using LC-MS-Based Technologies (Affinity Chromatography) 119
Martin Giera and Hubertus Irth

Advanced GC–MS and LC–MS Tools for Structure Elucidation in Effect-Directed Analysis 143
Pim E.G. Leonards, Rikke Brix, Damià Barceló, and Marja Lamoree

Computer Tools for Structure Elucidation in Effect-Directed Analysis 167
Emma Schymanski, Tobias Schulze, Jos Hermans, and Werner Brack

Effect-Directed Analysis of Mutagens in Ambient Airborne Particles 199
John L. Durant and Arthur L. Lafleur

Effect-Directed Analysis of Endocrine Disruptors in Aquatic Ecosystems 237
Corine J. Houtman, Juliette Legler, and Kevin Thomas

Effects-Directed Studies of Pulp and Paper Mill Effluents 267
Mark Hewitt

Effect-Directed Analysis of Ah-Receptor Mediated Toxicants, Mutagens, and Endocrine Disruptors in Sediments and Biota 285
Markus Hecker and John P. Giesy

Ecological Relevance of Key Toxicants in Aquatic Systems 315
Mechthild Schmitt-Jansen, Peter C. von der Ohe, Stephanie Franz, Stefanie Rotter, Sergi Sabater, Dick de Zwart, and Helmut Segner

Index 341

Early Evolution of the Toxicity Identification Evaluation Process: Contributions from the United States Environmental Protection Agency Effluent Testing Program

Gerald T. Ankley, James R. Hockett, Donald I. Mount, and David R. Mount

Abstract During the 1980s, whole effluent toxicity testing was incorporated into the regulatory control program for municipal and industrial effluents in the USA, as a complement to chemical-specific limitations. While regulating effluent toxicity offered several advantages, it also required the development of means to identify and control sources of toxicity within effluents, which could include toxicants not previously monitored or even known. To meet this need, the US Environmental Protection Agency developed an effects-directed analysis procedure called "toxicity identification evaluation". This involved a suite of physical/chemical manipulations that are applied to aliquots of a toxic effluent sample, and the relative effects of these manipulations on effluent toxicity are used to infer the type of toxicant(s) responsible for toxicity, and to guide their isolation and analytical identification. This chapter provides an overview of these methods and their component phases: I – Characterization, II – Identification, and III – Confirmation. Case examples of toxicant identification in effluents from municipal and industrial sources are discussed, along with a broad summary of the types of toxicants identified, and the characteristics of those toxicants that helped guide their assessment.

Keywords Effluent, Toxicity, Toxicity identification evaluation

Contents

1 Brief History of Effluent Regulation in the USA 2
2 Overview of the EPA Toxicity Identification Evaluation Process 4
3 Conceptual Considerations in Effects-Directed Analysis 6
3.1 Bioassay Selection 6
3.2 Sample Manipulations 7
3.3 Interpreting Test Data 9

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W. Brack (ed.), *Effect-Directed Analysis of Complex Environmental Contamination*, Hdb Env Chem (2011) 15: 1–18, DOI 10.1007/978-3-642-18384-3_1,
© Springer-Verlag Berlin Heidelberg 2011

4 Illustrating the Concepts: Select TIE Case Studies . . . 9
4.1 Case Study 1: Diazinon . . . 9
4.2 Case Study 2: Surfactants . . . 10
4.3 Case Study 3: Hexavalent Chromium . . . 12
5 Summary Findings from Effluent TIEs . . . 13
6 Advantages of EDA/TIE over Other Toxicity Control Approaches . . . 16
References . . . 17

1 Brief History of Effluent Regulation in the USA

Prior to 1970, most water pollution control efforts in the USA were focused on sanitation and human health; there was little coordinated regulation of the discharge of toxic chemicals to surface waters, particularly for the purpose of protecting aquatic organisms. Many lakes and rivers in industrialized areas were extremely polluted. In the 1960s, the Federal Water Pollution Control Administration issued annual reports chronicling pollution-induced fish kills, and in 1966 listed industrial pollution and municipal wastewater as the two most important causes among the reported 436 pollution-related fish kills spread across 46 states [1]. Of the Cuyahoga River in Ohio, the August 1, 1969 issue of TIME magazine said:

> Chocolate-brown, oily, bubbling with subsurface gasses, it oozes rather than flows. "Anyone who falls into the Cuyahoga does not drown," Cleveland's citizens joke grimly. "He decays." The Federal Water Pollution Control Administration dryly notes: "The lower Cuyahoga has no visible signs of life, not even low forms such as leeches and sludge worms that usually thrive on wastes." It is also – literally – a fire hazard. A few weeks ago, the oil-slicked river burst into flames and burned with such intensity that two railroad bridges spanning it were nearly destroyed.

Through the Federal Water Pollution Control Amendments of 1972, the Clean Water Act of 1977, and other related legislation, the US Environmental Protection Agency (EPA) was charged with developing and implementing approaches and requirements to control the release of pollutants to the waters of the USA. Early efforts focused primarily on so-called conventional pollutants, such as biochemical oxygen demand, fecal coliform bacteria, and suspended solids, with later expansion to a set of "priority pollutants," which included several metals and common industrial chemicals. The approach to controlling these pollutants relied on "technology-based" treatment standards, which established the allowable amounts that could be discharged based on the category of industry and the degree that existing technology could remove those pollutants with reasonable expense.

While these early efforts were effective at reducing some important sources of aquatic pollution, their scope and structure was found to be insufficient to adequately protect surface waters from chemical pollution. Because discharge limits were based on the ability of treatment technology to remove chemicals rather than on maintaining adequate water quality in the water body receiving the

discharge, there was no assurance that treatment would render a discharge nontoxic to aquatic life. Further, these technology-based limits focused on a limited number of pollutants rather than the much larger range of chemicals potentially present in industrial and municipal effluents. In 1984, a major step forward was taken with the EPAs issuance of a new national policy, *Development of Water Quality-Based Permit Limitations for Toxic Pollutants* (US Federal Register, 49:9016, 1984). This policy included two very important new directions for effluent control: (a) acceptable discharges of individual toxic chemicals would be based on maintaining safe concentrations in the receiving stream; and (b) biological methods (e.g., toxicity tests) would be used, in addition to chemical-specific limitations, to establish limits on the release of toxic chemicals. Toxicity tests were an important addition to the regulatory approach because they address several shortcomings in a chemical-specific approach. As outlined in that policy, these shortcomings include (a) the great number of toxic chemicals that may potentially be discharged into receiving waters and the difficulty in their analysis, (b) the changes in toxic effects of a chemical resulting from reactions with the matrix in which it exists, and (c) the inability to predict the effects of exposure to combinations of chemicals.

Measuring the aggregate effects of all effluent constituents in what became known as “whole effluent toxicity” (WET) tests overcame these weaknesses, because a toxicity test measures the combined effects of all chemicals in the effluent, known and unknown, in the context of the water chemistry of the effluent and/or receiving water. This provides a critical backstop to chemical-specific approaches, because one does not have to know anything about the presence or toxicology of a chemical in order to monitor its potential effect on aquatic organisms.[1]

While the concept of WET testing as a monitoring tool was (and remains) very appealing, its comprehensiveness could be simultaneously its greatest strength and greatest weakness. Even though a WET test can detect an effect from virtually any chemical, the finding of an effect in a WET test is essentially generic with respect to its cause – almost any chemical, including unknown chemicals, could be the cause of an observed effect. Because treatment technologies are generally designed around knowledge of the type of pollutant to be controlled, simply knowing that an effluent was toxic provided very little direction toward the means by which toxicity could be controlled. To make control of effluent toxicity practical it was highly desirable, if not essential, to develop a means by which the specific cause of toxicity could be identified, so that targeted and cost effective means could be found to control that cause.

[1]While WET testing is a powerful tool, it is important to note that its ability to detect the effects of toxic chemicals is limited to the types of effects that can be measured in the toxicity test procedures used. As such, effects such as those on secondary consumers mediated through food chain transfer, on life stages not tested, or on organisms more sensitive than those tested, may not be detected and must be evaluated by other means.

It was in response to this challenge that the EPA developed an effects-directed analysis (EDA) approach to identifying the cause of toxicity in toxic effluents or ambient waters. This EDA process, termed a "toxicity identification evaluation (TIE)", is the focus of the remainder of this chapter. In the following sections, we provide a brief overview of the original TIE procedures developed, followed by a more detailed discussion of the philosophical basis of the approach relative to choice of biological system/endpoints, sample manipulations and interpretation of test results. Finally, to illustrate the TIE process, including the logic involved in data collection and interpretation, we describe several case examples in which specific chemicals/classes of chemicals were successfully identified as causative of toxicity in effluents.

2 Overview of the EPA Toxicity Identification Evaluation Process

The EPA toxicity-based approach to effluent regulation necessitated the development of EDA techniques that fundamentally differed from more limited methods used to that point in the field of environmental toxicology. Previously, EDA approaches had been applied with some success to simplifying complex environmental mixtures that caused mutagenicity in bacteria (e.g., [2–4]). However, application of these approaches to more complex responses associated with acute or chronic toxicity in higher organisms was less successful (e.g., [5–8]). There were multiple reasons for this lack of success, but the most important involved how the test samples (typically surface water, effluent, or sediment) were handled. Specifically, many of the manipulations involved extraction and/or fractionation using relatively harsh techniques, often with strong solvents. This confounded the EDA process from several perspectives, including (a) altering the bioavailability of contaminants in the original samples (e.g., extracting/concentrating compounds that did not contribute to toxicity in the intact sample), (b) loss of some classes of possible toxicants (e.g., labile or volatile compounds), and (c) production of artifactual toxicity emanating from the treatments themselves (e.g., residual solvent). The net result of these problems was loss of a linkage between the initial sample (and the chemicals responsible for toxicity) and the biological endpoint(s) of concern. To address this, researchers supporting the EPA effluent testing program developed EDA/TIE techniques designed to preserve, as much as possible, linkages between the original test sample and observed toxicity [9]. This work involved development not only of novel physical/chemical manipulations and test approaches, but a logic framework for conducting EDA/TIE analyses to support ecological assessments.

The TIE process developed by the EPA consists of three phases: characterization, identification, and confirmation [10–15]. Although Phases I, II, and III are described as discrete activities and logically proceed from one another in a linear

fashion, in reality they often are iterative and, depending on the nature of the toxicants, may occur simultaneously. Phase I characterization is conducted in response to an effluent being identified as toxic to one (or more) of the test species required for discharge monitoring (typically the cladoceran *Ceriodaphnia* or the fathead minnow, *Pimephales promelas*). Phase I consists of a variety of sample manipulations conducted in conjunction with toxicity testing (with the species/endpoint that triggered the TIE) to characterize the general physicochemical properties of the causative toxicants(s) [10, 13]. Sample manipulations include aeration and filtration of the sample at low, neutral, and high pH values, solid-phase extraction with a nonpolar (C18) resin, addition of substances (e.g., sodium thiosulfate, EDTA) designed to mitigate the toxicity of different classes of chemicals, and testing at a graduated range of (physiologically tolerable) pH values. At conclusion of a successful Phase I study, causative toxicants can be broadly classified with respect to polarity, volatility, and stability (all as a function of pH), reactivity with thiosulfate (oxidants) or EDTA (cationic metals), and ability to exert differential toxicity at different pH values (e.g., ammonia).

Observed physicochemical characteristics from Phase I dictate approaches taken in the Phase II identification portion of the TIE [11, 14]. For example, if EDTA removed sample toxicity, toxicant identification would focus on measurement of cationic metals and comparison of measured concentrations to existing (or generated) toxicity data for the species/endpoint that triggered the TIE. Phase II identification also could involve further sample manipulation and toxicity testing in the context of fractionation. For example, if Phase I indicated the presence of a nonionic organic toxicant (toxicity removal by the nonpolar resin), Phase II would consist of fractionation of the sample via reverse-phase, low- and/or high-pressure liquid chromatography (L/HPLC). To decrease potential for artifactual toxicity and/or loss of toxicants from solution, Phase II TIE methods advocated use of low-toxicity solvents with some degree of miscibility in water (e.g., methanol, acetone), thereby ensuring that toxicity tests could be conducted directly on test fractions from the chromatography steps. Concurrent instrumental analysis of toxic fractions, using techniques such as gas chromatography–mass spectroscopy (GC–MS), is used to identify discrete chemicals, which then can be evaluated with respect to known or measured toxicity.

Once potential toxicants have been identified, Phase III analyses are conducted to confirm that the suspect chemicals are indeed responsible for sample toxicity [13, 15]. Because even minimal handling/manipulation of test samples can cause unanticipated changes in toxicity, and because complex environmental samples (and effluents in particular) can exhibit considerable temporal variability (i.e., chemicals responsible for toxicity may change over time), confirmation is a critical step, especially if substantial resource commitments to mitigation are to be made based on TIE results. The tools of Phase III include correlation of sample toxicity with concentrations of suspect toxicants over some gradient of toxicity/time, evaluation of relative species sensitivity, observation of signs of toxicity in test animals, and addition to, or deletion of, suspect toxicants from the test sample.

3 Conceptual Considerations in Effects-Directed Analysis

Although the TIE framework/techniques described above were developed specifically for acutely toxic effluents, most basic considerations are germane to any type of EDA. The effluent TIE methods themselves were later expanded by USEPA to address chronic toxicity in effluents [16] and similar approaches have been applied to a variety of sample matrices (surface waters, sediments, hazardous wastes) and endpoints in both in vivo and in vitro biological systems (e.g., acute and chronic toxicity in fish and pelagic and benthic invertebrates, gene expression in cell lines; binding to nuclear hormone receptors, etc.). In this section, we discuss some of the key considerations that arise when conducting EDA/TIE work.

3.1 Bioassay Selection

The success of any EDA study depends on selection of a bioassay system that is both relevant to the risk assessment question at hand and compatible with the types of sample manipulations used for the analysis. In terms of the latter, considerations include (a) sample volume/mass needed to conduct tests (i.e., amounts of sample from some fractionation steps could be quite limited), (b) tolerance of the biological system to additives (e.g., solvents, resins) used for the TIE/EDA, and (c) ability to conduct many assays (to accommodate the large number of manipulated/fractionated samples that could be generated) in a comparatively short timeframe. In the case of the EPA effluent TIE work, the decision concerning the biological test(s) to use was relatively straightforward. Specifically, endpoints/species utilized were the same as those that triggered the analysis – survival of cladocerans and/or larval fish in acute (48–96 h) tests. This choice also was reasonable because the tests are relatively inexpensive and, because the animals are small, did not require large sample volumes. However, it still was necessary to make adjustments to test conditions (relative to the standard regulatory tests), in terms of reducing sample volume and replication to optimize their utility for TIE work. Further, it was necessary to thoroughly characterize the baseline sensitivity of the species to the types of sample manipulations (and additives) used for the TIE studies.

Unfortunately, for a variety of reasons (e.g., size, amenability to lab testing, availability), it is not always possible to directly use species/endpoints of concern for EDA studies. In these instances, selection of a “surrogate” test system is necessary. A recent successful example of this involves EDA studies with effluents in the UK focused on identifying chemicals that cause estrogenic responses in fish. Specifically, there was evidence from studies with both feral and caged fish of the presence in some complex effluents of estrogenic substances that caused feminization of males [17]. In terms of logistics it was not possible to use long-term bioassays with fish to track this type of estrogenic response in EDA studies.

Hence, a surrogate in vitro system based in responses in yeast cells containing the human estrogen receptor was used to help fractionate/identify estrogenic chemicals in the effluents [18]. More resource intensive follow-up studies with adult male fish exposed to the identified chemicals confirmed their estrogenic properties, and generated the necessary dose/concentration–response relationships to evaluate cause-and-effect relationships in the field [19].

Although use of surrogate test systems for EDA work is eminently reasonable, care must be taken in selecting assays to ensure that they reflect toxicological processes of concern in organisms of interest. For example, the Ames test (a bacterial assay) was a popular surrogate system for many early EDA studies with complex environmental samples focused on mutagens and carcinogens (e.g., [2–4]). However, the degree to which chemicals that are mutagenic in the Ames test affect eukaryotic organisms is debatable, so even when mutagenic chemicals were identified in EDA studies, the significance of this in terms of human health or ecological risk was/is uncertain.

3.2 Sample Manipulations

The basic goal of EDA is to manipulate/alter a complex sample in such a fashion that it is effectively simplified with respect to interpreting what chemical(s) may be causing toxicity. In many instances this involves removing (extracting) toxicants from the sample (e.g., via selective resins), or modifying conditions in situ such that properties of the toxicants are changed (e.g., through adding EDTA, thiosulfate, changing pH, etc.). This may or may not involve sample concentration steps. Sample extracts can be further simplified by fractionation (with concurrent testing) prior to toxicant identification. There are so many different techniques available in the engineering and analytical literature for selective extraction and fractionation of different classes of chemicals from wastewater, sediment, soil, etc., it would seem the options for EDA applications are boundless. Unfortunately, the great majority of these techniques are, in fact, of limited (or no) utility for EDA work for two reasons. First, many of these techniques are so efficient that they extract materials which are not – and perhaps never would be – bioavailable in sufficient amounts to cause toxicity in an unaltered sample. For example, Soxhlet extraction with hexane of sediment can produce toxic extracts which can be processed to yield fractions containing potent organic chemicals like polycyclic aromatic hydrocarbons, but these chemicals may not occur in situ at concentrations sufficient to cause toxicity under normal circumstances. Hence, care must be taken such that EDA manipulations do not cause changes in toxicity of samples due to extractions/alterations that would not occur in the environment.

The second reason that many existing analytical extraction and associated fractionation techniques are of limited use for EDA work is that the procedures themselves can introduce toxicity to the samples. This is especially problematic for some nonpolar solvents commonly used for environmental analytical chemistry

like hexane and methylene chloride, which are very toxic to most biological systems even at trace concentrations. And, efforts to completely remove high-toxicity solvents from test extracts/concentrates, for example by evaporation, can result in loss of chemicals responsible for sample toxicity. In general, extraction of samples with selective sorbents/resins has proven less problematic than use of solvents in terms of producing artifactual toxicity and/or losing toxicants of concern. A sorbent that has proven quite useful for effluent TIE work is C18, which effectively removes nonpolar chemicals from the aqueous phase (hence, ostensibly, the fraction of chemical that is biologically available [20]), while introducing little artifactual toxicity [21, 22]. The C18 resins also can be used for fractionation work with relatively nontoxic solvents like methanol or acetone. Other types of resins (e.g., XADs) also have been used successfully for EDA work, although less frequently than C18 sorbents.

In this short paper it is impossible to completely discuss all sample manipulations that have been or could be used for EDA work; however, we do have two general recommendations in this area. First, it is best to use the least intrusive sample manipulation available to achieve desired results. This helps avoid the types of substantial alterations that could release nonbioavailable chemical and/or cause artifactual toxicity. Unfortunately, there is no straightforward way to judge a priori which sample manipulations might be effective, versus those that are problematic. Hence, development and application of new types of sample manipulations is very much a “trial and error” process. For example, in our lab we have experimented with different XAD resins for the selective removal of different classes of chemicals from aqueous solutions in TIE studies; some of the resins have proven quite useful for this, while others impart toxic materials to the test samples (unpublished data). Based on what we were able to learn about the XAD resins from manufacturers, there was no obvious difference between those that appeared useful for the EDA work versus those which were not.

Our second general recommendation concerning sample manipulations for EDA work involves use of appropriate blank and control treatments. A substantial amount of effort needs to be devoted to testing blanks and controls associated with various sample manipulations and fractionation steps. For example, in the EPA Phase II TIE process, one common step involves the generation of 26 HPLC fractions (of decreasing polarity) from one toxic extract. Not only must all these fractions be tested for toxicity, but 26 corresponding blanks (generated before sample has been applied to the column) need to be tested as well, in addition to clean-water controls. Since testing all appropriate blanks associated with a sample manipulation often can result in a doubling of effort, there is an understandable desire to limit the number of blanks tested in EDA work, especially if a particular manipulation has not been problematic in the past relative to introducing artifactual toxicity. However, columns, solvents, and other reagents can change over time and from batch to batch, and interactions with different test samples can be unexpected. Hence, based on experience, a decision to conserve resources through limiting testing of all appropriate blanks and controls can, in the end, be very costly if the EDA/TIE study pursues false leads (artifactual toxicity).

3.3 Interpreting Test Data

Most biological and chemical measurements made in conjunction with environmental assessments are straightforward relative to interpretation; e.g., a chemical is present at a particular concentration in an effluent, a sediment sample causes toxicity to a model invertebrate species in the lab, a desirable fish species is present at a field site, etc. In some instances interpretation of TIE data also can be relatively straightforward, particularly when commonly encountered toxicants are present. For example, only limited Phase I and Phase II data often are needed to implicate ammonia as a causative toxicant in effluents [23]. But, in other instances, interpretation of TIE/EDA test data is not routine, especially when unusual (not previously encountered) or multiple causative toxicants (that might or might not be additive) are present.

Evaluation of environmental chemistry and toxicity data often is guided by formal hypothesis testing but, at least in early stages of an EDA study (e.g., Phase I of a TIE), there may be little reliance on statistical analysis for data interpretation. Because TIE work involves using data resulting from a suite of manipulations to decide on next steps, the analyst(s) needs to rely heavily on inferential reasoning based on what can – in some instances – be a relatively complex set of facts. This dictates that those involved in the work have in-depth knowledge not only of the biology involved in the study, but the chemistry as well. As such, most successful EDA studies emanate from multidisciplinary teams of scientists who work together in an integrated manner.

Unfortunately it is impossible to be entirely prescriptive in describing how to conduct EDA/TIE studies, in part because a critical component of the work involves iterative testing to evaluate possible insights provided by initial observations. This might involve repeating manipulations of samples multiple times under slightly different conditions, or it could involve developing altogether different approaches/manipulations. For example, in *Case Study 2* below it was possible to learn whether causative toxicants were volatile or not by rinsing and testing aeration glassware for toxicity.

4 Illustrating the Concepts: Select TIE Case Studies

4.1 Case Study 1: Diazinon

One of the first examples of a complete (Phases I, II, and III) effluent TIE using the EPA protocols was conducted with an effluent from the southeastern US [24]. Samples of the effluent were consistently toxic to *Ceriodaphnia* but not the fathead minnow. The only Phase I manipulation that markedly affected toxicity was passage of the sample over a C18 column; toxicity subsequently was recovered in a 100% methanol elution of the column.

Phase I results suggested the presence of a nonpolar organic toxicant in the effluent, so the emphasis in Phase II was on extraction, fractionation, and

concentration of the unknown chemical(s) using C18 columns. Phase II studies showed that toxicity removed from the C18 column was consistently recovered in the 80% methanol/water fraction, with occasional toxicity observed in adjoining (75 and 85%) fractions. Toxic fractions subsequently were concentrated, tested to ensure toxicity was retained, and subjected to GC–MS analysis. Library searches of the GC–MS data revealed the presence of several chemicals in the toxic fractions. However, with one exception, predicted or known toxicity of the identified chemicals was low relative to their concentrations in the effluent. That one exception was the organophosphate pesticide diazinon, which was frequently present in the effluent at concentrations higher than the *Ceriodaphnia* LC50 for the chemical of 0.35 μg/L.

Phase III of the TIE used several approaches to confirm diazinon as the chemical responsible for effluent toxicity [24]. For example, a strong relationship was developed between measured and predicted effluent toxicity (based on diazinon "toxic units") in samples collected over time. Additional confirmatory evidence came through consideration of relative species sensitivity. Specifically, cladocerans are very sensitive to organophosphate pesticides such as diazinon, while fish are not, a relationship reflected in observed toxicity of the effluent.

Prior to this TIE work, there was little suspicion that diazinon might be an important toxicant in effluents. However, a subsequent survey of effluents from municipal waste water treatment plants throughout the southeastern US revealed that diazinon was a relatively common contributor to toxicity. At the time, diazinon was approved for a wide variety of both indoor and outdoor pest control applications, and so undoubtedly was entering waste streams from multiple sources. In the USA, regulations subsequently have restricted usage scenarios of the pesticide thus decreasing the potential for its occurrence/toxicity in effluents.

4.2 Case Study 2: Surfactants

Occasionally Phase I results can be confusing in terms of clearly defining the physicochemical nature of toxicants in a sample, but additional manipulations that logically build on the initial Phase I observations can nonetheless produce a successful characterization. An example of this involves TIE studies with several samples of a primary (untreated) municipal waste water facility effluent that was acutely toxic (lethal) both to fathead minnows and *Ceriodaphnia* [25, 26]. Filtration of the effluent through a 1 μm glass-fiber filter reduced, but did not completely eliminate toxicity. Subsequent passage of the sample through a C18 column completely removed toxicity, some of which could be recovered by eluting the column with 100% methanol. Phase 1 studies also showed that toxicity of the effluent sample could be removed by aeration of the sample. Finally, it was found that holding the sample at 4°C for more than a few days also reduced toxicity of the effluent.

Consideration of this suite of Phase I results suggested, perhaps, that effluent toxicity was caused by a volatile, nonionic organic chemical. However, this proved not to be the case. Further fractionation studies showed that toxicity extracted from

the effluent by C18 could be eluted from the column in several fractions of different polarity, ranging from 80 to 100% methanol/water, suggesting the presence of a mixture of toxic chemicals. And, efforts to recover a volatile compound(s) were completely unsuccessful. What was discovered, however, was that when the aeration glassware was rinsed (with control water) and the rinse water tested, toxicity was recovered. These observations, in conjunction with the initial Phase I data, suggested the presence of surfactants in the effluent. A common technique for concentrating surfactants from aqueous samples for instrumental analysis involves sublation, a process whereby chemicals are removed from solution by aeration followed by deposition on glassware. A wide variety of anionic, nonionic, and cationic surfactants used for both industrial and domestic applications can occur at relatively large concentrations in untreated effluents, but tend to be biodegradable, which would explain why samples lost toxicity over time. Furthermore, surfactants adhere to a variety of surfaces, hence explaining the effects of filtration on toxicity. Finally, because surfactants would be expected to occur as a complex mixture of compounds with varying polarities in an effluent, it seemed reasonable to expect that they would elute from a C18 column in multiple fractions.

Due to the nature of the toxic chemicals suggested by the Phase I characterization, Phase II of the TIE did not focus on identification of discrete chemicals responsible for toxicity but, rather, the behavior and occurrence of surfactant mixtures. This was warranted both from a toxicological/analytical and mitigation perspective. Specifically, the primary manner through which surfactants produce acute effects is via a common toxicity pathway, narcosis, which should result in additive effects of mixtures of the compounds [27]. So, integrating contributions from what could potentially be hundreds of different structures (which would be a substantial analytical challenge to measure individually) represented a pragmatic approach to the toxicity identification. Further, if surfactants, as a broad class, could be established as likely responsible for sample toxicity, the initial mitigation option for all of them (in a primary effluent) would be similar, advanced treatment.

The Phase II identification studies focused on two areas: (a) defining how mixtures of known surfactants behaved in the TIE process, as basis for comparison to behavior of the test effluent; and (b) nonspecific measurement of broad classes of surfactants in the test effluent. Mixtures of surfactants were generated through combining commercial products and standards containing different classes of the chemicals, and spiked into clean water or a nontoxic effluent (to account for matrix effects), prior to conducting toxicity tests and the various sample manipulations described above [25]. These studies showed that the artificial mixtures behaved in an identical manner as the effluent toxicants with respect to extraction (including filtration and sublation), and fractionation, thus providing strong circumstantial evidence for surfactants as toxicants in the effluent. Finally, the occurrence of anionic surfactants and nonionic surfactants was evaluated using analytical approaches that measured the chemicals based on class rather than individual compounds. These measurements confirmed the presence of both anionic and nonionic surfactants in the effluent, as well as in toxic C18 fractions from the effluent, at concentrations well within the range necessary to cause toxicity to the test species [26]. Overall, although specific compounds

responsible for effluent toxicity were not identified in the Phase II analysis, a compelling "weight-of-evidence" case for surfactants causing toxicity was developed, and has served as the basis for subsequent TIE studies with municipal and industrial effluents containing surfactants [23].

4.3 Case Study 3: Hexavalent Chromium

A third case study involves a steel production and milling facility, which had experienced episodes of effluent toxicity to *Ceriodaphnia*, primarily appearing in chronic effluent toxicity tests [28]. The renewal of the facility's effluent discharge permit required no acute or chronic toxicity in 100% effluent, and in the second round of monitoring following permit renewal, a spike in toxicity was observed, expressed as pronounced acute toxicity (LC50 = 9%) in the first of the three samples used in the chronic toxicity test. Despite the marked toxicity to *Ceriodaphnia*, these same samples showed no acute or short-term chronic toxicity to the other test species, fathead minnow.

In response to finding this highly toxic sample, a Phase I characterization was conducted. Of all 14 manipulations included in the characterization procedure [13], none affected toxicity of the sample. While this lack of response did not directly implicate a group of toxicants, the finding was nonetheless informative, as it ruled out a number of possible toxicants including: nonpolar organics (no effect of solid-phase extraction); many common cationic metals (no effect of EDTA); volatile or sublatable compounds (no response to aeration); and sulfide, ammonia, or other pH-sensitive toxicants (no change in toxicity with altered pH). Based on this characterization pattern, the most likely candidate cause seemed to be inorganic ionic toxicants (other than those excluded above), although ionic organic compounds too polar to be removed by the solid-phase extraction with C18 resin were also possible. To further evaluate these possibilities, the effect of three additional manipulations was evaluated, activated charcoal, as a broader spectrum sorbent that might attract more polar organic compounds, along with cation- and anion-exchange resins (tested separately), which might indicate the charge of the toxicant. These follow-up tests showed no effect of carbon or cation-exchange on toxicity, but anion-exchange completely removed toxicity, suggesting that the causative toxicant was most likely an inorganic anion.

With that knowledge, the manufacturing operations at the facility were reviewed to determine if there were obvious sources of potentially toxic inorganic anions in the facility. One clear candidate was hexavalent chromium, which was used in chrome plating operations and exists in water as an oxyanion. Chemical analysis of existing samples showed the presence of hexavalent chromium at potentially toxic concentrations in the acutely toxic effluent sample, and these concentrations were far higher than those in other effluent samples that were not acutely toxic.

While these data strongly implicated hexavalent chromium as the cause of toxicity in the acutely toxic sample, that sample was also not typical of other

effluent samples that had been tested in the past, and could have represented an upset condition not reflective of the usual facility operation. This made it important to determine whether hexavalent chromium was the ongoing cause of toxicity in the effluent. To do this, a series of six rounds of confirmatory testing was planned, in which paired chronic *Ceriodaphnia* toxicity tests would be conducted on unmanipulated effluent and on effluent that had been treated by anion-exchange. Chemical analysis of hexavalent chromium was conducted on all samples. To help interpret these concentration data, toxicity tests were also conducted on effluent that was treated by anion-exchange, then spiked with hexavalent chromium. The purpose of these chromium spiking tests was to establish the toxicity of hexavalent chromium in the effluent matrix. Spiked solution toxicity tests were also conducted with fathead minnows; these tests confirmed that fathead minnows were far less sensitive to hexavalent chromium than were *Ceriodaphnia*, which was consistent with the previous findings that the effluent was neither acutely nor chronically toxic to fathead minnows, even when it showed high acute toxicity to *Ceriodaphnia*.

The first two rounds of confirmatory tests showed chronic toxicity to *Ceriodaphnia* that was removed by anion-exchange and was consistent with the magnitude of hexavalent chromium measured in the effluent samples. Separate from the confirmatory testing, the facility operations staff was conducting a wastewater characterization study aimed at evaluating treatment system performance and looking for sources of hexavalent chromium in the wastewater system. This study uncovered a leaking heat exchanger on a chromium plating bath that was allowing chromium plating liquor to leak into the wastewater system. Though the leak was small, the concentration in the plating bath was very high, and even the small leak was sufficient to create toxic concentrations in the wastewater. The leak was easily and immediately repaired. During the subsequent rounds of confirmatory testing, no hexavalent chromium was detected, and no further toxicity was detected during normal operations.

Several important lessons can be gleaned from this study. First, even though none of the Phase I characterization tests affected effluent toxicity, these "negative" results still provided considerable insight into the probable characteristics of the causative toxicant(s). Second, while standardized sets of characterization tests are often effective in pointing the investigation toward specific toxicants or toxicant types, the investigator must be prepared to supplement these standard manipulations with others to address toxicants not contemplated by those standard methods. Finally, one of the most powerful forms of toxicant confirmation is to be able to change the concentration of the toxicant in the effluent and observe a concomitant change in toxicity.

5 Summary Findings from Effluent TIEs

Table 1 summarizes findings from a total of 84 effluent TIE studies conducted between 1988 and 1993, over a range of effluent types (JRH and DRM unpublished data). Among municipal effluents, two toxicants were dominant, pesticides and

Table 1 Summary of findings from TIE studies on 84 effluents from the USA, studied between 1988 and 1993

Effluent category	Toxicants identified					
	Ammonia	Misc organics	Pesticides	Metals	TDS[a] (major ions)	Other
Municipal ($n = 30$)	16	1	20	2	1	Residual chlorine (3)
Refinery/chemical ($n = 20$)	7	14	0	0	7	Residual chlorine (1)
Mining ($n = 13$)	0	0	0	8	4	Treatment polymer (1)
Manufacturing ($n = 8$)	1	2	0	4	2	Fluoride (1), nitrite (1)
Pulp and paper ($n = 6$)	0	3	0	0	6	–
Oil/gas coproduced water ($n = 8$)	0	0	0	0	8	Sulfide (1)

Number of effluents in categories reflect the number of facilities or discharges, but replicate samples from the same facility or discharge were not considered separately. The total of toxicants identified may be greater than the number of effluents because more than one source of toxicity was identified in one or more individual effluents (JRH and DRM unpublished data)
[a]*TDS* total dissolved solids

ammonia. Most instances of pesticide toxicity were tied specifically to diazinon as described in *Case Study 1*, although chlorpyrifos, carbaryl, and malathion were also identified as the cause of toxicity in one or more municipal effluents. Finding toxicity caused by these chemicals was somewhat unexpected, as it was thought that they would be degraded as part of biological treatment. In cases where the sources were determined, they were usually diffuse, suggesting broad, low level inputs rather than specific, localized inputs (although there was one instance where malathion was traced to washing of application equipment by a commercial applicator).

Ammonia toxicity was most commonly found in municipal effluents, although it was also found in several industrial effluents. Toxicity caused by ammonia can be easily confirmed through a combination of characteristics, including (a) about threefold higher toxicity to fathead minnows than to *Ceriodaphnia*; (b) measured ammonia concentrations and observed toxicity consistent with species-specific LC50 values from the literature and the concurrent pH; (c) highly pH-dependent toxicity, decreasing with decreasing pH in a manner consistent with literature data; and (d) both ammonia and toxicity reduced or removed proportionally by passing the effluent through a zeolite (cation exchange) column. In some cases, the presence of ammonia toxicity would "mask" the presence of an additional toxicant, such that the second source of toxicity would be observed primarily at low pH, when the toxicity of ammonia was suppressed. This led to the development of methods enabling conduct of TIE studies under pH control, such as testing in a CO_2-enriched atmosphere [29]. In some cases, these same pH control techniques were incorporated into routine whole effluent testing, if doing so allowed the effluent to be tested under conditions typical of the actual discharge (pH of biologically treated effluents frequently rises during storage).

Particularly in refinery or petrochemical effluents, it was common to find toxicity associated with organic chemicals that were removable by solid-phase extraction, but were difficult to specifically identify. In most cases attempts to fractionate toxicity eluted from solid-phase extraction columns were unsuccessful, as the toxicity "spread" among multiple fractions to the point it was no longer detectable by the toxicity test. Broad-spectrum GC–MS analysis of toxic fractions generally showed large numbers of unresolved peaks, and the general interpretation of these data was that toxicity was caused by the aggregate effect of large numbers of closely related compounds, such as the naphthenic acids that have been found in some refinery effluents. It was also common to find that toxicity increased with decreasing pH, which is consistent with an acidic organic compound. Even though the identification of specific compounds was often stymied in these cases, knowledge of the toxicant characteristics, and the ability to quantify the aggregate potency through extraction and elution of C18 columns was sufficient to guide the evaluation of process or treatment alternatives, leading to control of effluent toxicity.

Interestingly, the single most commonly identified source of toxicity across all effluent sources was high concentrations of the major ions present in all natural waters – combinations of Na, K, Ca, Mg, Cl, SO_4, and carbonates (e.g., HCO_3). While the aggregate of these ions is typically referred to as total dissolved solids (TDS), further study has shown that the toxicity of elevated concentrations of major ions is not associated with a particular TDS concentration, but rather to the specific concentrations and ratios of these ions [30]. In other words, the toxicity of any one of the ions is dependent on the co-occurring concentrations of all. Indications of major ion toxicity include a failure of any Phase I manipulations to reduce toxicity coupled with relatively high sample conductivity (e.g., 1,000 uS/cm or higher), with *Ceriodaphnia* generally showing greater sensitivity than fish.

The frequency of TDS as an identified toxicant in Table 1 is influenced in part by the inclusion of several oil/gas coproduced waters, but it was found in at least one case in all effluent categories. Elevated TDS occurs in effluents for several reasons. In produced waters and some mining-related wastewaters, the source waters are naturally high in TDS. In other cases, ions were added as a byproduct of a manufacturing or other process, or as the result of evaporation, either in cooling water or as the result of water reuse/conservation efforts. Another factor that may have influenced the frequency with which major ions caused effluent toxicity is that *Ceriodaphnia*, perhaps the most widely used effluent testing organism in the USA, appears to be among the more sensitive aquatic organisms to major ion toxicity. It varies among discharge situations as to whether or not major ion toxicity is of regulatory concern. In some cases, discharges were allowed to switch their compliance testing to another cladoceran, *Daphnia magna*, which has slightly lower sensitivity to major ion toxicity [30], but is thought to be similarly sensitive to other chemicals potentially present in effluents.

Although metals have been known to be important environmental contaminants for a long time, they were not commonly found as causative toxicants in effluents, especially outside of mining-related effluents. This may be because the toxicity of

these chemicals has been well studied and, as a result, water quality criteria and chemical-specific permit limits are largely effective in assuring that effluents do not contain toxic concentrations of common metals. Among those metals that were identified as causative toxicants, copper and zinc were the most common.

One other finding from these TIE studies was that most effluents have a very small number of causes of toxicity. Of the 80 effluents shown in Table 1, 47 (59%) were found to have a single cause of toxicity, 32 (40%) were found to have two causes, and one effluent had three causes of toxicity. It is important to note that in this context, a “cause” was not necessarily a single chemical (e.g., ammonia or diazinon), but was sometimes a combination of closely related compounds with a common source, such as the organic toxicants often found in refinery effluents, surfactants, or TDS. All of these exert effects as the aggregate of multiple chemicals, but they can be characterized through TIE as a single cause of toxicity. The finding that toxicity was frequently caused by a small number of toxicants was probably important to the success of the TIE approach, as the ability to parse and identify causes of toxicity using EDA/TIE can generally be expected to decline as the number of causes increases. Among effluents with two causes of toxicity, the most common combinations involved ammonia and a second source. Because ammonia toxicity (a) is well understood and easily characterized; (b) generally appears to act independently from other toxicants; and (c) can be suppressed by testing at reduced pH, it was frequently not that difficult to identify a second source of toxicity even when ammonia was also present in toxic concentrations.

6 Advantages of EDA/TIE over Other Toxicity Control Approaches

At the time the application of EDA/TIE to effluent toxicity in the USA was being developed, there was often resistance to the approach. Some preferred approaching the problem of effluent toxicity using other methods, such as traditional treatment performance evaluations and, where necessary, treatability studies to determine what additional treatment processes could be employed to control toxicity. The relative effectiveness of this more treatment-oriented approach versus the toxicologically based EDA/TIE approach can be expected to vary according to the specifics of individual cases. For example, in the hexavalent chromium example above, it is very possible that the presence of hexavalent chromium in the effluent might have been flagged as unusual without TIE studies. However, there are many examples where TIE uncovered new groups of toxicants not previously thought of as chemicals of concern in effluents. Diazinon in municipal effluents is an excellent example; this was not a chemical typically thought of as being present in municipal effluents and, further, was thought to be readily degraded. Surfactants are another group of toxicants identified through EDA/TIE that would most likely not have been readily addressed through treatment performance or treatability studies, as the chemical analysis for those materials are difficult, their components are often

proprietary, and in fact they are often purposely added to effluents to facilitate certain treatment processes.

We believe it can be effectively argued that identifying the specific cause of toxicity through EDA/TIE provides the broadest spectrum of possible resolutions to effluent toxicity. Clearly, knowing the exact cause of toxicity makes possible the development of very focused solutions, such as product substitution, that are not easily invoked when the cause of toxicity is not known. In many cases we have been involved with, control of effluent toxicity was achieved without installing additional treatment, through product substitution, source control, adjusting the operation of the existing wastewater system, or even altering the conditions of the effluent toxicity test to better reflect the discharge conditions [29]. Use of EDA/TIE approaches does not preclude the use of treatment-based approaches if they are needed, and in fact treatments can be better selected if the chemical characteristics (if not the actual identity) of the cause of toxicity are known. Compared to the potential costs associated with adding more elements to a wastewater treatment process, the costs of conducting TIE/EDA studies as part of the overall investigation are small and the potential benefits large.

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Recent Developments in Whole Sediment Toxicity Identification Evaluations: Innovations in Manipulations and Endpoints

Robert M. Burgess, Kay T. Ho, Adam D. Biales, and Werner Brack

Abstract Whole sediment toxicity identification evaluation (TIE) methods were developed primarily in the late 1990s and early 2000s in research programs dedicated to developing manipulations and endpoints to characterize and identify causes of toxicity to benthic freshwater and marine organisms. The focus of these methods included nonionic organic contaminants, cationic and anionic metals, and ammonia. This chapter discusses innovations in whole sediment TIE manipulations and endpoints developed primarily over the last 10 years. Innovations such as the use of supercritical fluid extraction as a Phase III manipulation, Phase II methods for identifying pyrethroid, organophosphate, and carbamate pesticides, and the integration of genomic endpoints into the TIE structure are described. In North America, recently implemented environmental regulations require the diagnosis and identification of environmental stressors as part of the total maximum daily loading process. These regulations are likely to result in an increase in the conduct of whole sediments TIEs and encourage the development and application of more innovations.

Keywords Bioavailability, Genomics, Pesticides, Supercritical fluid extraction, Toxicity identification evaluation, Whole sediment

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W. Brack (ed.), *Effect-Directed Analysis of Complex Environmental Contamination*,
Hdb Env Chem (2011) 15: 19–40, DOI 10.1007/978-3-642-18384-3_2,
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Contents

1 Background 20
2 Recent Developments: Manipulations 23
2.1 Use of Supercritical Fluid Extraction 23
2.2 Methods for Pyrethroid, Organophosphate, and Carbamate Pesticides 26
3 Endpoints 31
3.1 Genomic Methods 31
4 Summary and Status of Whole Sediment TIEs 35
References 36

1 Background

There are billions of tons of contaminated sediments in the streams, rivers, ponds, lakes, estuaries, and coastal areas of the world's aquatic environments. These sediments represent a potentially significant cause of ecological and human health risk in the form of toxicity to benthic organisms, communities, and resources (i.e., seafood), and serve as a source of bioaccumulable contaminants which transfer throughout aquatic and terrestrial food webs [1, 2]. Determining how to prioritize the management and cleanup of these sediments is an enormous scientific and economic challenge. Tools that provide insights into which contaminated sediments represent the greatest ecological and human health risks are very valuable in addressing this challenge through the establishment of environmental management priorities and setting cleanup objectives. In North America, whole sediment toxicity identification evaluation (TIE) methods have been used in ecological health assessments for identifying causes of toxic effects. Further, TIEs have been used at contaminated sites for assisting in selecting remediation tools based on toxicant identification.

In the 1980s, in the USA, TIE methods were developed in order to address the effects on ecological health and environmental management of industrial and municipal effluents [3–6]. The objective of the TIE is to determine the causes of toxicity (generally, whole organism toxicity) in an environmental matrix. The first TIEs were performed on industrial and municipal effluents and receiving waters using freshwater and marine water column toxicity testing species [5, 7]. The TIE process uses a combination of whole organism toxicity endpoints and chemical manipulations to perform the assessments. In general, the chemical manipulations altered toxicant bioavailability [e.g., chelation of cation metals by ethylene diaminetetraacetic acid (EDTA) addition] or actually remove the toxicant from the aqueous sample [e.g., extraction of nonionic organic chemicals (NOCs) from aqueous solution by reverse phase chromatography]. Basic TIE structure involves three phases (Fig. 1). In Phase I, contaminants are characterized into broad groups (e.g., NOCs, cationic metals, anionic metals, ammonia) in order to better understand the general causes of toxicity. Then Phase II is conducted to identify specific active toxicants. For example, if Phase I concluded toxicity was being caused by a NOC, Phase II would seek to determine if specific polychlorinated biphenyls

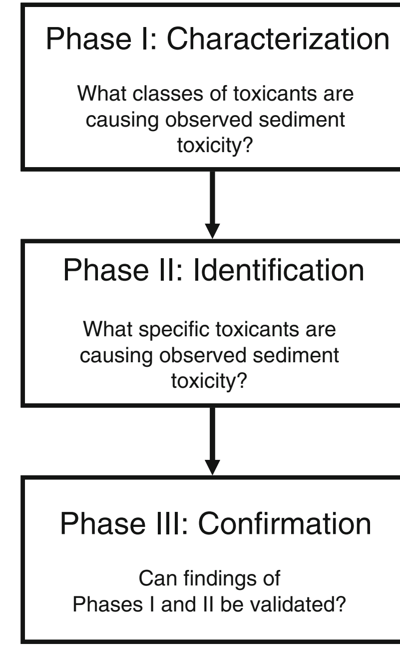

Fig. 1 Basic structure and questions asked in each phase of a toxicity identification evaluation (TIE)

(PCBs), polycyclic aromatic hydrocarbons (PAHs), or other NOCs (e.g., pesticides) were responsible for the observed toxic effects. In the final part of the TIE, Phase III, the results of the first two phases are confirmed. Generally, independent methods are used to perform the confirmation.

By the 1990s and early 2000s, the focus of TIE methods development was on their use with contaminated sediments [8–10]. Ho and Burgess [11] provide an overview of interstitial water and whole sediment TIE methods; consequently, only a brief review is presented here. Contaminated sediment TIE methods evolved initially from the earlier effluent and receiving water methods with the TIEs performed on sediment interstitial waters [8–10, 12]. Like the effluent and receiving water TIE methods, interstitial water TIEs used a combination of column chromatography techniques, the addition of chelating agents (e.g., EDTA), and ammonia-consuming algae (green macroalga, *Ulva lactuca*) (Fig. 2a) to characterize toxicity. However, for a variety of reasons, it was recognized that the use of interstitial water for conducting TIEs was not ideal and included several potential artifacts (e.g., oxidation of reduced metals, loss of NOCs to glassware, overexposure to interstitial waters). Consequently, by the late 1990s, both freshwater and marine research programs were developing whole sediment TIE methods which minimized or eliminated many of the interstitial water artifacts. In contrast to the earlier effluent, receiving water and interstitial water TIEs, whole sediment TIEs involved exposing the test organisms (now infaunal or epibenthic organisms) to sediments manipulated with TIE chemicals (e.g., EDTA). Figure 2b illustrates an array of manipulations

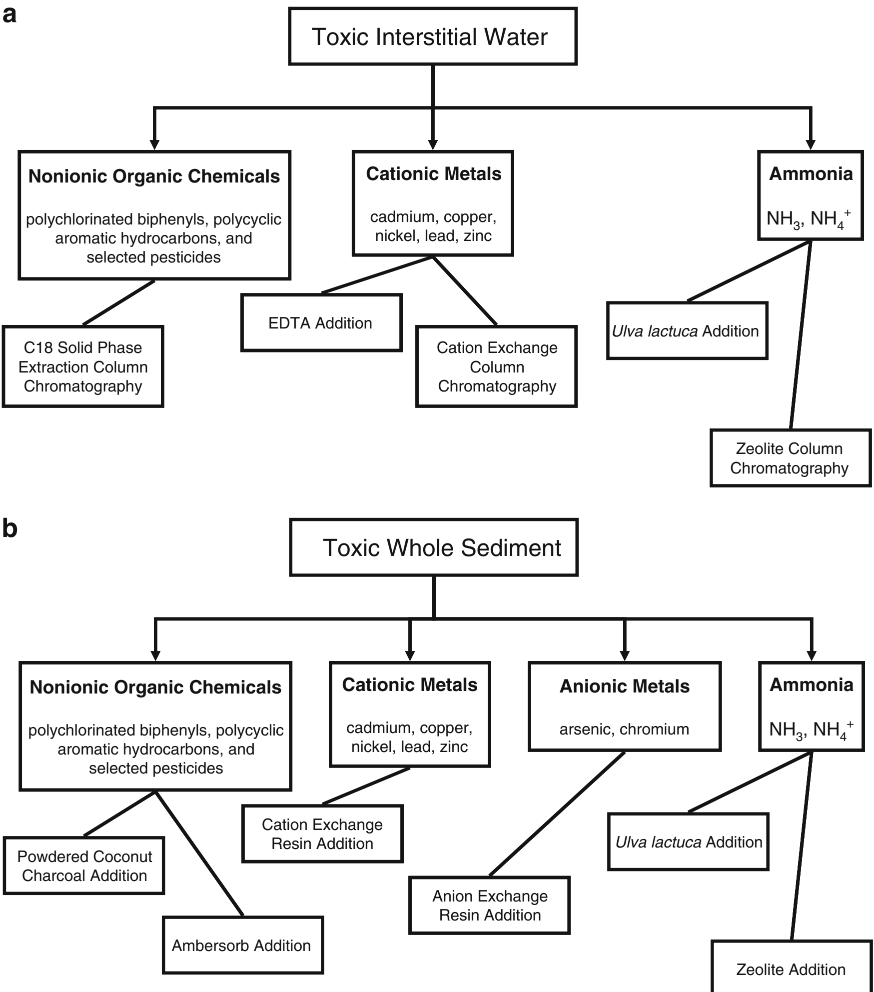

Fig. 2 Interstitial water (**a**) and whole sediment (**b**) toxicity identification evaluation (TIE) structures including toxicant classes, example contaminants, and manipulations

currently available for performing whole sediment TIEs [10]. These include the addition of powdered coconut charcoal or Ambersorb, cation exchange resins, anion exchange resins, and zeolite to characterize toxicity caused by NOCs, cationic metals, anionic metals, and ammonia, respectively. Like the interstitial water TIEs shown in Fig. 2a, the macroalgae *U. lactuca* can also be used to characterize ammonia-caused toxicity. In general, the whole sediment TIE methods shown in Fig. 2b operate either by reducing contaminant bioavailability (e.g., coconut charcoal addition or cation exchange resin addition) or altering the form of the toxicant to a less toxic form (e.g., zeolite addition) [11].

The objective of this chapter is to discuss the status of selected innovations in whole sediment TIEs beyond the established methods discussed previously. These innovations emphasize several new TIE manipulations as well as a novel biological endpoint that may be included in the TIE structure or serve to confirm TIE findings. As with most TIE methods, the innovations have advantages and disadvantages; however, when used in the structure of a whole sediment TIE they have or will have the potential to generate useful information for identifying the causes of toxicity.

2 Recent Developments: Manipulations

2.1 Use of Supercritical Fluid Extraction

2.1.1 Introduction

Supercritical fluid extraction (SFE) is a preparatory method for extracting NOCs from various solid matrices including soils, sediments, and tissues [13, 14]. The method uses pressurized carbon dioxide (CO_2) under supercritical fluid conditions as a substitute for standard organic solvents. Under supercritical conditions and at elevated temperatures, the CO_2 assumes the behavior of an organic solvent (e.g., hexane, dichloromethane) and the NOCs effectively partition from the competing matrix (e.g., soil, sediment) into the supercritical fluid. For analytical purposes, the extraction can be enhanced by including small quantities of organic solvents into the supercritical CO_2. Upon returning to ambient conditions, the supercritical CO_2 becomes a gas and the isolated NOCs are retained as an extract in a standard organic solvent. The method has several advantages including the minimization of organic solvent use and reduced exposure of laboratory staff to harmful solvents. For use in biological applications including TIEs, the method is also beneficial because of the lack of organic solvent residues in the extracted sample (assuming small amounts of organic solvents are not used) as any remaining CO_2 transfers to a gaseous state leaving the sample essentially intact. Bjorklund et al. [14] first proposed using SFE to evaluate the bioavailability of NOCs. They showed SFE treatments (12,000 KPa, 40°C for 1 h and 400 bar, 40°C for 1 h) reduced the amount of several measured PCBs bioaccumulated by a larval freshwater midge (*Chironimus pallidivitatis*) raised in the treated sediment [15, 16]. Following the SFE treatments, sediment PCB concentrations were reduced by 33–67% as compared to initial concentrations. Similarly, larvae tissue concentrations were reduced by 64–94% [15]. In a later study, Nilsson et al. [17] illustrated the utility of SFE for measuring and predicting the bioavailable fraction of PCBs to eels (*Anguilla anguilla*) exposed to lake sediments. Using SFE (36,800 KPa, 40°C for 2 h), they reported the sediment concentrations of eight PCBs were reduced by 38–69% and concentrations in eels exposed to post-SFE sediments were 72–87% lower than with untreated sediments. In several studies, SFE removal of PAHs was shown to be comparable to PAH

removal under field conducted bioremediation conditions [18–20]. They concluded the method could be applied to predict the effects of bioremediation after 1 year. Using three soil samples contaminated with PAHs, Hawthorne et al. [21] applied SFE and then exposed the treated soils to two terrestrial oligochaetes, *Eisenia fetida* and *Enchytraeus albidus*. Under mild SFE conditions (i.e., 20,265 KPa, 50°C for 40 min), concentrations of 2–6 ring PAHs were reduced by 7–97% compared to initial concentrations. It was also observed that smaller PAHs were being removed more effectively than larger compounds which were not being readily extracted. Further, worm survival increased from 0 to ≥95% for *E. fitida* and 0 to >40% for *E. albidus*. Based on this investigation and the earlier work examining the effects of SFE treatment on NOC bioavailability, Hawthorne et al. [21] proposed the use of mild SFE for performing soil TIEs.

2.1.2 SFE in Whole Sediment TIEs

In whole sediment TIEs, SFE may best serve in Phase III, where the results of Phases I and II are confirmed. In this role, SFE could be applied to confirm the findings of the more routine Phase I coconut charcoal addition which is intended to determine if NOCs are the cause of observed toxicity. One reason for using SFE in Phase III rather than Phase I or II is because SFE instruments can only extract relatively small amounts of soil or sediment at a time (i.e., <10 g dry). Consequently, to extract the amount of sample needed for conducting toxicity testing for Phase I is very time and labor intensive. Therefore, use of SFE in Phase I can be prohibitive and is best applied in Phase III to confirm the Phase I results. To this end, in a whole sediment TIE with highly PAH contaminated sediments from Elizabeth River (VA, USA), three SFE conditions were evaluated in a Phase III role [22]. In this investigation, SFE conditions included mild (20,000 KPa; 50°C), medium (40,000 KPa; 50°C), and strong (40,000 KPa; 150°C) treatments. On a chemical basis, all three SFE conditions reduced PAH concentrations on the sediments and in the interstitial waters (collected after the SFE treatment) by up to 100% (Fig. 3) [22]. As the strength of the extraction increased, the amount of removal in both the sediments and interstitial waters also increased. Further, in both media, the amount of removal decreased as the molecular weight of the PAHs became larger. For example, while nearly 100% of medium molecular weight compounds like phenanthrene, anthracene, and fluoranthene were removed in all three SFE treatments, very little if any of the higher molecular weight indeno[1,2,3-cd]pyrene, dibenz[ah]anthracene, or benzo[ghi]perylene were removed. These data suggest the higher molecular weight PAHs are associated with the sediments more strongly than are the smaller PAHs. Despite the lack of removal of the high molecular weight PAHs, as discussed below, toxicity was observed to decrease. Effects of SFE treatments on survival to the two marine species, the amphipod (*Ampelisca abdita*) and mysid (*Americamysis bahia*), ranged from 0% for both species to 100 and 90%, respectively, in the strong SFE treatment (Table 1). These results were confirmatory of the results of the Phase 1 coconut charcoal addition

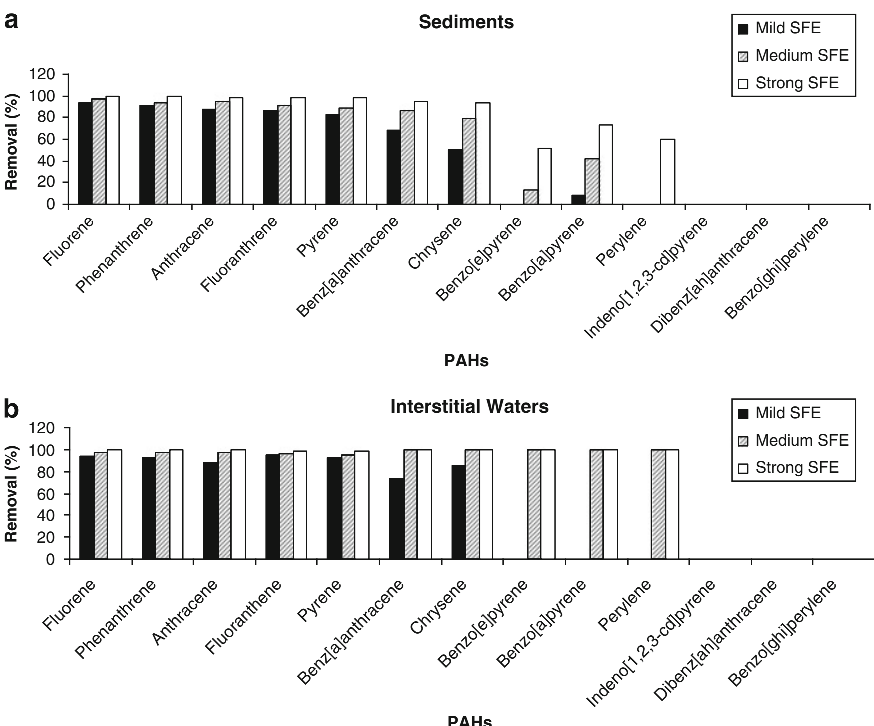

Fig. 3 Removal of selected PAHs from PAH contaminated (**a**) sediment and (**b**) interstitial water from Elizabeth River (VA, USA) under different SFE conditions (from [22])

Table 1 Results of supercritical fluid extraction (SFE) treatments to a PAH contaminated sediment from Elizabeth River (VA, USA) tested with two marine species and comparison to a whole sediment Phase I powdered coconut charcoal (CC) addition manipulation (from [22, 23])

Treatment	Survival (%)	
	Mysid *Americamysis bahia*	Amphipod *Ampelisca abdita*
No SFE	0 ± 0	0 ± 0
Mild SFE	0 ± 0	20 ± 28
Medium SFE	60 ± 0	40 ± 0
Strong SFE	100 ± 0	90 ± 14
CC addition	100 ± 0	93 ± 6

Mean and standard deviation reported

treatment [23]. The mild SFE and medium SFE treatments resulted in little to moderate increases, respectively, in survival compared to the no SFE treatment. As illustrated by the Elizabeth River sediment evaluation of SFE, it can be a useful Phase III manipulation. Burgess et al. [22] found this to be the case for three additional sediments, two were uncontaminated references sediments and one was contaminated (Patrick Bayou, TX, USA). However, they also treated one

contaminated sediment from New Bedford Harbor (MA, USA) in which PCB and PAH concentrations were shown to be reduced by SFE treatment but toxicity was not changed. The unexpected toxicity is suspected to have resulted from the release of initially nonbioavailable divalent cationic metals associated with the sediments during the harsh conditions of the SFE manipulation. Overall, the SFE manipulation looks promising as a whole sediment TIE method for application in Phase III, but given the results with the New Bedford Harbor sediments, care must be taken when using and interpreting SFE data. It is worth noting that the studies cited in this section used only the post-SFE extracted sediment and did not investigate the organic solvent extract generated by SFE. In principle, the SFE extract for a given sediment could be used in a Phase II TIE to determine the specific constituents causing observed toxicity. This is an area of potential future TIE research.

2.2 Methods for Pyrethroid, Organophosphate, and Carbamate Pesticides

2.2.1 Background

In the 1970s, the phase-out of organochlorine pesticides occurred due to their environmental persistence, bioaccumulation, and notable damage to raptor populations [24]. In their place, pyrethroid, organophosphate, and carbamate pesticides started to dominate the market and today play major roles in agricultural and urban use (Fig. 4). These pesticide classes are of environmental concern because of their widespread use and acute toxicity to both target and nontarget organisms. Despite their relatively rapid environmental degradation, they have been implicated in causing toxicity in a number of aquatic environments [9, 25–28]. Due to this ubiquitous environmental toxicity and their likely continued use, TIE methods for these classes of pesticides have been under development since the mid-1990s.

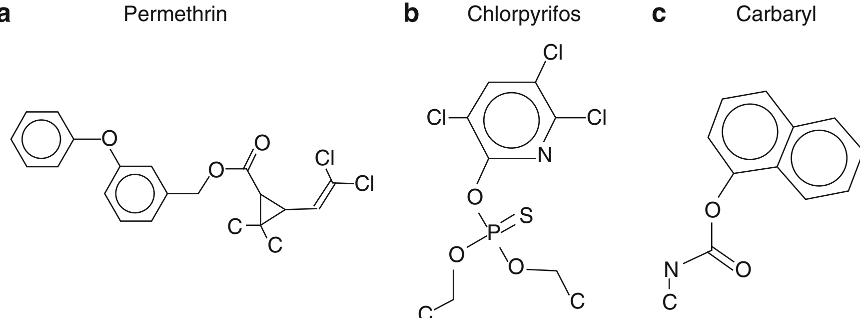

Fig. 4 Structures of example (**a**) pyrethroid (permethrin), (**b**) organophosphate (chlorpyrifos), and (**c**) carbamate (carbaryl) pesticides. Structures from ASTER – assessment tools for the evaluation of risk (http://cfint.rtpnc.epa.gov/aster/). For simplicity, hydrogens and most carbons are not shown

Understanding the history, mode of action, and chemistry of these pesticides is helpful in developing methods to characterize and identify them in complex environmental mixtures. Pyrethrins are naturally occurring compounds found in chrysanthemums. These natural compounds have insecticidal properties, most notably high “knock down” capabilities for flying insects but they also rapidly photo-degrade and oxidize under field conditions. In the 1960s, chemists developed synthetic pyrethroids modeled after pyrethrins and added chemical moieties to make them resistant to degradation. The mode of action in pyrethrins and pyrethroids is disruption of sodium channels in the nerve tissues which cause the nerves to fire continually, resulting in paralysis. Pyrethroids, in addition to being less prone to degradation, are often applied in combination with piperonyl butoxide (PBO). PBO is a compound that inhibits the mixed function oxidase (MFO) enzyme system that biologically degrades pyrethroids while in the target, or nontarget, organism. This inhibition prevents insects from clearing pyrethroids from their system which enhances paralysis and, ultimately, lethality. Hundreds of pyrethroids have been developed, and the trade names of a few common ones include permethrin, fenvalerate, cypermethrin, alethrin, bifenthrin, and cyhalothrin [29].

All organophosphate pesticides are synthesized from phosphoric acid. These pesticides tend to be relatively toxic to both target and nontarget mammalian species, but degrade rapidly in the environment. Organophosphate pesticides irreversibly inhibit the enzyme, acetylcholinesterase, which breaks down acetylcholine. Acetylcholine is the compound that transmits nerve impulses across synapses. When acetylcholinesterase is inhibited, acetylcholine builds up in the nerve junctions interfering with nerve impulse transmission, which may result in death of both target and nontarget organisms. Organophosphates are often categorized by those that need to be activated by the MFO enzyme system before they can inhibit acetylcholinerase, and those that do not. Common organophosphate pesticides include chlorpyrifos, diazinon, dichlorvos, and malathion [29].

Carbamate pesticides are those that contain the carbamate functional group (R^1-O-(CO)-N-R^2). Carbamate pesticides include carbofuran, aldicarb, and carbaryl (Sevin). Carbamates tend to degrade under most field conditions, undergoing hydrolysis, photodegradation, and microbial degradation. These pesticides, like organophosphates, inhibit the acetylcholinesterase enzyme. However, they do not need to be activated by the MFO enzyme system prior to inhibition. In addition, their metabolic activity is reversible (unlike organophosphates) so they are generally less toxic [29].

Pesticide manufacturing comprises a multibillion dollar industry creating millions of tons of pesticides annually (http://www.epa.gov/oppbead1/pestsales/01pestsales/sales2001.htm). These pesticides are present in aquatic systems in North America [30], Australia [31], and Europe [32, 33]. In North America, they are suspected of causing toxicity in many tributaries [34–38]. In response, TIE methods have been developed and refined since the mid-1990s to help characterize and identify specific pesticides and pesticide groups. The following section is divided into TIE manipulations that target the above pesticide classes.

2.2.2 Piperonyl Butoxide Addition

The addition of PBO can be used to determine the presence of both pyrethroids and organophosphates. Many organophosphate compounds must be activated by MFOs before they can inhibit the acetocholinesterase enzyme. Researchers demonstrated that the addition of PBO, which inhibits the MFO system, decreased the toxicity of the metabolically activated organophosphate pesticides – parathion, methyl parathion, diazinon chloropyrifos, azinophos-methyl, and malathion [39, 40]. They also demonstrated that organophosphate pesticides that do not require metabolic activation, such as dichlovos, chlorofenvinphos, and mevinphos, were not affected by PBO addition. Bailey et al. [41] also confirmed that the toxicity of the organophosphate pesticides, chloropyrifos and diazinon, decreased when PBO was added, however, carbofuran (a carbamate) toxicity was not changed. Carbamates do not need to be activated by the MFO system before they exert toxic action so they would behave like organophosphates that do not require metabolism.

In addition to decreasing the toxicity of organophosphates, PBO also generally increases the toxicity of pyrethroid insecticides by inhibiting the MFO system which degrades pyrethroids. Researchers have demonstrated that the toxicity of bifenthrin, permethrin, cypermethrin, and L-cyhalothrin increased to the freshwater amphipod *Hyalella azteca* when PBO was added to field sediments and in whole sediment toxicity tests [42–44]. Because of the opposing effects of PBO on organophosphates and pyrethroids, interpretation of changes in toxicity in field samples should be performed with care [26, 45]. Amweg and Weston [26] demonstrated that PBO-induced toxicity was not observed when equimolar concentrations of chloropyrifos and pyrethroids were present; however, they concluded that chloropyrifos field concentrations were generally not high enough to mask PBO-induced toxicity. In addition, they demonstrated that the toxicity of the metal cadmium and the PAH fluoranthene did not change when PBO was added to a test sediment. In whole sediment exposures, PBO is effective when added to overlying water in the test system. Methodology details can be found in a number of publications (e.g., [9, 26, 27, 35]). The success of this method is dependent, in part, upon the amount and type of MFO present in the test organism, as well as knowledge of the metabolic pathway of the pesticide.

2.2.3 Carboxylesterase Addition

Carboxylesterases hydrolyze ester containing compounds such as pyrethroids to their relatively nontoxic alcohol and acid components. This addition has been shown to be an effective method to detect the presence of pyrethroids in interstitial waters [9, 35, 46, 47] and whole sediments [28, 44, 45, 48]. Further, this method has been shown to be very useful in whole sediment TIEs with field sediments containing bifenthrin [44]; bifenthin and L-cyhalothrin [43]; permethrin, bifenthrin, and L-cyhalothrin [9]; and permethrin, bifenthrin, L-cyhalothrin, and cypermethrin [48]. Researchers have also shown that when esterases are added to the overlying water

of whole sediment tests, they selectively hydrolyze pyrethroids and do not change the toxicity of organophosphate pesticides [46, 48]. While esterases have the theoretical capability to hydrolyze carbamates [49], there has been no research performed on the efficacy or selectivity of this manipulation with carbamates in environmental samples. Esterases are generally derived from pigs or rabbits and the effective concentration differs from batch to batch. Esterase addition is generally performed with a dissolved organic matter control [i.e., bovine serum albumin (BSA)], as it has been shown that an increase in dissolved organic matter can substantially reduce the toxicity of NOCs by reducing their bioavailability. Researchers are developing engineered enzymes to specifically hydrolyze pyrethroids and organophosphates. These enzymes have promise in aqueous substrates but have been shown to be less effective in whole sediments [50].

2.2.4 Temperature Change Manipulation

Temperature is another tool used in TIEs to distinguish pesticide toxicity in whole sediments [9, 45, 51, 52]. Generally, an increase in temperature is positively correlated with an increase in toxicity [53] and this trend has been demonstrated for organophosphates [51] and carbamates [54]. Pyrethroids are generally an exception to this rule as researchers have shown that decreasing temperature is associated with increasing pyrethroid toxicity [52, 55–57]. Confounding factors using the temperature change manipulation with field sediments include effects on other toxic chemicals known to increase in toxicity with decreased temperature such as the DDTs. In addition, toxicants that have a strong positive toxicity correlation with temperature, such as cadmium, may mask the negative correlation of toxicity with temperature for pyrethroids [57]. Finally, this correlation is not necessarily consistent across all pyrethroids with all organisms [45]. Wheelock et al. [45] found that freshwater fathead minnows (*Pimphales promelas*) demonstrated almost no correlation between temperature and toxicity for the pyrethroids permethrin, bifenthrin, cypermethrin, esfenvalerate, and cyfluthrin. This is in comparison to the freshwater daphnid, *Ceriodaphnia dubia*, which generally demonstrated a strong negative temperature–toxicity correlation for the same pyrethroids except cypermethrin. Despite these confounding factors, researchers have shown that a negative temperature toxicity correlation is generally indicative of pyrethroid toxicity in whole sediment TIEs with field sediments [44, 57]. These results indicate the temperature change effect relationships can be a useful addition to the weight-of-evidence approach to characterizing and identifying these types of pesticides.

2.2.5 Summary

In general, PBO addition, esterase additions, and temperature change are effective methods for characterizing and identifying pyrethroids, organophosphates, and carbamates when used within a TIE design. Wheelock et al. [46] developed

a flowchart of how TIE methods can be expanded to include esterase and PBO addition. We have modified their flowchart to include temperature addition to an overall TIE schematic (Fig. 5). These manipulations, like all TIE manipulations, should include careful use of controls (e.g., appropriate organic carbon controls for esterase activity) and data interpretation. In addition, organism tolerance to

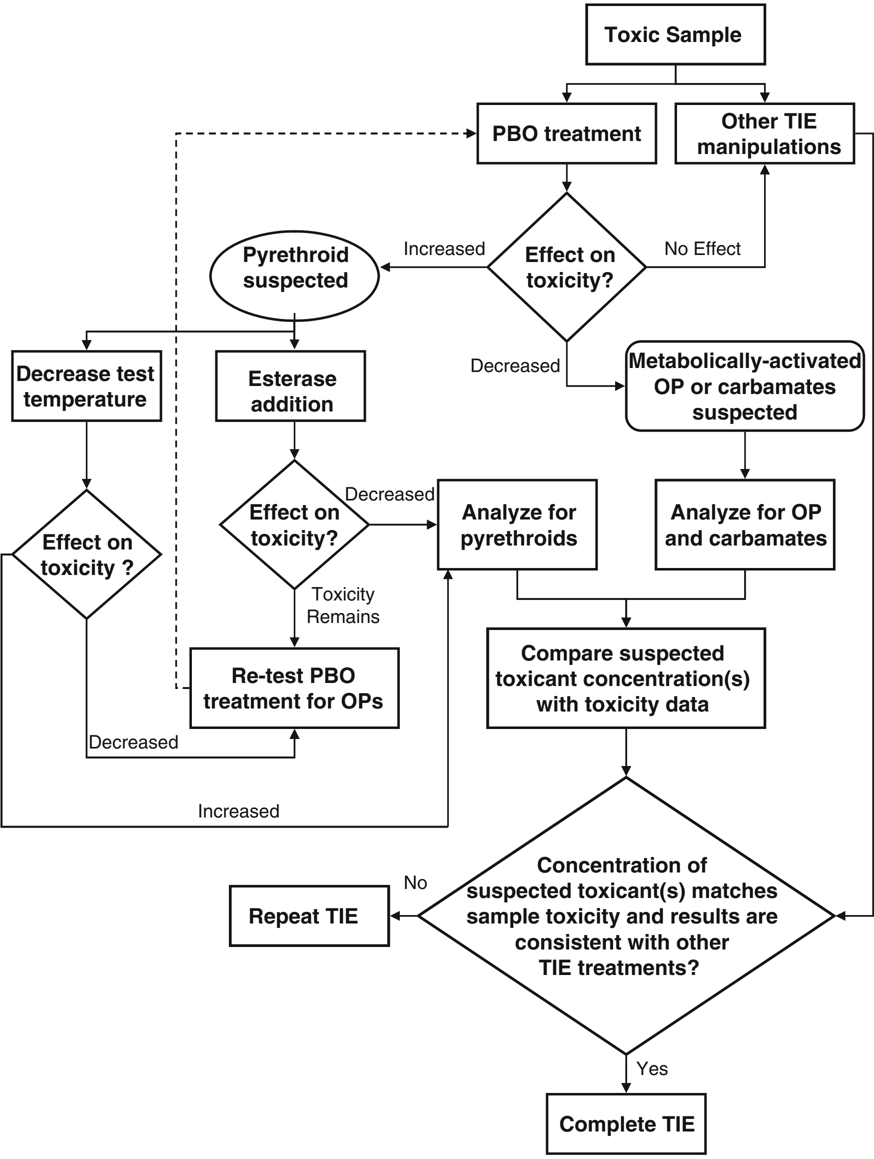

Fig. 5 Phase 1 TIE manipulation flowchart for pyrethroid, organophosphate, and carbamate pesticides as adapted from [46]

differing temperatures, the presence and type of MFO system in the test organism, and whether metabolic activation of the pesticide is necessary for toxic action all need to be considered. As noted above, because of confounding factors, including conflicting interactions of pyrethroids and organophosphates with PBO addition, exceptions to the inverse temperature–toxicity correlation with pyrethroids are likely to occur. Consequently, these manipulations are not standalone but should be used within a weight-of-evidence approach which includes at least two independent lines of evidence which point to the same toxicant with no contradictory lines of evidence [10].

3 Endpoints

3.1 Genomic Methods

3.1.1 Introduction

In recent years, there has been an increasing focus on genomic-based technologies within ecotoxicology [58]. Traditional ecotoxicology endpoints include relatively "broad" endpoints, such as mortality or reduced measures of growth and reproduction. Though these endpoints are clearly biologically important, and may have impacts at the organismal, population, or community levels, as biomarkers, in general, they are relatively uninformative by themselves in terms of identifying the causes of adverse effects. To produce measurable changes in these biological endpoints, unknown samples must be relatively toxic or exposures must occur for significantly long periods. Conversely, gene expression-based endpoints are often more sensitive and are useful for identifying exposures at sublethal concentrations. For example, male zebra fish (*Danio rerio*) exposed to 50 ng/L of the synthetic estrogen 17-α ethinylestradiol (EE2) displayed reduced fecundity after 6–10 days of continuous exposure [59], whereas they displayed elevated levels of the egg precursor protein, vitellogenin, a commonly used genomic biomarker of estrogenic exposure, following an 8-day exposure at concentrations of EE2 as low as 3.6 ng/L [60].

The worth of genomic endpoints is predicated on the idea that the direct interaction between an organism and the environment occurs on the cellular level. The result of this interaction is often an alteration of the cell's transcriptome or proteome, terms which refer to the total number and species of mRNAs or proteins within the cell at a given time. The species of mRNA and proteins present within a cell are indicative of the current state of the cell, thus perturbations divergent from daily maintenance and growth represent a new challenge that the cell must mediate (reviewed in Snell et al. [61]). Because alterations of the transcriptome and proteome are often the first and immediate responses of cells to environmental challenges, they are often specific and directed, since inappropriate activation of genes and proteins frequently results in pathologies evident at higher biological levels.

This biological cost underlies the need for an accurate response, thus necessitating tight regulation of mRNA and protein expression. Expression changes are specific to varying degrees for a chemical class or a mode of action [62]; therefore, they are often referred to collectively as an expression signature, and have been proposed as a new class of biomarker. There are several examples of expression signatures in ecotoxicology [63], as well as analogous uses of expression signatures in human health linking expression to disease states [64]. It is these expression signatures that show promise for use in or to confirm the results of whole sediment TIEs.

The identification of gene expression signatures can be accomplished through the use of technological platforms that allow wide-range monitoring of the entire transcriptome or proteome. The two most often used platforms are microarrays and two-dimensional polyacrylamide electrophoresis (2D PAGE) for transcriptional and proteomic inquiries, respectively. Microarrays consist of single-stranded DNA "probes" that are complementary to known mRNA sequences. The probes are arrayed on a solid substrate, usually a glass microscope slide. A typical experiment involves the isolation of RNA from a control group and a treatment group. Isolated RNA is then labeled with either a Cyanine-3 (Cy3) or Cyanine-5 (Cy5) fluorescent dye. The differentially labeled RNAs are then pooled, mixed, and allowed to hybridize to their corresponding probes on the microarray. Two images are captured, one each of the Cy3 dye and Cy5 dye channels. The resulting images are then overlaid and the color of each probe in the resulting composite image, which is a blend of the Cy3 and Cy5 channels, is used to determine the expression level of that probe relative to the control. Microarrays are somewhat limited in that they can only be used to quantify RNA for which probes exist. This may be problematic in cases where there is little or no genetic information available for the organisms being studied, as is often the case for ecotoxicologically important aquatic organisms. Therefore, there must be a significant preparatory effort to characterize these organisms on the molecular level. This often involves the use of cDNA library construction or, more recently, massive parallel sequencing to obtain transcriptional sequence information [65], both of which are cost and labor intensive. An alternative approach to transcriptional characterization is to interrogate the proteome using 2D PAGE. This technology requires no a priori knowledge of the proteome in the study organism, so it is readily suitable for poorly characterized organisms. There are additional advantages to focusing on the proteome, such as an increased biological relevance of identified changes that comes with analysis at a higher biological level. In 2D PAGE, proteins are independently isolated from control and treated samples. Sample proteins are then separated based on their physicochemical properties, generally isoelectric point and molecular weight. To visualize the results, the proteins are either fluorescently labeled prior to separation (i.e., difference gel electrophoresis [66]) or poststained using any number of available stains. Images are then taken of the separated proteins and analyzed in a manner similar to that of microarrays. One of the major drawbacks of this technique is its reported high degree of analytical variability, which can mask important changes (discussed below). Both 2D PAGE and microarrays have been shown to reliably identify toxicant response genes. However, reliable identification of

expression signatures often requires a significant reliance on complex statistical algorithms that generally are only available in expensive packages thus necessitating a significant bioinformatics investment.

3.1.2 Integration of Genomic Endpoints into TIEs

Once expression signatures have been established, they can be integrated into any number of existing toxicity tests in lieu of mortality, reproduction, or growth endpoints. In terms of TIEs, they can be integrated in both a “top-down” and “bottom-up” approach (Fig. 6). In the “top-down” approach, test individuals would be exposed to the initial or baseline sample as would normally be performed in Phase 1 of the TIE process. The transcriptome and/or proteome would then be examined for the presence of already established expression signatures for known toxicants (e.g., PCBs, metals, ammonia). If one or several were detected their relative contribution to the total toxicity of the sample could be confirmed during the characterization and identification phases of the TIE. In the “bottom-up” approach, the initial/baseline and control samples would again be evaluated as described above; however, the expression profiles of the organisms from the various TIE Phase I manipulation groups would also be evaluated. In this case, one would expect the expression patterns to shift in similarity away from the unmanipulated sample toward that of the control as the bioavailability of the toxic agents is altered during the TIE. Each of the two strategies has distinct advantages and disadvantages. The “top-down” approach would potentially allow the relatively cumbersome characterization and identification of manipulation exposures to be avoided, based on the reliability of the expression pattern. Thus, toxicants could potentially be confirmed immediately following Phase 1 using instrumental chemical analysis. The main disadvantage of this approach is that it

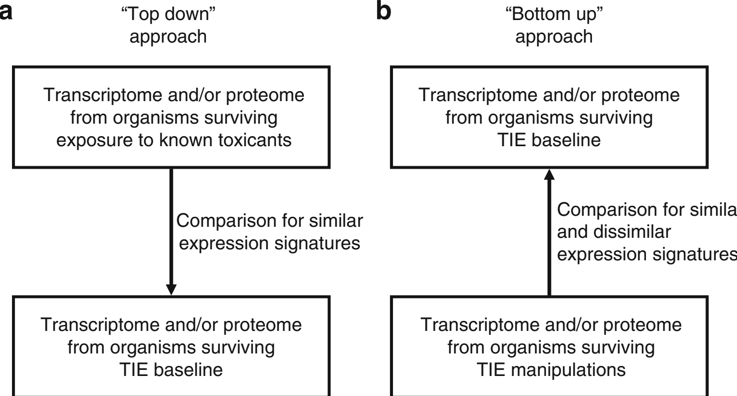

Fig. 6 Illustration of proposed (**a**) “top-down” and (**b**) “bottom-up” approaches for integrating genomic information into the whole sediment TIE

would require confidence that the expression signatures were unaffected by interactions with other contaminants contained in the environmental samples and that they remain intact under varying environmental conditions; an assumption difficult to test given the variability possible in environmental samples. The main disadvantage of the "bottom-up" approach is that it may require substantially more microarrays, adding more cost in terms of materials, time, and human resources. However, a major advantage of the "bottom-up" approach is that it does not necessarily rely on the a priori establishment of chemical specific expression signatures, as the shift in expression profiles away from the unmanipulated sample toward that of the control may be adequate to determine the toxicant class.

There are several factors that may affect the development and subsequent integration of expression signatures into TIEs. Unknowns in the degree of biological variability amongst test organisms within and across populations may be a limiting factor in the ability to determine expression signatures. The expression signature must be sufficiently stable and of a large enough magnitude to be detectable amidst background genetic variability. Therefore, as the degree of background variability increases, the quality of the expression signature decreases. This may be particularly problematic when using field-collected test organisms. For example, the marine amphipod, *A. abdita*, which is found along the east coast of the USA as well as in introduced populations on the west coast, is commonly used in sediment toxicity tests [67, 68]; however, there is no information publically available regarding the population genetics of this organism. Though a species may exhibit a continuous geographic distribution, it may contain discrete genetic subpopulations [69]. This suggests a knowledge gap in the understanding of what denotes a genetically homogenous population. For a population to be genetically homogenous there must be equal gene flow throughout, so that it is as likely that individuals on either geographic extreme of the population are equally likely to interbreed. For *A. abdita*, which is not considered highly mobile (as compared to pelagic fish), this would be unlikely. Because *A. abdita* has not been successfully cultured in the laboratory [70] it must be obtained from field collections for each toxicity test or TIE performed, and it may not always be known whether single or multiple populations are being sampled with each collection event. Even more potentially dramatic is trying to compare test organisms genomically when using populations from geographically separate areas (e.g., east coast vs. west coast of North America). Gene expression has been shown to vary among populations [71]; therefore, it is unclear how this alteration of the genetic background will affect the expression signature and the resulting efficacy of the TIE. An additional complication with the use of field caught organisms is the possibility of unknown or unanticipated contaminants in the collection area. These contaminants may act as a selective force further increasing variability among populations [72] and potentially resulting in organisms that exhibit adaptation to a given stressor and thus are relatively insensitive [73], and of limited use for detection of that stressor. Stressor resistance has been found to be inheritable in some fish species [74], suggesting a genetic underpinning, which may further complicate the use of genetic tools from uncharacterized organisms from unknown backgrounds. The timing of field collections

may also need to be considered when using field organisms in toxicological studies. It has been previously demonstrated that contaminant levels can vary widely with seasonal factors such as rain events (discussed in Luoma and Phillips [75]). However, careful planning and selection of field-collected test organisms can often avoid this sort of problem. Until the magnitude of these forms of genetic variability is quantified and assessed, many of the factors discussed previously can be side stepped through the use of laboratory cultured organisms. The previous discussion focused on the amphipod *A. abdita*, but many of the points noted are also likely to be true of many other commonly used field collected toxicity testing organisms. Despite current challenges to using genomic endpoints within the TIE context, the potential of these endpoints is extremely promising.

