Volume 212

David M. Whitacre
Editor

Reviews of Environmental Contamination and Toxicology

Springer

Reviews of Environmental Contamination and Toxicology

VOLUME 212

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Springer

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ISSN 0179-5953
ISBN 978-1-4419-8452-4 e-ISBN 978-1-4419-8453-1
DOI 10.1007/978-1-4419-8453-1
Springer New York Dordrecht Heidelberg London

Foreword

International concern in scientific, industrial, and governmental communities over traces of xenobiotics in foods and in both abiotic and biotic environments has justified the present triumvirate of specialized publications in this field: comprehensive reviews, rapidly published research papers and progress reports, and archival documentations. These three international publications are integrated and scheduled to provide the coherency essential for nonduplicative and current progress in a field as dynamic and complex as environmental contamination and toxicology. This series is reserved exclusively for the diversified literature on "toxic" chemicals in our food, our feeds, our homes, recreational and working surroundings, our domestic animals, our wildlife, and ourselves. Tremendous efforts worldwide have been mobilized to evaluate the nature, presence, magnitude, fate, and toxicology of the chemicals loosed upon the Earth. Among the sequelae of this broad new emphasis is an undeniable need for an articulated set of authoritative publications, where one can find the latest important world literature produced by these emerging areas of science together with documentation of pertinent ancillary legislation.

Research directors and legislative or administrative advisers do not have the time to scan the escalating number of technical publications that may contain articles important to current responsibility. Rather, these individuals need the background provided by detailed reviews and the assurance that the latest information is made available to them, all with minimal literature searching. Similarly, the scientist assigned or attracted to a new problem is required to glean all literature pertinent to the task, to publish new developments or important new experimental details quickly, to inform others of findings that might alter their own efforts, and eventually to publish all his/her supporting data and conclusions for archival purposes.

In the fields of environmental contamination and toxicology, the sum of these concerns and responsibilities is decisively addressed by the uniform, encompassing, and timely publication format of the Springer triumvirate:

> *Reviews of Environmental Contamination and Toxicology* [Vol. 1 through 97 (1962–1986) as Residue Reviews] for detailed review articles concerned with any aspects of chemical contaminants, including pesticides, in the total environment with toxicological considerations and consequences.

Bulletin of Environmental Contamination and Toxicology (Vol. 1 in 1966) for rapid publication of short reports of significant advances and discoveries in the fields of air, soil, water, and food contamination and pollution as well as methodology and other disciplines concerned with the introduction, presence, and effects of toxicants in the total environment.

Archives of Environmental Contamination and Toxicology (Vol. 1 in 1973) for important complete articles emphasizing and describing original experimental or theoretical research work pertaining to the scientific aspects of chemical contaminants in the environment.

Manuscripts for Reviews and the Archives are in identical formats and are peer reviewed by scientists in the field for adequacy and value; manuscripts for the Bulletin are also reviewed, but are published by photo-offset from camera-ready copy to provide the latest results with minimum delay. The individual editors of these three publications comprise the joint Coordinating Board of Editors with referral within the board of manuscripts submitted to one publication but deemed by major emphasis or length more suitable for one of the others.

Coordinating Board of Editors

Preface

The role of *Reviews* is to publish detailed scientific review articles on all aspects of environmental contamination and associated toxicological consequences. Such articles facilitate the often complex task of accessing and interpreting cogent scientific data within the confines of one or more closely related research fields.

In the nearly 50 years since *Reviews of Environmental Contamination and Toxicology* (*formerly Residue Reviews*) was first published, the number, scope, and complexity of environmental pollution incidents have grown unabated. During this entire period, the emphasis has been on publishing articles that address the presence and toxicity of environmental contaminants. New research is published each year on a myriad of environmental pollution issues facing people worldwide. This fact, and the routine discovery and reporting of new environmental contamination cases, creates an increasingly important function for *Reviews*.

The staggering volume of scientific literature demands remedy by which data can be synthesized and made available to readers in an abridged form. Reviews addresses this need and provides detailed reviews worldwide to key scientists and science or policy administrators, whether employed by government, universities, or the private sector.

There is a panoply of environmental issues and concerns on which many scientists have focused their research in past years. The scope of this list is quite broad, encompassing environmental events globally that affect marine and terrestrial ecosystems; biotic and abiotic environments; impacts on plants, humans, and wildlife; and pollutants, both chemical and radioactive; as well as the ravages of environmental disease in virtually all environmental media (soil, water, air). New or enhanced safety and environmental concerns have emerged in the last decade to be added to incidents covered by the media, studied by scientists, and addressed by governmental and private institutions. Among these are events so striking that they are creating a paradigm shift. Two in particular are at the center of everincreasing media as well as scientific attention: bioterrorism and global warming. Unfortunately, these very worrisome issues are now superimposed on the already extensive list of ongoing environmental challenges.

The ultimate role of publishing scientific research is to enhance understanding of the environment in ways that allow the public to be better informed. The term "informed public" as used by Thomas Jefferson in the age of enlightenment

conveyed the thought of soundness and good judgment. In the modern sense, being "well informed" has the narrower meaning of having access to sufficient information. Because the public still gets most of its information on science and technology from TV news and reports, the role for scientists as interpreters and brokers of scientific information to the public will grow rather than diminish. Environmentalism is the newest global political force, resulting in the emergence of multinational consortia to control pollution and the evolution of the environmental ethic.Will the new politics of the twenty-first century involve a consortium of technologists and environmentalists, or a progressive confrontation? These matters are of genuine concern to governmental agencies and legislative bodies around the world.

For those who make the decisions about how our planet is managed, there is an ongoing need for continual surveillance and intelligent controls to avoid endangering the environment, public health, and wildlife. Ensuring safety-in-use of the many chemicals involved in our highly industrialized culture is a dynamic challenge, for the old, established materials are continually being displaced by newly developed molecules more acceptable to federal and state regulatory agencies, public health officials, and environmentalists.

Reviews publishes synoptic articles designed to treat the presence, fate, and, if possible, the safety of xenobiotics in any segment of the environment. These reviews can be either general or specific, but properly lie in the domains of analytical chemistry and its methodology, biochemistry, human and animal medicine, legislation, pharmacology, physiology, toxicology, and regulation. Certain affairs in food technology concerned specifically with pesticide and other food-additive problems may also be appropriate.

Because manuscripts are published in the order in which they are received in final form, it may seem that some important aspects have been neglected at times. However, these apparent omissions are recognized, and pertinent manuscripts are likely in preparation or planned. The field is so very large and the interests in it are so varied that the editor and the editorial board earnestly solicit authors and suggestions of underrepresented topics to make this international book series yet more useful and worthwhile.

Justification for the preparation of any review for this book series is that it deals with some aspect of the many real problems arising from the presence of foreign chemicals in our surroundings. Thus, manuscripts may encompass case studies from any country. Food additives, including pesticides, or their metabolites that may persist into human food and animal feeds are within this scope. Additionally, chemical contamination in any manner of air, water, soil, or plant or animal life is within these objectives and their purview.

Manuscripts are often contributed by invitation. However, nominations for new topics or topics in areas that are rapidly advancing are welcome. Preliminary communication with the editor is recommended before volunteered review manuscripts are submitted.

Summerfield, North Carolina David M. Whitacre

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Biochemical Effects Induced by the Hexachlorocyclohexanes

Jesus Olivero-Verbel, Angélica Guerrero-Castilla, and Niradiz Reyes Ramos

Contents

1 Introduction

Hexachlorocyclohexane isomers (HCHs) are environmental contaminants produced as a result of the photochemical chlorination of benzene during the manufacture of technical HCH, which has been widely used for the control of agricultural pests and mosquitoes. Technical HCH is a mixture of five isomers: α-HCH (53–70%), β-HCH (3–14%), γ-HCH (11–18%), δ-HCH (6–10%), and ε-HCH (3–5%), of which only the γ-isomer (lindane) possesses relevant insecticidal activity (Langenhoff et al. 2002).

As a consequence of their widespread use, particularly in developing countries, HCHs residues have been incorporated as contaminants into the human diet through food products (Nigam and Siddiqui 2001), and proof of their distribution lies in the

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D.M. Whitacre (ed.), *Reviews of Environmental Contamination and Toxicology*, Reviews of Environmental Contamination and Toxicology 212,
DOI 10.1007/978-1-4419-8453-1_1,

observation that these compounds have been detected in different environmental niches (Corsolini et al. 2006; Ikemoto et al. 2008; Moisey et al. 2001; Simonich and Hites 1995), and in tissue samples from humans all over the world (Chu et al. 2006; Li et al. 2006; Molina et al. 2005). Although developed countries banned the use of HCHs more than 20 years ago, these compounds are still used in some developing countries for agriculture and public health programs (Bhatt et al. 2007). Even in the countries where technical HCH has not been used for several years, the problem of residual contamination remains for all isomers of HCH, because of their high environmental persistence and tendency to continually bioaccumulate in biota and humans (Fenster et al. 2006).

Humans become exposed to individual HCH isomers through variable routes, including ingestion of contaminated water or food, directly through the skin, or by inhalation of polluted air. Because of their liposolubility, HCHs are stored in fat-containing tissue, such as breast milk, as has been documented in studies conducted around the world (Pohl and Tylenda 2000). In fact, milk-containing HCHs from nursing mothers may lead to direct exposure of newborn children (Minh et al. 2004; Subramanian et al. 2007; Tanabe and Kunisue 2007). Although the ability of HCHs to induce neurotoxicity is well appreciated (Joy 1982; Shankland 1982), awareness of their toxic effects in other tissues has more recently become recognized, and the biochemical events leading to these effects are beginning to be uncovered. These compounds have also been suspected as possible risk factors for breast cancer (Mussalo-Rauhamaa et al. 1990; Steinmetz et al. 1996; Wong and Matsuura 2007), Non-Hodgkin Lymphoma (NHL) (Rafnsson 2006; Spinelli et al. 2007), and for reproductive disorders (Torres-Arreola et al. 2003).

The goal of this paper is to review the variety of biochemical responses elicited by the most commonly cited stereoisomers of HCH, with particular focus on alterations and activation of signal transduction pathways, and how these events may contribute to toxicity.

2 Structure of the Hexachlorocyclohexanes

HCHs have the general chemical formula $C_6H_6Cl_6$. They exist in the chair form, making possible the theoretical existence of 16 isomers. Some of these are mirror images and others can be interconverted into transitional forms. In technical mixtures, four isomers are the most common: the α-, β-, δ-, and γ-isomers (Fig. 1). The conformers differ as to the number and location of chlorine atoms on axial or equatorial positions, i.e., the C–Cl bonds are parallel to each other and project up or down from the ring structure, or are located around the perimeter of the ring, respectively. This simple geometric positioning has a tremendous impact on the physical, chemical, and biological properties of HCHs.

The common name for the γ-isomer is lindane, and this isoform has been the most extensively studied. Structural differences among HCH isomers are important for imparting varied properties toward biological systems, and their reactivity results not only from the presence of a particular pattern of chlorine substitutions, but definitively also from aspects such as molecular shape and global electronic distribution.

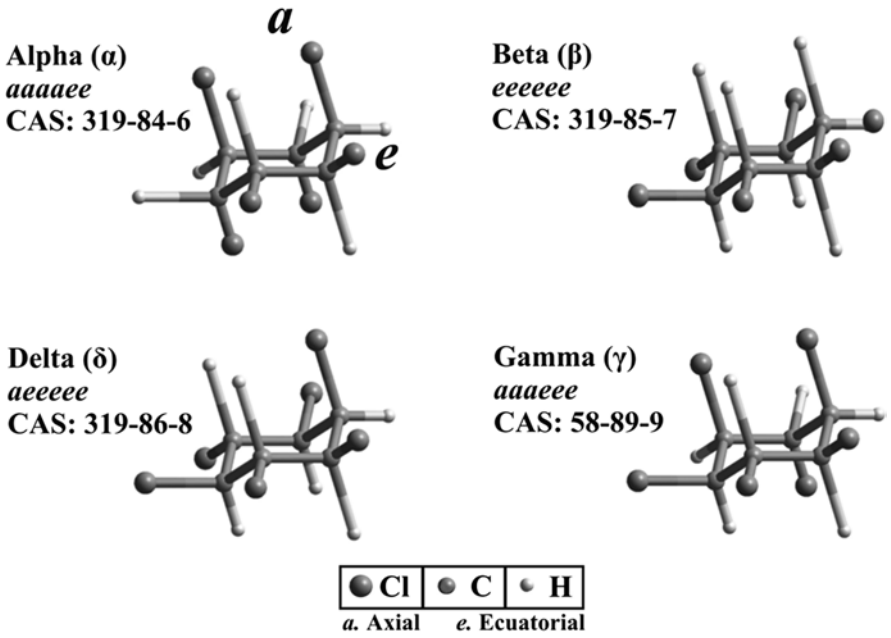

Fig. 1 Chemical structures of the most common hexachlorocyclohexane (HCH) isomers

3 Exposure, Distribution, and Metabolism

The most common modes of exposure to HCHs are via the oral and dermal routes. Food contaminated with the HCHs is the main source of these chemicals for humans, and their excretion in breast milk represents a serious health concern for breast-feeding children around the world, especially in developing countries (Ntow et al. 2008). The public health implications of exposure to HCHs via milk have been extensively reviewed (Pohl and Tylenda 2000), and most HCH isomers have been reported as residues in milk samples collected from around the world (Subramanian et al. 2007). Levels of HCHs found in food, as well as in human blood and milk samples obtained from different countries, are shown in Table 1. After dermal exposure, HCH isomers accumulate in different regions of the rat brain (Dick et al. 1997; Kumar et al. 1998). In the case of lindane, it accumulates in blood and is distributed throughout the body; at the cell level, it is incorporated into biological membranes following the order: mitochondria > sarcoplasmic reticulum > myelin > brain microsomes > erythrocytes (Sauviat and Pages 2002). β-HCH is highly accumulated in fat tissue and in the adrenals, whereas α-HCH and γ-HCH appear in high concentrations in fat tissue, kidneys, and the adrenals (Macholz et al. 1986). It has been suggested that α-HCH is transformed by hepatocytes into reactive species by a P450-dependent reaction (Mattioli et al. 1996); moreover, α- and γ-HCH but not β-HCH are reported to be dechlorinated by rat liver cytochrome P450 (Beurskens et al. 1991).