4 Summary and Status of Whole Sediment TIEs

Three relatively recent innovations in whole sediment TIE methods have been discussed in this chapter. Often, the methods have two common points: (1) promise for advancing the objectives of whole sediment TIEs and (2) challenges to address before wide range application especially on a routine basis. For example, SFE appears to be useful as a Phase III confirmatory tool but has demonstrated what may be occasional artifactual toxicity. Further, the pesticide methods are probably the most well developed of the discussed manipulations and are being used currently for performing whole sediment TIEs. Finally, the new genomic endpoint offers remarkable potential. If the "expression signatures" are ultimately developed successfully, they may be far more useful than simply confirming the results of conventional whole sediment TIEs. If they are made sufficiently specific, the expression signatures could replace the conventional whole sediment TIE. That is, environmental diagnostic investigations could include a genomic scan of organisms from areas suspected of being adversely affected by anthropogenic activity. The resulting expression signatures would constitute the dataset necessary to determine what stressors are causing toxic effects assuming adequate genomic libraries of individual toxicant expression signatures become available. In this scenario, the whole sediment TIE could be made redundant.

We should note that the topics discussed here are far from comprehensive. For example, we did not discuss the development of new conventional TIE testing organism endpoints or recent TIE-related studies showing much of the sediment toxicity previously associated with PAHs may actually be resulting from the presence of high concentrations of the oils [76]. These and other worthy developments will have to await a future book chapter or review article. For example, Brack and Burgess (see Chap. 3 in this volume [77]) discuss contaminant bioavailability issues as related to both TIE and EDA procedures.

With regard to the current status of whole sediment TIEs, as discussed previously, much of the whole sediment TIE development occurred in the late 1990s and early 2000s in North America. Since that time, research has continued relative to

whole sediment TIE methods with a focus on some of the topics discussed previously especially pesticides as well as assorted applications work. For example, these include the preparation of reports summarizing the findings of whole sediment TIEs (e.g., [23]) and guidance documents providing TIE methods [9, 10]. Further, valuable studies demonstrating the application of whole sediment TIE methods have been published [28, 44, 78–80].

While this research has been occurring, in North America, the need for reliable whole sediment TIE methods has also been intensifying. In the USA, with increasing frequency, federal and state organizations have been performing whole sediment TIEs for regulatory purposes. For example, the Clean Water Acts' total maximum daily loading (TMDL) process [81], which is designed to reduce the adverse effects of stressors on the country's water bodies, is very likely to result in the performance of more TIEs. This is because the TMDL process includes a diagnostic component for identifying toxic stressors in order to stop loadings or characterize sources, an ideal application for whole sediment TIEs. As a consequence of this future regulatory activity, the demand for whole sediment TIEs may significantly increase and with the increase, the need to develop new and more innovative TIE methods.

Acknowledgments The authors appreciate the insightful technical reviews provided by David Katz, Monique Perron, Jonathan Serbst, and Wayne Munns on this manuscript. This is NHEERL-AED, Narragansett Contribution AED-10-028. Mention of trade names or commercial products does not constitute endorsement or recommendation for use. This report has been reviewed by the US EPAs Office of Research and Development National Health and Environmental Effects Research Laboratory, Atlantic Ecology Division, Narragansett, RI, and approved for publication. Approval does not signify that the contents necessarily reflect the views and policies of the Agency.

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Considerations for Incorporating Bioavailability in Effect-Directed Analysis and Toxicity Identification Evaluation

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Abstract In order to avoid a bias toward highly toxic but poorly bioavailable compounds in the effect-directed analysis (EDA) of soils and sediments, approaches are discussed to consider bioavailability in EDA procedures. In parallel, complimentary approaches for making toxicity identification evaluations (TIEs) more capable of performing high resolution fractionation, toxicant isolation and identification are described. These approaches focus on three processes including bioaccessibility based on desorption kinetics from the abiotic matrix, activity driven partitioning into pore water and biota tissue or a biomimetic tool, and EDA and TIE in tissues and body fluids representing toxicological bioavailability including the toxicokinetics of the selected organism. Bioaccessibility may be addressed by extraction procedures that are designed to yield rapidly desorbing fractions including mild solvent extraction, desorption into water with subsequent adsorption to a competitive adsorbent such as TENAX® or cyclodextrin, supercritical fluid extraction, or biomimetic extraction with gut fluids of potentially affected organisms. While equilibrium partitioning-based extraction procedures may simulate partitioning into biota quite well they often fail to provide sufficient amounts of toxicants for subsequent EDA and TIE. Partition-based dosing, which may be combined with bioaccessibility-directed extraction methods, provides an excellent tool to simulate partitioning in sediments and to provide constant and well-defined concentrations in bioassays. EDA studies in fish and mussel tissues as well as in fish bile demonstrate the potency of the identification of bioavailable toxicants in biota. Continued research on the described approaches promises to improve the usefulness of both EDA and TIE in future applications.

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W. Brack (ed.), *Effect-Directed Analysis of Complex Environmental Contamination*,
Hdb Env Chem (2011) 15: 41–68, DOI 10.1007/978-3-642-18384-3_3,
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Keywords Bioaccessibility, Biota tissue, Cyclodextrin, Desorption kinetics, Partition-based dosing, Supercritical fluid extraction, TENAX®

Contents

1 Significance of Bioavailability in Effect-Directed Analysis and Toxicity Identification Evaluation 42
2 The Concept of Bioavailability 43
3 Bioaccessibility-Directed Extraction Procedures 46
3.1 Cyclodextrin Extraction 47
3.2 TENAX Extraction 48
3.3 Supercritical Fluid Extraction 51
3.4 Extraction with Biological or Biomimetic Fluids 53
3.5 Bioaccessibility-Directed Extraction in EDA and TIE 54
4 Equilibrium Partitioning-Based Extraction and Dosing 55
4.1 Equilibrium Partitioning-Based Extraction 55
4.2 Partition-Based Dosing 57
5 EDA in Biota Tissues 59
6 Conclusions 60
References 61

1 Significance of Bioavailability in Effect-Directed Analysis and Toxicity Identification Evaluation

Environmental analytical chemistry was developed with a particular focus on the detection and quantification of target analytes in environmental samples including biota, soil, sediment, air particulate matter, air, and water. In parallel, GC–MS nontarget screening methods have been developed [1]. However, both target analysis and nontarget screening methods are limited with respect to the assessment of the hazards of complex environmental mixtures. Fortunately, the emergence of new biological tools to detect chemicals via their adverse effects has offered promising tools for addressing these complex mixtures. About 30 years ago, these considerations inspired analytical chemists to focus chemical analysis on those components of complex mixtures causing adverse effects. This approach integrating bioassays, fractionation procedures, and chemical analysis in a sequential procedure was called bioassay-directed chemical analysis [2] or effect-directed analysis (EDA) [3]. In agreement with classical chemical analysis, EDA was normally based on exhaustive extractions, which were fractionated and dosed in bioassay-compatible solvents such as dimethylsulfoxide. Only recently there has been increasing concern that this analysis, particularly when applied to soils and sediments, ignores bioavailability and thus may produce a bias toward highly toxic but poorly bioavailable hydrophobic organic compounds which pose limited risks to biota while more bioavailable toxicants are overlooked [4, 5].

In parallel to EDA, the concept of toxicity identification evaluation (TIE) was developed and standardized in the USA for effluents and other water samples [6–8]

and later extended to sediments [9, 10]. While EDA has a focus on the identification and structure elucidation of individual toxicants typically based on in vitro effects, TIE has its origin and applications in whole effluent, receiving water, interstitial water, and whole sediment testing using in vivo toxicity tests. In TIEs conducted on water and sediment, a strong focus is given to the ecological relevance to whole organisms and environmentally realistic exposure conditions and thus on bioavailability; for example, the conduct of whole sediment toxicity tests [8]. This emphasis evolved from the strong linkage between the US Clean Water Act's goal of prohibiting discharge to the environment "of toxic pollutants in toxic amounts" to adverse effects in whole organisms (e.g., survival, reproduction, growth) [11]. However, this emphasis on whole organisms and environmentally realistic exposures has often limited the ability of the TIE to apply high resolution fractionation procedures and toxicant isolation and identification. Generally, these limitations result from the excessive costs and technical challenges related to the generation of sufficient fractionated sample to test whole organisms in an environmentally realistic fashion.

Involvement of bioavailability into EDA procedures aims to enhance ecological realism without reducing analytical power or enhancing costs. Thus, the procedures should be compatible with high throughput in vitro and in vivo assays as well as with high resolution separation procedures. So far, three different approaches have been developed to meet these requirements including bioaccessibility-directed extraction procedures, partition-based dosing, and EDA of tissue or body fluids of exposed biota. These approaches very well support a general concept of bioavailability as discussed later in this chapter. Relative to TIE, the incorporation of bioavailability is intrinsic to the approach, but as noted, limits the use of some of the more powerful fractionation, isolation and identification tools that the EDA approach uses so effectively. As discussed below, some of the approaches considered for involving bioavailability in EDA also offer promising tools for expanding the capabilities of TIE.

2 The Concept of Bioavailability

"Bioavailability is the degree to which chemicals present in the soil (or sediment) may be absorbed or metabolized by human or ecological receptors or are available for interaction with biological systems", according to the definition in ISO 11075: 2005. It is obvious that this comprehensive definition represents the complex interaction between a specific chemical, sediment or soil, and a specific organism depending on matrix and chemical properties as well as species type, life stage, metabolic activity, nutritional status, feeding behavior, and many more traits of the organism. Bioavailability is neither a straight forward measurable value nor an easily operationally defined parameter to include in EDA and TIE procedures. This problem may be approached by separating bioavailability into three steps according to ISO/DIS: 2006 and Harmsen [12] (Fig. 1).

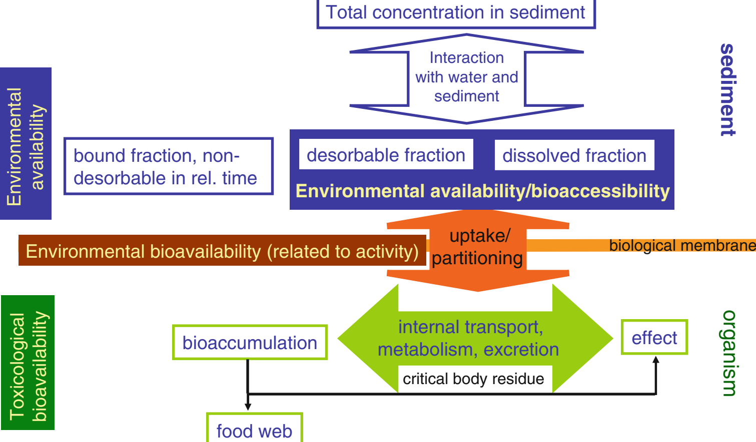

Fig. 1 Bioavailability in a sediment or soil system adapted from ISO/DIS: 2006 from [12] (rel. means relevant)

The first step focuses on processes in the soil or sediment that are independent of the biota. It is assumed that only those molecules (or ions in the case of some metals) may become bioavailable that are either dissolved in the interstitial water or can rapidly desorb. These fractions are often called environmentally available or bioaccessible. Molecules that are tightly bound and desorb in months to millennia, such as PAHs enclosed in soot carbon particles [13, 14], contribute very little to uptake into biota. A conceptual model of soil and sediment sorbent domains is given in Fig. 2 [13]. Bioaccessibility is a process determined by kinetics rather than by equilibrium partitioning. For example, ingestion and digestion of sediment particles by organisms may significantly enhance the uptake kinetics of a contaminant while the final concentration of the nonmetabolized hydrophobic organic chemical, at equilibrium, should be independent of the uptake route. To utilize this relationship, mild extraction methods have been developed to reflect the relevant desorption kinetics.

The second step may be termed environmental bioavailability and is characterized by equilibrium partitioning of freely dissolved or rapidly desorbing molecules between the dominant sedimentary phases (e.g., organic and soot carbon), the water phase, and biota [15]. This process is driven by chemical activity gradients between the different phases. For organic molecules, this principle may be operationally defined either by biomimetic extraction procedures to determine freely dissolved concentrations [e.g., solid phase micro extraction (SPME)] or by partition-based dosing techniques in toxicity testing, where chemicals of interest are dosed via a solid phase which emulates sediment or soil organic carbon. The concentration of every individual compound in the test medium and in the organism's tissues is a result of the equilibrium between the three phases.

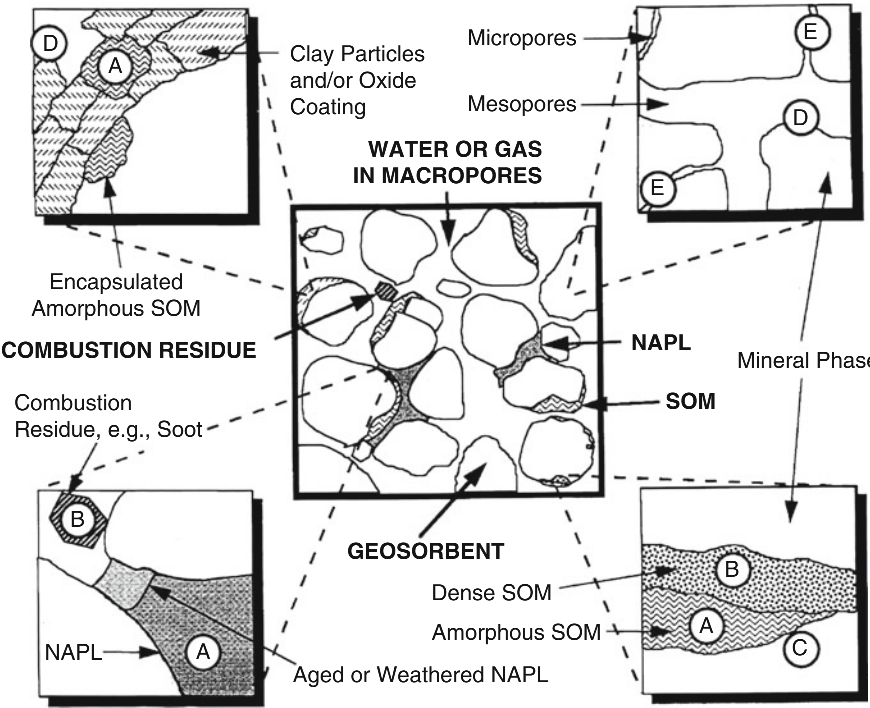

Fig. 2 Conceptual model of geosorbent domains. The *circled letters* refer to the following sorption mechanisms: A: absorption into amorphous natural sorbent organic matter (SOM) or nonaqueous-phase lipids (NAPL), B: absorption into condensed organic polymeric matter or soot, C: adsorption onto water-wet organic surfaces, D: adsorption to exposed water-wet mineral surfaces, E: adsorption into microvoids or microporous minerals with porous surfaces at water saturation below 100% (from [13] with permission)

The internal concentration in a real organism, however, is not only determined by equilibrium partitioning with the surrounding phases but also by toxicokinetic processes within the organism including internal transport, metabolism, and excretion [16]. In principle, exceeding the critical body residues or burden for a given chemical as a result of equilibrium partitioning and active toxicokinetic processes may result in adverse effects in the organism. Because these processes are organism specific they can be included in the EDA only if the organism itself is subjected to EDA. In contrast, in TIE, the steps outlined in Fig. 1 are built into the experimental design and, in fact, are used in the TIE manipulations. For example, the powdered coconut charcoal addition manipulation in whole sediment TIE (see Fig. 2B, [10]) introduces a large quantity of highly adsorbent black carbon to the test sediment [17]. If hydrophobic organic contaminants were contributing to whole organism toxicity, the addition of the charcoal results in the partitioning of the contaminants to the new sedimentary phase diminishing bioavailability by reducing the

bioaccessible concentrations of the contaminants [18]. A similar approach is used with the cation exchange resin addition but with the emphasis on toxic metal ions including cadmium, copper, nickel, lead, and zinc [19].

3 Bioaccessibility-Directed Extraction Procedures

The bioaccessible fraction of a contaminant in sediment is defined as the fraction that is readily desorbable from the sedimentary phase and thus accessible for partitioning with benthic organisms or for biodegradation. As a mechanism of the slow desorption of hydrophobic organic chemicals from natural organic carbon, diffusion limitations have been suggested to play a major role [20]. Temperature dependence of slow desorption allows for an estimation of activation enthalpy and suggests diffusion through the “polymer” (i.e., organic carbon or matter) rather than through pores as a mechanism for slow desorption [21]. Rapidly desorbing fractions are assumed to be present in the outer regions of sediment particles and to represent the fraction that is available for uptake into organisms and loss to biodegradation. There are controversial studies on whether desorption behavior can be correlated to sediment characteristics such as grain size, organic carbon, soot carbon, or the presence of young organic matter such as plant pigments, lipid, and lignin contents [22–24]. While the behavior of contaminants partitioned to soot carbon seems to support the existence of slowly and very slowly desorbing fractions, great proportions of young organic matter may enhance rapidly desorbing fractions. Competitive interactions of organic contaminants with sediment particles indicate that the number of slow sorption sites is probably limited. This is in accordance with nonlinear sorption of the slowly desorbing fraction [25].

We may distinguish generic approaches to extract bioaccessible fractions and those which try to mimic desorption in the digestive system of specific organisms. Bioaccessibility is generally associated with extraction procedures designed to yield rapidly desorbable and thus loosely bound contaminant fractions. Generic approaches include (a) mild solvent extraction using butanol [26], other organic solvents or mixtures thereof with water [27, 28], supercritical carbon dioxide [29–33], or subcritical water [34]; and (b) desorption into water with subsequent adsorption to a competitive adsorbent like TENAX® [35, 36], XAD2® [37, 38], or cyclodextrins [39, 40]. Recently, a fluidized-bed column method for the extraction of bioaccessible trace elements from solid wastes has been presented [41].

Despite attempts to understand the mechanisms behind bioaccessibility, rapidly desorbing fractions are still not predictable by sediment and contaminant properties alone. In addition, bioaccessibility is still a strongly operationally defined parameter and there are few studies comparing different methods (e.g., [27, 42]). All of the available studies suggest large differences between the extraction efficiencies of the different methods. However, only limited efforts have been focused on explaining these differences and on developing a comprehensive mechanism-based concept of bioaccessibility.

A good basis for a comprehensive mechanistic-based concept is with the discrimination of two steps in the extraction processes [43]. In the first step, the contaminant is desorbed from its original binding sites and diffuses through the sample matrix toward the particle surface. This process is determined by diffusion and desorption kinetics of the respective chemicals and thus the basis of the concept of bioaccessibility. The second step is the partitioning of the contaminants from the particle surface of the matrix into the solvent or the solid phase used for the extraction. In order to achieve a more mechanistic understanding of bioaccessibility extraction, methods are required which reflect but do not influence the diffusion step and while insuring that partitioning into the extracting medium is not the rate-limiting step. This partitioning step is highly dependent on the method (i.e., flow rate, amount of sorbent, pressure) and thus not related directly to processes in soils and sediments.

Independent of the type of extraction procedure, systematic modifications may help to identify optimum extraction conditions where diffusion and desorption are the rate-limiting step. According to this goal, a stepwise increase in the amount of or replacement frequency of the extracting solvent or agent should ultimately result in optimal desorption kinetics. Under such optimum conditions, different extraction procedures should provide comparable results. However, extraction conditions necessary to achieve this status may be contaminant-specific and matrix dependent and need to be validated before method application.

If it can be shown that the optimal method actually reflects desorption rather than method-dependent partitioning, extraction methods for bioaccessibility-based EDA and TIE may be selected according to practical requirements such as sufficient amounts of sample to be extracted, chemical and toxicological blanks, ease of separation of the extracting agent from the sediment suspension, risk of artifacts, and availability of instrumentation and required materials. A small selection of available potential methods will be discussed in the following section of this chapter. In all of the methods discussed, the process would involve using the selected method to extract the bioaccessible fraction from a sediment. Next, the extracting medium (e.g., cyclodextrin, TENAX, passive sampler) would itself be extracted and the resulting extract used for toxicity testing purposes in the EDA or TIE.

3.1 Cyclodextrin Extraction

Cyclodextrins are cyclic oligosaccharides with six (α-cyclodextrin), seven (β-cyclodextrin), or eight α-D-glucopyranoside units (γ-cyclodextrin) derived from starch. Cyclodextrins form a typical toroid structure with a hydrophilic exterior and a considerably less hydrophilic interior that allows for hosting hydrophobic organic molecules. The hydrophilic exterior is responsible for the large water solubility of the stable aqueous inclusion complexes. The cavity, and thus the size of the hydrophobic organic molecule that can be incorporated into the cyclodextrin, increases from α (diameter 4.7–5.3 Å) via β (6.0–6.5 Å) to γ-cyclodextrin

(7.5–8.3 Å) [44]. Recently, cyclodextrins and their derivatives with enhanced water solubility such as hydroxypropyl-β-cyclodextrin (HPCD) have become increasingly popular as nonexhaustive extraction methods focusing on bioavailable fractions of organic pollutants in soils and sediments. In situations where desorption of hydrophobic organic chemicals is controlled by aqueous boundary layer resistance, cyclodextrins enhance desorption processes [15, 45]. HPCD is thought to extract the rapidly desorbing fraction of soil- or sediment-associated hydrophobic organic compounds from the water phase and has been found to correlate with microorganismally bioaccessible and degradable fractions of 1–3 ring (poly)aromatic compounds [39, 40, 46–49]. A prerequisite for the formation of inclusion complexes and thus for an efficient extraction is that the size and shape of the target molecule fit into the HPCD cavity. Mechanistic investigations have shown a clear selectivity of different cyclodextrins for specific compounds [50].

Although HPCD extraction works quite well for low molecular polycyclic aromatic hydrocarbons (PAHs) such as naphthalene and phenanthrene, for larger PAHs, such benzo[*a*]pyrene, the target molecule and cavity sizes are in poor agreement. This is reflected by the ratio between the HPCD–water partition coefficient (K_{HPCD}) normalized over the organic carbon–water partition coefficient (K_{oc}) which is about 2 for naphthalene and 0.0072 for benzo[*a*]pyrene [40]. This means that HPCD extraction may result in an unwanted discrimination of PAHs according to inclusion into the HPCD cavity rather than according to desorption from sediments. This may be overcome only by using an excess of HPCD as compared to sediment organic carbon to achieve sufficient extraction efficiency. Despite this restriction, HPCD extractions have been successfully applied to predict biodegradation of a broad range of PAHs and hexadecane [51–53]. However, it is still an open question whether the range of compounds that can be extracted with sufficient efficiency with HPCD is broad enough to allow for application in EDA.

As a step toward a more realistic sediment or soil toxicity assessment, Fai et al. [54] suggested combining bioaccessibility-directed extraction using HPCD with direct application in toxicity tests such as cell multiplication inhibition with green algae. However, the presence of HPCD reduced the availability and thus toxicity of the tested herbicides by one to several orders of magnitude in a compound-specific but not particularly predictable way. This behavior excludes this technique as a promising approach for EDA. Other authors extracted the HPCD fractions with organic solvent prior to dosing the toxicity test, which may improve method performance [55].

3.2 TENAX Extraction

TENAX is considered an infinite sink for hydrophobic organic contaminants when used to extract contaminants in water–sediment suspensions. Cornelissen et al. [21, 56] established a multiple-compartment model describing the desorption kinetics

for hydrophobic compounds from sediments as determined by consecutive TENAX extractions with frequent replacement of the loaded TENAX (1). The model calculates sediment-associated amounts of a compound (S_t) after a time (t) as related to the amount at $t = 0$ (S_0). Further, the model considers a rapidly desorbing fraction F_{rap} with a desorption rate k_{rap}, a slowly desorbing fraction F_{slow} with a desorption rate k_{slow}, and a very slowly desorbing fraction F_{vslow} with a desorption rate k_{vslow}:

$$\frac{S_t}{S_0} = F_{\text{rap}} \times \mathrm{e}^{-k_{\text{rap}} \times t} + F_{\text{slow}} \times \mathrm{e}^{-k_{\text{slow}} \times t} + F_{\text{vslow}} \times \mathrm{e}^{-k_{\text{vslow}} \times t}. \quad (1)$$

In some cases, the three compartment model can be replaced by a two compartment model (i.e., rapid and slow). The procedure is based on the assumption that desorption from sediments is the rate-limiting factor rather than the uptake into TENAX. For an experimental design extracting chlorobenzenes, polychlorinated biphenyls (PCBs), and PAHs from sediments with ten times more TENAX than sediment organic carbon, Cornelissen estimated extraction rates three to ten times greater than rapid desorption rates [57]. The authors argued that frequent replacement of TENAX results in low concentrations on the resin compared to the sedimentary organic carbon. Further, estimated water concentrations in equilibrium with TENAX were 100–700 fold lower than water concentrations in equilibrium with the organic carbon. Consecutive extraction of sediments with TENAX has also been performed with other compounds like trifluralin. However, this work did not rigorously test whether the desorption model assumptions above also hold for these more polar compounds [58].

In order to simplify the method and to make it more appropriate as a routinely applicable extraction tool for EDA or TIE, one-step procedures have been developed and conducted for the evaluation of accessibility with chlorobenzenes, PCBs, PAHs, polybrominated diphenyl ethers (PBDEs), and DDT metabolites. The evaluated extraction times included 6 [36, 55, 59, 60], 14 [61], 24 [62–66], and 30 h [36]. Since F_{rap}, F_{slow}, and F_{vslow} desorb at the same time but with different and compound-specific rates, it is obvious that an extraction of 100% of F_{rap} excluding the other fractions is not possible. The proportions for F_{rap} from a large data set of chlorobenzenes, PCBs, and PAHs extracted after 6 h ranged from 12 to 263% with an average of about 50%, while $F_{30\text{ h}}/F_{\text{rap}}$ (expressed as a percentage) ranged from 33 to 770% with an average of about 140%. Schwab et al. [65] demonstrated that $F_{24\text{ h}}$ was a good estimate for F_{rap} for PAHs with a molecular weight of 202–252 while F_{rap} for smaller PAHs was slightly overestimated.

As might be expected, the F_{rap} of PAHs and PCBs has been found to be a valuable predictor for bioaccumulation in benthic and soil invertebrates as well as in bioremediation [35]. The fraction also exhibits linear or log–linear correlations with biota-sediment accumulation factors (BSAF) for different benthic organisms [67–69]. For example, BSAFs for PAHs and PCBs in sediments for different benthic organisms could be predicted within a factor of 2 using F_{rap} from consecutive TENAX extractions according to (2) [70, 71]:

$$\mathrm{BSAF} = \frac{K_{\mathrm{lipid}}}{K_{\mathrm{OC}}} \times F_{\mathrm{rap}}, \tag{2}$$

with K_{lipid} representing the lipid–water partition coefficient for given contaminants. Similarly, several authors found good linear [72] or log–log correlations between bioaccumulation of PCBs, PAHs, DDE, permethryn, and chlorpyrifos and $F_{6\ \mathrm{h}}$ or $F_{24\ \mathrm{h}}$. These correlations could be used in one relationship [59, 60, 63]. Further, removal of the rapidly desorbing fraction with TENAX significantly (i.e., factor 2–27) reduced bioaccumulation in benthic deposit feeders stressing the relevance of this fraction for estimating bioaccessibility and bioavailability [68].

However, it is clear that a one-step extraction can only be a very rough estimate for F_{rap} [67]. Further, there are also contradicting studies that did not find good relationships between F_{rap} and BSAF. This was the case for the very low F_{rap} values of sediment-associated polychlorinated dibenzo-*p*-dioxins, dibenzofurans, diphenylethers, and hydroxydiphenylethers [73]. The relationship between F_{rap} and bioaccumulation of spiked PAHs and PCBs by freshwater oligochaete *Lumbriculus* [74] also was not very strong. The authors suggested animal feeding behavior as a key factor limiting the predictability of bioaccumulation from F_{rap} [75]. In general, it should be kept in mind that desorption from sediments is only one step in the whole process of bioavailability (see Fig. 1) and neither considers the uptake in the organism as influenced by its behavior nor toxicokinetics of a specific contaminant in a specific organism.

EDA often attempts to characterize sediment or soil contamination without focusing on a specific organism. For this approach, exclusion of organism-specific factors is advantageous. A one-step TENAX extraction fits excellently into EDA procedures. However, more investigations with respect to the extraction domain are desirable, particularly for more polar organic compounds. TENAX extraction is thought to enhance environmental realism compared to exhaustive organic solvent extraction and allows for subsequent organism toxicity testing, fractionation, and chemical analysis. So far, applications of TENAX extractions for toxicity testing and EDA are rare and have not been reported for TIE. In one EDA example, Puglisi et al. [55] extracted bioaccessible fractions of sediment contaminants with TENAX and HPCD for subsequent testing with DR-CALUX for dioxin-like activity. In another study, Schwab et al. [64] performed a full EDA on sediment-associated algal toxicants using TENAX in comparison with exhaustive extractions. They could show a significant and site-specific influence of bioaccessibility on the relative importance of different fractions. In particular, PAHs were found to be of lower significance toward toxicity than conventionally thought when TENAX-extraction was applied while polar compounds gained in priority as suspected toxicants (Fig. 3). For TIE applications, the cyclodextrin and TENAX extractions offer potential tools for collecting sufficient quantities of bioaccessible contaminants that could be used in whole organism exposures. Perron et al. [76] found the use of exhaustive extractions with three field sediments from contaminated marine sites resulted in overestimations of toxicity in a passive dosing systems (see further discussion below) as

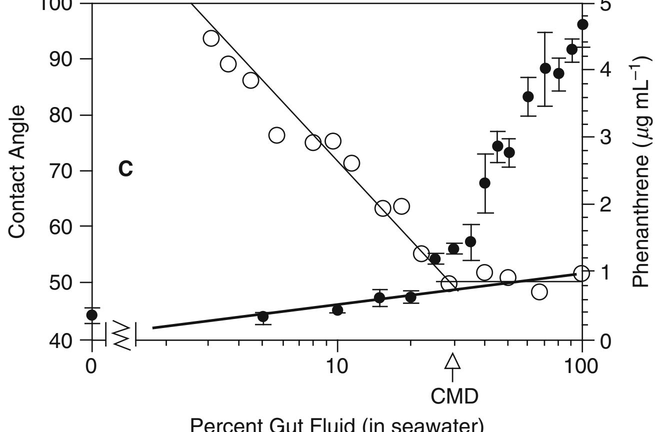

Fig. 3 Contact angle and pure PAH solubilization by lugworm (*Arenicola marina*) digestive fluids. *Abscissa* represents the dilutions of the gut fluid. *Left ordinate* and *open circles* represent the contact angle. *Right ordinate* and *black circles* represent the phenanthrene concentration solubilized ([86] with permission)

compared to whole sediment toxicity tests using marine invertebrates (mysid *Americamysis bahia* and amphipod *Ampelisca abdita*) performed on the same sediments. A cyclodextrin or TENAX extraction of the bioaccessible contaminants rather than all of the contaminants as captured by the exhaustive extraction is very likely superior but requires experimental demonstration.

3.3 Supercritical Fluid Extraction

An alternative approach to extract rapidly desorbing and more tightly bound sediment contaminants is supercritical fluid extraction (SFE). SFE with supercritical (SC) CO_2 allows for varying temperature and pressure conditions. The solubility of organic compounds in SC CO_2 increases with pressure and temperature; for example, phenanthrene at 60°C and 120 atm has a solubility of 479 mg/L at 310 atm the solubility is 10,860 mg/L [77, 78]. According to both the hot ball [79] and the finite slab models [80], it is assumed that the molecules diffusing out of the organic matter are transferred very quickly from the particle surface with a concentration at the surface of zero into the surrounding SC CO_2. Thus, the time-limiting factor is the diffusion through the organic matter. This conceptual model is in agreement with the theory behind TENAX and cyclodextrin extraction which both attempt to reduce the water concentration and thus the concentration at the particle surface to approximately zero. While fractions with different diffusion rates and related desorption kinetics in the cases of cyclodextrin,

TENAX, and other solid phases (e.g., XAD_2) are harvested at the selected extraction times, SFE exploits the temperature dependence of the diffusion coefficients according to Arrhenius (3):

$$D = D_0 \exp(-E_a/RT), \tag{3}$$

where D is the diffusion coefficient at a given temperature T, D_0 is the diffusion coefficient obtained when extrapolating to very high temperatures, E_a represents the activation energy, and R is the gas constant.

However, the ability of SC CO_2 to influence contaminant solubility [78] suggests that in contrast to the assumptions above, at least at low pressures extraction depends not only on diffusion through the sedimentary organic matter, which should not be influenced by pressure, but also on the dissolution process in the rather artificial solvent SC CO_2 (i.e., low pressure SC CO_2 is not a very good solvent). These low pressure conditions should be avoided by applying pressures that are sufficiently elevated to make the influence of this parameter negligible.

SFE has been applied in remediation studies attempting to predict PAH fractions that may be accessible to biodegradation [32, 81]. For example, Hawthorne et al. [32] found that only PAHs in the fast desorption fraction were significantly reduced by bioremediation of contaminated soils. PAHs from manufactured gas plant soils and sediments showed a similar desorption behavior in agreement with a two-site model when extracted with SFE and the resin XAD_2 in water [30]. Desorption rates were not predictable from water solubility and lower molecular weight PAHs often showed slower desorption than less soluble higher molecular weight PAHs.

Although SFE has not been applied to provide extracts for bioassays as part of an EDA, it seems to be a promising tool for bioaccessibility-directed soil and sediment extraction (for subsequent EDA). Relative to TIE, SFE was used successfully in three soil TIE-like procedures [82] in which survival of two terrestrial worm species (*Eisenia fetida* and *Enchytraeus albidus*) increased and PAH concentrations decreased following the SFE manipulation. Finally, Burgess et al. [10] successfully used SFE in two marine sediment TIEs as a Phase III manipulation to confirm the findings of the Phase I powdered coconut addition manipulation (see Chap. 2 in this volume [10]).

SFE has several advantages for use in EDA and TIE. The SC CO_2 evaporates after extraction without solvent residues in the sediment or soil or the extracted chemical mixture and allows for subsequent toxicity testing of both types of samples. A disadvantage to the application of TENAX, XAD_2, and cyclodextrin is the relatively limited use of these techniques in research and routine analysis laboratories. For SFE, the high cost and lack of the potential to scale-up extracted sample volumes to sufficient amounts for EDA and TIE are problematic. In contrast to the other approaches, the different fractions are predetermined by the selected extraction conditions while in the case of TENAX, for example, extracts are the result of modeling based on the desorption curves.

3.4 Extraction with Biological or Biomimetic Fluids

An alternative to generic and exclusively chemical-based extraction techniques for collecting the bioaccessible fractions of sediment or soil contaminants is in vitro digestive fluid extraction [83]. Three types of marine invertebrates have been used in this application including the holothuroid *Parastichopus californicus*, the echiuran *Urechis caupo* [84], and the polychaetes *Arenicola marina* and *Arenicola brasiliensis* [85]. While *A. marina* is about 20–40 cm in length and 2 cm in width, *A. brasiliensis* is a bit shorter and both species are frequently used. Digestive fluids are taken from the mid-gut of the dissected worms which offers the greatest amounts and the highest enzymatic activities. The organisms yield an average of 1 mL of digestive fluid per individual [84]. Weston and Mayer found some correlation between gut fluid solubilization of benzo[*a*]pyrene and different parameters representing in vivo bioavailability in *A. brasiliensis* including absorption efficiency, uptake clearance, and bioaccumulation factor although the variability was very high [85]. This variability is likely due to the fact that gut fluid solubilization does not incorporate any aspects of contaminant absorption across the gut wall as driven by chemical activity.

Two factors have been discussed to explain the increased solubilization of PAHs by gut fluid as compared to sea water. These are enzymatic and surfactant activity. There was no significant correlation of PAH solubilization with enzyme activity [84]. However, PAH solubilization experiments with an increasing percentage of gut fluid showed that above the critical micelle dilution (cmd), a rapid increase in PAH solubilization is observed (Fig. 3). The cmd was determined by recording surface tension (as contact angle) against percent gut fluid [86]. The cmd for the gut fluid with phenanthrene and benzo[*a*]pyrene was exceeded at 20 and 60% gut fluid, respectively. In contrast to other types of micelles (e.g., humic acids), contaminants associated with gut fluid surfactant micelles are bioavailable. Surfactant micelles may solubilize PAHs at concentrations 1,000 times greater than freely dissolved concentrations in seawater [86]. This may explain deviations from observed bioaccumulation as compared to equilibrium partitioning-based predictions of freely dissolved concentrations in the water phase. In addition, Voparil et al. found that hydrophobic contaminants and nutritional lipids impact the solubilization of each other [87], which is also not considered in simple equilibrium partitioning-based models. Further, increased bioavailability of PAHs via digestive fluids compared to equilibrium partitioning-based predictions has been confirmed for anthropogenic particles such as diesel soots, tire treads, and urban particulates [88].

In contrast to other bioaccessibility-directed extraction methods, in vitro digestive fluid extraction is not limited to hydrophobic organic chemicals but has been found to mimic the bioaccessibility of metals and other inorganic chemicals [89].

In contrast to the generic, chemical-based extraction procedures discussed above, in vitro digestive fluid extraction does not focus on desorption kinetics and rapidly desorbing fractions but tries to simulate conditions in the digestive systems of organisms potentially exposed to and affected by sediment contamination.

The approach integrates desorption from sediment particles and solubilization in surfactant-rich digestive fluids. In the cases of the other approaches using cyclodextrin, TENAX, or SFE, the impact of dissolution or absorption processes should be minimized since they are related to highly artificial systems whereas the digestive fluid extraction emulates a natural system.

Digestive fluid extraction within EDA and TIE has not yet been applied since the extractable amounts of contaminants are too small for subsequent toxicity testing, fractionation, and contaminant identification and confirmation. This may change if digestive fluids can be replaced by synthetic surrogates that are able to closely mimic the properties of the natural fluids. Sodium taurocholate, a vertebrate bile salt, together with bovine serum albumin was found to most accurately mimic *A. marina* gut fluid solubilization of individual PAHs [90]. However, in a recent study, the method has been found to be inadequate for describing PAH availability to bivalves [91]. Nakajima et al. used sodium dodecyl sulfate solutions as a "hypothetical gut fluid" for bioaccessibility-directed PAH extraction with subsequent toxicity testing and chemical analysis however method validation was not reported [92, 93]. In addition, it is obvious that the approaches discussed above need to be validated for a larger range of contaminants in order to become readily applicable in EDA and TIE.

3.5 Bioaccessibility-Directed Extraction in EDA and TIE

Bioaccessibility-directed extraction is a promising approach that is also compatible with whole sediment TIEs and may help to bridge the differences separating EDA and TIE. As noted above, the preferred solid-phase manipulation for hydrophobic organic contaminants in whole sediment TIEs is powdered coconut charcoal [8], a strong sorbent that is believed to rapidly reduce the bioaccessibility of a broad array of contaminants. Unfortunately, there is no method available to fully recover the charcoal from the sediment and the adsorbed compounds from the charcoal. Thus, although this procedure is useful for the characterization (TIE Phase I) of the hydrophobic organic contaminant contribution to total whole sediment toxicity, it is only of limited help for subsequent EDA.

A more promising approach may be the use of TENAX extraction together with whole sediment toxicity tests. TENAX was able to completely remove toxicity to the estuarine amphipod *Corophium volutator* exposed to sediments contaminated with organic toxicants as well as most of the peaks from the corresponding gas chromatograms [40]. TENAX extracts can be fractionated, analyzed and, in principle, redosed to extracted sediments or passive dosing systems opening new ways to perform EDA and TIE studies with whole sediment toxicity tests. However, like all of the other extraction methods, TENAX extraction suffers from the method having been applied primarily to PAH contaminated sediments, while the performance of TENAX with other organic chemicals is much less well documented. It has been shown that in many sediments and for many toxicological endpoints, chemicals with polar

constituents including nitro-, amino-, keto-, and hydroxy-groups play an important role. To what extent TENAX extraction can be applied to the bioaccessibility of these chemicals without method-related discrimination is still an open question.

4 Equilibrium Partitioning-Based Extraction and Dosing

4.1 Equilibrium Partitioning-Based Extraction

Bioaccessibility-directed techniques as discussed above are designed to extract those contaminants associated with soils, sediments, or other types of matrices that may rapidly desorb and thus are available for subsequent uptake by organisms. According to the equilibrium partitioning approach, in sediments, uptake is primarily a result of partitioning between the sedimentary partitioning phases (e.g., natural organic carbon and soot carbon), the surrounding aqueous phase, and the lipids of the organisms driven by chemical activity gradients between the phases, and provided the kinetics are sufficiently rapid to achieve equilibrium. Since desorption from sediment particles is often the rate-limiting step, it may be assumed that, in most cases, the rapidly desorbing fraction of a chemical takes part in equilibrium partitioning. This concept based on bioaccessibility and chemical activity as complementary approaches has been summarized recently by Reichenberg and Mayer [15].

The focal point of partitioning is the freely dissolved concentration in the aqueous phase in equilibrium with the (rapidly desorbing) concentration in the sedimentary phases and in biota lipids. The freely dissolved concentration, or estimates of that concentration, is considered as a surrogate measures for the chemical activity in the system. Classical liquid–liquid or solid-phase extraction of the aqueous phase (e.g., the interstitial water) does not discriminate between the freely dissolved concentration and the contaminants bound to small particles or colloids such as humic compounds [94, 95]. One of the most successful approaches for overcoming this challenge is the use of equilibrium passive sampling.

There are now several types of passive samplers used in environmental applications. For example, semipermeable membrane devices (SPMD) filled with triolein to mimic the accumulation of bioavailable hydrophobic chemicals from the aqueous phase to lipid tissues were introduced in 1990 [96] and are in routine use for water column monitoring today. In sediment EDA and TIEs applications, the use of passive samplers is still rare and often focuses on the use of depletive extraction rather than on equilibrium sampling [97]. More recently, it has been shown that low density polyethylene (LDPE) membranes (triolein-free SPMDs) are as efficient in the sampling of organic compounds as SPMDs although with lower sorption capacity [98, 99]. LDPEs have been used to mimic the uptake of PAHs and PCBs by the benthic polychaete *Nereis virens* [100, 101]. Solid-phase microextraction (SPME) fibers were also introduced in the early 1990s [102] and applied as matrix-SPME

utilizing the entire sediment matrix as a reservoir for equilibrium extraction [103, 104]. Depending on the hydrophobic organic contaminant, generally within 20–30 days equilibrium partitioning can be achieved. Several other polymer materials have been suggested as equilibrium passive samplers including different silicone rubbers (SR) [105, 106] and polyoxymethylene (POM) [107]. All of these tools are generally appropriate, although with different advantages and disadvantages. While POM has a hard and smooth surface facilitating the removal of small soot and sediment particulates following deployment and thus avoiding artifacts [107], diffusion in this material is very slow resulting in long equilibration times or insufficient equilibration [105]. In comparison, SRs exhibit much higher diffusion coefficients allowing for a faster equilibration. For most of the passive samplers, partition coefficients with water are linearly correlated with log K_{ow}.

One application of passive samplers is to mimic partitioning into biological membranes. In contrast to bioaccessibility-directed extraction, passive samplers do not attempt to exhaustively extract the desorbing fraction but to take part in partitioning without depletion of the matrix. Equilibrium is achieved when the chemical activity or the fugacity of a contaminant is the same in the sampler, in the sedimentary phase, and in the interstitial water. For hydrophobic sediment contaminants, the fraction of the contaminant remaining in the aqueous phase is negligible. The fraction $f_{i,\mathrm{PS}}$ of a compound i that is accumulated in the passive sampler may be calculated according to (4) [5]:

$$f_{i,\mathrm{PS}} = \frac{1}{1 + (1/(K_{i,\mathrm{PS,sed}} \times V_{\mathrm{PS}}/V_{\mathrm{sed}}))}, \tag{4}$$

$K_{i,\mathrm{PS,sed}}$ is the partition coefficient of the contaminant between the passive sampler and sediment and V_{PS} and V_{sed} represent the volumes of the passive sampler and of the extracted sediment, respectively. The extracted fraction depends on the volume ratio between the sampler and the sediment. At nondepletive conditions with $V_{\mathrm{PS}} \ll V_{\mathrm{sed}}$, the extracted fraction is proportional to the partition coefficient according to (5) and simulates equilibrium partitioning between the sediment, interstitial water and biota:

$$f_{i,\mathrm{PS,sed}} = K_{i,\mathrm{PS,sed}} \times V_{\mathrm{PS}}/V_{\mathrm{sed}}. \tag{5}$$

If large sampler volumes are used compared to the sediment volume (i.e., $V_{\mathrm{PS}} \gg V_{\mathrm{sed}}$), the fraction $f_{i,\mathrm{PS}}$ approaches 1. This means depletive extraction of the desorbing fraction is likely to occur similar to a TENAX extraction.

It is clear that equilibrium sampling in sediments is a powerful tool to predict bioavailable concentrations if the conditions discussed above are met. Unfortunately, for hydrophobic organic contaminants, this means that the total amount of extracted molecules is very low. This amount may be sufficient for chemical analysis but, in most cases, it is not sufficient for an EDA or TIE involving toxicity testing, fractionation, and structure elucidation. This limitation maybe addressed by working with very large volumes of sediment and passive samplers but logistically this can be

problematic. Further, the time to equilibration, especially for larger hydrophobic chemicals may be prohibitively long (i.e., weeks to months) and does not operate well with routine EDA or TIE requirements. For example, when working with field collected whole organisms for a TIE (as opposed to laboratory cultured organisms), it is desirable to use the same "batch" for a series of studies to reduce variability. Studies spread over several weeks or months can make using a single batch of test organisms very difficult. Thus, depletive extraction of rapidly desorbing fractions with subsequent consideration of equilibrium partitioning by partition-based dosing may be less elegant but appears to be more promising for use in EDA and TIE.

4.2 Partition-Based Dosing

As discussed above, the concentration of hydrophobic organic compounds in sediment interstitial water and accumulated in the lipids of benthic organisms may be described by the equilibrium partitioning of chemicals between the different phases assuming that desorption and uptake kinetics are fast enough to achieve equilibrium. This principle can be exploited for passive sampling (see above) or partition-based dosing. The latter approach, also called partitioning driven administration [108], partition controlled delivery [109], and passive dosing [110–112], applies a hydrophobic solid phase such as octadecyl empore disks [108], SPMDs [113], LDPE [114], and silicone in different configurations loaded with individual compounds or complex mixtures such as sediment extracts or fractions. Silicone has been applied as film [109, 115], stir bars [4, 116], and O-rings [110, 111] while in TIE LDPE has been used as a film (Fig. 4).

After being loaded with the appropriate extract, the passive dosing devices are equilibrated with the medium in a toxicity testing design. Depending on the design of the test and the dosing device, equilibrium can be achieved in the range of minutes to a few hours to days. In contrast to conventional dosing via toxicity testing-compatible solvents, such as DMSO, a rather constant exposure concentration, even of highly hydrophobic compounds, can be achieved. Desorption from the sampler

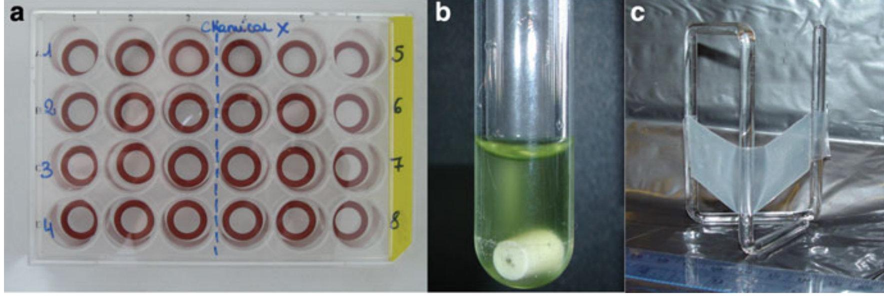

Fig. 4 Formats of passive dosing with various devices: (**a**) silicone o-rings and (**b**) silicone stirrer bars, and (**c**) low density polyethylene film ([114] with permission)

compensates for losses due to adsorption processes, uptake by the test organisms, degradation, or volatilization [109]. The concentration in the medium, and thus exposure, is well defined by the appropriate partition coefficient. Different passive sampler loading techniques depend on the purpose of the study. For example, tests of individual compounds may be loaded on the passive dosing devices by partitioning from methanol [110, 111] with stepwise addition of water if needed [112]. Regardless of the technique, complex mixtures extracted from sediments for EDA and TIE need to be transferred to the dosing devices without preferential losses. Therefore, direct loading using solutions in organic solvents is appropriate if a quantitative transfer is ensured and the formation of crystals is avoided [4, 114–116]. If silicone films are applied, the test compounds can be added to the prepolymer solution in a liquid state [109] similar to the use of triolein in SPMDs [96]. In sediment EDAs and TIEs, partition-based dosing of complex mixtures helps to mimic partitioning processes in sediments [113]. The passive samplers act as a surrogate for sediment organic carbon. Individual components partition into the medium according to their partition coefficients. This procedure is believed to simulate bioavailability to benthic organisms much better than solvent dosing where all compounds are forced into solution without regard for their partitioning behavior in sediments.

During the development of a LDPE-based passive dosing system for use in whole sediment TIEs, Perron et al. [114] were able to generate toxicity to the marine mysid *A. bahia* that matched the toxicity observed in whole sediment exposures (Fig. 5). The system was first tested with a clean sediment amended with several PAHs. When the system was evaluated with field contaminated sediments it failed to recreate whole sediment toxicity, often overestimating toxicity [76]. Because the system used an exhaustive extraction of the sediment to generate the extract loaded onto the LDPE, it is suspected that contaminants that were not bioaccessible (e.g., associated with soot carbon) were loaded onto the LDPE resulting in the overexposure. In the case of the initial clean sediment amended with PAHs in which the LDPE passive dosing system functioned, it is likely the

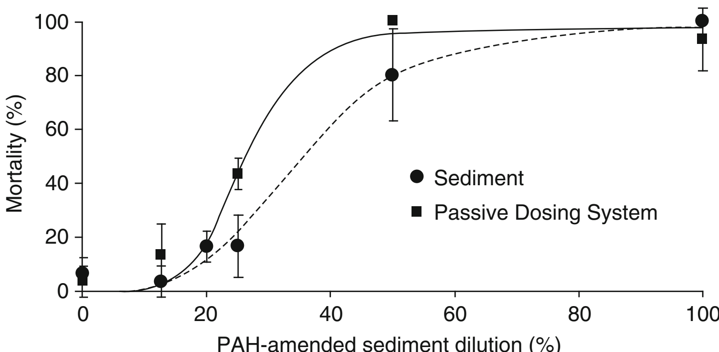

Fig. 5 Results of comparison of a passive dosing system and whole sediment toxicity testing using the marine mysid (*Americamysis bahia*) with reference sediments amended with polycyclic aromatic hydrocarbons (PAHs) (from [114] with permission)

amended contaminants were primarily in the rapid desorbing fraction. Consequently, the organisms in the whole sediment toxicity test were exposed to similar concentrations as the organisms in the passive dosing system. Once again, these data suggest the use of bioaccessibility-driven extraction (e.g. TENAX) with a passive dosing system would be very valuable for whole sediment TIEs. Along with insuring an accurate exposure, the method, if scaled properly, would allow for the generation of large amounts of bioaccessible chemical for fractionation in a TIE.

The application of partition-based dosing in sediment EDA provided a clear shift in designating toxic fractions (Fig. 6) when applying a recently developed fractionation procedure [117] that provided five fractions with hydrophobic aliphatic and small aromatic compounds, followed by six PAH fractions with increasing numbers of aromatic rings, and six more polar fractions, substantial toxicity to a green algae was observed in the PAH fractions when dosed with DMSO [4]. In contrast, using the same fractionation scheme, partition-based dosing resulted in the disappearance of toxicity from the PAH fractions while toxicity of the more polar fractions was maintained. The biocide triclosan, a component of personal care products, was found to be a dominant algal toxicant in this EDA.

5 EDA in Biota Tissues

The application of EDA procedures to tissues or body fluids from biota exposed to contaminated environments provides another alternative to chemical extraction or dosing techniques that were designed to mimic bioavailability. Tissue EDA covers

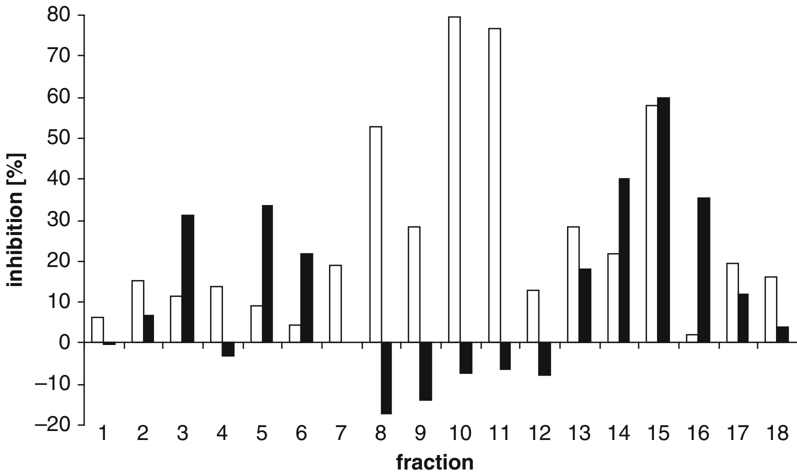

Fig. 6 Growth inhibition of the green algae *Scenedesmus vacuolatus* exposed to fractions of a sediment extract dosed with DMSO (*white bars*) and silicone stirrer bars (*black bars*) [4]. The fractions 1–5 coelute with aliphatic and small aromatic compounds including PCBs and PCDD/Fs [117]. The fractions 6–12 coelute with PAHs of increasing number of aromatic C-atoms, while fractions 13–18 are characterized by compounds with polar substituents

all of the processes shown in Fig. 1 that result in bioavailability including desorption from particles, uptake into biological membranes, and toxicokinetic processes. It provides a direct measure of hazards to specific organisms and the causes thereof, and is of great ecological relevance. In one investigation, Hewitt et al. investigated the hepatic tissues of white sucker (*Catostomus commersoni*) exposed to bleached kraft mill effluents for bioavailable ligands of arylhydrocarbon, estrogen, and androgen receptors as well as for sex steroid binding proteins [118–120]. They combined tissue extraction, reversed phase HPLC fractionation, and in vitro testing, and were able to detect significant bioactivities in different fractions. This activity could be removed by a clearance phase when fish were held in clean water. Houtman et al. focused on the identification of estrogenic compounds in deconjugated fish bile and was able to identify the natural hormones 17β-estradiol, estrone, and estriol as well as the contraceptive pill component ethynylestradiol as the dominant compounds in the ER-CALUX assay [121]. Since it was found that enhanced plasma vitellogenin concentrations in bream (*Abramis brama*) from some freshwater sites were well correlated with the ER-CALUX activity in the gastrointestinal contents, the latter were used for EDA of causative compounds confirming 17β-estradiol and estrone as the major estrogens in Dutch surface waters. Mussel tissues may also be a good matrix to investigate bioavailable and bioaccumulating compounds due to their great fat content and low metabolic activity. Donkin et al. investigated steam-distillation extracts of mussel tissues for effects on juvenile mussels combining toxicity testing with normal-phase HPLC fractionation [122]. They identified an unresolved mixture of PAHs as the cause of toxicity.

These studies may demonstrate the potential of the biota tissue EDA for the identification of bioavailable toxicants. However, the approach is selective for the specific organism under investigation with its unique toxicokinetic configuration and does not provide a characterization of the sediment as such. It is limited to organisms that provide sufficient biomass for subsequent toxicity testing, fractionation and analysis, and to compounds with sufficient persistence in the respective organism. The approach may be better used in monitoring programs to assess surface water risks using larger biomonitoring organism (e.g., mussels) rather than in sediment EDA or TIE applications.

6 Conclusions

The consideration of bioavailability in sediment EDA and TIE helps to avoid a fraction bias and contaminant prioritization toward chemicals with great toxic potential but limited exposure of biota. There are several approaches available mostly covering only a part of the bioavailability process (see Fig. 1) such as desorption from particles or equilibrium partitioning. Currently, the application of these approaches in EDA is still rare and in TIE even more rare. Most of the approaches have been applied extensively to a small range of chemicals, often

PAHs. Consequentally, a rigorous evaluation with a broader range of sediment contaminants including more polar ones is needed.

Despite this knowledge gap, the few EDA studies applying bioaccessibility-directed extraction or partition-based dosing techniques show that considering bioavailability will significantly shift the focus toward more polar contaminants and will increase the environmental realism of sediment EDA. EDA in benthic organism tissues provides an interesting supplement to sediment EDA with a direct focus on the organisms to protect although practical limitations may prevent the extensive application of this approach with sediments.

Sediment TIEs by design include bioavailability but are limited by sample size when working with whole organisms and other factors that hinder the extent of fractionation that can be performed to provide a complete identification of active toxicants. Most sediment TIEs performed so far demonstrate that hydrophobic organic chemicals are the principle causes of observed toxicity [123]. However, the lack of fractionation methods for whole sediment TIE limits these evaluations from proceeding to identify which specific chemicals are causing adverse effects. As discussed, the combination of TENAX-like extracts with passive dosing may simplify the TIE exposures to the point that greater identification is possible. This is an area that requires further research.

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Diagnostic Tools for Effect-Directed Analysis of Mutagens, AhR Agonists, and Endocrine Disruptors

Gisela Umbuzeiro, Miroslav Machala, and Jana Weiss

Abstract Environmental toxicants, such as mutagens and endocrine disruptors, can cause impact on human and environmental health and are distributed in different environmental matrices as complex mixtures. From the thousands of known toxic compounds, only a few are already regulated and monitored. There is evidence that several unidentified compounds are present in the environment due to the fact that when bioassays are performed the responses usually do not correlate with the analyzed target compounds. In order to minimize exposure of humans and biota to these compounds, it is necessary that they are accurately and clearly identified. This has always been a challenge to environmental chemists. For this purpose, analytical integrated strategies such as effect-directed analysis are useful. By combining differential extractions, chemical analysis, and bioassays it has been possible to identify important new chemical classes of environmental toxicants. This chapter describes bioassays that can be used in effect-directed identification studies, their advantages, and limitations.

Keywords Aryl hydrocarbon receptor, Dioxin-like compounds, Endocrine disruptors, Genotoxicity, Methods, Mutagenicity, *Salmonella* assay

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W. Brack (ed.), *Effect-Directed Analysis of Complex Environmental Contamination*, Hdb Env Chem (2011) 15: 69–82, DOI 10.1007/978-3-642-18384-3_4,
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Contents

1 Introduction 70
2 Bioassays Detecting Genotoxic and Mutagenic Effects 71
3 Bioassays Detecting Dioxin-Like Effects 74
4 Bioassays Detecting Endocrine Disruptors 75
5 Conclusions 78
References 78

1 Introduction

Some chemicals can interact with the genetic material (DNA) of living organisms causing different types of structural modifications. These chemicals are termed genotoxins. If the damage is not repaired, a permanent change in the DNA, *i.e.*, a mutation can occur. These changes can lead to adverse effects at the individual level such as aging,cancer, genetic and development disorders, and – at the population level – alterations in population fitness and offspring [1, 2]. Changes may also be neutral, *i.e.*, have no effect on the organism's survival or fitness, and, on rare occasions, the changes could be beneficial.

There are no mutagen-free environments. Mutagens are ubiquitous and some areas are present as hot spots of mutagenic activity derived from natural or anthropogenic sources. They are present in air, soil, natural water, sediments, and in the trophic chain. Both inorganic and organic compounds can cause DNA damage. Because only a limited number of inorganic chemicals causes DNA damage (*e.g.*, chromium), they can be easily detected in the environment or animal tissues using chemical analysis. For organic chemicals, on the other hand, detection of potential mutagenic compounds by chemical analysis is limited to selected candidates. Thousands of different organic environmental contaminants can cause mutations, not including possible abiotic and biotic transformation products.

The majority of the environmental characterization and monitoring studies are designed to include chemical analysis of target compounds and, more recently, bioassays. With respect to mutagenic assays, however, no good correlations have been observed between chemical analysis of target compounds and mutagenic activity. Hence, it is clear that the compounds that are causing the observed effect need to be identified. In this case, effect-directed analysis (EDA) seems to be a promising tool, combining chemical analysis directed by suitable bioassays [3, 4].

Many environmental chemical contaminants cause nongenotoxic effects leading to carcinogenesis, tumor promotion, endocrine disruption, or neurotoxicity. In previous years, several modes of action, underlying these adverse effects, have been recognized and cellular models and relevant endpoints have been established. Some of the models have been reported as promising tools for determination of specific toxic potencies in the EDA process, especially for their high sensitivity and high-throughput design.

Due to the relatively simple chemical structure and lipophilicity of steroids and hormones, their regulatory pathways can be readily modified by environmental contaminants. The receptor-based activation mechanism of both steroids and aryl hydrocarbon receptor (AhR) agonists allows the development of straightforward screening methods to measure endocrine disturbance, using the fact that transcription of target genes is induced after binding to specific DNA sequences in their promoter.

Dioxin-like compounds, acting via activation of AhR, are important nongenotoxic environmental toxicants; their detection is based on determination of activated AhR-dependent gene expression. Androgen and estrogen potencies are detected in a similar mode of action, on the basis of the activation of their respective gene receptor, whereas thyroid hormone disruption can be measured by the environmental pollutants' interference with their transport proteins.

2 Bioassays Detecting Genotoxic and Mutagenic Effects

There are genotoxicity assays that detect DNA damage (*e.g*., Comet assay, umu-test, Chromotest, levels of DNA adducts, etc.) and mutagenicity assays, whose endpoints are mutation and chromosome damage. The mutagenicity assays can be divided into those that detect point mutations (*e.g*., bacterial *Salmonella*/microsome assay, mammalian cell HPRT – hypoxanthine–guanine phosphoribosyl transferase assay) and those that detect chromosome aberrations including micronuclei. In general the tests that detect chromosome alterations are very powerful but time consuming, and therefore their application is limited in EDA studies.

The *Salmonella* assay, performed with different strains of bacteria, each one with a specific metabolic capability or a different type of repair or mutation, can provide useful information about the overall mutagenicity of the test sample and the class(es) of compounds present in the sample [5]. There are several classes of compounds that are present in the environment that can cause point mutations, *e.g*., nitro and/or oxy-arenes, PAHs, aromatic amines, anthraquinones, azo-dyes, aflatoxins, alkylating agents.

The *Salmonella* assay when combined with chemical fractionation of organic extracts can be a very powerful tool to identify mutagenic classes of compounds or individual mutagens [4]. Through the use of this approach, several important mutagens were discovered in environmental samples and other complex mixtures: PBTA – 2-phenilbenzotriazoles [6], MX **–** 3-chloro-4-dichloromethyl-5-hydroxy-2 (5H)-furanone [7, 8], PhIP – 2-amino-1-methyl-6-phenyl-imidazo[4,5-b]pyridine [9], and 3NBA – 3-nitrobenzanthrone [10].

The test is based on the ability of *Salmonella typhimurium* mutants to revert a mutation in the *his*$^{-}$ gene in the presence of a test agent. The *his*$^{-}$ mutation will not allow the cells to grow in the absence of the amino acid, histidine, but the mutant cells altered by the test chemical will be able to grow and form colonies. The number of *his* revertant colonies is a measure of the mutagenicity of the test

sample. The test has proved to be very sensitive, and for some compounds such as 3-nitrobenzanthrone, only a few picograms can provide a positive response.

Basically any environmental or biological sample that has been sterilized and is in a liquid form can be tested. Solvents such as water, DMSO, or methanol are mostly used. The maximum amount that can be tested is limited by its solubility and/or toxicity [11].

The test detects mutagens that interact directly with DNA, like methylmethanesulfonate, and also mutagens that require metabolic activation to be active. The bioactivation can be provided by the endogenous bacterial enzymes (*e.g.*, nitroreduction, acetylation, and azo-reduction) or an exogenous system, usually containing cytochrome P450 enzymes from the livers of rodents, humans, or fish (termed S9), or from plant homogenates. These metabolic reactions can be performed at oxidative or reductive conditions [12]. Some authors developed new *Salmonella* strains expressing isoforms of the P450 human enzymes [13]. This could be beneficial to EDA studies because of the relevance of information obtained regarding human exposure effects [14, 15].

The test can be performed using different protocols, such as plate incorporation, pre-incubation, microsuspension [11, 16, 17], and in a microplate format (MPF) [18]. The most widely used strains for environmental applications are TA98 and TA100 [3, 11, 19] which detect mutagens that cause frameshift and base pair mutations, respectively. Several other strains with different metabolic and repair capabilities are now available, and their responses can be very helpful in the identification of the classes of compounds that are responsible for the observed effect in a complex mixture [5] or to understand the metabolic pathways of these mutagens [20].

The assay can be performed with single doses or in dose–response experiments when the results can be quantified and expressed in a number of revertants per sample unit (*e.g.*, L, m^3, μg). Although the *Salmonella* assay potency does not correlate with the potencies in carcinogenicity tests [21], or with mammalian mutagenicity tests [22], the *Salmonella* assay provides useful information on potential mutagenic compounds present in EDA fractions.

By using a combination of the *Salmonella* strains (TA98 and TA100) with XAD4 resin and a blue rayon extraction procedure that is selective to polycyclic planar structures, Kummrow et al. [23] were able to distinguish industrial-derived contaminants from the halogenated disinfection by-products generated during water treatment. The origin of the sample and the chemical classes present were revealed when different strains of *Salmonella* were combined. The increased response observed for strain YG1041 in relation to TA98 and the differences in the responses with and without rat liver S9 indicated that a textile dyeing facility was the source of the river mutagenicity and that the compounds responsible for that effect were primary nitro-aromatics [5]. Chemical analysis confirmed that nitroaminoazobenzenes were the main mutagenic compounds contributing to the observed activity in those waters [24]. For aquatic sediments, although it was generally accepted that PAHs are the main compounds responsible for mutagenicity [25], it has become evident that other compounds such as nitro/aromatic amines are also accounting for mutagenicity in sediment samples [26, 27].

A comprehensive review on the study of the mutagenicity of air samples has been published by Marvin and Hewitt [4], and they concluded that the combination of extraction/fractionation, chemical analysis, and the *Salmonella* assay is a valuable approach for the toxicological characterization of airborne mutagens, and that more studies should be performed with the most polar fractions.

Courty et al. [28] analyzed the mutagenicity of soil and mutagenic PAHs. They noted that there is no clear correlation between the mutagenicity detected in soil extracts and the measured PAH content of the soils. In a situation such as this, EDA studies could help in the identification of the effect-causing compounds in the way it has been shown for other matrices. For instance, bioassay fractionation/chemical analysis revealed the five major mutagenic constituents of urban soils in Japan to belong to the nitro-PAH group probably deriving from combustion sources [29].

For the detection of mutagenicity several *Salmonella* tester strains are available [20], and new strains have been recently developed. The strains exhibit a differential sensitivity for certain type of mutagenic, for example, strain YG5161, which is very sensitive to benzo[*a*]pyrene-like compounds [30], and strain YG7108, which is sensitive to alkylating agents [31]. If these strains are used in combination with their parental strains, *i.e.*, TA1538 and TA1535, respectively, they can indicate and indirectly estimate how much of the mutagenicity can be accounted for by compounds of each class. Some examples of this type of study can be found in the literature [5, 32].

Some compounds such as aromatic amines usually require metabolic activation. In the past metabolic activation has been achieved by using liver homogenate supernatant (S9). However, the endogenous metabolic system of some of the newer *Salmonella* strains can produce positive results in the absence of liver S9, as shown by Kummrow et al. [83] for 2-aminoanthracene.

Table 1 presents how different tester strains and test conditions can be used along with chemical fractionation to indicate the classes of the mutagens present in environmental samples.

Table 1 Examples of responses in the *Salmonella* assay of environmental samples and the classes of mutagens that could account for the observed effects

Responses in the *Salmonella*/microsome assay	Possible compounds accounting for the observed mutagenicity
Positive response in the less polar fractions, with similar responses with TA98 and YG1041; mainly only with S9 bioactivation	Nonsubstituted PAHs such as benzo[*a*]pyrene
Positive responses in the more polar fractions both with and without S9 with TA98 and increased response with YG1041, sometimes with a greatly reduced response when S9 is added	Nitro-compounds such as nitroaminoazobenzenes, nitrobenzanthrones, nitropyrenes
Positive responses in the more polar fractions mainly with S9 for TA98 and a marked increase with the YG1041	Aromatic amines such as aminoazobenzenes, benzidines, and naphthylamines
Positive responses in the more polar fractions or in the extracts obtained after reducing the water pH to 2, and higher values with TA100 when compared to TA98, that decreases when S9 is added. The responses will be negative if blue rayon is used as adsorbent	Chlorinated compounds such as MX, chloral hydrate, and halogenated acids, among other chlorinated disinfection by-products

Source: Kummrow et al. [23], Ohe et al. [3], and Umbuzeiro et al. [5]

3 Bioassays Detecting Dioxin-Like Effects

Several mammalian and fish cellular models have been developed to determine activation of AhR, recognized as the key event associated with dioxin-like toxicity. Perturbation (chronic activation) of AhR signaling leads to a set of developmental, reproductive, and immunological defects and to chemical carcinogenesis and tumor promotion [33]. AhR activation potency of environmental samples is measured by quantification of the CYP1A and/or CYP1B1 mRNA or protein levels or the corresponding enyme activity 7-ethoxyresorufin-*O*-deethylase (EROD) [34]. The latter approach has been used to identify some nonpriority PAHs, halogenated aromatic hydrocarbons, and oxygen and sulfur heterocycles as CYP1 inducers in river sediments and suspended particulate matter [35–39]. Alternatively, competitive AhR binding assay has been used for determination of AhR agonistic activity, for instance, for polycyclic aromatic compounds in diesel exhaust particulate extracts [40]. Other approaches, such as the use of hepatoma cell lines stably transfected with an AhR-luciferase reporter gene construct, *e.g*., DR-CALUX™ (Dioxin Receptor-Chemically Activated LUciferase eXpression), are very suitable for high-throughput analysis, such as in the determination of dioxin-like compounds in total extracts of airborne and aquatic abiotic samples and their chromatographic fractions [41–48].

Interestingly, PAHs and not highly persistent dioxin-like compounds have been reported as major AhR agonists in many river and estuarine sediments [42, 48, 49]. Other studies [39, 46] also concluded that a great majority of the total AhR-mediated activity is attributable to (nonhalogenated) polyaromatic compounds. However, in some studies persistent dioxins and PCBs have been found to be responsible for most of the AhR-mediated activity in sediment extracts [47].

The commonly representative compounds causing dioxin-like effects are listed in Table 2.

Table 2 *In vitro* bioassays for specific mode of action

Responses in endocrine disrupting assays	Possible compounds accounting for the observed effect
Activation of AhR	PAHs, PCDD/Fs, and coplanar PCBs
Activation of ER	Natural estrogens and synthetic estrogens (estrone and estradiol); industrial xenoestrogens in sediments and water phase (alkylphenols, dialkyl phthalates, etc.); pyrethroids
Inhibition of ER	Highly chlorinated PCBs; synthetic antiestrogens
Activation of AR	Natural and synthetic androgens and their metabolites (dihydrotestosterone, androstenedione, androstanedione, 5β-androstane-3α,11β-diol-17-one, androsterone; and *epi*-androsterone, androstenone, and nandrolone)
Inhibition of AR	Musk compounds, PAHs, oxy-PAH, alkylphenols, organophosphates, phthalates, pesticides, and naphthenic acids
Competitive TTR-binding activity	PFCs, phenolic compounds (hydroxylated metabolites of PCBs and PBDEs, nonylphenol, tretrachloro/bromobisphenol A, aminobenzo[a]pyrene and benzo[a]pyrene

4 Bioassays Detecting Endocrine Disruptors

Activation of estrogen receptors (ER) and androgen receptor (AR) belongs to the most frequently studied modes of action associated with endocrine disruption [42, 48–51]. Among different modes of action, particularly endocrine disruptors with estrogenic potencies have been studied. Although estrogens can act via modulation of biosynthesis, transport, and catabolism of steroids, the major focus has been on the effects of xenobiotics on modulation of ER-mediated gene expression.

The most frequently used cellular bioassays include ER-CALUX^{TM} assay using human breast carcinoma T47D.Luc cells stably transfected with pEREtataLuc plasmid and luciferase as reporter gene [52]. Alternative reporter vectors, *e.g.*, with transfected MVLN cells, have also been explored [42, 50, 53].

The recombinant yeast estrogen screen (YES) assay [54] has been used to determine ER-mediated activity of pore water and solvent extracts of sediments. The bioassay-directed fractionation and GC/MS revealed natural estrogens, alkylphenols, bisphenol A, petrogenic naphthenic acids, and several other contaminants as ER agonists [55–59]. The YES assay use β-galactosidase as reporter and can be a time-consuming procedure as a result of long induction time and multiple steps in the assay protocol. Another, faster yeast-based assay has been developed, the bioluminescent yeast estrogen screen (BLYES), where a human estrogen receptor is expressed along with a bacteria luciferase system controlled by a hormone responsive promoter [60]. Other reporter gene assays employ green fluorescent protein in a yeast estrogen bioassay [61]; however, this system has not been widely used in bioassay-directed studies. However, cell-based assays have been found to be more sensitive than the yeast-based assays, but they are also more expensive and less suitable for high-throughput screening [51].

Antiestrogenic compounds can also be identified in ER-CALUX, MVLN, BLYES, or YES assays using cotreatment with a reference ER agonist (17β-estradiol) [60, 62].

Natural estrogens (17β-estradiol and estrone) and synthetic derivatives such as ethinylestradiol have been recognized as major contributors to estrogenic activity in river sediment extracts or fractions. They have been found, for instance, in fish bile and sediment samples in ER-CALUX-directed fractionation and GC/MS analysis [49, 63]. In sediment samples, the concentration of alkylphenols and bisphenol A, the known xenoestrogenic in aquatic environments, has been found to be below the detection limit for estrogenic activity in the used biotests [53]. Therefore, it has been concluded that the major part of estrogenic activity is not associated with any polar aromatic compound identified in sediment fractions ([53] and other studies).

Recently, transgenic fish strains that allow detection of estrogenic effects already in embryos have been developed. Such strains offer high-throughput possibility. An advantage of cellular systems could be the complexity, *i.e.*, they may be more similar to a complete adult organism and better integrate uptake and distribution of the compound. However, they have thus far not been used for EDA, but principally

could be very useful (*e.g.*, determination of inhibition of aromatase, the key enzyme of steroidogenesis in [64]).

For determination of androgen receptor (AR) interference, the two most commonly used *in vitro* bioassays are the yeast androgen screen (YAS) and the AR-CALUX™. The YAS is based on the human androgen receptor (hAR) and three androgen response elements coupled to a luciferase reporter gene [65]. In the presence of a ligand, *i.e.*, a natural steroid or a xeno-androgen, the androgen receptor bound to an androgen-responsive element on a plasmid, initiating transcription of the reporter gene LacZ, which produces a measurable color change of the sample [66].

Recently, the bioluminiscent yeast androgen screen (BLYAS) has been developed [67]. The yeast strain also contains a hAR, but the difference is that the reporter is based on the Luc reporter gene, which is producing a measurable light and has much shorter incubation time (hours). It has been reported that there are some difficulties measuring antagonism effects in the yeast assay.

Other reporter assays used, for instance, the AR-deficient PC-3 cells stably transfected with pSG5-puro-hAR, pMMTV-neo-Luc [68], or the breast cancer cell line MDA-MB-453, transfected with the MMTV luciferase neo reporter gene construct [69].

Both the YAS and AR-CALUX assays have been successfully applied in EDA studies to identify environmental androgens [59, 70–72]. Although the AR-CALUX has been reported to be a more sensitive assay, the applicability of the two assays to EDA is comparable and both assays have identified androgen activity in the same fractions [71].

Chemical analysis of the antiandrogenic active extracts reported the presence of compounds of a wide range of different groups, *e.g.*, bisphenol A, alkylphenols, *p*,*p*′-DDE, iprodione, musks, phthalates, organophosphates, PAHs, and naphthenic acids. It seems that the compounds causing the antagonistic androgenic response are structurally variable, whereas induction of the androgenic response is caused by natural androgens or structurally related compounds.

An EDA study of Thomas et al. [70] successfully identified the natural androgen metabolites, dihydrotestosterone, androstenedione, androstanedione, 5β-androstane-3α,11β-diol-17-one, androsterone, and *epi*-androsterone, to be responsible for 99% of the *in vitro* activity determined in an effluent. This is possibly the EDA study with the highest explanation factor.

Two studies reported total extracts of water and sediment samples showing only antiandrogenic activity, but after fractionations both androgenic and antiandrogenic activities were found in specific fractions [71, 72]. The awareness of this masking effect of antagonistic compounds is important for screening environmental samples for endocrine disrupting and other effects.

The thyroid-hormone-disrupting compounds (TDC), *e.g.*, those structurally and chemically resembling thyroid hormones (*e.g.*, thyroxine, T_4, or triiodothyronine, T_3), can target and interfere with the hypothalamus–pituitary–thyroid axis at different levels; binding to the transport proteins transthyretin (TTR) and replacing the natural hormones is one possible mode of action. A few different binding assays

have been used to determine the binding potency of environmental pollutants to the TTR. Examples of *in vitro* assays are: radioligand-binding assay (RLBA) [73], non-RLBA with the transport protein covalently bound to a Sepharose resin and HPLC analyzed [74], and a surface plasmon resonance (SPR)-based assay [75], where the thyroid hormone is covalently bound to a gold-layered chip and competes to transport protein binding with TDCs in a flow cell.

One of the most common TDC bioassays is the well-established radioligand TTR binding assay based on a method described by Somack and coworkers [73], used with several minor modifications by different laboratories [76–78]. It is a competitive binding assay for TTR where T_4 or T_3 (native and labeled) are used as competitor for TDCs. The assay successfully detected TTR-binding activity of several compounds (*e.g*., [76, 77, 79]) and contaminants in sediment extracts [80].

The assay has shown a coefficient of variation of less than 8% between analyses of the same sample at different time points [79]. The limit of detection is set to 20% of binding capacity (ca 16 nM T_4). No interlaboratory comparisons have been performed thus far and therefore QA/QC protocols for the technique are lacking.

It is difficult to compare the results among studies from different laboratories as different competitors (T_4 or T_3) as well as TTR originating from different species (human, bird, rodents, fish, and amphibians, both purified and recombinant TTR) are used. Different modifications of the methods between laboratories may also cause discrepancies between the reported results, *e.g*., incubation time and temperature, separation medium of bound and free compounds, purity of standards, and ligands and detection methods. Therefore, a standardized protocol should be established to support the interpretation of data in risk assessments. However, the technique shows great promise and provides a powerful tool in identifying compounds/extracts that can competitively bind with the TTR protein and hence be a potential EDC.

Major contributors of specific endocrine modes of action are presented in Table 2.

To assess the contribution of each individual contaminant to the overall toxic effect, chemical analysis are combined with the determination of a reference compound's equivalents (*e.g*. TCDD and estradiol) derived from concentration data and individual relative effective potencies (REP) related to a reference agonist or antagonist [81]. Potentially, development of REP values may be performed also for anti/androgens and competitors of TTR binding. In the EDA, an alternative strategy has been used – the biological response of a chromatographic fraction conduct chemical identification of potential toxicants and contribution of the individual identified compound to the observed effect should be confirmed by both analytical (target analysis) as well as biological (bioassays) tools [37, 49, 53].

In EDA studies, exhaustive extraction is usually applied in order to include all the compounds present in the mixture, but this approach can be misleading considering that under environmental conditions certain compounds may be more bioavailable than others. Hence, in the presence of both agonistic and antagonistic compounds, it is valuable to include a bioavailability aspect in the extraction step [82].

5 Conclusions

Combination of selected bioassays for genotoxicity, AhR-mediated activity, and endocrine disruption is effectively used for both rapid screening and biological effect-directed chemical identification of major toxicants. To this time, several *in vitro* bioassays have been developed for identification and/or quantitative analysis of mutagenic, AhR-, ER-, AR-mediated activities as well as for competitive TTR-binding activity. Nevertheless, there is a need for further development of high-throughput screening strategies to be used in EDA, including fish and invertebrate models.

EDA offers a possibility to identify the biologically relevant contaminants present in the environment, and therefore it will be possible to better choose priority compounds. However, for the estimation of their environmentally safe concentrations, it will be necessary to conduct a detailed analysis of their hazard assessment (including development of REP of individual compounds in specific bioassays). Even when this is accomplished, we still need to learn how to do risk assessment of mixtures, especially those that vary along time and location like water, sediment, air, and soil.

Acknowledgements The authors thank Fábio Kummrow and Errol Zeiger for the valuable comments and MODELKEY project [Contract-No. 511237 (GOCE)].

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Separation Techniques in Effect-Directed Analysis

Werner Brack, Nadin Ulrich, and Mahmoud Bataineh

Abstract The continuous development of new chemicals enhances the complexity of environmental analysis and poses a risk to environmental and human health. Awareness is increasing that together with the chemical products on the market, the enormous number of transformation and by-products may contribute to this risk. Effect-directed analysis (EDA) has been developed to identify major toxicants in such complex mixtures. Separation techniques in EDA are applied to reduce the complexity of environmental mixtures and provide valuable information on physicochemical and thus structural properties of candidate toxicants. Within the last decades, separation science provided an extensive understanding of processes and mechanisms in chromatography, developed novel stationary phases with specific separation properties, and introduced modeling tools such as linear solvation energy relationships (LSER) to predict retention. A selection of these tools is compiled in the present paper to support the exploitation of present knowledge on chromatography to enhance the ability to identify so far unknown toxicants in complex mixtures.

Keywords Fractionation, LSER, Mechanism, QSRR, Retention prediction, Stationary phase

Contents

1 The Need for Separation in Effect-Directed Analysis 84
2 Intermolecular Forces Supporting Separation 85
3 General Definitions and Important Parameters in Chromatography 86

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W. Brack (ed.), *Effect-Directed Analysis of Complex Environmental Contamination*, Hdb Env Chem (2011) 15: 83–118, DOI 10.1007/978-3-642-18384-3_5,
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4 Liquid Chromatography 88
4.1 Reversed Phase Liquid Chromatography 88
4.2 Normal Phase Liquid Chromatography 100
4.3 Hydrophilic Interaction Chromatography 102
4.4 Size Exclusion Chromatography 103
4.5 Affinity Chromatography 104
4.6 Planar Chromatography 104
4.7 Complex Fractionation Schemes 105
4.8 Combination of Fractionation Procedures and Biotesting 107
5 Gas Chromatography 108
5.1 Characterization and Prediction of Retention by QSSR 108
5.2 Characterization and Selection of Stationary Phases 110
5.3 Application in EDA 111
6 Conclusions 111
References 112

1 The Need for Separation in Effect-Directed Analysis

Despite increasing efforts to regulate the emission of chemicals, the number of chemicals that are released to the environment is constantly increasing. At present, a total of about 14 million chemicals are on the market, while the number of known chemicals is about 50 million (CAS), both increasing. This includes industrial chemicals, solvents, flame retardants, dyes, pesticides and biocides, personal care products, pharmaceuticals, detergents, food additives, incineration products, and many more, as well as their respective by-products and metabolites. These anthropogenic chemicals occur in the environment together with numerous natural chemicals produced by plants, animals, and bacteria including lipids and lipoids, amino acids, carbohydrates, carboxylic acids, terpenes, steroids, flavones, anthocyanes, vitamins, and so on. Not only anthropogenic chemicals but also some of the natural compounds such as many alkaloids, antibiotics, and mycotoxins may be highly toxic to different organisms.

The basic idea of effect-directed analysis (EDA) is to unravel these complex mixtures of chemicals in environmental and technical matrices and to isolate and identify compounds causing adverse effects. These matrices include ground- and surface waters, sediments, soils, air and air particulate matter, plant and animal tissues, technical products, and mixtures. Thus, it is obvious that separation techniques play a major role in EDA.

The primary separation that is applied in EDA is often an extraction and clean-up step separating the target chemicals from other compounds forming the sample matrix. The latter may include minerals, salts, water, and large biogenic organic molecules such as humic compounds, proteins, lipids and polysaccharides. Discrimination of chemicals and matrix is subjective, depends on the aim of the study and determines which compounds can be identified and which are excluded. After extraction and clean-up, chromatographic separation techniques are primarily applied in EDA. Preparative separation, here referred to as fractionation, aims to

sequentially reduce the complexity of a sample to yield fractions that can be subjected to biological and chemical analysis. The major requirements are that different compounds or compound classes are actually recovered in different fractions with acceptable and reproducible recoveries. Research in the early 1990s already indicated that selected fractionation techniques can significantly influence the detection or nondetection of effects [1]. Fractionation techniques should have a high throughput to provide sufficient amounts for biotesting, further fractionation, and chemical analysis. Analytical separation is closely linked to chemical analysis, including structure elucidation. Even after several steps of fractionation, mixtures may be still complex requiring high resolution separation prior to – in most cases – mass spectrometric analysis. Both preparative and analytical separation techniques in EDA should provide information that helps to characterize and identify the chemicals of concern. To achieve this goal, a sound understanding of the mechanisms of chromatography and the structure-dependent interactions between analytes and different mobile and stationary phases is required. This is crucial for selecting chromatographic systems with optimal selectivity to resolve a given mixture and for successful toxicant identification. Thus, this chapter will focus specifically on chromatography and how this can be applied for toxicant isolation and identification. There are numerous variables that can be used for the classification of chromatography including the state of aggregation of the mobile phase, mechanisms of retention, the application of columns or planar stationary phases, and the use of gradients [2]. In this chapter, we primarily classify chromatography according to the mobile phase with a focus on liquid chromatography (LC), often applied as high performance liquid chromatography (HPLC), and gas chromatography (GC).