Table 1 Levels of HCH (hexachlorocyclohexane) in foods and human serum and milk

Country/region	Year	Sample type or source	Mean ΣHCH (ng/g lipid)	References
Foods				
Brazil/Rio Grande	2007	Cow milk	8.6	Heck et al. (2007)
Canada/Ulukhaktok-Holman	2006	Ringed seal (Fat)	200	Addison et al. (2009)
China/Beijing	2006	Chiken (muscle)	6.96	Tao et al. (2009)
India/state of Haryana	1999	bovine milk	29.2[a]	Sharma et al. (2007)
Jordan	2009	Milk products	Fat milk: 83 Butter: 19 Cheese: 28 Yogurt: 43	Salem et al. (2009)
Mexico	2002–2003	Bovine muscle fat	64	Waliszewski et al. (2004)
Mexico	2009	Bottled drinking water	0.150[a]	Díaz et al. (2009)
Pakistan/Punjab Province	2007	Eggs and egret prey	Eggs: 344.3 Egret prey: 36.8	Malik et al. (2010)
Rumania	2004	Pork tissue (Liver)	Liver: 152.6 Muscle: 27.5	Covaci et al. (2004)
Turkey/Konya region	2007	Wheat	2.2	Guler et al. (2010)
Turkey/Konya	2006–2007	Fish	28.6	Kalyoncu et al. (2009)
Human serum and milk				
Africa/Guinea-Bissau	2007	Adult/blood serum	125	Linderholm et al. (2010)
China/Beijing and Shenyang	2009	Human milk	312	Yu et al. (2009)
China/Beijing Japan/Sendai, Kyoto and Takayama Korea/Seoul Vietnam/Hanoi	2007–2008	Human milk	Beijing/β-HCH: 570 Sendai/β-HCH: 190 Kyoto/β-HCH: 77 Takayama/ β-HCH: 49 Seoul/β-HCH: 110 Hanoi /β-HCH: 140 Population Mean 140 (9–1200)	Haraguchi et al. (2009)

China/North Eastern	2008	Children/blood Serum	α-HCH (0.0093)[b] β-HCH (0.0044)[b] γ-HCH (0.0055)[b] δ-HCH (0.0063)[b]	Lu et al. (2010)
China/Tianjin and Yantai	2006–2007	Human milk	586.7	Leng et al. (2009)
India/Agra region	2005	Human milk	α-HCH: 32 : 40 γ-HCH: 51	Kumar et al. (2006)
Italy	2010	Adult/blood serum	18	Ingelido et al. (2009)
Japan/Hong Kong	2002	Human milk	α-HCH: 0.6 β-HCH: 940 γ-HCH: 1.8	Hedley et al. (2010)
Korea/Seoul	2009	Adult/blood serum	β-HCH: 58.8	Kang et al. (2008)
Norway	2002–2006	Human milk	β-HCH: 4.7	Polder et al. (2009)
Spain/Catalonia	2002	Adult/blood serum (18–74 years)	159.4	Porta et al. (2010)
USA	2003–2004	Adult/blood serum (18 years old)	14	Patterson et al. (2009)

[a] Unit: ng/mL
[b] Unit: ng/L

4 Toxicity in Mammals

The most commonly reported effects of acute poisoning for HCHs in humans have been well documented for the neurotoxic lindane, especially in infants and young children (Singal and Thami 2006). Lindane toxicity has been the most studied among the HCH isomers. The toxicity symptoms of acute lindane exposure indclude seizures, hypoxemia, respiratory acidosis, and hypotension (Sudakin 2007). Lindane also decreases uterine contraction force in rats in a dose-dependent manner (Criswell et al. 1995; Criswell and Loch-Caruso 1995). At small doses, it increases the function of the resident macrophages of liver Kupffer cells. This activation has been proposed to contribute to liver injury (Videla et al. 1997). In human neutrophils, γ-HCH produces superoxide anion release (English et al. 1986; Kuhns et al. 1986).

In 1987, the International Agency for Research on Cancer (IARC) classified the HCHs as possibly carcinogenic to humans (group 2B), on the basis of having inadequate data on carcinogenicity to humans, sufficient evidence for carcinogenicity to animals (for technical grade and the α-isomer), and limited evidence for carcinogenicity to animals (for β- and γ-isomers) (WHO 1987).

Carcinogenesis studies indicate that the isomers are differentially associated with respect to cancer. For example, early animal studies showed that feeding with α-HCH (but not β-HCH or γ-HCH) induced liver tumors in rats and mice (Ito et al. 1973, 1975). More recent in vitro and in vivo animal studies (Steinmetz et al. 1996; Wong and Matsuura 2007) showed that the β-isomer is associated with increased breast cancer risk. However, despite the evidence shown in the animal studies and the mechanistic information provided by the in vitro studies, epidemiological data linking cancer risk with exposure to organochlorine pesticides, including specific HCH isomers, is conflicting. For example, while some case–control studies have found a link between plasma levels of β-HCH and breast cancer risk (Mussalo-Rauhamaa et al. 1990), other studies have not found such a link (Lopez-Carrillo et al. 2002).

On the other hand, some population-based studies have suggested that exposure to lindane may be a possible risk factor for NHL (Blair et al. 1998; Cocco and Brennan 2008; McDuffie et al. 2001; Purdue et al. 2007). In general, epidemiological studies that have assessed the contribution of organochlorine pesticides in cancer risk are limited by the limited information provided on individual pesticides, including the HCH isomers, since these studies are ones in which many different chemicals are evaluated in the same study population. Because these chemicals may differ in carcinogenic potency and mechanism of action, associations from these epidemiological studies can hardly be considered to have a cause–effect implication; thus, analyses of overall organochlorine usage may confound the true chemical-specific effects (Purdue et al. 2007).

Population studies, in which specific pesticide levels are analyzed in plasma and assessed for cancer incidence, also provided conflicting data. In some of these, an association was found between β-HCH or γ-HCH levels and increased risk of NHL (Steinmetz et al. 1996), whereas in others, no such association existed (Cocco and Brennan 2008). In view of the conflicting data, it is clear that prospective epidemiologic

studies, supported by detailed information on individual exposure to specific HCH pesticides are needed to address whether specific compounds are really carcinogenic to humans or not.

HCHs also target the reproductive system. Earlier studies in laboratory animals showed that treatment with HCHs decreased sperm number and induced spermatozoa damage and anomalies (Samanta et al. 1999a, b). These effects may be associated with the decrease in pregnancy rate detected in exposed animals (Beard et al. 1999). Animal studies with γ-HCH have shown that exposure to this chemical is linked to impairment of female fertility and implantation (Tiemann 2008), as well as with induction of reproductive abnormalities in male rats (Pages et al. 2002). In fact, lindane has been proposed as an endocrine disruptor, in that it produces both estrogenic and antiestrogenic effects in the rat, affecting testicular, ovarian and adrenal hormone synthesis in vivo and in vitro (Chowdhury and Gautam 1994; Oskarsson et al. 2006; Pages et al. 2002; Ronco et al. 2001). Endocrine-disruptors are complex environmental toxicants that mimic natural hormones such as estrogen and testosterone, interact with the body's endocrine system to exert their toxic effects, and interfere with normal proliferation and differentiation of target cells (Saradha and Mathur 2006).

Toxicants that affect testicular function have been shown to target intercellular junctions by either reducing the amount, or inducing aberrant intracellular localization of membranous proteins. In the SerW3 Sertoli cell line, some of these proteins are occludin, zonula occludens-1, N-cadherin, and gap connexin 43 (Fiorini et al. 2004). In this cell line, lindane has been shown to affect both gap and tight junction proteins, as it also does in the 42GPA9 Sertoli cell line (Defamie et al. 2001). In addition to endocrine disruption, defective sperm function and male infertility have been also associated with increased production of reactive oxygen species (ROS) to which spermatozoa are very susceptible. Lindane is one of the many contaminants known to disturb the pro-oxidant/antioxidant balance; in fact, exposure to oral lindane has been shown to induce oxidative stress and impair steroidogenesis in adult rat testis (Sujatha et al. 2001). Specifically, in adult rats this compound decreases the levels of antioxidant enzymes in the epididymis and epididymal sperm, contributing to an increase in oxidative stress (Chitra and Sujatha 2001). Thus, adverse effects of lindane on male reproduction may involve a combination of endocrine-mediated and ROS-mediated mechanisms. In support of this conclusion, it has been observed in pregnant rat myometrium in vitro that lindane-induced inhibition of gap junctional intercellular communication (GJIC) is mediated by an oxidative stress-dependent mechanism (Krieger and Loch-Caruso 2001).

5 Cell Signaling Induced by HCH

It has been suggested that one of the primary actions of HCH isomers is to disrupt plasma membrane architecture, thereby affecting cell viability (Bhalla and Agrawal 1998; Verma et al. 1992). However, as presented here, there is considerable evidence that supports more specific mechanisms of bioactivity and/or toxicity.

For example, during cell exposure to HCHs, several second messengers are elicited and enzymes are activated or released. Although it seems likely that the HCHs target many membrane-bound proteins, the number of biomarkers involve many other subcellular fractions. Some examples of reported enzymes that are changed in their expression/activity upon treatment with technical or individual HCH isomers are presented in Table 2.

In addition to changes in metabolic or biochemical markers, HCH isomers can differentially activate signal transduction pathways by several means: direct receptor activation/inactivation or via calcium homeostasis, phospholipases, and protein kinases, among others. Some of the most common of these are addressed below.

5.1 Gamma-Aminobutyric Acid Channel Function

GABA (γ-aminobutyric acid) is the main inhibitory neurotransmitter in the central nervous system. The inhibitory action of GABA is mediated by receptors present on the cell membrane and results in a reduction of neuronal excitability. Among the several organochlorine compounds that interact with the GABA receptor, HCHs have been the most extensively studied (Bloomquist 2003; Pomes et al. 1994). The great versatility of HCH isomers as molecular probes was documented in a paper by Woodward et al (1992). Using GABA channels expressed in *Xenopus* oocytes, two chloride currents were pharmacologically and electrically characterized: one mediated by $GABA_A$ receptors, that are ligand-gated chloride channels, and the other by atypical GABA receptors that are resistant to the channel blocker bicuculline, but not activated by the GABA agonist baclofen. The membrane current responses modulated by cerebral cortex $GABA_A$ receptors were suppressed by γ-HCH in a similar manner as was observed for picrotoxin, a $GABA_A$ channel blocker. This suggests that γ-HCH acts as a $GABA_A$ channel blocker. In contrast to γ-HCH, both α- and δ-HCH acted to potentiate GABA-activated currents. γ-HCCH interacted with the GABA channel in close proximity to the picrotoxin binding site; however, both α- and δ-HCH also were found to bind to this site, but with less potency than that expressed by γ-HCH. Interestingly, according to these results, it seems that the picrotoxin binding site may mediate both inhibitory and enhancement effects.

Different experiments have shown that the activities of HCHs on $GABA_A$ receptors may result from electrostatic interactions. For instance, the replacement of a transmembrane isoleucine residue by serine in the rho 1 subunit of $GABA_A$ receptors results in increases of up to tenfold the effect of δ-HCH on GABA responses (Belelli et al. 1999). The mechanism of this interaction is believed to result from the fact that δ-HCH acts as a positive allosteric modulator, and that γ-HCH acts as a noncompetitive antagonist of the $GABA_A$ receptor (Vale et al. 1997). Recently, Law and Lightstone (2008) have identified the potential binding sites of γ-HCH and β-HCH to the $GABA_A$ receptor, whose binding modes suggest a noncompetitive allosteric mechanism that is based on interruption of the channel gating mechanism,

Table 2 Effects of HCH stereoisomers on enzyme function

HCH isomer	Enzyme	Remarks	References
α- and γ-HCHs	↑ Cytochrome P-450 levels and superoxide dismutase activity	Rat liver	Barros et al. (1991)
α-HCH	↑ Catalase	Increased levels in rat liver	Barros et al. (1991)
α-HCH	↑ Cytidine triphosphate synthetase	In vitro. Liver cytosols	Seifert et al. (1978)
α-HCH	↑ CYP2B1, microsomal epoxide hydrolase, UDP-glucoronyltransferase, and glutathione S-transferase	In vivo. *Rat liver*	Lubet et al. (1992)
δ-HCH	↓ PI: inositol exchange enzyme ↓ 1,2-diacyl-sn-glycerol cholinephosphotransferase (75%) ↓ Brain and erythrocyte (Na^+, K^+)-ATPase (87 and 70%) ↓ Brain and erythrocyte Mg^{2+}-ATPase (38 and −5%) ↓ Brain 1,2-diacyl-sn-glycerol kinase (22%) ↓ Liver glucose 6-phosphatase (16%)	The inhibition pattern of different HCCH stereoisomers was similar to the their saturation level in phospholipid vesicles (δ>γ>α>β)	Parries and Hokin-Neaverson (1985)
β, γ-HCH	↑ Serum aminotransferases ↑ Hepatic glucose-6-phosphate dehydrogenase and aldolase activities. ↓ Liver glucose-6-phosphatase activity ↓ Liver microsomal Na^+, K^+-ATPases	Albino rats were fed with 800 ppm of both HCHs for 2 weeks inducing a higher rate of glucose oxidation and inactivation of gluconeogenesis in liver	Srinivasan and Radhakrishnamurty (1988)
γ-HCH	↓ Ca^{2+}-ATPase	Membrane bound	Janik and Wolf (1992)
γ-HCH	↓ Mg^{2+}-ATPase	Microsomal fraction of bovine endometrial cells. After a preincubation time of 30 min at 64 μM	Tiemann and Küchenmeister (1999)
HCH	↓ Na^+, K^+-ATPase, and acetylcholinesterase	In vivo. Rat red blood cells	Bhalla and Agrawal (1998)
γ-HCH	↑ Delta-aminolaevulinate synthase	Translational level. Porphyrinogen action, key enzyme in the synthesis of heme	Taira and San Martín de Viale (1998)
γ-HCH	↓ Enzymes involved in lipogenesis. Fatty acid synthase, citrate cleavage enzyme, malic enzyme, glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase	In vivo. Livers of 35-day-old male Wistar rats. Effect within 24 h of treatment. Transient decrease and recovery despite continuation of lindane feeding	Boll et al. (1995)

(continued)

Table 2 (continued)

HCH isomer	Enzyme	Remarks	References
γ-HCH	↓ Maltase activity	Rat Jejunum. Effect depends on dose and treatment duration	Moreno et al. (1996a)
γ-HCH	↓ Na^+-K^+-ATPase	Decrease intestinal basolateral enzyme activity. In situ treatment	Moreno et al. (1996b)
γ-HCH	↑ Oxygen free radical scavenging enzymes	Blood from poisoned people with several pesticides, including lindane	Banerjee et al. (1999)
γ-HCH	↑ Hepatic myeloperoxidase activity in rats (1.1-fold)	6.7-fold by lindane combined with iron. Iron alone (2.1-fold)	Junge et al. (2001)
γ-HCH	↑ Superoxide dismutase (SOD)	Red blood cells	Koner et al. (1998)
γ-HCH	↑ Activity of CYP-dependent 7-ethoxyresorufin-O-deethylase (EROD), 7-pentoxyresorufin-O-dealkylase (PROD) and N-nitrosodimethylamine demethylase (NDMA-d)	Brain and liver of offspring from pregnant Wistar rats exposed to low doses of lindane from gestation days 5 to 21	Johri et al. (2007)
γ–HXH	↓ Activity of SOD and Catalase, Cytochrome P450	In vivo. Rat liver. Reversible	Junqueira et al. (1997)
γ-HCH	↑ Cyclooxygenase 2	In vitro. Kupffer cells. Transcriptional and translational level	Kroll et al. (1999)
γ-HCH	↓ Gamma glutamyl transpeptidase	↓ Lymphoid system-rat thymus and macrophages	Koner et al. (1997)
		Rat thymus and macrophages	Chandar and Nagarajan (1984)
γ-HCH	↑ CYP1A1, CYP2B, and CYP3A7	In vitro. Fetal rat hepatocytes, quail hepatocytes, and Hep G2 cells, respectively	Dubois et al. (1996)
γ-HCH	↓ Hydroxysteroid sulfotransferase-a and aryl-sulfotransferase IV	Xenobiotic metabolism enzymes. Suppressed mRNA levels	Runge-Morris (1998)
γ-HCH	↑ Ornithine decarboxylase	Membrane-bound enzyme upon translocation. Increase in both mRNA and enzyme activity. Co-induction with c-fos. Changes were diminished by animal pretreatment with diazepan	Vendrell et al. (1991)
γ-HCH	↑ Nitroreductase	Effect observed in the rat small intestine after treatment for 5 weeks	Chadwick et al. (1990)

γ-HCH	↑ Dimethylnitrosamine demethylase I and II	Uninfected mice and in Schistosoma mansoni-infected animals	Mostafa et al. (1984)
γ-HCH	↑ Aminopyrine N-demethylase, NADPH-cytochrome c reductase, glutathione S-transferases and Haem oxygenase	Rat liver. Lindane increased the hepatic content of cytochrome P450	Puri and Kohli (1995)
γ-HCH	↑ NADPH-Cytochrome P450 reductase	Effect observed only in rat liver microsomes from hyperthyroid animals	Simon Giavarotti et al. (1998)
γ-HCH	↓ 3-Hydroxy-3-methylglutaryl coenzyme A reductase	In vivo. Rat liver. Decrease in the mRNA level	Jenke et al. (1988)
γ-HCH	↓ Acid phosphatase	Decreased enzyme activity In vitro. Mouse peritoneal macrophages. Concentrations greater than 10^{-4} M or after treatment of more than 12 h	Roux et al. (1978)
γ-HCH	↓ Benzo(a)pyrene hydroxylase	Decreased enzyme activity *In vivo*. Lungs of rats. Enzyme activity tended to return to normal 5 days after treatment	Khan et al. (1993)
γ-HCH	↓ Testicular hyaluronidase and 3-beta delta. 5-hydroxysteroid dehydrogenase	Decreased rat testicular enzyme activity In vivo	Chowdhury and Gautam (1994)
γ-HCH	↑ N-acetyltransferase	Increase the nighttime rise in pineal N-acetyltransferase (NAT) activity	Attia et al. (1991)
HCH	↓ Se-dependent and Se-independent glutathione peroxidase and catalase	In vivo. Cerebral hemisphere of rat	Sahoo and Chainy (1998)
HCH	↑ Gamma glutamyl transpeptidase	Rat liver	Chandar and Nagarajan (1984)
HCH	↑ Total testicular Ca^{2+}-Mg^{2+}-ATPase	Enzyme activity. Rats treated during critical stages of testicular development (6–30th day)	Samanta et al. (1999a)
HCH	↓ Cytosolic superoxide dismutase and catalase ↑ Glutathione peroxidase	Enzyme activity. Testis of both immature (15-day-old) and mature (90-day-old) rats	Samanta et al. (1999b)
HCH	↓ Mg^{2+}-ATPase, Na^{+},K^{+}-ATPase, and acetyl cholinesterase	Inhibited cerebral rat enzyme activity	Sahoo et al. (1999)

rather than directly blocking the channel. In addition, these authors found that the binding to γ-HCH (lindane) is more favorable than to β-HCH.

Effects of HCH isomers on GABA receptor–chlorine channel complexes have been observed in rat dorsal root ganglion neurons (Nagata and Narahashi 1995). Both γ-HCH and δ-HCH, when co-applied with GABA, acted in a biphasic manner, first slightly enhancing and then suppressing the GABA-induced chloride current. δ-HCH was more efficacious than γ-HCH during the last phase. The effect necessarily implies that these isomers bind to separate sites or act by different mechanisms. Because suppression of the current occurred slowly, it is supposed that the inhibitory effect may happen through an interaction with an intracellular component. The authors speculated that two mechanisms may be involved: alterations of the desensitization kinetics of GABA-induced current by action of protein kinases, or the presence of conformational changes induced by those compounds in the GABA receptor. In hippocampal slices, γ-HCH antagonized recurrent collateral $GABA_A$-mediated inhibition, altered the input/output coupling of excitatory input into pyramidal cells, and reversed the effects of propofol (Albertson et al. 1997; Joy et al. 1995), an anesthetic that potentiates $GABA_A$-mediated inhibition by increasing the probability of the $GABA_A$ receptor-chloride channel being open (Kitamura et al. 2004).

5.2 Calcium Homeostasis

HCH isomers alter Ca^{2+} movement through the cell membrane. α, β, and γ-HCH increased intracellular calcium concentrations ($[Ca^{2+}]_i$) in neurohybridoma cells with the following order of potency: $\alpha<\beta<\gamma$. This effect was seen at concentrations greater than those effective in antagonizing GABA-mediated inhibition (Joy and Burns 1988). In the case of γ-HCH, the increase in Ca^{2+}-induced neurotransmitter release from synaptosomes occurs through a mechanism involving an increase in the nonspecific synaptic membrane permeability to Ca^{2+} (Hawkinson et al. 1989). Both the δ- and γ-isomers of HCH-stimulated Ca^{2+} influx through voltage-gated Ca^{2+} channels (Rosa et al. 1997a). Dantrolene, a ryanodine receptor antagonist that inhibits Ca^{2+} release from sarcoplasmic reticulum and from unidentified internal Ca^{2+} stores in neurons, inhibits the intracellular Ca^{2+} release promoted by γ-HCH in cerebellar granule cells, but it is almost inactive against the same effect induced by δ-HCH in the same preparation. This behavior demonstrates that δ- and γ-HCH act on different receptors. γ-HCH affected Ca^{2+}-dependent, dantrolene-sensitive Ca^{2+} pools, whereas δ-HCH seemed to stimulate Ca^{2+}-release from nonspecific ryanodine-gated receptors (Rosa et al. 1997b). In the central nervous system, γ-HCH also induced $[Ca^{2+}]_i$ entry through voltage-dependent Ca^{2+} channels. A consequence of the increase in $[Ca^{2+}]_i$ is the increase in the expression of the proto-oncogene c-fos and the nuclear calmodulin concentrations (Vendrell et al. 1992). Conversely, δ-HCH significantly decreased c-fos basal levels in primary cortical cultures (Barron et al. 1995).

In human neutrophils, α, δ, and γ-HCH increase $[Ca^{2+}]_i$. In the case of γ-HCH, this arises from an intracellular source, the same pool as that released by the

chemotactic peptide formyl-methionyl-leucyl-phenylalanine (fMLP) (Kuhns et al. 1986). One mechanism by which fMLP releases intracellular calcium in neutrophils is through the activation of phospholipase C (PLC), with the subsequent generation of inositol 1,4,5-trisphosphate (IP_3), which then acts on an IP_3 receptor to release Ca^{2+}. The release of intracellular Ca^{2+} and the induction of capacitative Ca^{2+} entry by γ-HCH seem to be a common event in other cell types such as kidney cells (Lu et al. 2000). Interestingly, also in neutrophils, γ-HCH-induced increases in $[Ca^{2+}]_i$ were blocked by the Ca^{2+} antagonists nifedipine, verapamil, and diltiazem (Grigorian et al. 1988), suggesting that part of the increases in $[Ca^{2+}]_i$ depend on Ca^{2+} influx.

In rat atrial strips, δ-HCH is 30-fold more potent as a positive inotropic compound than is lindane (Pessah et al. 1992). Buck and Pessah (1999) have suggested that δ-HCH increases ionic permeability ($K^+ \gg Cs^+ > Na^+$) through Ca^{2+}-dependent mechanisms. One mechanism is through ryanodine receptors and the other acts through an ionophore-type mechanism. On the other hand, γ-HCH has been shown to inhibit Ca^{2+}- K^+-ATPases in the heart (Anand et al. 1995). These mechanisms may account for the toxic effects of HCHs in the cardiovascular system.

Structurally, the HCHs resemble inositol analogs. For instance, muco- and myo-inositol have the stereochemical configuration of γ- and δ-isomers of HCHs, respectively. δ-HCH did not compete with IP_3 for the IP_3 receptor, demonstrating that the δ-HCH-induced release of Ca^{2+} from IP_3-sensitive Ca^{2+} stores does not result from its similarity to myo-inositol-1,4,5-trisphosphate (myo-IP_3) (Mohr et al. 1995). In addition, δ-HCH interacts directly with the ryanodine receptor of cardiac sarcoplasmic reticulum (Buck et al. 1999; Pessah et al. 1992). It is interesting that this isomer also blocks Ca^{2+} influx. A question arises: Is there a specific unidentified binding site for δ-HCH in the IP_3-sensitive Ca^{2+} channels?

In summary, three of the most common isomers of HCH, α-, δ-, and γ-HCH, increase $[Ca^{2+}]_i$ in a variety of systems. Neither the mechanisms for these effects nor the molecular features are common to all of them. These observations suggest that there are particular electronic and geometrical molecular features that induce the specific effects of the HCHs.

5.3 Phosphoinositide Turnover

Phosphatidylinositol-4,5-bisphosphate (PIP_2) is a phospholipid located on the inner leaflet of plasma membranes. It is the source of active second messengers such as 1,2-diacylglycerol (DAG) and D-myo-IP_3 which play important roles in the activation of many cellular signaling pathways, functioning as second messengers for Protein Kinase C (PKC) activation and intracellular Ca^{2+} mobilization, respectively (Bishop and Pachter 1992). It has been shown that lindane stimulates the release of IP_3 from myometrial cells (Criswell et al. 1995). Lindane also induced the accumulation of *myo*-[2-^{3}H]inositol in rat brain cortex slices independently from stimulation of muscarinic and α_1-adrenergic agonists (Pajuelo et al. 1997). In rats exposed to HCHs for 3–6 months, phosphatidylinositol, phosphatidylinositol 4-phosphate,

and phosphatidylinositol 4,5-biphosphate were decreased in the erythrocyte membrane and cerebrum (Agrawal and Afaq 1995). In addition, γ-HCH reduced the incorporation of myo-[2-^{3}H] inositol into phosphoinositides that had better specificity, when compared to α, β, or δ-HCH (Carrero et al. 1996). Although a significant effect was observed at 100 μM γ-HCH, the data were evaluated at 500 μM, certainly a dose too large to establish a physiological relevance from those experiments. Because ethylenediaminetetraacetic acid (EDTA) prevented the effects of γ-HCH on Phosphoinositide (PI) synthesis, it was hypothesized that lindane enhances phosphatidylinositol synthesis by increasing intracellular Ca^{2+}. For instance, in macrophages, lindane directly triggers Ca^{2+} influx (Pinelli et al. 1994), and this Ca^{2+} can, in turn, activate a phospholipase C (PLC) to generate IP_3.

5.4 Activation of Phospholipases

In addition to activation of PLC, HCHs increase the activity of phospholipase A_2 (PLA_2). Specifically, it has been shown that the α-, δ-, and γ-isomers of HCH, but not the β-isomer, induced activation of PLA_2 in neutrophils (Olivero et al. 2002). The authors showed that an electrotopological motif shared by all the active compounds was required for the activation of the PLA_2, which consisted of a planar hydrophobic domain connected rigidly at a perpendicular angle to a halogen atom.

Using a rat renal tubular cell system, López-Aparicio et al. (1995) showed that, at concentrations larger than 50 μM, lindane activated a PLA_2 that prefers phosphatidylinositol rather than phosphatidylcholine as a substrate. PLA_2 activity in both soluble and membrane fractions was not modified by lindane (30–300 μM) over a 120 min period, suggesting that γ-HCH does not activate this enzyme directly but that other intracellular messengers play a role in PLA_2 activation. This activation of PLA_2, which releases arachidonic acid from cell membranes, may lead to changes in membrane permeability that result in cell injury observed by γ-HCH. Consistent with activation of PLA_2, γ-HCH induced release of glycerophosphoinositol and arachidonic acid from phospholipids in rat proximal tubular cell cultures (Senar et al. 1994). In macrophages, γ-HCH increased the production of arachidonic acid metabolites such as 6-ketoprostaglandin F1-alpha, prostaglandin E_2, leukotriene C_4, leukotriene B_4, and hydroxyeicosatetraenoic acid (Forgue et al. 1990).

5.5 Oxidative Stress

The HCHs produce reactive oxygen species in a number of tissues and cell types, and this effect appears to be structure dependent. For example, in neutrophils, α-, γ-, and δ-HCH, but not the β-isomer, are potent stimuli for superoxide anion production (Kuhns et al. 1986; Tithof et al. 2000). It has been reported that γ-HCH induces formation of reactive oxygen species from rat peritoneal macrophages (Bagchi et al. 1995) and liver (Barros et al. 1991; Hassoun et al. 1993), leading to enhanced lipid peroxidation and DNA damage. This has been suggested as a putative mechanism

for the carcinogenesis-promoting activity of this HCH isomer (Perocco et al. 1995). In addition, γ-HCH induces lipid peroxidation and increases superoxide dismutase activity in red blood cells (Banerjee et al. 1999). This effect is attenuated by simultaneous treatment with ascorbic acid (Koner et al. 1998).

5.6 Other Actions of HCH

Other actions of the HCHs have been reported. γ-HCH decreases, in a dose-dependent manner, the effect of forskolin, a direct activator of the adenylate cyclase catalytic subunit. This effect is isomer-specific since δ-HCH is indeed highly potent, whereas α- and β-HCH are poorly efficient on the inhibition of forskolin-dependent stimulation of adenylate cyclase activity (Carrero et al. 1999).

Although the diacylglycerol product resulting from PLC enzymatic action can activate protein kinase C (PKC), it has been demonstrated that lindane directly stimulates PKC activity (Bagchi et al. 1997; Moser and Smart 1989). This PKC activation by lindane would lead to phosphorylation of the adenylate cyclase catalytic subunit, inducing its activation (Carrero et al. 1999).

HCH isomers are not chemotaxins, compounds that attract phagocytes; however, δ- and γ-HCH inhibit chemotaxis, the process of recruiting these cells (Kaplan et al. 1988). Finally, ^{1}H NMR (Proton Nuclear Magnetic Resonance) studies revealed that γ-HCH disturbs the glycine synthesis pathway in ways that involve methionine, choline, and betaine (Descampiaux et al. 1997).

5.6.1 Effects on DNA Integrity

α-, β-, and γ-HCH increase hepatic DNA synthesis in liver and liver growth in a process associated with foci expansion and tumor promotion (Schroter et al. 1987). α-HCH acts as a liver promoter and induces nonbinucleating hepatocyte growth (Seglen 1997). The stimulation of DNA synthesis by α-HCH is independent of extracellular Ca^{2+} (Petronijevic and Edwards 1993) and has been suggested to be dependent on the activation of Na(+)/H(+) antiport activity (Lee et al. 2003). α-HCH also causes DNA breaks in rat and human hepatocytes, an effect explained with the assumption that α-HCH elicits short-path DNA repair (Mattioli et al. 1996). In male mice, α-HCH inhibits hepatic tumorigenesis, but in female mice, this isomer promotes hepatic tumor formation following chronic exposure (Siglin et al. 1995). γ-HCH also induces rat liver histone modifications (Sarkander et al. 1974), whereas β-HCH increases the risk of breast cancer slightly but not significantly (Hoyer et al. 1998).

5.6.2 Gap Junctional Communication

Gap junctions (GJs) make possible direct cell–cell communication through membrane channels that allow the diffusion of intracellular contents between cells.

GJs facilitate electrical and metabolic coupling of cells. Gap junctional intercellular communication (GJIC) is important for cell proliferation and differentiation as well as tissue homeostasis (Loewenstein 1979). Loss of GJ function is involved in neoplastic transformation and abnormal growth regulation. For example, it has been suggested that the promoting activity induced by γ-HCH in BALB/c 3T3 mice cells is due to dysfunction of the GJs that disrupt cell growth regulation (Perocco et al. 1995).

γ-HCH reduces GJ number, permeability, and expression. These effects are time- and treatment duration-dependent (Guan et al. 1995). γ-HCH-dependent loss of GJ in epithelial cells (WB-F344) results from endocytosis of the plaques and degradation of the GJ protein connexin 43-P2 (Cx43-P2), rather than from disaggregation of functional particles (Guan and Ruch 1996). This induced effect by γ-HCH is also seen in a Sertoli cell line (42GPA9). The process occurs in a time-dependent decrease and redistribution of Cx43 and ZO-1, a tight junction component associated with Cx43, from the membrane to the cytoplasmic perinuclear region. However, this does not occur for occludin, an integral tight junction protein (Defamie et al. 2001).

The signal transduction mechanisms involved in HCH-induced inhibition of GJ intercommunication have been studied. Although γ-HCH produces a concentration-dependent increase in cyclic AMP (cAMP), inhibition of adenylate cyclase does not reverse γ-HCH-induced loss of GJ intercellular communication, suggesting that the inhibition of GJ is unlikely to be related to increases in cAMP. Activation of a phospholipase that liberates arachidonic acid may be involved because the use of arachidonic acid-free conditions blocks the inhibition produced by γ-HCH (Criswell et al. 1995). This may be an effect of arachidonic acid rather than its prostaglandin or leukotriene metabolites, because the cyclooxygenase inhibitors, indomethacin and aspirin do not reduce the γ-HCH-mediated inhibition of GJ communication (Leibold and Schwarz 1993). Interestingly, vitamin E, a well-known free radical scavenger (Ikeda et al. 1999), prevents inhibition of GJ communication, suggesting a role for free radicals (Leibold and Schwarz 1993). This oxidative stress-dependent mechanism of lindane-induced inhibition of GJIC has also been observed in pregnant rat myometrium in vitro (Krieger and Loch-Caruso 2001). γ-HCH-induced inhibition of GJIC does not result from an increase in $[Ca^{2+}]_i$, as has been shown for the organochlorine insecticide 1,1,1-trichloro-2,2-bis(p-chlorophenyl)ethane (DDT) (Fransson et al. 1990), but rather is partly dependent on PKC activation (Criswell and Loch-Caruso 1995). Thus, effects of γ-HCH on GJ communication may be mediated through pathways involving arachidonic acid, PKC, and free radicals.