2 Intermolecular Forces Supporting Separation

In general, separation is supported by the fact that different chemical structures result in different interactions with surrounding molecules of the same type (e.g., in pure liquid) or of different type (e.g., in solution or when adsorbed to solid phases). These interactions include (a) nonspecific van der Waals interactions and (b) specific interactions as a result of particular molecular structures.

1. Van der Waals interactions are a superposition of several components, including London, Debye, and Keesom energies [3, 4].

   London dispersive energies are a result of attractions between time-varying, uneven electron distributions in adjacent molecules. The intensity of these interactions depends on the polarizability of the molecules. Polarizability is the relative tendency of a charge distribution, such as the electron cloud of an atom or molecule, to be distorted from its normal shape by an external electric field, which may be caused by the presence of a nearby ion or dipole. Higher polarizability means greater potential for dispersive interactions. Polarizability increases with increasing molecular size, with the occurrence of large atoms with non-binding electrons far from the nucleus, and with conjugated electron

systems. Polarizability is often determined via the refractive index. The refractive index of a medium is a measure of how much the speed of light is reduced inside the medium. At the microscale, an electromagnetic wave's phase velocity is slowed in a material, because the electric field creates a disturbance in the charges of each atom (primarily the electrons) proportional to the permittivity of the medium.

Debye energies result from dipole-induced dipole interactions. Molecules with atoms of different electronegativity next to each other exhibit permanent dipoles. If these dipoles are juxtaposed to a molecule with time-averaged even electron distribution, this will result in dipole induction to this molecule depending on the dipole moment (DIMO) of the first molecule and the polarizability of the second. Keesom energies are a result of the interaction of two permanent dipoles. The strength of the interaction depends on the product of the DIMOs of the interacting molecules.

2. Specific interactions result from particular molecular structures that allow strong interactions between permanently electron-poor and electron-rich parts of interacting molecules. The most important example is hydrogen bonding between permanently electron-rich sites of a molecule such as nonbonded electrons of atoms like oxygen and nitrogen acting as electron-donor or H-acceptor and permanently electron-poor parts of another molecule such as hydrogen bound to oxygen or nitrogen (electron-acceptor or H-donor). Hydrogen bonding is dependent on the specific atoms involved and on the orientation of the interacting molecules.

   These interactions, along with molecular size, geometric and steric factors, translate into the partitioning of a molecule between different solid, liquid, and gaseous phases and thus can be exploited to separate compounds in mixtures. While chromatographic approaches are of major importance, this includes also the separation of molecules binding to particles from dissolved compounds by (ultra)filtration [5], centrifugation, and sequential extraction techniques, such as hot pressurized water fractionation involving the sequential collection of high to low polarity fractions extracted with increasing water temperature [6–8]. Evaporation and distillation exploit partitioning between liquid and gaseous phase and may be used to separate volatiles from nonvolatile mixtures [9, 10]. The separation of acids, bases, and neutral compounds based on liquid–liquid, solid phase extraction (SPE), or stripping at different pH values or the application of ion exchangers is a valuable and frequently applied technique for a first fractionation of complex mixtures [11–19].

3 General Definitions and Important Parameters in Chromatography

Chromatographic separations are based on a continuous sequence of equilibrium partitioning steps of the analyte between a mobile phase and a stationary phase. Differences in partitioning behavior result in different migration velocities of the

analytes through the chromatographic bed. The migration velocity of the analyte i, u_i is defined as

$$u_i = u_0\left(\frac{1}{1+k_i}\right), \tag{1}$$

with u_0 representing the average migration velocity of the mobile phase and k_i representing the retention factor of compound i.

The retention factor is equal to the partitioning coefficient of the compound between the stationary phase (s) and the mobile phase (m).

$$k_i = \frac{c_i^{\mathrm{S}}}{c_i^{\mathrm{m}}} = \frac{V_{\mathrm{R}i} - V_{\mathrm{R}0}}{V_{\mathrm{R}0}} = \frac{t_{\mathrm{R}i} - t_{\mathrm{R}0}}{t_{\mathrm{R}0}}, \tag{2}$$

where c_i^{S} and c_i^{m} represent the concentrations of compound i in the stationary phase and the mobile phase, respectively. $V_{\mathrm{R}i}$ is the retention volume representing the volume that is required to elute a retained compound while $V_{\mathrm{R}0}$ represents the hold-up volume which is required for the elution of a nonretained compound. Expressed in terms of time, this involves the retention time of the retained compound i $t_{\mathrm{R}i}$ and the hold-up time that is required for a nonretained compound $t_{\mathrm{R}0}$. Compounds that are frequently used in RP-HPLC to determine hold-up times/volumes include uracil and thiourea.

The separation of two different compounds in a given chromatographic system is described by the selectivity factor α according to:

$$\alpha = \frac{k_2}{k_1}. \tag{3}$$

The resolution between two chromatographic peaks A and B is expressed in (4):

$$R = 2\frac{t_{\mathrm{R}B} - t_{\mathrm{R}A}}{W_{\mathrm{b}B} - W_{\mathrm{b}A}}, \tag{4}$$

where $t_{\mathrm{R}B}$ and $t_{\mathrm{R}A}$ are the retention times of peak A, and B, and $W_{\mathrm{b}B}$ and $W_{\mathrm{b}A}$ are the width of peaks A and B at baseline.

In terms of thermodynamics, the chemical potential μ_i of compound i in a chromatographic phase is given by the standard state chemical potential μ_i^0 assuming an infinite dilution of i and representing the intrinsic thermodynamic affinity of the compound to the system and a term related to entropy related to the dilution (concentration c_i) of the compound i.

$$\mu_i = \mu_i^0 + RT\ \ln\ c_i. \tag{5}$$

Equilibrium implies equality of chemical potential in the two chromatographic phases:

$$\mu_i^{\mathrm{S}} = \mu_i^{\mathrm{m}}. \tag{6}$$

Thus, the partitioning coefficient (retention factor) k_i can be expressed as

$$k_i = \frac{c_i^{\mathrm{S}}}{c_i^{\mathrm{m}}} = \exp(-\Delta\mu_i^0/RT). \tag{7}$$

Unfortunately, $\Delta\mu_i^0$ and thus k_i cannot be calculated using thermodynamics nor through a rigorous relationship to molecular parameters. Thus, according to Kaliszan [2], understanding and describing molecular equilibrium between phases requires a combination of experimental measurements, correlations by means of empirical equations, and approximate theories. In the following chapter, we will describe different approaches that may be used in EDA.

4 Liquid Chromatography

LC is the predominating fractionation technique in EDA and of increasing importance for analytical separation prior to mass spectrometry. There are several LC approaches relevant to EDA, involving different mechanisms of interaction between the analyte and the stationary and mobile phase. These include (a) reversed-phase (RP)-LC, (b) normal phase (NP)-LC, (c) hydrophilic-interaction chromatography (HILIC), (d) size exclusion chromatography (SEC), (e) ion-exchange chromatography (IEC), and (f) affinity chromatography. Most approaches apply chromatographic columns, while planar thin layer chromatography (TLC) only plays a minor role.

4.1 Reversed Phase Liquid Chromatography

Although chromatographic science started with NP-LC, nowadays RP-LC is the most frequently used LC technique. It can be applied as SPE, retaining hydrophobic compounds from aqueous solutions for subsequent sequential elution with organic solvent/water mixtures with decreasing water content. For high resolution separations, HPLC techniques are required, often applying gradients of water and methanol or acetonitrile as the mobile phase. If dissociating compounds are expected in a sample, the mobile phase pH needs to be adjusted. The greatest effects of alteration of pH in the mobile phase are observed within 1 pH unit of the pK_a value of the molecule. Assuming that only the nondissociated form partitions into the stationary

phase for acidic and basic molecules, the apparent partition coefficient K_{app} can be calculated from the partition coefficient of the nondissociated form k_i [(8) and (9)]. For acidic molecules:

$$K_{app} = \frac{k_i}{1 + 10^{pH - pK_a}}, \tag{8}$$

while for basic molecules:

$$K_{app} = \frac{k_i}{1 + 10^{pK_a - pH}}. \tag{9}$$

4.1.1 Characterization and Prediction of Retention by QSRR

The retention of analytes in a chromatographic system provides valuable information about the separated compounds for compound identification in EDA. Quantitative structure-retention relationships (QSRRs) can be used to link the chemical structure of the analyte to physicochemical properties and thus to retention. These models have been found to be helpful to better understand molecular mechanisms of separation, to characterize properties of analytes and stationary phases, and also to predict retention for a new analyte [20]. In EDA, there are particularly two applications for QSRRs: (a) to identify stationary phases with complementary selectivity for a multistep fractionation procedure and (b) to provide classifiers for structure elucidation that help to rank or exclude suggested structures according to the agreement between the observed and predicted retention.

Unfortunately, no strict quantitative retention model allows a precise prediction of retention for an individual solute for given chromatographic conditions [2]. However, there are several approaches and many descriptors that help improve the understanding and prediction of chromatographic behavior. Descriptors include empirical ones [including the measured physicochemical parameters log K_{ow} (RP-HPLC) or boiling points (GC)] and solvatochromic parameters derived from linear solvation energy relationships (LSER), see below. In addition, many nonempirical structural descriptors are available, based solely on the structural formula. Examples include CLOGP (octanol–water partitioning) calculated by fragmental methods; molar refractivity and polarizability calculated by the summation of the atomic, the group or the bond-type increments, molar volumes, and van der Waals surface areas; quantum chemically derived structural parameters such as the total energy, the DIMO, and energies of the highest occupied molecular orbital (HOMO) and the lowest unoccupied molecular orbital (LUMO); and molecular shape parameters for considering steric effects on retention [2]. It is beyond the scope of this chapter to give a comprehensive review of QSRR methods. Thus, only some examples are given below which might be of particular relevance for EDA. Although the exploitation of structural information from retention behavior of

analytes in EDA is limited so far, the potential of QSRRs for the identification of unknowns is obvious and might be one of the triggers to further advance these techniques.

Octanol-Water Partition Coefficients

Retention prediction in RP-HPLC via the octanol–water partition behavior (log K_{ow}) is the simplest and most frequently applied QSRR for a plausibility check of structure suggestions of unknowns in EDA, using simple regressions based on retention time or factor:

$$k_i = a + b \log K_{ow}. \tag{10}$$

Octanol–water partition coefficients are available for many compounds and can be estimated for every organic compound with freely or commercially available softwares. However, users should be aware that the software selected for log K_{ow} prediction has an influence on prediction quality. Baczek and Kaliszan tested three different software packages for log K_{ow} prediction with a model series of 15 well-known simple compounds and a test series of 47 compounds including nonpolar aromatic compounds, aromatic carboxylic acids, chloroanilines, PAH quinones, and others [21]. The authors achieved regression coefficients between measured retention times and those predicted from log K_{ow} estimates of 0.894 – 0.944 depending on the software used. The mean relative error in retention prediction was 19 – 27%. Even for relatively simple compounds, retention prediction based on log K_{ow} provides only a first approximation, but this is better than nothing, when no other information is available.

Linear Solvation Energy Relationships

LSER models are used to interpret the properties of compounds based on molecular interactions. In liquid chromatography, the Abraham equation describes the partitioning behavior of a solute between the liquid mobile and the solid stationary phase (11) [22].

$$\mathrm{SP} = aA + bB + sS + eE + vV + c. \tag{11}$$

Here, the solute property SP represents a specific solvent-dependent property [e.g., retention factor (log k) or a partitioning coefficient (log P)], while the variables with upper case letters specify possible interactions of the solute and the lower case letters interactions of the solvent.

The solute descriptor V is the McGowan volume, which can be calculated easily using characteristic atomic volumes (V_i), the number of atoms (n_i), and molecular bond counts (m) (12) [23].

$$V = (\Sigma n_i V_i) - (m)\ 6.56\ \mathrm{cm^3 mol^{-1}}. \tag{12}$$

The descriptor E is the molar excess refraction and can be calculated from the molar refraction (MR_x) and the refraction index of the pure liquid at 20°C (η) (13) using McGowan volumes (V) instead of molar volumes, minus the molar refraction of an alkane with the same V (14) [4].

$$MR_x = 10\left[\frac{\eta^2 - 1}{\eta^2 + 2}\right] \cdot V \tag{13}$$

$$E = MR_x - (MR_x)_{alkane} = MR_x - 2.83 \cdot V + 0.526 \tag{14}$$

The descriptor S represents the polarizability and dipolarity of the analyte, A the hydrogen bond donor strength, and B the hydrogen bond acceptor strength. In RP-LC, the solute volume and the refractivity contribute to retention, and solute dipolarity, polarizability, and hydrogen bonding favor elution.

For phase parameters this is reversed, that is, a describes the hydrogen bond acceptor strength of the solvent and b the hydrogen bond donor strength. Again, the dipolarity and polarizability are also represented by s. The parameter e represents the van der Waals interactions and v characterizes the cavity formation of the solvent. The intercept c is a system-specific constant [22]. The phase parameters reflect only a comparison of the different interaction possibilities of the two phases (stationary and mobile).

To set up the model for log k in isocratic liquid chromatography, a training set of compounds with known descriptors is used to determine retention times for at least five different columns. These results, converted to log k, can be used in a multilinear regression to get the phase parameters of the different columns. It is wise to choose the training set (aliphatic and aromatic compounds with different substituents) and columns (reversed phase, normal phase, and HILIC) very carefully to cover a large spectrum of interactions. Equation 14 can be used to calculate the descriptors of the analytes, for example, the excess molar refraction E and McGowan volume V. Only the descriptors S, A, and B have to be determined by a multilinear regression [24, 25].

Today, E, S, A, B, and V values are available for many solutes including numerous functional groups [25–30], which is an important prerequisite for an application of LSER in EDA. In addition, system parameters e, s, a, b, and v have been determined for many chromatographic systems including C8, C18, F13C9, PBB, PYE, CN, and many more columns by multiple regression [31, 32]. Interestingly, Abraham et al. [33] found one general equation for six different C18 stationary phases and several different mobile phase compositions. For 11 other C18 phases, the ratios v/Q and $(v + c)/Q$ were constant, where Q is the quantity of stationary phase per unit surface area [33].

Although LSERs may be the most promising approach for retention prediction at present, there are still significant shortcomings. These include, for example, the neglect of contributions to retention from shape selectivity, cation-exchange and related ionic interactions, and π–π complexation [34] and thus limited precision. Uncertainties are particularly notable for the estimation of Abraham's molecular descriptors from structure [35], which hampers its application in EDA.

Chromatographic Hydrophobicity Index

The most common model in a gradient system is the determination of the hydrophobicity index *CHI*, which can be related to log K_{ow} [36]. For the determination of *CHI*, a set of training compounds is used first in isocratic measurements at different eluent compositions, resulting in a linear regression between the percentage of organic solvent *x* and the retention factor log *k* (15, Fig. 1). Extrapolation to 100% water provides the intercept log k_w. For the training compounds, a CHI_0 value is determined as the quotient of the slope and the intercept (16). CHI_0 represents the percentage of organic solvent required to achieve an equal distribution of a compound between the mobile and the stationary phase.

$$\log k = S \cdot x + \log k_w, \tag{15}$$

$$CHI_0 = -\log k_w / S. \tag{16}$$

In the next step, the *CHI* of the target analyte(s) is determined based on the retention time t_R at the selected gradient by applying the linear correlation between CHI_0 and the retention time in the gradient system [36, 37].

The chromatography-based *CHI* value shows a correlation with log K_{ow}. However, it can be significantly improved by adding simple H-bond donor counts (HBC) or Abraham H-bond donor strength *A* [37, 38]. While the water saturated octanol phase may act as an H-bond acceptor, chromatographic C18 stationary phase has no polar functionality if we assume an extensive coverage of silanol groups.

QSRRs Based on Analyte Structural Descriptors

Retention in RP-HPLC can be predicted based on quantum chemical indices and/or on analyte structural descriptors from calculation chemistry [20]. A frequently

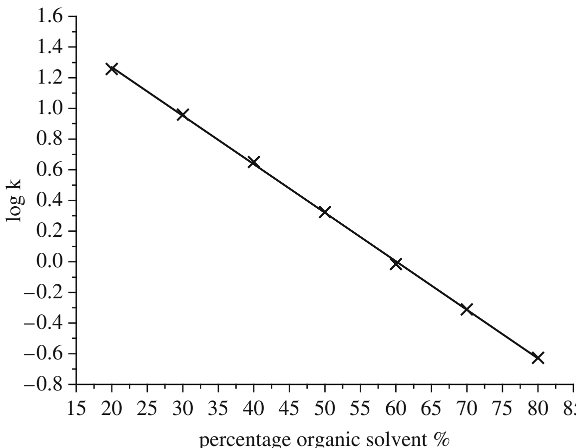

Fig. 1 Linear regression between log *k* of indole and the percentage organic solvent (*S*) for determination of CHI_0 (own unpublished data)

applied approach is based on the following descriptors: (a) total dipole moment μ accounting for the dipole–dipole and dipole-induced dipole attractive interactions of the analytes with stationary and mobile phase; (b) the electron excess charge of the most negatively charged atom δ_{Min} reflecting the local, fragmental analyte polarity and hence its ability to participate in polar interactions with the phases including submolecular dipole–dipole, charge-transfer, and hydrogen-bonding interactions; and (c) the water-accessible molecular surface area A_{WAS}, describing the strength of dispersive interactions of the analyte with the molecules forming the chromatographic phases (17) [20].

$$t_{\mathrm{R}} = k'_1 + k'_2\mu + k'_3\delta_{\mathrm{Min}} + k'_4A_{\mathrm{WAS}}. \tag{17}$$

Applying this method to the same set of compounds already explored with the log K_{ow}-based approach by Baczek and Kaliszan resulted in a somewhat better model for the model series ($R = 0.9870$) but not in a better prediction of the test series ($R = 0.8913$) [21]. Thus, the translation of structural formulas into sets of numerical descriptors still needs further improvement.

The same RP stationary phases that have been tested with the set of 15 compounds for the applicability of the log K_{ow}-based model have been also tested for this model. The correlation for all phases was slightly better with R-values between 0.969 and 0.988, demonstrating the applicability of this model.

Recently, a novel QSRR model for retention in RP-LC on different C18 columns has been developed [39]. The authors applied a hybrid method of Partial Least Square – Multiple Linear Regression – Self Training Artificial Neural Network. It is based on six calculated nonempirical descriptors including the molecular mass M, the van der Waals volume V, and the quantum chemical descriptors partial charge of the most negative atom (NPCH), partial charge of the most positive atom (PPCHH), dipole moment (DIMO), and highest occupied molecular orbital (HOMO). These parameters have some correlations with the empirical solvatochromic parameters used in LSER. HOMO represents a measure of the ability of a molecule to interact with the n- and π-electron pairs of other molecules. DIMO is considered as a parameter for the ability of a molecule to take part in dipole–dipole interactions. PPCHH and NPCH are measures for acidity and basicity of the molecule, respectively. The combination of the parameters M and V considers contributions from solvent cavity formation and dispersion interactions.

Molecular Shape as a Parameter Determining Retention

Except in affinity chromatography, where steric fitting is decisive for the process of molecular recognition, steric effects of retention is of minor importance for retention in most chromatographic systems compared to polar and dispersive interactions. However, in EDA, small differences in retention of isomers with different molecular shapes may be very valuable for their identification. An important

parameter in this context is the shape parameter η, which is defined as the ratio of the longer to the shorter side of the rectangle with the minimum area required to envelope the molecule [2]. Particularly retention of parent and Me-PAHs on polymeric C18 phases was found to be determined very much by the shape of parent and substituted PAHs characterized by the length-to-breadth ratio η [40, 41] and the existence of bay regions and substituents in bay and peri positions [42].

4.1.2 Characterization and Selection of Stationary Phases

While the possibilities to influence the selectivity of RP-HPLC using different mobile phases are limited, a wide range of stationary phases is available (examples in Fig. 2). However, despite the multitude of different RP-phases on the market, RP-HPLC fractionation in EDA has focused almost exclusively on the use of C18 columns [43, 44]. Although well-established approaches to characterize the selectivity of stationary phases are available and the use of stationary phases with

Fig. 2 Example stationary phases. NPE: nitrophenylethyl, PBB: 3-(pentabromobenzyloxy)propyl, PYE: 2-(1-pyrenyl)ethyl

Table 1 Column properties and reflection by chromatographic parameters according to the Tanaka protocol [46, 48]

Column property	Chromatographic parameter
Surface area and surface coverage	Retention factor for pentylbenzene
Hydrophobic selectivity α_{CH2}	Retention factor ratio between pentylbenzene and butylbenzene
Shape selectivity $\alpha_{T/O}$	Retention factor ratio between triphenylene (T) and o-terphenyl (O)
Hydrogen bonding capacity $\alpha_{C/P}$ (number of available silanol groups)	Retention factor ratio between caffeine (C) and phenol (P)
Total ion-exchange capacity $\alpha_{B/P}$	Retention factor ratio between benzylamine (B) and (P) at pH 7.6
Acidic ion-exchange capacity $\alpha_{B/P}$	Retention factor between B and P at pH 2.7
Aromatic selectivity (π-acidity) $\alpha_{PB/O}$	Retention factor between n-pentylbenzene (PB) and O
Aromatic selectivity (π-basicity) $\alpha_{TNB/NB}$, $\alpha_{DNT/NB}$, $\alpha_{TNB/DNT}$	Retention factor ratios between 1,3,5-trinitrobenzene (TNB) and nitrobenzene (NB), 2,4-dinitrotoluene (DNT) and NB and TNB and DNT

complementary selectivity in multi-step fractionation procedures may be very helpful, stationary phase selection in EDA based on systematic selectivity characterization is very rare. One of the most powerful approaches for column characterization in RP-HPLC is the Tanaka protocol [45–47]. Selectivity differences of columns are recorded on the basis of six variables obtained experimentally by measuring a set of standard compounds at defined chromatographic conditions. The parameters and their chromatographic reflection are given in Table 1 [46, 48]. Additional parameters are provided by Euerby and Petersson [45] and Neue et al. [49].

Euerby and Petersson tested 135 commercially available RP columns (examples in Fig. 3). Hydrophobic selectivity ranged between 1 (cyano columns, for example, Discovery CN, Supelco) and 1.6 (some octadecyl columns, e.g., Targa C18, Higgins). Shape selectivity was highest for a polymeric C18 column (4.1, Astec Polymer C18, Astec) and lowest for perfluorohexyl columns (0.6, Fluofix, Neos). Some columns such as Platinum C18 EPS (Alltech) are designed to provide dual-mode separation by allowing controlled exposure to nonbonded silica groups reflected by hydrogen bonding capacity of 2.6, while Astec Polymer C18 has a $\alpha_{C/P}$ of only 0.15. Principle component analysis of stationary phases based on selectivity parameters helps to identify similar or complementary phases [45]. Stationary phases with aromatic ring systems bonded through alkyl spacers to silica provide an interesting aromatic selectivity based on π–π interactions between the stationary phase and the solute [48]. This interaction is favored when the stationary phase is electron rich and thus a soft Lewis base, while the solute is electron deficient due to inductive (−I) and mesomeric (−M) effects of substituents and thus a soft Lewis acid. Aromatic stationary phases with aromatic selectivity include phenyl- [48], diphenyl- [50], naphthalenyl- [51], fluorenyl- [52], anthracenyl- [53] and pyrenyl- [51], and naphthylimide-type [54] phases.

Turowski et al. [31] identified stationary phases with high selectivity factors for solutes with different polarizability, such as the 3-(pentabromobenzyloxy)propyl

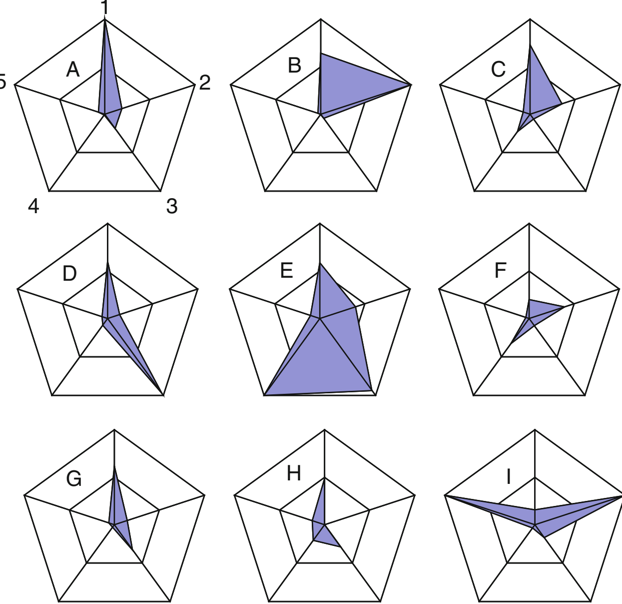

Fig. 3 RP-phases (selection) characterized by Euerby and Petersson [46] applying the Tanaka protocol: (**a**) Targa C18 (Higgins), (**b**) Astec Polymer C18 (Astec), (**c**) Aquasil C18 (polar endcapping, Hypersil), (**d**) Zorbax SB-C3 (Agilent), (**e**) Platinum C18 EPS (extended polar selectivity, high level of silica exposure, Alltech), (**f**) Inertsil CN3 (cyano phase, Hichrom), (**g**) Luna Phenyl-Hexyl (Phenomenex), (**h**) Fluofix (perfluorohexyl, Neos), I: Luna NH2 (amino phase, Phenomenex). The numbers represent 1: hydrophobic selectivity α_{CH2}, 2: shape selectivity $\alpha_{T/O}$, 3: hydrogen bonding capacity $\alpha_{C/P}$, 4: total ion exchange capacity (pH 7.6) $\alpha_{B/P,\ pH\ 7.6}$, 5: acidic ion exchange capacity (pH 2.7) $\alpha_{B/P,\ pH\ 2.7}$. Scales for Parameters are normalized

phase (PBB) and 2-(1-pyrenyl)ethyl (PYE), while fluoroalkane phases [e.g., 4,4-di (trifluoromethyl)-5,5,6,6,7,7,7-heptafluoroheptyl ($F_{13}C_9$)] showed extremely low dispersion interaction compared with C18 columns. For example, PBB differentiates very clearly between compounds containing sulfur (highly polarizable) instead of oxygen, while $F_{13}C_9$ does not (Fig. 4). Fluorinated alkyl phases have been shown to exhibit a unique orthogonal selectivity for geometrical isomers compared with C18 and C8 [55, 56]. Benskin et al. successfully separated perfluorinated acids (PFA) and PFA-precursor isomers in a technical mixture and in human serum using a linear perfluorooctyl stationary phase and an acidified mobile phase [56].

For the separation of aromatic compounds with electron-withdrawing substituents such as chlorine- or nitro groups, PYE provides excellent separation of isomers based on electron–donor–acceptor interactions with a preferential retention of symmetrical substitution or charge distribution, which results in a greater electron-deficiency in the aromatic ring [57]. This allows, for example, a fractionation of polychlorinated dibenzo-*p*-dioxins and furans (PCDD/F) separating the highly toxic and symmetric 2,3,7,8-substituted isomers from less toxic asymmetric ones. In contrast, 2-(nitrophenyl)ethylsilyl- and 3-(*p*-nitrophenoxy)propylsilyl-bonded

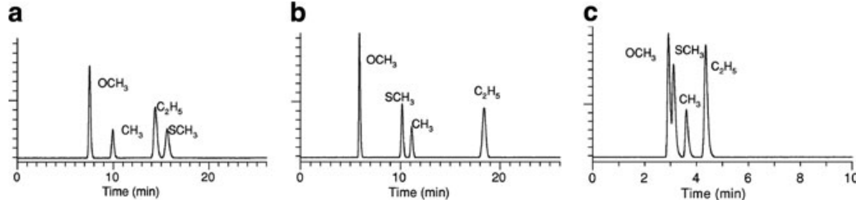

Fig. 4 Separation of anisole (–OCH_3), thioanisole (–SCH_3), toluene (–CH_3) and ethylbenzene (–C_2H_5) on PBB (**a**), C18 (**b**), and F13C9 (**c**) (from [31], with permission)

phases with an electron-deficient ring show preferential retention of asymmetrically substituted isomers with greater dipole character, for example, with crowded chlorine substituents. A combination of different stationary phases with different electron donor-acceptor properties allows for the separation of all PCDD/F, PCB, and PCN isomers [58].

Polar-embedded and ion exchange-embedded stationary phases can provide an alternative and complementary separation for many analyses performed on C8 or C18 columns. These polar groups are generally incorporated in the alkyl ligand close to the surface silica. A variety of polar functional groups including amides, carbamates, ureas, and ethers have been embedded. Amide-embedded phases were originally introduced commercially for their ability to deactivate silanol interactions with basic analytes [59]. Polar-embedded stationary phases exhibit lower hydrophobicity, while the retention of analytes with polar functional groups increases compared to conventionally alkyl-bonded phases [60]. Columns combining a C18 phase with a hydrophilic end-capping (e.g., Aquasil C18 column) offer a unique material with alternative selectivity and up to twice the retention for polar compounds.

Recently, embedded ion-exchange alkyl phases have been introduced commercially by SIELC Technologies and Oasis mixed-mode (MCX/MAX) which is sold by Waters. These mixed-mode phases are composed of a hydrophobic alkyl chain and either a hydrophilic acid group or a protonated base group. Both the alkyl chain and the ion-exchange functionality are contained in a single-bonded phase ligand. The ion-exchange group can be activated or deactivated depending on the pH of the mobile phase, and if the analyte has some polar or ion-exchange character, its interaction with the column can be pH adjusted. The mixed mode stationary phase was used by Venkatramani et al. for the separation of hydrophobic components from charged species [61].

LSERs are an appropriate way to characterize RP-HPLC systems [62]. Using the Abraham approach, Valko et al. [63] could show that selectivity is not only dependent on the stationary phase but the selection of organic mobile phase modifiers such as methanol, acetonitrile, or trifluoroethanol also strongly influences selectivity. It has been shown that the major difference between different RP-HPLC systems is their sensitivity toward H-bond donor compounds. This sensitivity is a result of the H-bond acceptor properties of the stationary relative to the mobile phase [38].

An alternative approach, called the hydrophobic subtraction approach, has been presented considering five solute-column interactions as contributions to compound retention and column selectivity: (a) hydrophobic interaction, (b) shape selectivity, (c) hydrogen bonding of acidic solutes to basic column groups, (d) hydrogen bonding of basic solutes with acidic column groups, and (e) cation exchange with ionized silanol groups [34, 64–66]. The model is based on (18) for column selectivity α.

$$\log \alpha = \log\left(\frac{k}{k_{\mathrm{EB}}}\right) = \eta' H - \sigma' S^{*} + \beta' A + \alpha' B + \kappa' C \tag{18}$$

where k is the retention factor of a given solute and k_{EB} is the retention factor of ethylbenzene as a nonpolar reference solute. The parameters H, S^*, A, B, C characterize the stationary phase indicating hydrophobicity (H), steric resistance to insertion of bulky solute molecules into the stationary phase (S^*), column hydrogen-bond acidity (A), column hydrogen-bond basicity (B), and column cation-exchange activity (C). The corresponding solute parameters include solute hydrophobicity (η'), molecular bulkiness (σ'), hydrogen-bond basicity (β'), hydrogen-bond acidity (α'), and approximate charge on the solute molecule (κ'). The solute parameters are relative to values for ethylbenzene, while the column parameters are relative to a hypothetical average pure silica C18 column. The model is a powerful tool for characterization of column selectivity and for selection of columns with very different selectivity for multistep fractionation procedures. A great number of commercially available RP-LC columns has been characterized in this way, and the results have been compiled by Snyder et al. [34]. While the column parameters are approximately the same for different separation conditions, the solute parameters vary with conditions such as the mobile phase, temperature, etc. Thus, the method is less promising for the characterization of solutes. Although correlations have been found between η' and log K_{ow}, σ' and molecular shape, length and thickness, and β', α', and κ' with molecular structure, no models are available at this stage to predict these parameters from molecular structure and thus to rank or exclude candidate compounds in EDA.

In cases where isomers with similar physicochemical parameters need to be separated, shape selectivity is of increasing importance and polymeric C18 [40, 41] or cyclodextrin (CD) [67, 68] may be used as stationary phase. Cyclodextrins consist of six (α-CD), seven (ß-CD), or eight (γ-CD) glucopyranose units forming a hydrophobic cavity and a hydrophilic surrounding with hydroxy groups (Fig. 5) [67]. Cavity diameters are about 5.0, 6.3, and 8.0 Å. The inclusion complex depends on geometry, size, and physicochemical properties of the analyte. Hydrophobic interactions predominate in the cavity. In addition, H-bonding occurs with the outer OH-groups. Good separation has been observed for different Me-BaPs [67] and ortho-, meta-, para-substituted benzenes related to the stability of the cyclodextrin-compound inclusion complex [68]. Cyclodextrin-bonded phases work as RP-HPLC for mobile phases with low organic content, where the mobile phase is more polar than the stationary phase. At high organic contents, the mobile phase is less polar than

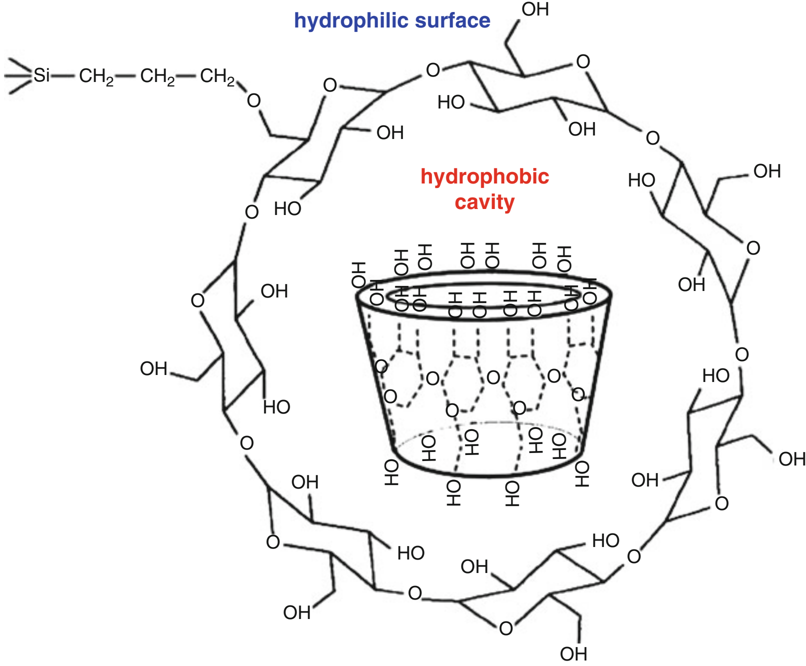

Fig. 5 Cyclodextrin as a stationary phase in chromatography

the stationary phase and the separation similar to HILIC for mobile phases with high organic content (MeOH or ACN) [69].

4.1.3 Application in EDA

RP-SPE represents the classical way to extract aqueous samples for subsequent EDA and may be exploited for separation by sequential elution with aqueous organic solvent mixtures with decreasing water contents [44, 70–81]. This technique has been suggested as a method for aquatic toxicity identification evaluation (TIEs) by the US-EPA [82] and reviewed by Lukasewycz and Durhan in the early 1990s [83]. Major SPE techniques involved XAD resins as polymeric adsorbents on a styrene divinylbenzene basis and octadecyl (C18) phases [84]. Within the last 10 years, additional phases such as the polystyrene-based polymer Isolute ENV+ [85–88] and poly(styrene-divinyl benzene) [88–90] came into use. Fiehn and Jekel tested three different SPE cartridges at different pH values for recovery of a broad array of compounds expected in industrial wastewater [91]. They suggested poly(styrene-divinylbenzene) as a sorbent for the extraction of hydrophilic aromatic compounds. To achieve good recovery for all compounds and as a primary approach for fractionation, the sequential use of different phases was propagated [88, 91–96].

RP-HPLC provides an excellent tool for preparative and analytical scale separation of mixtures. RP-HPLC is the predominating technique for preparative separation of complex mixtures from aqueous samples and provides an important tool also for fractionation of samples derived from soils, sediments, air particulate matter, and other solid matrices. In analytical HPLC-MS, RP-HPLC is the method of choice. It provides a powerful tool for separation but also valuable classifiers for compound identification by evaluating the retention behavior of a compound of interest.

Most EDA studies of effluents, surface waters, and other aqueous samples [44, 85, 93, 97–106] but also some EDAs of extracts of sediments [107], compost [108], fish bile [109] and hepatic tissue [110–112], pesticide formulations [113, 114], phototransformation products [115–117], and biological metabolites [118] were based on RP-HPLC fractionation.

Most of the RP-HPLC fractionations applied exclusively C18 phases. This is in agreement with the TIE procedure suggested by the US-EPA [82], provides a reasonable separation for many solutes, and allows for a calibration of the fractionation procedure according to log K_{ow} [109, 110, 119, 120]. Watanabe and co-workers [121, 122] successfully applied phenyl-hexyl phases after C18 fractionation to isolate mutagenic aromatic compounds such as phenylbenzotriazole-derivatives, 3,3′-dichlorobenzidine, and nitrated PCB derivatives from Japanese river waters exploiting the aromatic selectivity of this phase. Beck et al. [123] focused on the isolation and identification of estrogenic compounds in marine surface waters and tested five different RP-phases for separation of estrone and 17α-ethynylestradiol as examples for estrogenic steroids. They found Synergy™ Polar-RP (Phenomenex), an ether-linked phenyl phase with polar endcapping, to provide optimal separation.

Despite some examples for the use of specific RP-selectivities for fractionation in EDA, it is obvious that EDA could benefit greatly from exploiting the multitude of commercially available stationary phases with different selectivities to fractionate, isolate, and identify toxicants in complex mixtures.

4.2 Normal Phase Liquid Chromatography

Since the beginning of the twentieth century, NP-LC has been used for the separation of colored plant constituents [124]. For many years, NP-LC on silica, alumina, and other polar adsorbents using a nonpolar mobile phase was the only approach in chromatography and thus later called normal phase. Although today RP techniques have the leading role in chromatography, NP approaches may be very helpful for the (particularly preparative) separation of hydrophobic mixtures such as soil and sediment contaminants. The basic phenomenon behind NP-LC is adsorption in contrast to RP-LC where solvation is the discriminating step. In NP-LC, adsorption is based on a range of interactions including electron–donor–acceptor interactions, hydrogen bonding, and others [124, 125]. All these interactions may be understood as interactions between permanent or induced dipoles described by the Coulomb forces. Retention may be understood according to the adsorption-displacement

model expecting solute and solvent molecules to compete for positions in a monomolecular layer formed on the surface of the stationary phase [125].

4.2.1 Characterization and Prediction of Retention by QSRR

Compared with RP-LC, fewer efforts have been made to develop retention models for NP-LC. In general, retention in NP-LC can be described by LSER according to the Abraham equation (12), although the correlations obtained are worse than those for RP-LC [126]. In NP-LC, dipolarity, polarizability, and hydrogen bond abilities increase retention, whereas in RP-LC, the solute volume and the refractivity contribute to retention and solute dipolarity, polarizability, and hydrogen bonding favor elution.

4.2.2 Characterization and Selection of Stationary Phases

NP-LC offers a multitude of stationary phases with diverse selectivity. Most common in NP-LC are cyanopropyl-, diol-, and aminopropyl-bonded phases [127]. Li et al. [126] investigated these columns for selectivity using LSER (11) and principal component analysis. The solute's hydrogen bond basicity was shown to be the parameter with the predominant contribution to retention. The corresponding parameter for the chromatographic system *b* describes the specific interaction between a solute hydrogen bond acceptor and the stationary phase as a hydrogen bond donor. While the other parameters differed only slightly between the NP-phases investigated, *b* was strongly phase dependent with large values for the diol- and amino phase and small ones for the cyano phase. In contrast to the results by Li et al. [126], our own findings suggest highest *b* values for the amino phase [128]. The phase dependence of *b* explains the differences in selectivity of several orders of magnitude for polar benzene derivatives between these phases [126]. Salotto et al. [129] summarized that diol and amino columns each preferentially retain hydrogen bond accepting solutes such as esters and ketones vs. dipolar solutes (nitro and nitrile derivatives) when compared with a cyano column. Amino phases retain acidic solutes strongly. In addition to the NP-phases discussed above, Ballschmiter and Wößner [124] identified 55 modified silicas that have been used for NP-LC of PAHs and heterocyclic polyaromatic compounds, pesticides, and other compounds. This offers a wide range of selectivity for the separation of complex mixtures.

4.2.3 Application in EDA

NP-HPLC techniques are predominantly used for the separation of contaminant mixtures extracted from solid phases, particularly airborne particles, soils, and sediments. The latter are the major sink of lipophilic organic compounds in aquatic systems. In all three matrices, lipophilic compounds predominate, which are poorly dissolvable

in RP mobile phases, while excellent group separation using NP-HPLC methods are possible. In some cases, solid phase extracts from water have also been fractionated primarily with NP-HPLC, for example, to isolate estrogenic compounds [130, 131].

In contrast to C18 in RP-HPLC, there is no single predominant stationary phase in NP-HPLC fractionation. Separation techniques based on classical NP-phases such as alumina [132–137] or silica [133, 138, 139] have been used frequently in EDA. However, because of reproducibility problems, for example, due to traces of water, because of potential irreversible sorption of polar compounds [140], and because of limited selectivity, they were replaced more and more by organic modified silica phases. Popular columns for primary fractionation include aminopropyl silica [141–145], cyanopropyl silica [18, 140, 146–158], and cyano-amino-bonded silica [159–163]. No procedure for selectivity-based stationary phase selection has been reported. Which of the phases was selected was linked rather to the preferences of the individual group than to specific requirements of the mixtures to be separated. Generally, all of these phases provide reasonable separation, with amino groups providing greater selectivity to acids and basis. Although not performed in the context of toxicant identification, fractionation procedures for mixtures extracted from crude oils may be helpful for EDA by providing NP-LC group separation techniques for resins, nonpolar compounds, saturated carboxylic acids, phenols, and polyfunctional acids from petroleum [164–166].

Nitrophenylpropyl silica phases were frequently used for the separation of PAHs, often as a second fractionation step, for example, in sediment EDA. The electron-deficient ring system has a high selectivity for large π-electron systems such as in polycyclic compounds [135–137, 153, 154, 157, 167–170]. Scheurell et al. [89] fractionated the nonpolar fraction of drinking water samples with nitrophenylpropyl silica. For the separation of electron-deficient aromatic compounds, particularly PCBs, PCDD/Fs, and PCNs, PYE phases are frequently used in EDA and conventional chemical analysis of sediments and other environmental samples [134, 153, 170–174]. A PYE column separates these compounds according to planarity and the degree of chlorination and allows for a separation of dioxin-like nonortho-chlorinated compounds from nondioxin-like PCBs (Fig. 6). Alternative stationary phases with similar elution characteristics include porous graphitized carbon [154, 175, 176], C_{60}/C_{70} fullerenes bound to polystyrene divinylbenzene [177], and C18 dispersed PX-21 activated carbon [178]. Porous graphitized carbon was also applied quite successfully for the separation of nonylphenol isomers [179].

4.3 Hydrophilic Interaction Chromatography

In the early 1990s, a novel NP-separation technique for very hydrophilic compounds was developed called HILIC [69]. Strongly hydrophilic stationary phases are combined with mobile phases of ACN/H_2O or MeOH/H_2O. The stationary phase attracts water as the more polar component of the mobile phase, creating a stagnant aqueous layer on the surface [180]. The major retention mechanism is the partitioning of the analyte between this layer and the more hydrophobic bulk eluent. Retention

retention time

Fig. 6 Separation of PCBs, PCNs and TCDD on a PYE column

increases with decreasing water content. The elution order is more or less opposite of elution in RP-HPLC. HILIC is particularly useful for very hydrophilic compounds that are not retained on RP columns. Stationary phases used in HILIC include nonmodified silica together with aminopropyl-, amide-, polysuccinimide-, and diol-bonded silicas and cyclodextrins. In addition, zwitterionic phases are increasing in popularity. They are based on a grafted polymeric layer with sulfoalkylbetaine zwitterionic moieties of 3-sulfopropyldimethylalkylammonio inner salt as functional groups on a wide-pore silica with a strong ability to bind water to the surfaces [180]. Zwitterionic HILIC has been applied, for example, for the separation of shellfish toxins [181], aminoglycosides [182], and morphine derivatives [183]. Although a first review paper addressed separation efficiencies in HILIC [184], the mechanistic understanding seems to be too limited to provide classifiers for structure elucidation in EDA at this stage. At present, the applicability of HILIC for fractionation purposes is also limited due to the restriction to analytical dimensions of many commercially available HILIC columns. Thus, HILIC has not been applied so far in EDA. However, with increasing focus on polar toxicants in EDA and with technical progress, HILIC may become a relevant tool for EDA in the future.

4.4 Size Exclusion Chromatography

Another special case of liquid chromatography is SEC or gel permeation chromatography (GPC). This technique tries to minimize enthalpic interactions between

the stationary phase and the target molecules by using combinations of stationary and mobile phases that allow an almost complete partitioning into the mobile phase. The stationary phase is usually composed of hydrophilic- or lipophilic-reticulated organic polymers or minerals with pore diameters of 4–500 nm. Under local equilibrium, the driving force of separation is confinement entropy [185]. The analytes are separated according to their hydrodynamic molecular size, since the porous structure of the stationary phase allows small molecules to penetrate a bigger volume of the mobile phase including the interstitial volume external to the pores and the pore volume, while big molecules are restricted to the interstitial volume only [185]. This technique is frequently applied in EDA studies as a first separation step to remove elemental sulfur, long chain aliphatic compounds, humic substances, or biological macromolecules such as lipids and proteins [110, 111, 149, 150, 186–194]. SEC was also applied for toxicity-directed fractionation of tannery wastewater with regard to molecular weight [5], as well as for sediment extracts [151] and airborne particles [152]. Alternative methods to remove or fractionate large molecules from complex mixtures are steam distillation, for example, applied for mussel extracts [195, 196], techniques based on dialysis [191, 197–200], and ultrafiltration [201]. In specific cases, SEC can also separate closely related target analytes with different molecular sizes such as polychlorinated naphthalenes, biphenyls, dioxins, and furans [153, 172, 202]. Heisterkamp et al. applied SEC for fractionation of sewage treatment plant effluent extracts in order to isolate estrogenic compounds [203].

4.5 Affinity Chromatography

Affinity chromatography can be defined as a liquid chromatographic technique based on highly specific biological interactions such as those between a receptor and a ligand. Since often the first step in the sequence of events that may eventually lead to an adverse affects of chemicals on biota is binding to a receptor, affinity chromatography is a promising tool in EDA. It has been presented under the term bioresponse-linked instrumental analysis [204] and is based on the concept that key processes of adverse effects of environmental contaminants can be determined at the molecular and subcellular level. Hyphenated technologies using receptor affinity chromatography followed by LC-MS/MS for the isolation and subsequent identification of estrogenic compounds have been suggested [205, 206]. Affinity chromatography is discussed in more detail in Chapter 6 [207].

4.6 Planar Chromatography

Planar chromatography, also known as TLC, represents a niche in LC using a liquid mobile phase that migrates by capillary action through a solid stationary phase fixed

on a rectangular plate. This technique has been also automated (automated multiple development AMD-TLC) and used in EDA [101, 208–210]. The technique offers the possibility to detect toxicity directly on the TLC plates, for example, using luminescent bacteria [211]. However, low resolution of TLC and the difficult recovery of the fractions from the TLC plate prevent the wider use of this technique in EDA.

4.7 Complex Fractionation Schemes

While one single fractionation procedure may suffice for EDA of relatively simple mixtures, for example, of transformation products of a well-defined mother compound [115–117], in the case of complex environmental mixtures several separation steps with orthogonal selectivity are often required. The combination of different selectivities also helps to identify the isolated compounds by providing additional classifiers. Multistep procedures have been used especially for sediment extracts, combining different NP-fractionation steps often with RP fractionation. Durant et al. [152] applied a four-step fractionation procedure using three cyanopropyl-based NP-fractionation steps followed by SEC to separate human lymphoblast mutagens in urban airborne particles. Powerful separation procedures have been developed for Ah-receptor-mediated toxicants in sediment extracts. After separation of nonpolar aromatic compounds from the extract with gravity column NP chromatography on alumina, they were separated by NP-HPLC on nitrophenylpropyl silica according to the size of the aromatic ring system, yielding a first fraction containing compounds with two aromatic ring systems (diaromatic fraction) including PCBs, PCNs and PCDD/Fs, and several PAH fractions [134–136, 153]. The diaromatic fraction was subjected to NP-HPLC using a PYE column exploiting the electron–donor–acceptor interactions of PCBs, PCNs, and PCDD/Fs caused by the different degree of halogenation and planarity. This allowed a group-specific separation of less toxic nonplanar from the dioxin-like coplanar PCBs. Later, eluting fractions coeluted with PCNs and PCDD/Fs with four and more chlorine atoms. A separation of both groups became possible with SEC with PCDD eluting before PCDF and PCNs. PAH fractions were subfractionated with RP-HPLC on C18 followed by RP-HPLC on PYE exploiting its specific selectivity for PAHs [135]. Automated HPLC methods with coupled columns have been developed for the separation of Ah-R-mediated toxicants. Zebühr et al. [143] suggested a system applying a combination of an aminopropyl column with a carbon column. After nonpolar aliphatic and monoaromatic compounds, PCBs and PCDD/Fs were eluted from the aminopropyl column and subsequently concentrated and fractionated on the carbon phase applying a back-flush approach for the strongly adsorbing coplanar PCBs and PCDD/Fs. In a later study, the same group replaced aminopropyl with nitrophenylpropyl silica for better reproducibility and the carbon column with a PYE column [170]. Aiming at an automated fractionation of major groups of sediment-associated polycyclic aromatic compounds, Lübcke-von Varel et al. [154]

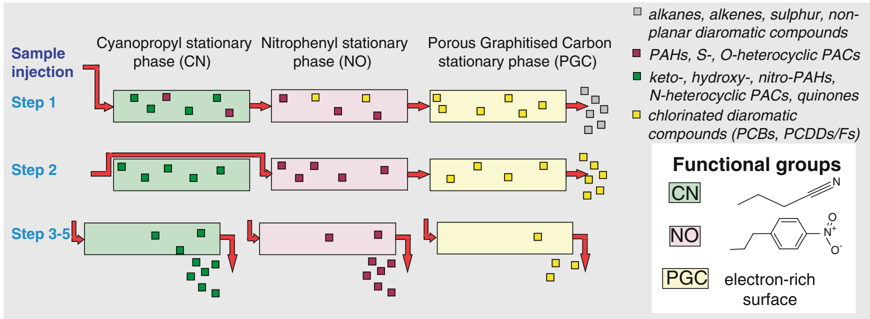

Fig. 7 Multistep fractionation procedure for polycyclic aromatic compounds [154]

developed a system using three-coupled NP-HPLC columns (Fig. 7). Polar compounds are trapped on a cyanopropyl column, while nonpolar compounds are eluted toward a nitrophenylpropyl column where PAHs are retained. Aliphatic and mono- to diaromatic are eluted quickly to a graphitized carbon column retaining PCBs, PCNs, and PCDD/Fs, while nonpolar aliphatic and monoaromatic compounds leave the system almost nonretained. Sequentially fractionated elution of the three columns provides 18 fractions containing major groups of sediment contaminants. The method has been applied in several studies quite successfully [155–157].

The approach of pumping a complex mixture through several stationary phases, trapping specific groups of compounds and leaving others almost unretained, followed by sequential fractionated elution of the columns is limited to NP-HPLC. For RP-HPLC, the differences in selectivity between available phases are much smaller since hydrophobicity is always the major driving force of separation. Thus, two- or multidimensional automated fractionation procedures based on RP-HPLC have not been used in EDA so far. However, there are analytical scale approaches that may offer opportunities for future developments.

Two-dimensional RP-HPLC approaches may be classified into two different groups [212]: (a) Comprehensive two-dimensional LC providing the transfer of all or a representative portion of all analytes composing the initially injected sample to all further separation modes and (b) heart-cutting methods providing only the transfer of a small number of fractions containing the analytes of interest from one separation mode to the next.

In comprehensive two-dimensional RP-HPLC, discrete fractions of mobile phase elute from first dimension of separation on regular intervals to the next separation dimension throughout the entire multidimensional run. The interval in which a fraction is to be transferred from one separation dimension to the next is determined according to the width of the peaks eluting from the earlier separation dimension. Venkatramani and Zelechonok pointed out the importance of tuning a two-dimensional system in order to minimize cross correlation between the two dimensions [213]. While the first dimension RP-chromatography provides a separation according to hydrophobicity, in the second dimension much higher solvent strength is applied to

provide separation according to more subtle differences in component interaction with the two phases to dominate the secondary column retention. The authors studied combinations of C18 with other C18, amino and cyano columns. Even for similar columns (both C18) in the second dimension polarity differences between the columns dominated the retention, rather than lipophilicity of the analytes.

A nice example for a heart-cutting procedure was provided for the isolation of mutagenic 3-nitrobenzanthrone (NBA) from diesel exhaust particulate matter extracts by three-dimensional HPLC [214]. Compounds from a methanolic extract were concentrated and separated on a nitrophenylethyl column using an additional pump to increase the water content. The NBA-containing fraction was concentrated for a second time on a C18 column again after adding additional water. The final separation step was performed on a PYE column with a strong retention of NBA, which could be eluted only with dichloromethane. Another example is the determination of hydroxybenzo[*a*]pyrene isomers using a column switching HPLC approach with a primary fractionation on an alkylamide column trapping the fraction of interest with additional water on C18 and subsequent separation on a β-cyclodextrin stationary phase [215].

Automatic combinations of NP- and RP-HPLC approaches are very rare, although not impossible. Tian et al. [216], for example, provided an innovative approach of a two-dimensional LC system combining NP- and RP-LC. After NP-LC on a cyano column, the solution was trapped in a heated vacuum evaporation loop where solvents were completely removed. Subsequently, RP-solvent dissolved the residue in the loop for subsequent RP-LC on C18. The method was applied to PAHs resulting in good recovery of lowly volatile compounds but significant losses of small PAHs and thermolabile compounds such as anthracene.

An interesting automatic column-switching HPLC system combining HILIC with RP-LC for the separation of compounds with a broad range of polarity has been presented by Wang et al. [217]. Very polar compounds can be hardly separated with RP-LC, since highly aqueous mobile phases may cause several problems such as inadequate phase wetting and thus the risk of folding down of alkyl chains and expulsion of the eluent from the pore space. Lipophilic compounds are not retained in HILIC. Thus, a combination of both provides great opportunities. The two techniques show highly orthogonal selectivity and use compatible mobile phases. The procedure starts with separation in the HILIC mode flushing non-retained lipophilic compounds to a specific interface, where they are mixed with a highly aqueous transfer solvent for trapping on a short C18 column and subsequent RP-LC.

4.8 Combination of Fractionation Procedures and Biotesting

While the direct connection of chromatography to chemical detection, for example, by mass spectrometry is a common procedure, direct connections to effect detection are rare. They may help to accelerate the procedure and provide direct links between toxic effects and detected compounds. Automated links can be provided by collecting

fractions, for example, in microtiter plates that are subjected to biotesting without further concentration or solvent exchange. This holds for the ToxPrint approach by Bobeldijk et al. [218, 219] who developed an online procedure linking RP-SPE, RP-HPLC fractionation and collection on a microtiter plate with UV-detection, and subsequent genotoxicity testing. Genotoxic fractions were thus selected for chemical characterization/identification by tandem mass spectrometry. An alternative approach applied integrated enzyme inhibition as a toxicological endpoint directly into the chromatographic procedure [220]. After separation on C18, the enzyme acetylcholinesterase was added before the mixture entered a reaction coil where Ellman's reagent was added for colorimetric detection of enzyme activity. In order to avoid band broadening in the reaction coils, the mobile phase flow was segmented into small reaction volumes with air bubbles at regular time intervals that were removed again by a bubble filter before photometric color detection.

5 Gas Chromatography

Gas chromatography (GC) is the predominant chromatographic tool to analyze mixtures of compounds that can be evaporated at temperatures up to about 400°C without destruction of the chemical structure. Thus, GC is a good separation tool for many non- and medium-polar environmental compounds, while highly polar compounds either need a derivatization step to render them sufficiently volatile or need to be separated by liquid chromatography. In EDA, GC may be used for both analytical and fractionation purposes.

5.1 Characterization and Prediction of Retention by QSSR

There are several approaches to characterize and predict retention in GC, which may help to select appropriate stationary phases and to use retention as a classifier in structure elucidation. These include boiling point-based estimates, QSRR based on analyte structural descriptors from calculation chemistry, and LSER.

5.1.1 Boiling Points

Boiling points (*bp*) have been presented as an estimate for retention indices [221, 222]. Eckel and Kind used Lee retention indices (*RI*) of a broad range of compounds separated on 95% methylsiloxane and 5% phenylsiloxane to derive a correlation between retention and the boiling point (19) (Fig. 8) [222].

$$bp = 0.98 \cdot RI + 24.36. \tag{19}$$

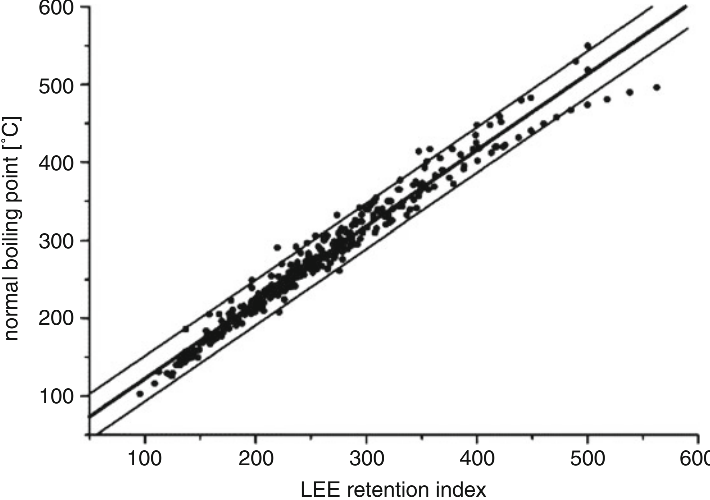

Fig. 8 Relationship between boiling points and Lee retention index for 370 compounds (from [222], with permission)

The authors concluded that an unknown compound with a particular *RI* will have a boiling point of $RI - 10$ (°C) to $RI + 50$ (°C). Thus, suggested structures with boiling points outside that range may be rejected. Schymanski et al. [223] demonstrated for the example of 14 constitutional isomers of formula $C_{12}H_{10}O_2$ that using this method five compounds could be excluded. Thus, the method is an appropriate tool to eliminate candidates with very different retention behavior. For more similar compounds, more sophisticated prediction techniques are required.

5.1.2 Linear Solvation Energy Relationships

Similarly to HPLC, LSER using the Abraham equation can be applied to GC by replacing the cavity-related term vV in (11) by the logarithmic gas-to-hexane partition coefficient L [224].

$$\mathrm{SP} = aA + bB + sS + eE + lL + c. \tag{20}$$

L is dependent on the intristic volume V_{I}, dipole moment μ, and molar refraction MR_x (21) [4].

$$\log L = 0.16 - 3.25 \cdot V_I + 0.16 \cdot MR_x + 0.053\,\mu. \tag{21}$$

For gas chromatographic systems, isothermal measurements at different temperatures are common. It is important to know that there are only a few columns

with a *b* phase parameter. This means that *B* descriptors, which describe the hydrogen bond acceptor strength, cannot be determined as easily as for liquid chromatography [225, 226].

5.1.3 QSRR Based on Analyte Structural Descriptors

There are many approaches to predict GC retention on the basis of structural descriptors, so some selected examples are included here. Katritzky et al. [227] used a set of 152 chemicals and 37 quantum chemical and conventional molecular descriptors derived with the CODESSA program (COmprehensive DEscriptors for Statistical and Structural Analysis) to identify polarizability and the minimum valency of a H atom, describing the dispersional and hydrogen-bonding interactions between the compound and the stationary phase, respectively. Good six parameter regressions including three additional quantum chemical and two conventional parameters with R^2 of 0.955 were presented. A QSRR developed recently used a data set of 846 compounds and ended up with 15 descriptors to predict retention indices using multiple linear regression [228]. The selection of these 15 descriptors out of 1,497 generated by the software *Dragon* was based on mathematical criteria and not all of them could be interpreted in terms of chromatography.

5.2 *Characterization and Selection of Stationary Phases*

While the major factor determining retention in temperature gradient gas chromatography is the boiling point [222], the specific selectivity of the stationary phase is determined by its ability to undergo dispersive dipole–induced dipole, dipole–dipole, and hydrogen bonding with the analytes. While nonpolar compounds such as alkanes are separated well on strongly dispersive, for example, polymethoxy stationary phases, polar phases are more selective for polar or polarizable (e.g., aromatic) compounds. LSER is the most promising approach to characterize stationary phases in GC, to identify complementary selectivities, and to predict retention-index windows of distinct compounds [see (20)]. Commercially available GC columns cover a reasonably wide range of dipolarity/polarizability *s* and interaction with hydrogen-bond acids *a* (hydrogen bond basicity) [224]. While squalane neither acts as hydrogen bond donor nor as acceptor and shows very small dipolarity/polarizability, 1,2,3-tris(2-cyanoethoxypropane) (TCEP) columns are both considerably dipolar/polarizable and hydrogen-bond basic. Stationary phases with substantial hydrogen-bond acidity (*b*) are so far limited to custom synthesized phases, for example, using liquid organic salts as listed by Abraham et al. [224]. The same authors applied multivariate statistics to unravel similarities and dissimilarities of GC stationary phases. However, it should be considered that system parameters may change significantly with temperature due to the fact that polar intermolecular interactions in general decrease with an increase in temperature.

5.3 Application in EDA

In EDA, GC separation is applied analytically prior to MS analysis and preparatively for fractionation. Retention estimates based on boiling points [222] and on LSER [224] may serve as classifiers in structure elucidation to reduce the number of possible structures. LSER-based column characterization may help to identify the optimal stationary phase for analytical and preparative separation and to identify complementary phases if a two-step separation process is required. Comprehensive two-dimensional gas chromatography applying two stationary phases with different selectivities is used increasingly to separate highly complex environmental mixtures [229, 230].

So far the application of preparative capillary gas chromatography (pcGC) in EDA is rather limited. The major application of pcGC has been the isolation of individual preselected components from complex environmental samples for radiocarbon analysis [231–235] and environmental fate assessment by compound-specific chlorine-isotope analysis [236, 237]. Some groups used pcGC to isolate naturally produced bioactive compounds for structure elucidation [238, 240]. Only recently, first attempts were made to use pcGC in EDA of complex mixtures focusing on the separation of technical mixtures of nonylphenol [239] and genotoxic groundwater contaminants [241]. Fractions are trapped after chromatographic separation either dry at low temperature or in solvent-filled traps [242]. While temperatures to achieve good recoveries depend on the vapor pressure of the analytes when trapped dry, the use of solvent-filled traps helped to establish a procedure that was applicable to a wider range of compounds, which is more useful for EDA. Dichloromethane was identified as a trapping solvent providing good recovery for many compounds.

6 Conclusions

Separation is one of the key issues in EDA and is a prerequisite for biological diagnosis and compound identification in complex mixtures. Separation science and particularly chromatographic science has been very productive within the last century and provided plenty of tools that can be applied in EDA. This includes well-characterized LC and GC stationary phases with different selectivity, tools to predict retention in chromatographic systems, as well as offline and online multi-step separation procedures. A rigorous evaluation and exploitation of the state of the art in separation science is a key to further progress in EDA. Despite the enormous knowledge available, there are still significant research needs to satisfy the separation needs of EDA. These include more comprehensive automated fractionation techniques as well as a better prediction of chromatographic behavior from chemical structure.

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Simultaneous Screening and Chemical Characterization of Bioactive Compounds Using LC-MS-Based Technologies (Affinity Chromatography)

Martin Giera and Hubertus Irth

Abstract The analyst faces a couple of challenges when screening complex mixtures. Over the past decades, several strategies were developed to overcome these problems. The review presented here provides an overview of the different strategies on the integration of separation sciences, mass spectrometry, and bioactivity screening in a single platform to allow the simultaneous screening and characterization of complex mixtures. The applied strategies can generally be categorized into precolumn and postcolumn principles. While the precolumn methodologies mainly include affinity-based screening, the postcolumn strategies can also employ enzyme activity assays. The different subtypes of these philosophies will be discussed and examples for each of the techniques are presented.

Keywords Bioaffinity, LC-MS, Screening

Contents

1 Introduction 120
2 Affinity-Based Screening Technologies 121
2.1 Frontal Affinity Chromatography 122
2.2 Affinity Capturing 124
2.3 (Pulsed) Ultrafiltration-Based Methods 125
2.4 Size Exclusion-Based Methods 127
3 Postcolumn Bioactivity Detection 128
3.1 Off-Line Bioactivity Screening 128
3.2 On-Line Bioactivity Screening 129
3.3 In-Line Bioactivity Detection 134
4 Conclusion 136
5 Perspectives 137
References 138

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W. Brack (ed.), *Effect-Directed Analysis of Complex Environmental Contamination*,
Hdb Env Chem (2011) 15: 119–142, DOI 10.1007/978-3-642-18384-3_6,
© Springer-Verlag Berlin Heidelberg 2011

Abbreviations

Ach	Acetylcholine
AChE	Acetylcholinesterase
AD	Alzheimer's disease
ALIS	Automated ligand identification system
AMQI	7-acetoxy-1-methylquinolinium iodide
BCD	Biochemical detection
EDA	Effect-directed analysis
EGFR	Endothelial growth factor receptor
ESI	Electrospray ionization
ESI-MS	Electrospray ionization mass spectrometry
FAC	Frontal affinity chromatography
hERα	Human estrogen receptor α
hERβ	Human estrogen receptor β
HMQI	7-hydroxy-1-methylquinolinium iodide
HPLC	High performance liquid chromatography
IAM	Immobilized artifical membrane
K_d	Dissociation constant
LC	Liquid chromatography
LC-MS	Liquid chromatography mass spectrometry
MMP3	Human matrix metalloprotease 3
MS	Mass spectrometry
NET	Norethisterone
PAB	Paramagnetic affinity beads
PDA	Photo diode array
SDH	Sorbitoldehydrogenase
SEC	Size exclusion chromatography
SMT	System monitoring trace
SPE	Solid phase extraction
TIC	Total ion chromatogram

1 Introduction

In today's drug discovery environment, complex mixtures are becoming more and more important. As an example, nature is still the most important resource for antibiotic substances since the beginning of the field [1]. However, not only in the field of drug discovery but also and especially in effect-directed analysis (EDA), the analyst is challenged with highly complex mixtures and the need to find the active substance(s) [2, 3]. When complex mixtures such as natural extracts [4, 5], combinatorial libraries [6, 7], or environmental samples [8, 9] are subjected to biological screening, it has always been challenging to identify the substance(s) causing the

biological effect. Complex samples demand some kind of fractionation/separation in order to track back the biologically active compounds, but not only the shear complexity of the samples can be very challenging, fluorescent or UV absorbing substances in mixtures can interfere with the applied biological screening strategies as well. Even if all these obstacles are conquered, the final structure elucidation can still be very difficult [10, 11].

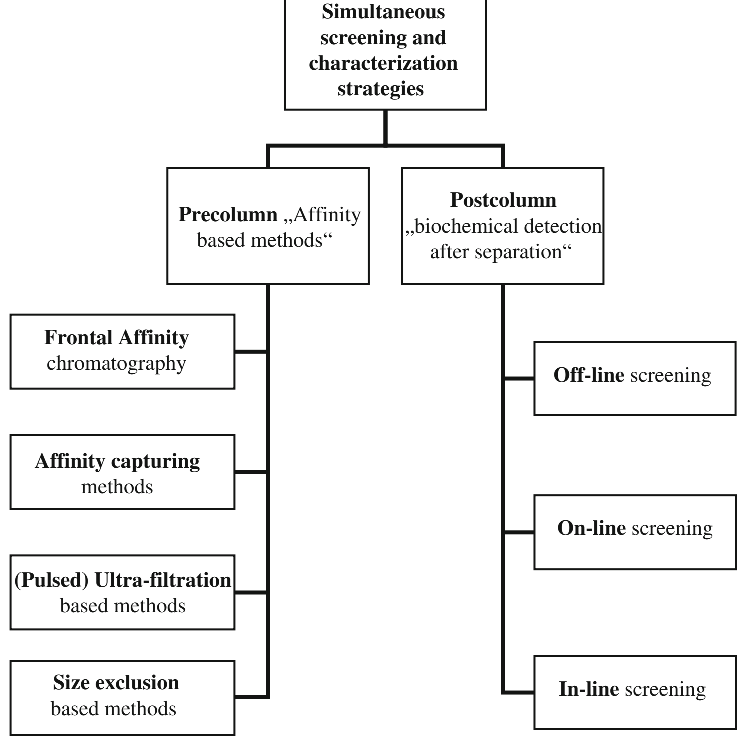
Over the past decades, several strategies have evolved to overcome problems related to complex samples in screening processes and to provide tools capable of fast and reliable substance detection and identification.

The present review describes strategies for the integration/combination of separation sciences, mass spectrometry (MS), and biological screening in order to allow more reliable and faster screening procedures of complex substance mixtures.

In general, the different strategies can be categorized into a precolumn-affinity-recognition and a postcolumn-affinity/activity-recognition mode. The term precolumn-affinity-recognition refers to the fact that bioactive substances in a complex mixture interact with a target enzyme (protein) before they are separated by a liquid chromatography (LC) step (affinity chromatography) [12–14]. In the case of postcolumn-affinity/activity-recognition, the complex mixture is first separated in an LC separation step after which biological activity or bioaffinity is determined for the previously separated substances. The precolumn strategy can be subdivided into (a) frontal affinity chromatography, (b) affinity capturing, (c) (pulsed) ultrafiltration-based methodologies, and (d) size exclusion-based methods. For the postcolumn-approach, three basic principles can be applied for the subsequent biological screening: (a) so called off-line approaches which are based on the fractionation and the subsequent biological testing in platereader formats [15, 16]; (b) on-line systems directly combining the analyte separation and the biological screening in an integrated system [17–21]; and (c) the in-line addition of all necessary biochemical reagents, a hybrid technique between the two aforementioned principles [22]. An overview is given in Scheme 1.

2 Affinity-Based Screening Technologies

The precolumn-affinity principle is based on the noncovalent-binding of a ligand to a (protein) target and the subsequent analysis of the bound protein ligands. As seen in Scheme 1, this precolumn principle can roughly be classified into four different categories. An advantage of affinity-based screening methods is the fact that only limited knowledge of the target is required, since binding and not the function of ligand(s) is determined. This is also a disadvantage of the method, as an influence on the biological function cannot be concluded directly from this type of analysis. Therefore, ligand-binding assays are mainly used in very early stages of drug discovery as they demand subsequent functional assays to confirm the biological relevance of the ligands found. A common problem of all affinity-based screening technologies is nonspecific binding and, consequently, the ability to decide whether



Scheme 1 Schematic overview of hyphenated screening and substance characterization strategies

a substance shows specific or unspecific binding. An example of the affinity screening principle is given in Fig. 1, showing the automated ligand identification system (ALIS), which is based on on-line size exclusion chromatography (SEC) to separate protein-bound from nonbound substances [23]. Direct affinity selection techniques, where the covalent or noncovalent protein–ligand complex is analyzed directly using mass spectrometric techniques, are not described here, for an overview see [23, 24].

2.1 Frontal Affinity Chromatography

In frontal affinity chromatography (FAC), the target protein is immobilized on a stationary phase and a sample containing potential ligands is infused continuously onto the column [25–28]. The order of substance elution is indicative of their affinity to the target protein, with the strong binders having the longest elution times. The eluting compounds can subsequently be identified using mass spectrometry (MS).

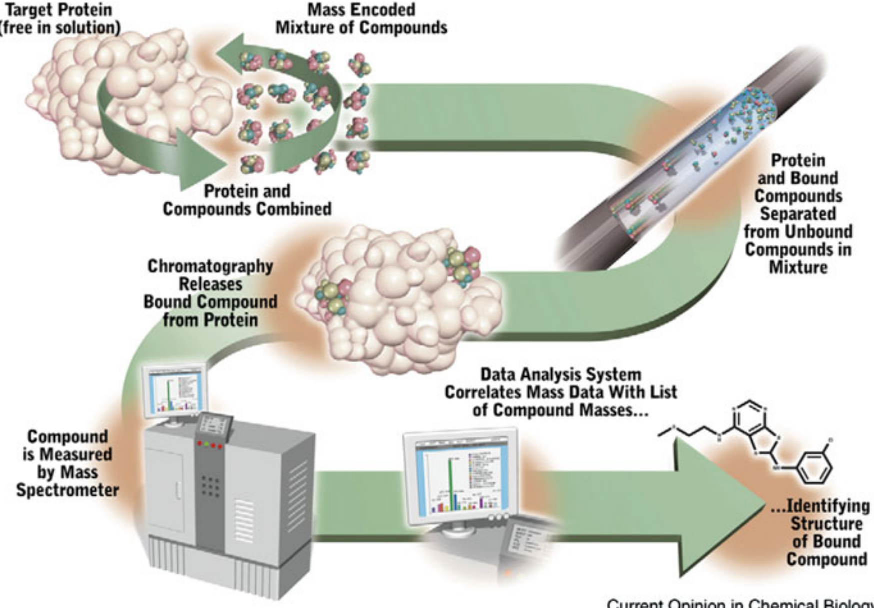


Fig. 1 ALIS as an example for a affinity based screening procedures (taken from [23]).

The coupling of FAC with MS, ultimately allowing the simultaneous determination of bioactivity and structural features, was described by Schriemer et al. [25]. Moreover, FAC also allows the determination of dissociation constants (K_d) [29]. An example of a FAC MS profile is shown in Fig. 2. The target enzyme sorbitol dehydrogenase (SDH) was biotinylated (reacted with biotin) by modification of the primary amine groups. About 15 pmol of the enzyme was immobilized on a column packed with streptavidin beads. K_d values were determined for different ligands.

A very interesting application for highly complex natural extract samples was developed and applied by the group of Xu [31, 32]. To screen for ligands of the epidermal growth factor receptor (EGFR), they used a polyclonal antibody instead of the target protein itself. This could be achieved by immunizing rabbits with an antigen consisting of the known EGFR inhibitor piceatannol and bovine serum albumin. Following collection, purification, and immobilization, the polyclonal antibodies were used as a stationary phase in the FAC MS analysis of complex mixtures.

The critical step in this type of affinity chromatography is certainly the need to modify the protein target in order to ensure a proper immobilization. This of course might alter the protein–ligand interaction. However in the last few years, noncovalent immobilization techniques such as streptavidin/biotin complexes and the immobilized artificial membrane technology (IAM) have proven to be very useful for critical targets, such as membrane-bound receptors [27, 33]. Another problem that can occur is coelution, which severely influences selectivity and accuracy due to ion suppression in the MS detection step.

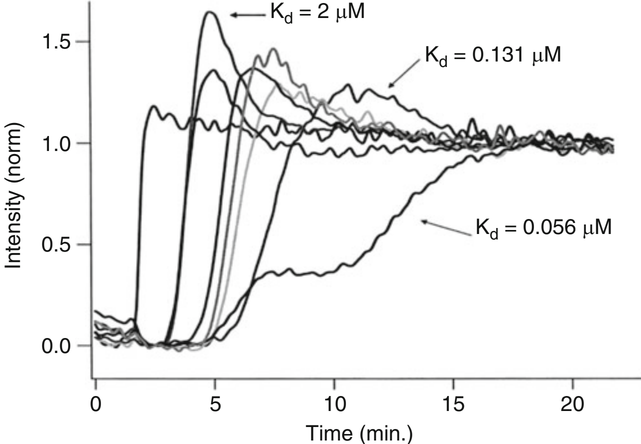


Fig. 2 FAC-MS profiles of compounds eluting from a SDH column as measured using single ion monitoring of molecular ions. A non-binding compound elutes first as the void marker because it shows no affinity to the target. The elution order of the eight components analyzed as a mixture reflects their relative binding strengths, as confirmed by the IC_{50} and K_d values (taken from [30])

2.2 *Affinity Capturing*

Affinity capturing is based on the immobilization of a (protein) target on a solid support (i.e., magnetic beads, gellan beads). The solid support generated in this way can then be incubated in solution phase with substances of interest. This has the advantage that the incubation between target and ligand is carried out under native conditions and is therefore as close to a "real-life" situation as possible. Another advantage of this technique is a significant increase in selectivity when compared with FAC. After incubation, the solid support is thoroughly washed in order to remove unbound substances, and finally the bound compounds can be analyzed using mass spectrometry [34–37]. This principle can also be applied for target identification (so-called protein fishing). In this case, a small molecule ligand is immobilized and bound proteins are subsequently identified using liquid chromatography mass spectrometry (LC-MS) analysis following trypsin digestion [38]. A limitation of the methodology is the nonspecific binding to the affinity sorbent, which results in a decreased ability to detect low-affinity binding substances.

2.2.1 On-Line Example: Development of a Magnetic Bead Protein Affinity LC-MS Assay

In order to combine affinity capturing and the subsequent ligand analysis as an on-line method, Jonker et al. developed a system that was based on the use of magnetic nanoparticles. The use of cobalt(II)-coated paramagnetic affinity beads

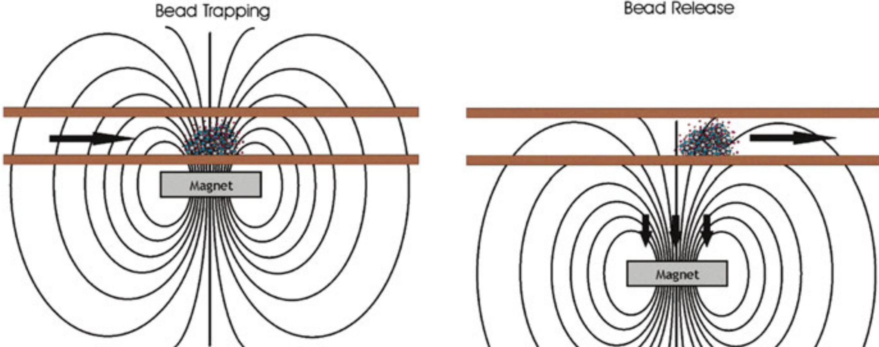


Fig. 3 Magnetic bead trapping principle (taken from [13])

(PAB) allowed the combination of the on-line selection and isolation of protein–ligand complexes with LC-MS analysis [13]. After the His-tagged (poly-histidine tag) protein is incubated with the substance mixture, the protein–ligand complexes are mixed with cobalt(II) PABs. Due to the His-tag, the protein–ligand complexes are bound to the paramagnetic beads and can be retained in a magnetic field of a trapping device, while unbound substances are washed away. Using a pH shift, bound ligands are eluted on-line toward the LC-MS system. In the final step, the protein PAB complexes are flushed to waste by temporarily lowering the magnetic field (see Figs. 3 and 4).

The methodology was applied successfully in a screening assay employing the human estrogen receptor α (hERα). As can be seen in Fig. 5, the assay was able to distinguish binders and nonbinders from a substance mixture.

2.3 *(Pulsed) Ultrafiltration-Based Methods*

Ultrafiltration-based applications employ the isolation of protein–ligand complexes using separation based on molecular mass. Unbound substances are not retained by the ultrafiltration membrane and can be washed away, as their molecular weight is lower than the cutoff of the membrane. The protein–ligand complexes typically have a high molecular weight (>5 kD) and are therefore retained by the membrane. The bound low-molecular weight substances are subsequently dissociated from the protein-target using, for example, methanol or a pH shift and detected using electrospray ionization mass spectrometry (ESI-MS) [39, 40]. Ultrafiltration membranes are normally categorized according to their nominal molecular weight cutoff, that generally refers to the smallest molecular weight species for which the membrane displays more than 90% rejection [41]. The membranes can be made from a wide range of different materials, that is, polyethersulfone, regenerated cellulose, or others.

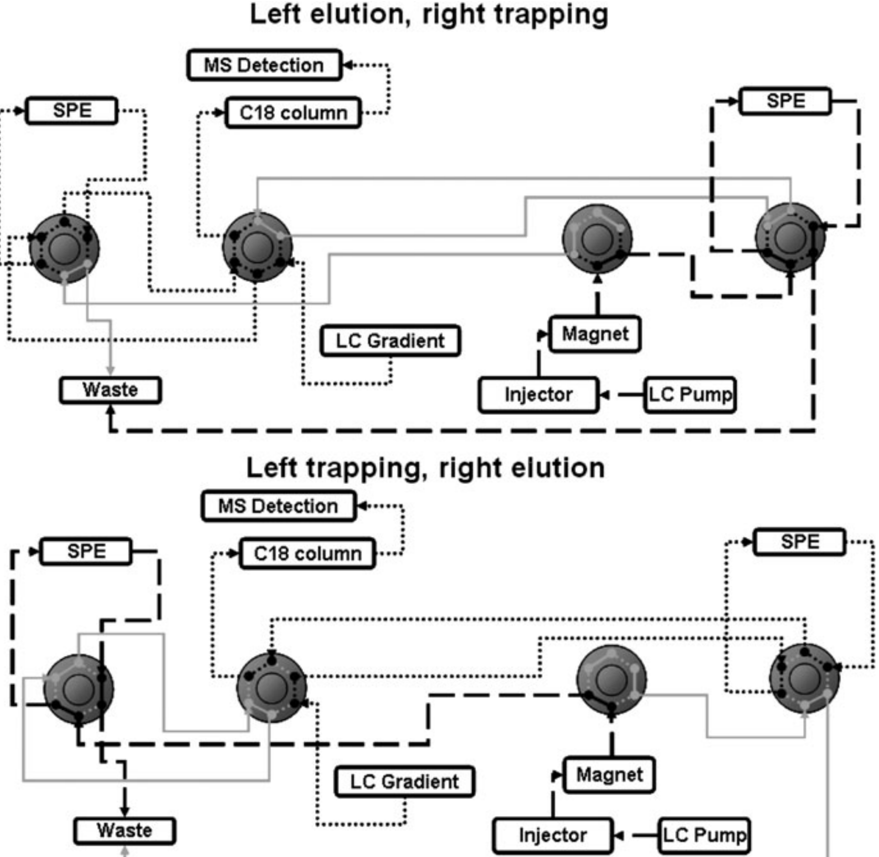


Fig. 4 Flow scheme used for the simultaneous magnetic protein immobilization and solid phase extraction (SPE)-LC-MS (taken from [13]). After injection, the complex of ligand(s), beads and protein is retained by the magnet, while unbound substances are washed away. This is followed by a denaturizing step of the retained protein, eluting bound substances to one of the SPE cartridges. Finally the protein-bound fraction which was retained on the SPE cartridge can be analyzed using LC-MS

Zhang et al. applied this methodology to screen a combinatorial library and cultured cell extracts for ligands of the enzyme DNA topoisomerase I [42]. Comess et al. applied this technology for the screening of ligands interacting with the essential *Streptococcus pneumoniae* bacterial cell wall enzyme MurF. They screened 45 mixtures of approximately 2,700 compounds within a single day and identified about 400 potential hits [43]. Drawbacks of this method are the establishment of an efficient separation of bound and unbound substances; moreover, unspecific binding to the membrane by either the protein or the ligand can be an important issue.