GJIC is known to play a critical role in the control of cell growth, and its disregulation constitutes a hallmark of tumor malignancy. Mograbi et al. (2003) have reported that γ-HCH impairs GJIC by promoting the intracellular localization of Connexin 43 (Cx43), which is considered to be a tumor suppressor. The mechanism involved endocytosis with no alteration of Cx43 partition in lipid rafts, and it was accompanied by Cx43 phosphorylation and activation of extracellular signal-regulated kinases (ERK), but not of Jun N-terminal kinase (JNK) and p38 mitogen-activated protein kinases. The authors suggested that this altered activation of the mitogenic MAPK pathway, and inactivation of the tumor suppressor Cx43 by γ-HCH, may participate in the promotion of neoplastic cell growth.

5.6.3 Other Effects of HCHs on the Neuronal System

In addition to the effects that lindane has on GABA receptors, other receptor channel activation possibilities have been explored. For instance, Zisterer et al. (1995) showed that PK11195, a ligand of the peripheral-type benzodiazepine receptor, reduced the convulsions induced by γ-HCH. It was concluded that the anticonvulsive protection was not from the action of PK11195 in the central nervous system but was the result of the effect on the gastrointestinal tract to delay absorption of γ-HCH into the bloodstream. Other authors have suggested that the cytotoxic action of γ-HCH in neurocortical neurons is mediated by the interaction with allosterically coupled GABA-benzodiazepine recognition sites at the $GABA_A$ receptor (Vale et al. 1997, 1998).

HCHs also affect other subcellular components of the neuronal system. Because (+)MK-801, a blocker of the NMDA-operated cation channel, antagonizes γ-HCH-induced seizures, it has been suggested that γ-HCH acts on these channels (Tusell et al. 1992). γ-HCH increases and δ-HCH decreases the Ca^{2+}-dependent K^+-evoked release of ^{3}H-noradrenaline, whereas α- and β-HCH did not produce any effect (Cristofol and Rodriguez 1991, 1993). This property has been shown to be dependent on changes of Cl^- membrane permeability and intracellular Ca^{2+} homeostasis (Cristofol and Rodriguez 1994).

5.7 HCHs and Estrogenicity

One of the most intriguing aspects of the HCHs has been their identification as possible estrogen-like chemicals (Roy et al. 1997). For example, γ-HCH has weak estrogenic activity in rats (Raizada et al. 1980), and the HCHs decrease serum testosterone levels, epididymal sperm count, and sperm motility (Prasad et al. 1995). Estrogen increases uterine weights in ovariectomized rodents by increasing protein synthesis and promoting both cellular hyperplasia and hypertrophy. Estrogen-like activity of β-HCH has been evaluated, and has shown increases in uterine weights (Bigsby et al. 1997). This compound produces an uterotrophic response (Van Velsen et al. 1986), suggesting that it is a weak estrogen. When the responses between animals with active (fasted) and inactive (fed) lipolysis were compared, the response was greater in fasted animals compared to those receiving food. Although it was concluded that this difference resulted from a release of β-HCH during lipolysis, the observed effect could have been the result of changes in metabolic pathways leading to an increase in estrogen precursors or any other uterotrophic endogenous mediator. These data are consistent with the notion that β-HCH-mediated stimulation of cell proliferation and gene expression is estrogen-receptor (ER) dependent, but its action is not through the classic pathway of binding and activating the ER. β-HCH may represent a new class of xenobiotic chemicals that produces estrogen-like effects through nonclassic mechanisms and, therefore, may be of concern with regard to breast and uterine cancer risk.

One of the most important effects of HCHs isomers is the reduction in hormone production and other related reproductive disorders. It has been observed that

γ-HCH affects steroidogenesis in mice by inhibiting the cholesterol side-chain cleavage activity of the ovary, the rate-limiting step in steroid biosynthesis in ovarian tissues. This blocking avoids the conversion of cholesterol to pregnenolone and subsequently to progesterone (Sircar and Lahiri 1990). It has been shown that γ-, α-, and β-HCH inhibit steroidogenesis in an adrenocortical cell line. However, only γ-HCH binds to the peripheral-type benzodiazepine-binding site, which is thought to regulate the rate-limiting step in steroidogenesis (Zisterer et al. 1996).

Walsh and Stocco (2000) reported that, in the mouse MA-10 Leydig tumor cell line, α-, δ-, and γ-HCH inhibit steroidogenesis by reducing the expression of the steroidogenic acute regulatory (StAR) protein, that mediates the intramitochondrial transfer of cholesterol to the side-chain cleavage cytochrome P450. Interestingly, Leydig cells from mature male rats incubated with human chorionic gonadotropin and exposed to γ-HCH showed a dose-dependent inhibition of testosterone production, process accompanied with a half-reduced LH/hCG receptor (luteinizing hormone/human chorionic gonadotrophin receptor) number and a decrease in cAMP production (Ronco et al. 2001).

In vitro studies with human cell lines have shown evidence that γ-HCH and β-HCH have weak estrogenic properties, which elevates concern that these chemicals may act as tumor promoters through hormonally mediated effects (Wong and Matsumura 2006) or through protein kinase activation (Enan and Matsumura 1998). Ratneswaran et al. (1997) tested the possible estrogenic and antiestrogenic activity of γ-HCH by measuring its ability to induce or prevent hepatic expression of Estrogen-Regulated mRNA Stabilizing Factor (E-RmRNASF), in response to estrogen exposure. The expression of this protein was quantified by detecting its ability to prevent the endonucleolytic degradation of apoII mRNA in vitro, in cell-free liver cytosolic extracts. Results were that γ-HCH failed to show estrogenic or antiestrogenic activity. More recently, it has been postulated that γ-HCH can be considered as an endocrine disruptor, because it modulates female reproductive development through direct interaction with ER-β (Maranghi et al. 2007).

It has been speculated that organochlorine compounds induce breast cancer through their action as estrogen agonists. Although different mechanisms have been proposed to define how estrogen-like compounds produce cancer, data are currently inconclusive. β-HCH stimulated proliferation in a dose-dependent manner in the ER-positive cell lines MCF-7 and T47D but not in the ER-negative lines MDA-MB231, MDA-MB468, and HS578T. This isomer also caused an increase in the steady-state level of pS2 mRNA in MCF-7 cells. These responses were equal in magnitude to the maximal effect of estradiol, and they were inhibited by inclusion of the antiestrogen ICI164384. However, when tested in a competitive binding assay, β-HCH did not displace 17-beta-[^{3}H]estradiol from the ER even at a concentration that was 40,000-fold higher than the tracer steroid (Steinmetz et al. 1996). These authors suggested that β-HCH stimulation of cell proliferation and gene expression is ER dependent, but its action is not through the classic pathway of binding and activating the ER. β-HCH may represent a new class of xenobiotic that produces estrogen-like effects through nonclassical mechanisms and, therefore, may be of concern with regard to breast and uterine cancer risk.

6 Summary

The hexachlorocyclohexanes (HCHs) are synthetic compounds that have been widely used for the control of pests. The most common HCH isomers are the α-, β, δ-, and γ-HCH. Although they have the same chlorine substitution pattern, the spatial orientation of chlorine atoms is different on each one of them, resulting in unique structures that have distinct molecular properties. Humans are exposed to individual HCH isomers through various routes, including ingestion of contaminated water or food, absorbed through the skin or by inhalation, and because of their liposolubility, these chemicals are mostly stored in fat-containing tissues. The isomer-specific spectrum of biochemical actions for these compounds has been well characterized for different endpoints such as enzyme activation, calcium homeostasis, gap junctional intercellular communication, endocrine disruption, and cancer, among others. The interaction with the GABA receptor has been one of the most extensively studied properties of the HCHs. For instance, γ-HCH acts as a $GABA_A$ channel blocker, whereas α- and δ-HCH potentiate GABA-activated currents, all working as allosteric modulators of the receptor. The changes in calcium homeostasis elicited by HCHs are both isomer and cell-type specific. For example, in neurons, both the δ- and γ-isomers of HCH stimulate Ca^{2+} influx through different voltage-gated Ca^{2+} channels. In human neutrophils, α-, δ-, and γ-HCH, but not β-HCH, increase intracellular Ca^{2+} concentrations. This isomer-dependent behavior is also similar to that observed for phospholipase A_2 activation and also correlates with oxidative stress generation. On the other hand, there are several lines of evidence suggesting that HCHs alter genomic integrity, and, therefore, these compounds have been classified as possibly carcinogenic to humans. Finally, HCHs have been reported to be endocrine disrupters. In fact, γ-and β-HCH have been shown to have weak estrogenic activity, and together with the α- and the δ-isomer, also interfere with steroidogenesis. In short, the HCH isomers are good examples of structurally related chemicals, for which the geometrical patterns present in each one of the different conformers create structures that possess specific mechanisms of action and toxicological properties.

Acknowledgments We gratefully acknowledge the University of Cartagena and the Vice-rectory for Research. Incentive Program for Research Groups. 2009–2010.

References

Addison RF, Muir DCG, Ikonomou MG, Harwood L, Smith TG (2009) Hexachlorocyclohexanes (HCH) in ringed seal (*Phoca hispida*) from Ulukhaktok (Holman), NT: Trends from 1978 to 2006. Sci Total Env 407: 5139–5146.

Agrawal D, Afaq S (1995) Influence of hexachlorocyclohexane on phosphoinositides in rat erythrocyte membranes and brain. Toxicology 95: 135–140.

Albertson T, Walby W, Stark L, Joy R (1997) The effects of lindane and long term potentiation (LPT) on pyramidal cell excitability in the rat hippocampal slice. Neurotoxicology 18: 469–478.

Anand M, Meera P, Kumar R, Gupta G, Tripathi O, Srimal R (1995) Possible role of calcium in the cardiovascular effect of prolonged administration of gamma-HCH (lindane) in rats. J Appl Toxicol 15: 245–248.

Attia AM, Richardson BA, Rodriguez C, Mostafa MH, Soliman SA, el-Sebae AH, Reiter RJ (1991) Lindane may enhance nocturnal pineal N-acetyltransferase activity via beta-adrenergic receptors. Brain Res 554:253–256.

Bagchi D, Bagchi M, Hassoun E, Stohs S, (1995) In vitro and in vivo generation of reactive oxygen species, DNA damage and lactate dehydrogenase leakage by selected pesticides. Toxicology 104: 129–140.

Bagchi D, Bagchi M, Tang L, Stohs S (1997) Comparative in vitro and in vivo protein kinase C activation by selected pesticides and transition metals salts. Toxicol Lett 91: 31–37.

Banerjee B, Seth V, Bhattacharya A, Pasha S, Chakraborty AK (1999) Biochemical effects of some pesticides on lipid peroxidation and free-radical scavengers. Toxicol Lett 107: 33–47.

Barron S, Serratosa J, Tusell J (1995). Regulation of c-fos expression by convulsants and hexachlorocyclohexane isomers in primary cultures of cortical neurons. J Neurochem 64: 1708–1714.

Barros S, Simizu K, Junqueira V (1991) Liver lipid peroxidation-related parameters after short-term administration of hexachlorocyclohexane isomers to rats. Toxicol Lett 56: 137–144.

Beard A, Bartlewski P, Rawlings N (1999) Endocrine and reproductive function in ewes exposed to the organochlorine pesticides lindane or pentachlorophenol. J Toxicol Environ Health 56: 23–46.

Belelli D, Pau D, Cabras G, Peters J, Lambert J (1999) A single aminoacid confers barbiturate sensitivity upon the GABA rho 1 receptor. Br J Pharmacol 127: 601–604.

Beurskens J, Stams A, Zehnder A, Bachmann A (1991) Relative biochemical reactivity of three hexachlorocyclohexane isomers. Ecotoxicol Environ Safety 21:128–136.

Bhalla P, Agrawal D (1998) Alterations in rat erythrocyte membrane due to hexachlorocyclohexane (technical) exposure. Hum Exp Toxicol 17: 638–642.

Bhatt P, Kumar MS, Chakrabarti T (2007) Assessment of bioremediation possibilities of technical grade hexachlorocyclohexane (tech-HCH) contaminated soils. J Hazard Mater 143: 349–53.

Bigsby R, Caparell A, Madhukar B (1997) Xenobiotics released from fat during fasting produce estrogenic effects in ovariectomized mice. Cancer Res 57: 865–869.

Bishop WR, Pachter JA (1992) Regulation of phospholipid hydrolysis and second messenger formation by protein kinase C. Adv Enzyme Regul 32: 177–192.

Blair A, Cantor K, Zahm S (1998) Non-hodgkin's lymphoma and agricultural use of the insecticide lindane. Am J Ind Med 33: 82–87.

Bloomquist JR (2003) Chloride channels as tools for developing selective insecticides. Arch Insect Biochem Physiol 54: 145–156.

Boll M, Weber LW, Stampfl A (1995) The effect of gamma-hexachlorocyclohexane (lindane) on the activities of liver lipogenic enzymes and on serum lipids in rats. Z Naturforsch C 50: 135–42.

Buck E, Pessah I (1999) Mechanisms of delta-hexachlorocyclohexane toxicity: II. Evidence for Ca^{2+}-dependent K^{+}-selective ionophore activity. J Pharmacol Exp Ther 289:486–493.

Buck E, Lachnit W, Pessah I (1999) Mechanisms of delta-hexachlorocyclohexane toxicity: I. Relationship between altered ventricular myocyte contractility and ryanodine receptor function. J Pharmacol Exp Ther 289: 477–485.

Carrero I, Recio M, Prieto, J, Perez, M (1996) Effect of g-hexachlorohexane in phosphoinositide synthesis in rat enterocytes is calcium-mediated. Pesticide Biochem Biophys 56: 79–87.

Carrero I, Rodriguez N, Guijarro L, Recio M, Perez M, Prieto J (1999) Lindane decreases forskolin-stimulated cyclic AMP accumulation but does not modify Gs in rat enterocytes. Cell Signal 5: 453–462.

Chandar N, Nagarajan B (1984) Induction of gamma-glutamyl transpeptidase by hexachlorocyclohexane. Biochem Int 8: 41–48.

Chadwick RW, Chang JJ, Gilligan PH, Forehand LR, Long JE, Duffy MC (1990) Effect of lindane on nitroreductase and dechlorinase enzyme activity in the gastrointestinal tract. Toxicol Lett 50: 299–308.

Chitra KC, Sujatha R (2001) Effect of lindane on antioxidant enzymes in epididymis and epididymal sperm of adult rats. Asian J Androl 3: 205–208.

Chowdhury AR, Gautam AK (1994) Steroidogenic impairment after lindane treatment in male rats. J UOEH 16: 145–152.

Chu S, Covaci A, Schepens P (2006) Levels and chiral signatures of persistent organochlorine pollutants in human tissues from Belgium. Environ Res 93: 167–176.

Cocco P, Brennan P (2008) Plasma polychlorobiphenyl and organochlorine pesticide level and risk of major lymphoma subtypes. Occup Environ Med 65: 132–140.

Corsolini S, Covaci A, Ademollo N, Focardi S, Schepens P (2006) Occurrence of organochlorine pesticides (OCPs) and their enantiomeric signatures, and concentrations of polybrominated diphenyl ethers (PBDEs) in the Adélie penguin food web, Antarctica Environ Pollut 140: 371–382.

Covaci A, Gheoghe A, Schepens P (2004) Distribution of organochlorine pesticides, polychlorinated biphenyls and a-HCH enantiomers in pork tissues. Chemosphere 56: 757–766.

Cristofol R, Rodriguez E (1991) Differential presynaptic effects of hexachlorocyclohexane isomers on noradrenaline release in cerebral cortex. Life Sci 49: 1111–1119.

Cristofol R, Rodriguez E (1993) Modulation of noradrenaline release from hippocampal slices by hexachlorocyclohexane isomers: Effects of GABAergic compounds. Brain Res 606: 237–243.

Cristofol R, Rodriguez F (1994) Hippocampal noradrenaline release is modulated by gamma- and delta-hexachlorocyclohexane isomers: Which mechanisms are involved? Eur J Pharmacol 252: 305–312.

Criswell K, Loch-Caruso R (1995) Lindane-induced elimination of gap junctional communication in rat uterine myocytes is mediated by an arachidonic acid-sensitive cAMP-independent mechanism. Toxicol Appl Pharmacol 135: 127–138.

Criswell K, Loch-Caruso R, Stuenkel E (1995) Lindane inhibition of gap junctional communication in myometrial myocytes is partially dependent on phosphoinositide-generated second messengers. Toxicol Appl Pharmacol 130: 280–293.

Defamie N, Mograbi B, Roger C, Cronier L, Malassine A, Brucker-Davis F, Fenichel P, Segretain D, Pointis G (2001) Disruption of gap junctional intercellular communication by lindane is associated with aberrant localization of connexin43 and zonula occludens-1 in 42GPA9 Sertoli cells. Carcinogenesis 22: 1537–1542.

Descampiaux B, Imbenotte M, Desenclos V, Vermeersch G, Lhermitte M, Erb F (1997) 1H NMR investigation of toxic effects of lindane and paraquat on Hep 3B and Hep G2 human hepatoma cell lines. Chem Res Toxicol 10: 34–40.

Díaz G, Ortiz R, Schettino B, Vega S, Gutiérrez R (2009) Organochlorine pesticides residues in bottled drinking water from Mexico City. Bull Environ Contam Toxicol 82:701–704.

Dick I, Blain P, Williams F (1997) The percutaneous absorption and skin distribution of lindane in man. Hum Exp Toxicol 16: 652–657.

Dubois M, Plaisance H, Thomé JP, Kremers P (1996) Hierarchical cluster analysis of environmental pollutants through P450 induction in cultured hepatic cells. Ecotoxicol Environ Saf 34: 205–215.

Enan E, Matsumura F (1998) Activation of c-neu tyrosine kinase by o,p'-DDT and b-HCH in cell-free and intact cell preparations from MCF-7 human breast cancer cells. J Biochem Mol Toxicol 12: 83–92.

English D, Schell M, Siakotos A, Gabig T (1986) Reversible activation of the neutrophil superoxide generating system by hexachlorocyclohexane: Correlation with effects on a subcellular superoxide-generating fraction. J Immunol 137: 283–290.

Fenster L, Eskenazi B, Anderson M, Bradman A, Harley K, Hernandez H, Hubbard, A, Barr DB (2006) Association of in utero organochlorine pesticide exposure and fetal growth and length of gestation in an agricultural population. Environ Health Perspect 114: 597–602.

Fiorini C, Tilloy-Ellul A, Chevalier S, Charuel C, Pointis G (2004) Sertoli cell junctional proteins as early targets for different classes of reproductive toxicants. Reprod Toxicol 18: 413–421.

Forgue M, Pinelli E, Beraud M, Souqual M, Pipy B (1990) Chemiluminiscence response and arachidonic acid metabolism of macrophages induced by g-hexachlorocyclohexane (lindane). Food Addit Contam 7: 97–99.

Fransson R, Nicotera P, Warngard, L, Ahlborg U (1990) Changes in cytosolic Ca^{2+} are not involved in DDT-induced loss of gap junctional communication in WB-F344 cells. Cell Biol Toxicol 6: 235–244.

Grigorian M, Akopov S, Gabrielian, E (1988) Regulation by the protein kinase C activator phorbol ester of calcium channels in polymorphonuclear leukocytes. Biokhimiia 53: 1462–1466.

Guan X, Ruch R (1996) Gap junction endocytosis and lysosomal degradation of connexin43-P2 in WB-F344 rat liver epithelial cells treated with DDT and lindane. Carcinogenesis 17: 1791–1798.

Guan X, Bonney W, Ruch R (1995) Changes in gap junction permeability, gap junction number, and connexin43 expression in lindane-treated rat liver epithelial cells. Toxicol Appl Pharmacol 130: 79–86.

Guler GO, Cakmak YS, Dagli Z, Aktumsek A, Ozparlak H (2010) Organochlorine pesticide residues in wheat from Konya region, Turkey. Food and Chemical Toxicology 48: 1218–1221.

Haraguchi K, Koizumi A, Inoue K, Harada KH, Hitomi T, Minata M, Tanabe M, Kato Y, Nishimura E, Yamamoto Y, Watanabe T, Takenaka K, Uehara S, Yang HR, Kim MY, Moon CS, Kim HS, Wang P, Liu A, Hung NN (2009) Levels and regional trends of persistent organochlorines and polybrominated diphenyl ethers in Asian breast milk demonstrate POPs signatures unique to individual countries. Environ Int 35: 1072–1079.

Hassoun E, Bagchi M, Bagchi D, Stohs, S (1993) Comparative studies on lipid peroxidation and DNA-single strand breaks induced by lindane, DDT, chlordane and endrin in rats. Comp Biochem Physiol 104: 427–431.

Hawkinson J, Shull L, Joy R (1989) Effects of lindane on calcium fluxes in synaptosomes. Neurotoxicology 10: 29–39.

Heck MC, Sifuentes dos Santos J, Bogus S, Costabeber I, Emanuelli T (2007) Estimation of children exposure to organochlorine compounds through milk in Rio Grande do Sul, Brazil. Food Chem 102: 288–294.

Hedley AJ, Hui LL, Kypke K, Malisch R, Van Leeuwen FXR, Moy G, Wong TW, Nelson EAS (2010) Residues of persistent organic pollutants (POPs) in human milk in Hong Kong. Chemosphere 79: 259–265.

Hoyer A, Grandjean P, Jorgensen T, Brock J, Hartvig H (1998) Organochlorine exposure and risk of breast cancer. Lancet 352: 1816–1820.

Ikeda K, Kajiwara K, Tanabe E, Tokumaru S, Kishida E, Masuzawa Y, Kojo S (1999) Involvement of hydrogen peroxide and hydroxyl radical in chemically induced apoptosis of HL-60 cells. Biochem Pharmacol 57: 1361–1365.

Ikemoto T, Tu NP, Watanabe MX, Okuda N, Omori K, Tanabe S, Tuyen BC, Takeuchi I (2008) Analysis of biomagnification of persistent organic pollutants in the aquatic food web of the Mekong Delta, South Vietnam using stable carbon and nitrogen isotopes. Chemosphere 72: 104–114.

Ingelido AM, Abballe A, Marra V, Valentini S, Ferro A, Porpora MG, Barbieri PG, De Felip E (2009) Serum concentrations of beta-hexachlorocyclohexane in groups of the Italian general population: a human biomonitoring study. Ann Ist Super Sanita 45: 401–408.

Ito N, Nagasaki H, Aoe H, Sugihara S, Miyata Y, Arai M, Shirai T (1975) Development of hepatocellular carcinomas in rats treated with benzene hexachloride. J Natl Cancer Inst 54: 801–805.

Ito N, Nagasaki H, Arai M, Sugihara S, Makiura S (1973) Histologie and ultrastructural studies on the hepatocarcinogenicity of benzene hexachloride in mice. J Natl Cancer Inst 51: 817–826.

Janik F, Wolf HU (1992) The Ca(2+)-transport-ATPase of human erythrocytes as an in vitro toxicity test system – acute effects of some chlorinated compounds. J Appl Toxicol 12: 351–358.

Jenke HS, Löwel M, Berndt J (1988) Modes of action and combination effects of polychlorinated biphenyls and gamma-hexachlorocyclohexane on the regulation of rat liver 3-hydroxy-3-methylglutaryl coenzyme A reductase. Chem Biol Interact 65: 175–186.

Johri A, Yadav S, Dhawan A, Parmar D (2007) Overexpression of cerebral and hepatic cytochrome P450s alters behavioral activity of rat offspring following prenatal exposure to lindane. Toxicol Appl Pharmacol 225: 278–292.

Joy R, Burns V (1988) Exposure to lindane and two other hexachlorocyclohexane isomers increases free intracellular calcium levels in neurohybridoma cells. Neurotoxicology 9: 637–643.

Joy R, Walby W, Stark L, Albertson T (1995) Lindane blocks GABAA-mediated inhibition and modulates pyramidal cell excitability in the rat hippocampal slice. Neurotoxicology 16: 217–228.

Joy RM (1982) Mode of action of lindane, dieldrin and related insecticides in the central nervous system. Neurobehav Toxicol Teratol 4: 813–823.

Junge B, Carrion Y, Bosco C, Galleano M, Puntarulo S, Tapia G, Videla LA (2001) Effects of iron overload and lindane intoxication in relation to oxidative stress, Kupffer cell function, and liver injury in the rat. Toxicol Appl Pharmacol 170: 23–28.

Junqueira VB, Koch OR, Arisi AC, Fuzaro AP, Azzalis LA, Barros SB, Cravero A, Farré S, Videla LA (1997) Regression of morphological alterations and oxidative stress-related parameters after acute lindane-induced hepatotoxicity in rats. Toxicology 117: 199–205.

Kalyoncu L, Agca I, Aktumsek A (2009) Some organochlorine pesticide residues in fish species in Konya, Turkey. Chemosphere 74: 885–889.

Kang JH, Park H, Chang YS, Choi JW (2008) Distribution of organochlorine pesticides (OCPs) and polychlorinated biphenyls (PCBs) in human serum from urban areas in Korea. Chemosphere 73: 1625–1631.

Kaplan S, Zdziarski U, Kuhns D, Basford R (1988) Inhibition of cell movement and associated changes by hexachlorocyclohexanes due to unregulated intracellular calcium increases. Blood 71: 677–683.

Khan MA, Matin MA, Beg MU (1993) Effect of intratracheally administered lindane on aldrin and benzo(a)pyrene contents in lungs of rats. Toxicol Lett 69: 63–67.

Kitamura A, Sato R, Marszalec W, Yeh JZ, Ogawa R, Narahashi, T (2004) Halothane and propofol modulation of gamma-aminobutyric acidA receptor single-channel currents. Anesth Analg 99: 409–415.

Koner BC, Banerjee BD, Ray A (1997) Modulation of gamma glutamyl transpeptidase activity in lymphoid system by organochlorine pesticides in rats. Indian J Exp Biol. 35: 1132–1134.

Koner B, Banerjee B, Ray A (1998) Organochlorine pesticide-induced oxidative stress and immune suppression in rats. J Exp Biol 36: 395–398.

Krieger TR, Loch-Caruso R (2001) Antioxidants prevent gamma-hexachlorocyclohexane-induced inhibition of rat myometrial gap junctions and contractions. Biol Reprod 64: 537–47.

Kroll B, Kunz S, Klein T, Schwarz LR (1999) Effect of lindane and phenobarbital on cyclooxygenase-2 expression and prostanoid synthesis by Kupffer cells. Carcinogenesis 20: 1411–6.

Kuhns D, Kaplan S, Basford R (1986) Hexachlorocyclohexanes, potent stimuli of O_2- production and calcium release in human polymorphonuclear leukocytes. Blood 68: 535–540.

Kumar R, Pant N, Srivastava S (1998) Hexachlorocyclohexane and its isomers: regional brain levels in the rat after dermal exposure. Arch Environ Contam Toxicol 35: 674–679.

Kumar A, Dayal P, Shukla G, Singh G, Joseph PE (2006) DDT and HCH residue load in mother's breast milk: A survey of lactating mother's from remote villages in Agra region. Environment International 32: 248–251.

Langenhoff AAM, Staps JJM, Pijls C, Alphenaar A, Zwiep G, Rijnaarts HHM (2002) Intrinsic and stimulated in situ biodegradation of hexachlorocyclohexane (HCH) Water, Air Soil Poll. Focus 2: 171–181.

Law RJ, Lightstone FC (2008) Gaba receptor insecticide non-competitive antagonists may bind at allosteric modulator sites. Int J Neurosci 118: 705–34.

Lee CH, Cragoe EJ, Edwards M (2003) Control of hepatocyte DNA synthesis by intracellular pH and its role in the action of tumorpromoters. J Cell Physiol 195: 61–69.

Leibold E, Schwarz L (1993) Inhibition of intercellular communication in rat hepatocytes by phenobarbital, 1,1,1-trichloro-2,2-bis(p-chlorophenyl)ethane (DDT) and g-hexachlorocyclohexane (lindane): Modification by antioxidants and inhibitors of cyclo-oxygenase. Carcinogenesis 14: 2377–2382.

Leng JH, Kayama F, Wang PY, Nakamura M, Nakata T, Wang Y (2009) Levels of persistent organic pollutants in human milk in two Chinese coastal cities, Tianjin and Yantai: Influence of fish consumption. Chemosphere 75: 634–639.

Li QQ, Loganat A, Chong YS, Tan J, Obbard JP (2006) Levels of persistent organic pollutant residues in human adipose and muscle tissues in Singapore. J Toxicol Environ Health 69: 1927–1937.

Linderholm L, Biague A, Månsson F, Norrgren H, Bergman Å, Jakobsson K (2010) Human exposure to persistent organic pollutants in West Africa – A temporal trend study from Guinea-Bissau. Environment International. 36: 675–682.

Loewenstein W (1979) Junctional intercellular communication and the control of growth. Biochim Biophys Acta 560: 1–65.

López-Aparicio P, Merino M, Recio M, Perez M (1995) Lindane enhances a specific phospholipase A2 activity in rat renal tubular cell culture. Pesticide Biochem Physiol 52: 90–97.

Lopez-Carrillo L, Lopez-Cervantes M, Torres-Sanchez L, Blair A, Cebrian ME, Garcia, RM (2002) Serum levels of beta-hexachlorocyclohexane, hexachlorobenzene and polychlorinated biphenyls and breast cancer in Mexican women. Eur J Cancer Prev 11: 129–135.

Lu CH, Lee KC, Chen YC, Cheng JS, Yu MS, Chen WC, Jan CR (2000) Lindane (gamma hexachlorocyclohexane) induces internal Ca2+ release and capacitative Ca2+ entry in Madin Darby canine kidney cells. Pharmacol Toxicol 87: 149–155.

Lu Y, Zhou SB, Li BX (2010) Exposure to Environmental Hexachlorocyclohexane (HCH) and Dichlorodiphenyltrichloroethane (DDT) among Rural Children in North Eastern China. Biomed Environ Sci Jun 23: 230–233.

Lubet RA, Dragnev KH, Chauhan DP, Nims RW, Diwan BA, Ward JM, Jones CR, Rice JM, Miller MS (1992) A pleiotropic response to phenobarbital-type enzyme inducers in the F344/NCr rat. Effects of chemicals of varied structure. Biochem Pharmacol 43: 1067–1078.

Macholz RM, Bleyl DW, Klepel H, Knoll R, Kujawa M, Lewerenz HJ, Muller D, Plass R (1986) Comparison of the distribution and toxicity of alpha-, beta-, and gamma-hexachlorocyclohexane (HCH) following 30 days of administration to rats. Nahrung 30: 701–708.

Malik RN, Rauf S, Mohammad A, Eqani SA, Ahad K (2010) Organochlorine residual concentrations in cattle egret from the Punjab Province, Pakistan. Environ Monit Assess Mar 7. [Epub ahead of print].

Maranghi F, Rescia M, Macrì C, Consiglio E, De Angelis G, Testai E, Farini D, De Felici M, Lorenzetti S, Mantovani A (2007) Lindane may modulate the female reproductive development through the interaction with ER-beta: an in vivo-in vitro approach. Chem Biol Interact 169: 1–14.

Mattioli F, Robbiano L, Adamo D, Federa R, Martelli A, Brambilla G (1996) Genotoxic effects of alpha-hexachlorocyclohexane in primary cultures of rodent and human hepatocytes. Mutagenesis 11: 79–83.

McDuffie HH, Pahwa P, McLaughlin JR, Spinelli JJ, Fincham S, Dosman JA, Robson D, Skinnider LF, Choi NW (2001) Non-Hodgkin's lymphoma and specific pesticide exposure in men: cross-Canada study of pesticides and health. Cancer Epidemiol Biomarkers Prev 10: 1155–1163.

Minh NH, Someya M, Minh TB, Kunisue T, Iwata H, Watanabe M, Tanabe S, Viet PH, Tuyen BC (2004) Persistent organochlorine residues in human breast milk from Hanoi and Hochiminh City, Vietnam: contamination, accumulation kinetics and risk assessment for infants. Environ Pollut 129: 431–41.