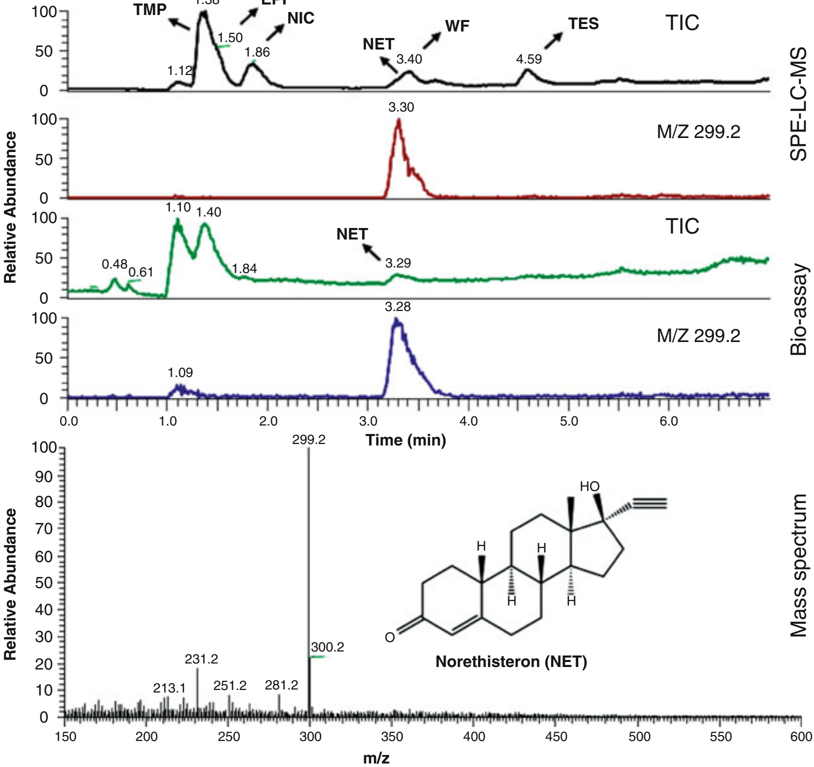


Fig. 5 Screening of a mixture containing norethisterone (NET) (binder) and five other non-binding compounds. The top trace shows a reference experiment showing the separation and detection of all six substances. The ion *m*/*z* 299.2 refers to NET. The bioassay trace clearly shows that only NET is bound to the hERα and is, therefore, being found after affinity capturing and on-line elution (taken from [13])

2.4 Size Exclusion-Based Methods

Size exclusion affinity screening methods rely on the separation of small molecules and protein–ligand complexes using SEC. After the incubation of target and ligand under native conditions, substances of interest are bound to the macromolecular target protein. The protein–ligand complex can easily be separated from nonbinding substances in a mixture using SEC [44]. The protein fraction containing the ligands of interest can then be analyzed using LC-MS for detection and identification of protein–ligands [14, 45]. Muckenschnabel et al. [14] applied this principle based on 96 well SEC plates in their so-called *SpeedScreen* platform. They incubated up to 400 compounds per well with a single protein, allowing the screening of up to 600,000 compounds as mixtures of 400 against one target protein within

a single day. Another prominent SEC-based screening system is the so-called ALIS platform which differs from the above-described *SpeedScreen* principle by the fact that the SEC step is integrated on-line into the screening platform [23]. An additional on-line variant of a SEC method was described by Blom et al. for the screening of a combinatorial mixture for the target protein human matrix metallo-protease (MMP3) [46]. A clear advantage of this method is the relatively simple applicability and the very high throughput that can be generated. Problems include the coelution of substances and/or ion suppression in the applied ion source, therefore, leading to selectivity problems or false negatives.

3 Postcolumn Bioactivity Detection

In this section, the following postcolumn biochemical detection strategies: off-line, on-line, and in-line will be discussed. The postcolumn principle differs from the affinity principle discussed previously in that the initial step is separation of the substance mixture. The column effluent is subsequently analyzed for affinity or bioactivity in either an on-line, off-line, or in-line mode.

3.1 Off-Line Bioactivity Screening

Off-line bioactivity screening is certainly the most widely employed and accepted strategy for the determination of bioactivity in complex mixtures. In the process of bioactivity-guided fractionation, a complex substance mixture is being fractionated while the bioactivity of each fraction is established in an off-line, usually, plate reader-based biochemical assay [47, 48]. A special case is the separation of a mixture of enzyme activity monitoring substances, such as the enzyme substrates, which do not get converted due to enzyme inhibition. After a single substance is incubated in a whole cell system (thereby covering multiple targets), the incubation mixture is, after sample pretreatment, separated to determine changes in the substrate/product pattern compared to control incubations [49–51]. The off-line strategies have several disadvantages. First of all the fraction size is usually in the minutes range and therefore a single fraction can still contain a large number of substances [52, 53]. In other words, long fractionation times do not allow the maintenance of the resolution that is achieved by high performance liquid chromatography (HPLC). Second, fraction drying, which is usually applied, can cause not only logistical problems but mixing problems can also occur when the biochemicals are added to the dried fractions in a microtitre plate. This is especially true for 384 or 1,536 well formats [54]. Therefore, the on-line or in-line combination of substance separation and bioactivity determination has several advantages.

3.2 *On-Line Bioactivity Screening*

3.2.1 Principle

In on-line bioactivity screening, the substance separation is postcolumn directly combined with the biochemical reaction detection (BCD) [55–57]. An example of such a system is shown in Fig. 6. An in-depth review of on-line postcolumn bioactivity screening can be found in [58].

A complex substance mixture (1) is separated over a HPLC column (2). The enzyme solution is added to the effluent stream (3) and the enzyme inhibitors possibly present bind to the protein in the first reaction coil (4). The time required for this step is normally rather short (<30 s) and can be controlled by the volume of the reaction coil (4). To this enzyme/substance mixture, the enzyme substrate or a fluorescent probe are added subsequently (5). Both enzyme and substrate are then allowed to react in a second reaction coil to form a reporter molecule (6) which can be continuously monitored using ESI-MS [59], fluorescence measurement [60], or any other technique applicable for a continuous reporter molecule measurements. The data obtained comprises of the chromatographic data (substance identification) and the simultaneously measured BCD trace (bioaffinity information). Correlation of the chromatogram and the bioactivity trace allows direct identification of a bioactive substance in a complex mixture.

In summary, on-line bioactivity screening has certain advantages over off-line systems, namely in logistics, resolution (with respect to the biochemical detection trace), and in assigning bioactive substances with simultaneous identification using LC-MS-based methods. Drawbacks of on-line bioactivity screening systems are, for example, the basic requirement of short incubation times (<5 min). Longer incubation times are very difficult to establish without a serious loss in resolution. This is basically caused by the extra column band broadening which is a result of the long incubation times [61]. Another critical aspect for on-line bioactivity screening is the establishment of the maximum organic modifier content, as different enzymes/proteins can react very differently to organic modifiers; this value has to be determined for every investigated enzyme. A counter-gradient system to overcome organic modifier related problems was developed by Schebb and Heus et al. [62], making it possible to keep the organic modifier content constant throughout the analyses.

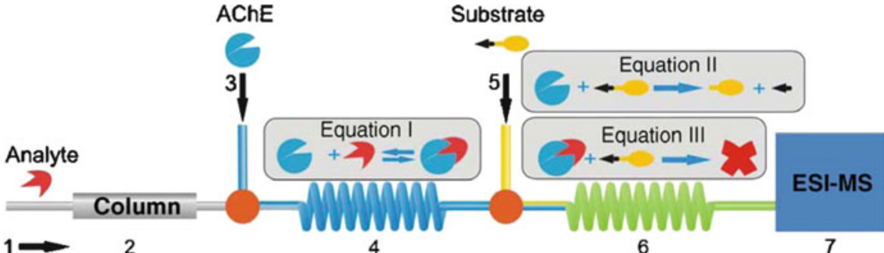


Fig. 6 Example of an on-line continuous-flow system with an ESI-MS read out principle, the numbers are described in the text (taken from [18])

3.2.2 Example: On-Line Acetylcholinesterase Assay

The enzyme Acetylcholinesterase (AChE) promotes the esterification of the neuro-transmitter acetylcholine. AChE is believed to be a promising target for the treatment of Alzheimer's disease (AD) [63]. In principle, there are two readout methods suitable for the on-line monitoring of the AChE activity. These are (A) the fluorescent substrate 7-acetoxy-1-methylquinolinium iodide (AMQI) [57], thereby monitoring the fluorescent product 7-hydroxy-1-methylquinolinium iodide (HMQI) and (B) the direct continuous ESI-MS monitoring of the native substrate acetylcholine (ACh) [18]. Figure 7 shows the screening of a *Narcissus* extract and the on-line detection of an AChE inhibitor. Trace B shows the continuous monitoring of the reaction product choline in the corresponding mass trace m/z 104, the substrate concentration (trace C) and a system monitoring compound (trace D) are monitored simultaneously. To make sure that a negative peak in trace B is related to an inhibition of the AChE, the substrate monitoring trace should show a corresponding positive peak, and moreover the system monitoring trace (SMT) (D) should be unaffected. If the SMT shows a negative peak, this is most likely caused by ion suppression in the ESI source, thereby also influencing the detection of the product choline. As the negative peak in trace B does show a positive peak in trace C and no negative peak in trace D, it corresponds unmistakably to a lower enzyme activity, hence to an inhibition of the target enzyme AChE. As it can be seen from Fig. 7 the negative inhibition peak in the product trace B corresponds to an extracted ion with a m/z value of 288, the substance showing this m/z value could be traced back to be galanthamine, a natural AChE inhibitor known to be present in *Narcissus* extracts [64].

3.2.3 Example: Dual On-Line Estrogen Receptor α/β Assay

In many drug discovery projects, target selectivity is an integral question which has to be addressed. Well-established examples are the human estrogen receptors α and β (hERα and hERβ). De Vlieger et al. described a dual on-line assay based on fluorescence enhancement, allowing the simultaneous assessment of estrogen receptor α and β affinity [65]. This ultimately allowed them to screen two targets simultaneously in an on-line manner and to estimate the target selectivity of active components. A schematic of the described system is shown in Fig. 8.

3.2.4 Miniaturization: A Chip-Based On-Line Assay

In order to minimalize the consumption of expensive enzymes/substrates, de Boer et al. miniaturized an on-line screening assay to a chip format [66]. A microfluidic chip with two microreactors (1.6 and 2.4 μL) for enzyme inhibition and substrate conversion, respectively, was designed (Fig. 9). The system was based on an ESI-MS

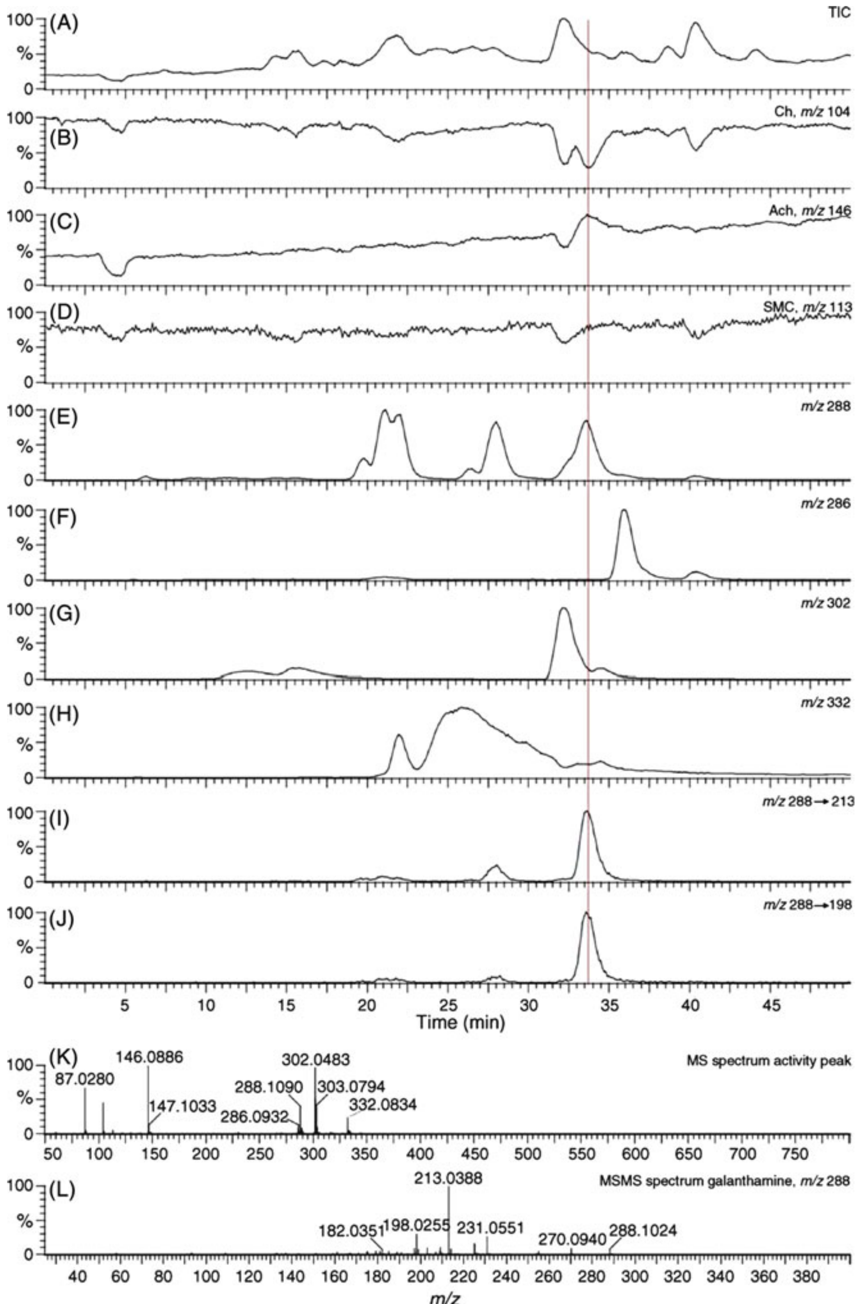


Fig. 7 Analysis of a Narcissus extract by HPLC coupled to the MS-based AChE assay. Trace A, total ion current (TIC); Trace B, product trace (choline) *m*/*z* 104; Trace C, substrate trace (ACh) *m*/*z* 146; Trace D, system monitoring compound (SMC) detected at *m*/*z* 113, E–J, MS traces, K and L MS and MS–MS spectra for the bioactive compound detected at an elution time of 37.5 min (taken from [18])

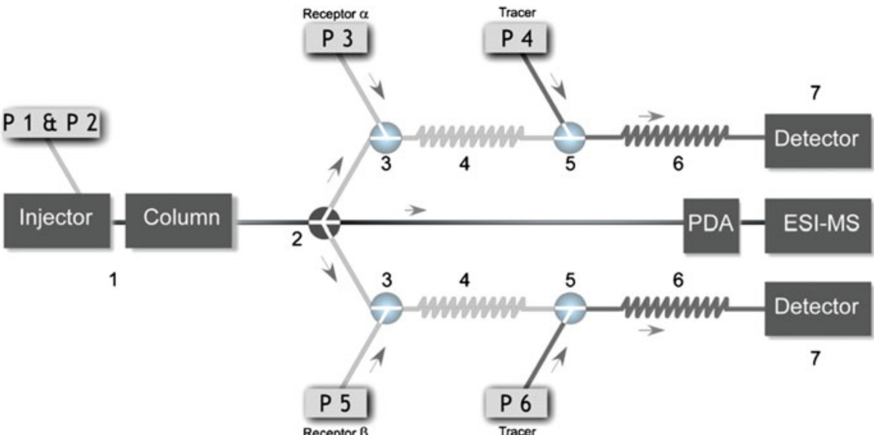


Fig. 8 Schematic overview of a dual on-line assay. (1) sample injection followed by gradient HPLC [P1 and P2], (2) splitting the flow to PDA (photo diode array detector)-ESI-MS and the two receptor affinity detection systems (hERα and hERβ), (3) infusing hERα and hERβ via superloops and reagent pumps [P3 and P5], (4) binding of receptor and potential ligands in reaction coils, (5) infusion of tracer solution via superloops and reagent pumps [P4 and P6], (6) binding of fluorescent tracer to receptor in reaction coils, (7) detection of receptor–tracer complex by fluorescence (taken from [65])

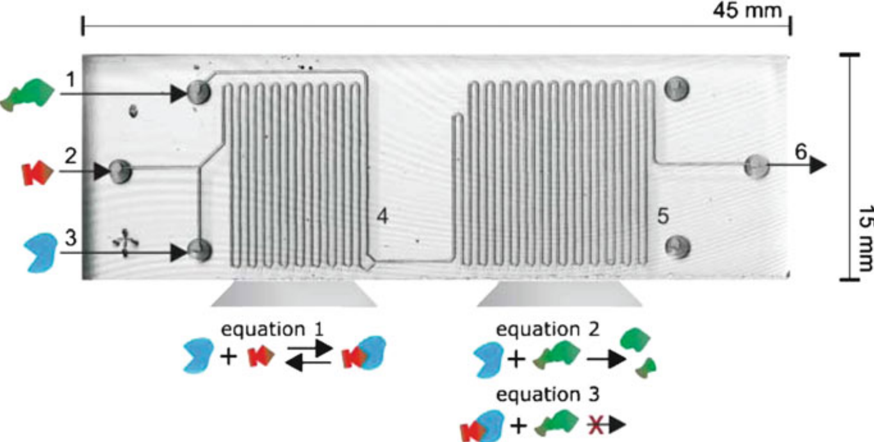


Fig. 9 The microfluidic chip as used for bioactivity screening: (1), substrate solution, (2) LC effluent, (3) enzyme solution, (4) open tubular microreactor with a volume of 1.6 μL, (5) open tubular microreactor with a volume of 2.4 μL, (6) flow towards mass spectrometer. The enzyme hydrolyses the substrate into products (equation 2) if no bioactive compound is eluting from the column. Bioactive compounds present in the eluate bind to the enzyme (equation 1), resulting in a decrease of substrate turnover (equation 3) (taken from [66])

read out and was developed successfully for the cysteine protease cathepsin B. The overall flow rate of the chip-based on-line assay was 4 μL/min. The screening of a green tea extract spiked with antipain [67] and E64 [68], two known inhibitors of cathepsin B, proved the applicability of the developed system to screen for cathepsin B inhibitors (see Fig. 10).

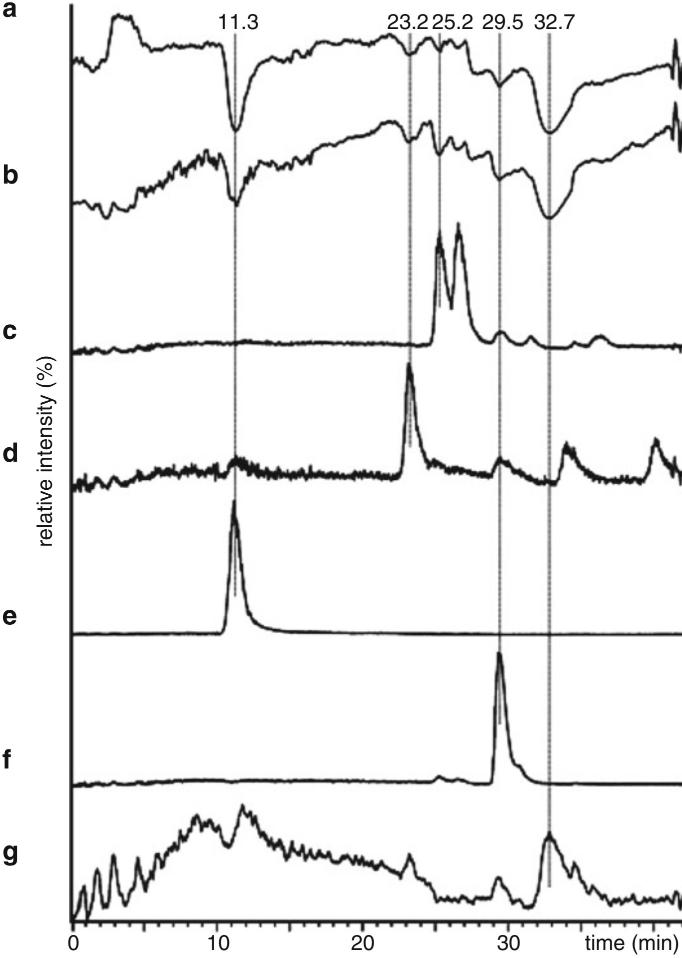


Fig. 10 Screening of a green tea extract spiked with antipain and E64. A, mass chromatogram of product AMC, *m*/*z* 176.1; B, mass chromatogram of product Z-Phe-Arg-OH, *m*/*z* 456.2; C, extracted-ion chromatogram of the negative peak at 25.2 min; D, extracted-ion chromatogram of the negative peak at 23.2 min; E, extracted-ion chromatogram of the negative peak at 11.3 min (E64); F, extracted-ion chromatogram of the negative peak at 29.5 min; and G, extracted-ion chromatogram of the negative peak at 32.7 min (antipain) (taken from [66])

3.3 *In-Line Bioactivity Detection*

As discussed above, the main advantage of on-line bioactivity detection lies in its direct coupling of separation and bioactivity determination, therefore overcoming resolution-related problems and allowing simultaneous data acquisition. To overcome the problem of long reaction times discussed previously, Giera et al. have recently introduced an in-line principle [22]. One part of the column effluent is split into the mass spectrometer allowing substance identification. The other part of the column effluent is directed toward a mixing device where all necessary reagents required for the biochemical assay are added and microfractionated into a 384 or 1,536 well microtiter plate at fractionation times as low as 1.5 s (Fig. 11). After incubation, readout reagents can be added in the same manner (or by other devices) and finally, the readout is performed by a suitable microtiter plate reader. This procedure allows generation of sufficient "biochemical data points" to maintain the resolution achieved by the LC separation, thus not compromising resolution through long incubation times. Furthermore, the in-line addition of all biochemical reagents and its subsequent microfractionation overcomes mixing problems and

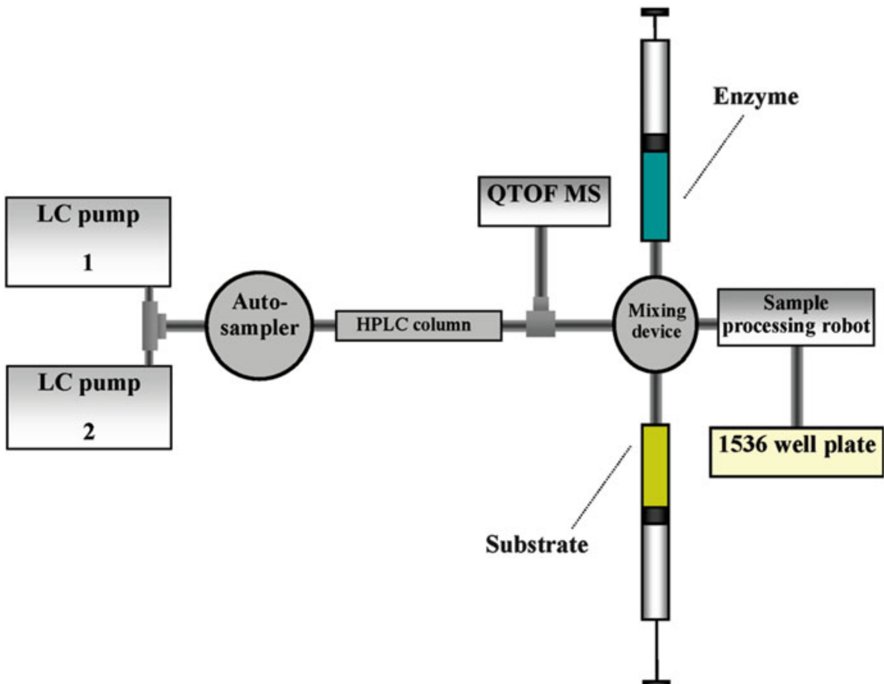


Fig. 11 Schematic overview of an in-line micro-fractionation screening system (taken from [22])

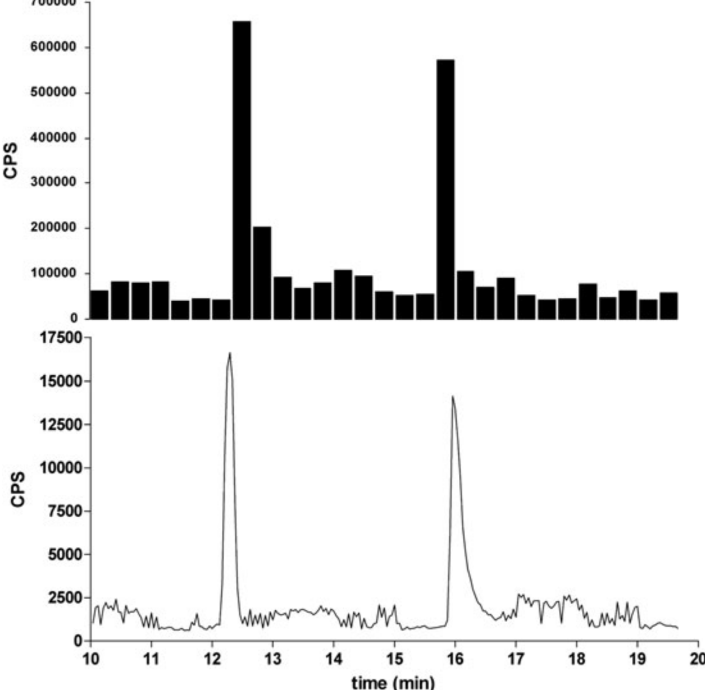


Fig. 12 Comparison of a 96 well off-line fractionation (above, 20 s fractions) and a 1,536 well in-line fractionation (below 2.5 s fractions). Two protein kinase A inhibitors PKI 5-24 (12 min) and staurosporine (16 min) were fractionated. After the addition of a read out reagent the chemiluminescence of each fraction was determined and plotted against the time (taken from [22])

other challenges related to the highly miniaturized 1,536 well format which was applied. A comparison of a traditional off-line and an in-line fractionation into a 1,536 well plate is shown in Fig. 12.

In a study expanding the scope of this principle, Giera et al. also investigated the incorporation of a living organism (*Escherichia coli*) into such an in-line system. As bacterial assays need extremely long incubation times when compared with enzymatic assays (18 h in the cited study), the in-line principle, of course, is the only possibility for the direct coupling of a bacterial growth assay to substance separation/identification using LC-MS. The authors prepared a shotgun mixture of new *N*-alkylated neomycin derivatives by reductive amination with octanal and sodium cyanoborohydride. This mixture could then be separated and screened simultaneously for bioactivity (see Fig. 13). High-resolution MS^n experiments using an ion trap time-of-flight mass spectrometer even allowed full structure elucidation of the *N*-alkyl derivatives formed, requiring only microgram amounts of the substances to be investigated [69].

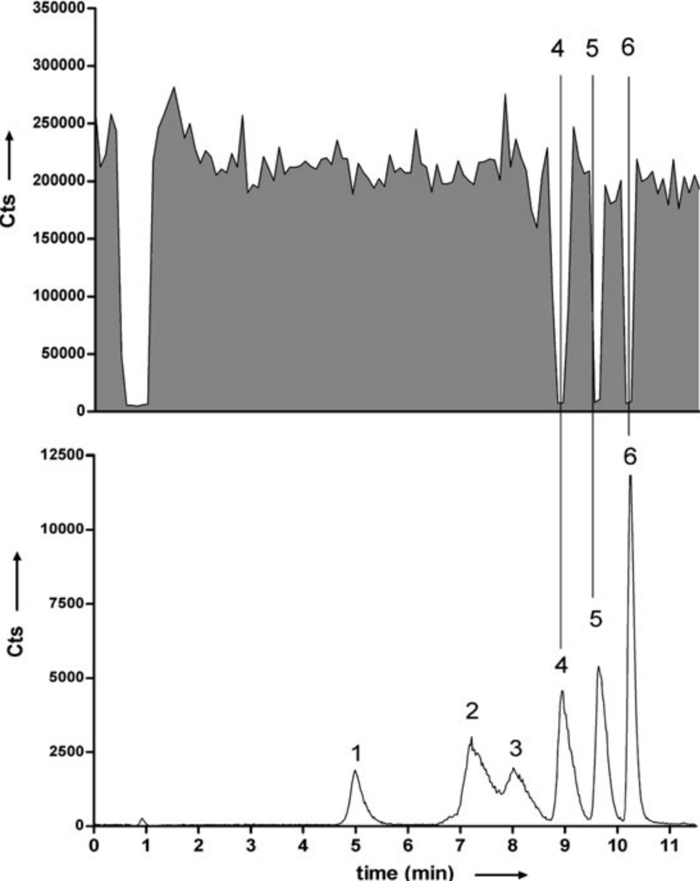


Fig. 13 Simultaneous separation/detection and bioactivity measurement of a neomycin shotgun mixture, employing the described in-line principle described. The numbers refer to the six neomycin regioisomers formed (taken from [69])

4 Conclusion

The combination of bioactivity screening with separation sciences and mass spectrometry results in fully integrated screening platforms. The integration of the bioactivity measurement can either be accomplished as a precolumn or postcolumn step. Precolumn strategies are mainly based on protein–ligand binding (affinity) with a subsequent analysis of the bound ligand(s), largely using ESI-MS. These affinity-based methods are primarily used in very early stages of screening, as no information about the targets functionality can be gathered. The advantage of the affinity-based methods clearly lies in their relatively easy principle and the very high throughput which is possible even when complex mixtures are screened.

Postcolumn strategies for the integration of the bioactivity determination involve so called off-line (traditional approach), on-line, or in-line modes. The on-line coupling shows clear advantages in resolution, assessment of bioactive compounds, and logistics when compared to off-line approaches. A disadvantage of on-line systems, which could be overcome by the introduction of the in-line principle, was the limited incubation period.

5 Perspectives

Overall the methodologies described in this review provide useful additions and alternatives to classical screening processes, especially for the screening of complex substance mixtures. Therefore, their implication into EDA might show several advantages over solely fractionation-based principles and could possibly help to overcome certain problems [70]. For example, the here described dual assay for estrogen receptor binding substances could be used for the screening of xenostrogens [71] in environmental samples, detecting pollutants which show bioaffinity to either hERα or hERβ. Moreover, the in-line screening principle could possibly be used in combination with a cell viability assay to screen for toxic pollutants. Taken together, several enzymatic targets which have been successfully implicated into on-line or in-line screening platforms are also interesting in the context of EDA, namely: AChE, being affected, for example, by organophosphate and carbamate pesticides [72] or the acetylcholine-binding-protein (AChBP) [73] which is a model protein for the nicotinic acetylcholine receptor. For this receptor, it was described that it is affected by the pollutant butyl benzylphthalate [74]. Other target proteins like protein kinases [75] or cytochrome P450s [76] might also be investigated in the context of EDA to screen for pollutants affecting their function. A critical aspect in relation to the implementation of on-line or in-line technologies certainly is the toxicants' amount found in crude environmental samples. Hence, it would be advantageous to combine on-line or in-line-based technologies with fractionation-based enrichment and classification of environmental samples, in order to guarantee toxicant concentrations displaying detectable biological effects. In conclusion, the here presented on-line and in-line screening technologies especially in combination with already established fractionation schemes [77], could be helpful to chase and identify bioactive pollutants, their metabolites, or degradation products in environmental samples.

The future challenges for postcolumn screening technologies certainly lie in the integration of more complex biological systems, such as fungal or human cells or G-protein-coupled receptors. A critical factor for the implication of the aforementioned targets in postseparation assays is without doubt the organic modifier content needed for substance separation. High temperature chromatography as well as supercritical fluid chromatography might be alternatives to overcome this problem. Moreover, it will be very interesting to see if chemical synthesis steps can also be integrated into such systems and if on-line and in-line screening approaches will find useful applications in the field of EDA. This might particularly be the case in

EDA-based screening campaigns aiming to identify metabolites or degradation products of known pollutants in environmental samples.

Acknowledgments The work of the author M. Giera was supported by the German Academic Exchange program (DAAD).

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Advanced GC–MS and LC–MS Tools for Structure Elucidation in Effect-Directed Analysis

Pim E.G. Leonards, Rikke Brix, Damià Barceló, and Marja Lamoree

Abstract An important step in effect-directed analysis (EDA) is the identification of the compound(s) causing the biological response of the bioassay. The combined use of gas chromatography (GC) and liquid chromatography (LC) with mass spectrometry (MS) is a powerful, complementary approach for identification of unknown compounds in EDA. In the last decade, MS techniques have evolved considerably with respect to high sensitivity scanning and non-target screening. These new techniques, often with high mass resolution, generate large amounts of data, making the evaluation of the data for further prioritization and selection of the peaks of interest a challenging task. The development of LC–MS strategies for structure elucidation of unknown compounds requires a major effort, as current LC–MS libraries are very limited. Comprehensive two-dimensional GC (GC × GC) coupled to low-resolution rapid-scanning MS is an established technology for the separation and identification of compounds in complex mixtures. However, to enable the empirical formula assignment of unknown compounds, it is required that GC × GC is combined with rapid-scanning accurate mass spectrometers.

Keywords EDA, GC × GC, GC–MS, Identification, LC–MS, LC–MS, Mass spectrometry, Structure elucidation

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W. Brack (ed.), *Effect-Directed Analysis of Complex Environmental Contamination*, Hdb Env Chem (2011) 15: 143–166, DOI 10.1007/978-3-642-18384-3_7,

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Contents

1 Introduction 144
2 Mass Spectrometry 145
3 Gas Chromatography Coupled to Mass Spectrometry 148
4 Comprehensive Two-Dimensional GC (GC × GC) 149
5 Structure Elucidation in EDA by Gas Chromatography Coupled to Mass Spectrometry 151
5.1 Deconvolution of Peaks 151
5.2 Elemental Search Approach 153
6 Structure Elucidation in EDA by Liquid Chromatography Coupled to Mass Spectrometry 153
6.1 Peak Detection 154
6.2 Spectral Database Search 154
6.3 Generation of Elemental Composition 155
6.4 Chemical Databases 155
7 Examples of Non-target GC–MS and LC–MS Screening of Environmentally Relevant Samples 155
7.1 Non-target GC–MS Screenings in EDA 155
7.2 Non-target LC–MS^n Screenings in Natural Samples 157
7.3 Effect-Directed Analysis (EDA) Employing LC–MS/MS 159
8 Conclusions and Future Challenges 162
References 162

1 Introduction

In effect-directed analysis (EDA), a biological response is used to direct the chemical analysis of a complex environmental sample. Active samples are fractionated and the fractions are re-tested by the bioassay to determine active fractions. Active fractions are usually further fractionated (using a different fractionation method) and biologically/toxicologically tested. The active sub-fractions are then analysed using coupled chromatographic methods to identify the key toxicants and, if applicable, are confirmed by bioassays (Fig. 1). Often, chemical identification is a process of comparing the experimental data (e.g. the mass spectra) of the unknown compound with the properties of compounds that are included in databases. The identification step is rather challenging as the fractionated sample often still contains a multitude of compounds. Gas chromatography (GC) in combination with mass spectrometry (MS) is frequently used for the structure elucidation of unknown compound(s), with the advantage that large compound libraries can be searched for the identification of the unknowns (e.g. Wiley, NIST). Within the last decade, liquid chromatography coupled with high-resolution mass spectrometry (LC–MS), with the ability to perform mass scans with high sensitivity has become available. This has led to the possibility of performing non-targeted screening of a wide spectrum of compounds, including those that are polar and thermo-labile. However, due to the different ionization patterns obtained with different interfaces, there are so far no substantial MS libraries available as yet and it is up to the individual researcher to deal with the large amount of data generated from non-target screening using LC–MS.

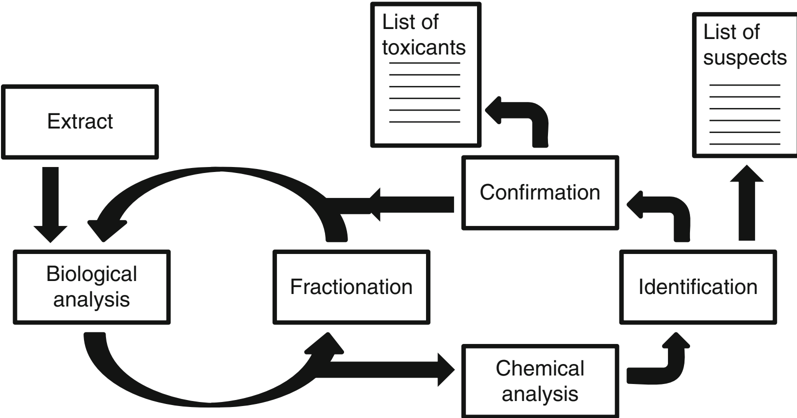

Fig. 1 Effect-directed analysis process including the chemical analysis and identification phase of the toxicants

Other advanced techniques that have evolved in the last decade are comprehensive two-dimensional GC (GC × GC) in combination with mass spectrometry, and high resolution MS coupled to GC. Both techniques also generate large amounts of data. GC × GC software is now available to streamline the quantification and identification process. In analogy, comprehensive two-dimensional LC (LC × LC) has been described, but this technique is still very much in its infancy.

In this chapter, the identification of unknown compounds in EDA extracts by coupling GC or LC to mass spectrometry for the structure elucidation of unknown compounds will be addressed. The EDA concept, the bioassays involved, and the fractionation techniques before the identification process are described in [1–3].

The next sections start with an overview of mass spectrometric techniques. Next, the strategies for identification with GC–MS and LC–MS are described, and the use of mass spectral and database libraries is discussed. This chapter will end with a number of examples of EDA studies.

2 Mass Spectrometry

The history of mass spectrometry dates back to the late 1800s. An overview of the most common used spectrometers is shown in Table 1. The first mass spectrometers were developed by Sir J.J. Thomson [4] in the first decade of the nineteenth century and later Dempster in 1918 [5], closely followed by Aston in 1919 [6]. Mass spectrometers can be divided into integrating and non-integrating analysers, where the integrating analysers detect all ions in a specified range and non-integrating only detect previously specified ions. The time of flight (ToF), ion cyclotron cell (ICR) and Orbitrap are all integrating analysers and the magnetic sector, quadrupoles and

Table 1 Overview of characteristics of mostly commonly used mass spectrometers in environmental chemistry

Mass spectrometer	Dynamic linear range	Mass accuracy	Mass resolution
Magnetic sector	10,000	1–2 ppm	100,000
Single quadrupole	10,000	100,000 ppm	4,000
Triple quadrupole	10,000	100,000 ppm	5,000
Quadrupole ion trap	1,000	100,000 ppm	7,000
Linear ion trap	10,000	50–200 ppm	1,000
Time of flight	100	5 ppm (with lock mass)	15,000
FT–ICR	>5,000	>1 ppm	500,000
Orbitrap	>5,000	5 ppm 1–2 (with lock mass)	200,000

The values in the table are only guiding and vary depending on the model and company of the mass analyser. Recently, triple quadrupoles with high mass accuracy have been developed

ion traps are non-integrating [7]. The different mass spectrometers will be described briefly below.

The magnetic sector mass spectrometer was the first commercial mass spectrometer. It typically consists of a curved tube, where a magnetic field bends the trajectories of the ions as they pass through the mass analyzer, according to their mass-to-charge ratios. Lighter ions are deflected more and heavier ions are deflected less. It is, thus, possible to choose a field strength which only allows a specific m/z to pass through the tube to the detector [8]. In environmental research, the sector instrument is regularly used for target analysis (e.g. dioxins), and occasionally for the structure elucidation of unknown compounds. The magnetic sector MS has the advantages of low detection limits (low fg range) and accurate mass. The detection limit is, however, dependent on the unit of resolution and is normally in the range of 100 pg for full scan mode and 10–100 fg for selected ionization mode (SIM). The instrument is more complex than quadruple MS, and can more easily be contaminated than quadrupole instruments and is costly. The double focusing instrument with high resolution is able to separate ions with small mass differences. This characteristic can be used to elucidate the possible elemental composition of unknown compounds, which however is not often used in environmental chemistry probably because of the relative high limit of detection (LOD) in full scan mode.

The first use of a linear quadrupole mass filters was reported by W. Paul et al. in 1954 [9]. The quadrupole consists of four parallel metal rods. Each opposing rod pair is connected electrically and a radio frequency voltage (RF) is applied between one pair of rods and the other. One pair of rods are charged positively and the other negatively, causing the ions to oscillate. A direct current voltage (CV) is then superimposed on the RF voltage, moving the ions towards the detector. By choosing the correct voltages, only the desired m/z will pass all the way through to the detector [10]. The linear quadrupole coupled to GC or LC is the most used mass spectrometer in environmental research and mainly applied for target analyses using selected ion monitoring. The sensitivity of this MS is decreased when the detector is running in scan mode. The latest generation of linear quadrupole can operate in both selected ion and scan mode at relative high sampling frequency (e.g. 50 Hz).

By now the most common configuration using quadrupoles is the triple quadrupole mass spectrometer, which is a linear series of three quadrupoles. In this instrument, the first and third quadrupoles are used as mass filters, and the middle quadrupole is employed as a collision cell. This collision cell is an RF-only quadrupole (non-mass filtering). This allows for the selection of a precursor (or parent) ion in the first quadrupole, fragmentation of the ion in the collision cell and filtering of the correct product (or daughter) ion in the third quadrupole. This process is called multiple reaction monitoring (MRM) or selected reaction monitoring (SRM) and leads to an increased selectivity compared to the single quadrupole [8]. The QqQ instruments are easy to use and with fewer problems with interfering compounds and matrix than single quadrupole MS. The main advantage for the structure elucidation of unknown compounds is the ability to determine the relationship between the parent and daughter ions which can provide information on substructures of the compound.

Quadrupole ion trap mass spectrometers, originated by W. Paul, is also called the Paul Trap [11, 12]. It is a three-dimensional version of the linear quadrupole and consists of a doughnut-shaped ring electrode and two end-capped electrodes. Ions can be stored inside the device in a stable trajectory for seconds to minutes, but by increasing the RF on the ring electrode, the trajectories of ions of increasing m/z become unstable and the ions are expelled from the trap towards the detector [7]. The ion trap detector (ITD) is easy to operate, the costs are relative low, the ion source is easy to clean and it is possible to perform MS^n. A disadvantage is that ITD mass spectra are not always identical to those produced by quadrupole and magnetic sector instruments. The detection limits are similar as with quadrupole MS, that is in the range of 1 pg, and can be further reduced in the MS/MS mode to the fg range, for example for PCB77 LOD is 60 fg [13]. The MS^n option that separates the ionization between parent and daughter ions in time is an interesting technique for structure elucidation of unknown compounds.

A linear quadrupole ion trap is similar to a quadrupole ion trap, but it traps ions in a two-dimensional quadrupole field, instead of a three-dimensional quadrupole field as in a quadrupole ion trap. The design has the advantages of higher trapping efficiency of trapped ions and the ability to trap more ions, which results in greater sensitivity compared to the cylindrical ion trap [14].

The concept of time of flight mass spectrometry (ToF MS) was developed in 1946 by William E. Stephens of the University of Pennsylvania [15]. In the time-of-flight mass spectrometer, charged particles are analysed by their mass-to-charge ratio, which is determined by measuring the ToF of the charged particles between two given points, for example between the ion source and the ion detector. To obtain a longer flight tube (and thus higher mass resolution), many instruments have a reflectron (a constant electrostatic field) at the end of the flight tube, causing the ions to travel back towards the detector which is placed opposite the entry point. Ions will, thus, fly in a V-shaped pattern [16]. Newer instruments have three reflectrons causing the ions to travel in a W-shape, quadrupling the length of the original flight tube. Two types of ToF systems exist (1) high resolution (5–10 ppm) but with a moderate scan speed (10 Hz) and (2) high-speed scanning instruments

(100–500 spectra/s) usually with unit-mass resolution. The advantage of ToF is the low limits of detection (1–10 pg) achieved under full scan conditions, which is highly interesting for the identification of unknown compounds.

The principle of operation of ion cyclotron resonance (ICR) cells and Fourier transform (FT) mass spectrometry is based on the fact that ions rotate in a plane perpendicular to a superimposed magnetic field in a direction defined by the so-called "right-hand rule" at a frequency dependent on their m/z. The rotating ions can be detected based on an image current that is induced in detector plates positioned outside of the cyclotron cell. Fourier transform analysis is used to convert the complex transient signal from a time-dependent to a frequency-dependent function, which is the basis for generation of the mass spectra [7, 17].

The Orbitrap mass spectrometer was invented by Alexander Makarov and first published in 2000 [18]. It consists of an outer barrel-like electrode and an inner spindle-like electrode. Ions rotate about the inner electrode and oscillate harmonically along its axis (the z-direction) with a frequency characteristic of their m/z values. An image current transient of these oscillations is converted to a frequency spectrum using a Fourier transformation [18–20].

3 Gas Chromatography Coupled to Mass Spectrometry

GC–MS is a well-established technique that collects structural data from all types of chemicals amenable to GC analysis, except those that have a very high boiling point or thermally labile compounds. The most common MS systems for GC are (1) quadruople, (2) ion trap, (3) ToF, or (4) magnetic sector instruments. The first two techniques are often low-resolution mass spectrometers. ToF mass spectrometers are available in low (mass unit) medium (mass resolution about 5,000) and high resolution, while magnetic sector instruments are high-resolution mass spectrometers.

Various sources are used for the ionization of molecules, but electron impact (EI) is the most widely used ionization technique. EI provides structural information of the compound, often the molecular ion and fragment ions of the analytes. For low resolution EI spectra, extensive libraries of MS spectra are available (refs to NIST, Wiley for example), mainly from quadrupole instruments, which are very useful for automatic library searches and identification of compounds. Most organic compounds yield good EI responses. However, thermo-labile compounds often give weak responses and no molecular ion. Chemical ionization (CI) is a good alternative in these cases, with the appropriate ionization gas (methane, isobutane and ammonia are the most common). CI is gentler than EI ionization and little or no fragmentation of the molecular ion occurs. Chemical ionization is less frequently used than EI ionization in environmental chemistry; electron capture negative ionization (ECNI) is a more common alternative to EI. In ECNI, the compounds capture electrons and form negative ions with high selectivity. ECNI can also improve the LOD for specific compounds, for example chlorinated and brominated

compounds, by decreasing the background signal. A sensitivity increase of 100 times compared with EI has been reported, with detection limits as low as 20 fg for some PCBs [21]. ECNI is also very sensitive for the detection of compounds containing N, S and O. The technique is, therefore, very useful for the identification of compounds containing Cl, Br, N, S and O in the full scan mode.

Recently, Time-of-Flight (ToF) mass spectrometry in combination with GC has gained much attention. Two types of ToF systems exist (1) high resolution (5–10 ppm) but with a moderate scan speed (10 Hz) and (2) high speed scanning instruments (100–500 spectra/s) usually with unit-mass resolution. The advantage of ToF is the low limits of detection (1–10 pg) achieved under full scan conditions, which is highly interesting for the identification of unknown compounds.

4 Comprehensive Two-Dimensional GC (GC × GC)

Comprehensive two-dimensional GC (GC × GC) was invented in the early 1990s and is now an established technique with a tremendous capability to separate and identify volatile organic compounds in complex environmental samples [22]. In the last decades, the focus was on the technical aspects of GC × GC, and it has developed into a complete chromatographic system with dedicated software for quantification and identification. In GC × GC, a first GC column, typically an apolar phase, is connected by interface called modulator with a second column which is often a polar of shape-selective phase (Fig. 2). The modulator will separate the eluate of the first column in very small fractions, typically a few seconds, by trapping, refocusing and launching the fractions to the second column. The separation in the second dimension is very fast and typically takes 2–15 s. The separation mechanism between the columns should be different so that an orthogonal separation is carried out. Two types of modulators, the cryogenic modulation and capillary flow technology (CFT), are currently used to couple the two GC columns. For cryogenic modulation the first dimension column is a typical high-resolution capillary GC column (e.g. 15–30 m × 0.25–0.32 mm ID × 0.1–1 μm df column), and in the second dimension a much shorter and narrower column is used (e.g. 1–2 m × 0.1 mm ID × 0.1 μm df). Cryogenic jet-based modulators, using either CO2 or liquid N2 for cooling, and recently cryogen free thermal modulator have been developed. Extensive comparisons of five types of modulators have been

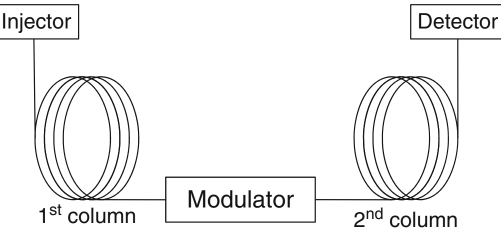

Fig. 2 Schematic overview of GC × GC system. Shown are the two GC columns that are connected with a modulator

carried out [23]. These studies showed that all cryogenic modulators can be applied for a wide range of applications, from alkanes to PCBs. Optimization of the modulator conditions is a crucial factor to obtain satisfactory modulations and GC × GC chromatograms. CFT modulation uses two high-resolution columns with equal diameter, of which the second column is shorter (5 m) than the first column (15–30 m), and which are connected by a capillary flow plate and a three-way system valve is used for flow switching [24]. The capillary flow plate consists of a thin metal plate (3 × 6.2 × 0.1 cm) and is employed as a microfluidic Deans switch. Optimization of the gas flows is the crucial factor for this modulator system.

The GC × GC chromatograms are typically plot as contour plots in which the x-axis shows the first dimension retention time and the y-axis the second dimension time (Fig. 3). As an example, a contour plot of a shrimp sample analysed by GC × GC coupled to ToF–MS is shown in Fig. 3.

For a long time, GC × GC analysis was hampered by the lack of suitable software, but today impressive packages are available that generate two-dimensional contour plots, automatically deconvolute the peaks and perform peak identification by comparing the MS spectra with library spectra.

GC × GC not only gives more resolving power than single column GC, but it also has the advantage that compounds are grouped in bands with the same chemical characteristics, which can be useful for the identification of unknown compounds. Impressive results have been obtained in terms of separation efficiency and compound classification of structurally related compounds. Examples are the group separation of paraffins, naphthenes and mono-, di- and triaromatics in petrochemical products [25–27].

Because the peak widths in the second dimension of GC × GC are in the order of only a few hundreds of milliseconds, it is necessary to couple this technique with a rapid-scanning mass spectrometer. The most appropriate detector is the ToF–MS with a scan speed of 100 spectra per second, which includes in general low resolution machines. The higher resolution systems are unable to acquire the scan speeds required, with the exception of a recently introduced HRToF–MS. An alternative option is the rapid-scanning quadrupole MS, which is less expensive but with the disadvantage that only a limited mass range (200–300 Da) can be

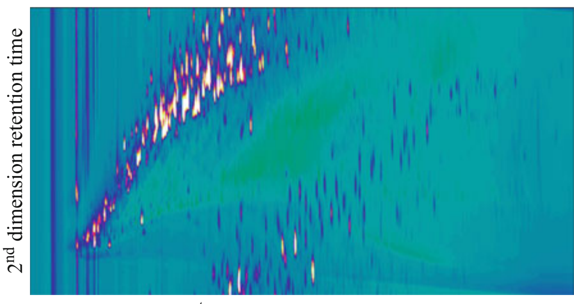

Fig. 3 Contour plot of a GC × GC-ToF-MS chromatogram of a shrimp sample from the Western Scheldt estuary (Netherlands). Each dot indicates a compound. Shown are the first and second dimension retention times.

scanned to obtain the desired scan speed [28]. The drawback of this MS system is that a limited range of the compounds of interest can be only scanned.

The separation power of the GC × GC–ToF–MS system is shown in the analysis of cigarette smoke in which more than 30,000 peaks were detected [29]. A contaminated sediment from the river Elbe (Czech Republic) that was subjected to a nondestructive extraction and fractionation method and analysed by GC × GC–ToF–MS showed the complexity of this sample but also the identification power of this system [30]. The identification strategy was based on peak deconvolution with AMDIS followed by a search in the NIST library. More than 400 compounds were tentatively identified including many polycyclic aromatic hydrocarbons (PAHs), such as quinones, dinaphthofurans and chlorinated and alkylated PAHs.

Another advantage of GCxGC is the possibility to separate interfering matrix, for example humic acids or lipids, from the compounds of interest in the two-dimensional space, which was nicely shown for sediment by Korytar et al. [28]. In addition, the intra-group separation of contaminants was demonstrated for 12 classes of halogenated compounds [31].

5 Structure Elucidation in EDA by Gas Chromatography Coupled to Mass Spectrometry

Identification of unknown compounds by GC–MS starts with the deconvolution of peaks of the chromatogram (Fig. 4). This step is followed by the structure elucidation by (1) comparing the mass spectrum of an unknown compound with the mass spectra of a spectral library, (2) using computer tools for structure elucidation [32] or (3) generating the elemental composition of the unknown compound using high-resolution MS. The first approach is a straightforward process and the identified compound can be confirmed by the analysis of an analytical standard of the compound and the determination of the retention time and mass spectrum. The elemental composition approach is more complicated and the list of possible elemental formulas generated needs further reduction.

5.1 Deconvolution of Peaks

An important aspect of the identification of compounds in complex mixtures is the deconvolution of peaks in the chromatogram. For this step, the software Automated Mass Spectral Deconvolution and Identification System (AMDIS) is frequently used, which is user friendly, freely available and suitable for both GC–MS and LC–MS data, but is working in low resolution mass spectra mode

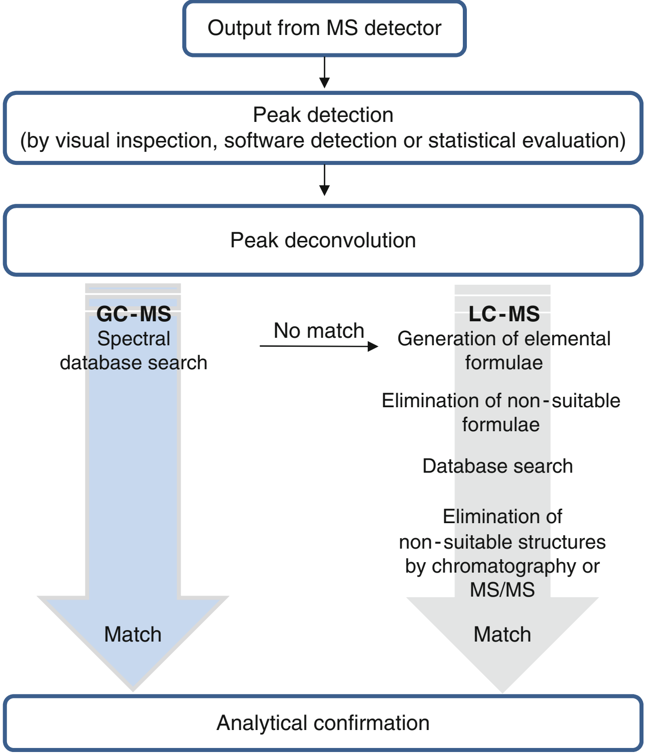

Fig. 4 Typical strategies for structure elucidation using GC–MS and LC–MS. The typical identification routes of both systems are highlighted

only. The program automatically extracts the component mass spectrum from the background and interfering peaks. In general, four steps are carried out, starting with analysis of the background noise level, followed by component perception that searches for increases of special ions. In the third step, the spectrum ions with the same retention time are assumed to belong to the same peak or compound. The peak is then modelled. Finally, the deconvoluted mass spectrum is searched in libraries to identify the compound. For GC–MS large spectral libraries are available such as NIST and Wiley, which together contain about 796,000 spectra of 667,000 compounds [33]. In addition, these libraries contain GC–MS retention time data of approximately 44,000 compounds.

The library search GC–MS approach can be performed using low- and high-resolution MS. However, if no good matches between spectra can be found, the second approach based on generation of elemental formulae of the unknown compound can be followed but this requires-high resolution MS. The elemental formulae can be searched in, for example Chemspider [34] or computer tools can be used to generate (sub)structures (see [32]).

5.2 Elemental Search Approach

In EDA, GC coupled to accurate mass spectrometry for the assessment of the elemental composition of an unknown compound is less frequently used than in LC–MS. The accurate mass of the molecular ions of the unknown compound can be used to generate the elemental formulae. Both the ToF–MS and Orbitrap are appropriate systems as they are available in high resolution mode and can scan at high sensitivity, compared to a magnetic sector instrument. The limited use of GC accurate mass spectrometry is partly due to the restricted number of commercially available interfaces that can couple GC to high-resolution MS. The further development of electrospray (ESI) or atmospheric pressure ionization (APCI) interfaces for GC would be a great benefit for EDA but also for other research fields such as metabolomics.

6 Structure Elucidation in EDA by Liquid Chromatography Coupled to Mass Spectrometry

LC–MS is employed with increasing frequency in environmental analysis due to the polar nature of many emerging contaminants, making them unfit for analysis by GC–MS. The most common LC–MS configuration is the triple quadropole, followed by the Q-trap for target-analysis in multi-residue methods. On the other hand, the Q-ToF, Orbitrap and ICR-FT are generally used for structure elucidation of unknown compounds due to their high mass accuracy.

The main ionization methods used for LC–MS are: electrospray ionization (ESI), atmospheric pressure chemical ionization (APCI) and atmospheric pressure photoionization (APPI).

Electrotrospray ionization, where the mobile phase is sprayed from a charged capillary, is the most commonly encountered ionization technique. It is applicable to molecules of moderate to high polarity and has the advantage that it can ionize molecules from a mass range of ~60–10,000 Da. However, it is not appropriate for the ionization of molecules with low polarity. ESI is used for LC–MS analysis of a wide range of compounds, including pesticides, pharmaceutical compounds, endocrine disrupting compounds and personal hygiene products.

Atmospheric pressure chemical ionization is a technique in which the analytical sample is subjected to a corona discharge. APCI is suitable for moderately polar substances, in the mass range of ~40–1,000 Da. APCI is applied to LC–MS analysis of compounds such as phthalates, oxysterols and steroid glucuronides.

APPI is a relatively new ionization technique in which samples are ionized using ultraviolet light. APPI achieves good ionization with low to moderate polarity compounds; however, this technique has a rather narrow mass range of ~20–500 Da. APPI has been applied, amongst other things, to the analysis of free and esterified phytosterols and polycyclic aromatic compounds (PACs).

The interfaces APCI and APPI makes LC–MS suitable for compounds which could traditionally only be analysed by GC. Selection of the ionization method is based on the sample properties.

Structural elucidation by LC–MS is not as straightforward as with GC–MS, mainly due to the lower sensitivity in full scan mode, the lack of spectral libraries and simple fragmentation interpretation rules [35]. However, due to the polarity of many emerging compounds, it is not sufficient to perform non-target screenings using GC–MS alone. Hence, an increasing number of publications deal with structure elucidation based on LC–MS. An overview of the most common strategies applied in the process is shown in Fig. 2.

6.1 Peak Detection

The majority of EDA studies using LC–MS non-target screening are based on visual inspection for the identification of peaks; however, three studies utilize software for the identification of peaks [36, 37], although visual inspection is usually still part of the identification process. In proteomics and metabolomics, it is common practice to use software and quite a few commercial and free software packages exist, for example Kegg pathway database [38] and human metabolome database [39].

6.2 Spectral Database Search

The NIST spectral library (http://webbook.nist.gov/chemistry) is a widely used, commercially available spectral library. The library was created for GC–MS and contains mass spectra for over 192,108 compounds, and combined with Wiley 667,000 compounds. Typical searches are performed using AMDIS, as discussed above. NIST can also be searched using spectra from liquid chromatography, but because the ionization is different with the softer ionization techniques used, matching is more difficult. Furthermore, many polar compounds will not be present in the database, as they are not detected by GC–MS.

Several attempts have been made to construct commercial spectral libraries for LC–MS; however, because there can be large differences in ionization, fragmentation and chromatographic retention, there are no generally recognized libraries so far. One example of an mass spectral library is the CI-CID mass spectral library from Institute for Inland Water Management and Wastewater Treatment in the Netherlands (RIZA, now MinVenW), which has been employed by Bobeldijk et al. [40]. The library contains spectra of several hundred compounds and was opened to all users by the possibility of downloading an excel sheet [41]; however, the link is no longer available. Another example is the spectrum library constructed by Kiwa in the Netherlands. The library contains around 3,000 compounds and has been employed several times in the literature [35, 37, 40]. This library is, unfortunately, not available to the public.

Marquet et al. have created an in-house LC–ES–MS spectral library with 1,600 compounds utilizing in-source fragmentation [42]. Using this library, Saint-Marcoux et al. [43] compared the results of screening 51 serum samples by LC–MS, LC–UV and GC–MS. In total, 46 compounds were identified, whereof 39 were identified by LC–MS, 32 by LC–UV and 27 by GC–MS. The authors conclude that not one technique could identify all compounds; however, 38% of the identified compounds were identified by all techniques.

6.3 Generation of Elemental Composition

Due to the different relative abundances of isotopes for the different elements, it is possible to exclude some elemental compositions based on a comparison of their calculated isotopic pattern to the one obtained from the unknown compound. Several examples of this practice can be found in the literature, for example Thurman [44] and Ibáñez et al. [45]. The majority of publications evaluate the isotope pattern of the full elemental composition. However, Hogenboom et al. [37] purely use the $^{13}C/^{12}C$ isotope ratio to calculate the maximum number of possible carbon atoms, which is only possible with very stable mass spectra.

6.4 Chemical Databases

Once the number of possible elemental compositions has been reduced, corresponding structures are found from searches of available databases. The most commonly used are: the Merck index, the NIST chemistry webbook, the Sigma-Aldrich search engine, the PubChem database and ChemSpider [34]. This step is a possible pitfall, because the searches are limited by the available structures in the database. To minimize this problem, the majority of authors search several databases in parallel.

7 Examples of Non-target GC–MS and LC–MS Screening of Environmentally Relevant Samples

7.1 Non-target GC–MS Screenings in EDA

Non-target screening using GC with low-resolution MS is the most frequently used tool for the identification of unknown organic compounds in EDA studies. A number of EDA case studies are described briefly to highlight the importance but also the limitations of GC–MS as an identification technique for unknown pollutants.

An EDA study was conducted on water produced from oil and gas platforms in the North Sea, as it was found that these waters contained compounds that bind to the aryl hydrocarbon receptor (AhR, dioxin-like toxicity) and estrogenic receptor (ER) [46]. Extracts of samples were fractionated with HPLC to reduce the complexity of the sample and re-analysed with the in vitro assays. These showed AhR and ER activity in some fractions. The most active fractions were analysed by an array of techniques – GC–EI–MS, GC–ECNI–MS, GC–ToF–MS, GC $\times$ GC–ToF–MS – to identify the responsible compounds in the complex samples. In total, 63 compounds were identified in the estrogenic fractions, among them several alkylphenols, alkylated methoxybenzenes and multi-aromatic petrogenic compounds. The estrogenic potency of the identified compounds was predicted with a QSAR model (COPEDA) and 34 of the 65 compounds were assessed to be estrogenic agonists. For the AhR activity, 41 compounds could be identified of which five were evaluated to be AhR agonists.

In another EDA study on waters produced offshore, the effective use of high resolution GC–MS was demonstrated [47]. The water showed estrogenic and anti-androgenic potency [48–50]. About 35% of the in vitro estrogenic activity could be ascribed to alkylphenols that were identified by low-resolution GC–MS [46, 48]. The low-resolution MS was, however, unable to identify another group of active compounds, the naphthenic acids, which is a highly complex group of compounds. Besides PAHs, known to be anti-androgenic, the high resolution GC–ToF–MS screening of the offshore waters identified a range of alkyl-substituted phenols and a complex mixture of petrogenic naphthenic acids [47]. It was found that certain naphthenic acids were weakly estrogenic as well as anti-androgenic and could explain 65% of the in vitro estrogenic potency [47].

Despite the large MS libraries for GC–MS, the identification of unknown compounds can be limited. This limitation is shown in the work by Weiss et al. [51]. In this work, the androgenic potency of sediment from the river Scheldt was studied. Sediment was fractionated with reversed and normal phase HPLC and tested in vitro for androgenic and anti-androgenic activity. The fractions were analysed using GC quadrupole low-resolution MS in full scan mode. Identification of the peaks was performed with AMDIS in combination with the NIST library and Kovats Retention Indices (KRI). Seventeen compounds could be identified, such as PAHs, nonyl phenol and dibutyl phthalate, while 71 peaks remained unidentified. This study not only shows the limitations of the MS libraries, despite the large number of compounds, but also the limitation of low-resolution MS. The development of commercial interfaces for high-resolution GC–MS would be beneficial for EDA. Recently, some studies were carried out to couple GC with MS using an ESI [52] or a microchip APCI interface [53], which shows stable mass spectra, good limits of detection and good quantitative performance.

Several EDA studies have focused on genotoxicity, mutagenicity and AhR-mediated effects of air, sediment and waters [e.g. 54–56]. PAHs were frequently identified by GC–MS as the major mutagenic and AhR effective compounds. PAHs

are easily analysed by GC–MS; however, the exact structure elucidation of the PAH responsible for the effects is more difficult as many isomers generate the same mass spectrum or have the same retention time. The advantage of GC × GC–ToF–MS for the separation and the identification of PAHs is seldom used in EDA, where the importance of the exact PAH structure is highly important to explain the toxicity. The separation possibility for PAHs is known from petrochemical studies in which a width range of different PAH groups and isomers could be separated [e.g. 25]. Although GC × GC–ToF–MS is seldom used in EDA, it is frequently applied in other fields such as metabolomics.

7.2 Non-target LC–MS^n Screenings in Natural Samples

As both EDA and structure elucidation by LC–MS are relatively new approaches, only a few examples combining these methods can be found in the literature. Table 2 gives an overview of some of these studies.

Bobeldijk et al. [40] developed a procedure for screening water extracts with LC–Q-ToF using model compounds. The procedure was based on automatic switching between MS and MS/MS, followed by visual identification of relevant peaks in the chromatogram. The exact masses were used for a search in the Merck index, the NIST library, an in-house database containing 2,500 known water pollutants and a CI-CID library constructed by the Institute for Inland Water Management and Wastewater Treatment in the Netherlands. In the spiked water samples, they were able to identify all model compounds and four unknown compounds. Of these four compounds, structures were proposed for three (*N,N*-di-cyclo-hexyl-*N*-methyl-amine, carbamazepine and triphenylphosphine oxide), but the identity was not confirmed.

In an example of a non-target screening of pesticides, Thurman et al. [44] identified three pesticides in an extract of tomato-skin. The authors visually identified four major peaks in the extract by LC–MS (ToF) and generated empirical formulae. By evaluating the isotope pattern, one elemental formula was selected for each peak. The Merck index was searched for these formulae, but no hits were found. A search in ChemIndex lead to possible structures and the suitability of these was evaluated by ion trap MS^n, and final confirmation of identities was achieved by the injection of pure standards for three out of the four peaks. The identified compounds were all pesticides: carbendazim, thiophanate methyl and buprofezin.

By a similar procedure, Ibáñez et al. [45] analysed natural water samples by online SPE–LC–MS/MS (Q-ToF). After visual inspection, the isotopic pattern was used to eliminate candidate elemental compositions and the resulting elemental compositions were searched in the Merck Index and the NIST database. Proposed structures were evaluated by MS/MS and three compounds (enilconazole, terbutryn and diuron, all pesticides) were identified and confirmed by the use of standards.

Table 2 Overview of non-target screening studies applying LC–MS^n

Matrix	Bio-assay	Fractionation	LC-MS^n	Identified compunds	Confirmed in bio assay	Reference
Water	–	–	LC–Q-ToF	*N*,*N*-di-cyclo-hexyl-*N*-methyl-amine, carbamazepine and triphenylphosphine oxide (tentative identification)	÷	[40]
Tomato skin	–	–	LC–ToF	Carbendazim, thiophanate methyl and buprofezin	÷	[44]
Water	–	–	LC–Q-ToF	Enilconazole, terbutryn and diuron	÷	[45]
Water	Embryo toxicity assay with zebrafish	RP–HPLC	LC-Orbitrap	9-Methylacridine 4-azaapyrene	+	[37]
Water	*Daphina* toximeter		LC–Q-ToF	Isoproturon, hexa(methoxymethyl)melamine (HMMM), penta(methoxymethyl)melamine and 3-cyclohexyl-1,1-dimethylurea	+	[35]
Urine	In-vitro estrogen bioassay	RP–HPLC	LC–Q-ToF	Equol, nonylphenol and pentamethylchromanol	+	[57]
Sediment	UmuC genotoxicity	NP/RP–LC	LC–FTICR	1,6-dinitropyrene, 3-nitrobenzanthrone, 6-nitrobenzo[a]pyrene, 1-nitropyrene, 1,6-pyrenequinone, 1,8-dinitropyrene, Cyclopenta[cd]pyrene-3[4H]-one, 7-nitrobenz[a] anthracene and 1,6-pyrenequinone	÷	[36]
Water	Umu genotoxicity	RP–HPLC	LC–Q-ToF	9-hydroxy-acridine-*N*-oxide/9,?-dihydroxy-acridine and 9-amino-hydroxyacridine (tentative identification)	÷	[58]
Marmalade	DR-CALUX	RP–HPLC	LC–QqQ	Bergapten	+	[59]
Water	In-vitro estrogen bioassay	SEC	LC–QqQ	Estrone, Bisphenol A and nonylphenol	Attempted	[60]
Sediment	AR-CALUX	NP/RP–LC	LC–Orbitrap	Musks, steroids, phosphor flame retardants, O-PAH	+	[61]

+ compound confirmed; ÷ compound not tested in bioassay for confirmation

7.3 Effect-Directed Analysis (EDA) Employing LC–MS/MS

The objective of EDA studies is to identify which compounds are responsible for a certain toxic effect. Ideally, the non-target identification would lead to an exhaustive list of compounds. However, in practice, this is almost impossible and it is even more difficult to decide when the list is complete. This point has been demonstrated by Mohamed et al. [62]. The authors spiked water and urine samples with 38 known compounds. A blind sample, spiked water and urine were separated by two retention mechanisms in parallel LC–MS runs. The resulting data were analysed by principal component analysis (PCA) and organized by principal component variable grouping. This lead to a reduced list of *m/z* candidates and possible structures were found by database searching in two metabolomics databases [38, 39]. Reduction of the list of possible structure candidates was performed by studying the MS/MS fragmentation pattern. It is interesting to note that of the 38 spiked compounds, 13 were eliminated from the further study due to poor ionization, high endogenous levels or because they did not show a trend in the data treatment. Of the remaining 25 compounds, only 12 (i.e. $<50\%$) were identified in the first run of this study. The example demonstrates the previously mentioned difficulties in obtaining a full list of the compounds present and, thus, the causative key toxicants.

Using an Orbitrap in the data-dependent-acquisition (DDA) mode, Hogenboom et al. [37] performed a target screening of 14 effluent, surface, ground- and drinking water samples. Their in-house mass library consisted of 3,000 water pollutants and 17 pharmaceutical and illicit drugs were identified. In the screening process, deconvolution of chromatograms was performed with software called Formulator and several unknown peaks were identified. The isotope ratio $^{13}C/^{12}C$ was used to calculate the number of carbon atoms, and the double-bond equivalent (DBE) of potential elemental compositions was evaluated against the UV-trace. Several tentative identifications were achieved and the identification of one compound was confirmed. The authors further performed a toxicity identification evaluation (TIE) of an extract of soil from a former municipal landfill. The sample was fractionated three times by reverse-phase HPLC and tested with an *in vivo* embryo toxicity assay with zebrafish (*Danio rerio*). Active fractions were analysed by GC–MS and the most polar of the active fractions was further analysed by LC-Orbitrap. One compound (9-Methylacridine) was tentatively identified by both GC–MS and LC–MS. LC–MS further showed the presence of another unknown compound which was identified and confirmed as 4-azaapyrene. The two compounds were tested in the bioassay and yielded responses similar to the active fraction.

The effect-driven study conducted by De Hoogh et al. [35], although it does not involve fractionation, is another good example. Because of several alarms in a Daphnia-toximeter [63] used for monitoring of drinking water quality, a thorough investigation of the possible causative compounds was carried out. By comparing LC–MS (Q-ToF) chromatograms from the alarm events to non-alarm events, it was possible to visually identify four peaks. Three of the four peaks were identified by the use of an in-house library; the identified compounds were isoproturon,

hexa(methoxymethyl)melamine (HMMM) and penta(methoxymethyl)melamine. Possible structures that fitted both the exact mass and fragmentation pattern of the fourth peak were searched in Merck, NIST, Sigma-Aldrich and various internet sites. Careful evaluation led to a reduction from twenty to two possible structures, and the final identification (3-cyclohexyl-1,1-dimethylurea) was carried out by injection of standards. The compound 3-cyclohexyl-1,1-dimethylurea was found at concentrations up to 5 μg/l. It was attempted to verify that 3-cyclohexyl-1,1-dimethylurea was the cause of the alarms and laboratory tests showed that it did indeed affect behaviour of the Daphnia, but at 3–10-fold higher concentrations than found in the environment. It is concluded that the alarms were due to the additive effects of 3-cyclohexyl-1,1-dimethylurea and other pollutants present in the water sample.

In a search for illegal estrogen residues in calf urine, Nielen et al. [57] developed a system based on a combination of an in-vitro estrogen bioassay and LC–MS/MS (Q-ToF). In the set-up, the effluent from the chromatographic column is split between the mass spectrometer and a 96-well fraction collector, each well containing effluent from a 20 second window. The bioassay GreenScreen [64] for estrogenic activity was then applied to the 96 wells and it was, thus, possible to pinpoint the relevant time windows in the LC–MS chromatogram. As the majority of estrogenic compounds are detected in the negative ionization mode, only this mode was applied in the present study. The relevant spectra were scrutinized and possible elemental compositions were calculated for relevant exact masses. Potential elemental compositions were filtered by the criteria of negative ionization and estrogenic activity. The resulting compositions were searched in the Merck index and the Sigma-Aldrich search engine. Possible structures were again filtered by the criteria of negative ionization and estrogenic activity. By this strategy, the authors were able to make four identifications of which three were confirmed: equol, nonylphenol and pentamethylchromanol. It was possible to confirm that equol and nonylphenol were the causative compounds by testing their activity in the bioassay. It is interesting that pentamethylchromanol did not yield estrogenic response in the bioassay, and it is speculated that the high presence of this compound either cause ion-suppression of the responsible estrogenic compound or that the latter ionize poorly.

An example of non-target identification in active fractions from an EDA study can be found in Bataineh et al. [36]. In this study, sub-fractions of a mutagenic sediment sample were obtained by normal phase, followed by reverse phase chromatography. The authors used classifiers from fiftyfive PACs to eliminate possible candidate structures based on retention time and ionization pattern. Spectra were deconvoluted using the software MZmine [65]. Possible empirical formulae were generated for the exact mass of possible candidates and these empirical formulae were the basis for searches in the PubChem database. Twenty-six compounds were tentatively identified, and the identity of nine compounds (see Table 1) was later confirmed by comparison to standard solutions.

Bobeldijk et al. [58] examined industrial wastewater for the presence of possible genotoxic compounds. Using the Umu genotoxicity test, they were able to identify three interesting fractions out of fortyfive in total. By LC–MS/MS (Q-ToF) in both

positive and negative ionization mode, they were able to show the presence of four unknown compounds in one of the fractions. Calculated elemental compositions were searched in NIST, the Merck index and InfoSpec® (a GC-MS database supervised by Kiwa) and structures were evaluated based on the fragmentation pattern. This lead to the tentative identification of 9-hydroxy-acridine-N-oxide or 9,?-dihydroxy-acridine and 9-amino-hydroxyacridine; however, these were not confirmed due to the lack of standards. Most acridine derivatives are known to be genotoxic and these are considered to be the source of the observed genotoxicity.

In an interesting case, although not using an environmental sample, Van Ede et al. [59] were able to identify AhR agonists in marmalade solely by the use of a triple quadrupole instrument (LC–MS/MS). Hexane extracts of marmalade were fractionated into six fractions. The fraction which showed activity in the DR-CALUX bioassay was further analysed by first HPLC coupled to a photodiode array (PDA). This analysis showed that there were at least four compounds present in the active fraction and this fraction was fractionated into five sub-fractions. The active sub-fraction was analysed by LC–MS/MS, which showed the presence of bergapten. However, testing of a standard with DR-CALX showed that it was possible that bergapten was not responsible for the full response obtained from the active fraction.

Similarly, using only targeted analysis with a triple quadrupole instrument (LC–MS/MS), Heisterkamp et al. [60] investigated the presence of estrogenic compounds in water samples. When applying a recombinant yeast estrogen screen (YES) [66], to a river sample and the effluent of a WWTP, the authors found acute toxicity to the yeast cells. This problem was overcome by fractionation of the sample with size exclusion chromatography (SEC). The active fraction was target-analysed for the presence of eight known estrogens. Of the eight, only estrone, bisphenol A and nonylphenol were found in the active fraction. It was attempted to confirm that these three compounds were responsible of the observed effects, but this was not possible. This study highlights the importance of non-target chemical analysis for the identification of all relevant responsible compounds.

Using an LTQ-Orbitrap, Weiss et al. [61] were able to identify eight biologically active androgen disruptive compounds in sediment from a tributary to the river Scheldt in Belgium. After extensive sample extraction, clean-up and two-dimensional LC-fractionation, the fractions were tested in the AR-CALUX assay for the assessment of androgenic and anti-androgenic potency. For the identification of unknown toxicants in the responsive fractions, a data handling strategy was developed consisting of nine steps using a variety of different software tools. The accurate mass of the observed peaks served as the starting point in the identification pipeline, combined with prior knowledge from the fractionation procedure on the log Kow range fitting the fraction under analysis. Compounds that were identified and whose biological activity was confirmed in the bioassay included polycyclic musk compounds used in personal care products, high production volume organophosporus acids used as polymers and flame retardants, steroids and an oxygenated PAH originating from, for example, combustion processes.

8 Conclusions and Future Challenges

The combined use of GC–MS with LC–MS analysis is a powerful and complementary approach for identification of unknown compounds in EDA. From the examples described in this chapter, it is clear that the application of high-resolution mass spectrometry, either in combination with GC or with LC, is the most valuable tool for the identification of unknown pollutants. However, for the routine application of high-resolution MS techniques, the main angle for further exploration is the data handling strategy that is required to be able to deal with the large amount of data generated by the high resolution and fast screening techniques. Strategies to further prioritize the tentatively identified compounds using multivariate techniques such as PCA also need further development.

Novel approaches such as comprehensive two-dimensional LC (LC × LC) may be used to separate complex mixtures of compounds, which are often found even after extensive fractionation procedures in EDA. Today, LC × LC can be coupled with ToF–MS or rapid-scanning quadrupole instruments (5 Hz scan speed is needed) [67]. Applications with ESI and APCI sources have been described and used for the separation of, for example peptides, triacylglycerols, drugs and organic acids. The possibilities of LC × LC–MS for EDA may be found in the combination of the fractionation and the identification step, but also for the separation and identification of complex mixtures in the final extracts.

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Computer Tools for Structure Elucidation in Effect-Directed Analysis

Emma Schymanski, Tobias Schulze, Jos Hermans, and Werner Brack

Abstract The identification of unknown compounds isolated during Effect-Directed Analysis (EDA) is often a hurdle on the way to the successful outcome of these studies. Ever-improving separation, analytical, and biological techniques allow the isolation of more compounds and effects; however, not all of the compounds contributing to sample toxicity are easily identified. The advancement of database search strategies and publishing of online databases has improved tentative identification of many compounds in recent years, but many chemicals and their transformation products are still not captured within such databases. Structure generation, where the analytical information is used to identify substructures present and absent, provides an alternative strategy to database searching. Where multiple structures matching an unknown spectrum are possible, candidate selection becomes critical to successful identification. The main steps in candidate identification and selection are discussed in this chapter, including examples of programs and strategies available. Improvements in the ability to share data between institutes and the selection criteria for candidate structures are needed to take advantage of recent analytical developments and further enhance structure elucidation in EDA studies.

Keywords Databases, Identification of unknowns, Spectrum prediction, Structure elucidation, Structure generation

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W. Brack (ed.), *Effect-Directed Analysis of Complex Environmental Contamination*, Hdb Env Chem (2011) 15: 167–198, DOI 10.1007/978-3-642-18384-3_8,
© Springer-Verlag Berlin Heidelberg 2011

Contents

1 Introduction 169
2 Database Tools 170
  2.1 Mass Spectrometry and Databases 170
  2.2 Databases Based on Exact Mass 174
  2.3 Databases Including Unknowns 174
3 Structure Generation Tools 177
  3.1 Concept of Structure Generation 177
  3.2 Substructure Identification 178
  3.3 Prediction of Molecular Formula 179
  3.4 Structure Generation 184
4 Candidate Selection 185
  4.1 Prediction and Comparison of Spectra 185
  4.2 Retention Properties 187
  4.3 Partitioning Properties 188
  4.4 Abraham Parameters 191
  4.5 Additional Methods 192
5 Summary/Conclusions 194
References 195

Abbreviations

ACD	Advanced Chemistry Development Inc.
AMDIS	Automated Mass Spectral Deconvolution and Identification System
AQI	Assignment Quality Index
BP	Boiling Point
CAS Number	Chemical Abstract Services Registry Number
Da	Dalton (atomic mass unit)
EDA	Effect-Directed Analysis
EI-MS	Electron Impact Mass Spectrometry
EEI	Even Electron Ions
ElCoCo	Elemental Composition Computation
EMPOMASS	Database of Mass Spectra of Unknown or Provisionally Identified Substances (NORMAN)
ESI–FT–MS	Electrospray Ionization–Fourier Transform–Mass Spectrometry
ESI–QToF-MS	Electrospray Ionization–Quadrupole Time-of-Flight Mass Spectrometry
GS–MS	Gas Chromatography–Mass Spectrometry
2HA	2-hydroxy-9,10-anthraquinone
HR-MS	High Resolution Mass Spectrometry
IQ	2-amino-3-methyl-3H-imidazo(4,5-f)quinoline

K	Kelvin
KRI	Kovat's Retention Index
LC	Liquid Chromatography
LSERs	Linear Solvation Energy Relationships
LRI	Lee Retention Index
"M"-peak	Mass Spectrometric Molecular Ion Mass Peak
MALDI–ToF-MS	Matrix-Assisted Laser Desorption/Ionization–Time-of-Flight Mass Spectrometry
MODELKEY	Models for Assessing and Forecasting the Impact of Environmental Key Pollutants on Marine and Freshwater Ecosystems and Biodiversity (EU project)
MOLGEN	Molecular Structure Generator
MS	Mass Spectrometry
MS-MS	Tandem Mass Spectrometry
MS^n	Multistage Mass Spectrometry
MV	Match Value
m/z	Mass-to-charge ratio
1NP	1-nitropyrene
NIST	National Institute of Standards and Technology
NORMAN	Network of Reference Laboratories for Emerging Pollutants
OEI	Odd Electron Ions
PAH	Polycyclic Aromatic Hydrocarbons
QPID	Quality Peak Identification Database
QSAR	Quantitative Structure-Activity Relationship
Q-ToF	Quadrupole Time-of-Flight Spectrometry
RDB	Ring and Double Bond
RI	Retention Index
RP-HPLC	Reversed Phase High Performance Liquid Chromatography
TIE	Toxicity Identification Evaluation

1 Introduction

Effect-Directed Analysis (EDA) often results in the isolation of unknown compounds, where the identity of these compounds could have a large impact on the outcome of the study. The fractionation procedures used in isolating potentially toxic compounds means that only small amounts of sample are available for analysis, such that chromatographic methods (e.g., gas and liquid chromatography) and the subsequent method(s) of detection (e.g., mass spectrometry) are often the only analysis techniques possible. Ever-improving separation and analysis techniques have increased the number of compounds detected; however, identification of these compounds is not always straightforward. The concentration of toxicologically significant compounds may be close to detection limits, or not detectable at all with the method applied, while those compounds identified relatively easily

(e.g., priority pollutants) may not be relevant to the type of toxicity of the sample. The amount of data that accompanies most analyses these days renders manual data processing near impossible and while most instruments come with helpful software, several other programs and strategies have also been developed to assist in structure elucidation.

This chapter looks at various computer tools for structure elucidation in EDA studies, in three general sections. Firstly, we cover the use of databases and how to retrieve as much information from these as possible, including the incorporation of unknown compounds into the database itself. Secondly, we look at the concept of structure generation and how this can be useful, especially in the absence of database matches. Finally, we look at candidate selection, which is needed for both database searches containing multiple matches and for structure generation. Although it is not possible to mention all available programs within this book chapter due to the large number of commercial or proprietary releases with restrictive access, we have endeavored to tabulate example programs with which to apply the structure elucidation strategies within the relevant sections. As a result, this chapter aims to outline strategies and use examples to illustrate these, rather than being a comprehensive discussion of all programs available for structure elucidation.

2 Database Tools

2.1 Mass Spectrometry and Databases

The mass spectrum of a compound is often considered to be its fingerprint, which may easily be used for identification purposes. However, this is not necessarily true for a mass spectrum by definition. Mass spectra generated with a hard ionization technique, such as Electron Impact (EI) ionization, often used in combination with gas chromatography (GC), produce spectra with a high identification power, because this technique has the potential to produce many fragments and therefore often unique and/or easily identifiable spectra. Mass spectra generated with soft ionization techniques such as Chemical Ionization, Desorption Ionization, and Atmospheric Nebulization Ionization, however, generally do not contain fragments but only yield molecular mass information. Nevertheless these techniques can also be very useful in combination with high-resolution mass spectrometry (HR-MS), as discussed later. Atmospheric Nebulization Ionization is often applied together with liquid chromatography (LC). If a higher specificity is needed in combination with soft ionization, tandem MS (MS–MS) is usually the method of choice. This technique isolates the molecular ion of interest and fragments it by collision-induced dissociation with an inert gas, resulting in a spectrum with additional fragment ions. These spectra, however, are less reproducible compared with spectra produced by electron impact ionization.