Mograbi B, Corcelle E, Defamie N, Samson M, Nebout M, Segretain D, Fenichel P, Pointis G (2003) Aberrant Connexin 43 endocytosis by the carcinogen lindane involves activation of the ERK/mitogen-activated protein kinase pathway. Carcinogenesis 24: 1415–1423.

Mohr C, Alojipan S, Dunston S, Pessah I (1995) The delta isomer of hexachlorocyclohexane induces rapid release of the myo-inositol-1,4,5-triphosphate-sensitive Ca2+ store and blocks capacitative Ca2+ entry in rat basophilic leukemia cells. Mol Pharmacol 48: 512–522.

Moisey J, Fisk AT, Hobson KA, Norstrom RJ (2001) Hexachlorocyclohexane (HCH) isomers and chiral signatures of alpha-HCH in the Arctic marine food web of the Northwater Polynya. Environ Sci Technol 35:1920–1927.

Molina C, Falcón M, Barba A, Cámara MA, Oliva J, Luna A (2005) HCH and DDT residues in human fat in the population of Murcia (Spain). Ann Agric Environ Med 12: 133–136.

Moreno MJ, Pellicer S, Fernández-Otero MP (1996a) Effects of different subchronic treatments with lindane on some brush border enzymes in rat jejunum. Rev Esp Fisiol 52: 37–44.

Moreno MJ, Pellicer S, Fernández-Otero MP (1996b) Effects of in situ and systemic lindane treatment on in vivo absorption of galactose and leucine in rat jejunum. Arch Toxicol 70: 767–772.

Moser G, Smart R (1989). Hepatic tumor-promoting chlorinated hydrocarbons stimulate protein kinase C activity. Carcinogenesis 10: 851–856.

Mussalo-Rauhamaa H, Hasanen E, Pyysalo H, Antervo K, Kauppila R, Pantzar P (1990) Occurrence of beta-hexachlorocyclohexane in breast cancer patients. Cancer 66: 2124–2128.

Mostafa MH, el-Bassiouni EA, el-Sewedy SM, Akhnouk S, Tawfic T, Abdel-Rafee A (1984) Hepatic microsomal enzymes in Schistosoma mansoni-infected mice: I. Effect of duration of infection and lindane administration on dimethylnitrosamine demethylases. Environ Res 35:154–159.

Nagata K, Narahashi T (1995) Diferential effects of hexachlorocyclohexane isomers on the GABA receptor-chloride channel complex in rat dorsal root ganglion neurons. Brain Res 704: 85–91.

Nigam U, Siddiqui MK (2001) Organochlorine insecticide residues in dairy milk samples collected in Lucknow, India. Bull Environ Contam Toxicol 66: 678–682.

Ntow WJ, Tagoe LM, Drechsel P, Kelderman P, Gijzen HJ, Nyarko E (2008) Accumulation of persistent organochlorine contaminants in milk and serum of farmers from Ghana. Environ Res 106: 17–26.

Olivero J, Bezdecny SA, Ganey PE (2002) Molecular motif required for the activation of rat neutrophil phospholipase A(2) by organochlorine compounds. Chem Res Toxicol 15: 153–159.

Oskarsson A, Ulleras E, Plant KE, Hinson JP, Goldfarb PS (2006) Steroidogenic gene expression in H295R cells and the human adrenal gland: adrenotoxic effects of lindane in vitro. J Appl Toxicol 26: 484–492.

Pages N, Sauviat MP, Bouvet, S., Goudey-Perriere F (2002) Reproductive toxicity of lindane. J Soc Biol 196: 325–338.

Pajuelo L, Sanchez J, Del Hoyo N, Pulido J, Perez M (1997) Non-muscarinic and non-adrenergic-mediated effects of lindane on phosphoinositide hydrolysis in rat brain cortex slices. Neurochem Res 22: 57–62.

Parries GS, Hokin-Neaverson M (1985) Inhibition of phosphatidylinositol synthase and other membrane-associated enzymes by stereoisomers of hexachlorocyclohexane. J Biol Chem 260: 2687–2693.

Patterson DG Jr, Wong LY, Turner WE, Caudill SP, Dipietro ES, McClure PC, Cash TP, Osterloh JD, Pirkle JL, Sampson EJ, Needham LL (2009) Levels in the U.S. population of those persistent organic pollutants (2003–2004) included in the Stockholm Convention or in other long range transboundary air pollution agreements. Environ Sci Technol 43: 1211–1218.

Perocco P, Colacci A, Del Ciello C, Grilli S (1995) Cytotoxic and cell transforming effects of the insecticide, lindane (γ-hexachlorocyclohexane) on BALB/c 3 T3 cells. Res Commun Mol Pathol Pharmacol 89: 329–339.

Pessah I, Mohr F, Schiedt M, Joy R (1992) Stereoselective modulation of ryanodine-sensitive calcium channels by the d-isomer of hexachlorocyclohehaxe (δ-HCH). J Pharmacol Exp Ther 262: 661–669.

Petronijevic T, Edwards A (1993) Role of Ca2+ in stimulation of DNA synthesis by epidermal growth factor and tumor promoters in cultured rat hepatocytes. J Cell Physiol 155: 44–53.

Pinelli E, Cambon C, Tronchere H, Chap H, Teissie J, Pipy B (1994) Ca2+-dependent activation of phospholipase C and D from mouse peritoneal macrophages by a selective trigger of Ca2+ influx, γ-hexachlorocyclohexane. Biochem Biophys Res Commun 199: 699–705.

Pohl HR, Tylenda CA (2000) Breast-feeding exposure of infants to selected pesticides: A public health viewpoint. Toxicol Ind Health 16: 65–77.

Polder A, Skaare JU, Skjerve E, Løken KB, Eggesbø M (2009) Levels of chlorinated pesticides and polychlorinated biphenyls in Norwegian breast milk (2002–2006), and factors that may predict the level of contamination. Sci Total Environ 407: 4584–4590.

Pomes A, Rodriguez E, Sunol C (1994) Effects of organochlorine pesticides on 36Cl-flux in primary neuronal cultures. Neurotoxicology 15: 745–749.

Porta M, Gasull M, Puigdomènech E, Garí M, Bosch de Basea M, Guillén M, López T, Bigas E, Pumarega J, Llebaria X, Grimalt JO, Tresserras R (2010) Distribution of blood concentrations of persistent organic pollutants in a representative sample of the population of Catalonia. Environment International 36: 655–664.

Prasad A, Pant N, Srivastava S, Kumar R, Srivastava S (1995) Effect of dermal application of hexachlorocyclohexane (HCH) on male reproductive system of rat. Hum Exp Toxicol 14: 484–488.

Purdue MP, Hoppin JA, Blair A, Dosemeci M, Alavanja MC (2007) Occupational exposure to organochlorine insecticides and cancer incidence in the Agricultural Health Study. Int J Cancer 120: 642–649.

Puri S, Kohli KK (1995) Differences in hepatic drug metabolizing enzymes and their response to lindane in rat, rabbit and monkey. Pharmacol Toxicol 77: 136–4.

Rafnsson V (2006) Risk of non-Hodgkin's lymphoma and exposure to hexachlorocyclohexane, a nested case-control study. Eur J Cancer 42: 2781–2785.

Raizada R, Misra P, Saxena P, Datta K, Dikshith T (1980) Weak estrogenic activity of lindane in rats. J Toxicol Environ Health 6: 483–492.

Ratneswaran R, Meldon T, Carole P (1997) Modulation of the hepatic expression of the estrogen-regulated mRNA stabilizing factor by estrogenic and antiestrogenic nonsteroidal xenobiotics. Biochem Pharmacol 53: 1425–1434.

Ronco AM, Valdes K, Marcus D, Llanos M (2001) The mechanism for lindane-induced inhibition of steroidogenesis in cultured rat Leydig cells. Toxicology 159: 99–106.

Rosa R, Sanfeliu C, Rodriguez E, Frandsen A, Schousboe A, Sunol C (1997a) Properties of ryanodine receptors in cultured cerebellar granule neurons: effects of hexachlorocyclohexane isomers and calcium. J Neuros Sci 47: 27–33.

Rosa R, Sanfeliu C, Sunol C, Pomés A, Rodríguez E, Schousboe A, Frandsen A, (1997b). The mechanism for hexachlorocyclohexane-induced cytotoxicity and changes in intracellular Ca2+ homeostasis in cultured cerebellar granule neurons is different for the γ- and δ-isomers. Toxicol Appl Pharmacol 142: 31–39.

Roux F, Puiseux-Dao S, Treich I, Fournier E (1978) Effect of lindane on mouse peritoneal macrophages. Toxicology 11: 259–269.

Roy D, Palangat M, Chen Ch, Thomas R, Colerangle J, Atkinson A, Yan Z (1997) Biochemical and molecular changes at the cellular level in response to exposure to environmental estrogen-like chemicals. J Toxicol Environ Health 50: 1–29.

Runge-Morris M (1998) Regulation of sulfotransferase gene expression by glucocorticoid hormones and xenobiotics in primary rat hepatocyte culture. Chem Biol Interact 109: 315–27.

Sahoo A, Chainy GB (1998) Acute hexachlorocyclohexane-induced oxidative stress in rat cerebral hemisphere. Neurochem Res 23: 1079–1084.

Sahoo A, Samanta L, Das A, Patra SK, Chainy GB (1999) Hexachlorocyclohexane-induced behavioural and neurochemical changes in rat. J Appl Toxicol 19: 13–18.

Salem NM, Ahmad R, Estaitieh H (2009) Organochlorine pesticide residues in dairy products in Jordan. Chemosphere 77: 673–678.

Samanta L, Roy A, Chainy G (1999a) Changes in rat testicular antioxidant defense profile as a function of age and its impairment by hexachlorocyclohexane during critical stages of maturation. Andrologia 31: 83–90.

Samanta L, Sahoo A, Chainy G (1999b) Age-related changes in rat testicular oxidative stress parameters by hexachlorocyclohexane. Arch Toxicol 73: 96–107.

Saradha B, Mathur PP (2006) Effect of environmental contaminants on male reproduction. Environ Toxicol Pharmacol 21: 34–41.

Sarkander H, Kemmerle M, Brade W (1974) Rat liver histone modifications and their relationship to DNA-dependent RNA polymerase activities during alpha-hexachlorocyclohexane induced liver proliferation. Naunyn Schmiedebergs Arch Pharmacol 284: 39–53.

Sauviat MP, Pages N (2002) Cardiotoxicity of lindane, a gamma isomer of hexachlorocyclohexane. J Soc Biol 196: 339–348.

Schroter C, Parzefall W, Schroter H, Schulte-Hermann R (1987) Dose-response studies on the effects of alpha-, beta-, and gamma-hexachlorocyclohexane on putative preneoplastic foci, monooxygenases, and growth in rat liver. Cancer Res 47: 80–88.

Seglen P (1997) DNA ploidy and autophagic protein degradation as determinants of hepatocellular growth and survival. Cell Biol Toxicol 13: 301–315.

Seifert J, Vácha J, Seifertová M (1978) Liver growth, biosynthesis of cytidine nucleotides and level of cytochrome P-450 in rat liver after administration of alpha-hexachlorocyclohexane. Chem Biol Interact 20: 227–238.

Senar S, Puente J, Lopez P, Recio M, Perez M (1994) Increased intracellular glycerophosphoinositol and arachidonic acid are biochemical markers for lindane toxicity Cell Signal 6: 915–921.

Shankland DL (1982) Neurotoxic action of chlorinated hydrocarbon insecticides. Neurobehav Toxicol Teratol 4: 805–811.

Sharma HR, Kaushik A, Kaushik CP (2007) Pesticide residues in bovine milk from a predominantly agricultural state of Haryana, India. Environ Monit Assess. 129: 349–57.

Siglin J, Weghorst C, Rodwell D, Klaunig J (1995) Gender-dependent differences in hepatic tumor promotion in diethylnitrosamine initiated infant B6C3F1 mice by alpha-hexachlorocyclohexane. J Toxicol Environ Health 44: 235–245.

Simon Giavarotti KA, Rodrigues L, Rodrigues T, Junqueira VB, Videla LA (1998) Liver microsomal parameters related to oxidative stress and antioxidant systems in hyperthyroid rats subjected to acute lindane treatment. Free Radic Res 29: 35–42.

Simonich SL, Hites RA (1995) Global distribution of persistent organochlorine compounds. Science 269: 1851–1854.

Singal A, Thami GP (2006) Lindane neurotoxicity in childhood. Am J Ther 13: 277–280.

Sircar S, Lahiri P (1990) Effect of lindane on mitochondrial side-chain cleavage of cholesterol in mice. Toxicology 61: 41–46.

Spinelli JJ, Ng CH, Weber JP, Connors JM, Gascoyne RD, Lai AS, Brooks-Wilson AR, Le ND, Berry BR, Gallagher, RP (2007) Organochlorines and risk of non-Hodgkin lymphoma. Int J Cancer 121: 2767–2775.

Srinivasan K, Radhakrishnamurty R (1988) Biochemical changes produced by beta- and gamma-hexachlorocyclohexane isomers in albino rats. J Environ Sci Health B 23: 367–386.

Steinmetz R, Young PC, Caperell-Grant A, Gize EA, Madhukar BV, Ben-Jonathan N, Bigsby RM (1996) Novel estrogenic action of the pesticide residue beta-hexachlorocyclohexane in human breast cancer cells. Cancer Res 56: 5403–5409.

Subramanian A, Ohtake M, Kunisue T, Tanabe S (2007) High levels of organochlorines in mothers' milk from Chennai (Madras) city, India. Chemosphere 68: 928–939.

Sudakin DL (2007) Fatality after a single dermal application of lindane lotion. Arch. Environ Occup Health 62: 201–203.

Sujatha R, Chitra KC, Latchoumycandane C, Mathur PP (2001) Effect of lindane on testicular antioxidant system and steroidogenic enzymes in adult rats. Asian J Androl 3: 135–138.

Tanabe S, Kunisue T (2007) Persistent organic pollutants in human breast milk from Asian countries. Environ Pollut 146: 400–413.

Taira MC, San Martín de Viale LC (1998) Effect of lindane and heptachlor on delta-aminolaevulinate synthase and its regulation. Arch Toxicol 72: 722–730.

Tao S, Liu WX, Li XQ, Zhou DX, Li X, Yang YF, Yue DP, Coveney RM (2009) Organochlorine pesticide residuals in chickens and eggs at a poultry farm in Beijing, China. Environ Pollut 157: 497–502.

Tiemann U, Küchenmeister U (1999) Influence of organochlorine pesticides on ATPase activities of microsomal fractions of bovine oviductal and endometrial cells. Toxicol Lett 104: 75–81.

Tiemann U (2008) In vivo and in vitro effects of the organochlorine pesticides DDT, TCPM, methoxychlor, and lindane on the female reproductive tract of mammals: A review Reprod Toxicol 25: 316–326.

Tithof PK, Olivero J, Ruehle K, Ganey PE (2000) Activation of neutrophil calcium-dependent and -independent phospholipases A2 by organochlorine compounds. Toxicol Sci 53: 40–47.

Torres-Arreola L, Berkowitz G, Torres-Sanchez L, Lopez-Cervantes M, Cebrian ME, Uribe M, Lopez-Carrillo L (2003) Preterm birth in relation to maternal organochlorine serum levels. Ann Epidemiol 13: 158–62.

Tusell J, Vendrell M, Serratosa J, Trullas, R (1992) Lindane-induced convulsions in NMRI and OF1 mice: antagonism with (+)MK-801 and voltage-dependent calcium channel blockers. Brain Res 593: 209–24.

Vale C, Damgaard I, Sunol C, Rodriguez E, Schousboe A (1998) Cytotoxic action of lindane in neocortical GABAergic neurons is primarily mediated by interaction with flunitrazepam-sensitive GABA(A) receptors. J Neurosci Res 52: 276–285.

Vale C, Pomes A, Rodriguez E, Sunol C (1997) Allosteric interactions between gamma-aminobutyric acid, benzodiazepine and picrotoxinin binding sites in primary cultures of cerebellar granule cells.

Differential effects induced by gamma- and delta-hexachlorocyclohexane. Eur J Pharmacol 319: 343–353.