As a result, EI-MS lends itself well to database techniques, as the spectra are also generally reproducible across different instruments and laboratories, such that finding a matching spectrum is often a very good indication of the compound structure. This is why currently the starting point for many EDA studies is a general gas chromatography–mass spectrometry (GC–MS) analysis, as this is readily available and relatively easy to perform [1]. Significant EI-MS databases exist (e.g., NIST [2] and Wiley [3], with over 600,000 spectra combined), allowing relatively quick identification of compounds within these databases. Most instruments come with software linked either to one of the commercial databases or to their own internal database, such that implementation of database searches is very easy for the user, often as simple as one click on the chromatogram. During a search, the measured mass spectrum is compared with those in the database, generating, in the case of NIST, a match factor, reverse match factor and a probability that this spectrum is the "right" match. The match factor and reverse match factor give an indication of how well the mass peaks (reported as m/z, the mass-to-charge ratio) and their magnitudes match, excluding and including m/z not measured in the experimental spectrum, respectively, so that similar spectra should have very high values for both. The probability, however, is relative to all other spectra in the database and as such is more subjective. If the experimental spectrum is distinctive and very similar to only one spectrum in the database (e.g., Fig. 1b), a match is usually associated with a high probability; however, if there are similar spectra for different compounds (often seen for isomers, e.g., polycyclic aromatic hydrocarbons (PAHs), Fig. 1c, d and substituted aromatics, e.g., Fig. 1e, f, only a low probability is possible because the match could be one of several spectra). If there are no exact matches but some similar spectra, it is also possible to have a high probability of a match for spectra that do not match well visibly, because the probability compares with the spectra available (see examples elsewhere [4]).

The down side of database searching based on EI-MS spectra is that the spectra are not necessarily unique (as eluded to above), while the mass peak ratios in spectra measured on different types of instruments (e.g., quadrupole versus ion trap) can vary dramatically, such that measurement differences can exceed differences between spectra of similar compounds. This may even happen for instruments of the same type when tuned for maximal sensitivity at a specific mass or wrong mass range. Differences in mass peak abundance also affect the database search results, as the search takes both mass peak presence and its relative abundance into account. Furthermore, the searching algorithms used are such that the results are quite trustworthy for compounds within the database but are less reliable outside the database domain. While additional data are given to assist in eliminating false matches (e.g., Kovat's retention index in NIST), the errors in some of these measurements are greater than the differences in values for similar compounds [5]. Further examples and discussion about database searching are given elsewhere [6, 7].

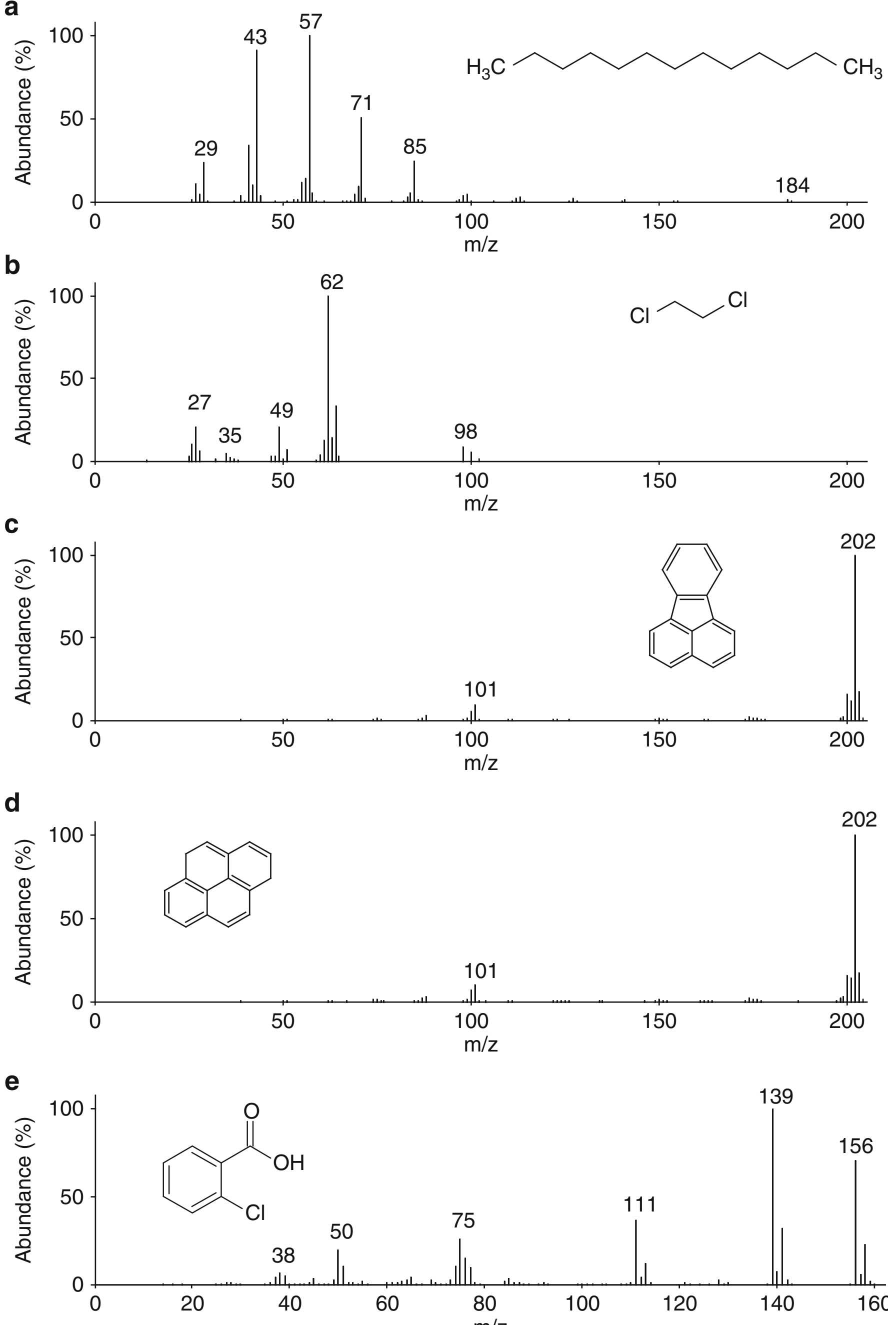

Fig. 1 (continued)

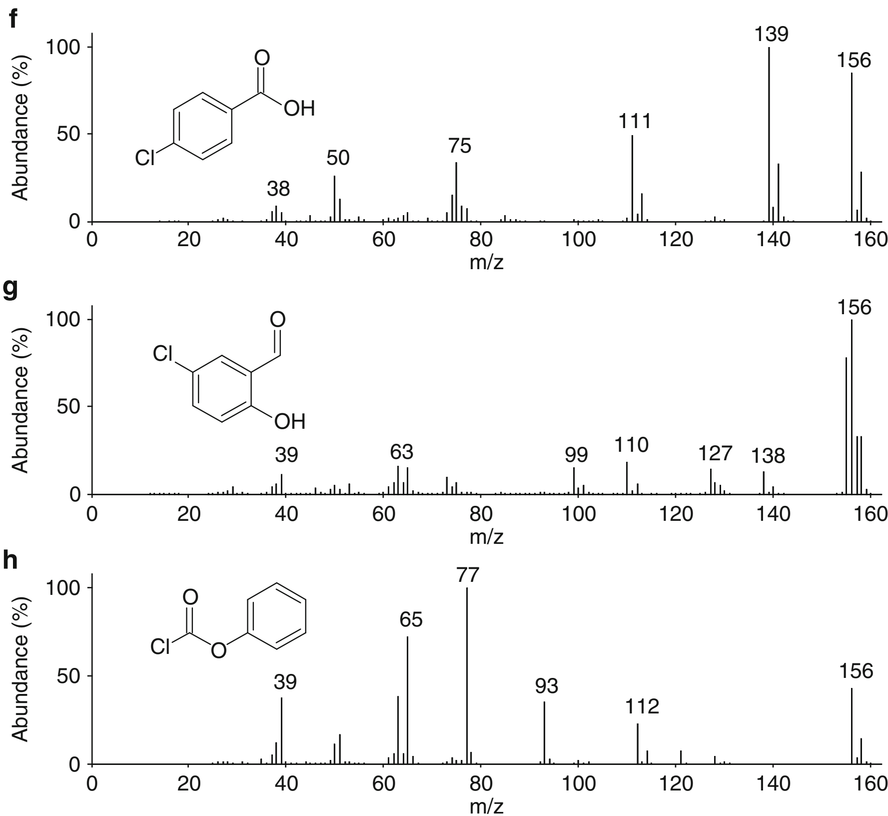

Fig. 1 Example mass spectra. (**a**) Tridecane, showing the distinctive alkane chain pattern. (**b**) 1,2-dichloroethane as an example of a distinct spectrum for the given formula. (**c**) Fluoranthene and (**d**) pyrene are almost identical spectra for different compounds of the same formula. Spectra (**e**) 2-chlorobenzoic acid and (**f**) 4-chorobenzoic acid differ from each other only in peak magnitudes, while compounds with the same formula (**g**) 5-chloro-2-hydroxy-benzaldehyde and (**h**) phenylchlorocarbonate have distinctly different fragmentation patterns in the spectra. Spectral data from the NIST05 database [2], spectrum numbers 61976, 114952, 228362, 227992, 228871, 228878, 231382, and 292166, respectively

Although 600,000 spectra in a database seem an impressive number, this is a tiny subfraction of the number of different chemicals produced, let alone their breakdown products and metabolites. Furthermore, many toxicologically significant compounds (i.e., those of interest in an EDA study) are not detected using GC–MS, due to low volatility, thermal instability, or too high polarity [8]. It is also estimated that the molecular ion (“M”) peak may be missing from up to 30% of all EI-MS spectra [9], which makes estimation of even the molecular weight of the unknown compounds challenging. Thus, while GC–MS coupled with a database search is a good starting point, more information is generally necessary for identifying all compounds of interest in an EDA study.

2.2 Databases Based on Exact Mass

With increasing use of high-resolution single, tandem and multistage mass spectrometry (MS^n) techniques (e.g., Q-ToF and Orbitrap), especially coupled with liquid chromatographic (LC) techniques, an increasing number of databases are available based on accurate mass of compounds. Measurement of the mass at higher resolution (e.g., 10 mDa or less) as opposed to low resolution (1 Da) restricts the possible elemental composition in many cases to one or a few formulae. The accurate mass is used to determine the chemical formula, which is then used to search the databases (e.g., those included in Table 1) for structures matching the input formula [8, 16]. Spectral data and/or additional substructural information or classifiers can then be used to select the most likely match. Such an approach is theoretically able to tentatively identify compounds if the database is sufficiently comprehensive (e.g., ChemSpider and PubChem, see Table 1), although many compounds relevant in environmental samples may still be missing from these databases. As it is, searching a single chemical formula in these databases results in many cases between tens and hundreds of matching structures, not counting the many more possible structures existing outside the databases. If any compound information is known (e.g., likely to be a pesticide or pharmaceutical), these searches can be restricted to a more likely compound domain, reducing the number of unwanted hits. The generation of too many hits means that separating these to choose the "correct" structure based on fragmentation and spectral data alone would be both difficult and time-consuming. Many strategies have been developed to narrow the number of database hits down into more manageable numbers, discussed in detail later on in this chapter.

2.3 Databases Including Unknowns

2.3.1 GC–MS Databases

The next step in database searching and the identification of unknown compounds in EDA studies is the development of databases containing EI-MS spectra from

Table 1 Selected databases available for compound searches, including both free access and subscription databases

Database	Entries	Access
ChemIndex [10]	1,000,000	Free, online
ChemINDEX [11]	75,000	Subscription
ChemSpider [12]	59,276,371	Free, online
Merck Index [13]	10,000	Purchase or subscription
NIST MS [2]	220,460	Purchase
NIST WebBook [14]	61,274	Free, online
PubChem Substance [15]	62,647,169	Free, online
Wiley Registry of MS [3]	662,000	Purchase

Number of entries includes duplicate compounds in some cases. Figures from March 2010

unknown compounds themselves. This means that even if the compound itself is not identifiable, information about its distribution and/or occurrence can be gathered. Furthermore, the combination of analytical information from multiple sources may assist in the ultimate identification of the compound.

The MODELKEY [17] project developed the Quality Peak Identification Database (QPID) as part of the efforts to identify key toxicants in three European river basins with the aim to easily separate interesting compounds from trivial ones by using filters [18]. EI-MS spectra from EDA case studies meeting given quality criteria, together with their Kovat's Retention Indices (KRI), were uploaded into the database, with three classifications: identifications, indications, and unknowns. Spectra were first deconvoluted using AMDIS [19] and then processed by a NIST database search for tentative identifications. Spectra with match probabilities above 80% and with matching KRIs within ±20 were considered identified, those outside the KRI bounds as indications and those failing both criteria as unknowns. The resulting database can also be uploaded into the NIST search domain, so subsequent NIST searches could also search the unknown spectra.

To increase the chance of identifying unknown compounds, the GC–MS analyses were performed under standardized analytical conditions for all samples by all data contributors to ensure a high grade of comparability between the different samples (Table 2).

For example, Weiss and coworkers [18] used the QPID database to evaluate EI-MS of fractionated sediment extracts from Scheldt River and found 71 unknown compounds in two fractions with androgenic and antiandrogenic activity that will be further investigated using other techniques (e.g., high-resolution LC–MS).

Table 2 Recommended GC–MS settings for EI-MS full scan analysis for identification of unknowns and calculation of Kovat's Retention Indices (KRI) used in MODELKEY

Parameter	Recommended value	Explanation of effects for the KRI
Scan range	50–500 amu	Regular range for environmental GC–MS measurements
Scan speed	1.5–2 scans/s	Processing procedures are optimized for 1.7 scans/s
Column type	DB5 (MS)	Column type highly determines retention behavior
Column dimensions	50 m × 0.25 mm × 0.25 μm	Influence on KRI is not examined but expected to be considerable
Carrier gas	Helium	Influence on KRI is not examined but not expected to be considerable
Constant flow	1.3 ml/min	Constant pressure will cause KRI deviations of ±10 units which is within our accepted tolerance (±20)
Oven start Temperature	70°C (4 min static)	Together with solvent used not very critical for KRI > 900
Temperature rate	3°C/min	Temp rate of 10°C/min gives up to 50 units higher KRI values; so avoid the use of different rates
Oven end temperature	300°C (20 min static)	Especially important for KRI > 3,000

Furthermore, they tentatively identified known compounds with antiandrogenic activity such as tris(1-chloro-2-propoyl)phosphate, fluoranthene, benz[*a*]anthracene and a technical mixture of eight isomers of *p*-nonylphenol.

Another alternative is the databases set up by the NORMAN network of reference laboratories, research centers and related organizations [20]. This network, started with funding from the European Commission, is now self-sustaining and aims (amongst others) to enable the exchange and collection of data on environmental substances. Specifically of interest in EDA studies is the NORMAN EMPOMASS database of unknown and provisionally identified mass spectra [21]. Similarly to the QPID database, this provides information on the occurrence of substances not included in major monitoring schemes due to uncertainty about their identity and, as a result, helps define new emerging threats.

The web-based database MassBank [22] is another possible tool to search unknown spectra. Although the online version can be used to search available spectra, the database can also be installed locally (without data) to search user data, leaving the option to store known and unknown spectra. Developed originally for metabolomics data, MassBank can store a wide variety of spectra including EI-MS, ESI-QToF-MSMS, ESI-FT-MS, and MALDI-ToF-MS. Data upload from raw data files via the freeware Mass++ [23] also includes a spreadsheet-based record editor for the addition of compound and sample information (e.g., SMILES code, structure file, retention time and index, instrument settings, and analytical conditions) and an administrative upload tool. The database management system available for download retains the central functions of the online version, including a spectrum similarity, peak, molecular formula, and substructural searches.

The main features of the three databases are summarized in Table 3.

Over time, community contribution to these databases should assist in the identification of unknown compounds and providing some information as to their distribution. Each database has both advantages and disadvantages. While EMPOMASS and MassBank are open access, they do not include the bioassay features found in QPID, designed to help data processing during EDA studies. MassBank can accept multiple MS(^{n}) formats, making this extremely flexible for multiple instrument data; however, the number of entries is an order of magnitude lower than NIST for EI-MS. While the strict protocols associated with the QPID database restricts the amount of information available for inclusion, the reliable (or consistent) data within the database and the ability to include the unknown spectra using the NIST search function is a definite advantage. Relaxing these protocols will result in the inclusion of more data (e.g., via EMPOMASS or MassBank) but may in the end reduce the usability of this data, a problem which is being increasingly realized, for example, in the public access proteomic databases [24].

2.3.2 LC–MS Databases

Unlike EI-MS, LC–MS(^{n}) spectra are generally very instrument-specific and thus not often suitable for comparison between labs using the same tools as for EI-spectra. As LC–MS techniques are applicable to a much wider range of compounds

Table 3 Comparison of three databases for known and unknown spectra

	QPID	EMPOMASS	MassBank
Availability	Offline database, MODELKEY members only	Online (login to search, members/data providers can also add/edit)	Online (with data) Free download (without data)
Spectral information	GC–MS only, RT, RI, structure	GC–MS only, area, RT, standards, structure	GC–MS, LC–MS(^{n}), RT, RI, structure
Additional data and filters	Chemical, analytical and bioassay data; EDA/TIE-based filters	Chemical and analytical data only	Chemical and analytical data only
Search domain	Within QPID or NIST (local)	Online	Online or local data
Search functions	Via NIST (library, formula, name, peaks, molecular weight) or filters	Simple filters (location, matrix, laboratory, RT, main peaks)	MassBank (library, quick, peak, substructure, advanced searches)
	Optimized for EI-MS		Optimized for ESI-MS^2
Upload function	AMDIS (processing of raw data) and spreadsheet	Manual entry web (single) spreadsheet (bulk)	Mass++ (processing of raw data) and spreadsheet

than GC-based techniques, the development of databases for environmental compounds will be critical to the use of these methods for identification of compounds in EDA studies. The key in using LC/MS^n spectra in databases is to release the *m*/*z* peak ratio information from a spectrum because these ratios especially differ from instrument to instrument and also on the same instrument using slightly different measurement conditions. The processing of proteomic and/or metabolomic datasets, for example, includes procedures such as open-access databases, spectral matching from previously identified components and hybrid methods [24, 25]. Statistical methods are also being developed to address the data quality issues associated with the open-access databases. A “downscaling” of the spectral matching tools already developed to the smaller molecules expected in the environmental contaminant area should be possible. Although MassBank (mentioned above) was originally developed for metabolomics datasets, this may be suitable for this challenge as the search functions are already optimized for ESI-MS^2 measurements [22].

3 Structure Generation Tools

3.1 Concept of Structure Generation

Structure generation provides a completely different approach to dealing with unknown compounds. This approach is database independent, i.e., the outcome does not depend on the number or quality of spectra within a database. All possible

structure candidates are considered, which allows the user to see how many structures fit the data, rather than taking the first (or "best") match and thereby overlooking another candidates merely because they were absent from the database. However, sufficient substructural information is required (e.g., based on MS fragmentation patterns) to avoid the generation of thousands to millions of possible structures. In general, the larger the molecular formula, the greater the number of structural possibilities, so where insufficient information is available from the spectrum, candidate selection and elimination become critical to a successful tentative identification.

Structure generation itself requires only a molecular formula (or even several) as a bare minimum. For this simplest case, basic structure generation programs can be used. One such program is MOLGEN (several versions available [26, 27]), which generates all mathematically possible structures for the input formula(e). Although it may be interesting to know how many structures could be possible for an unknown, this is not generally very useful for identifying unknowns and the number of possible structures can in fact be overwhelming. Thus, including as much information as possible into the structure generation procedure becomes necessary to avoid data overload.

3.2 Substructure Identification

In terms of analytical techniques applicable for EDA studies, structure generation methods for EI-MS have been under development for many years, as substructure identification based on common patterns within the spectra is possible due to the reproducibility of the spectra. Several years ago, Varmuza and coworkers developed database-independent substructure identifiers (or "classifiers") [28, 29]. These classifiers assign percentage likelihood to the presence or absence of given substructures, based on the experimental spectrum. Due to their database-independence, these are now included in a number of programs such as the freeware AMDIS [19] for spectral deconvolution and MOLGEN-MS [30], which combines EI-MS interpretation and structural generation in one program. One hundred and sixty of these database-independent classifiers are currently implemented in MOLGEN-MS. The search for substructures is performed by both programs in a very short time frame. If performing structure generation using MOLGEN-MS, the classifier information is automatically loaded into the next stage of the program, whereas information from AMDIS can be included manually into structure generation.

The NIST database also incorporates a substructure search, which assigns probabilities to the presence/absence of substructures based on the experimental spectrum. While these are, in our experience, more accurate than the database-independent classifiers [4], this applies only to spectra that are within or similar to those within the database domain. The presence of substructures for spectra very dissimilar to any within the database is difficult to ascertain, although in many cases the absence of many substructures allows the exclusion of several candidate

structures. The NIST database includes 541 substructures at this stage. Additionally, NIST also estimates the number of chlorine and bromine present and molecular weight probabilities. The substructure information also includes chemical elements as well as Ring and Double Bond counts [RDB, see (1)], both of which are useful for structure generation purposes.

For both sets of substructure identifiers, 95% probability of presence/absence is a good starting point for investigations. While probability of absence is generally a lot more reliable than presence, a 95% probability of absence already includes a large number of substructures in most cases and including more at lower probability becomes counter-productive. There are a few exceptions – for some reason bromine, for example, always appears at 94% in the NIST database, even for alkane spectra. While 95% probability of presence is also a good starting point, for many cases, this will need to be lowered where, for instance, the spectrum is dissimilar to others in the database and any substructural information is needed to reduce the number of structures generated. The lower the probability, however, naturally the lower the certainty and this has to be taken into account in the postprocessing of data.

The combination of the database-independent classifiers within MOLGEN-MS and the NIST substructure search has been shown to reduce the number of structural candidates generated by several orders of magnitude in many cases [4]. While this reduced the number of candidates from tens to only a few in some cases, in others hundreds of candidates still remain, necessitating further tools.

Although these methods have been very useful in many GC–MS studies to provide more information about unknowns, or even identifying them, increasing use of LC methods coupled with higher resolution MS(n) means that other methods are needed. While the accurate mass of the compound is readily available, only limited fragmentation peaks are available to provide sufficient structural information for structural generation. Problems with the reproducibility of these methods between labs and instruments has also prevented the development of comprehensive substructure identifiers as for EI–MS thus far. As the compounds measured using LC methods are generally higher mass, the lack of structural information combined with a far greater number of possible structures means there are far too many structural candidates for a successful outcome. As far as we are aware, this information has only been used successfully to narrow database hits in order to tentatively identify compounds, but not using structure generation. Expansion of LC–MS techniques to MS^n and subsequent improvements in fragmentation information and data processing will hopefully increase the chances of using structural generation to identify unknowns soon.

3.3 Prediction of Molecular Formula

One of the major difficulties with EI-MS, as mentioned above, is the determination of the molecular ion and subsequently the molecular formula. While the molecular ion may be missing in up to 30% of EI-MS spectra [9], many more spectra have

Table 4 Example programs for calculating molecular formulae from MS data

Program	Mass/ Isotope	Odd/Even ion display	RDB	Elements	Availability
MolForm	Both	Yes	No	C,H,N,O,S,Si, P,Br, Cl,F,I	Purchase as part of MOLGEN-MS [30]
ACD Formula Generator	Both	Yes	Filter	Max. 10, user selection	Purchase as part of MS Manager or similar [31]
ChemCalc.org	Mass	No	Yes	C,H,N,O,Br,Cl,F,I	Free, online [32]
NIST Formula Generator	Mass	Yes	Yes	Max 10, user selection	Purchase as part of database [2]
Xcalibur	Mass	No	Yes	All, user selection	Purchase or supplied with instrument [33]
Uni Cambridge MF Search	Mass	No	No	C,H,N,O or all. No restriction possible	Free, online [34]

The Mass/Isotope column indicates whether the isotope pattern or mass difference is used to distinguish candidates, odd/even ion display whether the program displays the ion information, RDB whether it takes RDB information as input to restrict the generated candidates

absent or only very small isotope peaks, such that many candidate molecular formulae exist – and the number of candidate structures increases with every formula. Higher resolution MS helps avoid this problem by recording a more accurate mass, so that determination of the molecular ion and subsequently the molecular formula of the compound is far more successful. There are a number of different programs available to assist in the prediction of the molecular formula for both low- and high-resolution data, ranging from simple online calculations through predictions based on the whole spectrum incorporated into spectral interpretation software. A selection is shown in Table 4.

The number of elements considered by the software and the ability of the user to select these influences the outcome of calculations considerably. While some programs restrict their selection to selected elements only (e.g., NIST Formula Generator and MOLGEN-MS with 10 or 11 elements respectively), others offer no restrictions at all. Both strategies have their advantages and disadvantages. Naturally, the more elements are included, the greater the possibilities, so choosing which elements to include or exclude becomes critical. Excluding possible elements too early without taking this information from the spectrum or other analytical data reduces the number of possible formulae but also the objectivity of the unknown determination, whereas including all elements possible results in the generation of possibly hundreds of incompatible and highly unlikely formulae.

For low-resolution MS data, determination of the molecular formula depends generally on the presence of isotope peaks for either the molecular ion ("M" peak) or fragment peaks, as only a few, low molecular weight compounds have only one formula possible for a given molecular weight. If the "M" peak and its isotopes are sufficiently abundant, calculation of the molecular formula is relatively straightforward using, for example, MolForm or ACD Formula Generator (see Table 4).

Searching for the formula based purely on mass difference is not usually sufficient to isolate the correct formula and is an arbitrary criterion for low-resolution data, as the mass is only determined within one Da and therefore the closeness to the integer value not necessarily indicative of composition.

Including any substructural or elemental composition information from the spectrum or other analytical techniques prior to calculation is instrumental in reducing the number of formulae possible in many cases. This can also include, for example, substructural information retrieved from the NIST database search or the classifiers in AMDIS or MOLGEN-MS (as discussed above). As well as elemental information, another useful criterion for eliminating unlikely formulae is the Ring and Double Bond (RDB) count, otherwise known as the hydrogen deficiency or degree of insaturation. Equation (1) demonstrates how to calculate this based on 11 elements with the following valences: H, Br, Cl, F, I $= 1$; O, S $= 2$; N $= 3$; Si, C $= 4$ and P $= 5$.

$$\mathrm{RDB} = \frac{(2 + 3n_{\mathrm{P}} + 2(n_{\mathrm{C}} + n_{\mathrm{Si}}) + (n_{\mathrm{N}})) - (n_{\mathrm{H}} + n_{\mathrm{Br}} + n_{\mathrm{Cl}} + n_{\mathrm{F}} + n_{\mathrm{I}})}{2}, \qquad (1)$$

where n_{x} represents the number of atoms of element “X” in the molecular formula. Alternative valences for P, S, and N can be considered by adjusting the above equation accordingly.

The NIST substructure information contains relatively reliable RDB indications. Furthermore, if it is known that certain substructures are present (e.g., benzene, RDB $= 4$), formulae with incompatible RDBs can be eliminated (e.g., those with RDB < 4). Several of the programs listed in Table 4 come with RDB filters.

Table 5 shows the number of formulae and the rank of the correct formula calculated for three compounds (see Fig. 2) using the programs listed in Table 4 and the different strategies discussed here and below. The table shows clearly that for these compounds the use of the isotope pattern of the “M” peak alone is insufficient to isolate the correct formula (ranked between 6th and 117th of all possible formulae). The inclusion of substructural information from the NIST database search of the EI-MS spectra, however, improved the calculation substantially, with the correct formula ranked first or second in many cases and 11th in the worst case. The number of formulae generated was also significantly lower (from over 100 matching formulae in most cases to a maximum of 32 formulae).

In some EI-MS spectra, the isotope peaks of the “M” peak are not available, or of such low abundance that the calculations based on these would be inaccurate. An alternative is to calculate the formula based on the isotope peaks of fragment signals, if these have a better pattern (e.g., Fig. 1b 1,2-dichloroethane, where the strong peaks at 62 dominate the “M” peak at 98). The peak abundances can be used similarly to those based on “M” peak isotope patterns but note that the calculation needs to consider even electron ions (EEIs) as well as odd electron ions (OEIs) to account for the loss of atom(s), not just electrons. An example calculation of the formula for 1,2-dichloroethane based on the fragment isotope peaks at 62 compared with the “M” peak at 98 using MolForm is shown in Table 6. In this case the correct

Table 5 Calculation of the molecular formula of IQ, 1NP, and 2HA (see Fig. 2) using selected programs (see Table 3)

		IQ	1NP	2HA
Exact mass				
ACD	N. Formulae	17	26	24
	Rank of Correct	1	5	2
Cambridge	N. Formulae	60	60	60
	Rank of Correct	2	5	4
ChemCalc	N. Formulae	268	515	394
	Rank of Correct	1	4	1
MolForm	N. Formulae	7	14	12
	Rank of Correct	1	4	1
NIST	N. Formulae	2	5	3
	Rank of Correct	1	3	2
Xcalibur	N. Formulae	10	6	4
	Rank of Correct	2	6	3
Isotope pattern "M" peak				
ACD	N. Formulae	187	330	266
	Rank of Correct	117	98	44
MolForm	N. Formulae	76	126	116
	Rank of Correct	4	8	13
Isotope pattern "M" peak with substructural information				
ACD	N. Formulae	32	2	9
	Rank of Correct	11	1	4
MolForm	N. Formulae	12	2	9
	Rank of Correct	2	2	5
Whole spectrum calculation				
ElCoCo	N. Formulae	3	7	7
	Rank of Correct	1	6	3

"N. Formulae" indicates the number of possible formulae generated for the data by the given program, whereas "Rank of Correct" indicates where the correct formula was in relation to the others, based on the sorting criterion (exact mass, isotope pattern, whole spectrum match – see subheadings). All calculations considered C, H, N, and O only for consistency between programs. Exact mass calculations were based on a measured mass of 199.0989 (IQ), 248.0724 (1NP), and 225.0560 Da (2HA), recorded during standard measurement by C. Gallampois. Isotope patterns were taken from the EI-MS spectra retrieved from the NIST database (spectrum numbers 138963 (IQ), 101258 (1NP), and 132776 (2HA). Substructural information from the NIST database was used to restrict element numbers in the isotope peak calculations. All calculations took less than 1 s. MolForm calculations considered odd electron ions only

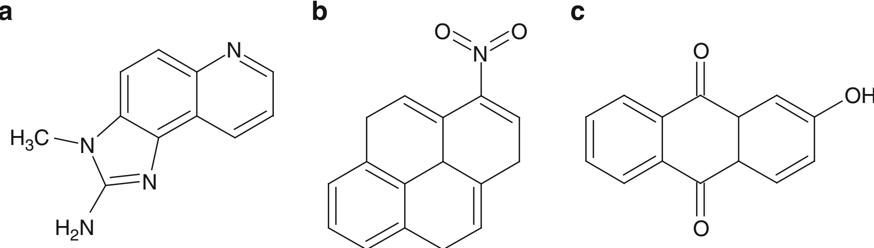

Fig. 2 Structures of (**a**) 2-amino-3-methyl-3H-imidazo(4,5-f)quinoline (IQ), (**b**) 1-nitropyrene (1NP), and (**c**) 2-hydroxy-9,10-anthraquinone (2HA)

Table 6 Calculation of the formula for 1,2-dichloroethane (Fig. 1b) based on fragment isotope peaks (left) and the "M" peak using MolForm (middle), sorted based on isotope pattern match, including all ions. The column to the right shows a calculation based on the whole spectrum using ElCoCo, sorted according to match value

MolForm		ElCoCo
Partial formula based on fragment isotopes at $m/z = 62$	Formula based on isotopes at "M" peak, $m/z = 98$	Formula based on whole spectrum, $MW = 98$, precision 95%
C_2H_3Cl	Cl_2N_2	***$C_2H_4Cl_2$***
CHClN	CCl_2O	C_2H_7Cl
H_6Si_2	CH_2Cl_2N	C_2H_4Cl
H_2SSi	***$C_2H_4Cl_2$***	
CH_6OSi	H_3Cl	
(top 5 of 40)	(top 5 of 183)	(only 3 results)

All calculations performed without any restriction to element numbers, based on C, H, O, N, S, Si, P, Cl, Br, F, I

partial formula (shown in bold italic type) is top of the list for $m/z = 62$, while the complete formula is only fourth on the list for $m/z = 98$. While fragment peaks may help identify a more accurate partial formula, this will then need to be completed by the user, or alternatively the elemental composition of the partial formula can be used as input into a calculation similar to the above (Table 5) to determine the rest of the formula.

If all else fails, calculations based on the whole spectrum, including substructural information, are also possible. This is needed, for example, when the "M" peak is missing, only limited or no isotope information is available or if the calculations were inconclusive. This calculation (e.g., using the ElCoCo module of MOLGEN-MS) is more flexible than those outlined above but is also more time-consuming (e.g., seconds to minutes, as opposed to almost instantaneous), with the time increasing significantly with decreasing restrictions and increasing molecular weight. An example calculation for 1,2-dichloroethane is also shown in Table 6, while the summary in Table 5 indicates that ElCoCo can yield better results in some cases than calculations based on the isotope peaks of the "M" peak alone.

Calculation of the molecular formula based on accurate mass (i.e., high-resolution data) is similar conceptually to the strategies mentioned above for low-resolution data, the main difference being that the formulae are assessed on the closeness to the exact mass, rather than the isotope patterns. The exact masses of the elements are used to determine the combination of elements closest to the mass measured. Although the calculation based on accurate mass is a straightforward calculation, the six programs used in Table 5 came up with quite different results for the same input masses and elements, shown in Table 5 as the difference in ranking of the correct formula. As for the calculations based on isotope patterns, restriction of the numbers of elements can be critical in reducing the number of candidate formulae to manageable levels. Any fragmentation information available can also be used to determine which elements are likely or unlikely. Examples include the neutral loss of NO from nitro-PAHs or the ionization of acidic groups in negative ion mode

compared with the basic groups in positive mode. Furthermore, if there is isotope information available, a combination match value can be calculated with some programs, based on match to the exact mass and to the isotope pattern.

3.4 Structure Generation

Once the molecular formula has been determined and all possible substructural information retrieved from the spectrum, structure generation can begin in earnest. The MOLGEN series of structure generators are a good starting point, see Table 7. While it is possible to use a basic structure generator (e.g., MOLGEN 3.5) to perform this task, this means that input of the substructural information will need to occur manually – easy for simple cases but time-consuming for other cases with many substructures to include or exclude. As many EDA studies start with GC–MS measurements, MOLGEN-MS offers a streamlined alternative, where the spectral interpretation, formula calculation and substructure information are all performed in one program. Work has also been undertaken to incorporate as much information as possible from the NIST database [2] into MOLGEN-MS. As mentioned earlier, however, MOLGEN-MS only caters for 11 elements, whereas MOLGEN can take any element as input. Although it is possible to compute for more than one formula at once, it may be easier for the user to keep the formulae separate to assist in separating the candidates later on.

As well as requiring sufficient substructural information for structure generation to be effective, there are a number of other "tricks" that can be used to eliminate "unlikely" candidates. Carefully defining the valence states of atoms (if this is known) is important, e.g., multiple valence states of P and S can increase the number of candidates by several orders of magnitude. Additionally, as structure generators rely on mathematically possible structures, this includes all possible ring and double/triple bond possibilities. Thus, cases suspected to be highly unlikely in an environmental context could be eliminated (being EDA-based, one would assume the compounds responsible for toxicity would need to be reasonably stable in the environment). This could include, for instance, three- and four-membered rings, two consecutive double bonds, triple bonds within a ring, O–O bonds, etc. Many structure generators already have "unlikely" combinations saved in a permanent exclusion list that can be selected if desired. It is also possible to add further

Table 7 Comparison of the MOLGEN series of structure generators

Program	Features
MOLGEN 3.5	Basic structure generation, all elements, all valences (one at a time), fast calculation. User interface, for Windows. [26]
MOLGEN 5.0	More flexibility, command line only. Windows or Linux [27]
MOLGEN-MS	Streamlines basic structure generation with spectral interpretation. Only 11 elements, multiple valences in one calculation. Structure generation is orders of magnitude slower than MOGLEN 3.5 [30]

structures to these lists. A feature that is currently absent (as far as we know) and would be of great use in environmental applications is the criterion of aromatic planarity, i.e., that one could generate only flat aromatic structures. As it currently stands, any alkyl substituent could also form a “bridge” over the aromatic ring – which is relatively unlikely in environmental situations but results in the generation of thousands of additional potential structures. One way of eliminating these structures post-generation is discussed further below.

4 Candidate Selection

Once either the database search or structure generation has resulted in a number of possible candidates, further methods are needed to separate the likely from unlikely structures. As confirmation requires the use of an orthogonal method to prove the identity and/or purchase/synthesis of the compound, this can quickly be both expensive and time-consuming if several structural candidates are still possible. Thus, the sections below outline a number of methods that can be used in some cases, either in combination or in isolation, to assist in the elimination of structural candidates from consideration.

4.1 Prediction and Comparison of Spectra

An important method to match structures to an unknown spectrum is to predict the mass spectra of the potential structures and compare this with the experimental spectrum. General EI-MS fragmentation rules have been developed and published over several years and have been incorporated into a number of programs, both commercial and research-based releases. These have been extended in many programs to incorporate other forms of MS, such that the predictive capacities also extend to protonation and deprotonation, cluster ion formation, alkali metal adducts, and chemical ionizations. Several fragmentation options are then available for the user to select. In addition to general fragmentation rules, most programs also incorporate a library feature of some description, giving the user the choice of using the library developed by the software firm (where applicable) or also developing their own library. The more rules included in the calculation, the longer the calculation, so while this may improve the prediction, it also increases the time taken. Other options to improve the prediction in some programs include the number of reaction steps and the reactions limit. Similar to the library rules, including more steps and reactions not only improves the prediction (i.e., more fragments are predicted) but also increases the calculation time significantly. Selected programs containing some or all of these features are summarized in Table 8.

A recent comparison of three of these programs, ACD MS Manager [31], Mass Frontier [37] and MOLGEN-MSF [38] showed that although increasing these parameters may improve the prediction of the individual spectrum, it improved

Table 8 Selected programs available for the prediction and comparison of spectra

Program	Features
ACD MS Fragmenter	Part of most ACD suites (e.g., [31]). Predicts how well fragments of given structure matches uploaded spectrum (not spectrum independent). Comparison summarized visually and using the AQI value. Implementation of batch function requires programming in python [35].
FiD	Combinatorial approach to prediction of high-resolution tandem or MS^n spectra. Only tested on a limited number of compounds so far [36].
Mass Frontier	Spectral prediction based on structure fragmentation then comparison with spectrum. Basic and/or library fragmentation rules, visual demonstration of results (no calculated value). Batch function for fragmentation [37].
MOLGEN-MS	Spectral interpretation, structure generation, and spectral prediction all in one. 11 elements only. Graphical user interface for Windows automatically calculates results for all structures. Results summarized in a match value (MV), see Equation (2).
MOLGEN-MSF	The spectral prediction component of MOLGEN-MS as a stand-alone command prompt-based program [38]. Predicts spectrum of input structure(s) then compares predicted fragments to an experimental spectrum. Magnitudes taken from spectrum – see Equation (2).

this for all structural candidates, true or false, such that the increased calculation time is not necessarily warranted in terms of identifying unknowns by their spectrum – and in some cases even detrimental to the overall placement of the true structure in relation to the false [39].

While many programs predict spectra, a number of these limit their prediction to a mostly visual-based display of the results. Although this allows the user to see and interact with the results, it can be a very subjective way to judge the different structural candidates and becomes impractical with several structural candidates. Some programs, e.g., ACD MS Fragmenter (incorporated into ACD MS Manager and other packages) also calculate how well the predicted matches the experimental, in this case in the form of an Assignment Quality Index (AQI). There is little information, however, on how exactly this value is calculated and as such can only be used for the fragments predicted by the ACD software, excluding alternative programs. Although the AQI apparently takes into account peak magnitudes, the results for simple spectra are sometimes surprising [39] and should be interpreted cautiously. Other commercial releases, e.g., Mass Frontier refrain from performing this calculation, due to the uncertainties involved in estimating predicted peak magnitudes. MOLGEN-MSF uses a third way of assessing the predicted spectra, by assigning peak magnitudes to the predicted peaks based on the experimental spectrum. The "match values" generated [see (2)] can be calculated easily and thus used to compare predicted spectra from all programs that export their predictions in some form of text output.

$$\mathrm{MV} = 1 - \sqrt{\frac{\sum_m (I(m) - x(m)I(m))^2}{\sum_m (I(m))^2}}, \tag{2}$$

where MV is the match value, m is the mass-to-charge (m/z) ratio of the fragment, $I(m)$ is the intensity of the experimental mass spectral peak at m (scaled to the base peak to a value between 0 and 1) and $x(m)$ indicates the presence/absence of predicted fragments such that $x(m) = 0$ if there is no predicted fragment for m and $x(m) = 1$ if there is a predicted fragment for m.

Further details on this calculation are available elsewhere [39].

Once match values have been calculated for all possible structures, the structures can then be sorted from high to low match value, such that those with the highest match value are considered to be the "better" matches to the experimental spectrum, while those with lower match values are poorer matches and thus less likely to be the "correct" structure for the experimental spectrum. While this sounds easy enough, the predicted spectra are not very selective sorting criteria at this stage. Of the three programs mentioned above, the best case had the correct structure on average in the top 27% of all possible structures only, whereas with some options (e.g., more fragmentation steps) this increased above 50%. This was, however, without using substructure information to generate possible candidates, it remains to be seen whether inclusion of substructural information may improve this or in fact be detrimental.

In terms of excluding candidates, none of the spectral prediction methods are yet sufficiently accurate to be used exclusively to select possible candidates. However, it is useful in many cases to indicate whether all structures are a very poor match (e.g., if incorrect substructural information has been used) or to isolate structures with distinctly higher match values than others. In combination with other selection criteria outlined below, this provides some supporting information toward selection of possible candidates.

4.2 *Retention Properties*

The retention time of a compound is a common starting point to assist in confirming the identity of an unknown in GC–MS. This is typically expressed as a retention index (RI), which is less instrument- and parameter-dependent. The application domain of the two common indices varies according to the columns used and compounds investigated. The Kovat's RI (KRI) is based on the C_6- to C_{36}-alkanes, while the Lee RI (LRI) is based on four two- to five-ring PAHs [40]. RIs calculated from experimental data with the appropriate standards can be compared with documented RIs of known compounds and thus provide additional evidence to confirm structural identity where both the MS and RIs match. This strategy, based on the KRI, is currently implemented in the QPID database developed as part of MODELKEY.

The prediction of the RI of unknown compounds (i.e., without standards) requires specialized knowledge and a large computation effort [41], although generalized prediction strategies are available for both KRI and LRI. A compound class-based prediction of KRI is available for all compounds in the NIST database,

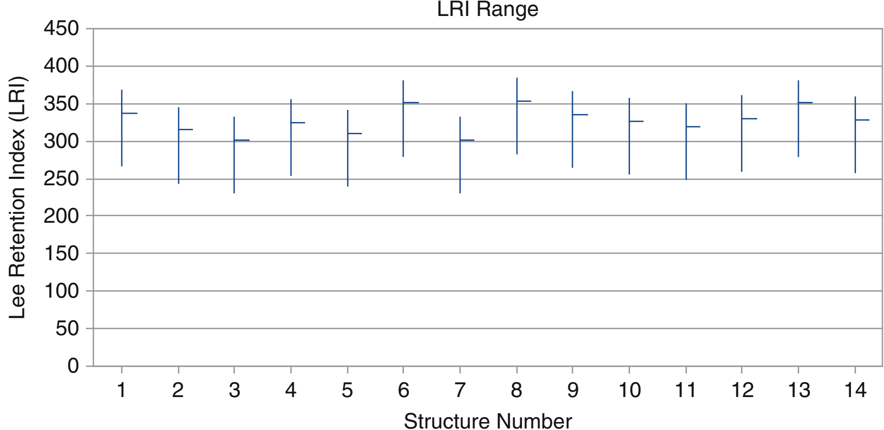

Fig. 3 Lee Retention Indices (LRIs) predicted from the boiling point and the associated range for 14 structures of formula $C_{12}H_{10}O_2$ [5]. The molecular structure and CAS numbers are given in Table 9

accompanied by an error interval and although this may not be useful for all possible structures resulting from structural generation strategies, this is of assistance in separating database matches. The errors in this prediction, however, are quite large and are often larger than the differences between RIs of similar compounds [5], see Fig. 3. This is most likely due to the fact that there are no clear quality criteria on the uptake of the retention data into these databases and these are often just taken from any literature source.

A prediction of the LRI based on boiling point (BP) data has been used by Eckel and Kind [41] to eliminate library matches for unknowns with BPs outside the range (LRI − 10) to (LRI + 50)°C. As BP predictions are generally more accurate and available for many structures (e.g., using EPISuite™ [42]) not just those within a database, this can be used to eliminate structures with retention properties markedly outside that of the unknown. As EPISuite™ has an average absolute error of 20.4°C, those compounds with BPs outside the range (BP−70.4) to (BP + 30.4)° C can be eliminated from further consideration [5]. As for the KRI prediction, these error margins are still quite large (see Fig. 3) and are more useful to assist in eliminating clearly incorrect structures rather than selecting the correct one.

4.3 Partitioning Properties

In a method similar to that described above, partitioning coefficients can also be used to eliminate structure candidates with very different properties to the unknown compound. The octanol–water partitioning coefficient (K_{ow}, often expressed in the logarithmic form log K_{ow}) is a general starting point, rating the hydrophilicity or lipophilicity of the compound. If the compound was found in a water sample, for

Table 9 Structure number, structure, and CAS number for the 14 molecules in Fig. 3

Structure number	Structure	CAS number	Structure number	Structure	CAS number
(1)		574-19-6	(8)		7028-41-3
(2)		713-68-8	(9)		21421-61-4
(3)		830-81-9	(10)		2197-57-1
(4)		56542-38-2	(11)		15971-29-6
(5)		1013-99-6	(12)		63509-76-2
(6)		19310-98-6	(13)		581-96-4

(*continued*)

Table 9 (continued)

Structure number	Structure	CAS number	Structure number	Structure	CAS number
(7)	O, OH	2459-25-8	(14)	O, CH_3, O	5409-32-5

example, highly lipophilic candidate structures (high log K_{ow}) may not be expected (depending on sample preparation and handling) and could thus be eliminated.

Where fractionation in EDA is undertaken using reverse phase high performance liquid chromatography (RP-HPLC) – using columns packed with a stationary phase containing long hydrocarbon chains (e.g., C_8, C_{18}) [43] – a correlation between the retention time and the log K_{ow} can be used to estimate the log K_{ow} range of each fraction and hence a range for the compounds within each fraction. The general relationship to determine the log K_{ow} for isocratic and linear gradient conditions is shown in (3), however deviations from a linear relationship may occur [43, 44]. The parameters A and B are determined by the linear regression of the logarithmic capacity factor k' of several standard compounds, calculated according to (4) [43]:

$$\log K_{ow} = A + B \times \log k', \tag{3}$$

$$k' = \frac{t_R - t_0}{t_0}, \tag{4}$$

where t_R is the retention time of the standard compound and t_0 the average time of solvent molecule passing the system obtained using an unretained organic reference compound (e.g., thiourea) [43].

Once A and B have been determined, the log K_{ow} range for each fraction can be calculated, which can be used to assign a log K_{ow} range for peaks within that fraction. Similarly to the boiling point calculation, the log K_{ow} for each candidate structure can also be calculated using EPISuite™ and thus used to eliminate structures widely outside the fraction range. There are, however, errors associated with the calculations and previous studies show that an error margin of at least ±1 unit was required for most cases, with greater margins needed for compounds with many functional groups [4]. Even the pH conditions in the biotest system should be considered for the estimation of log K_{ow} using RP-HPLC, because some compounds may be ionized at these pH conditions, such that prediction of the appropriate pH-dependent octa-nol–water distribution coefficient log D_{ow} using the quantitative structure-activity relationships (QSAR) in EPISuite™ is impossible [45]. In this case, other software must be used for the log D prediction such as ACD/Log$D$ [46].

Thus, as for RI data, partitioning coefficients are useful for eliminating structures with a broad range of values, but are less applicable in selecting the exact match where many structures come into consideration. Improvement to the prediction of partitioning coefficients, as well as separation techniques on different columns will increase the usefulness of this method immensely.

4.4 Abraham Parameters

Another, potentially more accurate way of predicting retention properties of compounds measured in both GC- and LC-systems are the Linear Solvation Energy Relationships (LSERs), established by Abraham and coworkers [47]. The equations account for relevant interactions between the analyte and the mobile and stationary phase, including nonspecific (e.g., van der Waals and cavity energy) and specific intermolecular interactions (e.g., electron donor/acceptor properties and dipolarity/polarizability). Each interaction term is a product of two descriptors, one representing the properties of the chromatographic system and the other the complementary properties of the analyte, as shown in (5):

$$\mathrm{SP} = aA + bB + sS + eE + vV + c, \tag{5}$$

where SP represents a solvent-dependent property such as the retention factor (log k), A the measure of the ability of the analyte to donate H, B the measure of the ability of the analyte to accept H, S the dipolarity/polarizability descriptor, E the excess molar refraction of the analyte, V the molecular volume from the McGowan algorithm, c a constant, the SP for a reference compound. a, b, s, e, v are regression coefficients for the chromatographic system (formed by stationary and mobile phases). See [48].

Although the equations were originally developed for isocratic/isothermal conditions, they can now be applied to nonisocratic/isothermal conditions [5]. Analyte descriptors are tabulated for around 2,000 compounds, while the system descriptors are determined by regressing the equations to around 50 experimentally determined sorption coefficients for a diverse set of calibration compounds with known compound descriptors. Where these conditions are met, the prediction accuracy is, in principle, sufficient to substantially reduce the list of potential structural candidates. This is, however, possible only with the experimentally determined compound descriptors, and as this is only available for a limited number of organic compounds, this is unlikely to help in the context of identifying unknown compounds.

As for RIs and partitioning coefficients, there are also methods for predicting the descriptors from the molecular structure, such that this could be calculated for all proposed structures. The incremental methods published by Platts et al. [49] have been shown to be too inaccurate for predictive purposes [50], but prediction based on quantum chemical approaches have shown promising results [50] and when

extended to all parameters may improve the applicability of this technique in separating candidate structures.

4.5 Additional Methods

4.5.1 Steric Energy

As discussed above, structure generation can often lead to mathematically possible but energetically unlikely structural candidates. While some of these can be excluded easily, others such as "bridging structures" formed during generation of polycyclic compounds are extremely difficult to eliminate objectively. Calculating the steric energy for all candidate structures could then allow elimination of these "unlikely" structures and reduce the candidate subset substantially. A suitable algorithm is, for example, MM2 [51], which is no longer available in original form but is implemented in slightly modified form in the Chem3D suite (forming part of ChemBioOffice [52]) offered by CambridgeSoft. Bulk calculations can be used to perform energy minimizations on all structural candidates, although molecules far from "normal" can pose a problem (which in itself indicates that the structure is unlikely). An example is shown in Fig. 4, which indicates that the "likely" candidates are within one order of magnitude, with the less likely structures having significantly higher energies. The values themselves are less important, for this context, than the differences between the "likely" and less likely.

Although other, more sophisticated energy calculations are available, they are correspondingly much longer and computationally intense. While this would lead to more accurate results in many cases, the additional computation time makes these less suitable as a quick filtering criterion as the calculations for hundreds of possible structures would then take days rather than hours. As the aim of the

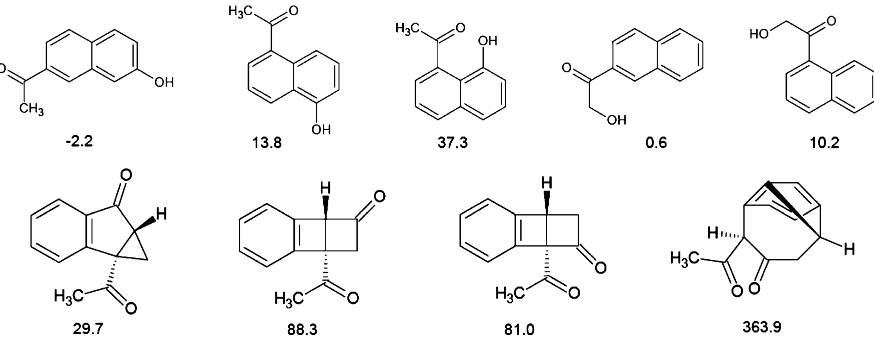

Fig. 4 Selected candidate structures for the formula $C_{12}H_{10}O_2$ with calculated steric energies using ChemBioOffice in kcal/mol

calculation is to separate highly unlikely structures from consideration, the quick MM2 algorithm should generally suffice.

4.5.2 Toxicity Information

Turning toward the biological side of EDA studies, it is possible in some cases to incorporate toxicity information from the biotest into the elimination or selection of possible structures. Structural alerts or toxicophores have been developed for a limited number of biotests, including *Daphnia magna* [53] and mutagenicity-based tests [54]. Structural alerts can be used to determine whether compounds may exhibit excess toxicity or baseline (narcotic) toxicity. Narcotic toxicity can be predicted based on the partitioning properties of the compound, so if a compound may exhibits toxicity well above that predicted from its log K_{ow}, it may be suspected of being excess toxic. Should this be the case, structural alerts compatible with the molecular formula can then be added (one-by-one where more than one possible exists) to the inclusion list during structural generation. If the opposite is suspected (i.e., no excess toxicity), these substructures could then be added to the exclusion list so that potentially excess toxic compounds are not generated.

4.5.3 Precursor Information

As some EDA studies involve known compounds (e.g., degradation studies), information on the precursors can be used to eliminate candidate structures incompatible with the precursors. If this information can be suitably expressed as substructures, this can be added to inclusion or exclusion lists during structure generation. A diclofenac degradation product was tentatively identified using such information and subsequently confirmed by synthesis and purchase of the standard and repeating the analysis [45].

4.5.4 Compound Class

Two-dimensional GC techniques have been shown recently to improve compound separation with the result that some distinct compound groups are formed [55]. This has been used in an EDA study of the Elbe basin to tentatively identify compounds and it is conceivable that this information could also be used to select or eliminate candidate structures to identify unknowns.

4.5.5 Spectral Families and Networks

As the mass spectral information generated from LC–MS methods are generally less reproducible using different instruments and therefore less predictable than, say,

EI-MS, computer methods have focused on ways of narrowing primary database hits. As compounds containing common substructures are likely to produce common fragment ions in tandem MS, all compounds sharing one fragment ion (within 5 mDa) can be classified into a family. Networks can then be formed by bridging components present in two or more families. Hao et al. used this approach to positively identify 87 compounds from a Chinese herbal medicine, without the availability of much structural information [56].

5 Summary/Conclusions

The identification of unknown compounds, especially those suspected to show significant toxic effects, is crucial to the successful completion of EDA studies. Without even a tentative identification available for suspected toxicants, full confirmation cannot take place. While EI-MS databases such as NIST and Wiley play an important role as a first step toward identifying compounds within a sample, the current contents of approximately 600,000 spectra does not even cover the numbers of chemicals released in the environment, let alone degradation products, metabolites and even natural products present. The availability of significantly larger open-access online databases (e.g., ChemSpider) will hopefully herald the start of a new open-access era in identification and data sharing. While increasing the size and access to databases is an important step in increasing the number of tentative identifications, the creation of databases containing unknown spectra is also important to allow stakeholders to get an impression of the distribution of some of these chemicals, rather than these falling into the "too-hard basket" as has tended to happen in the past. The QPID, EMPOMASS, and MassBank databases offer a way of storing these unknown spectra so that gradually a picture of the distribution and occurrence of these unknowns will appear and the availability of analytical information from more than one source may assist in the eventual identification of some of these chemicals. In order for these databases to be successful, data ownership issues need to be addressed to ensure that researchers have the benefit of sharing data without compromising their ability to publish their own data. Furthermore, uploading of data into these databases should be as simple as possible to ensure users are not put off by a time-consuming process. Supporting tools (e.g., standalone programs or plug-ins for spreadsheets) for the data processing and upload to the database are therefore required. It is also recommended that data processing of the mass spectra occurs in a commonly available nonproprietary mass spectra format (e.g., MSP, mzXML) to ensure a standardized delivery to the database without the loss of information.

If no matching database entries are available, structural generation provides an alternative method of identifying unknowns. Where sufficient substructural information can be obtained from the spectrum (e.g., for EI-MS), tentative identifications can be made, allowing further progress toward completion of the EDA study

[45, 57]. Substructural information is missing for many cases, however, such that further research is needed to improve the use, for EI-MS and other MS methods. The use of selection criteria mentioned above – as well as any others that bring any valid way of choosing the correct match from false – is very important in ensuring the usefulness of structure generation. Further research on the improvement of the various prediction techniques (e.g., partitioning behavior, Abraham parameters, etc.) will also help here.

Although many EDA studies are restrained by limited sample amounts, more than one analytical technique should be used where possible to obtain more information, even if this requires sending samples to an alternative laboratory. The development of consistent data formats for reporting data would help the transfer of information between laboratories, as many instruments save data in their own formats and export only limited data in text. Here again databases such as QPID, EMPOMASS, and MassBank, allowing upload and evaluation of information from a variety of sources, also address this issue.

As the advances of chromatographic-mass spectrometric systems result in the generation of much more information for structure elucidation within one instrument run, the development and testing of computer-based predictive techniques is necessary to allow the user the opportunity of comprehensively processing all the data obtained. The myriad of programs available promise many features to assist in identification but the users should investigate the results carefully rather than using these as black-box aids in structure elucidation as the results can sometimes be surprising. The more the results relating to unknown compounds expected to be potentially toxic during EDA/TIE studies are published and/or communicated, both successes and failures, the greater are the chances of successfully identifying these compounds.

Acknowledgments This chapter was supported by the European Commission through the Integrated Project MODELKEY (Contract No. 511237-GOCE). Our thanks go to Dr. M. Meringer, Prof. A. Kerber, and colleagues for on-going use and development of the MOLGEN series of programs, to C. Gallampois for the measurement data and to Dr. K. Thomas for reviewing the document.

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Effect-Directed Analysis of Mutagens in Ambient Airborne Particles

John L. Durant and Arthur L. Lafleur

Abstract This chapter reviews the major advances and challenges in effect-directed analysis (EDA) of mutagenic chemicals in ambient airborne particles. Mutagens are chemicals that can cause mutations – inheritable changes in the genetic code that can give rise to adverse health effects. The majority of studies dealing with EDA of mutagens in airborne particles combine liquid chromatographic fractionation of particle extracts with short-term mutagenicity assays and chemical analysis by gas chromatography–mass spectrometry. A variety of bacterial and human-cell lines with different metabolic capabilities have been used, allowing the measurement of different classes of chemical mutagens. The mutagens most frequently detected in non-polar and semi-polar fractions of airborne particles include unsubstituted polycyclic aromatic hydrocarbons (PAH) and substituted PAH, such as alkyl-PAH, nitro-PAH, hydroxynitro-PAH, nitro-PAH lactones, and PAH ketones. These compounds account for <20–25% of the total mutagenicity of unfractionated samples. The remaining mutagenicity is present in fractions containing more polar compounds. Analytical challenges that have slowed the pace of mutagen identification include the chemical complexity of particle extracts and their fractions, relative paucity of reference standards (particularly polar compounds), and interaction effects among sample constituents. The use of other genotoxicity measures, such as DNA-adduct formation and DNA damage, may help to identify the most important genotoxic compounds in airborne particles. Similarly, EDA studies of size-fractionated airborne particles may help to identify mutagens and other genotoxic chemicals in those size fractions most relevant to human health.

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W. Brack (ed.), *Effect-Directed Analysis of Complex Environmental Contamination*,
Hdb Env Chem (2011) 15: 199–236, DOI 10.1007/978-3-642-18384-3_9,
© Springer-Verlag Berlin Heidelberg 2011

Keywords Airborne particles, Bioassay-directed fractionation, Effect-directed analysis, Mutagen, Polycyclic aromatic compounds, Products of incomplete combustion

Contents

1 Introduction ... 200
2 Methods Used in EDA of Mutagens in Airborne Particles ... 201
2.1 Sample Collection ... 202
2.2 Sample Preparation ... 205
2.3 Fractionation ... 208
2.4 Mutagenicity Testing ... 211
2.5 Chemical Analysis ... 213
3 Mutagens Identified ... 217
3.1 Polycyclic Aromatic Hydrocarbons ... 218
3.2 Nitro-PAH, Hydroxynitro-PAH, and Nitro-PAH Lactones ... 219
3.3 Oxygen-Containing PAH ... 222
4 Analytical Challenges in the EDA of Airborne Particles ... 223
4.1 Identification of New Mutagens ... 223
4.2 Interaction Effects ... 225
5 Conclusions and Implications for Future Research ... 226
References ... 227

1 Introduction

It was discovered in the 1940s and confirmed many times since that chemical extracts of urban airborne particles can cause cancer in laboratory animals [1–6]. These findings have motivated research to identify individual carcinogens in airborne particles and thereby provide a better basis for understanding risks to human health as well as controlling sources of carcinogenic air pollution. However, urban airborne particles are composed of complex mixtures of thousands of different organic and inorganic compounds, the majority of which have not been identified. Furthermore, carcinogenicity assays often take months to complete. These two constraints have complicated efforts to identify individual carcinogens in airborne particles and necessitated the use of new tools that allow more rapid assessment of genotoxicity. One example is mutagenicity assays. Mutagenicity assays measure the ability of chemicals to cause mutations, inheritable changes in the genetic code that can eventually lead to cancer, and can be done in vitro over the course of a few days. To date hundreds of studies have reported using mutagenicity assays in analyzing airborne particles [7–9]. Another example is effect-directed analysis (EDA). EDA involves separating complex mixtures into chemical fractions, which are then tested for biological activity. The most biologically-active fractions are subjected to further fractionation and analysis. The goal is to facilitate the chemical analysis of complex mixtures by creating a small number of fractions that contain the majority of the biological activity of the sample, but which are relatively

free of non-biologically-active compounds that could interfere with chemical analysis. This methodology is often referred to as bioassay-directed chemical analysis or bioassay-directed fractionation [10–13]. By using mutagenicity as the measure of biological activity in EDA of airborne particles, many new mutagens have been identified [13–32].

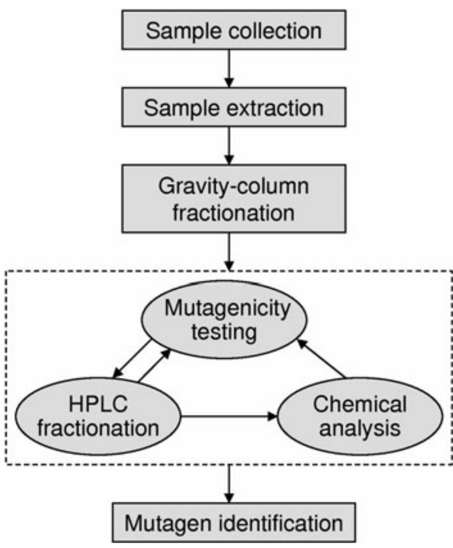
Recently, several very detailed review articles have appeared in the literature summarizing (1) efforts to characterize the genotoxicity of airborne particles in bacteria [8] and other cell lines [9, 33], and (2) advances in fractionation techniques and chemical analysis for EDA [34]. The objectives of this chapter are not to re-review this literature, but rather to focus specifically on the major advances and challenges in using EDA to identify mutagenic chemicals present in ambient airborne particles. We chose to focus our effort on particle-phase mutagens as opposed to gas-phase mutagens because more research has been done on EDA of particle-phase mutagens. Similarly, we place greater emphasis on mutagenicity as opposed to other genetic endpoints simply because studies involving EDA of mutagens are more abundant in the literature.

A systematic literature search in Pubmed and Science Direct was performed to trace studies related to EDA of mutagens in airborne particles. We used search phrases such as “mutagens in airborne particles”, “EDA of airborne particles”, and “bioassay-directed fractionation of airborne particles”, and then performed expert searches within the initial search results. This was supplemented by papers included in the reference lists of the traced papers and papers that were already known to us based upon previous EDA work. We only included papers in the English language. Although this should not be considered a comprehensive review of all the literature, we attempted to review most of the critical publications, both recent and historical. Primary emphasis was placed on reviewing studies involving EDA of particulate matter in outdoor ambient air; however, some discussion of combustion emissions was also included because many combustion-derived mutagens and mutagen precursors are present in ambient air.

2 Methods Used in EDA of Mutagens in Airborne Particles

EDA of environmental samples consists of three interconnected steps: (1) complex mixture fractionation, (2) biological testing, and (3) chemical analysis (Fig. 1). The ultimate goal of fractionation is to isolate individual bioactive chemical species within an individual; however, this is not routinely achievable for complex samples even when the most efficient chromatographic methods are used [35]. Although a number of greatly simplified fractions are usually obtained at the conclusion of a given separation protocol, most studies show that fractions typically contain multiple mutagens and thus require extensive chemical analysis. This section describes the methods commonly used in EDA of mutagens in airborne particles.

Fig. 1 General protocol for effect-directed analysis of mutagens in airborne particles. HPLC fractionation, mutagenicity testing, and chemical analysis of mutagenic fractions must often be repeated several times depending on the complexity of the samples



2.1 Sample Collection

EDA requires large amounts of airborne particles – on the order of several hundred milligrams or more of total particle mass – so that both biological and chemical analysis can be performed with the particles. Samples are typically collected by vacuum filtration onto pre-cleaned and pre-weighed filters, which are then stored frozen prior to EDA. Glass fiber filters are most often used because they have been demonstrated to be free of mutagenic artifacts [36, 37]. In some studies high-volume samplers ($\geq$ 300 L/min) have been run for several hours per day over the course of one or more days at individual sites until sufficient particle mass has been collected [18, 25, 38, 39]. In other studies, 24-h samples have been collected on a regular schedule (e.g., every 6th day) throughout the year to allow seasonal effects to be measured [29, 40–46]. While the majority of work has focused on EDA of airborne particles from urban and industrial areas, particles from rural areas have also been studied to characterize background conditions and measure rural–urban differences [27, 29, 41, 42, 47, 48]. As shown in Table 1, a range of different particle sizes has been analyzed. Total suspended particle (TSP) samples are commonly analyzed because they can be collected relatively easily and quickly. Size-segregated samples – PM_{15}, PM_{10}, PM_3, and smaller sizes – have been collected to allow EDA of particles in the respirable size range. There is considerable interest in sub-micron particles due to their toxicity. For example, Monarca et al. [49] analyzed individual size fractions (<10 μm, 0.5–10 μm, and <0.5 μm) of a size-segregated aerosol sample collected in an industrialized area in northern Italy and reported that the majority of the mutagenicity was contained in the smallest size fraction. Similarly, Pagano et al. [50] found that mutagenicity of

Table 1 Summary of EDA of chemical mutagens in particles from ambient air

Particulate material[a]	Fractionation method[b]	Cell line[c]	Chem. anal. method[d]	Mutagens identified[e]	Ref.
Southern California (USA); PM_{10}	NP-LC, NP-HPLC, RP-HPLC	TA98 (−S9)	GC–MS	N-PAH	[14]
Philadelphia (USA); $PM_{1.7}$	Silica gel CC, NP-HPLC	TA98 (+S9/−S9)	EI + NICI HR-GC–MS	HN-PAH, HN-PAK	[15]
Kokkola (FIN); PM_{15}	Silica gel CC	TA98, TA100 (+S9/−S9); SCE-CHO	HR-GC–MS	PAH, PAK	[16]
Southeast Michigan (USA); TSP	TLC	TA98, TA98NR, TA98DNP	HPLC-F	N-PAH, DN-PAH	[17]
Riverside, CA (USA); PM_{10}	NP-LC, NP-HPLC	TA98 (−S9)	GC–MS	N-PAL	[18]
Ohmuta City, Fukuoka City (JPN)*	NP-LC	TA98, TA1538 (+S9/−S9)	GC–MS	PAH	[19]
Washington DC (SRM 1649); TSP	SSE	TA98, TA100 (+S9/−S9)	GC–MS	PAH	[20]
Tokyo (JPN)*	NP-HPLC	TA98** (−S9)	GC–MS	N-PAL	[21]
Barcelona (ESP)*	GPC, NP-HPLC, RP-HPLC	TA98, TA98NR, TA98DNP (+S9/−S9)	GC–MS	PAH, PAK, N-PAH	[13]
Teplice (CZE); PM_{10}	ABP, NP-HPLC	TA98, YG1041 (+S9/−S9)	GC–MS, HPLC-F	PAH	[22]
Osaka (JPN)*	NP-HPLC, RP-HPLC	TA98, TA100 (+S9/−S9)	GC–MS	Azaarenes, PAH	[23]
São Paulo (BRA); PM_{10}	SSE, NP-HPLC	TA98 (+S9/−S9)	GC–MS	PAH	[24]
Urban and rural sites in Netherlands*	LLE, NP-LC, NP-HPLC	TA98, TA98NR, TA98DNP, TA100, TA97 (+S9/−S9)	HPLC-F HPLC-C	PAH, N-PAH	[25]
Osaka; TSP	None	YG1024 (−S9)	GC-NP, GC–MS	N-PAH	[26]
Flanders (BEL); PM_{10}	None	TA98 (+S9/−S9)	HPLC-F-UV	PAH	[27]
Los Angeles (USA); PM_3	NP-HPLC, RP-HPLC, SEC	Human cells (h1A1v2)	GC–MS	PAH, PAK, N-PAH	[28]
Eastern USA; PM_3. Washington DC (SRM 1649); TSP	NP-HPLC, RP-HPLC, SEC	Human cells (h1A1v2)	GC–MS	PAH, PAK	[29, 30]

(*continued*)

Table 1 (continued)

Particulate material[a]	Fractionation method[b]	Cell line[c]	Chem. anal. method[d]	Mutagens identified[e]	Ref.
Teplice, Prague (CZE); PM_{10}	None	TA98, YG1041 (+S9/−S9)	HPLC-F	PAH	[31]
Copenhagen (DNK); $PM_{2.5}$	Silica Gel CC	TA98, YG1041, YG5161 (+S9/−S9)	HPLC-F	PAH	[32]

[a] PM_x particulate matter $\leq x$ micrometers in diameter; *TSP* total suspended particles; *SRM 1649* standard reference mixture – urban airborne particles from Washington, DC. *Particle size not reported

[b] Multiple techniques for a particular study are listed in order that fractionation was performed. *CC* column chromatography; *SSE* sequential solvent extraction; *LC* liquid chromatography; *HPLC* high performance liquid chromatography; *NP* normal phase; *RP* reversed phase; *SEC* size-exclusion chromatography; *TLC* thin-layer chromatography; *GPC* gel permeation chromatography; *ABP* acid–base partitioning into acid, base, and neutral fractions; *LLE* liquid–liquid extraction

[c] See Sect. 2.4 for more information on some of these cell lines. *TA98*, *TA97 S. typhimurium* strains used for measuring different frameshift mutations; *TA100 S. t.* strain used for measuring base-pair substitution mutations; *YG1024 S. t.* strain that possesses high *O*-acetyltransferase activity; *YG1041 S. t.* strain that possesses high nitroreductase and *O*-acetyltransferase activity; *TA98NR*, *TA98DNP* nitroreductase deficient strains of *S.t.*; *+S9* tested in the presence of mammalian metabolizing enzymes; *−S9* tested without mammalian metabolizing enzymes; *SCE-CHO* sister chromatid exchange assay based on Chinese hamster ovaries; *h1A1v2* human white blood cells transfected with a plasmid coding for CYP1A1. **transfected with a plasmid that confers 3–100 more nitroreductase metabolizing enzyme activity than parent TA98 cell line

[d] *GC–MS* gas chromatography–mass spectrometry; *EI* electron ionization; *NICI* negative ion chemical ionization; *HR* high resolution; *F* fluorescence detection; *C* = chemiluminescence detection; *IC* ion chromatography; *GFAAS* graphite furnace atomic absorption spectrophotometry; *NP* nitrogen phosphorus detection; *UV* ultraviolet-visible light detection

[e] *PAH* polycyclic aromatic hydrocarbons; *PAK* polycyclic aromatic ketones; *HN* hydroxyl nitro; *N* nitro-substituted; *DN* dinitro-substituted; *PAL* polycyclic aromatic lactones

size-segregated urban airborne particles increased inversely with size, with <0.4 μm particles being the most mutagenic per unit mass. Particle size-dependent genotoxicity has also been reported in other studies [51–55].

2.2 Sample Preparation

The purpose of sample preparation in EDA of airborne particles is to extract or solubilize components of interest that are incorporated into the solid matrix of the particles. Once the components are in solution, they can be separated using liquid chromatography or transferred to other solvents appropriate for biological testing.

2.2.1 Soxhlet Extraction

The most frequently utilized method of sample extraction for EDA has been Soxhlet extraction [34]. Traditional use of Soxhlet extraction along with current trends in updating the technique has recently been reviewed by deCastro and Garcia-Ayuso [56]. With Soxhlet extraction, the choice of extraction solvent is an important consideration. Solvents with low boiling temperature ($<100°C$) (e.g., dichloromethane, methanol, acetone) are often used because concentration of the solvent extract by evaporation can be done at relatively low temperatures thus minimizing loss of volatile components. Factors other than temperature can also affect the choice of solvent. For example, aliphatic ketones such as acetone can undergo aldol condensation in the Soxhlet apparatus to produce a mixture of undesirable contaminants ranging from diacetone alcohol to 1,3,5-trimethylbenzene. Aldol condensation is common when acetone is used to extract soils and sediments because their mineral components can serve as catalysts for this reaction [57].

Soxhlet extraction has been compared with several newer techniques for the analysis of urban airborne particles and diesel exhaust particles [58–60]. These studies suggest that Soxhlet extraction is inadequate for the complete extraction of polycyclic aromatic hydrocarbons (PAH) and their derivatives from PM samples. Furthermore, even the newer and more aggressive extraction procedures are not 100% efficient in recovering PAH from carbon-based particles. This is important for EDA because PAH and their polar derivatives make up a significant fraction of mutagenic material in PM samples.

2.2.2 Alternative Extraction Techniques

The introduction of microwave-assisted extraction (MWAE) and pressurized fluid extraction (PFE) now opens the possibility of using a much wider range of solvents than before. Reviews of MWAE have appeared in the literature [61–64], and the technique has also been compared with other extraction methods including Soxhlet

extraction [65]. Although pressurized liquid extraction (PLE) is technically a subset of PFE, the two terms are often used interchangeably and refer primarily to the process of extracting samples by exposing them to solvent at high pressure and sometimes high temperature. Further complicating the nomenclature is the fact that instrumentation for PFE was introduced commercially over a decade ago and the manufacturer refers to its function as accelerated solvent extraction (ASE), a term currently used by a number of authors [66]. Applications of PLE have recently been reviewed by Perraudin et al. [67], Schantz [68], and Primbs et al. [69]. In addition to MWAE and PFE, other sample-extraction techniques have also been developed in the last decade, including subcritical water extraction (SWE), which is described in comparison with PFE in a recent review [70]. Other alternative extraction techniques (e.g., supercritical fluid extraction, solid-phase extraction, novel fluid-phase partitioning methods) have also been reviewed [71]. To date, these alternative techniques have been utilized primarily for target compound analysis or trace-level analysis and their use for EDA of airborne particles has yet to be described in the literature.

2.2.3 Sample Cleanup Methods for Mutagen Identification

The interpretation of spectral data is made easier if compounds of interest can be completely separated from interfering impurities prior to analysis. This is especially true for the identification of airborne PAH by mass spectrometry because their mass spectrum primarily consists of just two peaks (i.e., a molecular ion and a doubly-charged ion having half the mass-to-charge ratio as the molecular ion), whereas the vastly more abundant matrix of impurities in which they are found consists largely of aliphatic compounds producing abundant ions at a range of masses [72]. Interestingly, spectrophotometry with UV and visible light (UV–Vis) is unaffected by most of these impurities because aliphatics show little absorbance and do not generally interfere with the UV–Vis absorbance spectrum of PAH and other arenes. This is also true for fluorescence-based analysis because weak absorbance means weak fluorescence, so the effect of the aliphatic impurities on fluorophores of interest is negligible.

Researchers have had success in utilizing the multimode separation behavior of poly(divinylbenzene) (PVDB) HPLC columns to isolate mutagenic PAH fractions from airborne particle extracts. Although these columns can separate a wide range of compounds in a size-dependent mode, the use of dichloromethane as the eluent causes PAH to exhibit non-size retention and elute late in the chromatogram where they can be isolated as a single fraction [73]. An example of this multimode separation applied to the separation of a PAH fraction from diesel particles (SRM 1650) is shown in Fig. 2 [note that the total ion chromatogram (TIC) of the PAH fraction is much less complex than that of the unfractionated sample]. The PAH fraction consists mainly of unsubstituted planar PAH and their oxygenated and nitrated derivatives. Alkylated PAH generally elute earlier than the unsubstituted PAH and therefore can be collected in a separate fraction containing aliphatic impurities. The results of using this same PDVB cleanup procedure with urban airborne particles (SRM 1649) are shown in Fig. 2. Although the results obtained to

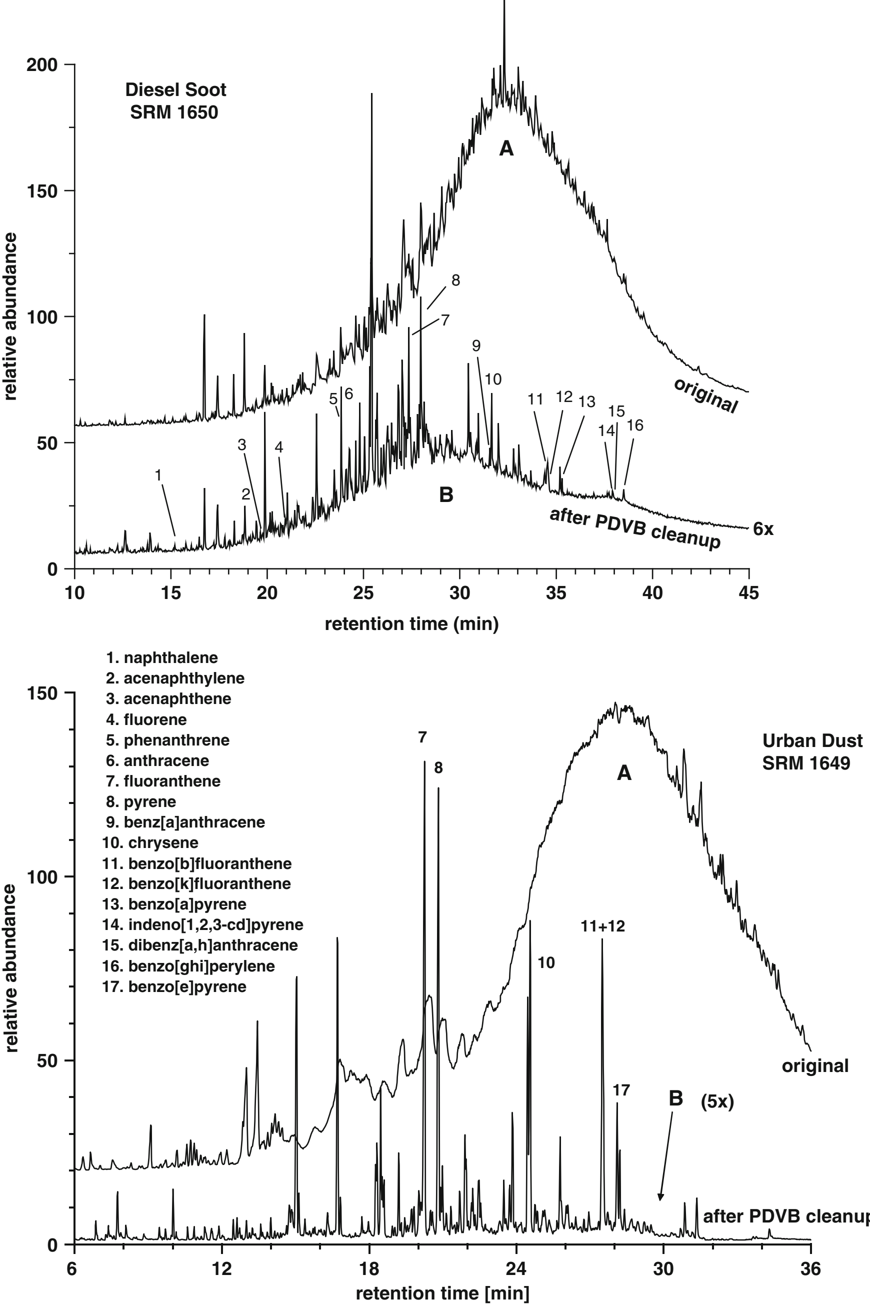


Fig. 2 *Upper panel* Curve A is the GC–MS total ion chromatogram (TIC) of a dichloromethane extract of SRM 1650 diesel particles. Curve B is the TIC of the PAH fraction obtained from the multimode separation of the extract on a poly(divinylbenzene) (PDVB) HPLC column. Peaks

date with PDVB look promising, more work needs to be done with a range of airborne particle samples to determine the separation efficiency and reproducibility of the method.

2.3 Fractionation

A wide range of fractionation methods have been used for the EDA of mutagens in airborne particle extracts (Table 1). Much of the literature describing these methods has been reviewed recently by Brack [35] and Marvin and Hewitt [34]. Often the first fractionation step is based on gravity-flow solid-sorbent columns and follows elution protocols designed to elute components of increasing polarity. These types of columns have been utilized for chromatographic separation of mixtures for over 50 years and they remain useful for a wide range of separations [74]. Newer chemically-bonded silica sorbents have largely replaced the traditional silica and alumina columns, and high-pressure liquid chromatography has replaced the gravity-flow separations [34, 35]. While a detailed description of the fractionation of extracts is beyond the scope of this work, we describe two practical examples of fractionation strategies for the separation of particulate matter samples. These examples highlight some of the challenges associated with complex mixture fractionation.

2.3.1 Fractionation Based on Alumina and Cyanopropyl Gravity-Column Chromatography

The fractionation and chemical analysis plan for an oil furnace combustion exhaust sample analyzed by Leary et al. [75, 76] is shown in Fig. 3. Particles were collected on glass fiber filters and Soxhlet extracted in dichloromethane. A four-solvent alumina fractionation was performed on the extract and the fractions were tested for mutagenicity in a Salmonella forward mutation assay [77]. Two bioactive fractions were obtained, X2 and X3. Fraction X2 contained 90% of the mutagenic activity of the whole sample and therefore underwent further separation, with normal-phase HPLC, to obtain a less complex fraction retaining all of the bioactivity (Fig. 3).

The X2-B and X3 fractions were chemically characterized using a range of techniques including HPLC with spectrophotometric UV–Vis detection, high resolution mass spectrometry (HRMS), and GC–MS. Based on the chemical analysis and bioassay data, it was determined that the majority of the mutagenic activity of X3 fraction was attributable to a single PAH ketone, phenalen-1-one. However, the

Fig. 2 (continued) 1–16 are the 16 EPA-listed PAH. This figure is modified from Jiao and Lafleur [74]. *Lower panel* Curve A is the GC–MS TIC of a dichloromethane extract of SRM 1649 urban dust; curve B is the TIC of the PAH fraction obtained from multimode separation of the extract on a PDVB HPLC column

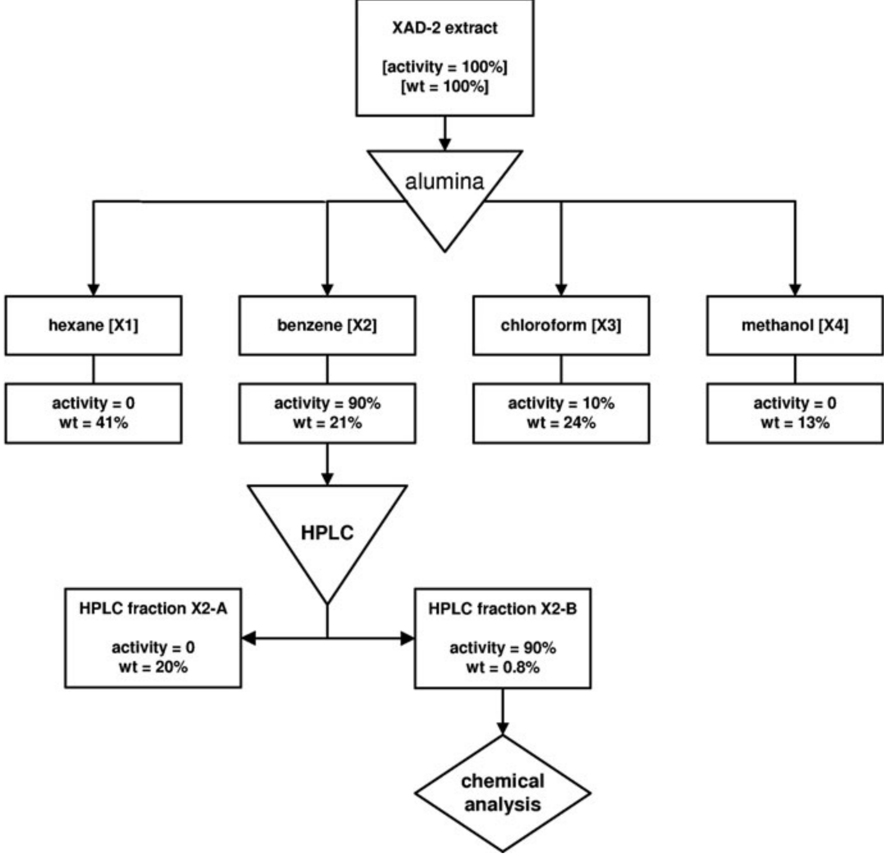


Fig. 3 Fractionation of a domestic oil furnace exhaust sample using alumina-column chromatography

more mutagenic X2-B fraction presented a separation challenge, one that is often encountered in EDA. The chemical analysis data showed this fraction consisted of a complex mixture of nitrated fuel components, predominantly alkyl-nitronaphthalenes, alkyl-nitrobiphenyls and alkyl-nitrophenanthrenes. Individual structures could be tentatively identified or approximated from the combined analytical data and by the consideration that the low ring number of the molecules observed limited the number of plausible molecular structures. However, conclusive identification of the individual components responsible for the mutagenic activity of the fraction was not performed because it would have required (1) mutagenicity tests of numerous reference compounds available only via organic synthesis, (2) HPLC separation of the fraction into its constituents and additional chemical analysis to determine peak purity, (3) mutagenicity testing of all single-component peaks to detect the mutagens, and (4) further separation of all peaks containing more than one component followed by repeating steps 1–3. This additional work was beyond the scope and budget of the study.

SRM 1649, an urban airborne particle reference standard, has often been used in the development and validation of protocols involving the EDA of mutagens [12, 20, 44, 78, 79]. For example, the first separation step in a fractionation scheme developed for the EDA of human-cell mutagens in SRM 1649 is shown in Fig. 4 [30]. This

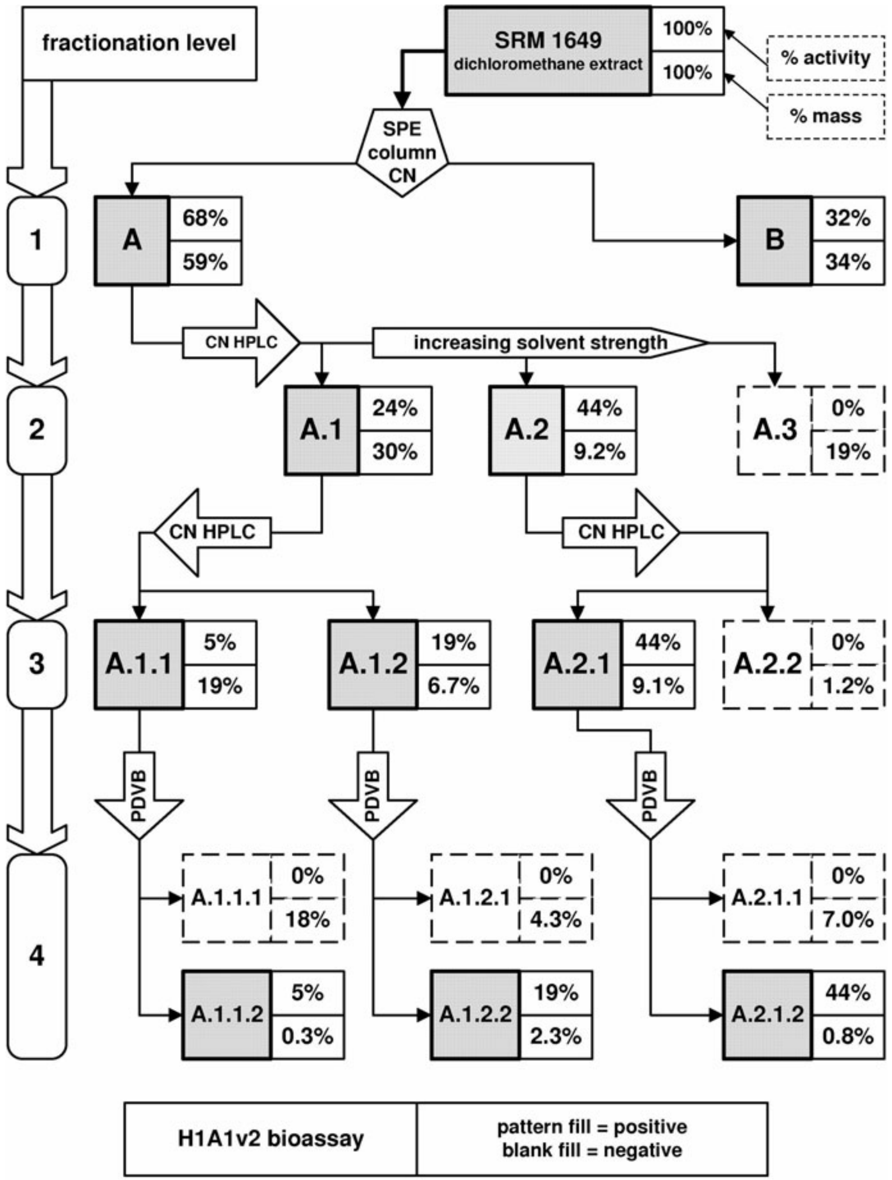


Fig. 4 Multi-level fractionation protocol for SRM 1649 (ambient airborne particles) using cyanopropyl-column chromatography. Modified from [30]

separation was performed using a 1-g gravity column packed with cyanopropyl-bonded silica sorbent. Cyanopropyl (CN) was used because it produces high recoveries of bacterial mutagenicity when compared with other common sorbents [80]. The goals of the first separation step were to (1) simplify the mixture by creating two fractions – A and B – of differing polarity, and (2) remove substances that could affect the performance of the HPLC separation used in subsequent steps.

The multi-level protocol for further separation of fraction A, which contained 68% of the mutagenicity of the sample, is shown in Fig. 4. The level-2 and level-3 fractions were created using a semi-preparative CN HPLC column; the level-4 fractions were created using a PDVB column. The sample naturally separated into three well-resolved parts; therefore, this unforced separation formed the basis for the generation of three fractions. Higher level fractions were generated in the same way. Most fractions exhibited mutagenic activity in the human-cell line used. Because of the high complexity of the level-4 fractions it was not cost-effective to isolate all of the individual mutagens.

Although the chromatographic isolation of individual mutagens has been demonstrated for some combustion samples (e.g., Thilly et al. [81]), airborne particle samples have proved generally much more chemically complex, and the chromatographic isolation of individual mutagens from these types of samples typically requires an exceptional amount of effort [35]. Instead of using chromatography to obtain increasingly greater numbers of fractions, many studies have generated a limited number of fractions followed by identification and quantification of known mutagens in each fraction using standard chemical analysis techniques [28–30]. This method requires access to databases of bioactive reference compounds associated with the samples. For example, Graedel et al. [7] and Claxton et al. [8] compiled a database for the more than 500 compounds found in atmospheric particles that have been tested for mutagenicity in Salmonella. Similarly, dozens of chemicals in combustion emissions and in airborne particles have been tested in human-cell mutagenicity tests [30, 81–89].

2.4 *Mutagenicity Testing*

This section describes the development and use of mutagenicity assays based on bacteria and human cells.

2.4.1 Bacteria Assays

The most widely used mutagenicity assays in EDA studies of airborne particles are based on various strains of the bacterium *Salmonella typhimurium* [9] – collectively referred to herein as Salmonella assays or Salmonella tests. Salmonella assays offer several advantages (1) they are relatively inexpensive and simple to perform, (2) they only require small amounts of sample material, (3) several different tester strains have been developed to allow characterization of a wide range of mutagens, and (4) over 70% of Salmonella mutagens are carcinogenic [90]. The most often

used Salmonella tests are reverse mutation assays based on histidine-dependent Salmonella strains that carry different mutations in genes coding for histidine [91]. When these mutant strains are grown in media containing low levels of histidine, only those bacteria that revert to histidine independence are able to form colonies. However, in the presence of mutagens, the number of revertant colonies is increased, typically in a dose-dependent manner.

Several Salmonella strains have been used in EDA of airborne particles (Table 1). For example, strain TA100, which measures base-pair substitution mutations, and TA97 and TA98, which measure frameshift mutations, are particularly sensitive to unsubstituted PAH [91, 92]. TA98NR and TA98/1,8-DNP_6 (or simply TA98DNP) are nitroreductase-deficient derivatives of TA98 that are highly sensitive to nitropyrene, dinitropyrene (DNP) and other nitro-PAH [93]. YG1024 and YG1041 are TA98 derivatives that contain high levels of *N*-hydroxyarylamine *O*-acetyltransferase activity and are thus very sensitive to aromatic amines and nitro-PAH [94, 95]. Because some chemicals (e.g., PAH, aromatic amines) require metabolic activation before they are able to react with DNA and bacteria lack the required metabolic capability, enzyme systems deriving from rodents are added to the assays. These enzyme systems consist of the supernatant fraction of rat liver cell homogenates that have been centrifuged at $9{,}000 \times g$ (S9). Airborne particle extracts are often tested in both the presence and absence of S9 to more fully characterize the range of mutagens present.

Salmonella reverse mutation assays can be done using the standard plate incorporation assay protocol in which airborne particle extracts, S9, growth media and $\sim 10^8$ bacteria cells are mixed together (~3 mL total liquid volume), and then poured onto plates containing glucose-agar medium. The plates are incubated for 48 h, after which colonies are counted [91]. To increase the sensitivity of the assay for detecting the low concentrations of mutagens typically present in airborne particle samples, Kado et al. [96] developed a preincubation step in which tenfold higher level of bacteria (10^9 cells) are mixed with particle extracts and S9 mix or a buffer (−S9) in a small volume (0.20 mL) for ~90 min. This step offers two advantages (1) the smaller volume results in higher effective concentrations of bacteria and sample; and (2) short-lived mutagenic metabolites have longer contact (reaction) time with bacteria compared to when the mixture is plated immediately. Kado et al. [96] reported a tenfold increase in the sensitivity of the microsuspension assay for detecting mutagens in particle extracts compared to the standard assay.

2.4.2 Human-Cell Assays

Human-cell assays have also been used – although less frequently compared to bacteria assays – for EDA of mutagens in airborne particles. For example, a mutagenicity assay based on human white blood cells (h1A1v2 cells) has been used in several studies [28–30, 42, 97, 98]. h1A1v2 cells are AHH-1 TK+/− cells to which two copies of cytochrome P4501A1 (CYP1A1) cDNA have been added [99, 100]. CYP1A1 is known to be important for the activation of many promutagens

and is constitutively expressed in h1A1v2 cells at levels ~50-fold higher than the basal level and ~threefold higher than the fully induced level of the parent cell line. h1A1v2 cells are sensitive to a variety of chemicals commonly found in airborne particles including PAH, nitro-PAH, nitro-PAH ketones, nitro-PAH lactones, and oxygenated PAH [88, 89]. In addition, testing of atmospheric transformation products has also been done in assays based on human MCL-5 cells [87, 89]. MCL-5 cells are genetically very similar to h1A1v2 cells (MCL-5 cells derive from L3 cells which derive from AHH-1 TK+/− cells); however, MCL-5 have been engineered to constitutively overexpress five cytochrome P450 enzymes (including CYP1A1) as well as microsomal epoxide hydrolase.

In the h1A1v2 assay, exponentially growing cells (initially 1.8×10^6 cells/mL) are incubated for 3 days in the presence of the sample material. Treatment is terminated by centrifuging the cells and resuspending them in fresh medium. One day later, the cultures are diluted to 2.0×10^5 cells/mL, and then allowed to grow for an additional 2 days without dilution to allow for the phenotypic expression of mutations. Next, the cultures are plated in 96-well microtiter plates in the presence (mutagenicity) and absence (colony forming efficiency) of the selective agent, trifluorothymidine, and incubated for 13 days and scored for the presence of a colony in each well. This assay protocol allows measurement of point mutations (i.e., base-pair additions, deletions, transitions, and transversions) and other events (e.g., nonlethal recombination and chromosomal loss) leading to the loss of heterozygocity at the *thymidine kinase* gene locus in h1A1v2 cells. Additional details of the assay are described elsewhere [84, 85].

2.5 Chemical Analysis

2.5.1 Gas Chromatography–Mass Spectrometry

Perhaps the most common chemical analysis method used for EDA of airborne particles is gas chromatography–mass spectrometry (GC–MS). It is a widely available method that can be adapted to a broad range of compound types [34, 35]. The development of GC–MS instrumentation has been reviewed from a historical perspective [101].

PAH were some of the first airborne chemicals suspected of being mutagens and carcinogens [72], and are now known to be major contributors to the mutagenicity of airborne particles [33, 79]. For the past several decades, much effort has been devoted to their characterization using a range of GC and GC–MS techniques [72, 102]. Many studies have shown that fractions containing polar PAH-derivatives are responsible for a significant percentage of the total mutagenicity of airborne particle samples [8, 28–30, 98, 103]; therefore, GC–MS analytical methods have been adapted for the characterization of more polar analytes [34, 35].

HRMS has also been used either as a stand-alone technique utilizing some form of direct sample introduction or in combination with gas chromatography (GC-HRMS)

[75]. Nitrated PAH (N-PAH) pose a greater analytical challenge than unsubstituted PAH because they are inherently more labile and more difficult to separate via gas chromatography. The preponderance of the work involving the identification of N-PAH in airborne particles did not involve EDA studies; however, most of the methods developed for N-PAH analysis would be applicable to EDA of N-PAH. A comprehensive introduction to the analytical chemistry of N-PAH has been compiled by White [104]. A more recent review of the formation, occurrence and analysis of environmental N-PAH has also been published [105]. Another review discusses state-of-the-art negative ion chemical ionization (NICI) mass spectrometric methods, such as GC–NICI–MS–MS, for N-PAH analysis [106]. Application of NICI–MS techniques to the analysis of nitrated and oxygenated PAH present in airborne particles and in SRM 1649 are described by Albinet et al. [107] and Bezabeh et al. [108].

2.5.2 High-Performance Liquid Chromatography

GC–MS techniques have proven very useful for the characterization of PAH with $\leq$ five aromatic rings. However, larger PAH are more difficult to analyze by GC–MS for two main reasons: (1) as the aromatic carbon number (C_n) increases, the number of isomers increases dramatically (e.g., there are 16 $C_{20}H_{12}$ isomers and 64 $C_{24}H_{14}$ isomers [72]); and (2) multiple isomers often do not readily separate by GC causing considerable overlapping of peaks. As an alternative to GC-MS analysis, HPLC with spectrophotometric diode-array detection (HPLC-DAD) can be used to identify individual PAH, which possess highly characteristic UV–Vis spectra [109, 110].

The HPLC-DAD chromatogram of an ethylene combustion sample is shown in Fig. 5. The separation is based on a ternary non-aqueous reversed-phase method developed by Fetzer and coworkers [111, 112]. It can be seen that C_n is related to the elution volume in a relatively simple manner. However, it should be noted that this correlation works well here because the PAH in the sample were generated at a high temperature (1,300 K) in a research combustor [113, 114], and these conditions result in flame-generated PAH with similar length-to-width (aspect) ratios near unity. The effect of aspect ratio on reversed-phase separation of PAH has been the subject of numerous studies [72, 115, 116]. If the full range of isomers were present for every possible PAH species, there would be a significant overlap between adjacent PAH species at every carbon number. An example of how UV–Vis spectral data can shed light on the structures of isomeric PAH is illustrated in Fig. 6, where spectra are shown for the three dicyclopentapyrene (DCPP) isomers in Fig. 5 (peaks A, B, and C). The availability of reference standards made the identification of the DCPP isomers possible without the need for other analytical techniques [117]. The HPLC-DAD data were displayed as a total-absorbance chromatogram because it was observed that the total sum of the absorbance bands for a range of PAH was generally proportional to the amount of PAH present, and that therefore this parameter would make it possible to approximate the amount of

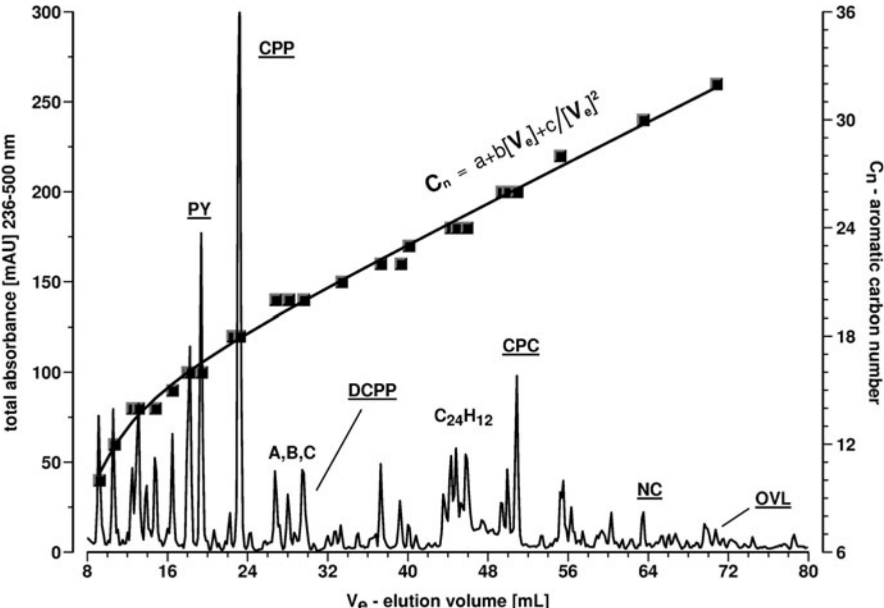


Fig. 5 HPLC-DAD total absorbance chromatogram of a combustion effluent from a research combustor. *PY* pyrene, *CPP* cyclopenta[*cd*]pyrene, *DCPP* dicyclopentapyrene isomers, $C_{24}H_{12}$ isomers of coronene, *CPC* cyclopenta[*bc*]coronene, *NC* naphtho[8,1,2-*abc*]coronene, *OVL* ovalene. The three DCPP isomers are as follows: A = dicyclopenta[*cd,fg*]pyrene, B = dicyclopenta [*cd,mn*]pyrene and C = dicyclopenta[*cd,jk*]pyrene

novel PAH present in a sample when reference standards are unavailable [118]. The most abundant PAH constituent of the combustion-generated sample in Fig. 5, cyclopenta[*cd*]pyrene (CPP), is a very potent mutagen and carcinogen, and it has been widely reported in combustion effluents and ambient airborne particles [28, 30, 81, 82, 86, 119–122]. Despite this, CPP is not included as one of the US EPA 16 priority PAH, but it is included as one of the European Union 15+1 priority PAH.

Another useful method for identifying PAH in particle samples is atmospheric pressure chemical ionization–liquid chromatography mass spectrometry (APCI–LC–MS) [123]. Figure 7 shows the APCI–LC–MS TIC of an ethylene combustion sample whose corresponding HPLC-DAD chromatogram is shown in Fig. 5. It is seen that there is good agreement between the two chromatograms in terms of the peaks present and their relative sizes. Therefore, both methods provide a good approximation of the relative abundance of PAH in the sample. The APCI method used to obtain the data in Fig. 7 utilized a heated pneumatic nebulizer (HPN) interface [117]. With the HPN interface, PAH are characterized by the production of a single pseudo-molecular ion consisting of the protonated parent molecule having a mass 1 unit higher than the PAH nominal molecular weight. By comparison, simple molecular ions (radical cations) created in the absence of the HPN are much less abundant and thus more difficult to detect and quantify. The use of HPN–APCI–LC–MS for the analysis of PAH has been described by Anacleto et al. [124].

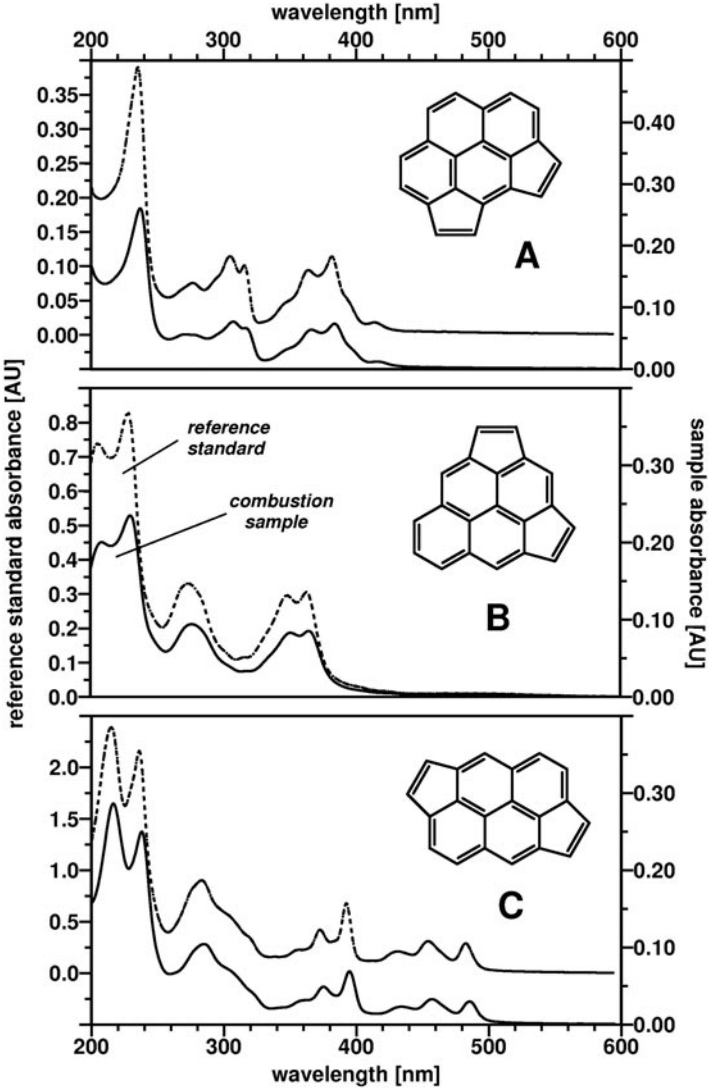


Fig. 6 UV–Vis spectra for three dicyclopentapyrene isomers (see DCPP triplet at 28 mL in Fig. 5) from an ethylene combustion sample. These spectra were obtained on-the-fly using HPLC-DAD

APCI–LC–MS allows for the detection of PAH with molecular mass exceeding 1,000 Da [117]. These large PAH are beyond the tolerable volatility range of standard GC–MS analytical methods. However, it has been demonstrated recently that GC columns and operating conditions can be optimized to extend the mass range of quantifiable PAH to include the important carcinogenic PAH isomers at 302 Da [125] as well as PAH with molecular mass up to 376 Da [126]. Much recent work has been aimed at developing improved LC–MS methods for nonpolar

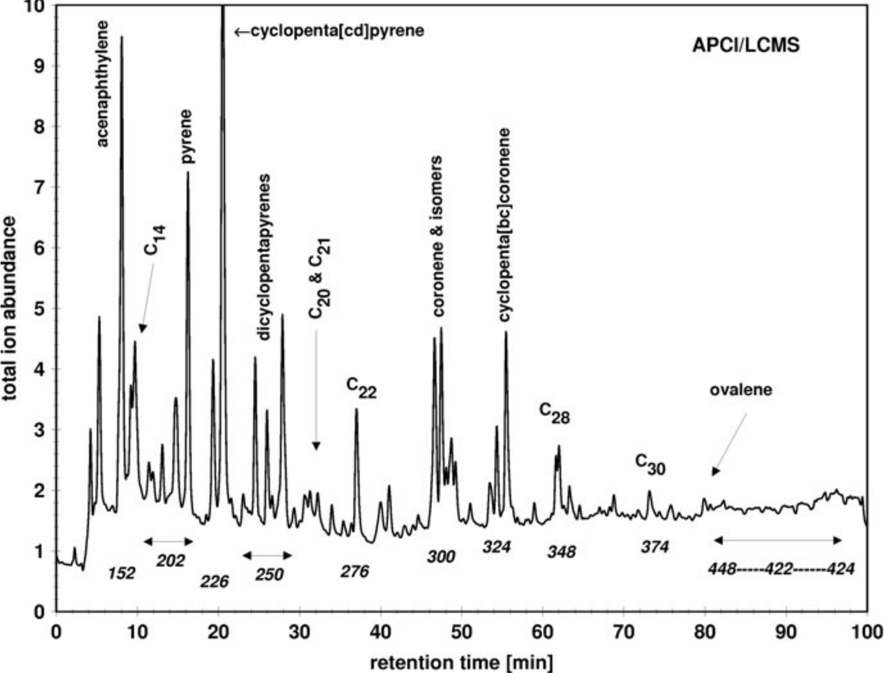


Fig. 7 APCI–LC–MS total ion chromatogram of the research-combustor effluent shown in Fig. 5. Numbers in italics are the molecular weights (g/mol) of the compounds under the corresponding peaks

analytes (reviewed in Hayen and Karst [127]). Also, work has been done in the development of atmospheric pressure photoionization (APPI) as an ionization method for LC–MS [128, 129].

In addition to analysis of nonpolar compounds, LC-based methods are also useful for analysis of polar compounds. For example, polar PAH derivatives found in AP have been characterized by LC–MS–MS [130]. Also, organic acids in ambient aerosols have been analyzed using HPLC coupled with a time-of-flight mass spectrometer utilizing negative-ion electrospray ionization [131]. These methods should be readily adaptable to EDA as suggested by Moriwaki [132], who reviewed the use of LC–MS in the analysis of polar (and nonpolar) environmental mutagens.

3 Mutagens Identified

The mutagens most frequently detected in the EDA of airborne particles include PAH, nitro-PAH, hydroxynitro-PAH, nitro-PAH lactones, and PAH ketones (Table 1, Fig. 8). In most studies, these compounds account for <20–25% of the

Cyclopenta[*cd*]pyrene Benzo[*a*]pyrene Dibenzo[*a,e*]pyrene

7*H*-Benz[*de*]anthracen-7-one 6*H*-Cyclopenta[*cd*]pyren-6-one

3-Nitro-7*H*-benz[*de*]anthracen-7-one 2-Nitro-6*H*-dibenzo[*b,d*]pyran-6-one

Fig. 8 Structures of selected mutagenic polycyclic aromatic compounds found in airborne particles

total mutagenicity of the unfractionated samples, while the remainder is attributed to fractions containing semipolar and polar compounds. As noted in the reviews by Gradel et al. [7] and Claxton et al. [8] many aliphatic organic compounds, cycloalkanes, monocyclic and bicyclic aromatic hydrocarbons, and halogenated organic compounds have been detected in airborne particles, but with few exceptions these compounds are nonmutagenic. This section reviews the major classes of mutagens identified by EDA of airborne particles.

3.1 Polycyclic Aromatic Hydrocarbons

It is well known that many PAH are mutagenic and carcinogenic [133]. Indeed, it was discovered in the early days of chemical carcinogenicity research in the 1930s that benzo[*a*]pyrene, a common constituent of airborne particles, is a particularly

potent carcinogen [134]. Formed by the incomplete combustion of organic matter, PAH are present in particulate emissions from a variety of combustion processes and as a result are widely distributed in the environment. The majority of EDA studies in Table 1 report that PAH, both unsubstituted and alkyl-substituted, account for a relatively small fraction of airborne particle mutagenicity. Pedersen et al. [29] and Durant et al. [30] reported that PAH accounted for 15–20% of the human-cell mutagenicity (h1A1v2 cells) in airborne particles from Boston and Washington DC (SRM 1649); Hannigan et al. [28] found that six mutagenic PAH accounted for as much as 6% of the h1A1v2-cell mutagenicity of airborne particles from Los Angeles. Similar results have been achieved with Salmonella assays in the presence of metabolizing enzymes (S9). For example, Du Four et al. [27] reported that PAH accounted for only 3% of the +S9 mutagenicity (TA98) in airborne particles from rural, urban, and industrial areas in Belgium, while de Raat [25] found that PAH in airborne particles from cities in the Netherlands accounted for as much as 5–20% of the +S9 mutagenicity depending on the Salmonella strain used. The most commonly identified mutagenic PAH in airborne particles are four- to six-ring compounds including chrysene, cyclopenta[*cd*]pyrene, benzo[*a*]pyrene, benzofluoranthenes, indeno[1,2,3-*cd*]pyrene, benzo[*ghi*]perylene, dibenzo[*a,e*]pyrene, and naphtho[2,1-*a*]pyrene (Fig. 8). Although PAH with $\leq$3 rings are often abundant in airborne particles, few have been identified as potent mutagens. Due to the challenges of analyzing large PAH in airborne particles (i.e., poor chromatographic separation, high number of isomers, low levels), PAH with >6 rings have yet to be ruled out as being important mutagens.

Additional evidence that PAH contribute significantly to airborne particle genotoxicity – as measured by DNA adduct formation and DNA damage – is reported in several recent studies (Table 2). Binkova et al. [31] and Sevastyanova et al. [135] found that the PAH content of extracts of PM_{10} was highly correlated with DNA adduct levels in different cell types exposed to the extracts. In particular, Binkova et al. [31] showed that eight four- to five-ring PAH in PM_{10} extracts accounted for as much as 48% of the measured DNA adducts. de Kok et al. [54] observed that PAH levels correlated with both DNA adducts and oxidative DNA damage in fish cells exposed to extracts of airborne particles. Interestingly, Gutierrez-Castillo et al. [136] found that metals in airborne particles played a role in causing DNA damage, and studies by Sharma et al. [32] and de Kok et al. [54] indicated that metals and PAH in airborne particles may interact in causing DNA damage. Taken together, these studies and the others summarized in Table 2 indicate the value of non-mutagenicity-based assays in measuring the genotoxicity of PAH and suggest a role for DNA-adduct and DNA-damage testing as complements to mutagenicity testing in the EDA of airborne particles [32, 54].

3.2 *Nitro-PAH, Hydroxynitro-PAH, and Nitro-PAH Lactones*

Several EDA studies have shown that nitro-PAH, hydroxynitro-PAH, and nitro-PAH lactones, which are potent mutagens in Salmonella assays, are present in airborne particles. For example, Lewtas et al. [137] reported that nitro-PAH contributed as much

Table 2 EDA of airborne particles based on DNA-damage, DNA-adduct and toxicity assays

Particulate material[a]	Bioassay[b]	Chem. anal. methods[c]	Genotoxins identified[d]	Results[e]	Ref.
Mexico City (MEX); PM_{10}, $PM_{2.5}$	DNA damage in A549 human lung epithelial cells	IC, GFAAS, GC–MS	V, Ni, Cu, PAH	Water extracts caused more DNA damage than DCM-extracts Transition metals were a factor in DNA damage	[136]
Catania (ITA); PM_{10}, $PM_{3.5}$, $PM_{1.2}$, $PM_{0.42}$, $PM_{0.14}$, $PM_{0.05}$	Chromosomal aberrations in CHEL cells	GC–MS, GC–NCI–MS	PAH	HIGHEST levels of PAH were found in 0.14–1.2 m particles Genotoxicity likely due to $PM_{1.2}$	[55, 137]
Prague (CZE), Kosice (SVK), Sofia (BGR); PM_{10}	DNA adducts in human HepG2, HEL and THP-1 cells	^{32}P-postlabeling	PAH	DNA adduct levels were highly correlated with PAH levels in particles Higher DNA adduct levels in winter	[138]
Prague, Kosice, Sofia; PM_{10}	DNA adducts in acellular calf thymus DNA (+S9/−S9)	^{32}P-postlabeling	PAH	DNA adduct levels were highly correlated with levels of 8 PAH in particles	[39]
Teplice (CZE) + Prague; PM_{10}	DNA adducts in acellular calf thymus DNA; chick embryotoxicity	^{32}P-postlabeling	PAH	8 PAH accounted for 30% and 48% of DNA adducts measured in summer and winter samples, respectively	[31]
Teplice + Prachatice (CZE); PM_{10}	DNA adducts in rodent cells	^{32}P-postlabeling	PAH, N-PAH	Highest DNA adduct levels were found in nonpolar (PAH) and polar (N-PAH) fractions Three–four-fold higher DNA adduct levels in winter	[139]
Maastricht (NLD); TSP, PM_{10}, $PM_{2.5}$	DNA adducts + oxidative DNA damage in fish cells (+S9/−S9)	^{32}P-postlabeling, HPLC-EC, ICP-AES, GC–MS	Metals, PAH	PAH levels correlated with adducts + oxidative DNA damage Interaction of PAH + metals correlated with DNA adduct formation	[54]

Copenhagen (DNK); $PM_{2.5}$	DNA damage, cytokine expression + cytotoxicity in A549 human lung epithelial cells	ICP-MS, HPLC-F	PAH	PAH and metals levels correlated with DNA damage and cytokine expression	[32]

[a] *PM_x* particulate matter $\leq x$ micrometers in diameter; *TSP* total suspended particles
[b] *+S9*= tested in the presence of mammalian metabolizing enzymes; *−S9* tested without mammalian metabolizing enzymes; *CHEL* Chinese hamster epithelial liver; *HepG2* a hepatoma cell line; *HEL* diploid lung fibroblasts; *THP-1* acute monocytic leukemia cells
[c] *GC–MS* gas chromatography–mass spectrometry; *NCI* negative chemical ionization; *HR* high resolution; *F* fluorescence detection; *IC* ion chromatography; *GFAAS* graphite furnace atomic absorption spectrophotometry, *EC* electron capture detection, *ICP-AES* inductively coupled plasma, atomic emission spectrometry; *^{32}P-postlabeling* method for detecting DNA adducts and other DNA lesions that involves enzymatic digestion of a DNA sample followed by enrichment of the adducts, radiolabeling the adducts with ^{32}P-orthophosphate, chromatographic separation of labeled adducts, and quantification by radioactive decay
[d] *PAH* polycyclic aromatic hydrocarbons; *N* nitro-substituted
[e] *DCM* dichloromethane

as 6% of the −S9 mutagenicity (TA98) of SRM 1649. Arey et al. [14] found that 1–8% of mutagenicity of samples from Torrance and Clairmont (California) was attributable to specific mono-nitrofluoranthenes and mono-nitropyrenes. de Raat et al. [25] reported that 13–24% of the −S9 mutagenicity (TA98) of particles from The Netherlands was attributable to mononitro-PAH. Siak et al. [17] reported that ~50% of the −S9 mutagenicity (TA98) of samples from the Detroit area was attributable to fractions containing nitro-PAH, and that as much as 3% of this mutagenicity was attributable to 1-nitropyrene and 1,6- and 1,8-dinitropyrene. Helmig et al. [18] reported that the nitro-PAH lactone, 2-nitro-6*H*-dibenzo[*b,d*]pyran-6-one (2-NDBP; Fig. 8), an atmospheric transformation product, could account for nearly 50% of the total −S9 mutagenicity (TA98) of samples from Claremont, CA. Nishioka et al. [15] reported that hydroxynitro-PAH, in particular hydroxylated nitropyrenes and nitrofluoranthenes, were among the most important mutagens (TA98) in samples from Philadelphia.

In contrast, in EDA studies involving human h1A1v2 cells much less of the mutagenicity of particle samples is attributable to nitro polycyclic aromatic compounds [28–30]. This may be due to differences in the types and amounts of metabolizing enzymes present in h1A1v2 cells and the various Salmonella assays used in EDA studies. *S. typhimurium* naturally produce reductase enzymes that are very efficient in converting nitro-PAH into aryl-hydroxylamines, the ultimate mutagenic metabolites of nitro-PAH [93]. Rosenkranz and Mermelstein [138] observed that 1-nitropyrene, 1,3-dinitropyrene, 1,6-dinitropyrene, and 1,8-dinitropyrene were 200–100,000 times more mutagenic in TA98 (−S9) than benzo[*a*]pyrene, a potent +S9 mutagen. When 1-nitropyrene and the three dinitropyrene isomers were tested in strains deficient in nitro-reductase (TA98-NR) and dinitro-reductase (TA98-DNP) enzymes, their mutagenicity was substantially reduced compared to the parent cell line (TA98) [139]. Likewise, when these same nitro-PAH were tested in Salmonella assays in the presence of S9, a tenfold reduction in the mutagenicity of 1-nitropyrene and about a 1,000-fold reduction in the mutagenicity of the dinitropyrenes was observed [140–142]. However, it should be noted that human-cell lines have different sensitivities to nitro polycyclic aromatic compounds. For example, Busby et al. [84] showed that mono- and dinitropyrenes are highly mutagenic to MCL-5 cells. Also, Phousongphouang et al. [89] observed that 3-nitro-7*H*-benz[*de*]anthracen-7-one (Fig. 8) and a mixture of other nitropyrene lactones induced different mutation frequencies depending on which cell line (MCL-5 and h1A1v2) was used and which gene was targeted, and Grosovsky et al. [143] reported very similar findings for 2-NDBP. Thus, as was observed in EDA studies involving Salmonella, the importance of nitro polycyclic aromatic compounds as human-cell mutagens in airborne particles is dependent on which cell lines and assay protocols are used.

3.3 Oxygen-Containing PAH

Compared to the relatively large number of EDA studies that have led to the identification of mutagenic PAH and nitro polycyclic aromatic compounds in

airborne particles, fewer studies have identified individual mutagenic oxygen-containing PAH (oxy-PAH). This is due in part to the chemical complexity of fractions containing oxy-PAH as well as the lack of commercially available, oxy-PAH reference standards. Another factor is that few of the most abundant oxy-PAH identified in mutagenic fractions are potent mutagens. For example, Durant et al. [30] reported that a semi-polar fraction of SRM 1649, which accounted for ~50% of the h1A1v2-cell mutagenicity of the sample, contained many different classes of oxy-PAH including ketones, quinones, coumarins, and carboxylic acid anhydrides. However, of the chemicals identified in this fraction only three PAH ketones – phenalenone, 7*H*-benz[*de*]anthracen-7-one, and 6*H*-benzo[*cd*]pyren-6-one – accounted for a measurable percentage (~0.5% total) of the mutagenicity of the sample. Very similar results were reported by Hannigan et al. [28] and Pedersen et al. [29] who used the same EDA methods in analyzing particle samples from Los Angeles and Massachusetts (USA), respectively. Likewise, oxy-PAH do not appear to be significant mutagens to Salmonella strains. With the exception of benzopyrene quinones [144, 145] and certain low molecular weight oxygen heterocycles, which are not widely reported in airborne particles [8], very few oxy-PAH are mutagenic in Salmonella assays.

4 Analytical Challenges in the EDA of Airborne Particles

This section describes some of the analytical challenges in identifying airborne particles.

4.1 Identification of New Mutagens

When an EDA fractionation effort results in the creation of a mutagenic fraction that contains just a few suspected, but unknown mutagens, what additional effort is required to unequivocally identify the isolated components and to determine their mutagenicity? The answer depends primarily on (1) the availability of high purity reference compounds, (2) the complexity and time needed to perform the selected mutagenicity assay, and (3) the number of isolated unknown compounds. Some light can be shed on the scope and nature of the required effort by considering the steps taken to fully characterize just one unknown component in SRM 1649 [30]. The four-part fractionation of this sample was described in Sect. 2.3 and the fractionation protocol is illustrated in Fig. 4.

During our work on the identification of polar mutagens in SRM 1649, it was found that one component (compound **I** in Fig. 9) accounted for about approximately 50% of the total mass of the most mutagenic fraction (fraction A.2.1.2 in Fig. 4). The structural elucidation of **I** was tentatively made by GC–MS, but component **I** could not be unequivocally identified nor assessed for

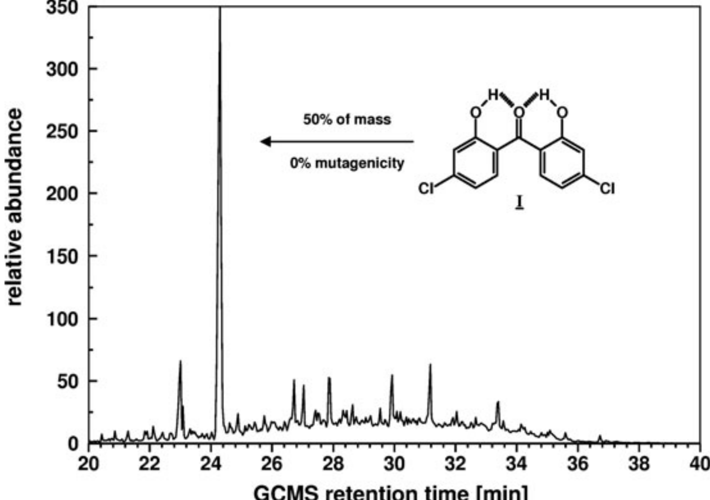


Fig. 9 Component of interest in a level-4 fraction (fraction A.2.1.2) of SRM 1649 (see Fig. 4)

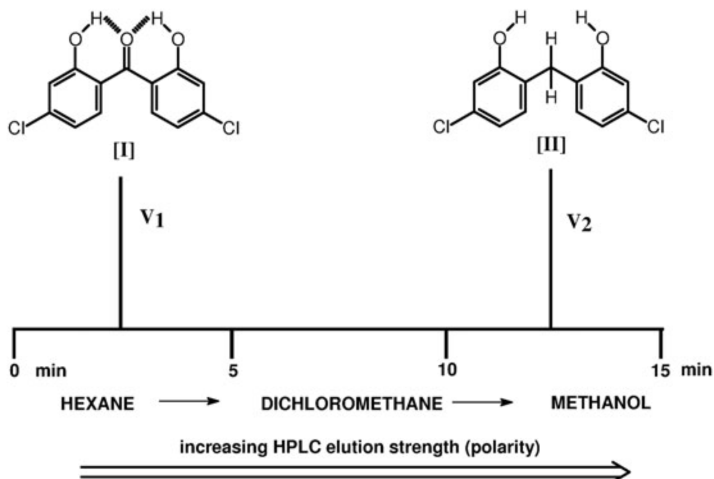


Fig. 10 Effect of intramolecular *H*-bonding on apparent polarity of components of a polar fraction (A.2.1.2) of SRM 1649 (see Fig. 4)

mutagenic potency because a reference standard was not available. This component was ultimately identified as 4,4′-dichloro-2,2′-dihydroxy-benzophenone (DDB), a keto derivative of dichlorophen(methylenebis[4-chlorophenol], CAS No. 97-23-4), whose structure is shown as **II** in Fig. 10. Dichlorophen, a commercial fungicide, was identified in a more polar fraction (Fraction B) of SRM 1649. After a time-consuming effort to synthesize a reference standard of DDB, it was subjected to mutagenicity testing in h1A1v2 human cells, but it was found to be completely

non-mutagenic. That meant component **I** was not the hoped-for major mutagen and that the potent mutagenicity observed for this higher-order (level-4) subfraction was most likely due to the presence of one or more minor components.

An important result of this extensive work – which involved the isolation, identification, synthesis, and mutagenicity testing of an unknown component – was the realization that chemical mixtures in airborne particle samples were more complex than previously thought, and that strategies that worked so well with sediment [146] and combustion [81] samples are likely insufficient for identifying mutagens in airborne particle samples containing low levels of hundreds of potential mutagenic components. New strategies need to be developed to facilitate finding the most important mutagens in these samples in a more efficient way.

Further complicating the identification of unknown mutagens is the difficulty involved in predicting how a mixture of apparently similar components will separate into fractions. Highly complex airborne particle samples may not strictly follow empirical separation rules for chromatography [147]. For example, increasing solvent strength might not always yield fractions with increasing number of polar substituents. The more complex the molecule, the more unpredictable the elution behavior can become. This fact is illustrated in Fig. 10. The benzophenone derivative, DDB (**I**), has three polar substituents, a carbonyl group and two hydroxyl groups, while dichlorophen (**II**) has only two hydroxyl groups. Nevertheless, DDB behaves as a much less polar compound in liquid chromatographic separations. The reason for this becomes clear when the structures of the two compounds are examined. The effect of the carbonyl substituent is seen as a means by which the DDB molecule can undergo intramolecular hydrogen bonding, thus nullifying much of the chromatographic polarity normally expected by the presence of two hydroxyl groups.

4.2 Interaction Effects

Another challenge in identifying individual mutagens in airborne particle extracts is interaction effects – inhibition and enhancement of mutagenicity due to interactions between sample constituents. For example, Iwado et al. [148] reported that the TA98 (±S9) mutagenicity of airborne particle extracts was substantially higher following fractionation on blue cotton. It was hypothesized that long-chain fatty acids (palmitic, stearic, oleic, and linoleic acids) in the fraction that did not sorb to the blue cotton inhibited the mutagenicity of PAH in the fraction that sorbed to the blue cotton. Iwado et al. suggested that the fatty acids inhibited the mutagenicity of the PAH by trapping them in miscelles and/or by interfering with metabolizing enzymes necessary for PAH to exert their mutagenicity. Similar inhibition effects were observed by Hermann [149], who tested benzo[*a*]pyrene (B[*a*]P) in TA98 (+S9) in binary mixtures of different PAH. Hermann found that at high doses some PAH (e.g., benzo[*e*]pyrene and naphthacene) inhibited the mutagenicity of B[*a*]P. Interestingly, Hermann [149] also observed that the mutagenicity of B[*a*]P was

enhanced (i.e., the response was greater than additive) when tested along with relatively low doses of these and other PAH. Hermann hypothesized that both the inhibition and enhancement effects were attributable to enzymes involved in PAH metabolism. These kinds of interaction effects are difficult to detect and quantify, and could lead to inaccurate estimation of the mutagenicity of particle extracts and fractions as well as individual mutagens. Therefore, the results of these two studies highlight the importance of employing fractionation methods that allow separation of individual mutagens and nonmutagens in complex mixtures.

5 Conclusions and Implications for Future Research

A considerable amount of work has been done in the past 35 years to develop artifact-free sampling and fractionation methods for detecting organic mutagens in airborne particles. In addition, many different cell lines and mutagenicity testing protocols have been developed allowing identification of a variety of mutagens. The most commonly identified mutagens include PAH and PAH derivatives, some of which are atmospheric transformation products. However, despite these advances only about 20–25% of mutagenicity in samples has been attributed to known mutagens. The remaining mutagenicity appears to be in polar fractions containing oxygenated and nitrated PAH and other polar organic compounds. Identification of mutagens in polar fractions has been slowed due to challenges imposed by the chemical complexity of these fractions and the lack of readily available chemical standards required to conclusively identify new mutagens.

Using the current EDA paradigm, large amounts of airborne particles need to be collected at each sampling site, extracted, and fractionated into as many fractions as possible to avoid interaction effects and isolate individual mutagens. The fractions must then be tested for mutagenicity and refractionated and retested if necessary, and then chemically analyzed. Finally, suspected mutagens must be found from commercial sources or synthesized and tested for mutagenicity. Because airborne particles in the submicron size range ($PM_{1.0}$) are generally much more mutagenic than larger particles, emphasis should be placed on EDA of mutagens in this size range. In light of these considerations and the chemical complexity of airborne particles, further significant advances in the EDA of mutagens in airborne particles will likely require focused and sustained efforts by interdisciplinary teams that include aerosol scientists, analytical chemists, toxicologists, and synthetic chemists.

Application of emerging tools, such as automated bioassays and computational methods, may help to accelerate the pace and productivity of EDA studies. For example, Brinkman and Eisentraeger [150] describe the development of an automated bioassay system for measuring genotoxicity in Salmonella strain TA1535/pSK1002. The system contains a programmable robotic pipetting station that allows rapid throughput of prepared samples. Although such systems have yet to be perfected for routine analysis, it would appear their development is not far off.

Computational methods may be used to identify mutagens in complex mixtures. Foremost among these is quantitative structure-activity relationships (QSAR), a method (principally developed by Hansch several decades ago) to predict reaction rates based on knowledge of chemical structure [151]. QSAR methods have undergone significant improvement since their inception [152, 153], and are now at the stage where they could be considered for inclusion in EDA experiments. For example, in a recent EDA study to identify mutagenic compounds in ground water, Meinert et al. [154] analyzed MS spectra of chemicals in a ground water sample using a computer-based structure generation tool called MOLGEN-MS to identify unknown compounds. In addition, a model for mutagenicity prediction (ChemProp) was used to identify candidate mutagens. Using this combination of methods, a total of ten compounds tentatively identified in the sample were predicted to be mutagenic. Although none of the ten was actually found to be mutagenic based on testing of authentic standards, this study suggests that combining traditional EDA with computational methods has the potential to yield more rapid characterization of genotoxicants in complex samples compared to EDA alone. In another study, Eide et al. [155] developed structure-mutagenicity models based on GC–MS data and mutagenicity testing (TA98/−S9) of whole (unfractionated) extracts of combustion-derived particles. The models were developed using multivariable linear regression and the model with the highest correlation had an r^2 of 0.62 based on 41 variables. Work is currently being done to improve the predictive capabilities of computational methods and thereby obviate the need for detailed EDA studies. Seen in this light, EDA is perhaps just one step on the pathway leading to complete characterization of complex mixtures based solely on computerized analytical methods. However, until such methods are developed and shown to be reasonably accurate, research is needed to elucidate the most important chemical toxicants in complex mixtures, and EDA is presently one of our best tools for achieving this goal.

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Effect-Directed Analysis of Endocrine Disruptors in Aquatic Ecosystems

Corine J. Houtman, Juliette Legler, and Kevin Thomas

Abstract The topic of endocrine disruption in the aquatic environment is a clear example of a problem-driven research area. Field observations of endocrine abnormalities in wild life have prompted the growth of scientific attention and concern about the topic. Multiple studies have reported the presence of endocrine disrupting activities in various compartments of the aquatic environment, without, at the time, knowing the cause of the observations.

The application of effect-directed analysis (EDA) has shown to be a valuable approach in investigating the nature of the compounds responsible for endocrine disrupting activities in environmental samples. Various research groups have applied EDA approaches and thereby successfully identified compounds responsible for endocrine disrupting effects. The research field of endocrine disruption is thus one of the research areas that has extensively experienced the benefits of EDA. This chapter describes the issue of endocrine disruption in the aquatic environment and discusses examples of the application of EDA for the identification of responsible compounds.

Keywords 17a-ethynylestradiol, Androgen, CALUX, Estradiol, Feminisation, Glucocorticoid, Hormone, Progesterone, Thyroid-hormone, Yeast Oestrogen Screen

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W. Brack (ed.), *Effect-Directed Analysis of Complex Environmental Contamination*, Hdb Env Chem (2011) 15: 237–266, DOI 10.1007/978-3-642-18384-3_10,

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Contents

1 Endocrine Disruption 238
1.1 Endocrine Disruption: A Threat for Wildlife and Humans? 238
1.2 Examples of Endocrine Disrupting Compounds and Their Effects in Wildlife 239
2 Oestrogenic Compounds 240
2.1 Oestrogenic Effects in Wildlife 240
2.2 Oestrogenic Hormones, Functions and Mechanism 241
2.3 Compounds with Oestrogenic Activity 242
3 Other Types of Endocrine Disruptors 244
3.1 (Anti-)androgenic, Progestagenic and Glucocorticoid-Like Compounds 244
3.2 Thyroid Hormone-Like Compounds 245
4 EDA Methods for the Analysis of Endocrine Disruptors in Aquatic Matrices 245
4.1 Bioassays for the Detection of Endocrine Disrupting Compounds 245
4.2 Effect-Directed Analysis 247
4.3 How to Deal with Mixture Effects in EDA 249
5 Examples of EDA Studies on Endocrine Disrupting Compounds 250
5.1 Oestrogens in STP Effluents and Process Water Samples 250
5.2 Oestrogens in Fish Bile and Gastrointestinal Content Samples 251
5.3 Oestrogens in Sediment 252
5.4 Oestrogenic Compounds Identified in the Environment 253
5.5 Androgenic Compounds and Anti-androgenic Compounds 254
5.6 Progestagens and Glucocorticoids 255
5.7 Thyroid Hormone-Like Compounds 255
6 Evaluation of Effect-Directed Analysis Approaches for the Identification of EDC 256
6.1 Advantages of EDA for EDC 256
6.2 Aspects to be Developed Further 256
6.3 Future Applications of EDA Approaches 257
References 258

1 Endocrine Disruption

1.1 Endocrine Disruption: A Threat for Wildlife and Humans?

In the early 1990s, alarming adverse effects on male reproduction in wildlife species were reported. Impaired fertility and associated population declines were observed for a wide range of wildlife species, including seagulls [1, 2], panthers [3], turtles, frogs [4] and fish [5]. Such observations were collectively named 'endocrine disruption', and led to the hypothesis that these could have arisen from exposure of wildlife to chemicals in the environment (endocrine disruptors) capable of interfering with the physiological processes under hormonal (endocrine) control [6, 7].

In 1996, a European Union Workshop on this topic was held in Weybridge, UK. One of the main outcomes was agreement on the definition of an endocrine disruptor:

> An endocrine disrupter is an exogenous substance that causes adverse health effects in an intact organism, or its progeny, secondary to changes in endocrine function [8].

In addition, the Weybridge Report also expressed concerns about human reproductive health [8]. In humans, adverse health trends affecting the reproductive organs of both males and females had been reported. For males, lowered sperm counts, increased incidences of other reproductive disorders related to infertility (hypospadias and cryptorchidism) and testicular cancer were associated with exposure of children to endocrine disruptors prior to or after birth [9]. In women, a link between environmental contaminants and increased incidences of breast cancer was suggested [10]. Although insufficient evidence was available to definitively establish a causal link between adverse health effects in humans and exposure to endocrine disruptors, trends were serious enough to raise concerns [11–13]. These concerns were heightened further following the publication of the book 'Our Stolen Future', by Colborn and co-workers [14]. Endocrine disruption is a topic to which much research effort is now being dedicated [15–20].

1.2 Examples of Endocrine Disrupting Compounds and Their Effects in Wildlife

Hundreds of different chemicals have been identified as potentially having endocrine-disrupting effects. Endocrine disruption can occur via multiple mechanisms and in different life stages. For instance, contaminants have been shown to alter: (1) hormone production at its endocrine source, (2) the release of stimulatory or inhibitory hormones from adrenal glands such as pituitary or hypothalamus, (3) enzymatic biotransformation of hormones and (4) free hormone concentrations in serum by alteration of concentrations or functioning of transport proteins [21]. This paragraph gives a few examples of field observations that were linked to exposure to endocrine disrupting chemicals. Different classes of endocrine disrupting compounds and their effects are discussed separately in the next sections.

Organochlorine pesticides are perhaps the most widely known examples of endocrine disruptors. The abundantly applied insecticide dichlorodiphenyltrichloroethane (DDT), for which in 1948 the Swiss scientist Paul Müller was rewarded with a Nobel Prize, appeared to cause feminisation of male birds [1, 2]. Also, its metabolite *p,p′*-DDE inhibited prostaglandin synthesis leading to eggshell thinning in birds and thus in reduced reproductive success [22]. An extensive spill of *p,p′*-DDE into Lake Apopka, Florida, resulted in a wide range of reproductive and other abnormalities, such as reduced penis size and altered testis structure in alligators [23].

One of the most dramatic illustrations of endocrine disrupting effects in the environment concerns tributyltin. This compound, the active ingredient of antifouling paint used on ships, exhibited masculinising or androgenic effects in female molluscs, particularly the growth of penis and the occlusion of the oviduct by the development of a vas deferens [24]. The mechanism behind this condition, referred to as imposex, is the inhibition by tributyltin of the enzymatic formation of the female hormone estradiol from the male hormone testosterone, allowing high levels

of the latter to prevail. The resulting reduced fertility led to severe declines in the numbers of molluscs in the North Sea [25].

2 Oestrogenic Compounds

2.1 Oestrogenic Effects in Wildlife

The most notable example of endocrine disruption in the aquatic environment concerns the so-called feminisation of male fish. In wild fish populations, a high occurrence of ovotestis (or intersexuality, a condition in which oocytes are formed in the testicular tissue, Fig. 1) and other testicular abnormalities have been observed in rivers, coastal waters and estuaries in the United Kingdom [26–29] and fresh-waters sites in other countries [30–33]. Remarkably, intersexuality was most often seen in waters receiving effluents of sewage treatment plants (STPs). Investigations further showed that male trout and carp produced the egg yolk protein vitellogenin (VTG) when exposed to STP effluent [5, 34]. This protein is normally produced in response to oestrogens only by mature female fish and is a very sensitive and specific biomarker for oestrogen exposure [35]. The observed oestrogenic effects in fish have thus been associated with exposure to oestrogenic chemicals. These compounds could be natural oestrogenic hormones, synthetic analogues thereof or xenobiotic chemicals, capable of mimicking the action of the female oestrogenic hormone and thereby disturbing the internal endocrine balances in fish. Indeed, the presence of oestrogenic compounds in STP effluents has been demonstrated

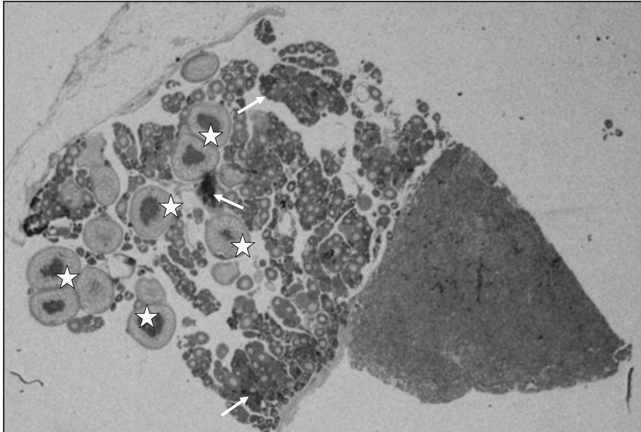

Fig. 1 Histological section of an intersexual gonad of male bream (Abramis brama). Primary oocytes (*asterisks*) are found in male gonadal tissue (*arrows*) (courtesy of Anton A.M. Gerritsen, Deltares, The Netherlands)

[36, 37]. Fish exposure to (xeno-) oestrogens in laboratory experiments, as extensively reviewed by Mills and Chichester [38], supported the hypothesis that oestrogenic compounds in the aquatic environment can cause feminisation of various fish species.

2.2 Oestrogenic Hormones, Functions and Mechanism

Oestrogenic hormones, also referred to as female steroid hormones, are steroidal molecules structurally based on the phenanthrene ring. They are produced from cholesterol primarily in the ovaries of females in response to signals from the brain or other organs and, albeit in lower quantities, in testes of males. The main naturally occurring oestrogens in all classes of vertebrates are 17β-estradiol (17β-E2), 17α-estradiol (17α-E2), estrone (E1) and estriol (E3). Their chemical structures are given in Fig. 2. Oestrogens play pivotal roles in sexual development and are, e.g. responsible for the development of feminine secondary sexual characteristics, control of reproductive cycles and fertility [39]. In addition, they also have functions in the nervous system, vasculatory system and in the regulation of bone density [40].

In most fish species, sex is genetically determined. Sexual differentiation of the gonads is believed to be comparable with the mammalian situation, in which the presence or absence of a testis determining factor directs female or male differentiation. Steroid hormones are involved in the subsequent expression of secondary characteristics. However, it has long been known that exposure to steroid hormones at critical developmental life stages can reverse the sex from its genetic predisposition to a different phenotype [41]. This shows the critical role steroid hormones may have in these processes. Another major role of oestrogens in oviparous (egg-laying) fish is to stimulate the production of VTG in the liver. This precursor of egg yolk is subsequently stored in the oocytes. Normally, only in mature females are oestrogen levels high enough to induce VTG, but environmental exposure to oestrogenic chemicals can trigger this response in juvenile females as well as males [38]. Clearly, inappropriate exposure to oestrogens, in the wrong animal, or at the wrong time in the life cycle, or at the wrong concentrations, may adversely influence critical processes.

Oestrogens act by a receptor-mediated mechanism depicted in Fig. 3. They are transported to their target organs bound to transport proteins such as albumin and sex hormone binding globulins. After dissociation from these proteins, free hormones can enter cells by diffusion through the cell membrane. In the cell, hormones can bind to oestrogen receptors. In fish, two subtypes of oestrogen receptors (ER) have been identified, ERα and ERβ, with ERβ existing in the functional forms ERβ1 and ERβ2 [42]. Oestrogen receptors differ in their ligand binding affinity, transcriptional capacity and distribution among tissue types. The main tissue types in which they are expressed are brain, pituitary, liver and gonads [42]. After binding of an oestrogen to the ER, a homodimer of two receptor–oestrogen complexes is

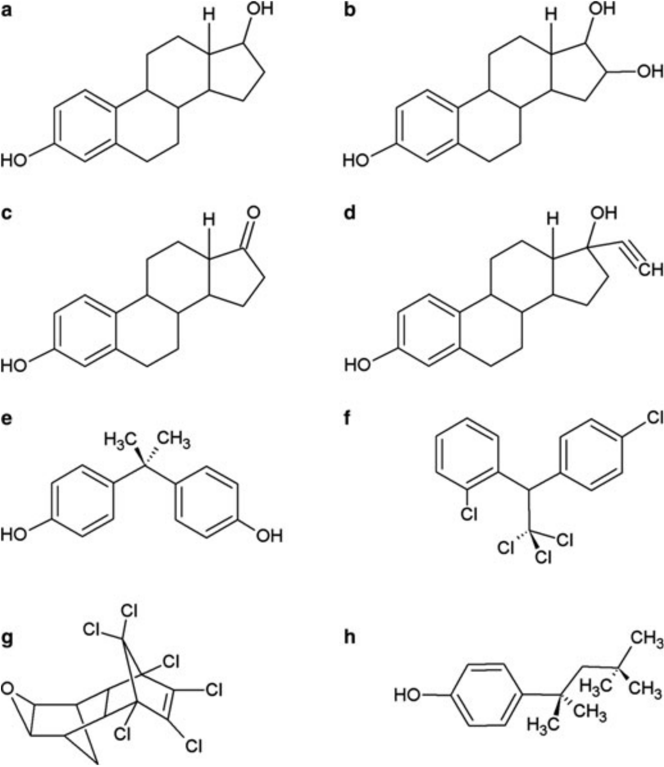

Fig. 2 Molecular structures of the natural estrogenic hormones (**a**) 17β-estradiol, (**b**) estriol, (**c**) estrone, the synthetic oestrogen (**d**) 17α-ethynylestradiol, the xeno-oestrogens (**e**) bisphenol A, (**f**) *o,p′*-dichlorodiphenyltrichloroethane, (**g**) dieldrin, and (**h**) 4-*tert*-octylphenol

formed and translocated into the nucleus, where the complex binds to the DNA at oestrogen responsive elements (ERE), the regulatory regions of oestrogen-responsive genes. Once bound to the ERE, the homodimer complex recruits transcription factors to the target gene promoter, which leads to increased gene transcription to messenger RNA. The production of proteins, e.g. VTG, following translation of messenger RNA results in the ultimate effect that oestrogens can have on cellular functioning and on physiology.

2.3 Compounds with Oestrogenic Activity

The natural oestrogenic hormones have strong affinities for the oestrogen receptor, with 17*β*-E2 being the most potent natural oestrogen. In addition to the endogenous ER ligands, affinity for oestrogen receptors has been found for large numbers of

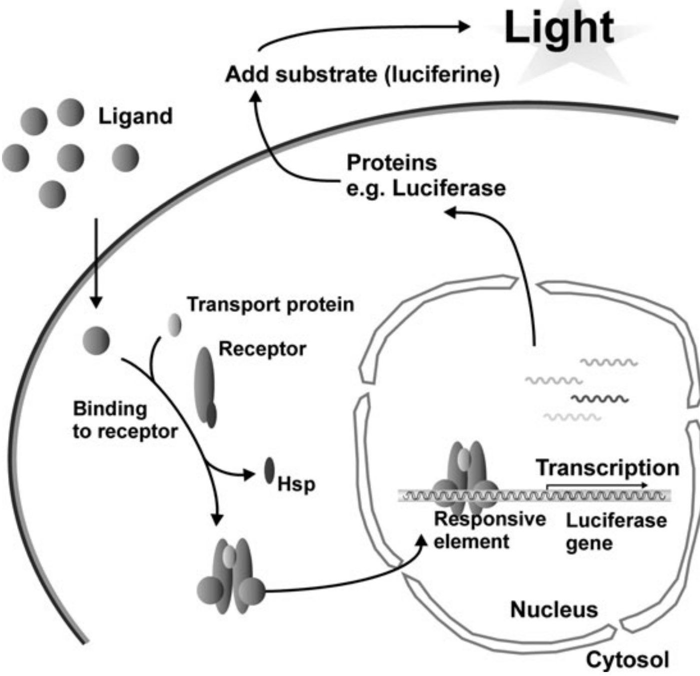

Fig. 3 Receptor-mediated mechanism of action of steroid hormones. Steroid hormones enter the cell, bind to steroid receptors, which subsequently homodimerize and translocate to the cell nucleus. There, the complex binds to specific sites (responsive elements) on the DNA and induces transcription of steroid hormone-inducible genes. Subsequent protein synthesis eventually leads to the cellular and physiological response to that specific hormone. In Chemical Activated Luciferase gene eXpression (CALUX) bioassays, cells are stably transfected with the firefly luciferase gene. Exposure to compounds with hormone(-like) activity leads to transcription of luciferase that is measured in a light reaction after addition of its substrate luciferine

structurally very diverse compounds of natural, synthetic or xenobiotic origin. The capacity of ER to bind a wide variety of ligands has been attributed to the shape of its ligand binding domain, which has an accessible volume of nearly twice that of 17β-E2 and large unoccupied cavities [43]. Activation of oestrogen receptors by chemicals in the environment is believed to be a major mechanism of oestrogenic disruption.

Chemicals known to have the potential to bind and activate oestrogen receptors include natural compounds such as phyto-oestrogens, myco-oestrogens and synthetic oestrogen analogues, such as the potent pharmaceutical 17α-ethynylestradiol (EE2, the active ingredient of contraceptive drugs). A wide selection of xenobiotic, industrial chemicals, referred to as xeno-oestrogens, have also proved to be capable of binding to the ER, although with binding affinities that are generally much lower than those of the natural hormones. Examples of xeno-oestrogens include organochlorine pesticides such as *o,p*′-DDT and dieldrin, alkylphenolic

compounds such as 4-nonylphenol and 4-*tert*-octylphenol primarily derived from non-ionic surfactants and diphenyl derivatives such as bisphenol A, a synthetic chemical used in plastic production, and some phthalates used as plasticisers [44]. Examples of xeno-oestrogenic compounds are shown in Fig. 2.

3 Other Types of Endocrine Disruptors

Chemicals in the environment can interfere with endocrine systems by multiple mechanisms. In addition to oestrogens, nowadays research also focuses on other classes of hormones and compounds with comparable or opposite activities such as anti-oestrogens, androgens (male sex hormones), anti-androgens, progestagens (female pregnancy hormones) and glucocorticoids (hormones controlling energy balance and inflammation), and compounds with thyroid hormone-like activities. Several surveys have demonstrated their presence in the environment [45–48].

3.1 (Anti-)androgenic, Progestagenic and Glucocorticoid-Like Compounds

Androgens are male steroid sex hormones with testosterone and dihydrotestosterone (DHT) as main representatives [49, 50]. Androgens are also the precursor of all oestrogens, the female sex hormones. Androgens stimulate and control the development and maintenance of masculine characteristics. They exert their action by binding and activating the androgen receptor [51]. Activation of the androgen receptor leads to transcription of genes responsible for androgenic effects, such as development and maintenance of male secondary sex characteristics [52], and anabolic effects such as promotion of muscular growth [53]. Synthetic analogues of androgens (anabolic androgenic steroids) are notorious for their illegal use in sports as doping compounds to increase muscle mass and performance. Other applications are to increase meat quantity of live stock and in clinical androgen replacement therapy.

Triggered by experience with oestrogens, several studies have investigated the occurrence of androgenic compounds and effects in the environment [54, 55]. Exposure of fish to androgens has been linked with masculinisation of females, skewed sex ratio, and disturbed relative weight and histopathology [56]. In addition, many manmade compounds such as the pesticides vinclozolin and dichlorvos and industrial chemicals, appear to be able to interfere with the androgen system of humans and wildlife, mainly by acting as anti-androgen [57].

Many hormone-like compounds are used as pharmaceuticals and might enter the environment via similar routes as oestrogens. For example, amounts of androgens and progestagens that are excreted by humans via urine are estimated to be several

orders of magnitude higher than that of oestrogens [58]. The exposure to glucocorticoids has been associated with impairment of the immune system, reproduction and development, and various hormones are known to be used by fish as reproductive pheromones, i.e. chemical compounds excreted to communicate with other fish [45].

3.2 Thyroid Hormone-Like Compounds

Thyroid hormones are non-steroidal hormones that are primarily responsible for the regulation of metabolism. The major form of thyroid hormone in the blood is thyroxin (T4). Disruption of thyroid hormone homeostasis could have major adverse effects in aquatic species such as fish [59] and amphibians [60]. Thyroid hormone disrupting compounds, e.g. those structurally and chemically resembling thyroid hormones, can target and interfere with the hypothalamic-pituitary-thyroid axis at different levels, among others by binding to the transport proteins and thus replacing the natural hormone. Thyroid hormones are weakly bonded (not covalently bound) to transport proteins such as transthyretin (TTR). This complex functions as a circulating reservoir to buffer changes in thyroid hormone levels such as thyroxin (T4), by making T4 available for deiodination to the more active form, triiodothyronine (T3). TTR is not only a highly conserved plasma protein and the main T4 carrier in cerebrospinal fluid, but also important in the serum of most mammalian species and birds.

A limited number of studies have demonstrated the occurrence of compounds with thyroid hormone-like activity, for example in sediments [46] and household dust [61].

4 EDA Methods for the Analysis of Endocrine Disruptors in Aquatic Matrices

4.1 Bioassays for the Detection of Endocrine Disrupting Compounds

Several in vitro biological assays have been developed to screen compounds for endocrine disrupting activities and to test environmental samples. As a result, the occurrence of endocrine disrupting activities in the aquatic environment has been demonstrated on a virtually global scale [55, 62–66].

In vivo assays for endocrine activities use a variety of endpoints, such as organ weights, cell proliferation and protein expression in different organisms [67]. In vivo assays have the advantage of assessing a true impact of endocrine action on a target species, but, on the other hand, have the disadvantages of a lack

of specificity, high costs and unsuitability for large-scale screening purposes [68]. Furthermore, there are ethical objections against the large-scale use of animals in exposure experiments.

In vitro alternatives include competitive ligand binding assays, cell proliferation assays, recombinant receptor/reporter gene assays and yeast-based screens [67]. Each assay measures different aspects of the cascade of events between exposure and the ultimate effects. In addition to compound screening, in vitro bioassays also have shown their value for the analysis of endocrine disrupting activities in complex environmental samples. While chemical analysis can be used to identify and quantify known endocrine disrupting compounds in environmental samples, assessment of total toxic activities in these samples is complicated, because of the large structural differences between endocrine disrupting compounds, possible interactions between compounds and possible contributions of as yet unknown compounds. In such cases, bioassays offer an integrated measure of the combined potencies of mixtures of compounds present in environmental samples, without the necessity of knowing the identity of all contributing compounds beforehand.

Oestrogenic and anti-oestrogenic activity can be measured in vitro using a number of established bioassays [56, 68, 69], including the reporter gene assays yeast oestrogen screen (YES, [70]) and Estrogen Receptor mediated Chemical Activated Luciferase gene eXpression (ER-CALUX) [71]. Reporter gene assays consist of a cell line that is transfected with a gene under control of the response element of the investigated receptor. Exposure of the cells to a compound or sample leads to receptor activation and synthesis of the reporter gene product. Reporter gene assays are biologically relevant in that they cover all events involved in receptor transactivation from uptake of the compound by the cell to the synthesis of protein. The mechanism of action of CALUX bioassays is illustrated in Fig. 3.

The YES and ER-CALUX bioassays are fully validated and have both been used in monitoring and EDA-type studies on oestrogen receptor activity in various environmental matrices [37, 46, 72–76].

In vitro bioassays for androgenic activity have also been developed, e.g. a binding assay using androgen receptor ligand binding domains [77], yeast androgen screens (YAS) [78] and the AR CALUX bioassay [79]. The high degree of representation of the in vivo situation of the latter was reflected in the excellent correlations observed between androgenic activities in the AR CALUX bioassay and other in vivo and in vitro screening models for androgenic activities [80]. Both the YAS and AR-CALUX are suitable for the detection of androgenic as well as anti-androgenic compounds and have been successfully applied to characterise environmental androgens in the past [47, 55, 81].

In addition to the ER and AR CALUX bioassays, CALUX bioassays have been developed for progesterone and glucocorticoid receptor interacting compounds [47, 79, 82]. Progestogenic activity has also been tested with assays developed to test the binding of chemicals to human progesterone receptors (PR) as well as PR isolated from various non-mammalian species [83–85]. Similar to PR CALUX, a number of other mammalian cell-based PR transactivation assays have been

developed and have demonstrated progestogenic activity in a wide range of environmental chemicals [86–89].

There are a few different binding assays in the literature used in the determination of binding potency of environmental pollutants and extracts to the thyroid hormone transport protein TTR. Examples of in vitro assays include radioligand binding assay (RLBA) [90–95], non-radio ligand binding assay with TTR covalently bound to a sepharose resin and high-performance liquid chromatography (HPLC) [96] and surface plasmon resonance based assay [97]. In the latter, T4 is covalently bound to a gold chip. It competes with tested compounds in a flow cell for TTR binding. The radio ligand TTR binding assay is a well-established method [94] that has been used for many years, although with some modifications between laboratories [90–93, 95]. It is a competitive binding assay where T4 (cold and labelled) is used in a mixture with the competitor (compounds or extracts) to bind to human TTR. The assay successfully detected TTR-binding activity of reference materials, e.g. [90, 92, 98] and in sediment extracts [46].

4.2 Effect-Directed Analysis

Linking endocrine disrupting activities to the exposure to particular causative agents is often problematic because of the large numbers of different compounds present in the environment. Bioassays can serve to analyse activities in samples, but cannot identify compounds. At the same time, present-day chemical analytical techniques provide excellent sensitivity in the analysis of known compounds, but they cannot give information on potency and will easily miss compounds not explicitly looked for.

The effect-directed analysis (EDA) approach has been introduced to overcome these difficulties. In this approach, analytical chemistry is combined with bioassay analysis to isolate and ultimately identify the compounds in a complex sample that are responsible for the observed effects. Originally referred to as toxicity identification and evaluation (TIE), but currently also known as bioassay-directed fractionation, effect-directed identification or EDA, this integrated approach was launched by the US Environmental Protection Agency in 1988 in a handbook for fractionation of acutely toxic effluents [99]. Since then, comparable procedures have been developed for various toxic activities and matrices [55, 100–109]. In the UK, Desbrow and colleagues used the approach successfully to identify natural oestrogenic hormones as the main contributors to the oestrogenic activity observed in domestic STP effluents [37].

Basically, in all applications EDA aims to eliminate compounds that do not contribute to the activity of a sample and isolate active compounds in a series of fractionation and separation steps. After each fractionation step, all fractions are tested in a bioassay to investigate in which fraction(s) the active compounds are isolated. This is repeated until the complexity of the active fractions is sufficiently reduced to enable identification of the active compounds by chemical analysis.

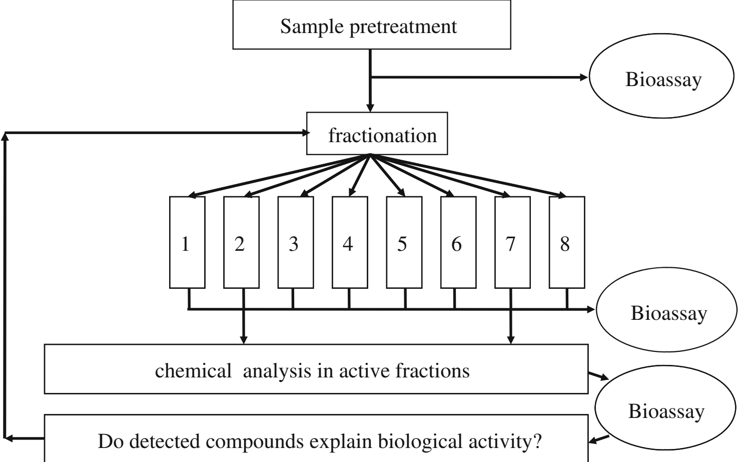

Fig. 4 Schematic representation of effect directed analysis approach for endocrine disrupting compounds in environmental samples. After sample pretreatment, an environmental sample is tested in the bioassay for endocrine disrupting activity. If activity is measured, the sample extract is fractionated. Each fraction is tested again in the bioassay to investigate in which fraction the active compounds are isolated. This is repeated until the complexity of the active fractions is sufficiently reduced to enable identification of the active compounds by chemical analysis, e.g. with massaspectrometric techniques

Eventually, pure standards of the identified compounds are tested in the bioassay to confirm their activity and possible involvement in the activity of the investigated sample. The advantage of effect-directed analysis is that no presumptions about the identity of the responsible chemicals are made beforehand, thus as yet unknown compounds with biological activities might also be identified. A basic EDA is shown in Fig. 4.

In EDA, most often a non-destructive clean-up procedure is used which enables the inclusion of as many compounds in a sample as possible that exert a specific mode of action in a bioassay. The procedure typically starts with sample pretreatment, which may include sieving, freeze-drying, homogenisation and pooling. The second step consists of the extraction of organic compounds from the matrix. The main aims of extraction are to concentrate the compounds of interest and to transfer them to a solvent that is compatible with analytical detection techniques.

The extract is tested, after an additional clean up step if necessary, in a bioassay to investigate the presence of endocrine disrupting activities in the total extract. If significant activity is found, the extract is then separated into several fractions to isolate active compounds.

Fractionation is undertaken often by means of HPLC. Two commonly used types of HPLC systems are reversed phase (RP) and normal phase (NP) HPLC. In RP-HPLC, separation and isolation of compounds according to their polarity is achieved. In NP-HPLC, compounds are separated according to their chemical

functionalities such as planarity. In general, HPLC enables efficient and non-destructive separation of compounds based on their molecular properties. Aliquots of the eluate can be collected in separate fractions. Each fraction is tested in the bioassay to determine in which fractions activity has been isolated. A second round of fractionation and testing might be necessary before chemical analysis of responsible compounds in active fractions is possible.

If the fractionation behaviour of the activities fits well to the chromatographic properties of certain known compounds, their presence and contribution to the activity can be investigated in a targeted analysis of the concerned compounds, e.g. using gas chromatography (GC) or liquid chromatography (LC) separation combined with mass spectrometric (MS) detection.

When the observed endocrine disrupting activity is attributed to so far unknown compounds, screening techniques, e.g. GC–MS operated in full scan mode, are used for this purpose. Obtained spectra are compared with reference spectra from a spectral library for tentative identification. Despite successive rounds of fractionation and separation, analysed fractions are often still very complex.

Testing pure standards of identified compounds in the bioassay reveals whether the compounds indeed could be fully or partially responsible for the endocrine disrupting activity in the investigated sample. Based on the concentration found in the sample, its potency and knowledge of combined behaviour of endocrine disruptors, the contribution of each identified compound to the observed activity in the fraction can then be calculated.

4.3 How to Deal with Mixture Effects in EDA

Receptor-mediated effects of steroid receptor agonists can be described by the concept of concentration addition (CA) [110]. This concept, which was introduced by Loewe and Muischneck in 1926, assumes that chemicals act according to a similar mechanism. It therefore states that equal levels of effects can be achieved by full or partial replacement of a component with other components in a mixture. The contribution of each component to the total effect of the mixture is considered to be proportional to its potency and its concentration in the mixture. The CA concept has been shown to be valid for the prediction of mixture effects of oestrogenic compounds in the YES [111–113] and ER-CALUX [114] assays and for androgenic compounds in the AR-CALUX [50]. One of the consequences of the additive behaviour of steroid-receptor agonists is that CA can be used to compare endocrine activity in a sample with activity that can be calculated based on individual compounds detected in the same sample. Detected concentrations are multiplied with their potencies relative to a reference compound, such as 17β-E2 or DHT, and expressed as 17β-E2 equivalents (EEQ) or DHT-equivalents (DHT-eq). In this way, it can be calculated to what extent compounds identified with EDA are responsible for the effect observed in the bioassay.

5 Examples of EDA Studies on Endocrine Disrupting Compounds

5.1 *Oestrogens in STP Effluents and Process Water Samples*

The first EDA study on oestrogens was performed by Desbrow and co-workers in 15 sewage effluent samples collected from the southeast of the UK [37]. Large volumes of sewage (20 L) were extracted by octadecyl silica (C18) solid phase extraction (SPE) and subsequently tested using the YES. The samples were first eluted from the SPE columns using different concentrations of methanol in water to elute fractions of different polarities. The fractions containing oestrogenic compounds were further fractionated by RP-HPLC using two fractionations in series to yield 90 fractions of which three contained oestrogenic compounds. GC–MS analysis was used to identify E1 and 17β-E2 in all of the sewage samples collected and EE2 in 7 of the 15 samples as compounds responsible for the majority of the activity observed. This tied in with laboratory studies which showed that these compounds had feminising effects at environmental levels [115].

Thomas et al. further refined Desbrow's procedure by removing the coarse fractionation steps when eluting from the C18 SPE column and using a generic RP HPLC fractionation procedure to provide 30 fractions. They applied this refined procedure to effluents discharging into UK estuaries in which concentrations of oestrogenic activity up to 24 ng EEQ/L were detected. 17β-E2, androsterone, nonylphenol and (tentatively) *bis*(2-ethylhexyl)phthalate, as well as unknown compounds, were responsible for the activity in these samples [74]. Pore waters were tested from the same locations, but activity was only measured at one of the locations [116]. Application of the same fractionation procedure used for STP effluent showed the presence of oestrogenic compounds in the pore water samples which were more polar than the compounds identified in the STP effluents. GC–MS analysis was unsuccessful in identifying the compounds responsible; however, it was confirmed that they were not steroids or known phyto-oestrogens [116].

Cespedes and co-workers combined LC–MS and a recombinant yeast assay to estimate the loads of oestrogenic compounds in influent and effluent waters of an STP discharging in the Spanish River Llobregat. Although strictly speaking their approach was not EDA, they were able to correlate results of chemical analysis and bioassay data and concluded that nonylphenol contributed to more than 90% to the predicted oestrogenic activity in most samples [117].

A similar approach to that of Cespedes was followed by Quiros et al. to investigate ng/L concentrations of oestrogenic activity in 183 water samples from Portuguese rivers [118]. For two sites, concentrations of alkylphenols and bisphenol A were high enough to explain the oestrogenic activity detected in the bioassay. For other samples, the causative compounds remained unknown. However, the contribution of natural and synthetic hormones could not be elucidated as they were present at levels too low to be detected by the bioassay.

In 2004, Aerni et al. published a study on oestrogenic compounds in five STP effluents and receiving waters from Switzerland and France. Oestrogenic activities in these samples, detected with the YES assay, were up to 6 ng EEQ/L. Correlation of chemical data and YES assay results showed that natural and synthetic steroid hormones were the major contributors to the oestrogenic activity. Alkylphenols and nonylphenolethoxylates, though detected at μg/L concentrations in effluents from STP, did not contribute significantly to the oestrogenic activity, because of their low relative oestrogenic potencies [119].

Besides WWTP effluents and receiving waters, oestrogenic activity has also been characterised in water from an off-shore platform in the North Sea. Levels up to 90 ng EEQ/L were detected [75]. Using normal phase fractionation, complex mixtures of non-polar compounds in produced water were separated and isomeric mixtures of C1–C5 and C9 alkylphenols were identified as major contributors to the oestrogenic activity measured in the samples. The NP fractionation procedure used a cyano-amino-bonded silica HPLC to isolate oestrogenic compounds from the North Sea off-shore produced water extracts followed by full-scan GC-electron-impact MS to identify them [75]. Interestingly around 45% of the activity in these samples was unidentified [120] with mixtures of naphthenic acids recently being identified as responsible for the remaining activity [121]. In these studies extensive effort was made to identify the remaining causes with techniques such as comprehensive GC. It was noted that much of the activity was not being recovered from the normal phase fractionation column and that recovery of this material and subsequent derivatisation prior to high-resolution GC-MS analysis successfully showed the presence of complex mixtures of naphthenic acids.