Van Velsen F, Danse L, Van Leeuwen F, Dormans J, Van Logten M (1986) The subchronic oral toxicity of the beta-isomer of hexachlorocyclohexane in rats. Fundam. Appl Toxicol 6: 697–712.

Vendrell M, Zawia NH, Serratosa J, Bondy SC (1991) c-fos and ornithine decarboxylase gene expression in brain as early markers of neurotoxicity. Brain Res 544: 291–296.

Vendrell M, Pujol M, Tusel J, Serratosa J (1992) Effect of different convulsants on calmodulin levels and proto-oncogene c-fos expression in the central nervous system Mol Brain Res 14: 285–292.

Verma S, Rastogi A, Lin P (1992) Hexachlorocyclohexane pesticides reduce survival and alter plasma membrane structure of chinese hamster V79 cells. Arch Biochem Biophys 298: 587–593.

Videla L, Troncoso P, Arisi A, Junqueira V (1997) Dose-dependent effects of acute lindane treatment on Kupffer cell function assessed in the isolated perfused rat liver. Xenobiotica 27: 747–757.

Waliszewski SM, Gomez-Arroyo S, Infanzon RM, Carvajal O, Villalobos-Pietrini R, Trujillo P, Maxwell M (2004) Persistent organochlorine pesticide levels in bovine fat from Mexico. Food Addit Contam 21: 774–80.

Walsh LP, Stocco DM (2000) Effects of lindane on steroidogenesis and steroidogenic acute regulatory protein expression. Biol Reprod 63: 1024–1033.

Wong PS, Matsumura F (2006) Serum free BG-1 cell proliferation assay: a sensitive method for determining organochlorine pesticide estrogen receptor activation at the nanomolar range. Toxicol In Vitro 20: 382–394.

Wong PS, Matsuura F (2007) Promotion of breast cancer by beta-hexachlorocyclohexane in MCF10AT1 cells and MMTV-neu mice. BMC Cancer 7:130.

Woodward R, Polenzani L, Miledi R (1992) Effects of hexachlorocyclohexanes on gamma-aminobutyric acid receptors expressed in Xenopus oocytes by RNA from mammalian brain and retina. Mol Pharmacol 41: 1107–1115.

World Health Organization/International Agency for Research on Cancer (1987) IARC monographs on evaluation of carcinogenic risks to humans. Overall evaluations of carcinogenicity: An updating of IARC monographs. Lyon: International Agency for Research on Cancer: 220–221.

Yu Y, Tao S, Liu W, Lu X, Wang X, Wong M (2009) Dietary intake and human milk residues of hexachlorocyclohexane isomers in two Chinese cities. Environ Sci Technol 43: 4830–4835.

Zisterer D, Sunol C, Moynagh P, Williams D, Rodriguez E (1995) PK 11195 reduces the brain availability of lindane in rats and the convulsions induced by this neurotoxic agent. Life Sciences 57: 2359–2364.

Zisterer D, Moynagh P, Willians D (1996) Hexachlorocyclohexanes inhibit steroidogenesis in Y1 Cells. Biochem Pharmacol 51: 1303–1308.

The Use of Cholinesterases in Ecotoxicology

Bruno Nunes

Contents

1 Introduction

The need for reproducible and accurate biomarkers in Environmental Toxicology has led researchers to implement methods to evaluate the physiological effects caused by contaminants. Such methods are of particular biological importance and ecological interest if they allow the measurement of direct impairment of key endpoints in the test organisms or nontarget species. Neurotransmission impairment via cholinesterase

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D.M. Whitacre (ed.), *Reviews of Environmental Contamination and Toxicology*,
Reviews of Environmental Contamination and Toxicology 212,
DOI 10.1007/978-1-4419-8453-1_2, © Springer Science+Business Media, LLC 2011

ChE inhibition is the target of two important classes of modern pesticides, the organophosphates (OPs), and the carbamates (CBs). Because of their extensive use in modern agriculture, these two classes of compounds are widely employed. Metcalfe et al. (2002) estimated for the California Department of Food and Agriculture that the ban from current agricultural use of such compounds would cause the loss of 209,000 jobs and would result in a national economic loss of $17 billion. Therefore, it is not difficult to conclude that these chemical agents will continue to be used, despite the fact that humans and many nontarget organisms are exposed to them (spray drift from crop application, run off from agricultural fields that contaminate adjacent water bodies, residues in food, etc.) (Vermeire et al. 2001).

Protecting agroecosystems from pesticide effects commonly entails measuring the levels of pesticides that occur in manifold environmental media. The inhibition of cholinesterases (ChEs) is poised to assume a leading role as a tool for monitoring the presence of those classes of pesticides or other agents that are ChE inhibitors, and their use may assist in determining the effects of such compounds on nontarget organisms. Denoyelle et al. (2007) have already provided evidence for what is possible in using such methods, by assessing the effects in apple orchards of certain pesticides on earthworms (*Allolobophora chlorotica*).

The prospective overall importance of ChEs to modern ecotoxicity assessment is high, particularly acetylcholinesterase (AChE). AChE acts to terminate the nervous impulse at the synaptic cleft and is directly involved in the intoxications caused both by the OP and CB pesticides. The toxic effect elicited by both types of pesticides has assured this biomarker a leading role in future environmental analyses. Although the OP pesticides may be hazardous, they are still extensively used in agriculture, and they exhibit certain favorable characteristics; in addition to successfully controlling many economic pests, and the OPs have low environmental persistence (when compared with organochlorine insecticides), low bioaccumulation (Sánchez-Hernandez 2001). These are the main reasons that justify their wide and continuing use, despite the fact that they also have high, nonspecific acute toxicity that can lead to frequent intoxication of nontarget organisms.

ChEs are extremely complex; namely, they display differential forms, expression, biologic functions, location, and catalytic activity. In addition to their involvement in neurodegenerative disorders, ChEs have other biologic functions (Small et al. 1996). These authors enumerated some of the functions that may be attributed to ChEs, including regulation of neurite growth, cell proliferation, tumorigenic processes, cell adhesion molecules, and megakaryocytopoiesis. Mammalian brain AChE (an enzyme that is released by presynaptic neurons during the neuronal communication process), for example, is mainly an enzyme located in postsynaptic neurons, is anchored to the cellular membrane by a single glycosylated protein, and is specialized in the hydrolysis of the neurotransmitter acetylcholine (Boschetti and Brodbeck 1996). AChE is of fundamental importance in terminating the nervous impulse; consequently, the regulation of its synthesis depends on, through a mechanism of feedback, the activation of nerve cells by propagation of an action potential. Schweitzer (1993) showed that AChE, which is mainly located in the postsynaptic cell, is constitutively expressed when nerve cells are resting; however, activation (by depolarization) of these cells leads to the release of AChE molecules to the extracellular space. Furthermore, this author found that the

secretion and release of AChE from nerve cells could be of two types: (1) a constitutive, and (2) a regulated pathway that can be activated following neuronal activity. These two pathways differ not only in the amount of AChE released but also in the preferential (asymmetric and globular) forms that are released. The expression of human AChE is controlled via a number of distinct pathways, one involving cAMP (Wan et al. 2000; Tsim 1998), which can be regulated by a calcitonine gene-related peptide (Tsim 1998).

The physiological role attributed to AChE is not limited only to the regulation of neurotransmission. The involvement of nerve growth factor in the synthesis of AChE was first demonstrated by Greene and Rukenstein (1981), indicating that the activity of this enzyme could also affect the neuronal differentiation processes. More recently, other research has produced evidence of this association. The role of AChE in neurodifferentiation was shown by Deschênes-Furry et al. (2003) and Choi et al. (1998). Yang et al. (2002) demonstrated the potential activity of AChE at the onset of apoptosis in nerve cells, with its potential implication in the neurodegenerative pathophysiology of certain diseases, such as Alzheimer's Disease. The increased AChE activity observed in neurodegeneration (a characteristic of Alzheimer's disease) is associated with the presence of high concentrations of reactive oxygen and nitrogen species (ROS and RNS, respectively). These results were published by Melo et al. (2003), whose work established a direct linkage between oxidative stress and the enzymatic activity of AChE of the human brain.

Butyrylcholinesterase (BChE), the other ChE present in the majority of vertebrates, is found mainly in the plasma and has an unclear function (Jbilo et al. 1994). However, BChE is thought to be involved in regulation of cell proliferation and the early stages of neuronal differentiation (Mack and Robitzki 2000). In addition to its presence in body fluids, BChE can exist in hematopoietic cells, liver, lung, heart, at cholinergic synapses, in the central nervous system, in tumors and in developing embryonic tissues (Mack and Robitzki 2000).

The use of ChE inhibition has thus become increasingly common among the batteries of biomarkers used in ecotoxicological assessment. Its use is favored because of several attributes, such as sensitivity to a large number of significant environmental contaminants, low cost, easy performance of quantification assays, adaptability to a vast number of species from distinct ecosystems, good reproducibility, and biological/ecological relevance. This latter factor is, indeed, of major importance. Padilla (1995) showed that the assessment of ChE in body fluids of organisms exposed to OP compounds correlated well with levels of ChE inhibition in target organs (namely, the central nervous system), and was closely accompanied by clinical symptoms and behavioral modifications. However, the time course of the intoxication must be assessed frequently, when ChE is employed as a biomarker. The possibility of establishing a direct relationship between ChE inhibition, and the behavioral/biochemical/physiological changes that occur in exposed organisms, is an important factor to consider in environmental analysis. The reason is that altered behavior may have severe population implications that result from potential impairment of reproduction, migration, and predator avoidance.

Inhibition of cholinesterasic activity is also relevant ecologically, because mortality of exposed organisms is a potential outcome. Fulton and Key (2001) highlighted the fact that cholinesterasic inhibition was closely accompanied by a rise in mortality;

much research demonstrates that the survival of aquatic organisms is impaired when inhibition from anti-cholinesterase compound exposure approached 70%. The potential impact of anticholinesterasic agents on animal behavior was shown by Chebbi and David (2009), following exposure of *Cyprinus carpio* to quinalphos. These authors observed that subsequent to a significant reduction of AChE activity, a major modification occurred in the swimming pattern of the mentioned fish species, such as erratic, darting, and burst-swimming. Pronounced impairment of behavior may constitute an adverse outcome that has evident ecological repercussions. Similarly, data obtained by Sandahl et al. (2005), after they exposed Coho salmon (*Oncorhynchus kisutch*) to chlorpyrifos, showed that a good correlation was obtained by plotting AChE inhibition against spontaneous swimming and feeding behaviors. Furthermore, this team found a remarkable result: ecologically relevant concentrations of chlorpyrifos could cause significant AChE inhibition and, simultaneously, cause clearly evident behavioral changes. The behavioral changes that result from AChE inhibition are not always obvious (Bain et al. 2004). These authors indicate that the anticholinesterasic effects caused by fenitrothion on the lizard species *Pogona vitticeps* were not followed by any significant modification in prey-capture ability.

The effects that occur concurrently with ChE inhibition may be important at the population level (Duquesne 2006). This author showed that after exposure of the crustacean *Daphnia magna* to paraoxon-methyl, a transient phase occurred during which ChE inhibition was noticeable, and simultaneously, several individual life traits were affected. These traits included survival, reduction in reproductive performance, and a decrease in body size. These population effects were a direct consequence of the toxic effects, and resulted from a reduced rate of population growth.

The neurotoxicity of xenobiotics may also produce effects at the cellular level. After exposure, unicellular organisms may display altered cell morphology and functionality, with consequences at the population level (lower number of cells). Falugi et al. (2002) showed that exposure of the protozoan species to basudin, a neurotoxicant, could result in cholinesterasic inhibition, followed closely by effects such as altered cell morphology, lower cell density, and impairment in the formation of aggregates.

The aim of the present review article is to address practical issues associated with the use of cholinesterase inhibition as a tool to determine the effects that anthropogenic contaminants have on nontarget wild species. In this article, we will also address the following points vis-a-vis ChEs: the need for previous characterization of cholinesterasic forms, the validity of use for field vs. laboratory quantification, main drawbacks as a biomarker, the future potential for use, and relevance for use with several classes of contaminants.

2 Types of Cholinesterases

The nature of the ChEs that exist in vertebrates and invertebrates are quite different. Vertebrates usually possess several distinct molecular forms of the enzyme, whereas invertebrates typically exhibit only one form (Massoulié and Bon 1982).

Fournier et al. (1988) showed that the native AChE form present in the insect *Drosophila melanogaster* was characterized by noncovalent association of two dimeric subunits, resulting from proteolysis of a precursor encoded for by the *Ace* locus. In contrast, Talesa et al. (1997) demonstrated the presence of two acetylcholinesterasic forms in the annelid species *Spirographis spallanzanii*; these differed in several characteristics, such as relative amounts present, the way in which they anchored to the membrane and pharmacological properties (namely, different sensitivities to edrophonium or procainamide). Both are amphiphilic globular forms, and the main distinguishing feature between the two enzymes was their different manner of attachment to the cellular membrane. Whereas the predominant form established strong electrostatic interactions with the membrane, the other one was anchored through a phosphatidylinositol linkage. Unlike the forms reported by Fournier et al. (1988), the acetylcholinesterasic forms found by Talesa et al. (1997) were more likely to result from expression of different genes. The acetylcholinesterasic forms found in tissues of *Octopus vulgaris* by Talesa et al. (1995a) were more complex. These authors found that AChE from this species could be divided into amphiphilic, dimeric, and hydrophilic tetrameric forms that share a common pharmacological relationship with heparin. This tendency was similar for both forms that existed in *O. vulgaris*, and pointed to the existence of a single gene underlying the expression of AChE.

The ChE forms found in the mollusc species *Mytilus edulis*, *M. galloprovincialis,* and *Corbicula fluminea* were investigated by Mora et al. (1999). These authors reported that the cholinesterasic forms identified in *M. edulis* and *M. galloprovincialis* were almost identical in terms of mass (180 kDa), whereas the form found in the other mollusc species, a clam, had a higher mass (240 kDa). However, all forms shared a similar membrane anchorage in that they were connected by a glycosyl inositol phosphate residue. More recently, representatives of one of these same mussel species (*M. galloprovincialis*), collected in the Adriatic Sea, was analyzed by Talesa et al. (2001), who found three forms of ChE in its tissues. They found two hydrophilic, spontaneously soluble forms (accounting for approximately 80% of all hydrolytic activity) in the hemolymph that had dimeric and globular tetrameric structures. The third form was mainly an amphiphilic globular dimer attached to the cellular membrane by a phosphatidylinositol tail insertion. This form promptly interacted with detergent (such as Triton X-100 and Brij 96) and was likely to undergo self-aggregation.

The existence of highly variable polymorphism of AChE forms in vertebrates was illustrated by Rocío Marcos et al. (1998). The authors isolated, identified, and characterized the AChE present in sheep platelets, and showed the presence of several forms that could be differentiated according to their relative solubility in low- and high-level saline solutions and in detergents. Furthermore, the authors observed that the physical–chemical characteristics of the different forms could account for the differential solubility: the AChE of sheep platelets could be extremely hydrophilic (soluble in aqueous salt solutions) or could be amphiphilic. The amphiphilic, globular, membrane-bound form is thought to be the initial stage in which AChE is found in sheep platelets; however, it can be cleaved as a consequence of the activity of endogenous proteases and phospholipases, or by endogenous chemical reduction.

The cholinesterases present in the medicinal leach, *Hirudo medicinalis*, were also characterized by Talesa et al. (1995b). They found two main types of ChE: a low salt-soluble hydrophilic monomer and a detergent-soluble amphiphilic glycolipid-anchored dimer that differed markedly in terms of substrate preference and inhibitor efficacy.

3 Characterization of Cholinesterases

Different forms of ChEs can produce distinct types of hydrolytic capacity. Vertebrates show two main types of enzymes with cholinesterasic activity: AChE and BChE (Massoulié et al. 2008). Both cholinesterasic forms also exist in humans and are simultaneously present in the human brain. These two distinct forms are differentiated on genetic, structural, and kinetic bases. Age also affects the relationship between AChE and BChE, because their relative content varies with progressing age (Giacobini 2004). Other forms of variability also exist. Different AChE forms may result from the alternative splicing of a single gene (Massoulié et al. 2008). Aquatic organisms are also likely to produce different cholinesterasic forms, whose existence may be manifested through their selective affinity for specific inhibitors and/or substrates (Kozlovskaya et al. 1993).