5.2 *Oestrogens in Fish Bile and Gastrointestinal Content Samples*

Stimulated by the success of EDA studies in water, the approach has also been applied to other matrices such as fish tissue and bile, as these may provide an indication of internal exposure levels.

In the Dutch River Dommel, high plasma-VTG concentrations and a high prevalence of intersexuality of unknown cause had been observed in male bream [33]. As plasma VTG is strongly correlated with oestrogenic activity in bile of male breams from this location [122], Houtman et al. [108] identified compounds responsible for the oestrogenic effects in the bile of wild breams from this site. Bile was deconjugated, extracted with ethylacetate and fractionated with RP-HPLC into ten fractions. Oestrogenic activity was measured with the ER-CALUX bioassay. Chemical analysis with GC-MS/MS identified the natural oestrogens 17*β*-E2 and E1 as the main contributors to the oestrogenic activity in male bream bile. EE2 was detected in effective concentrations as well. In addition to the identification of active oestrogens, GC-MS screening of bile fractions resulted in the detection of relatively high concentrations of xenobiotic chemicals such as the disinfectants triclosan, chloroxylenol and clorophene.

Subsequent work was initiated to investigate the endocrine health status of breams in Dutch freshwaters and the exposure to and effects of oestrogenic compounds 4 years later [76]. Oestrogenic activity in gastrointestinal content of male breams was strongly correlated with VTG concentrations in plasma, while oestrogenic activity in plasma and liver was not. Therefore, oestrogenic activity in gastrointestinal content was chosen as an indicator of internal exposure and used for the investigation of recent internal exposure to oestrogenic compounds. The EDA work in gastrointestinal content confirmed the important role natural oestrogens might have in the occurrence of oestrogenic activities in Dutch freshwaters and the results of the study again showed that 17β-E2 and E1 were the main contributors.

Gibson et al. performed EDA of fish bile to analyse the uptake of oestrogenic compounds from effluents in fish [123]. They benefited from the fact that, due to bioconcentration, levels of oestrogens in fish bile may be over a 1,000-fold higher than in effluents and are thus easier to detect. Rainbow trouts and roaches were exposed for 10 days in flow-through tanks with STP effluents. Later, bile was collected, extracted with SPE, fractionated with RP-HPLC into 60 fractions and analysed with YES and GC-MS/MS. Most of the activity could be attributed to 17β-E2 and E1; however, EE2, alkylphenols and nonylphenolethoxylates and an equine oestrogen were also identified [124].

5.3 *Oestrogens in Sediment*

Research on oestrogenic compounds in the environment was until recently predominantly focused on the water compartment. However, sorption studies [125] and bioassay studies have shown that oestrogenic compounds are lipophilic enough to bind to sediment [46, 126, 127]. Because numerous aquatic species live in close contact with sediment, this compartment might thus be an important source of exposure.

Examples of EDA on oestrogens in sediment show that identification of active compounds in sediments is even more challenging than in water, due to the complex composition of sediments. Khim et al. fractionated extracts of sediments from Masan Bay, Korea on Florisil columns [128]. By treating some fractions with concentrated sulphuric acid, the authors studied both acid-stable and acid-labile compounds. Oestrogenic activity, measured with the MVLN (MCF-7 human breast carcinoma stably transfected with an oestrogen receptor controlled luciferase) reporter gene assay, was detected in acid-labile polar and in acid-stable mid-polar fractions. Oestrogenic activity in the polar fraction could partly be explained by the presence of alkylphenols and bisphenol A, whereas active compounds in the latter were not identified.

Thomas et al. could identify nonylphenol, cinnarizine and cholest-4,6-dien-3-one as active oestrogens in sediment extracts from the UK River Tyne. However,

these compounds only accounted for $<1\%$ of the overall activity and the main contributors to oestrogenic activity in this sample remained unidentified [116].

In 2003, Fenet and co-workers performed a correlation study with the MELN assay and alkylphenol analysis in sediment extracts from French rivers. It was concluded that oestrogenic activity in sediments from industrial, rural and urban sites was largely explained by alkylphenols, whereas in sediments from agricultural sites alkylphenols poorly contributed [62].

Peck et al. investigated sediments from UK rivers receiving STP effluents. GC–MS analysis of extracts fractionated into hundred fractions, revealing that, like in STP-effluent samples, E1 and 17β-E2 were the major active compounds. In addition, a number of unidentified oestrogens were present [129]. In addition, at sites not influenced by STP effluents, natural oestrogenic hormones may play an important role. This was shown for sediments collected at the inner harbour of the small Dutch town Zierikzee. In addition, at this site remote from STPs, E1 and 17β-E2 accounted for the majority of the observed oestrogenic activity, although some oestrogenic activity associated with more nonpolar compounds remained of unknown identity [109].

Schlenk et al. performed an EDA of marine sediments that was directed by an in vivo VTG bioassay. Although 17β-E2 was detected, it did not correlate with VTG expression and it was concluded that other as yet unknown natural and/or xeno-oestrogens may have contributed to the oestrogenic activity [130].

5.4 *Oestrogenic Compounds Identified in the Environment*

The majority of EDA studies for oestrogens have addressed natural and synthetic steroidal oestrogens as the primary causative agents of oestrogenic activity in the aquatic environment, and, more specifically, of feminising effects in fish. Laboratory experiments confirm that very low concentrations of natural and synthetic oestrogenic hormones suffice to induce oestrogenic effects in fish (reviewed by [38]). In general, many organisms, including all classes of vertebrates and many plants, synthesise oestrogenic hormones. EE2 is used on a large scale in female oral contraceptive pill treatments and hormone replacement therapies. Excreted hormones and metabolites collected in sewer systems end up in STPs, where their incomplete removal from the influent as well as hydrolysis by bacteria can result in the release of oestrogenic effluent in the environment. Oestrogens excreted by livestock often do not pass STPs and may easily enter the environment by field drains and headwater streams on farms [131]. The growing human and animal populations and more intensive farming have thus made oestrogen excretion by humans and livestock an important source of oestrogen influx into the environment [132] and may explain the detection of natural and synthetic oestrogens as main active oestrogens at sites both in the vicinity and remote from STP effluent discharges.

Many EDA studies have, however, shown that not all of the activity found can be attributed to steroid oestrogens. At certain specific locations, often those associated

with industries, oestrogenic activities have been (at least partly) attributed to high concentrations of alkylphenols instead of oestrogenic hormones [62, 74, 118, 133]. In addition, a variety of other compounds causing oestrogenic activity have been found, ranging from rather unknown compounds such as cinnarizine [116], oxybenzone [130] and naphthenic acids [121]. In summary, natural and synthetic steroidal oestrogens could generally be considered as the most likely causes of oestrogenic activities in the environment [134], although at certain locations other compounds are also involved.

5.5 Androgenic Compounds and Anti-androgenic Compounds

The first reported EDA study to identify androgens used the YAS assay to detect androgenic compounds in samples collected from UK estuaries [55]. Following detection of androgens in surface water samples (<2–9 ng DHT-eq/L), androgenic activity was detected in sediment pore waters (<45 ng DHT-eq/L), sediment solvent extracts (<454–15,300 ng DHT-eq/kg) and selected STP effluents (34–635 ng DHT-eq/L). An EDA study of one of the effluents using the YAS assay successfully identified the natural steroids and steroid metabolites DHT, androstenedione, androstanedione, 5β-androstane-3α,11β-diol-17-one, androsterone and epi-androsterone as responsible for 99% of the in vitro activity [55]. This is possibly one of the most successful EDA studies published to date.

More recently the YAS assay has been used to identify androgens in river water and sediments from Italy [135]. Subsequent screening of samples such as water effluents produced at offshore platforms did not show androgenic activity above the detection limits of the assay [75].

The first reports on anti-androgenic compounds in environmental samples have reported their occurrence in sewage effluents, surface waters, sediments and produced-water effluents [81, 135]. Concentrations, expressed as flutamide (FLU; a potent anti-androgen) equivalents, ranged from 1.34 to 17.1 μM FLU-eq for rivers and 20 to 8,000 μg of FLU-eq/L for produced-water effluents. EDA of an offshore produced water sample identified alkylphenols, polycyclic aromatic hydrocarbons and naphthenic acids as responsible for this activity [121].

In general, both YAS and AR-CALUX provide comparable androgenicity data. Weiss et al. [48] used the AR-CALUX assay to profile the androgenicity and anti-androgenicity in sediment samples from the rivers Elbe, Llobregat and Scheldt. Both androgenic and anti-androgenic effects were detected. The first screening of sediment samples revealed androgenic activities in Llobregat (2 pmol DHT-eq/g) but not in Elbe or Scheldt sediment. After further fractionation, however, two fractions of the Scheldt sediment extract showed a total androgenic activity of 1.3 pmol DHT-eq/g sediment, suggesting that anti-androgenic compounds in the other fractions may have suppressed the androgenic potency of the whole extract. Similar suppression of anti-androgenic potency by androgenic compounds was observed for the whole Scheldt extract, with an anti-androgenic potency of

220 nmol FLU-eq/g sediment. After fractionation, the total potency of the anti-androgenic fractions summed up to 1,250 nmol FLU-eq/g. One fraction had both increasing androgenic and anti-androgenic effects compared to the whole extract, which can be explained by partial agonist activity, i.e. when a weak agonist has an antagonistic effect in the presence of a strong agonist. Furthermore, around 200 nmol FLU-eq/g was found in the Elbe sediment [48].

5.6 Progestagens and Glucocorticoids

Studies regarding the presence of progestagens and glucocorticoids in the environment are limited, and EDA studies actually identifying causal agents have not yet been carried out to our knowledge. In a recent study by Van der Linden and colleagues [47], progestagenic and glucocorticoid-like activities measured with CALUX assays were found in extracts prepared from effluents from industry, hospital and municipal STPs. In addition to low progestagenic activity, glucocorticoid-like activity was detected in all samples, at elevated levels ranging from 0.39 to 1.3 ng dexamethasone (DEX; a potent synthetic glucocorticoid)-eq/L in surface water to 11–243 ng DEX-eq/L in effluents. These results expressed in cortisol (a natural glucocorticoid less potent than dexamethasone) equivalents would range up to 2,900 ng cortisol-eq/L. A subsequently performed correlation study based on instrumental analyses and relative potencies of individual glucocorticoids supported the conclusion that triamcinolone acetonide, dexamethasone and prednisolone are the main contributors to the glucocorticogenic activity in the investigated wastewater extracts [136]. Various glucocorticoids (prednisone, prednisolone, cortisone, cortisol, dexamethasone and 6R-methylprednisolone) have also been found in STPs and receiving rivers in China [45, 137]. The levels of DEX and cortisol in this Chinese study averaged 1.2 and 39 ng/l, respectively, suggesting much lower levels of cortisol in effluents [137].

5.7 Thyroid Hormone-Like Compounds

A few studies have demonstrated thyroid hormone-like activity in environmental samples. Houtman et al. [46] showed the occurrence of TTR-binding compounds in sediment extracts. A study of drinking water sources in the greater Paris area showed the presence of compounds in extracts of STP influents, but not STP effluents or surface water, which could activate the thyroid hormone receptor in an in vitro mammalian reporter gene assay [138]. A recent Japanese study demonstrated that TTR-binding compounds are also found in the non-aquatic environment [95] In indoor dust, levels up to 300–5,000 pmol T4-eq/g (median 1,000 pmol T4-eq/g), which are 30–550 times higher than those reported in sediments [46], were observed. In a subsequent EDA study, Suzuki and colleagues [61]

demonstrated that 2,4,6-tribromophenol and 2,3,4,5,6-pentachlorophenol were potent TTR-binding compounds in all dust samples and accounted for about 40–70% of experimental T4-eqs detected in indoor dusts.

6 Evaluation of Effect-Directed Analysis Approaches for the Identification of EDC

6.1 Advantages of EDA for EDC

In the discussed studies, the EDA approach has proven to be a powerful tool for the investigation of specifically acting endocrine disrupting compounds, in complex environmental samples.

One of the main advantages of the approach is the direct link that can be established between a biological effect and identified compounds, due to the combination of bioassays (activity measurement) and analytical chemistry in the same fraction. In this way, compounds are identified based on their potential hazard and not just on their occurrence in the environment.

A second advantage is that the portion of the activity that is explained by the presence of specific identified compounds can be quantified. Because mixtures of compounds behave additively in bioassays such as CALUX [50, 114] and yeast screens [112, 113], determination of potencies of pure standards of identified compounds and measurement of their concentrations in a sample suffice to calculate the proportions of activity that can be assigned to individual compounds.

Another advantage of the applied EDA approach is that, as activity is the only director of the identification, the analysis is in principle unbiased, i.e. all active compounds can be detected, not just compounds on a precompiled target list. This is exemplified by the unexpected identification of oestrogenic hormones as compounds responsible for oestrogenic activity in the environment.

A fourth advantage is that, although principally aimed at the analysis of, e.g. oestrogenic or androgenic compounds, application of sophisticated chemical screening techniques can also provide other analytical information on the sample or its fractions.

6.2 Aspects to be Developed Further

Certain aspects of the EDA approach could benefit from further development. A first aspect is that chemical screening techniques do not always succeed in the detection of active compounds present at low levels. For example, in the presented studies target analysis was often indispensable to detect endocrine disrupting compounds (e.g. the natural oestrogens), because their concentrations were too low in the complex fractions to be detected by the applied chemicoanalytical

screening technique. Furthermore, several studies reported that in some fractions activities could not be explained [48, 62, 74, 108, 109, 128–130]. This might indicate the presence of very potent compounds in concentrations below detection limits. Yet another explanation might be the presence of active compounds that cannot be detected with applied GC- or LC-based analytical techniques, e.g. because of very low volatility or high polarity.

The main problem for identification, however, might be that fractions remained very complex or that chemical screening techniques do still suffer from a lack of identifying and interpretative power to identify low concentrations of active compounds in complex environmental samples.

From a biological perspective, the translation of in vitro results to the in vivo situation, i.e. the level of a whole organism or population, should be considered. In most EDA studies, identifications were directed by in vitro bioassays. For certain assays, e.g. CALUX, there are strong indications that in vitro measurements are indeed indicative of the in vivo situation [80, 139]. However, in vitro assays do not include the toxicokinetics of an intact organism. Therefore, considerable differences between in vitro and in vivo potencies do occur [139]. This could imply that relative contributions of each compound to in vitro activity as calculated in a bioassay-directed identification might deviate from the in vivo situation. Therefore, the relative contributions of identified active compounds to the in vitro effect in a EDA study predominantly indicate whether all contributing compounds have been elucidated or whether one should look for other contributors. To calculate the individual contribution of identified compounds to in vivo effects, as necessary for the risk assessment of the individual compounds, a correction for differences between in vitro and in vivo potencies might be necessary.

A possible way to circumvent differences between in vitro and in vivo measurements is to direct the identification by an in vivo bioassay, e.g. [130]. In some studies, aspects of toxicokinetics were included to some extent by the choice of the matrices investigated, e.g. using fish bile or gastro-intestinal content as indicators of internal exposure [76, 108, 124]. From an ethical point of view and from cost and efficiency considerations, however, limiting the use of test animals in in vivo assays is preferred.

6.3 Future Applications of EDA Approaches

Although further development of the approach is recommended, the EDA approach is an appropriate means to investigate effects in the environment that are associated with exposure to unknown chemicals. The approach can be applied in scientific as well as regulatory settings.

The occurrence of various types of endocrine disrupting activities in the aquatic environment evokes the question for the elucidation of causative agents. Until now, answers to this question have been found for oestrogenic and androgenic compounds, and some first results are obtained for glucocorticoid and thyroid-like

compounds. Comparably, EDA approaches could in the future be applied to investigate other types of activities in the environment that are of concern from an ecotoxicological point of view, such as progestagenic compounds and all kinds of receptor-mediated activities.

Bioassays can be used to monitor the occurrence of potentially toxic activities in the environment caused by polluting compounds. In case current chemical analyses do not lead to the identification of responsible compounds of activity at specific locations, the EDA approach can be applied to elucidate unknown causative agents and their combined effects. The identification of active agents is then a first and vital step towards the identification of pollutant sources, and to measures for the restoration of the environmental quality at the location concerned.

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Effects-Directed Studies of Pulp and Paper Mill Effluents

Mark Hewitt

Abstract The history of effects-directed investigations of pulp and paper mill effluents has been driven primarily by the environmental effects associated with mill discharges. The first effect to confront the industry was acute toxicity to aquatic biota. Through a series of effects-directed studies in the 1970s and 1980s the causative agents were elucidated, subsequent regulations enacted, and effluent treatment technologies implemented to reduce the loadings of resin acids, chlorophenolics and other toxic compounds in mill discharges. Effects-directed investigations in the pulp and paper sector have since focused on other endpoints, primarily mutagenicity and endocrine disruption. Identification of these active substances has proven to be much more challenging due to the evolving complexities of effluent matrices and the intricacies of the responses themselves. Residual lignin in final effluents remains a significant barrier to the isolation and identification of low molecular weight bioactive substances. The evolution of analytical techniques (e.g., from XAD resins to SPE cartridges) coupled with new approaches (e.g., studying in-mill waste streams and fish tissue burdens of active substances) have nevertheless provided insights into the sources and identities of mutagens and endocrine disruptors. Active chemicals have been identified primarily using GC–MS with recent limited applications of LC–MS. A high proportion of substances originating from wood feedstocks have been identified or implicated in the effects studied. Differences in the patterns of effects between North and South American mill effluents may be reflective of the different tree species utilized. Careful selection of the endpoint used to direct such investigations, its reproducibility, robustness and linkages to whole organism responses and regulatory applications are emphasized.

Keywords Androgen, Effects-directed analysis, Endocrine disruption, Estrogen, Fish, Investigation of cause, Pulp and paper mill effluent

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W. Brack (ed.), *Effect-Directed Analysis of Complex Environmental Contamination*, Hdb Env Chem (2011) 15: 267–284, DOI 10.1007/978-3-642-18384-3_11,

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Contents

1 Introduction . . . 268
2 Acute Toxicity . . . 273
3 Endocrine Disruption in Fish . . . 274
3.1 Induction of Detoxification P450IA1 enzymes . . . 274
3.2 Effects on Fish Reproduction . . . 275
4 Mutagenicity . . . 279
5 Bioassay Considerations for Effects-Directed Investigations . . . 280
6 Regulatory Applications . . . 280
References . . . 281

1 Introduction

The effects of pulp mill effluents on aquatic environments have been examined for over 40 years, and published studies on effects-directed investigations of mill effluents have been conducted since the late 1960s (Table 1). During this period, environmental effects have been observed, regulations have been implemented in pulp-producing nations, and the industry has responded to these regulations resulting in significant reductions in acute toxicity [6], as well as other effects on fish populations and benthic communities [44, 45]. However, other environmental responses, such as effects on fish reproduction have persisted [46] and have become the main focus of cause and effect research concerning mill effluents since the early 1990s [47]. Because of effluent complexities and changes in effluent compositions over the last 3 decades, this matrix has represented one of the greatest analytical challenges in identifying bioactive substances in complex environmental mixtures.

Pulp and paper mill effluents contain material from wood feedstocks of different species (e.g., terpenes, phytosterols, trace metals), process derivatives or compounds formed during pulping/bleaching (e.g., dimethyldisulfide formed during Kraft pulping), additives (e.g., polymeric formulations used as slimicides), nutrients supplemented to biotreatment systems, and (partially) biodegraded products of the above. Concerning the molecular weight distributions of mill effluents, investigators in different countries have tackled this problem over the past 2 decades, with controversy surrounding the results obtained with different methods. What does emerge from this work is evidence for a peak distribution of chlorinated organic substances between 200 and 800 Da [48, 49] with nonchlorinated substance peak molecular weight distributions at much higher ranges, such as 6,000–8,800 Da [50] and even 25 kDa [51]. In contrast, much of the chemical characterization of mill effluents was conducted during the 1980s and 1990s [52, 53]. Following process modifications over the last decade, effluent compositions have changed markedly since the previous characterizations and this lack of information has impeded effects-directed studies of present-day effluents.

Historically, effects-directed studies of final effluents from pulping operations have proven to be quite challenging. Difficulties encountered include: (1) fractionation experiments conducted on "grab" samples of effluent which do not reflect

Table 1 Summary of effects-directed studies of mill effluents and associated matrices

Citation	Endpoint(s)	Matrix	Mill process, Feedstock, Location	Isolation method	Characterization method	Substances identified, *ID Level attained*
Das et al. [1]	Acute toxicity: salmonids	C, E stage effluents	Bl. Kraft, softwood, North America	Solvent extraction, cation exchange, lyophilization, TLC	GC–FID, UV–VIS, NMR, MS	Tetrachloro-*o*-benzoquinone, *Confirmed*
Leach and Thakore [2–4]	Acute toxicity: salmonids	C, E stage effluents	Bl. Kraft, softwood, North America	XAD, silica gel, TLC	IR, UV, NMR, GC–MS	Resin acids, Fatty acids, Chlorinated phenolics, Juvabione, *Confirmed*
McKague et al. [5]	Acute toxicity: salmonids	C stage effluent	Bl. Kraft, softwood, North America	XAD, TLC, silica gel, preparative GC	IR, NMR, GC–MS (incl. high res)	3,4,5-trichlorocatechol, tetrachlorocatechol, 2,6-dichlorohydroxyquinone, *Confirmed*
Kovacs et al. [6]	Acute toxicity: fish, invertebrates	Final effluents	Chemical, mechanical, multiprocess types, hardwood and softwoods North American mills (32)	Diagnostic tests based on hypothesized toxicity causes (e.g., pH adjustment, chelation)	Applied as necessary according to cause (e.g., GC–MS for resin acids)	Fish: ammonia, carbon dioxide and resin acids, Cu, Mn, polymeric additives, *Confirmed* Invertebrates: polymeric formulations (defoamers), *Confirmed* Nonylphenol ethoxylates, *Tentative*
Reyes et al. [7]	Acute toxicity, invertebrates	E stage effluent	Bl. Kraft, feedstock not provided, South America	Phase I TIE manipulations [8] (pH, chelation), anion & cation exchange, activated carbon columns	UV–VIS, GC–MS	Cu^{+2}, *Confirmed*
McKague [9]	Mutagenicity: *S. typhimurium* TA100-S9	C stage effluent	Bl. Kraft, feedstock not provided, North America	XAD, silica gel	GC–MS	Tetra, penta and hexa-chloroacetones, *Confirmed*
Kinae et al. [10, 11]	Mutagenicity: *S. typhimurium* TA98, 100, 1537 ±S9	Wild sea trout liver extracts, sediments	Mill type, feedstocks not provided, Japan	Soxhlet, HPLC	GC–MS	9,10-epoxystrearic acid, 2,4,6-trichlorophenol, 3,4,5,6-tetrachloroguaiacol, dehydroabietic acid, *Confirmed*
Holmbom et al. [12]	Mutagenicity: *S. typhimurium* TA100-S9	C stage effluents	Bl. Kraft, softwood, North American mills (4)	Solvent, XAD, silica column, HPLC, TLC	IR, GC–MS (high res)	MX, 3-chloro-4-(dichloromethyl)-5-hydroxy-2(5H)-furanone, *Confirmed*

(*continued*)

Table 1 (continued)

Citation	Endpoint(s)	Matrix	Mill process, Feedstock, Location	Isolation method	Characterization method	Substances identified, *ID Level attained*
Perez-Alzola and Santos [13]	Mutagenicity: *S. typhimurium* TA100 + S9	Bleachery effluents	Bl. Kraft, biobleaching, softwood, South America	Solvent extraction	GC–MS	Biobleaching ineffective in reducing known mutagens, creates new active, *Unknowns*
Burnison et al. [14, 15]	P4501A1 induction, in vitro, R. trout in vivo	Final effluent	Bl. Kraft, soft/ hardwood North America	Tangential flow filtration, SPE, HPLC	GC–MS (including high res)	Trichloropterostilbene, *Confident*
Martel et al. [16]	P4501A1 induction, R. trout in vivo	TSE, condensates, wood feedstock	TMP, softwood North America	Solvent extraction, flash chromatography, TLC	GC–MS	Juvabione, Dehydrojuvabione, *Confirmed*
Coakley et al. [17]	P4501A1 induction, R. trout in vivo	Bleachery filtrates	Bl. Kraft, softwood and hardwood North American mills, (2) laboratory simulations	N/A	N/A	First bleaching stages more potent, hardwood more potent than softwood, paper mill wash water also a source
Higashi et al. [18], Cherr et al. [19]	Sea urchin sperm motility, marine mussel embryonic development, English Sole mortality, in vivo	Final effluent	Bl. Kraft, softwood, North America	Lyophilization, HPLC, ultrafiltration, SDS-PAGE	GC–MS, UV–VIS, NMR	40–400 kDa fraction, high carboxylate, no aromatic content, polysulfonated
Zacharewski et al. [20]	P4501A1, estogenicity, anti-estrogenicity in vitro	Black liquor	Bl. Kraft, Soft/hardwood North America	Tangential flow filtration, lyophilization, solvent extraction	N/A	Multiple estrogenic, dioxin-like ligands present but not identified, *Unknowns*
Parrott et al. [21]	Circulating and gondal production of sex steroids, Goldfish in vivo	TSE, 13 mill process wastes, final effluent	Bl. Sufite/GW, soft/hardwood North America	N/A	N/A	Biotreatment identified as source; responsible compound not identified, *Unknowns*

Dube and MacLatchy [22, 23]	Plasma/gonadal testosterone, mummichog in vivo	TSE, 6 process wastes, final effluent	Bl. Kraft soft/hardwood North America	N/A	N/A	Chemical recovery condensates identified as source; responsible compounds-*Unknowns* Plant sterols not involved, reverse osmosis treatment removes effects
Rickwood et al. [24]	Reproduction, fathead minnows in vivo	TSE, 5 process wastes, final effluent	Bl. Kraft soft/hardwood North America	N/A	N/A	Combined alkaline stream identified as source of decreased spawning events, egg production and ovipositor development in males.
Hewitt et al. [25], Belknap et al. [26], Shaughnessy et al. [27]	Plasma/gonadal testosterone, mummichog in vivo	Chemical recovery condensates	Bl. Kraft, hard/ softwood North America	SPE (sequential), GPC (sequential), HPLC	GC–MS	6 hydroxylated diterpenoids, sesquiterpenoids, and a stilbene associated with hormone depressions-*Tentative* –Additional semivolatile terpenes and/or phenolics causative, *Unknowns*
Jenkins et al. [28–30]	Androgenicity, in vitro	River water, sediment	Bl. Kraft, feedstock not provided, North America	SPE, HPLC	LC–MS–MS (triple quad)	Androstenedione, androstadienedione, progesterone *Confirmed*
Parks et al. [31], Durhan et al. [32]	Androgenicity in vitro, mosquitofish in vivo	River water	Bl. Kraft, feedstock not provided, North America	SPE, HPLC	Testosterone radioimmunoassay, GC–MS	Androstenedione *not* causative, additional androgens, *Unknowns*
Ellis et al. [33]	Androgenicity, in vivo mosquitofish, in vitro	Final effluent	Bl. Kraft/TMP, soft/ hardwood New Zealand	Filtration, SPE, soxhlet	GC–MS, LC–MS	Dissolved fraction androgenic in vitro not in vivo; additional, androgens, *Unknowns* –Androstenedione and testosterone *not* detected in active extracts
Larsson et al. [34]	Androgenicity, in vitro	Primary, final effluents	Bl. Kraft, softwood Sweden	SPE, semipermeable membranes, GPC (sequential) HPLC (sequential)	GC–MS	Progesterone partially associated with androgenicity, *Confirmed* –Identified 37 additional candidate androgens, 200–400 Da, carbonyl/ hydroxyl moieties, *Unknowns*
Parrott et al. [35], Hewitt et al. [36–38]	Androgenicity, estrogenicity, P4501A1 in vitro	Fish liver tissue extracts	Bl. Kraft, Bl. Sulfite hardwood + softwood North American mills (3)	Soxhlet, HPLC	GC–MS (high res)	Multiple ligands for estrogen, androgen and aryl hydrocarbon receptors with range of K_{ow} accumulated via waterborne exposure, *Unknowns*

(*continued*)

Table 1 (continued)

Citation	Endpoint(s)	Matrix	Mill process, Feedstock, Location	Isolation method	Characterization method	Substances identified, *ID Level attained*
Carson et al. [39]	Androgenicity, in vitro	Pine feedstocks, bark, needles	N/A	Solvent extraction, SPE, HPLC	LC–MS–MS	Progesterone identified as androgenic precursor of additional effluent androgens, *Confirmed*
Terasaki et al. [40]	Anti-androgenicity, estrogenicity, in vitro	Final effluent	Mill type, feedstock, not provided, Japan	SPE (sequential)	GC–MS	Seven resin acids identified as anti-androgens, *Confirmed*
Orrego et al. [41]	P4501A1, estrogenicity, R. trout in vivo	Untreated, primary, final effluents	Bl. Kraft, soft/ hardwood, South American mills (2)	SPE	GC–MS	All effects decreased after effluent treatment
Basu et al. [42]	Neurochemical receptors and enzymatic activities, in vitro	Primary, final effluent	TMP, softwood, North America	Sequential solvent extraction, polyphenol extraction	N/A	Activities in polar fractions before and after secondary treatment, *Unknowns*

XAD polystyrene extraction resins, *TSE* toxicity source evaluation, *Bl. Kraft* bleached kraft pulping, *Bl. Sulfite/GW* bleached sulfite with groundwood pulping, *GW* groundwood pulping, *TMP* thermomechanical pulping, *ECF* elemental chlorine free bleaching effluent, *D stage* chlorine dioxide bleaching stage effluent, *C stage* elemental chlorine bleaching stage effluent, *E stage* caustic extraction bleaching stage effluent, *ER* estrogen receptor, *AR* androgen receptor, *AhR* aryl hydrocarbon receptor, *HPLC* high pressure liquid chromatography, *SDS-PAGE* sodium dodecyl sulfate-polyacrylamide gel electrophoresis, *GPC* gel permeation chromatography, *NMR* nuclear magnetic resonance spectroscopy, *GC–FID* gas chromatography-flame ionization detection, *GC–MS* gas chromatography–mass spectrometry; high res: $>$ 10,000 mass resolving power, *TLC* thin layer chromatography, *UV–VIS* Ultraviolet–Visible spectrophotometry, *Unknown* lack of available spectral matches to speculate on structural identity, *Tentative* reasonable match between sample mass spectra and literature or library spectra [43], *Confident* no authentic standard available but MS and chromatographic data closely approximate experimental and/or published data of authentic standards [43], *Confirmed* match between mass spectral and chromatographic data between samples and authentic standards [43]

temporal fluctuations in active chemicals, (2) the effect of storage conditions (temperature, container type) on effluent toxicological potency, (3) the large amount of residual lignin material present that functions as a significant interference when investigating low molecular weight (<600 Da) biologically active extractives, (4) the complexity (temporal, within-mill, between mills) of the low molecular weight effluent extractives themselves, and (5) uncertainties regarding the bioavailability of identified bioactive components as influenced by the high proportion of lignin degradation products present.

This chapter examines the analytical approaches and results obtained from effects-directed analysis of pulp and paper mill effluents from the perspective of (1) the isolation and characterization techniques used to tackle various effluent matrices, (2) the evolution of the endpoints used to direct effects-directed investigations, and (3) the success levels attained (specific chemicals, classes or sources identified). These aspects are considered according to the effects of interest that span acute toxicity, chronic toxicity, mutagenicity, and endocrine disruption (Table 1).

2 Acute Toxicity

Early studies on the acute toxicity of effluents from pulping operations were largely successful in that specific causative agents were confirmed. One of the first effects-directed investigations concerning pulp mill effluents was by Das et al. [1] who indirectly implicated tetrachloro-*o*-benzoquinone and other chlorodihydroxybenzenes in the acute toxicity of kraft chlorination liquors to fish (Table 1). Studies conducted during the 1970s and 1980s continued to focus on kraft mill process streams, particularly the chlorination and extraction stages of bleaching, and the chemicals responsible for acute toxicity to salmonid fish [2, 54]. These were thorough investigations that utilized XAD resins for extraction, acid partitioning with aqueous base, and fractionation using silica gel and/or preparative TLC. From these investigations, resin acids, unsaturated fatty acids, and chlorinated phenolics were determined to be the major sources of acute toxicity, the results of which were confirmed with authentic standards. Diterpene alcohols, pitch dispersants, juvenile insect hormone analogues, and unidentified neutral compounds also contributed to lesser degrees (Table 1). These discoveries led to increased attention to these compounds [55], and the subsequent discovery of dioxins and furans led to regulations restricting their discharge in whole (adsorbable organic halide) or in part (dioxins and furans) [56]. As a result, the industry adopted process changes and effluent treatment throughout the 1990s to reduce the loadings of these compounds to the environment.

In Canada, the incidence of acute toxicity regulatory non-compliance from the mid-1990s to the mid-2000s has ranged from 10 to 25% [6]. Investigations of the cause(s) of these infractions has included effects-directed analyses in hypothesis-based diagnostic testing that included information on mill operating conditions and bioassay responses that provided additional information about the cause. Effluent manipulations that facilitated toxicity identification included filtration, addition

of chelating agents, solvent extraction using methyl-*t*-butyl ether with compound identification using gas chromatography–mass spectrometry (GC–MS). Using these techniques, the toxicants were identified in 70% of the cases involving >80 separate investigations of 32 mill effluents [6]. Toxicities for fish were largely attributed to biotreatment performance (e.g., ammonia), whereas invertebrate toxicities could be attributed largely to polymeric formulations used in pulp production (e.g., defoamers; Table 1).

With the emergence of the pulp and paper industry in South America over the last decade, investigations of the sources of acute toxicity of mill effluents of relevant tree species (*Pinus radiata* and *Ecualyptus globulus*) have been undertaken. In an approach used in other investigations (see Sect. 3), final effluents can be investigated by applying effects-directed techniques on individual process wastes that comprise combined mill effluent. Investigating individual process wastes is a phase of effects-directed studies applied to mill effluents known as Toxicity Source Identification (TSE) [57]. Using a combination of ion exchange resins, activated carbon and EDTA additions, it was shown that Cu^{2+} was the chief causative compound within the first alkaline extraction stage (E1) of the bleaching sequence at a Chilean Kraft mill toward *Daphnia magna* acute toxicity [7].

While obvious environmental benefits have been accrued from efforts and resources that the industry has applied to effluent treatment and reductions in loadings of chlorinated organics, suspended solids and biological oxygen demand, subtle effects on fish reproduction, first noticed in Scandanavia in the late 1980s [58], have persisted to the present day in Canada (reviewed in [59], the United States [60], Sweden [61], Finland [62] and New Zealand [33] with more recent evidence of effects emerging from Chile [41, 63, 64]. These include a broad spectrum of effects in both wild fish as well as laboratory bioassays (reviewed in [47]).

3 Endocrine Disruption in Fish

Despite the level of effort over the last 15 years, and with the experience gained from the identification of acutely toxic compounds in mill effluents, the compounds responsible for the persistent reproductive changes in fish have remained elusive. In addition to the obstacles confronting chemical aspects of effects-directed investigations of mill effluents, there has been a high level of uncertainty surrounding which biological endpoint to use in directing fractionations. This uncertainty is chiefly derived from the complexity of the responses, and the lack of a defined mechanism causing the effect(s) [47].

3.1 Induction of Detoxification P450IA1 enzymes

Although the mechanisms involved in the reproductive effects of mill effluents have been difficult to establish, bioassay-directed compound identification has

nevertheless progressed using responses observed from effects assessments of wild fish populations, namely induction of P450IA1 enzymes and reductions of gonadal sex steroids. Other investigations have employed in vitro assays such as binding to sex steroid receptors and androgen-responsive cell lines (Table 1).

Burnison et al. [14] attempted to directly isolate chemicals inducing P450IA1 activity in fish by following an effects-directed approach on final effluent from two Canadian bleached kraft mills. Using centrifugation, tangential flow filtration, and C18 solid phase extraction (SPE), effluents after secondary treatment were investigated using a 4-day rainbow trout in vivo bioassay. It was determined that methanol extracts of particulates/colloidal material and SPE fractions contained active substances. Work focused on the particulate material and showed that activity could be isolated using methanol extractions. High-pressure liquid chromatography (HPLC) isolations determined that the active substances were present in a relatively nonpolar region of the chromatographic separation, with a log K_{ow} of 4.6–5.1. As a result of follow-up studies using rainbow trout exposures and incubations with a rat hepatic carcinoma cell line (H4IIE) which directed HPLC fractionations of the methanol extract of the high molecular weight material, a chlorinated lignin-derived pterostilbene structure was postulated for an unknown compound strongly associated with induction [15]. This was significant in that it showed a natural product, modified in the bleach plant, was eliciting the biological response (Table 1).

In a comprehensive study, Martel et al. [16] determined the source and identities of two substances associated with induction present in the primary-treated effluent of a newsprint thermomechanical pulp (TMP) mill. To determine the sources of activity within the mill, the authors used a TSE approach and rainbow trout exposures to condensate, deinking, paper machine effluents, TMP whitewater, and various process effluents sampled throughout the mill. Contaminated TMP steam condensates were identified as the major process source of P450IA1-inducing substances. Using conventional liquid/liquid extraction, silica gel fractionation and preparative thin-layer chromatography (TLC) procedures, an inducing fraction was isolated. The major constituents were identified by gas chromatography/mass spectrometry (GC–MS) as juvabione, dehydrojuvabione, and manool, which are naturally occurring extractives in balsam fir. After extraction and isolation from balsam fir and TMP condensates using the methodology developed, trout exposed to juvabione and dehydrojuvabione exhibited significant induction. These results were consistent with previous results in that they also showed natural products prior to bleaching were causing the effect of interest.

3.2 Effects on Fish Reproduction

Subsequent studies from the mid-1990s to the present day have attempted to address the more complex issue of reproductive effects in wild fish. This approach of focusing on in vivo effects of biota in the receiving environment and then

working toward cause and effect solutions represents a unique and highly appropriate application of effects-directed investigations. Although ecologically relevant, reproductive dysfunction in wild fish has represented a much greater challenge to address because the mechanisms involved are not understood and likely involve pertubations in multiple pathways [65].

In the late 1990s, development of suitable bioassays, such as fish-specific sex steroid receptor assays [66, 67], life cycle tests [68] and short-term in vivo tests for steroid effects [69, 70], has provided the opportunity to couple mechanistically linked endpoints to chemical fractionations. This has led to the ability to formulate questions regarding the characteristics of bioactive substances, their relationship to production type, and whether compounds associated with sex steroid depressions are related to other reproductive impacts.

In the late 1990s, Parrott et al. [71] used caged fish to investigate the uptake of aryl hydrocarbon receptor (AhR) ligands from effluent from a bleached kraft mill. Ligands were recovered from hepatic tissues using successive methanol and dichloromethane (DCM) extractions and EROD induction in H4IIE cells as the indicator. In these investigations, the approach focused on what was bioavailable to the organism by using controlled exposures. One of the advantages of focusing effects-directed investigations on tissue residues is that it takes into account additional modification processes that may be involved in the responses, such as modification after mixing of effluent process streams, modifications during secondary treatment, modifications after release into the receiving environment, and metabolic modifications after accumulation.

In an extension of this approach, both unexposed wild fish and fish collected adjacent to the effluent outfall were held in a concentrated effluent stream (50% v/v) for 4 days at a bleached kraft mill known to cause reproductive dysfunction in wild fish [37]. Hepatic tissue extracts from exposed fish were soxhlet extracted with DCM, and fractionated according to lipophilicity using reverse phase HPLC where elution conditions were calibrated to achieve a linear relationship between K_{ow} and capacity factor (K'). Fractions were tested for the presence of ligands for the AhR in H4IIE cells, rainbow trout hepatic estrogen receptors (ER), goldfish testicular androgen receptors (AR), and goldfish sex steroid binding protein (SSBP) [37]. These results showed fish rapidly accumulate multiple non-dioxin ligands across discreet ranges of K_{ow} for the AhR and fish sex steroid receptors after a 4-day exposure. PCDD/DF equivalents measured by EROD activity in H4IIE cells and by high-resolution GC–MS showed that in all fish historically exposed to effluent, the contributions to total toxic equivalents (TEQs) from TCDD was >80%, and that naïve fish held in effluent accumulated 1,2,3,7,8-pentachlorodibenzofuran that accounted for a major portion of TEQ [37]. This study also showed that when fish normally residing in the effluent plume leave for a brief period to spawn in an uncontaminated stream, hepatic burdens of all ligands decrease to background.

Follow-up studies at a bleached sulfite/groundwood mill found the pattern of accumulated substances was very similar to that previously obtained at the bleached kraft mill [36]. A third study involved wild fish collected from the receiving

environment at another bleached kraft mill [38] and found detectable levels of hormonally active substances were present in hepatic tissues. HPLC fractionations of male and female hepatic tissues showed gender-based differential accumulations. Collectively, the bioaccumulation model is an excellent foundation for use in effects-directed investigations of substances of concern, with the added advantage of beginning with a mixture of lesser complexity.

Several researchers have investigated individual waste streams within the papermaking process to determine the source(s) of hepatic EROD induction and compounds affecting steroid levels in fish. Black liquor was the subject of investigations involving EROD activity and hormonal endpoints. The pulping process digests lignin, the complex phenolic polymer that binds cellulose fibers together. The spent cooking liquor, known as black liquor, contains the degradation products of lignin and cellulose as well as wood extractives such as resin and fatty acids. Zacharewski et al. [72, 73] found that the methanol extract of black liquor particles and colloids > 0.1 μm from a bleached kraft mill contained AhR ligands which also displayed anti-estrogenic effects via the AhR in vitro. Hodson et al. [73] investigated the potential of black liquor from hardwood and softwood pulping at a bleached kraft mill to induce EROD activity in rainbow trout and found significant activity. A higher-potency liquor was associated with alcohol digestion of wood chips, as well as solvent extracts of wood.

In the late 1990s an extensive investigation was conducted at a bleached kraft mill in New Brunswick, one of a handful of pulp mills in Canada that does not employ secondary treatment. This work successfully resulted in the identification of chemical recovery condensates as a primary source of substances that depress circulating sex steroids in fish [69] and focused subsequent bioassay-directed studies. Minimal high molecular weight material was found in the condensates, facilitating fractionation work [25]. Using steroid depressions in mummichog, a sequential SPE method using styrene divinylbenzne and reversible graphitized carbon solid phases was developed which completely recovered the active chemicals from the condensates in two fractions [25]. GC–MS profiles of both fractions revealed unknowns consistent with lignin degradation products and terpenoids originating from the wood furnish [74]. Further effects-directed studies have been confounded with an inability to measure hormone depressions previously observed [27]. Such observations may be the result of losses due to condensate handling procedures. They may also be the result of changes in mill operating conditions and performance, which underscores the dynamic nature of effluent compositions and having to “chase a moving target” in effects-directed studies (Fig. 1).

While the majority of effects-directed investigations have focused on fish reproduction, other reproductive effects have been investigated. Higashi et al. [75] used early embryonic development in marine echinoderms and mollusks to direct manipulations of effluents from a bleached kraft mill in northern California. In a particularly thorough study, final effluents were pH adjusted, filtered and lyophilized, and the residues sequentially extracted with DCM followed by acetonitrile. The solvent extracted residue was processed through an ultrafiltration membrane

Fig. 1 Typical bleached Kraft pulp and paper mill from North America (photo by J. Bennett, 2006)

and the retentate (>10 kDa) was lyophilized and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) [76]. These investigations have determined that lignin-derived macromolecules bind to the plasma membrane of the sperm head of purple sea urchins (*Strongylocentrotus purpuratus*), thereby blocking the acrosome reaction and preventing fertilization [77].

Since 2001, a series of effects-directed approaches to identify compounds causing masculinization effects in mill effluents have provided some controversial results (Table 1). Using in vitro tests, several attempts have been made to determine the cause of effluent masculinization in Florida, USA. The recipient river receiving bleached kraft effluent was found to contain androgen receptor agonists. In one case, further fractionation work identified androstenedione (AED) and progesterone as the causative agents [29]. However, an earlier study in which SPE was followed by HPLC fractionation identified a fraction without AED that caused androgenic activity [32]. Further evidence for progesterone being a source of androgenicity in mill effluents was provided in a follow-up study where pine feedstocks (including bark and pine needles) were subjected to SPE, fractionation with HPLC and characterization using LC–MS–MS [39]. Fractionations directed by an in vitro androgen receptor transcription assay isolated androgenic activity in approximately three fractions of the wood materials investigated; one of these fractions contained progesterone. Other androgens were unidentified.

Multi-tiered bioassay-directed fractionation experiments of primary and bio-treated effluents from a chlorine-free Swedish kraft mill directed by androgen receptors isolated from Atlantic croaker (*Micropogonias undulates*) ovaries showed that unidentified compounds possessing diterpenoid skeletons are present in multiple fractions exhibiting binding affinities and that a receptor-mediated pathway is the primary route via which masculinization effects occur [34]. Progesterone was

identified as an androgen in primary effluent but was absent following biological treatment. Collectively, this evidence suggests that progesterone may be a source of androgens in mill effluents that are reduced or eliminated, depending on biotreatment efficiency.

Work has continued in the area of endocrine disruption by incorporating new assays (anti-estrogenicity, neuroendocrine receptors) to direct fractionations, thus shedding light on new mechanisms involved in the overall effects of mill effluents. In a recent successful study, seven resin acids and two esterified resin acids were found to account for 72% of the anti-estrogenic activity of Japanese mill effluents. The investigators used sequential SPE (C18 followed by Florisil) extraction to isolate the bioactive substances and obtained chemical confirmation by GC–MS [40]. Basu et al. [42] fractionated primary and secondary effluents from a Canadian softwood TMP mill using sequential liquid–liquid (hexane followed by ethyl acetate) and polyphenolic extraction approaches and concluded that mill effluents contain chemicals that function as ligands for neurotransmitter receptors and affect neurotransmitter metabolism involved in fish reproduction. While the chemicals themselves were not identified they were contained in polar extracts of effluent before and after treatment. This discovery may explain the rapid onset and dissipation of some reproductive effects of mill effluents and is significant in that a new, plausible mechanism for these effects has been provided.

4 Mutagenicity

In contrast with effects-directed studies of endocrine disruption, studies on mutagenicity have used commonly accepted genotoxic bioassays with well defined endpoints (Table 1). From the early 1980s until the mid-1990s, the mutagenic activities of mill effluents have employed extraction techniques based solely on XAD resins. Most of the mutagens are derived from polar compounds produced from individual waste streams, with corresponding weak evidence of final effluents containing mutagenic substances. Efforts were first directed toward chlorination stage effluents of mill bleach plants that provided the strongest activity and led to the association of chloroacetones with mutagenic activity [9]. Kinae et al. [10] detected genotoxins in livers of wild fish collected from areas receiving pulp mill wastes, indicating the potential for exposure and bioaccumulation. Holmbom et al. [12] used a combination of ethyl acetate and XAD-4 resins to quantitatively recover 70–90% of the mutagenic activity from chlorination bleachery effluents. The majority of the recovered activity was removed by partitioning with aqueous $NaHCO_3$. Preparative thin layer chromatography (TLC) was used as a first step in isolation, followed by C8 RP-HPLC, further preparative TLC, C18 HPLC, and a final TLC step that allowed the isolation of a compound labeled “MX.” As this compound was not amenable to GC–MS analyses, methylated, acetylated and trimethylsilyl derivates were synthesized and analyzed to facilitate structural interpretation.

5 Bioassay Considerations for Effects-Directed Investigations

Since this chapter considers a variety of toxicological endpoints used to direct chemical manipulations, it is necessary to highlight some considerations surrounding endpoint selection. Of primary importance is the scale of the bioassay, which dictates the scale of the separations. This will influence not only fractionation method development, but preparation for bioassay testing. Factors for consideration are the ratio of organism biomass:test solution, toxicity of exposure chamber materials, dissolved oxygen in the test solutions, and dilution of test solutions upon transfer of the organisms. Additional considerations for the bioassay response itself include: consistency, reliability, replication and time for response. Also important and related to the scale of the bioassay is its relevance to the whole organism response being tested. Is it an in vitro or an in vivo bioassay? Obviously an in vivo assay has greater relevance to detecting an effect in an organism, as opposed to an in vitro test which ultimately requires validation in vivo.

The scale of the bioassay has obvious effects on the choice of chemical manipulations; micro-scale in vitro assays require less material to test and less effluent to be initially extracted. Larger scale in vivo bioassays involve preparative techniques that can be quite laborious and require large effluent volumes (>10 L) to be processed. This can become tedious when additional fractionation levels are added, which can inflate costs. Bioassays are typically destructive in their consumption of isolates, necessitating either a continuous supply of active material or the preparation of a "stock" at the outset of experiments.

Often cytotoxicity of in vitro tests are not reported. The interference of cytotoxicity, perhaps due to physicochemical characteristics of an effluent sample, is a highly relevant phenomenon in toxicity testing. Dead cells do not exhibit effects, and cytotoxicity may result in false negatives. It is therefore important to evaluate cytotoxicity by testing a dilution series of the sample or by providing some measurement of cell viability. In vitro tests offer distinct advantages in that they do not kill large numbers of laboratory animals, provide rapid responses with adequate replication and are relatively inexpensive.

6 Regulatory Applications

It is worth noting the importance of effects-driven fractionation studies conducted on pulp and paper mill effluents and its incorporation into regulatory practice in Canada. Environmental Effects Monitoring (EEM) programs in Canada have been developed for the pulp and paper and metal mining industries, where cyclical evaluations of the health of biota in receiving environments determine whether effects exist when facilities comply with existing regulations. Investigation of cause (IOC) is a specific stage in EEM that involves determining the sources and causes of effects observed in the receiving environment of a discharger. Several levels of

effort have recently been described that can be undertaken for cause identification [78]. The framework includes levels to define whether there is an effect, whether it is related to the effluent discharge facility, and whether response patterns in the receiving environment are characteristic of a particular stressor type. The next tier of the framework involves investigating individual process wastes within the mill to determine the components contributing to final effluent effects. In contaminant-focused causal investigations, questions progress along a continuum which first asks if the source within the mill can be identified, and to effects-driven identification to the compound classes and ultimately, the specific chemicals involved. The fundamental question driving the investigations is whether sufficient information has been generated to define the effect such that a mitigative solution can be found. Effects-directed questions within the framework have been tailored so that the investigation may be halted when that information is attained [78].

As reviewed here, numerous laboratory tools exist to investigate the reproductive effects of mill effluents on fish, however there has been no consensus as to which are the most appropriate for use in effects-directed work. The regulatory EEM studies in Canada have identified metabolic disruption in fish as a national response pattern that includes reduced gonad size [44]. At present, a practical test for gonad size does not exist and it is not known if other measures of fish reproduction (e.g., sex steroid levels, androgenicity; Table 1) are related to gonad size or are predictive of the gonad size reductions observed in wild fish. An evaluation of available tests is therefore necessary to determine what linkages exist across these levels of biological organization involved before actual causes and solutions can be found [47].

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Effect-Directed Analysis of Ah-Receptor Mediated Toxicants, Mutagens, and Endocrine Disruptors in Sediments and Biota

Markus Hecker and John P. Giesy

Abstract Sediments and associated biota represent important sources for the exposure of aquatic organisms to environmental toxicants including dioxin-like compounds, genotoxic chemicals, and endocrine disruptors. One of the key challenges that environmental toxicologists and risk assessors are facing is the characterization and assessment of toxicological risks associated with such complex matrices such as sediments. Therefore, approaches have been developed supplementing chemical analysis with bioanalytical techniques that make use of the specific properties of certain groups of chemicals to interfere with specific biological processes. This type of analysis has been coined effect-directed analysis (EDA), and is based on a combination of fractionation procedures, biotesting, and subsequent chemical analyses. In this chapter, we review the current state of the art of EDA regarding the assessment of sediment and biota samples for dioxin-like, genotoxic, and endocrine disrupting potentials. We discuss in vivo and in vitro screening concepts that are used in combination with fractionation and chemical analytical techniques to aid in the risk assessment of these chemical groups in sediments and biota. Advantages and disadvantages of current EDA strategies are considered, and recommendations for more realistic and relevant EDA approaches are given. Specifically, these include the use of optimized biotest-batteries covering a broad range of different endpoints as well as the inclusion of in vivo tests, and the parallel assessment of ecologically relevant parameters such

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W. Brack (ed.), *Effect-Directed Analysis of Complex Environmental Contamination*,
Hdb Env Chem (2011) 15: 285–314, DOI 10.1007/978-3-642-18384-3_12,
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as benthic community structure. Furthermore, the need for refinement and standardization of current sediment EDA approaches that allow capturing and assessing exposures to unknown or emerging chemicals such as endocrine disruptors, perfluorinated compounds, or polybrominated and mixed halogenated dibenzo-*p*-dioxins and -furans is discussed.

Keywords Bioassay, Complex mixtures, Dioxin-like chemicals, Endocrine activity, Fractionation, Genotoxicity, In vitro, Toxicity identification and evaluation

Contents

1 Background ... 286
1.1 Toxicity Identification and Evaluation vs. Effect-Directed Analysis ... 287
1.2 Dioxin-Like Activities ... 289
1.3 Genotoxicity ... 291
1.4 Endocrine Disruption ... 292
2 Effect-Directed Analysis in Sediments and Biota ... 293
2.1 In Vivo Bioassays ... 294
2.2 In Vitro Bioassays ... 295
2.3 Bioassay-Directed Fractionation ... 296
2.4 Relative Potency and Potency Balance Analysis ... 299
3 Limitations and Perspectives of Bioassay-Directed EDA ... 302
4 Conclusions ... 305
References ... 306

1 Background

Since the middle of the twentieth century there has been increasing concern over the potential adverse effects of xenobiotics present in the environment on human and wildlife populations. Three groups of toxicants that are of particular interest relative to potential environmental health effects are dioxin-like, genotoxic, and endocrine active chemicals. Some of these ubiquitous compounds are hydrophobic, lipophilic, and resistant to biological and chemical degradation. These properties impart persistency and a propensity to bioaccumulate and biomagnify to concentrations that can cause deleterious effects. Sediments, especially, represent long-term sinks for some contaminants [1–8], which can become bioavailable through remobilization events such as floods or dredging, or through benthic or bottom-dwelling organisms [9–13]. Severely contaminated sediments have been reported to occur in rivers and lakes, and have been shown to be acutely and chronically toxic to fish and benthic invertebrate species [8, 14–16]. Under certain conditions, toxicity of sediments has been reported to contribute to decreases in reproductive success, recruitment, and alterations in community structure, effects which could potentially cause changes in population structure. For example, amphipod abundance in San Francisco Bay was found to be inversely proportional to contamination and measured toxicity of sediments [14]. Studies have shown that toxic sediments can affect aquatic species. Zebrafish

embryos exposed to sediments from the Danube River showed impaired development and decreased hatching rates compared to control fish [8]. Sediments have been classified as genotoxic, mutagenic, endocrine disrupting, or recognized for dioxin-like effects [8, 15, 17, 18]. In addition to their acute or chronic toxicity to benthic invertebrates, accumulated residues in sediments can be a pool that can be accumulated through food webs and have subsequent toxic effects on the predators.

Chemicals typically occur as mixtures in environmental matrices such as sediments and biota, and can include different congeners and isomers of both natural and anthropogenic origin. Concentrations and toxic potencies of compounds present in mixtures can range over several orders of magnitude. In addition, interactions among different classes of compounds, such as estrogenic vs. anti-estrogenic, can modulate the toxic potential. Exposure to mixtures and the potential for greater and less than additive effects of mixtures complicates hazard evaluation and risk assessment of complex mixtures of xenobiotics. So, even if complete information is available about the concentrations of all inorganic and organic residues, it is difficult to predict the effects of the mixture. However, this complete knowledge is seldom the case. In fact there might be residues present that have not yet been described in the literature. There are contaminants and/or their degradation products that can cause toxicity for which there are no analytical methods or authentic standards. Furthermore, toxic effects of some contaminants, even those, which are analytically determined, are not well characterized. There are potentially significant classes of contaminants for which little or no information on their effects on organisms is available. In other words, chemical analysis has been used to identify and quantify only those chemicals for which analytical techniques and standards are available. In environmental monitoring, chemists find what they look for. If they do not know to look for a chemical, then it will not be quantified. Finally, instrumental analyses do not account for interactions among the chemicals in mixtures and provide little information on their biological availability and provide no information on their effects. Thus, relying on quantification of individual residues by instrumental analyses, while useful for source identification, can underestimate the potential risks posed by these chemicals and some toxicologically important compounds could be overlooked. In summary, analysis of the vast number of chemicals typically present in an environmental sample would not only be prohibitively expensive but simply impossible due to limits in the available analytical methodologies for many chemicals, especially since often no a priori knowledge of the chemicals present in the sample exists.

1.1 Toxicity Identification and Evaluation vs. Effect-Directed Analysis

To overcome some of the above discussed limitations, bioanalytical approaches have been developed to supplement instrumental chemical analysis. These bioanalytical techniques make use of the specific properties of certain groups of chemicals

to affect biological systems. Such bioassays are often based on in vitro responses of cells or even cell-free responses of biomolecules but can also utilize whole organism in vivo systems. The use of bioassays in chemical identification has several advantages relative to instrumental analyses. First, the assays “read out” directly in terms of a biological response. This could be a molecular change or even something as simple as lethality. The bioassay responds to all of the chemicals in the mixture so even unidentified chemicals that cause a particular endpoint are measured even if they cannot be identified. These assays also respond proportionally to the aggregate effects of mixtures and account for all of the interactions among chemicals. In fact, bioassays can be used in conjunction with instrumental analyses in a “potency balance” approach that helps determine if there are unidentified active compounds. In particular, bioassays can be used with a fractionation scheme to direct instrumental analyses to identify unidentified active chemicals in a mixture.

In the late 1980s, one of the first standardized effect-directed analysis (EDA) procedures, the toxicity identification and evaluation (TIE) approach, had been established by the US-EPA. This approach focuses primarily on the identification and evaluation of organic or inorganic contaminants in aqueous samples using a combination of in vivo tests, fractionation, and chemical analysis, and is characterized by the following three steps (reviewed in [19]):

1. Toxicity characterization by assignment of toxicity to general groups of toxicants (typically bioassay directed analysis)
2. Identification of suspected toxicants (chemical analytical determination)
3. Confirmation of the suspected cause of toxicity

The basic concept of the TIE approach is the removal of compound groups from the tested matrix until the toxicity of the sample disappears. Suspected chemicals are then identified by analytical chemistry, and their toxicity is confirmed by means of the same bioassay used in the initial characterization phase. Approaches like TIE have been an important step towards improving environmental risk assessment (ERA) focusing on surface waters. It has been increasingly recognized, however, that particle-bound contaminants such as suspended matter or sediments, or those accumulating through the food chain might be of greater ecotoxicological relevance with respect to moderately or strongly lipophilic compounds. While there have been some recent developments of standardized EPA techniques and procedures for sediment TIE [20, 21], there are still a number of uncertainties associated with this approach. For example, it is often difficult to construct an artificial mixture of the suspect compounds identified in the characterization phase, because these may not always be commercially available. Furthermore, exposure of tests organisms as part of the confirmation step is problematic since it requires spiking of clean or artificial sediments with the suspected chemicals, and which often alters the toxicological properties of the tests matrix because of differing adsorption and/or bioavailability properties. Finally, TIE primarily relies on whole organism tests that often do not provide information on the specific mode of toxicity of a sample, and which can be helpful in identifying the compound groups responsible for the measured effect.

A recent approach that aims to overcome some of the shortcomings of TIE is the EDA procedure as reviewed by Brack [19, 22]. Like TIE, the principle of EDA is to use a biological response to direct the identification of causative agents in a complex matrix such as sediment. During the conduct of an EDA, however, the causal substances are identified by analyzing different extracts that were prepared from the original sample, e.g., by separation based on lipophilicity, polarity, size, etc., and not by a retrospective step-by-step exclusion of certain compound groups as done in TIE. Furthermore, during EDA the biological analysis phase is typically dominated by in vitro or in vivo mechanistic assays that enable identification of the properties of a sample to interact with specific biological pathways or targets, and thus, can narrow the group of suspected chemicals (e.g., dioxin-like chemical that can be detected by means of an AhR assay as described in Sect. 2 in [23]). Thus, EDA has some distinct advantages over sediment TIE by better intertwining the specific biological (toxicity) and analytical pathways. However, the TIE approach is considered to be of greater ecological relevance because it does not require alteration of the sample through extraction, and utilizes whole organisms that are tested in direct contact with the matrix of concern.

1.2 Dioxin-Like Activities

Chemicals that elicit toxic effects similar to that of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), known as dioxin-like chemicals, are of concern due to their ability to cause hepatotoxicity, embryotoxicity, teratogenicity, immunotoxicity, dermal toxicity, lethality, carcinogenesis, wasting syndrome, and tumor promotion in many different species at relatively small concentrations [24, 25]. A number of studies have demonstrated that several toxic and biochemical effects caused by dioxin-like chemicals are mediated through the aryl hydrocarbon receptor (AhR) [26, 27] and associated dioxin responsive elements (DREs) on DNA. The AhR, which belongs to the basic helix-loop-helix protein family [28], is a ligand-dependent transcription factor, complexed with heat shock proteins located in the cytosol. The strength with which congeners bind to the AhR is directly proportional to the toxicity, enhanced gene transcription, and enzyme activities mediated by the AhR mechanism [29]. The role of the AhR in mediating toxic and biological effects of dioxin-like chemicals has been well documented in a number of studies, even though the exact biochemical mechanism leading to the pleotrophic toxic responses is yet to be elucidated [22]. After binding of ligands to cytosolic AhR, heat shock proteins dissociate from the complex, the receptor ligand complex is activated and translocated to the nucleus, where it forms a dimer with the Ah receptor nuclear translocator (ARNT) protein and possibly other factors. The heteromeric ligand: AhR:ARNT complex binds with high affinity to specific DREs on DNA. Binding of the transformed ligand-AhR-ARNT complex to DREs results in DNA bending, disruption of chromatin and nucleosome, and thus increased promoter accessibility and transcriptional activation of adjacent responsive genes (Fig. 1) [31, 32].

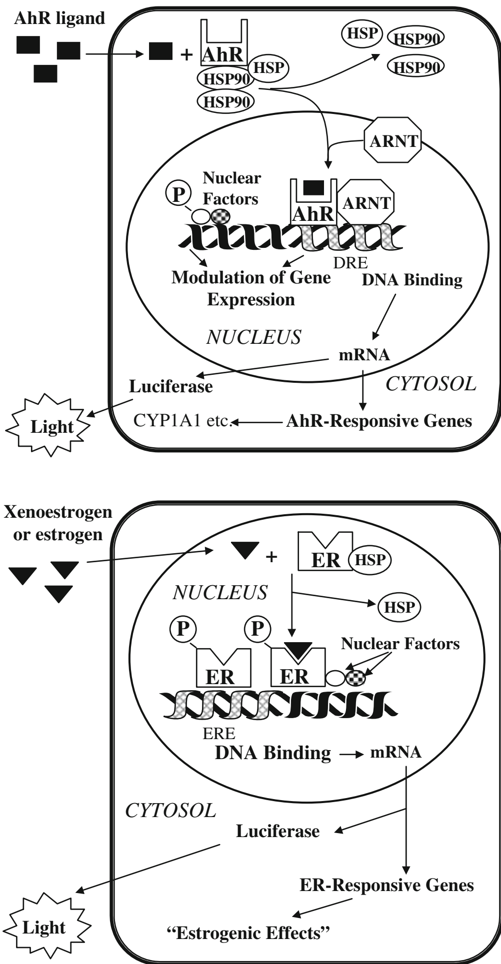

Fig. 1 Mechanism of AhR- or ER-receptor-mediated response in cells (adapted from [30]). For description see text. *HSP* heat shock proteins, *P* = phosphates: phosphorylation is an important regulatory factor for receptor function

The traditional, well-known ligands for AhR have been described as hydrophobic aromatic compounds with planar structure of a particular size of the molecule or a part of the molecule, which fits the binding sites [33, 34]. Thus, the ability of these ligands to bind to the AhR and to cause toxic effects greatly depends on their structure and substitution pattern. These include planar congeners of polychlorinated dibenzo-*p*-dioxins and polychlorinated dibenzofurans (PCDDs and PCDFs), chlorinated azobenzenes and azoxybenzenes, polychlorinated biphenyls (PCBs), several polycyclicaromatic hydrocarbons (PAHs) and polychlorinated napthalenes (PCNs) [35]. Other chemicals that have been suggested as potential AhR agonists due to their stereochemical configuration, but not yet experimentally confirmed, include polybrominated and chloro-/bromo- analogs of the previously listed classes of compounds [36], alkylated-chlorinated dioxins and furans, chlorinated

dibenzothiophenes, chlorinated xanthenes and xanthones [37], polychlorinated diphenylthiophenes (PCDTs), anisols (PCAs), anthracenes (PCAN), fluorenes (PCFL), and others [38]. Recently, new types of relatively weak AhR ligands or inducers (compared to TCDD) have been identified, which include both natural and synthetic compounds [31]. These compounds deviate from the traditional criteria of planarity, aromaticity, and hydrophobicity. The natural compounds that bind to the AhR include, among others, indoles, tryptophan-derived products, oxidized carotinoids, and heterocyclic amines. Some pesticides or drugs with various structures, such as imidazoles and pyridines, also possess the AhR binding ability. These ligands act as transient inducers and bind to the AhR with weak affinity and are rapidly degraded by the induced detoxification enzymes.

1.3 Genotoxicity

Chemicals in the environment can cause overt toxicity, but they can also cause subtle changes that may not result in immediate toxicity. One such effect is genotoxicity. Chemicals, such as biotransformation products of some polycyclic aromatic hydrocarbons (PAH), which are common contaminants in sediments, can bind to DNA where they cause a number of types of damage. The resulting DNA adducts can result in point mutations or strand breaks or other types of reorganization of the DNA that can result in adverse effects in germ cells and can result in decreased fitness of individuals in subsequent generations [39].

A number of in vitro and in vivo techniques have been developed to screen for these effects. Here we provide three examples of tests that have been found to be useful in screening of sediments for genotoxic potentials. The first measures point mutations or mutagenicity in vitro, while the second is an in vivo test that measures the occurrence of DNA strand breaks. The Ames assay uses the TA 98 *Salmonella typhimurium* bacteria strain to measure frame-shift mutations and the TA 100 *S. typhimurium* bacteria strain to measure base pair substitutions [4]. These strains are mutants that cannot produce histidine. A colorimetric measure of the number of back-mutated cells that are able to produce histidine is used as a measure of the mutagenic potency of a pure chemical or sediment extract.

Some chemicals need to be metabolically activated to cause mutagenicity. Because *Salmonella* do not possess the metabolic machinery to bioactivate molecules such as certain PAHs, S9 microsomal preparation can be added to samples. The Ames assay can be conducted using different bacteria strains, the most used of which are the mutated strains (TA 98 and TA 100) and with and without preincubation of the extract with the S9 microsomes to enable a comprehensive assessment of the mutagenic potential of a sample.

Another method to determine genotoxic effects measure DNA fragmentation [40]. This assay, which is variously called the alkaline DNA unwinding assay or the comet assay, measures small fragments of DNA that occur due to breaks in the DNA. Under alkaline conditions double-stranded DNA will unwind, such that when

separated by polyacrylamide gel electrophoresis (PAGE), the smaller fragments of DNA migrate more quickly than the larger strands of DNA. The more fragmentation, the more bands that can be identified. Because of the pattern formed on a two-dimensional PAGE that represents a comet, this assay is often referred to as the "Comet" assay [41]. DNA strand breaks can be studied in either in vitro or in vivo assays.

A third type of genotoxicity assay that is commonly used to assess genotoxicity of chemicals or complex matrices such as sediments is the micronucleus test. The assay measures the formation of a micronucleus during the metaphase/anaphase transition of mitosis, e.g., as the result of an acentric chromosome fragment detaching from a chromosome after breakage, and which coalesce into bodies of chromatin material referred to as micronuclei. The number of micronuclei present in cells is directly proportional to DNA damage [42].

1.4 Endocrine Disruption

Over the past 2 decades, there has been increasing concern about the possible effects of chemicals in the environment on the endocrine and reproductive systems in humans and wildlife [43, 44]. Such compounds have the potential to disrupt normal reproduction or developmental processes which can lead to adverse health effects such as compromised reproductive capacity, breast and testicular cancer, reproductive dysfunction such as feminization or demasculinization of males, and other adverse effects. A range of classes of compounds including natural products, pharmaceuticals, pesticides, and other industrial chemicals have been shown to affect endocrine systems of wildlife and humans. The manner by which these chemicals can interact with the endocrine system is manifold, and in general it is distinguished between compounds that elicit their response through binding to hormone receptors, and those that act through other mechanisms such as interference with steroidogenesis. While any chemical that causes an organism to be unable to maintain homeostatic regulation could be classified as an endocrine disrupting chemical (EDC), chemicals classified as EDCs have typically been those that are either able to bind to hormone receptors or can modulate the expression of steroid hormones such as estrogens or androgens or thyroid hormones. There are both natural and artificial chemicals that can modulate the endocrine system. These chemicals can be direct-acting and cause effects as receptor agonists or antagonists or can have indirect effects that ultimately modulate expression of genes that lead to effects that are similar to those caused by direct-acting effects. For example, a chemical that can induce the activity of CYP19 (aromatase) can result in the production of more endogenous estradiol (E2), and subsequently would cause an estrogenic effect even though it might not bind to the estrogen receptor (ER).

Much of the research in the area of environmental endocrine disruption has been focused on chemicals that can bind to hormone receptors including the estrogen receptor (ER) and androgen receptor (AR) as either agonists or antagonists.

However, various modes of actions have been reported, which include binding of chemical to other nuclear receptors, which then interact with an estrogen responsive element; acting through other receptors and/or signal transduction pathways; modulations of steroidogenesis and catabolism of active steroid hormones [45–48]. Estrogenic compounds are characterized by their ability to bind to and activate the estrogen receptor, which is a transcription factor belonging to the steroid receptor family. While there are structural similarities among some compounds that are ER agonists, other ER-active compounds do not share similar structures. Upon binding of an estrogenic compound to the ligand binding domain of the ER (located predominantly in the nucleus), the associated heat shock protein complex, which masks the DNA binding domain, dissociates and subsequently the ligand occupied receptor dimerizes. The homodimer complex interacts with specific DNA sequences referred to as estrogen response elements (EREs) located in the regulatory regions of estrogen-inducible genes. ER complexes bound to an ERE recruit additional transcription factors, leading to increased gene transcription and synthesis of proteins required for expression of hormonal action (Fig. 1) [49]. A series of natural and synthetic endocrine disrupting compounds have been identified by different in vivo and/or in vitro methods. Unlike chemicals that can directly interact with the nuclear hormone receptors, there is a multitude of different ways by which chemicals can interact with other endocrine processes such as steroidogenesis. For instance, substances such as some imidazole-like fungicides and phyto-flavonoids have been shown to modulate hormone production by affecting activities of the steroidogenic enzymes aromatase (CYP19) and 17β-hydroxysteroid-degydrogenase (17β-HSD), respectively [45, 46, 50]. Other chemicals such as naphthenic acids can inhibit estradiol metabolism, and thereby increase estradiol concentrations in vitro [51].

EDCs such as pesticides, plasticizers, plant sterols, PAHs, etc., have all been measured in sediments and have been shown to disrupt the endocrine system in in vitro and in vivo assays [46, 52, 53]. For example, known estrogen receptor agonists, such as 17α-ethinylestradiol (EE2), 17β-estradiol (E2), and bisphenol A, have been measured in sediments in several ecosystems [54–56]. Sediment extracts from the Upper Danube River produced estrogenic-like responses in a transcriptional ER assay [8]. It has also been reported that the same sediments caused embryo toxicity, disruptions in hatching rates and time to hatch in *Danio rerio* embryos [8]. Other endocrine effects that were caused by sediment-associated contaminants were changes in the expression of key genes involved in steroidogenesis [57], and alteration in the production of the sex steroids testosterone (T) and E2 [18] using the H295R cell line.

2 Effect-Directed Analysis in Sediments and Biota

Specific testing systems have been developed for the detection of dioxin-like, genotoxic, and endocrine active potential in environmental samples. These systems can be separated into two general categories: (1) in vivo assays using whole

organisms, or (2) in vitro tests utilizing cellular or sub-cellular systems that detect interactions with specific biological functions. These bioassays are used to assess the net effects of a complex sample to an animal or in vitro system. Organisms are predominantly used to identify effects of sediment-bound pollutants in direct contact assays with the unaltered sample on apical endpoints such as growth, reproduction, and survival. They are typically utilized to assess the biological risk of a given exposure but often do not allow pinpointing the effect to specific contaminants in a sample. Therefore, whole organism assays are often paired with a parallel exposure assessment by means of a combination of in vitro assays and analytical chemistry. In vitro bioassays are based on the responses of either wild type or genetically altered eukaryotic or prokaryotic cells that enable the assessment of potencies of individual chemicals or complex mixtures of environmental contaminants in extracts to cause effects specific to the exposure with certain chemical groups. Specifically, either endogenous responses or specific exogenous induced alterations incorporated into a cell are used for the measurements. The induction of specific responses following the exposure of cells to specific chemicals or mixtures of compounds can be assessed by measuring endogenous or engineered responses such as mutations, DNA strand breaks, protein expression, enzyme activity, etc., depending on the test system and endpoint.

2.1 In Vivo Bioassays

There are a number of organisms that are amenable to determine the toxicity of sediments [58]. Here we will focus on those that are most useful for studying the three classes of chemicals discussed in this chapter. While there are a number of hypotheses and recommendations for the use of invertebrate systems to assess the endocrine-disrupting potential of sediments to date, only very few studies have used this approach relative to EDA [59, 60]. One of the key issues associated with the use of different species is uncertainties regarding the predictability of endocrine effects to vertebrates because they have different hormone systems. Also, since most invertebrates do not express the AhR, they are essentially unresponsive to dioxin-like compounds. Finally, while benthic invertebrates might represent useful sentinels for the assessment of genotoxic potentials of sediments [61], they are rarely used in this context.

For application in the EDA process described here, therefore, we will focus on vertebrate assays because they have been successfully used in EDA of sediments. There have been numerous efforts to use fish species such as sanddab (*Citharichthys stigmaeus*), California halibut (*Paralichthys californicus*), flounder (*Platichthys flesus*), or trout (*Oncorhynchus mykiss*) to assess exposure to dioxin like or EDCs in sediments [54, 62–64]. However, most of these studies utilized large organisms in time- and cost-intensive experiments. One promising test is the zebrafish (*D. rerio*) embryo sediment contact test [15, 65].

The zebrafish is a small, easily cultured freshwater fish that reaches sexual maturity in approximately 3 months. They produce between 50 and 200 eggs every 2–3 days and the embryos develop rapidly. The eggs have a transparent chorion, which makes it relatively easy to monitor development of the embryo. The size of the zebrafish allows exposures to be performed in 24-, 48-, or 96-well culture plates. The protocol for using these fish is fairly simple. Zebrafish embryos 1–2 h postfertilization are exposed directly to sediments or to diluted sediment extracts in 96-well plates in 100 μL ISO water containing 20 mM $CaCl_2 2H_2O$, 5 mM $MgSO_4 7H_2O$, 7.5 mM $NaHCO_3$, and 0.037 mM KCl. Embryos are covered with an oxygen permeable cover and incubated at 27°C for 48 h. The embryos can be monitored for various endpoints, including lethality and deformities, but can also be used in subsequent measures of gene expression and enzyme activities such as CYP1A, which is under the control of the DRE, and genotoxicity, e.g., by means of the comet assay [15, 66]. The only shortcomings of embryo-based assays, such as the zebrafish egg contact test, are its limitation to measure effects on the endocrine system due to the lack of sexual differentiation at this life stage.

2.2 *In Vitro Bioassays*

In vitro bioassays have been used to assess dioxin-like, genotoxic, and endocrine activities in a variety of environmental matrices, including sediments and biota. Various environmental samples, such as sediments [4, 18, 52, 53, 67, 68] or particulate matter [69, 70], sludge [71, 72], and animal tissues, have been assessed regarding their potential to cause toxicity in vivo or in vitro. Significant dioxin-like activity has been observed in egg extracts of birds such as herring gull, cormorant, and great blue heron [73, 74] as well as in birds at different stages of development [75]. Among other animals, extracts of fish (white sucker, juvenile whitefish) [37, 72], bivalves [76], and otter [77] have also been tested. Different than in tests with live organisms, in vitro assays typically require clean up and extraction of the original sediment or tissue samples prior to testing. This is usually done through extraction by organic solvents. The solvent of choice needs to be compatible with the cell system, not causing any effect by itself, but enabling distribution of the extracted material to the cells. Extracts can be cytotoxic, which is caused by some compounds present in complex mixtures. For example, sulfur is a major cytotoxic constituent in sediment extracts, which should be eliminated prior to performing dioxin-like or estrogenic activities. The measurement of cell viability/cytotoxicity is essential in all bioassays dealing with complex mixtures as well as single compounds. Cell bioassays with multi-well plate formats enable the measurement of several samples at the same time. In addition, current procedures allow subsequent measurement of viability index, enzyme activity, and protein content in the same multi-well plates [35].

A number of different measurement endpoints are used to assess the exposure to dioxin-like, genotoxic, and endocrine active chemicals. Exposure to chemicals that exhibit dioxin-like properties can be measured by increased expression and induced

activity of cytochrome P4501A1 and its monooxygenase activities, such as 7-ethoxyresorufin *O*-deethylase (EROD) or aryl hydrocarbon hydroxylase (AHH) [63, 78]. Genotoxicity can be assessed by measuring a variety of endpoints, including DNA strand breaks using, e.g., the comet assay, micronucleus formation, and mutations [40, 79–81]. The potential of chemicals or environmental samples to interact with the endocrine system is typically assessed by means of three endpoints: (1) binding to the estrogen receptor (ER), (2) binding to the AR, and (3) alteration of sex steroid production through interaction with steroidogenic pathways. Determination of the potential of a chemical to interact with the ER or AR is either conducted by means of direct receptor binding assays or by transcriptional assays using genetically modified cells [49]. The latter are typically obtained by transfection of the so-called wild type cells with recombinant expression vectors, which contain selective responsive elements upstream of a reporter gene. The most common reporter genes are firefly luciferase (luc), alkaline phosphatase (PAP), chloramphenicol acetyl transferase (CAT), or β galactosidase (LacZ) [82, 83]. Effects on steroidogenesis can be measured at the gene, protein, or end-product level. Common assays include the quantification of changes in steroidogenic or metabolic genes [84], CYP19 aromatase enzyme activity (transforms androgens into estrogens) [46], or quantification of steroid hormones [47, 85]. A list of some bacterial, yeast, animal, and human cell lines used for the detection of in vitro TCDD-like, genotoxic, or endocrine activity is shown (Table 1).

2.3 Bioassay-Directed Fractionation

As discussed previously, in vitro bioassays as well as certain in vivo tests such as the *D. rerio* embryo sediment toxicity assay can be used in support of the characterization of complex mixtures in sediments or biota. Specifically, bioassays can be used in combination with specific analytical techniques in a tiered approach, which is termed bioassay-directed fractionation methodology. This approach provides information needed for monitoring and risk assessment of the compounds with specific modes of action and may lead to identification of novel classes of environmental toxicants [70]. Specifically, a complex sample (e.g., sediment or tissue extract) is first analyzed using one (for specific questions such as the characterization of dioxin-like chemicals) or a combination of multiple (nontarget analysis) bioassays representing different modes of biological action. Parallel to the assessment of effects on specific biological processes, the general toxicity of a sample (e.g., cytotoxicity for a cell-based bioassay) that could mask a specific response is evaluated. If a sample has been identified as having a significantly altered biological activity it is then subjected to fractionation, separating the chemicals contained in it by chemical–physical properties, such as polarity, molecular weight, or any other physical–chemical properties or combinations thereof [19]. These fractions are then again analyzed for their potential to interfere with biological processes using both acute and mechanism specific bioassays. This procedure is repeated until the activity can be pinpointed to one or

Table 1 Examples of cell lines used in effect directed analyses of sediments and biota

Effect type	Cell line	Source	Toxicity endpoints	References
AhR receptor ligands	H4IIE-Luc	Rat hepatoma cell line	Ah receptor-mediated luciferase reporter gene assay	[30, 86]
	RTL-W1	Rainbow trout liver cell line	7-ethoxyresorufin O-deethylase (EROD) activity (CYP1A1)	[68, 87]
Genotoxic chemicals	DNA-repair-deficient DT40	Chicken DT40 B-lymphocyte line	Screening and characterizing the genotoxicity	[88]
	Ames test	Salmonella TA98 strain	Prokaryote cell assay to assess the potential of chemical compounds to cause point mutations	[80]
		Salmonella TA100 strain	Prokaryote cell assay to assess the potential of chemical compounds to cause frame shift mutations	[80]
Endocrine disrupting chemicals	H295R	Human adrenal cancer cells	Endocrine disrupting activities: modulation of steroidogenesis	[84, 85, 89]
	MVLN-assay	Transformed MCF-7 human breast cancer cell line	Estrogen receptor-mediated luciferase reporter gene assay	[90]
	T47D-KBluc	Transformed T-47D human breast cancer cell line	Estrogen receptor-mediated luciferase reporter gene assay	[91]
	MDA-KB	Transformed MDA human breast cancer cell line	Androgen receptor-mediated luciferase reporter gene assay	[92]
	YES	Transformed yeast cell	Estrogen Lac-Z reporter gene assay	[93]
	YAS	Transformed yeast cell	Androgen Lac-Z reporter gene assay	[94]

multiple specific fractions that contain chemicals of certain properties. These fractions are then subjected to chemical analysis for suspected compounds. One possible strategy for toxicants identification and evaluation (TIE) in complex mixtures is shown (Fig. 2). The general steps are as follows: (1) Screening of the whole extract – to determine the samples containing significant toxic potencies, which require further chemical analysis. If no significant response is observed, there is no need to conduct expensive, time- and material-consuming chemical analysis. Since the method detection limit is known for the bioassay, an upper limit of concentration of toxic equivalents in the sample can be defined. (2) Fractionation of the samples that were active in bioassays and chromatographic analysis can be used to determine the most

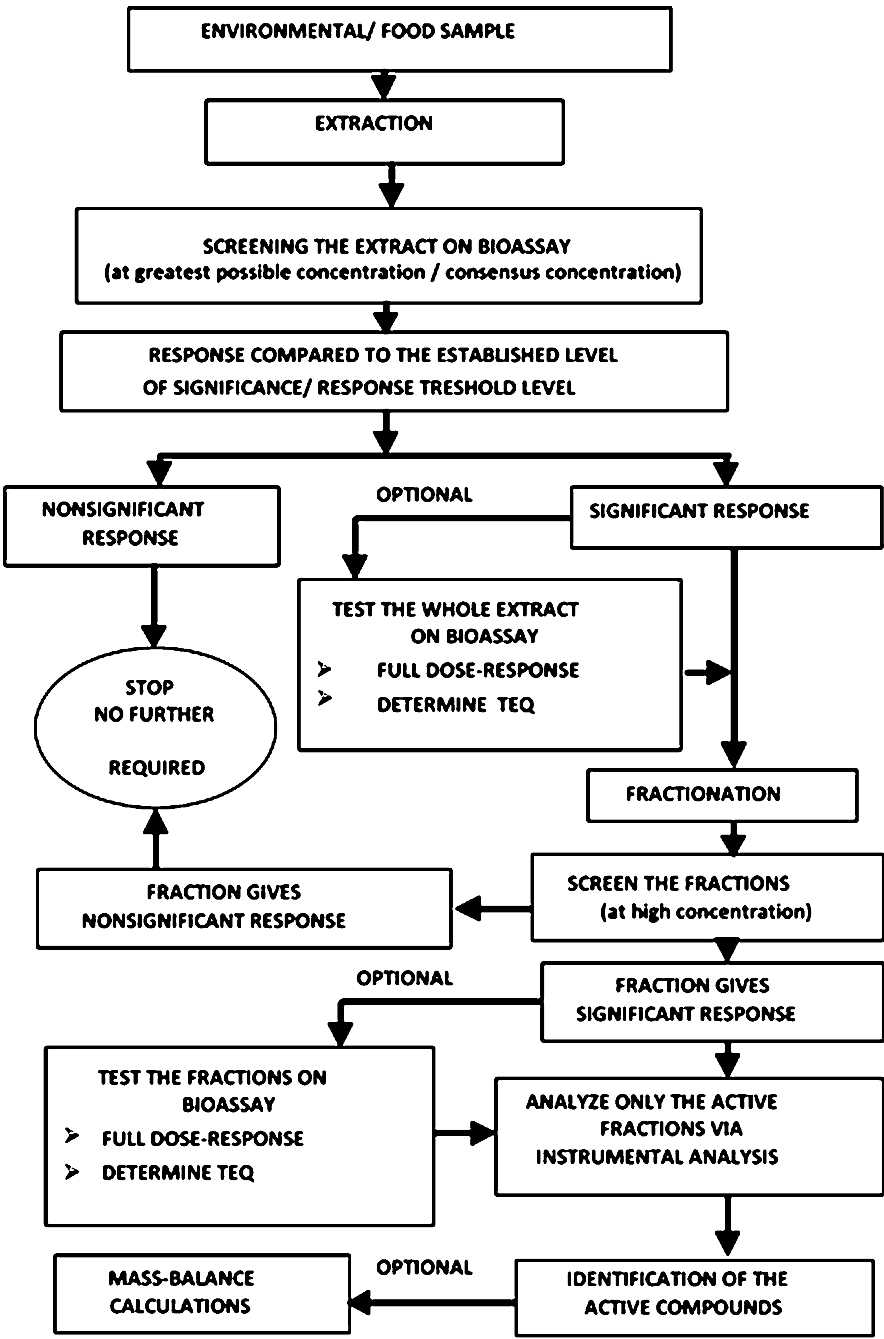

Fig. 2 Screening system: Toxicant identification and evaluation strategy (adapted with permission from [30])

probable contributors to the total activity. (3) Generating the full dose–response relationship of the unfractionated sample or fractions thereof, so that the total activity of the sample can be determined as response equivalents. It should be noted, however, that such a mass-balance approach is only feasible for chemicals for which potencies relative to a positive control, such as 2,3,7,8-TCDD for AhR-mediated responses or E2 for ER binding assays, can be calculated. For more complex assays such as the H295R Steroidogenesis assay that enables the determination of multiple parallel endpoints such as effects on androgen and estrogen production as well as changes in enzyme activity, relative potency approaches have yet to be developed. In such cases, data is simply expressed in terms of changes relative to the applicable solvent control [85].

Where applicable, calculation of the potency balance is accomplished by comparing the potency of a mixture observed in a bioassay with the potency calculated as the sum of the products of concentrations of individual, compounds quantified by instrumental analyses and their RP vales. If the values derived and fractionation does not indicate that there were antagonistic effects in the whole extract, it can be concluded that all of the significant contributors to the total complex mixture have been identified. However, if the total activity determined from the bioassay is significantly greater than those predicted from the instrumental analysis it can be inferred that there are unidentified compounds or that there is synergism. By comparing the activity of the whole extract to that of the various fractions, it is possible to determine if the difference is due to the presence of unidentified compounds or synergism. Also, depending on the fraction where an unknown compound occurs gives insight into the most appropriate instrumental analysis to apply for identification and quantification of active chemicals in a fraction. Antagonisms can occur, particularly between non-AhR-active and AhR-active PCB congeners [38]. These antagonisms will affect the potency balance and need to be considered.

Finally, some nonactive parent compounds in complex mixtures contained in environmental matrices can be metabolically activated to potent inducers of specific biological responses; alternatively the active compound can be biotransformed to nonactive metabolites. This phenomenon can also influence mass-potency balance analysis, metabolic activation or deactivation of chemicals would result in an apparent synergism or antagonism of the chemicals detected in the mixture. For most compounds, the activity of their metabolites is unknown. Some of the cell lines possess a number of metabolic capabilities and upon prolonged duration of exposure they can partly simulate in vivo biotransformation of some compounds. This fact can be used analytically by use of selective inhibitors.

2.4 Relative Potency and Potency Balance Analysis

To apply the potency balance approach with complex mixtures, species- and endpoint-specific relative potencies (RPs) or toxicity equivalency factors (TEFs)

need to be determined relative to validated standard chemicals previously shown to exert the desired effect.

2.4.1 Estimation of Relative Potencies of Complex Mixtures in Sediments and Biota

The RPs of samples are usually calculated as the amount of standard (reference toxicant) giving the same response as the sample, commonly based on the amount of sample needed to produce 50% of the maximal standard response (EC_{50}). The exogenous compound with the greatest known affinity as well as toxicity, 2,3,7,8-tetrachlodibenzo-*p*-dioxin, is used as a standard for AhR-mediated responses. The endogenous substrate E_2 serves as a standard for ER-mediated activity, and the endogenous nonaromatizable androgen dihydrotestosterone (DHT) is used for determining RPs in AR assays. Activities of samples are then expressed as bioassay-derived equivalents: dioxin equivalents (TCDD-EQ), estradiol equivalents (E_2–EQ), or DHT equivalents (DHT-EQ) per specified amount of sample. For calculating and comparing the equivalents, complete dose–response curves from step-wise diluted extracts and standards should be obtained. This is rather difficult with mixture extracts obtained from environmental matrices such as sediments or biota. Common problems encountered when determining the relative potencies of complex mixtures include different efficacy (maximal induced response), nonparallel slopes, cytotoxicity at greater concentrations or insufficient mass of agonists to reach full efficacy or the occurrence of partial agonists that do not attain the maximum possible response. These limitations must be taken into account when calculating the relative potency of the sample. There is always variation in the EC_{50} in replicates measured on different days due to differences in plating density. For some cell lines, the normalization for protein content can solve this problem. For endogenous enzyme activities, the normalization to protein content is necessary. In some transgenic cell lines, normalization to the amount of protein present has been inadvisable because of increased variability of the normalized results. Protein normalization is not recommended in cell lines used for estrogen-receptor mediated activity, where response induction correlates with estrogen-induced protein synthesis [95].

2.4.2 Potency Balance Calculations

In the potency-balance approach, the potency of a mixture to elicit a particular measurement endpoint determined by a bioassay is compared with the sum of potencies of individual compounds determined by instrumental analysis and corrected by application of a RP. This strategy has been widely used for dioxin-like [24, 96] and estrogenic compounds [29]. Toxic equivalents (TEQs) are calculated by multiplying the relative potency (RP) for the specific assay (if available) or the international toxic equivalency factor (TEF) by concentration of the specific

congener giving total sum toxic equivalents per mass unit. For calculating the TEQs from chemical data, effects are assumed to be additive (Equation 1).

$$\mathrm{TEQ} = \sum_{I=1}^{N} \mathrm{CONC.\ OF\ COMPOUND}_i \times \mathrm{TEF}_i \tag{1}$$

TEFs are species-, endpoint-, and assay-specific determination of potency expressed relative to the standard, they can vary widely depending on the species and endpoint. The relative potencies (RPs) should be used for bioassay-directed potency-balance calculation for complex mixtures that are specific for studied endpoint and assay [96]. The international TEFs are consensus values, based on many different types of assays [97, 98] including multiple in vitro and in vivo endpoints for multiple species. TEF values are order-of-magnitude estimates suitable for risk assessment purposes. Because of the differences in RPs among species, specific sets of international TEFs have been established for mammals, fish, and birds [97, 98]. Currently, TEFs and RPs are available for dioxins, furans, some PCBs and PAHs from a number of assays. There are many compounds with potential AhR-mediated activity for which RPs are unavailable and TEFs have not been established. Therefore, those compounds cannot be included in the mass-balance calculations.

Limited RP values are available for estrogenic compounds. RPs have been established only by use of in vitro bioassays for a few alkylphenolic compounds, phytoestrogens, some pesticides, PCBs, and PAHs [95, 99]. In this case, by calculating the E_2-EQs based on analytical results, one can estimate the proportion of the total activity determined by bioassay that is represented by the compounds which have been quantified and have known relative potencies. There are several limitations of calculating TEQs from analytical results: (1) RPs or TEFs are available for only a limited number of chemicals; for some compounds there are no endpoint-specific nor consensus values for TEFs available; (2) TEFs derived for other species, usually from mammals, where the most research has been conducted, may not be suitable for use with nonmammalian species due to the interspecies differences in sensitivity; (3) there may be some compounds not routinely detected whose contribution to the activity would be overlooked; (4) application of the additive approach is routinely used in the total activity calculation; potential interactions among compounds in a mixture, such as synergism or antagonism are neglected; and (5) detailed analysis of trace contaminants require specialized equipment such as HRGC/HRMS (high resolution gas chromatograph/mass spectrophotometry), which is not available in all laboratories and may be prohibitively expensive. Toxic equivalents estimated based on analytical data are correlated with the bioassay results in some situations, depending on the composition of the complex mixture of compounds in the samples. For biota, highly significant correlations have been found between bioassay derived EROD activity and instrumentally measured TEQs in extracts of fish or bird samples [37, 73]. However, toxic activities determined in the bioassays and concentrations of known dioxin-like or

xenoestrogenic compounds are sometimes not correlated. For instance, data obtained from bioassays may be an independent parameter that is predictive of ecotoxicological effects. Besides nonadditive (synergistic or antagonistic) interactions among individual ligands, differences between TEQs derived in bioassays and those calculated from concentrations of individual compounds may be caused by the following events: (1) there are some other active compounds present, which were not identified by the chemical analysis [100]; (2) noncomplete dose responses or cytotoxicity disabling accurate estimations of toxic equivalents; (3) the RPs or TEFs used may not be appropriate. Potencies of mixtures determined by use of bioassays have ecotoxicological relevance because they represent an integrated biological response, but are limited in that the TEQ concentration cannot be "moved" among trophic positions. While the REP can correct for the relative potency of a chemical it does not embody any information on partitioning behavior or degradation or rates dissipation, which are factors that influence disposition of chemicals in the environment.

3 Limitations and Perspectives of Bioassay-Directed EDA

It is necessary to point out disadvantages and limitations of bioassays to be used in support of EDA of sediments and biota. While they provide a greater degree of realism and enable assessment of biological relevant exposures, the main limitations associated with benthic in vivo tests are the lack of specificity of the response and the often resource and time intensive nature of studies including whole organisms, especially vertebrates. The former is particularly of concern in the context of EDA because it limits the characterization of specific types of exposure, which is the core component of bioassay-directed EDA. However, there are currently efforts under way such as the DanTox project that aims to overcome these limitations by utilizing high throughput and mechanism-specific in vitro bioassay formats, including exposure of eggs or embryos [66]. In contrast, in vitro bioassays do not account for the pharmacokinetics, tissue distribution, and biotransformation that may occur in vivo. If cell lines possess only limited metabolic activities, substances active after bioactivation may not be detected by in vitro system [101]. Bioassays do not identify the individual compounds causing the response and are often limited in scope because they focus on specific compounds, e.g. those who act through a specific receptor-mediated mechanism of action. This is especially true in context with the assessment of endocrine disrupting potentials of environmental samples. The majority of studies that were conducted to characterize exposure with endocrine-active chemicals in sediments, effluents, and surface waters focus on the presence of compounds that can interact with the nuclear hormone receptors, namely the ER or AR. Recent studies have demonstrated that relying on a few "popular" endpoints such as ER-mediated effects can provide a false assessment of the actual exposure and are prone to miss relevant effects [18, 102]. In this context, it seems to be commonly misunderstood that the phenomenon of endocrine

disruption is not limited to chemicals that can mimic hormones such as estrogens or androgens. Endocrine disruption appears to be a much broader issue that cannot be seen separated from other classic toxicological issues. This is especially true with regard to impacts on the synthesis or metabolization of sex steroids, processes that primarily depend on P450 enzymes and can be affected by many different chemicals including PCBs, pesticides, etc. [46, 103, 104]. In fact, induction or inhibition of the production of estradiol or testosterone can have much greater impacts than exposure to estrogen or androgen mimics because they are among the most potent receptor agonists. This indicates the need for more comprehensive and integrative approaches in support of EDA of environmental samples such as sediments or biota. Potentials of residues in sediments from the Danube River, Germany would not have been identified if the sediments had been examined solely for ER-modulating potential [18]. To address these issues it is becoming increasingly popular to include a variety of biossays detecting multiple different types of effects in EDA of environmental samples [18, 102]. Furthermore, incorporation of high throughput microwell format, in vivo tests, such as the *D. rerio* embryo assay into such test batteries has the potential to overcome the above discussed concerns regarding the lack of realism of in vitro systems [66, 102]. We strongly encourage expansion of on these recent trends by further broadening the spectrum of endpoints utilized in biotest batteries as well as to integrate in vivo tests such the above discussed *D. rerio* embryo assay used in support of EDA of sediment or biota samples. Similarly, it was recently suggested that EDA could be used as an additional line of evidence in comprehensive weight-of-evidence studies [105], aiming at the identification of the unknown substances responsible for the biological effects in the bioassay paired with parallel assessment of the ecological relevant endpoints such as benthic invertebrate community structure (Fig. 3).

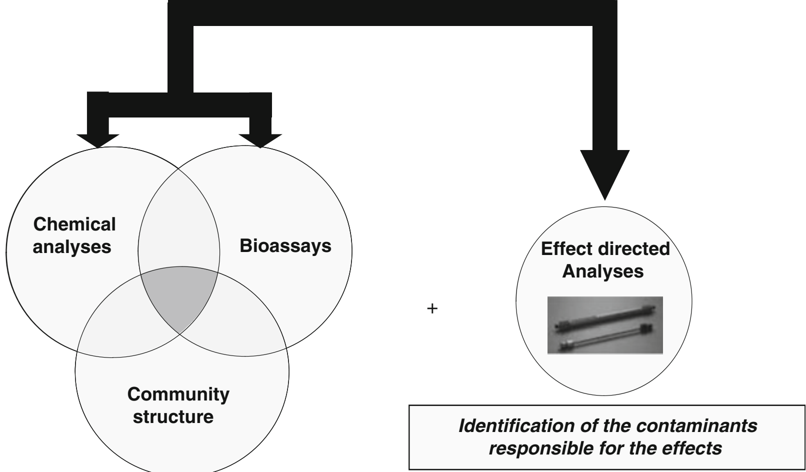

Fig. 3 Recommended combination of the triad approach with EDA (adapted from [105])

There are increasing concerns about emerging contaminants including perfluorinated compound [106, 107] as well as polybrominated and mixed halogenated dibenzo-*p*-dioxins and -furans [108]. For these compounds, no, or only a limited number of sufficiently specific bioassays are available, and thus, such exposures often cannot be appropriately addressed. As a consequence, there is still a great need for refinement and standardization of current sediment EDA approaches that allow capturing and assessing exposures to these chemicals. Furthermore, while well established for its use in ERA of contaminants such as dioxin-like, genotoxic/mutagenic, or estrogenic substances, the continuing discovery of new contaminant groups of concern in the environment or new effect types pose new challenges to classical EDA approaches. These challenges include establishing bioassays that are specific to the biological activity of chemical groups of concern, as well as the identification and description of relative potencies to model compounds characteristic for these types of effects to enable the utilization of mass-balance approaches.

Another issue is the lack of correlation between the chemical analytical data and the biological response measured by the bioassay. For example, as discussed in one of the previous sections, the chemically detected TEQs can often not explain the biological activity measured with the bioassay (bio-TEQ) (e.g., [109, 110]). Similarly, attempts to correlate genotoxicity of complex environmental samples measured by tests such as the Ames, the micronucleus, or the comet assay with PAH concentrations often fail [111–115], suggesting a contribution of other non-regulated mutagens to the observed biological effect. In a number of studies assessing sediments by EDA it could be demonstrated, for example, that in addition to priority pollutants several nonregulated PAHs, including perylene and benzo[a]fluoranthene, 11H-indeno[2,1,7-cde]pyrene, a methylbenzo[e]pyrene and a methyl perylene, were present at significant concentrations in the analyzed samples [116]. Furthermore, Fernandez et al. [117] showed that more polar compounds, including several polycyclic quinones and nitroquinones, as well as nitro-PAHs, contributed significantly to the mutagenic effects of marine sediments. Furthermore, one of the key challenges for the assessment of the contribution of individual chemicals to the bioassay derived estrogenic potential (estradiol equivalents; EEQs) of a sample is the sensitivity of the utilized analytical method, as has been demonstrated by a study assessing estrogenic compounds in complex environmental samples in the catchment area of the River Neckar, Germany [118]. Those estrogenic chemicals that were detected at concentrations above the method detection limits, including nonyl- and octylphenol, phthalates, PCBs, bisphenol A, and DDT, were only able to explain 9–14% of the total Bio-EEQs. In contrast, when the method detection limits of chemicals that could not be detected by the utilized analytical methods, specifically 17β-estradiol and ethinylestradiol, were taken as a basis of estimation for the Bio-EEQs, 95% of the Chem-EEQ could be explained. Advanced analytical methods for natural and synthetic hormones with lower detection limits such as HRLC/HRMS (high resolution liquid chromatography/high resolution mass spectroscopy) are one way to reduce this problem [119–121].

EDA has been shown to have the potential as a powerful tool in support of ERA, and already is routinely utilized in environmental monitoring programs

[13, 109, 120, 122–125]. However, to date EDA is almost exclusively based on measurable effects in in vitro and in vivo biotests. To address current needs of regulators and risk assessors, therefore, an increasing focus should be on the integration of EDA into ERA. Specifically, there is need for the development of tools to confirm EDA-determined key toxicants as stressors in populations, communities, and ecosystems [120].

4 Conclusions

In summary, there have been increasing efforts over the past decade to improve current strategies in the assessment of ecological risks associated with the exposure to environmental contaminants of concern through sediments or biota. It has been recognized that classic approaches relying on a few endpoints or analysis types (e.g., analytical chemistry) alone are not only impractical but also tend to wrongly or incompletely assess a given exposure. This is particularly true with respect to exposure to complex mixtures of different types of chemicals that are typical for many aquatic environments. One example is the focus on chemicals that interact with the estrogen receptor (ER) as a representative of endocrine effects. As discussed in the previous section, identification of ER binding potentials represents only one facet of a much more complex issue and can lead to a wrong assessment of the true endocrine disrupting potential of a sample. To address these shortcomings, therefore, studies increasingly rely on advanced EDA concepts by using multiple-endpoint bioassay batteries in combination with fractionation techniques followed by confirmatory targeted chemical analysis.

Also, there are increasing concerns about emerging contaminants including EDCs, perfluorinated compounds, as well as polybrominated and mixed halogenated dibenzo-*p*-dioxins and -furans, for which no or only a limited number of sufficiently specific bioassays are available. As a consequence, such exposures often cannot be appropriately addressed, and there is still a great need for refinement and standardization of current sediment EDA approaches that allow capturing and assessing exposures to these chemicals. Furthermore, while well established for its use in ERA of contaminants such as dioxin-like, genotoxic/mutagenic, or estrogenic substances, the continuing discovery of new contaminant groups of concern in the environment or new effect types pose new challenges to classical EDA approaches. These challenges include establishing bioassays that are specific to the biological activity of chemical groups of concern, as well as the identification and description of relative potencies of model compounds characteristic for these types of effects to enable the utilization of mass-balance approaches.

There is need to increase the realism of environmental effect analysis to enable linking exposure to biological relevant outcomes that can ultimately predict effects at the population and/or community level. As discussed in this chapter, it is therefore recommended to integrate standard EDA practices based on mechanism-specific in vitro bioassays, high throughput in vivo tests, and analytical

chemistry into more comprehensive assessments of ecologically relevant endpoints such as benthic invertebrate community structure, e.g., as part of weight-of-evidence studies.

Acknowledgements We wish to acknowledge Drs. Klara Hilscherova and Henner Hollert and all the other people with whom the authors have cooperated over the years while developing the basis for this book chapter. Finally, we wish to thank Dr. Xiaowei Zhang for his help with providing information on cell based in vitro assays. Prof. Giesy was supported by the Canada Research Chair program, an at large Chair Professorship at the Department of Biology and Chemistry and State Key Laboratory in Marine Pollution, City University of Hong Kong, The Einstein Professor Program of the Chinese Academy of Sciences and the Visiting Professor Program of King Saud University.

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Ecological Relevance of Key Toxicants in Aquatic Systems

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Abstract The effect-directed analysis (EDA) methodology is an important component in site-specific risk assessment of contaminated aquatic systems, but improves its relevance in assessment strategies when confirmed on ecosystem level. Several approaches are available to confirm the ecological relevance of EDA results, but just a few studies exist, directly linking EDA processes with field studies in the aquatic environment. In this chapter, approaches on different assessment levels ranging from molecular responses to integrated community analysis are summarised in a multiple line of evidence approach to illustrate their potential use in EDA-confirmation procedures. Biomarkers were identified to have the potential to bridge bioassay-identified chemicals from EDA studies to effects in indigenous organisms on a mechanistic level. In situ bioassays use reference organisms to directly link exposure to EDA-identified chemicals to site-specific effects on organisms at realistic exposure conditions. For the sites of investigation, in-depth information on chemical concentrations and site-specific community data may be available from monitoring programs. These data can be used to confirm the relevance of EDA-identified chemicals by linking their concentrations to community-level effects. The SPEAR-index directly links changes in macroinvertebrate

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W. Brack (ed.), *Effect-Directed Analysis of Complex Environmental Contamination*, Hdb Env Chem (2011) 15: 315–340, DOI 10.1007/978-3-642-18384-3_13,
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communities to site-specific contaminants. Multivariate statistical tools and prognostic modelling based on the TU-approach or on ms-PAF modelling may reveal correlations of EDA-identified chemicals and provide prognosis on potential impacts on the ecological status. Next to the principles and examples of the suggested approaches constraints and challenges are discussed, when aiming to link the methodology of EDA with ecological-based site assessment.

Keywords Biomarkers, Community metrics, Environmental risk assessment, In situ bioassays, ms-PAF, Multiple line of evidence approach, PICT, Prognostic modelling, TRIAD, TUs

Contents

1 Introduction 316
2 Biomarkers for Confirming the Ecological Relevance of EDA Results 318
3 In Situ Experimental Approaches for Confirming the Ecological Relevance of EDA Results 322
4 Analysis of Aquatic Communities for Confirming the Ecological Relevance of EDA Studies 324
5 Prognostic Modelling for Confirming the Ecological Relevance of EDA Studies 328
6 Constraints and Uncertainties of Confirmation Approaches in EDA 330
7 Conclusion 334
References 335

1 Introduction

The assessment of a multitude of stressors, including chemical mixtures, driving the ecological status of an aquatic ecosystem calls for advanced approaches for identifying and prioritising those stressors. With respect to chemical stressors, the identification of site-specific contaminants is the basis for establishing cause–effect relationships between chemical contamination and ecosystem impairment. The methodology of effect-directed analysis (EDA) has been suggested for identification of site-specific contaminants [1, 2]. The EDA approach derives its diagnostic power from combining chemical fractionation of mixtures of contaminants present in an environmental sample, e.g. water, effluents or sediments with biological toxicity evaluation and chemical analysis. Several in vivo and in vitro bioassays were used in EDA studies, yet. These bioassays have to fulfil specific requirements to be suitable for EDA studies, e.g. they have to be cost-effective and small-scaled. Therefore, mainly acute and/or in vitro bioassays have been used in EDA studies as they are more suitable for high-throughput screening. This implies that chronic effects or effects, which could just manifest at ecosystem level, such as deriving from species interaction or biomagnification of compounds in the foodweb, are not reflected in the bioanalytical process of the EDA. Additionally, it remains unclear whether results from the in vivo bioassays performed on fractions from chemical

mixtures propagate to negative outcomes in ecosystems, as factors such as bioavailability, temporal and spatial variability of contamination in the ecosystem are not considered during the EDA process. Therefore, advanced evaluation of site-specific chemical and biological data and experimental approaches are needed to confirm the ecological relevance and potential risks of identified compounds towards the ecosystem. This is of special importance, when restoration efforts have to be allocated to selected contaminated sites and restoration success has to be monitored, afterwards.

A tiered approach has been suggested to confirm toxicants, which were identified by EDA [3]. The authors suggested confirmation on an analytical level comprising chemical-analytical and computer-based structure-elucidation methods, confirmation on the effect level based on bioassays and verification at the community level at realistic exposure conditions.

The focus of this chapter is to summarise approaches, which could reveal the ecological relevance of toxicants identified by EDA procedures. Two scenarios have to be considered, which may trigger an EDA study (Fig. 1): in retrospective site assessment an EDA study may have been motivated by an ecological deterioration in an aquatic system, e.g. a decrease of fish stock, a disturbed population structure or an indicated bad ecological status according to the EU-WFD (water framework directive). In this case EDA is used to identify the cause of the deterioration and bioanalytical tools for the EDA process, e.g. the trophic level of a bioassay or a specific biomarker are selected according to the observed effects in the field. In this case confirmation will focus on this particular problem, for instance by caging a selected fish species, which was identified to be affected before.

In a second scenario, water bodies exposed to complex contamination from diffuse or point sources need to be assessed for compounds posing a risk to the ecosystem and human health. Thus, the focus is on identification of key toxicants considering a broad range of endpoints including mechanism-based in vitro assays and integrated observation parameters, e.g. growth. Depending on the scenario

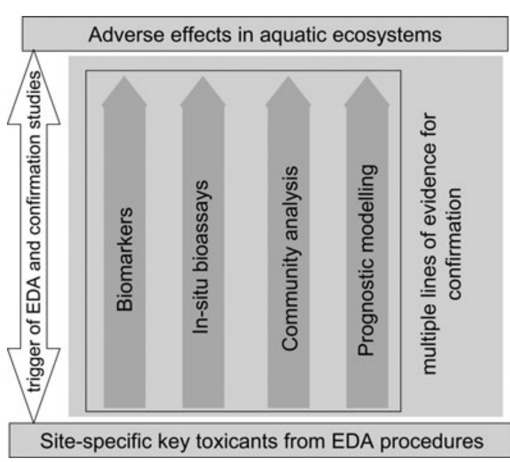

Fig. 1 Multiple lines of evidence approach to assess the ecological relevance of site-specific toxicants identified in EDA procedures

different confirmation strategies are required. To this aim, a set of applicable approaches is presented in this chapter.

These approaches follow a multiple lines of evidence (LoE) framework and demonstrate and confirm effects and potential risks caused by key pollutants at the ecosystem level (Fig. 1). They include approaches requiring additional experimental field work as well as approaches evaluating site-specific monitoring data, as far as available.

A designed set of experimental analysis could be applied for further diagnosis of site-specific risks of EDA-identified key toxicants. This includes the analysis of biomarkers (Sect. 2) or the application of in situ bioassays (Sect. 3) for exposure analysis. The multiple LoE-approach further considers the use of site-specific information on the chemical and biological status of an aquatic system, which may be available from monitoring programmes, e.g. within the EU-WFD national programmes. This comprises an in-depth analysis of site-specific community data by applying stressor-specific community indices, such as the TDI for diatoms or the SPEAR index for invertebrates [4], as well as multivariate statistical tools to identify potential cause-effect relationships (Sect. 4). Finally, the conversion of chemical data into toxicologically relevant information, such as toxic units (TU) [5] or multi-substance potentially affected fractions of species (msPAFs) [6] allow predictions of potential effects, e.g. the potentially affected ecological groups (Sect. 5). Final considerations address the uncertainties arising when aiming to link EDA approaches to ecological site assessment.

2 Biomarkers for Confirming the Ecological Relevance of EDA Results

Bioassays applied in EDA procedures often utilise molecular responses to identify chemicals with specific modes of action. Many of these molecular responses are also utilised as biomarkers for monitoring exposure to or effects of chemical pollutants. An example is the induction of cytochrome P4501A (CYP1A): in vitro bioassays expressing CYP1A are used in EDA studies to identify dioxin-like chemicals in environmental samples [1, 7–10], but CYP1A is also used as in vivo biomarker, to detect environmental exposure of organisms to dioxin-like chemicals (e.g., [9, 11–14]). This dual use of molecular endpoints in principal opens the possibility to take biomarker measurements in wildlife species exposed in situ to confirm the ecosystem relevance of biomarker-inducing chemicals identified in EDA. However, the relationship of the molecular biomarker response in the EDA study to the molecular biomarker response of the organisms exposed in situ is not that simple and straightforward as it may look at a first glance. In evaluating the relationship between the EDA and the field biomarker response, we have to consider, as already addressed above (see Fig. 1), the two scenarios that may have triggered the EDA study: In the first scenario, the bioanalytical EDA study is triggered by the findings from a field study observing elevated biomarker

expression in wildlife species, what leads to the question on the causative chemicals. In the second scenario, the starting point is the EDA identification of chemicals inducing a specific biomarker, what then leads to the question if this is of relevance for the biota in situ.

In the most simple case, the bioassay used for EDA is based on the same molecular event as the biomarker. An example illustrating how the elevated biomarker expression in wild fish can be traced back to the causative chemicals is provided by the study of Houtman et al. [15], in which the authors observed elevated levels of vitellogenin (VTG) in the plasma of male bream (*Abramis brama*) at several sites in Dutch rivers. VTG expression is regulated via the oestrogen receptors, and induction of VTG in fish is indicative of exposure to environmental compounds that are able to activate the oestrogen receptors. In order to evaluate if the VTG induction is indeed associated with the accumulation of oestrogen receptor-activating chemicals in the fish, Houtman et al. [15] performed a bioanalytical study on bream plasma, liver and gastrointestinal contents. The latter compartment was analysed because, on the one hand, fish accumulate hydrophobic contaminants via the dietary rote, and, on the other hand, fish eliminate metabolised and conjugated oestrogens (both endogenous and xenobiotic ones) mainly via bile fluid into the intestine. The bioassay used to determine total oestrogenic activity in plasma, liver and gastrointestinal contents was the ER-CALUX assay, a reporter assay with stably transfected T74D human breast cancer cells expressing reporter gene coupled oestrogen receptors [16]. Thus, the bioassay measured activation of the same molecular target, i.e. oestrogen receptors, as did the in vivo biomarker, vitellogenin. While samples from all analysed compartments (plasma, liver, gastrointestinal contents) of bream showed elevated oestrogenic activities in the ER-CALUX assay, only oestrogenic activities of the gastrointestinal tract correlated to the VTG levels of the fish. In other words: induction of the VTG biomarker in bream appeared to be related to the levels of oestrogen-active compounds in the gastrointestinal tract but not to oestrogenic compounds being present in liver or plasma. Houtman et al. [15] performed then a bioassay-directed fractionation of the gastrointestinal contents in order to identify the causative chemicals. The oestrogenic hormones 17β-oestradiol and its metabolite oestrone were identified as major contributors to the oestrogenic activities. In addition, the xenoestrogen bisphenol A was detected at high concentrations in the fractions, however, due to its low oestrogenic activity, the contribution to the total oestrogenic activity of the gastrointestinal contents was negligible. Thus, the authors succeeded in tracing back the observation of elevated VTG biomarker expression in wild bream to the levels of specific oestrogen-active compounds to which the fish were exposed via the dietary route.

A bioassay such as ER CALUX which is indicative of a specific molecular mode of action detects the sum of chemicals in a sample that exert that mode of action. This leads to the question how to identify the contribution of the individual compounds being present in a composite sample to the overall bioassay activity of the sample. This problem can be solved by using bioassay-derived relative potency estimates (REP) of the individual chemicals (see [17, 18]). The REP

compares the potency of an individual chemical to induce the analysed biological response relative to a standard. For instance, for chemicals activating the oestrogen receptor pathways and inducing VTG, usually the natural hormone, 17β-oestradiol, is used as standard. For chemicals activating the arylhydrocarbon receptor (AhR) pathway and inducing CYP1A mostly 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) is used as standard. The potency of the individual compound relative to the standard is the ratio of the concentrations of the standard and the individual compound, respectively, to elicit a defined magnitude of response, e.g. a 50% induction, or if the chemical or sample is highly cytotoxic, a corresponding lower-effect level (see [19]). The underlying assumption to this approach are parallel concentration–response curves of the standard and the compound of interest (an assumption that can create a conceptual problem – see [17]). Knowing the actual concentration of the individual compounds in a complex sample, and knowing the REPs of the individual compounds, the total potency of the sample can be calculated assuming that the individual chemicals in the sample act in an additive manner. If the calculation of the total bioassay activity of the whole sample comes to a value that is close to the actually measured bioassay activity of the sample, then it is likely that the effect-directed fractionation detected all active compounds in the sample. If, however, the calculated and the measured activities differ clearly, then additional, non-identified compounds must be active in the sample.

In the study of Houtman et al. [15], the biomarker VTG and the bioassay ER-CALUX were based on the same molecular event – activation of the oestrogen receptors – and thus, the conclusion that those chemicals identified in the EDA are those chemicals that cause VTG induction in the fish appears to be well defendable. The relation between the bioassay and the biomarker responses, however, is getting more complicated, if one of the two methods uses a "surrogate" endpoint (see [20]), i.e. the two endpoints measure different biological responses, which, however, share a mechanistic link. An example to that case comes from the studies on the chemicals causing feminisation in wild roach, *Rutilus rutilus*, in UK Rivers. Field studies have demonstrated that intersex gonads, i.e. gonads characterised by the presence of oocytes in apparently testicular gonad tissue, are widespread among roach populations in British rivers [21]. In order to identify the chemicals causing intersex formation, an EDA would be instrumental. However, this would require combining chemical fractionation with developmental or chronic fish tests in order to test for intersex induction, an approach that is not possible for practical reasons. Thus, Desbrow et al. [22] employed an in vitro oestrogen receptor bioassay as "surrogate" assay to identify intersex-inducing potential chemicals in the EDA approach. This approach was justifiable, as there exist good evidence, both from epidemiological and experimental studies that the formation of intersex gonads in fish is caused by exposure to oestrogen receptor-binding contaminants [21, 23]. Therefore, oestrogen receptor-binding assays may serve as surrogate assay to identify chemicals with the potential to induced intersex formation. With this approach, Desbrow et al. [22] identified natural – 17β-oestradiol, oestrone – and synthetic – ethinyloestradiol – hormones as main contributors to the overall oestrogenic activity of the effluents. Importantly, although this approach succeeded in revealing the major oestrogenic

compounds being present in the effluents, it does not yet prove that these compounds or their environmental mixtures are indeed responsible for intersex development in roach, despite the fact that the surrogate endpoint "oestrogen receptor activation" is mechanistically linked to the induction of intersex gonads. Therefore, in a follow-up study, Routledge et al. [24] went a step forward to narrow down the gap between the in vitro bioassay results and the in vivo effects observed in wild fish. These authors exposed roach to the oestrogenic compounds found in the EDA at concentrations as present in the effluent, and could show that this exposure scenario indeed triggers an oestrogenic response – induction of VTG – in the fish.

In situ biomarkers can also be helpful for the confirmation of field relevance of effects and toxicants identified in in vivo EDAs. For instance, EDA of a sediment may result in the identification of mutagenic contaminants in sediment – does this mean that benthic – or even pelagic – organisms living at the site will suffer genotoxic or cancerogenic effects? To answer this question, mutagenic or genotoxic biomarkers measured in sediment-dwelling or pelagic organisms should be helpful to confirm whether this expectation comes true or not. As put by Brack et al. [3], "the confirmation of in vitro effects of . . .. contaminants is possible on the basis of related in vivo effects and biomarkers". This can be done on a qualitative yes/no basis – in vitro bioassay detection of molecular event A correlates with in vivo biomarker detection of molecular event A – or it can be attempted on a more quantitative basis. If a concentration–response curve exists for the biomarker with the EDA-identified toxicant, it can be evaluated whether the intensity of the biomarker expression observed in organisms in situ corresponds to the analytically determined concentrations of the toxicant in situ. An example of this approach comes from the investigation of oestrogenic contamination in rivers from Catalonia [25].

Problems in the biomarker-based confirmation of the biological relevance of the contaminants identified by EDA arise if the biomarker data do not correlate with the bioassay results. A variety of factors can confound the relation between the two assessment levels. They include – to name a few – toxicokinetic processes such as chemical bioavailability, uptake and metabolism, combination effects from chemical mixtures, different time-effect profiles, or modulation of the biomarker response by physiological processes (compensatory responses, physiological changes related to reproduction, pathogen infection or nutrition, etc.). For instance, Whyte et al. [18] compared bioassay results on the accumulation of planar chlorinated hydrocarbons in livers of lake trout from the Great Lakes, to biomarker expression in these livers. The measured biomarker, the CYP1A-catalysed 7-ethoxyresorufin-*o*-deethylase (EROD) activity, indicated exposure to planar chlorinated hydrocarbons, and the same endpoint – EROD activity – was also used in the bioassays. Despite using the same molecular event for both the biomarker and the bioassay measurement, there existed no correlation between the in vivo EROD levels and the bioassay-detected EROD activities. The authors suggested that this discrepancy may be caused by exposure of trout to EROD-inducing chemicals such as polyaromatic hydrocarbons (PAHs) that do not bioaccumulate and therefore are not detected in the bioassay on tissue extracts. Also Schmitt et al. [26], in their study on contamination of the Rio Grande, suggested PAHs to explain the observed

discrepancy between the EROD biomarker response in fish and the EROD induction in the H4IIE bioassay on fish tissue extracts. This example may highlight that the use of in vivo biomarkers to confirm the ecological relevance of bioassay-identified chemicals from the EDA study is not always straightforward but a number of confounders can complicate the use of biomarkers to confirm bioassay findings.

3 In Situ Experimental Approaches for Confirming the Ecological Relevance of EDA Results

In situ approaches (field-based bioassays) provide valuable diagnostic tools for identification and confirmation of risks of toxicants towards resident communities at realistic exposure conditions. For this purpose, laboratory cultures or field populations from reference sites are transferred to polluted sites. This allows studying toxicant-induced physiological, biochemical, morphological or taxonomic changes directly in the field. In situ approaches have been included in several active biomonitoring programmes using organisms from different taxonomic and functional groups, e.g. molluscs, fish, macrophytes, or benthic algae. Changes in a variety of biological parameters have been observed, such as growth, histological effects, physiological changes and biomarkers or tolerance development [27–29]. These parameters are able to increase the diagnostic power in comparison to passive biomonitoring approaches or laboratory approaches significantly by several key advantages, which have been extensively discussed by de Kock and Kramer [28] and Crane et al. [27]. One of these advantages are that these approaches can be standardised by omitting effects from substrata (e.g. biofilms grown on glass discs) or differences in the sensitivity of sub-populations of a species, the gender or live stage of an organism, which may confound results from passive biomonitoring [30]. Statistical power of observations can be increased by a higher number of replicates or normalisation of effects to specific variables, e.g. biomass or dry weight.

There are specific features of in situ bioassays, which are especially beneficial for confirmation of EDA studies. It is possible to establish cause–effect relationships by running a reference approach. Transferred organisms can be placed at the site, where the contaminated material was taken to perform the EDA study. These organisms can be compared in their biological response to organisms, situated up-stream at a reference site. Additionally, it is possible to use test organisms, which offer an adequate observation parameter, sensitive to a certain mechanistic endpoint, e.g. a specific biomarker, which could be directly used to evidence this effect quality at the site of investigation (see Sect. 2). Furthermore, it has to be taken into consideration that varying environmental conditions can alter the toxicity of a substance, e.g. due to changes in bioavailability, bioaccumulation or metabolisation of the substances, seasonal variability or interactions deriving from multiple stressors.

Two examples are provided, both applying in situ approaches for confirmation of effects of site-specific toxicants on organisms.

The first investigation was a direct follow-up investigation after an EDA study to confirm effects of identified toxicants to local communities (in this case: microalgal biofilms). In a former EDA study applied to sediments from a polluted site in the Elbe basin, Germany, Brack et al. [31] identified several phytotoxic compounds. Among them, the herbicide prometryn and the industrial chemical phenyl-napthylamin (PNA) were identified to be causative for algal reproduction inhibition in the in vivo test during the EDA study. The concept of pollution-induced community tolerance (PICT, [32]) was applied to discern potential effects of these key toxicants on community level [29]. The concept is based on the assumption that sensitive species within a community are replaced by more tolerant species under toxicant exposure, resulting in an increased tolerance of the entire community towards the mode-of-action of the toxicants of exposure. Therefore, the development of PICT of biofilms grown at the contaminated site and a nearby reference location towards the key toxicants prometryn and PNA, identified by EDA, was surveyed. A clear PICT response was observed for prometryn. Although varying in magnitude during the year, tolerance could be observed during several seasons (Fig. 2). This was in accordance with parallel time-integrated analysis of water samples using polar organic chemical integrative samplers (POCIS) and spot sampling.

Whereas prometryn was shown to be causative for induced community tolerance, tolerance towards PNA was not detectable in a first screening (Schmitt-Jansen, unpublished data). The relatively high lipophilicity of this substance (log Kow 4.47) may be the reason for this finding. Solvent extracts from sediments were taken for algal reproduction testing in EDA studies, but under real environmental conditions this substance may be bound to the sediment and not be present in the water phase. This finding indicates that key toxicants, revealed by EDA studies using sediments need a confirmation step on site level, if their ecological impacts are to be considered.

In a second example, an organism reported to be specifically sensitive to a defined group of compounds was selected for investigations. The parthenogenic mudsnail *Potamopyrgus antipodarum* increases its reproduction after exposure to

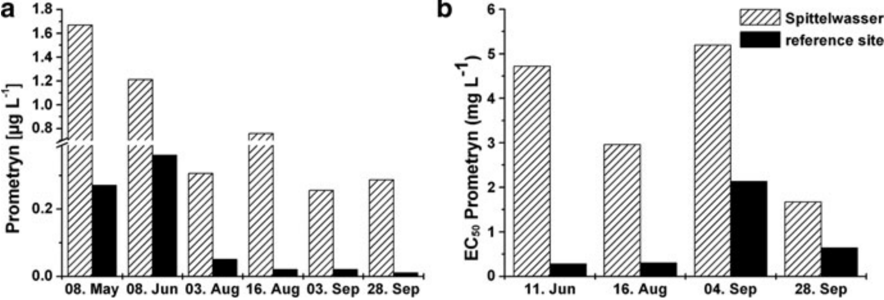

Fig. 2 Prometryn concentrations in μg L^{-1} from spot sampling during different seasons at a reference site and at the polluted creek Spittelwasser (Elbe basin, Germany) (A). Pollution-induced community tolerance (PICT) towards prometryn was quantified as EC_{50} for biofilm communities, grown for 4 weeks at the reference site and the Spittelwasser (B) [33]

oestrogens and decreases its reproduction after exposure to androgens [34, 35]. Schmitt et al. [36, 37] used this organism in several test approaches and stepwise increased the relevance for complex field situations.

In a first step, Schmitt et al. [36] applied a sediment contact test using *P*. *antipodarum* to evaluate the endocrine disrupting potency of sediments from three European rivers. Results were compared to effects from sediment extracts using the yeast oestrogen screen (YES) assay. They revealed a good accordance between the in vivo and in vitro assays and concluded that nonylphenol could be responsible for the observed oestrogenic activities in the Scheldt (The Netherlands). Some differences between the assays were related to a limited bioavailability of the sediment-born compounds and to limitations of the YES assay (e.g. no detection of anti-androgenic potencies).

In a further step, Schmitt et al. [37] aimed to confirm the results from the sediment contact test in complex field situations by caging laboratory cultures of *P*. *antipodarum* at contaminated sites. As this snail was reported to be quite insensitive to fluctuations in abiotic field conditions like temperature or salinity [38], it seemed a promising candidate for application in in situ experiments. Schmitt et al. [37] got a clear indication that nonylphenol could be responsible for an increase of reproduction of *P*. *antipodarum* under real in situ exposure.

As androgen-disrupting potencies are frequently studied in EDA studies, e.g. by using the above-mentioned YES assay [39], *P*. *antipodarum* seems to be an excellent candidate for confirmation of endocrine-disrupting compounds revealed by EDA in the field. It has even been shown that the test systems using *P*. *antipodarum* are more sensitive than the YES assay [40].

In conclusion these examples demonstrate that in situ methods have several advantages for site-specific risk assessment in comparison to laboratory approaches or passive biomonitoring which makes them excellent tools for confirmation of EDA outcomes. When combined with appropriate in vivo or in vitro bioassays in the related EDA studies addressing the same mechanism of action (e.g. the above-described algal reproduction test and a subsequent in situ study on algal communities, or a YES assay and an in situ investigation using *P*. *antipodarum*), a confirmation of EDA results in complex field situations seems possible.

4 Analysis of Aquatic Communities for Confirming the Ecological Relevance of EDA Studies

It was recognised that EDA-like approaches need to integrate with in-site ecosystem assessments for more ecological relevance [41]. However, the approaches being used by EDA and those by ecological site assessments (e.g. ecological status assessment based on community analysis) are different regarding the scales and processes involved. While EDA involves chemical extraction, fractionation and toxicity testing, community-based assessment in the field is based on passive biomonitoring and includes many variables (biological, environmental) that in

addition interact with confounding factors (responses to nutrients and a multitude of toxicants, for instance). Moreover, effects caused by physical and chemical stressors in river ecosystems may either be buffered or enhanced by complex biological interactions [42]. There is a high diversity of stressors (e.g. nutrients, dissolved organic matter, hydrologic discontinuities, temperature, alien species and hazardous toxicant) potentially affecting these communities. Thus monitoring the specific effects of toxicants, separately as well as related to each other, is a challenging task and requires specific methods. This can be approached by using a combination of various metrics and multivariate techniques. The present chapter describes abilities and constraints of community-level tools and their potential links to the EDA approach.

Nutrient concentrations, habitat degradation and toxicants are the most prevalent stressors in polluted systems, where they might be in excess and cause responses of stream-dwelling organisms [43, 44]. The response of the biological community can be considered as the summing up of the responses of every individual species, plus the effects of their interaction [45–47]. As such, responses of the species are not simple or unimodal, but highly difficult to predict.

Several metrics have been defined to summarise the information provided by the biological community. Therefore, correlation of integrated community metrics instead of individual species responses with chemical contamination, e.g. key toxicants revealed by EDA studies at a given site is possible. *Community diversity* derives from ecological theory, and considers the richness (species or taxa numbers) and frequency of their relative abundance. A number of indices have been developed to describe the diversity of biological communities, mostly for algae (diatoms), invertebrate and fish. The response may be dependent on the temporal status of a community (i.e. the particular stage of ecological succession they are in), when an increase in the degree of a stress could either increase or decrease diversity. In general, it is complicated to make a general prediction about how diversity may be affected by pollution in a natural community. As diversity patterns vary along environmental gradients (e.g. the size of a stream or river, its water flow, chemical contamination, etc.), changes in diversity can be analysed only by comparing sampling sites along a spatial contamination gradient (e.g. downstream of a site investigated with EDA compared to a related reference site) or by assessing historical data series at a given site [48]. It was even observed that biodiversity of algal communities increased at low concentrations but decreased in higher concentration ranges [49]. A comparative analysis in the Llobregat River, Spain showed that the taxonomic distinctness reflected the waterborne concentration of the herbicide diuron better than the usually used Shannon-Wiener or Simpson indices [48]. There was – according to this index – a significant decrease of diatom diversity with increasing diuron concentrations in the river (Fig. 3). This example illustrates that chemicals, when identified to be causative in bioassays in an EDA process, can be correlated with integrated community indices and therewith provide a first hint on community-level effects. However, these results need further confirmation for a clear causal interpretation, like studies suggested in the chapters before.

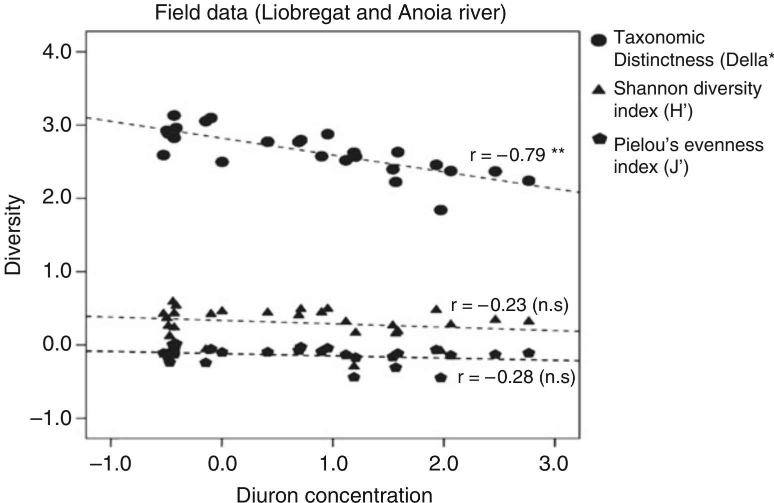

Fig. 3 Taxonomic distinctness (Delta*) compared to Shannon diversity and Pielou's diversity indices based on diatom communities in the Llobregat River, Spain [48]

Other indices not related to diversity can be more suitable to link the ecological status of the site of investigation to the chemicals revealed during the EDA process. Relevant and currently used diatom indices include the Specific Polluosensitivity Index (IPS; [50]), the Trophic Diatom Index (TDI; [51]), and variations of the saprobic indices. However, these indices have mainly been applied in eutrophication studies, yet, and their use to reflect the effect of toxicants is hampered by the interference between the responses of toxicants and variations in organic matter or nutrients. None of these indices has been combined with an EDA-like approach yet. However, an application of diatom indices to contaminated sites showed that they still might be appropriate to obtain a general overview of degradation and responded to changes in contamination in a translocation study of biofilms [29].

With regard to benthic macroinvertebrates, the trait-based SPecies-At-Risk (SPEAR) index [4] seems to be the most promising metric to evaluate community changes due to toxic stress, as indicated by EDA procedures. The species are classified to be at risk when being sensitive to organic chemicals according to their physiological sensitivity [4, 5] or their generation time [52]. The index expresses the share of all individuals of an invertebrate species sensitive to toxic stress compared to the total abundance of the community. Since it is based on biological traits and not on taxonomic composition, as many conventional bioassessment indices, it is relatively independent of confounding factors. Therefore, its application is not constrained by geographical and geomorphological factors and associated differences in biological communities and therefore more suitable for confirmation considerations of EDA results than indices developed for general degradation of aquatic systems. The SPEAR index correlated well to contamination expressed as toxic units for the crustacean *Daphnia magna* (see Sect. 5) and was

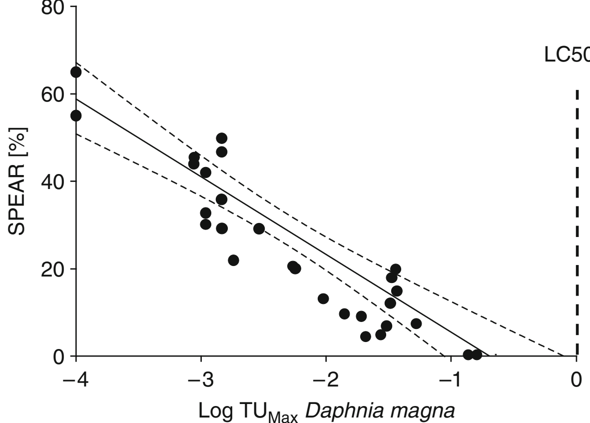

Fig. 4 Relationship between the maximum log TU_{max} *Daphnia magna* and the SPEAR index (linear regression, $p < 0.01$). A log TU of 0 represents a concentration equal to that of the LC_{50} for *D. magna*. The confidence band shows the 95% confidence limit of the respective mean [44]

successfully applied to detect pollution gradients and uncontaminated sites in different biogeographical regions [44, 52, 53]. Figure 4 shows an example of the Llobregat river, Spain, where the SPEAR index was applied [44]. This metric may be used to directly link bioassay-identified chemicals from EDA studies with ecological site assessments. By assessing whether concentrations of the identified compounds are likely to cause the observed community composition of macroinvertebrates at the site of investigation, it might confirm toxicity predictions, expressed as toxic units, based on site-specific chemical concentrations. Even a prioritisation of these toxicants seems possible. Moreover, results of the acute daphnid assay in EDA studies might be directly transformed into TU or effects on the SPEAR index, combining the three elements (chemistry to measure contamination, bioassay to measure toxicity, in situ biological assessment to measure effects such as benthic community alteration) in a TRIAD-like approach [54].

Multivariate techniques [55] have been widely used to assess the effects of disturbance and pollution in aquatic ecosystems [56] and are essential approaches for understanding cause-effect relationships between biological communities and stressors in polluted systems. As an example, the potential effect of pesticides and pharmaceuticals in the Llobregat River was analysed by means of Redundancy Discriminant Analysis (RDAs). The multivariate analysis combines the biological data with the environmental data, and allows accounting for the explained variance of groups of variables (e.g. nutrients vs. pollutants) on the species. In the Llobregat, a large number of pesticides and pharmaceuticals reach very high concentrations. The chemicals analysed in this study were evaluated within monitoring programmes but their measurements could also have been initiated by the results of an EDA study. Their potential effects had to be discerned from community changes caused by high nutrient concentrations and hydrological stress factors. Using multivariate tools it was possible to determine that the occurrence of triazine-type

herbicides specifically affected the algal community structure [43], but did not so for the invertebrate community. In contrast, the occurrence of pharmaceuticals in the river water affected the local benthic community structure [57], though in a different way than described for pesticides. Some anti-inflammatories and β-blockers were related to the abundance and biomass of two benthic invertebrates (i.e. *Chironomus* spp. and *Tubifex tubifex*); however, the response of the algal communities to the pharmaceutical products was very weak. Thus, multivariate analysis revealed a differentiated response pattern of the local communities to the contaminants, a result which follows an analogue principle as in EDA. Therefore, EDA might help to confirm the effects of identified mixtures by using appropriate bioassays that reflect the observed community effects.

In conclusion, there is a variety of approaches, complementary to each other, which account for the overall response of biological communities to stressors, including specific responses to groups of toxicants. Especially biological indices and multivariate analyses can be useful to determine the ecological status of a site [58] and can be applied to evaluate whether chemicals identified by EDA are candidates to cause a deterioration of the ecological status. However, determining the causality of a mixture of toxicants in the response of the biological community would require further steps.

5 Prognostic Modelling for Confirming the Ecological Relevance of EDA Studies

A typical outcome of EDA studies is a short list of candidate substances, which are assumed to cause the observed effects in the bioassay(s), which were applied to the environmental sample and their fractions. In this way, the selection of bioassays would largely determine if the observed changes in the biological communities (e.g. effects on ecological status) in the study area could be linked to substances identified in the EDA samples. While this is more difficult for bioassays based on cellular responses (e.g. mutagenicity), it would be easier for acute-based toxicity assays on whole organisms (e.g. 48 h LC_{50} for *D. magna*). Organism-based bioassays are also the basis of several prognostic models to predict the probability if EDA-identified chemicals and their mixtures exert toxic stress towards aquatic communities at a given site. Two straight forward approaches could be recommended here, to predict effects on biological communities of different trophic levels. The methods use experimental data for those very bioassays and compare them to the confirmed concentrations of the toxicants that were identified by the EDA process.

The first approach is the msPAF model, which could be used to predict the expected loss of species (in %) due to exposure to an environmental cocktail of toxicants as identified by EDA. The second is the Toxic Unit (TU) model, which quantifies toxic stress in the form of a hazard quotient and predicts changes in the

community structure for different trophic levels at concentrations far below the acute toxicity. Both methods address a more realistic risk of chemicals found in EDA by estimating bioavailable concentrations instead of using total concentrations.

While some EDA studies may consider the bioavailability of chemicals in the test design (see [59]), freely dissolved concentrations are usually not available from the subsequent target analysis. While organic contaminants may be bound to (organic) carbon, which is present in the sediment and suspended matter [60], the freely dissolved fraction of chemicals in the aqueous phase in equilibrium with the particulate phase is considered to be readily (bio)available for organisms uptake [61]. The equation from Di Toro et al. [60] could be used in the EDA context to consider the partitioning of organic chemicals between the water phase and organic carbon in the sample. Neglecting the difference in density between water and organic carbon in the sample, the bioavailable concentration can be estimated by the following formula in its simplest form:

$$\text{Bioavailable concentration} = \frac{C_{\text{Total}}}{1 + f_{\text{OC}} \times K_{\text{OC}}}$$

where C_{Total} is the total environmental concentration of a compound, f_{OC} is the w/w fraction of either particulate or dissolved organic carbon in the environmental sample, and K_{OC} is the partitioning coefficient between organic carbon and water. In this way, more realistic exposure concentration can be used for the following predictions of potential risks of EDA-identified toxicants.

The msPAF model is based on species sensitivity distributions (SSDs). These were derived from laboratory toxicity data [62, 63] by applying mixture toxicity modelling [6], and considering the bioavailability differences between sites caused by differences in water composition [64]. The use of SSDs allows estimating the potentially affected fraction of species (PAF) as a result of exposure to individual compounds, assuming that the sensitivity of test species used in the SSD is representative of the sensitivity of the species occurring in the field. The next step to achieve an msPAF is applying mixture toxicity models [6]. Compounds have been grouped for this purpose according to their most prominent toxic mode of action (TMoA), derived from literature sources [62]. The use of acute laboratory LC_{50} and EC_{50} data in the SSD modelling provides msPAF values that actually quantify an expected loss of taxa because of acute toxic effects. Hence, any acute effect that could be found in the EDA study with respective bioassays (e.g. *D. magna*) could be linked to the predicted loss of species according to msPAF.

For the purpose of quantifying sub-lethal effects on the community structure related to organic or metal pollutants, toxic units (TUs) are suggested, based on the TU approach by Sprague [65]. A separation between metals and organic compounds was made because different community effects are expected due to organic and metal compounds, at least for invertebrate species [5]. To derive respective TUs, the measured compound concentrations were scaled to the inherent toxicity (i.e. LC_{50}) of each compound towards a standard test organism (e.g. *D. magna*) and optionally added to an overall toxicity measure. The endpoint of the acute LC_{50} was

selected to be representative for observed community effects [4, 53, 66]. Concentrations below the Limit of Quantification (LOQ) were excluded from the computation of TUs in order to avoid overestimation of risks [67]. Using only the maximum TU (TU_{max}) derived from individual compound values allows assigning the minimum expected effect based on the most potent toxicants identified in the EDA. The lower end of the toxicity range was set to 1/10,000 of the acute LC_{50}, where no effects on the community structure would be expected. In contrast, sub-lethal concentrations above 1/1,000 of the acute LC_{50} would be expected to affect the biota when applying respective community metrics [4, 44, 53]. In this way, TUs could be used to predict effects on biological communities based on community metrics and hence, link EDA to ecologically more relevant endpoints, such as the SPEAR index (see Sect. 4).

To identify the stressor responsible for the observed effects in the applied EDA bioassay test-battery, which commonly cover different trophic levels, it is recommended to calculate respective TUs for each trophic group [44]. In this way, the results from the different bioassays used in the EDA could be addressed, as specific effects of toxicants, such as insecticides or herbicides, are not expected to act in a similar way on all trophic levels. For this purpose, the reference species with the most effect data available are suggested here: i.e. *D. magna* for macroinvertebrates [5], *Selenastrum capricornutum* for algae and *Pimephales promelas* for fish.

6 Constraints and Uncertainties of Confirmation Approaches in EDA

Despite the variety of approaches, all following different assessment strategies, as described before, remaining uncertainties have to be considered, when using these approaches for confirmation of EDA. Most relevant for the outcome of the EDA is the appropriate selection of the biological system used for biotesting. EDA can only reveal toxicants, whose mode of action is reflected in the bioassays. If aiming to relate EDA outcomes to impairment in ecosystems, there are several major restrictions from this perspective. There are effect qualities at community or ecosystem level, which only become evident when species interact with their abiotic or biotic environment. For instance, Paulsson et al. [68] identified a zinc-induced phosphorus deficiency in autotrophic biofilms and subsequent decrease in periphyton biomass in the River Göta Älv, Sweden. An additional example may provide the effects of PCBs on eggshell thinning of seabirds [69], which could have hardly be predicted from bioassays. Even extensively studied compounds with a well-understood mode of action like pharmaceuticals caused effects on population level, which were not predicted with the currently used risk assessment approaches. One prominent recent example is the decrease of vulture populations in India after renal failure caused by diclofenac [70].

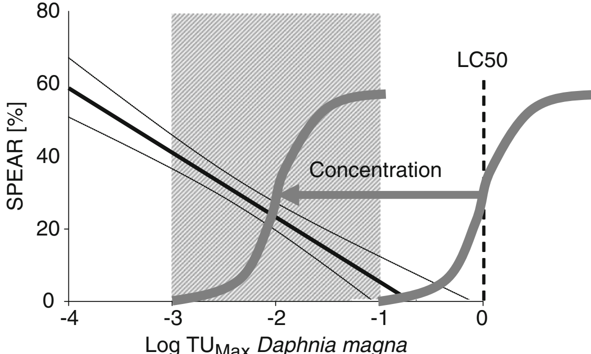

Fig. 5 Concentration- response curve of the acute *Daphnia magna* test in relation to the expected effects of organic toxicants, expressed as toxic units (TU), on the SPEAR index. The shaded area describes the concentration range, where effects on the community are expected, which would be overlooked, if the water sample is not concentrated. The *arrow* shows a concentration of at least 100 times in order to cover the *shaded* area

Another uncertainty remains related to the exposure. The commonly used biotest-batteries utilise several bioassays to consider different exposure routes (e.g. sediment or water) and trophic levels (algae, fish and invertebrates). However as the observed exposure levels often occur in sub-lethal concentration ranges below the acute LC_{50}, water samples would have to be concentrated in order to see any response in acute bioassays such as the 48 h *D. magna* test. Bioassays applied to non-concentrated water samples would only show effects in highly contaminated sites in the range of 1/10 of the acute LC_{50}. As a consequence, the majority of sites would be falsely assessed as unproblematic. However, at this concentration range, sensitive species would already be lost, according to the SPEAR index (Fig. 5). Hence, environmental samples would have to be concentrated at least by a factor of 100 to show effects in the acute bioassay. So far, this has never been tested, as large volumes of water would have to be extracted to allow for the biotesting of concentrated samples. On the other hand, the results of these bioassays could be used to confirm observed effects in local invertebrate communities. Moreover, the result might also be recalculated to toxic units to confirm the toxicity predictions based on observed concentrations of the key toxicants, identified by EDA. This approach generally also holds for the other trophic levels, like algae and fish (see Toxic Units above). For algae, the combination of bioassays with standard species as *S. capricornutum* and the field-based IPS metric could be used. In the case of fish, the standard test species *P. promelas* lacks of a respective index, as far as we know.

Finally, the translation of a biological effect addressed in a bioassay to an observable effect in ecosystems is not straight forward. This is especially difficult for bioassays using molecular responses like mutagenicity or genotoxicity (Table 1). These biological responses are of general concern in human and environmental toxicology and are therefore of relevance to be included in EDA studies. But it remains difficult to directly translate these responses in effects observable at

Table 1 Selection of in vitro and in vivo test systems used in EDA studies and effects potentially corresponding in aquatic ecosystems

Bioassay	Organism group	Level of observation parameter	Substances	Effect potentially corresponding in aquatic systems
In vitro				
YES/YAS	Yeast oestrogen screen	Molecular (binding to and activating of receptor)	Oestrogen/androgen receptor active substances	Reproductive and developmental disturbances
AR-Calux	Cell line	Molecular (binding to and activating of receptor)	Androgen receptor active substances	Reproductive and developmental disturbances
TTR-binding assay	Human thyroid transport receptor	Binding with thyroid hormone transporter protein TTR (molecular (binding to and activating of receptor)	Thyroid hormone receptor active substances	Developmental disturbances
DR CALUX	Cell line	Molecular (binding to and activating of receptor)	AhR receptor active substances	Reproductive and developmental disturbances
H4IIE, RTL-W1, PLHC	Cell line (fish, mammals)	Molecular (binding to and activating of receptor)	AhR receptor active substances	Reproductive and developmental disturbances
AMES, SOS Chromio	Bacteria	Interaction with DNA	Mutagenic/cancerogenic activity	Genomic changes/genmetic constitution, neoplastic changes
PAM-fluorescence of PS II	Primary producers	Photosynthetic activity	PSII-inhibiting substances	Primary production, plant growth
Antibiotic challenge bioassay, Premitest	*Bacillus stearothermophilus*	Growth inhibition	Antibiotics	Resistance in microbial communities
Bioluminescent bacteria test	*Vibrio fischeri*	Energy budget of cells	Biocides	Impairment of energy metabolism
In vivo				
Algal reproduction test	Primary producers	Population growth	Biocides, herbicides, metals	Phytoplankton/ microphytobenthos composition
Daphnia test	Zooplankton	Mortality	Biocides, insecticides	Zooplankton macrozoobenthos – populations

Danio rerio embryo test	Fish larvae	Embryonic development	Biocides oestrogen acting compounds	Fish – populations
Chenorabditis elegans	Nematodes	Population growth	Biocides	Nematode marcozoobenthos – populations
Choronomus riparius	Invertebrates	Population growth	Biocides	Marcozoobenthos – populations
Lumbricus variegatus	Invertebrates	Population growth	Biocides	Marcozoobenthos – populations
Potamopyrgus antipodarum test	Gastropode	Reproduction/Embryonic development	Endocrine disrupting compounds	Marcozoobenthos – populations

population or ecosystem level. An overview of bioanalytical tests used in EDA studies is given in Blasco and Picó [71]. In Table 1, a selection of bioassays, commonly used in EDA, is related to information they may provide to support understanding ecological effects. It may be concluded that the in vitro bioassays provide more mechanistic and compound-specific information, but their relationship to population or even ecosystem changes remains unclear. In vivo bioassays based on growth or population development are integrating several mechanisms of action. They can better be linked to observations in the ecosystem, for instance by population modelling, but are less specific in discriminating compounds with different modes of action.

Another factor that can cause disparate findings of bioassays used in EDA and confirmation steps is different scales of time integration in these approaches. Vermeirssen et al. [72], when assessing oestrogenic contamination of Swiss rivers, found no correlation between the VTG biomarker in fish and the yeast oestrogen receptor (YES) bioassay on extracts from water samples. The likely cause for this discrepancy is the different time scale: while the bioassay detected the actual oestrogenic potency being present in the grab sample of the river water, the VTG response integrates the prolonged exposure of fish to oestrogenic contaminants in the river. This explanation was corroborated by the findings of Burki et al. [73], who found a good agreement between the VTG responses of fish exposed to river water for 30 days and the bioassay-derived oestrogenic potency of semipermeable membrane devices being exposed to river water for the same time. Thus, the change from grab sampling to time-integrated sampling made the bioassay and the biomarker results comparable. Additional factors may complicate the relation between bioassay and biomarker findings. For instance, pathogenic infections or water temperature can modulate the biomarker response [74].

7 Conclusion

Very few studies directly combined EDA with an ecological-based site assessment, but several approaches are available to link these two assessment levels, in general. The risk of key toxicants to the aquatic environment may be best addressed by a multiple LoE framework, integrating, in a triad-like approach, methods from different scientific disciplines, namely from analytical chemistry, toxicology and community ecology [54] (Fig. 6).

In a weight of evidence approach, these individual lines of evidence are synthesised by quantitative or qualitative methods [75]. Aiming to increase causal evidence in this synthesis, several approaches could be integrated in this triad-like framework and therewith confirm EDA-identified chemicals to be causative for ecological impairment. Field observations of pollution-induced changes in communities indicated by structural metrics or multivariate statistics may be the strongest LoE to indicate ecological risks by key toxicants, but are mainly correlative and achieve causality just in combination with the other assessment levels.

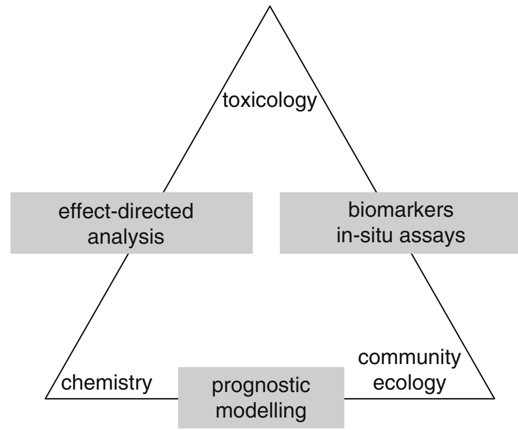

Fig. 6 Triad-like approach unifying components from chemistry, toxicology and field observations of communities, linked by additional lines of evidences (*grey* boxes) that are suitable to increase causal evidence within this multiple line of evidence framework

The EDA methodology reinforces its value in this multiple LoE framework by causality linking analytical chemistry and toxicology. Concentrations of contaminants, identified in EDA can be used for prognostic modelling to link chemistry to ecology. They can be converted into toxic units or msPAFs and give a first indication of potential risks towards field communities by considering mixture toxicity concepts and bioavailability. Finally, biomarkers induced in field organisms, in situ bioassays using reference organisms as well as laboratory bioassays using contaminated field samples causally link observed impairment of inherent communities (ecology) to indicated toxicity in bioassays (toxicology), as identified during the EDA process.

None of these LoE alone is able to ascertain causes of ecosystem impairment due to chemicals in a multiple stressed environment, but gain value when confirmed by each other. Therewith, the strength of the triad-like approach is its ability to discern direct toxicological effects from natural variation in habitat characteristics [77]. By a weight of evidence evaluation of the assessment components, a causal analysis can be derived, whether chemical contamination, identified by EDA, results in disturbance of local communities. This multi-criteria approach may provide a stronger basis for decision makers and water managers and increase the level of certainty for prioritisation and selection of areas for remediation.

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Index

A
Abraham parameters, 191
Abramis brama, 60, 240, 319
Accelerated solvent extraction (ASE), 206
7-Acetoxy-1-methylquinolinium iodide (AMQI), 130
Acetylcholine-binding-protein (AChBP), 137
Acetylcholinesterase (AChE), 27, 130
Affinity capturing, 124
Affinity chromatography, 104
Affinity-based screening technologies, 121
Ah receptor nuclear translocator (ARNT), 289
Airborne particles, 199
Alethrin, 27
Alkyl-nitrobiphenyls, 209
Alkyl-nitronaphthalenes, 209
Alkyl-nitrophenanthrenes, 209
Alkylphenols, 76
Ambersorb, 22
Americamysis bahia, 24, 51
2-Amino-1-methyl-6-phenyl-imidazo[4,5-*b*] pyridine, 71
2-Amino-3-methyl-3H-imidazo(4,5-*f*) quinoline (IQ), 182
9-Amino-hydroxyacridine, 161
Ammonia, 14
Ampelisca abdita, 24, 34, 51
Androgens, 237, 244, 267
  metabolites, 76
  receptors, 75
Androstenedione (AED), 278
Androsterone, 74, 76, 250, 254
Anguilla anguilla, 23
Anion exchange resins, 22
Anisole, 97
Anthracene, 24, 108, 291
APCI–LC–MS, 215
Arenicola brasiliensis, 53
Arenicola marina, 53
Aryl hydrocarbon hydroxylase (AHH), 296
Aryl hydrocarbon receptor (AhR), 69, 71, 276, 289
Atmospheric pressure photoionization (APPI), 217
Automated Mass Spectral Deconvolution and Identification System (AMDIS), 151

B
Base-pair substitution, 212
Benzo[*a*]pyrene, 48, 53, 218
Benzo[*ghi*]perylene, 24, 219
Benzofluoranthenes, 219
Benzopyrene quinones, 222
Bifenthrin, 27, 28
Bioaccessibility, 42
Bioaffinity, 119
Bioassays, 285
  in situ, 316
  in vitro, 295
  in vivo, 294
  selection, 6
Bioavailability, 19, 43
  environmental, 44
Bioluminescent yeast estrogen screen (BLYES), 75
Biomarkers, 316
Biomimetic fluids, 53
Biota-sediment accumulation factors (BSAF), 49
Biota tissue, 42
Bis(2-ethylhexyl)phthalate, 250
Bisphenol A, 76, 252, 293
β-Blockers, 328
Buprofezin, 157
Butyl benzylphthalate, 137

W. Brack (ed.), *Effect-Directed Analysis of Complex Environmental Contamination*, Hdb Env Chem (2011) 15: 341–346, DOI 10.1007/978-3-642-18384-3,
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C
C18 resins, 8
CALUX, 237, 246, 256
Carbamates, 29
Carbaryl, 14, 26
Carbendazim, 157
Carboxylesterases, 28
Cathepsin, B 133
Cation exchange resins, 22
Catostomus commersoni, 59
Ceriodaphnia spp., 5, 9, 29
Chironomus pallidivitatis, 23
Chironomus riparius, 333
Chloramphenicol acetyl transferase (CAT), 296
Chlorobenzenes, 49
Chlorobenzoic acid, 173
3-Chloro-4-dichloromethyl-5-hydroxy-2(5H)-furanone, 71
Chlorodihydroxybenzenes, 273
5-Chloro-2-hydroxy-benzaldehyde, 173
Chloroxylenol, 251
Chlorpyrifos, 14, 26
Cholest-4,6-dien-3-one, 252
Chromatography, 86
Chromium, 12, 22, 70
Chrysene, 219
Cinnarizine, 254
Citharichthys stigmaeus, 294
Clorophene, 251
Coconut charcoal, 22, 45
Combustion, incomplete, 199
Community diversity, 325
Community metrics, 316
Complex mixtures, 285
Concentration addition (CA), 249
Corophium volutator, 54
Cuyahoga River, 2
Cyanine-3/-5, 32
Cyanopropyl gravity-column chromatography, 208, 211
Cyclodextrin, 42, 46, 98
  extraction, 47
3-Cyclohexyl-1,1-dimethylurea, 160
Cyclopenta[*cd*]pyrene (CPP), 214, 219
Cyhalothrin, 27, 28
CYP19 aromatase, 76, 292, 296
Cypermethrin, 27, 28
Cytochrome P4501A (CYP1A), 212, 318

D
Danio rerio, 31, 159, 293
Daphnia magna, 193, 274, 326
Databases, 167
DDE 76, 239
DDT 49, 239
Desorption kinetics, 42
Dexamethasone, 255
Diazinon, 9
Dibenz[*ah*]anthracene, 24
Dibenzo[*a,e*]pyrene, 219
Dibenzofurans, 50
Dichlorobenzidine, 100
Dichloro-dihydroxy-benzophenone (DDB), 224
1,2-Dichloroethane, 183
Dichloromethane (DCM), 23, 107, 111, 205, 208, 276
Dichlorophen(methylenebis[4-chlorophenol], 224
Dichlorvos, 244
Dicyclopentapyrene (DCPP), 214
Dihydrotestosterone (DHT), 244, 300
Dimethylsulfoxide, 42
Dinaphthofurans, 151
Dinitroreductase (TA98-DNP), 222
Dioxin responsive elements (DREs), 289
Dioxin-like compounds, 69, 71, 285
Diphenylethers, 50
Dipole moment, 86
Discharge monitoring, 5
Diterpene alcohols, 273
DNA damage, 70, 199, 219, 291
DNA topoisomerase, 126
Dosing, passive, 58

E
EDTA, 5, 20
Effect-directed analysis (EDA), 42, 120, 144, 199, 247, 267
Effluents, 1, 105, 240, 250
  toxicity, 3, 17, 247, 273
Eisenia fetida, 24, 52
Elemental search, 153
Elizabeth River (VA, USA), 25
Enchytraeus albidus, 24, 52
Endocrine activity/disruption, 69, 75, 267, 285, 292
  bioassays, 245
Endpoints, 31
Environmental risk assessment (ERA), 288, 316
Epidermal growth factor receptor (EGFR), 123
Esfenvalerate, 29
Estradiol, 237, 241, 292
Estrogen responsive elements (ERE), 242, 293
Estrogenic chemicals, 6, 60, 100, 240
Estrogens, 75, 241, 267
  receptor, 75, 241, 292

7-Ethoxyresorufin-*O*-deethylase (EROD), 74, 296, 321
Ethylbenzene, 97, 98
17α-Ethynylestradiol (EE2), 31, 60, 237, 242, 293
Extraction, bioaccessibility-directed, 46, 54
  equilibrium partitioning-based, 55

F
Feminisation, 237
Fenvalerate, 27
Fish, 267
  reproduction, 275
Fluoranthene, 24, 173
Fractionation, 83, 208, 285
  bioassay-directed, 199
Frameshift mutations, 212
Frontal affinity chromatography (FAC), 122

G
Gas chromatography (GC), 109
GC, 143
GC × GC, 149
GC–MS, 5, 42, 143, 148, 171, 213
  databases, 174
Gel permeation chromatography (GPC), 103
Genomic endpoints, 31
Genomics, 19
Genotoxicity, 69, 201, 285, 291
Glucocorticoids, 237, 255
  receptor, 246

H
Heated pneumatic nebulizer (HPN), 215
Hepatic tissues, 276
Hexa(methoxymethyl)melamine (HMMM), 160
Hormones, 237
HPLC, 60, 85, 105, 128, 214, 247
HR-MS, 170, 208, 213
Human estrogen receptor α (hERα), 125, 130
Human-cell assays, 212
Hyalella azteca, 28
Hydrogen bonding, 86
Hydrophilic interaction chromatography (HILIC), 102
2-Hydroxy-9,10-anthraquinone (2HA), 182
Hydroxy-acridine-*N*-oxide, 161, 9
Hydroxydiphenylethers, 50
7-Hydroxy-1-methylquinolinium iodide (HMQI), 130
Hydroxynitro-PAH, 219
Hydroxypropyl-β-cyclodextrin (HPCD), 48

I
Identification, 143, 167
Immobilized artificial membrane technology (IAM), 123
Indeno[1,2,3-*cd*]pyrene, 24, 207, 219
Infertility, 239
Intersexuality, 240
Ion trap detector (ITD), 147
Iprodione, 76

J
Juvenile insect hormone analogues, 273

K
Kraft mill process, 273

L
LC–MS, 119, 143
  databases, 176
Ligand-binding assays, 121
Linear solvation energy relationships (LSER), 89, 90, 109, 191
Lines of evidence (LoE), 318
Liquid chromatography (LC), 88, 170
London dispersive energies, 85
LSER, 83
Lumbricus variegatus, 333

M
Magnetic bead protein affinity LC-MS assay, 124
Malathion, 14
Masculinization, 278
Mass, exact, 174
Mass spectrometry (MS), 143, 170
Matrix metalloprotease (MMP3), 128
Metals, 15
9-Methylacridine, 159
Methylmethanesulfonate, 72
Micropogonias undulates, 278
Microwave-assisted extraction (MWAE), 205
Molecular formula, prediction, 179
MOLGEN-MS, 180
MS–MS, 170
ms-PAF, 316
Multiple line of evidence approach, 316
Multiple reaction monitoring (MRM), 147
Musks, 76
Mutagenicity, 69, 200, 279
  airborne particles, 200
  testing, 211
Mutagens, 71, 199
MX, 71

N
Naphthenic acids, 76, 254
Naphtho[2,1-*a*]pyrene, 219
Nitroaminoazobenzenes, 72
3-Nitro-7H-benz[*d,e*]anthracen-7-one, 222
3-Nitrobenzanthrone (3NBA), 71, 107
2-Nitro-6H-dibenzo[*b,d*]pyran-6-one (2-NDBP), 222
Nitro-PAH lactones, 219
1-Nitropyrene (1NP), 182, 222
Nitro-reductase (TA98-NR), 222
Nonionic organic chemicals (NOCs), 20, 23
Nonylphenol, 250
Nonylphenolethoxylates, 251
Norethisterone (NET), 127
NP-HPLC, 101
NP-LC, 88, 100

O
Oncorhynchus mykiss, 294
Organochlorine pesticides, 239
Organophosphates, 27, 76
Oxybenzone, 254

P
P450IA1 enzymes, 274
PAHs *see* Polycyclic aromatic hydrocarbons
Paper mill effluent, 267
Paralichthys californicus, 294
Paramagnetic affinity beads (PAB), 124
Parastichopus californicus, 53
Partition-based dosing, 42, 57
Partitioning, 47, 188
PBTA (2-phenilbenzotriazoles), 71
PCDD/F, 96
Pentachlorodibenzofuran, 276
Pentachlorophenol, 256
Penta(methoxymethyl)melamine, 160
Permethrin, 26
Pesticides, 19, 26, 157
Phenalen-1-one, 208
Phenanthrene 24, 48
Phenylbenzotriazole derivatives, 100
Phenylchlorocarbonate, 173
Phenylnaphthylamine (PNA), 323
PhIP, 71
Phthalates, 76
Phyto-estrogens, 250
PICT, 316
Pielou's diversity, 326
Pimephales promelas, 5, 9, 29
Piperonyl butoxide (PBO), 27, 28
Pitch dispersants, 273
Planar chromatography, 105
Platichthys flesus, 294
Polar organic chemical integrative samplers (POCIS), 323
Pollution-induced community tolerance (PICT), 323
Polybrominated diphenyl ethers (PBDEs), 49
Polychlorinated biphenyls (PCBs), 20, 23, 49
Polychlorinated diphenylthiophenes (PCDTs), 291
Polychlorinated naphthalenes (PCNs), 290
Polycyclic aromatic compounds, 7, 199
Polycyclic aromatic hydrocarbons (PAHs), 21, 48, 58, 76, 151, 171, 199, 205, 218, 290, 321
  nitrated (N-PAH), 213, 219
  oxygen-containing, 222
Poly(divinylbenzene) (PVDB), 206
Polyoxymethylene (POM), 56
Postcolumn bioactivity detection, 128
Potamopyrgus antipodarum, 323, 333
Precolumn affinity, 121
Precursors, information, 193
Pressurized fluid extraction (PFE), 205
Pressurized liquid extraction (PLE), 206
Priority pollutants, 2
Progestagens, 244, 255
Progesterone, 237, 278
  receptors (PR), 246
Prognostic modelling, 316
Prometryn, 323
Pulp, 267, 268
Pyrene, 173
Pyrethrins, 27

Q
Quantitative structure-retention relationships (QSRRs), 83, 89, 101, 108
Quinones, 151

R
Radioligand binding assay (RLBA), 77, 247
Redundancy discriminant analysis (RDA), 326
Regulatory applications, 280
Relative potencies (RPs), 299, 319
Retention prediction, 83
RP-LC, 88

S
Salmonella assay, 69, 71, 211, 291
Sample cleanup methods, 206
Sample collection, 202
Sample preparation, 205

Screening, 119
Sediments, 4, 19, 72, 100, 151, 255
 estrogens, 252
Selected reaction monitoring (SRM), 147
Semipermeable membrane devices (SPMD), 55
Separation, intermolecular forces, 85
Sewage treatment plants (STPs), 105, 240, 250
 estrogens, 250
Sex steroid binding protein (SSBP), 276
Shannon diversity, 326
Silicone rubbers (SR), 56
Size exclusion chromatography (SEC), 103, 127
Solid-phase extraction (SPE), 86, 250
Solid-phase microextraction (SPME), 44, 55
Sorbitol dehydrogenase (SDH), 123
Soxhlet extraction, 205
SPecies-At-Risk (SPEAR) index, 326
Specific polluosensitivity index (IPS), 326
Spectrum prediction, 167
SRM 1649, 210
Stationary phases, 83, 94, 110
Steric energy, 192
Steroids, 71, 75, 241, 277, 293, 303
Streptococcus pneumoniae, 126
Strongylocentrotus purpuratus, 278
Structure elucidation, 143, 167
Structure generation, 167, 177
Subcritical water extraction (SWE), 206
Substructure identification, 178
Supercritical fluid extraction (SFE), 19, 23, 42, 51
Surfactants, 10

T
TCDD, 276, 289, 299, 320
Temperature change manipulation, 29
Temperature–toxicity correlation, 29
TENAX, 42, 46, 48
Testicular androgen receptors, 276
Testicular cancer, 239
Testosterone, 244
Tetrachloro-*o*-benzoquinone, 273
Thermomechanical pulp (TMP), 275
Thioanisole, 97
Thiophanate methyl, 157
Thiosulfate, 5
Thymidine kinase, 213
Thyroid, 237, 245
Thyroid-hormone-disrupting compounds (TDC), 76
Thyroid hormone-like compounds, 255
Time of flight mass spectrometry (ToF MS), 147
TLC, 105
Toluene, 97
Total dissolved solids (TDS), 15
Total ion chromatogram (TIC), 206
Total maximum daily loading (TMDL), 36
Toxic equivalents (TEQs), 276, 300
Toxicity, 1, 193
Toxicity equivalency factors (TEFs), 299
Toxicity identification evaluation (TIE), 1, 4, 19, 42, 285
Toxicity source identification (TSE), 274
Transport proteins transthyretin (TTR), 76
Transthyretin, 245
TRIAD, 316
Triazine-type herbicides, 327
Tribromophenol, 256
Triclosan, 251
Tridecane, 173
Trifluorothymidine, 213
Trophic diatom index (TDI), 326
Tubifex tubifex, 328
TUs, 316

U
Ultrafiltration, 104, 121, 125
Ulva lactuca, 21
Urechis caupo, 53

V
Van der Waals interactions, 85
Vinclozolin, 244
Vitellogenin, 31, 60, 240, 319, 334

W
Whole effluent toxicity (WET), 3

X
XAD resins, 8
Xanthenes/xanthones, chlorinated, 291

Y
Yeast androgen screen (YAS) assay, 76
Yeast estrogen screen (YES) assay, 75, 161, 237, 246, 324, 334

Z
Zeolite, 22