Because of the inherent variability of ChE morphology and hydrolytic activity, one of the mandatory pre-requisites for use of cholinesterase inhibition in environmental analysis is the full characterization of the cholinesterasic form that is present in the tissue of the test organism(s) selected for use. If the most prominent form of a ChE present is not identified, it is impossible to positively identify the cholinesterasic forms for which hydrolytic activity is being assayed. In general, cholinesterase characterization involves using specific inhibitors to establish an inhibition profile of the enzymatic form under study; concurrently, it also involves the study of the substrates that the enzyme prefers.

ChEs belong to the family of enzymes designated as esterases that retain the capability of hydrolyzing carboxylic esters. ChEs can be distinguished from other esterases in that they exhibit a preference for hydrolyzing choline esters rather than other carboxylic esters; ChEs are generally inhibited by physostigmine (eserine) at concentrations in the range of 10^{-5} M (Nunes et al. 2005; Eto 1984). Eserine can also inhibit other esterases (e.g., carboxylesterases) that may be present in the same tissue, and this inhibitory effect is dependent on the substrate used in the enzymatic determinations (Laguerre et al. 2009), as has been reported in the snail species *Xeropicta derbentina*.

ChEs can be classified according to their preference for specific substrates: AChEs have a strong preference for acetylcholine; BChEs prefer butyrylcholine; propionylcholinesterases are better at degrading propionylcholine. Accordingly, numerous studies have been conducted to define the most common types of ChEs that occur in living organisms. In spite of being present in virtually all organisms, marked differences in ChEs have been observed in terms of substrate preference and hydrolytic

activity. Drawing from the points made above, vertebrates and invertebrates possess different types of cholinesterases that must be fully characterized prior to their use as tools in environmental assessment.

3.1 Unicellular Organisms

Intermediate hydrolytic characteristics were found in the protozoan *Dictyostelium discoideum* (Falugi et al. 2002). These authors revealed that this protozoan had an enzymatic form with cholinesterasic activity, which preferred acetylcholine and propionylcholine as substrates, whereas butyrylcholine was not metabolized.

3.2 Earthworms

ChEs of different natures and structures may exhibit different properties, and the inhibitory effects of specific ones may differ from species to species. One must keep in mind the variability that exists among these enzymes when establishing ecotoxicological assessments, particularly when assessing the toxic effects of pesticides in distinct organisms. The first efforts to characterize ChEs were made during the late 1970s and early 1980s. Andersen et al. (1978) refers to the presence of a cholinesterasic form in tissues of *Eisenia foetida* that showed a marked preference for propionylthiocholine (PSCh) as a substrate; consequently, this enzyme could neither be classified as an AChE nor as a BChE. However, this enzyme was inhibited by the presence of pesticides (both OPs and CBs), a finding that allowed researchers to conclude that it was a B-esterase. Principato et al. (1978) identified a cholinesterasic form in *Allolobophora caliginosa* that was inhibited by eserine, and it showed higher catalytic activity when in the presence of the substrate acetylthiocholine (ASCh). These findings led to the conclusion that tissues of this species were likely to have a predominance of AChE, rather than any other form. However, Principato et al. (1989) also characterized the other form as a propionylcholinesterase that could also exist in the same species. Stenersen (1980) found that the tissues of the earthworm *E. foetida* were rich in two cholinesterasic forms; the predominant form was a propionylcholinesterase, whereas the other form displayed the properties of a nonspecific ChE.

Stenersen et al. (1992) reported that ChEs of three species of earthworms (*Eisenia fetida*, *E. veneta,* and *E. andrei*) could be distinguished by their tendency to be inhibited by the carbamate pesticide carbaryl. *E. andrei* and *E. fetida* had distinct cholinesterasic forms, and one of these could be completely inhibited by carbaryl. The remaining forms were extremely resistant to this pesticide, which may be accounted for by the lack of responsiveness of these species to carbamate exposure. Aamodt et al. (2007) reported the dual role of the ChEs in *E. fetida*. The authors distinguished two cholinesterasic forms, both of which were inhibited by carbaryl. However, one of the forms was promptly regenerated, whereas the second had not recovered, even after 21 days. Such findings are noteworthy, since toxicological

monitoring of anticholinesterasic effects in the wild account for such biological variations, which in turn may explain increased survivals of a particular species.

Modern soil ecotoxicologists may also assess pollution through the use of cholinesterasic inhibition. When doing so, they must provide a full characterization of the cholinesterasic forms present in the test organism that is to serve as the model for toxicological interaction. One example was described by Caselli et al. (2006), when attempting to characterize the cholinesterasic forms present in the earthworm *Eisenia andrei*. These authors discovered the hydrolytic preference of the ChE present was for ASCh and PSCh; simultaneous inhibition of this enzymatic form was more effective after exposure to BW284C51, a compound that strongly reduced ASCh and PSCh hydrolysis. This chemical, however, only caused a slight inhibitory effect when the substrate used was BSCh. Exposure to tetra(monoisopropyl) pyrophosphortetramide (iso-OMPA) did not elicit any significant inhibitory effect, for all tested substrates. These results suggest the presence of an intermediate cholinesterasic form that can, to a similar extent, simultaneously hydrolyze ASCh and PSCh, without affecting butyrylthiocholine (BSCh). A similar procedure was adopted by Rault et al. (2007), for characterizing the ChEs of several species of earthworms (namely, *Lumbricus terrestris*, *L. castaneus*, *Aporrectodea nocturna*, *A. caliginosa*, *Allolobophora chlorotica*, and *Aporrectodea rosea*). Some observations were extremely interesting: the enzymatic activities were stable during the sampling campaign (12 month), favoring the use of ChE inhibition as an effect criterion during all four seasons. All tested species, with the exception of *A. chlorotica*, had AChE as the predominant form. This is an extremely important finding, because the quantification of biomarkers, including cholinesterasic activity, is a promising tool in soil ecotoxicity assessment (Scott-Fordsmand and Weeks 2000). However, the use of ChE inhibition as an effect criterion, when assessed in earthworms, is novel and must be further validated under field conditions (Rodríguez-Castellanos and Sanchez-Hernandez 2007a). Earthworms respond to anticholinesterasic compounds, but their response is somewhat unpredictable; issues such as high inter-individual variation must be taken into account and clarified for the successful use of cholinesterasic inhibition with earthworms.

3.3 Crustaceans

Varó et al. (2002) showed that the main cholinesterasic forms present in the crustacean species *Artemia salina* and *A. parthenogenetica* were different, despite the phylogenetic proximity of the two species. In fact, the cholinesterasic forms present in these two invertebrates were classifiable neither as AChEs, nor as BChEs. Indeed, these two enzymes retained intermediate characteristics, since their maximum hydrolytic activity was recorded with PSCh.

Forget et al. (2002) studied the hydrolytic profile of the ChEs found in the estuarine copepod species *Eurytemora affinis*. The authors found that AChE was the predominant form among all ChEs, because it preferred ASCh as a substrate. Although it degraded PSCh and butyrylthiocholine, the hydrolysis rates were much slower.

Furthermore, a distinct profile existed for this enzyme form that exempted it from being a pseudocholinesterase (PsChE); it lacked responsiveness toward the inhibitor iso-OMPA, but was susceptible to significant inhibition by eserine. Similar results were obtained for the marine copepod *Tigriopus brevicornis* (Forget and Bocquené 1999). These results underscore the importance of using autochthonous species that were previously characterized for cholinesterasic activity in environmental studies conducted in marine and estuarine environments. Stefano et al. (2008) indicated that the most important cholinesterasic form in gill and muscle tissues of the marine scallop *Pecten jacobaeus* was AChE, and this form shared common characteristics with mammalian AChE. The study results showed that the substrate preferred ASCh, and that the hydrolytic activity was almost fully inhibited by BW284c51, which is a specific AChE inhibitor.

The muscle tissue of the crayfish species *Procambarus clarkii* was shown to be rich in both AChE and BChE (Escartín and Porte 1996) but had higher AChE activity.

Key and Fulton (2002) showed that the grass shrimp *Palaemonetes pugio*, possessed AChE as the predominant ChE form, demonstrated by a higher preference for ASCh as a substrate. Furthermore, AChE activity in this species was inhibited by eserine, BW284c51, but not by iso-OMPA, showing that AChE was predominant over other ChEs.

Frasco et al. (2006) identified the main cholinesterasic form present in the eye tissues of the prawn *Palaemon serratus* as AChE. Similarly, the results obtained by Xuereb et al. (2007) showed that the most important ChE in the crustacean *Gammarus pulex* was AChE, because it preferred ASCh as a substrate and was inhibited by BW284c51.

3.4 Molluscs

Talesa et al. (2001) characterized the ChEs of *M. galloprovincialis.* These authors found that all forms of ChEs in the tissues of this mussel were likely to prefer ASCh as a substrate, and they found one form that did not hydrolyze butyrylthiocholine. The ChEs found were barely inhibited by compounds such as eserine and paraoxon, and no inhibition was observed for propoxur and diisopropylfluorophosphate. However, these ChEs were especially sensitive to BW284c51, which is a specific inhibitor of AChEs. This finding points to the intermediate hydrolytic characteristics of these ChEs.

An extreme case has been reported by Valbonesi et al. (2003), who observed no cholinesterasic activity for the bivalve species *Tapes philippinarum*. However, the same work showed that AChE was the predominant cholinesterasic form in the tissues of *M. galloprovincialis* and *Ostrea edulis*. These results were an important part of an effort to define an adequate battery of indicator organisms for use in the assessment of agricultural chemical residues (that were rich in anticholinesterasic agents) in the area of the Mediterranean. The authors suggested that both bivalves, *M. galloprovincialis* and *O. edulis*, can be used as suitable test organisms for this

purpose; in contrast, *T. philippinarum* is definitely not an adequate species for such biomonitoring of marine contamination by OP or CB pesticides.

AChE was found by Brown et al. (2004) in the gill homogenates of *M. edulis*. Despite being an invertebrate species, *M. edulis* gill ChE activity was significantly inhibited by exposure to the AChE-specific inhibitor compound BW284c51.

3.5 Fish

The concurrent presence of distinct forms of ChEs in the same organisms also occurs. Varó et al. (2003) showed that the main cholinesterasic form in the brain tissue of the fish *Dicentrarchus labrax* was AChE, whereas muscle tissue simultaneously possessed AChE and BChE. Similar results were obtained by Solé et al. (2008), when assessing the esterasic activity of muscle tissue from the marine fish *Lipophrys pholis*. The authors concluded that the predominant cholinesterasic form present was AChE. A comparable conclusion was obtained by Rodríguez-Fuentes and Gold-Bouchot (2004) for the most predominant ChE form present in brain of the freshwater fish tilapia (*Oreochromus niloticus*). AChE was more abundant in nervous tissue, while atypical forms were more evident in liver and muscle tissues.

Similarly, the study conducted by Arufe et al. (2007) showed that the predominant cholinesterasic form in the gilthead seabream (*Sparus aurata*) larvae was AChE. The authors determined the substrate preference of whole-body cholinesterasic activity of yolk sac seabream larvae and observed a noticeable preference for AChE, followed by propyonylthiocholine, and finally BChE. Additionally, the enzymatic activity of homogenized tissues was almost fully inhibited by BW284c51, indicating the presence of AChE. Jung et al. (2007) also discovered that a considerable portion of the cholinesterasic activity in the muscle tissue of the sole species *Limanda yokohamae* was attributed to BChE. However, the most important form identified in the nervous tissue was AChE.

Sturm et al. (1999a) reported the presence of AChE as the dominant cholinesterasic form in brain tissue of several marine fish species, such as *L. limanda*, *Platichthys flesus*, and *Serranus cabrilla*. This was an indisputable finding because AChE is predominant among all cholinesterasic forms that exist in vertebrate species; however, muscle tissue is somewhat different, in that the simultaneous presence of butyryl- and acetylcholinesterase was observed. One of the major findings described in this paper for this tissue was the responsiveness of the characterized butyrylcholinesterase. This enzymatic form was much more sensitive toward OP pesticides than was AChE; this observation may be important in future marine risk assessment.

Sturm et al. (1999b) characterized the cholinesterasic forms present in the tissues of the freshwater fish species *Gasterosteus aculeatus*. They observed that this enzyme form's capacity to hydrolyze ASCh, and its near complete inhibition by eserine, indicated that this fish had a ChE in its muscle tissue. However, this

cholinesterasic form was intermediate in terms of substrate preference, since it could degrade both ASCh and BSCh. The authors postulated that *G. aculeatus* muscle ChE was an atypical PsChE that had intermediate sensitivity to the inhibitory compound BW284C51 (complete inhibitor of AChE in mammals).

Garcia et al. (2000) reported similar results when studying the ChE content of the freshwater fish species *Poecilia reticulata*. These authors found that the main cholinesterasic form present in head tissue of the mentioned species was AChE. In agreement with these results, Nunes et al. (2005) found that the most active cholinesterasic form that existed in nervous tissue of *Gambusia holbrooki* was AChE.

The characterization of the predominant ChE forms present in tissues of *Pomatoschistus microps* was performed by Monteiro et al. (2005). They reported that the ChEs present in the homogenized tissues from the head of the organism had a clear preference for ASCh as a substrate and the reaction was inhibited by BW284C51. The results obtained in the entire head homogenate with iso-OMPA (a common BChE inhibitor) showed that a significant inhibitory effect was possible. These two results are consistent with the possibility that an atypical form of ChE exists and seemed to indicate that there was an intermediate behavior of the ChE present in *P. microps* tissues.

ChEs may be present in tissues other than that of the nervous system and muscles. Wogram et al. (2001) showed that the responsiveness of the butyrylcholinesterase found in tissues (liver and axial muscle) of the freshwater fish species three-spined stickleback (*G. aculeatus*) was clearly higher than was AChE, following exposure to parathion. The importance of the significant inhibition of BChE in liver tissue is high: parathion requires a previous metabolic activation (with consequent formation of paraoxon, the active metabolite), namely, through cytochrome P450 oxidative activity to exert its anticholinesterasic activity. Because liver tissue is rich in cytochrome P450, it is natural that the hepatic BChE form becomes immediately inhibited after parathion metabolism. In general terms, hepatic BChE was 1,000-fold more sensitive than was AChE. This finding is important in terms of use of this species in environmental assessment, since extremely short periods of exposure can be better assessed by the quantification of BChE, rather than AChE activity.

The assessment of the anthropogenic impact on reef ecosystems was also the main concern that drove Leticia and Gerardo (2008) to characterize the ChEs of the fish species *Haemulon plumieri*. These authors reported higher enzymatic activities, specifically measured as AChE, in brain and liver tissues.

3.6 Reptiles

Bain et al. (2004) characterized the plasma cholinesterasic forms that existed in the lizard species *P. vitticeps*. The authors observed that the predominant form present was butyrylcholinesterase. A similar result was obtained by Sanchez-Hernandez and Sanchez (2002) in plasma of the reptile species *Gallotia galloti*. Despite the presence of butyrylcholinesterase in serum, a residual amount of AChE was also present.

The total amount of detected serum BChE constituted 74% of total cholinesterasic activity (Sanchez-Hernandez 2003). However, in this same study it was also shown that brain ChEs were exclusively composed of AChE.

An extensive study was conducted by Schmidt (2003) to characterize the ChE activity of reptiles, which included several species such as the spotted turtle (*Clemmys guttata*), river cooter (*Pseudemys concinna*), logerhead sea turtle (*Caretta caretta*), Texas hornet lizard (*Phrynosoma cornutum*), desert hornet lizard (*P. platyrhinos*), round-tailed hornet lizard (*P. modestum*), eastern cottonmouth rattlesnake (*Agkistrodon piscivorus*), western diamondback rattlesnake (*Crotalus atrox*), American alligator (*Alligator mississippiensis*), and Morelet's crocodile (*Crocodylus moreletii*). For all tested species, brain ChE was predominantly composed of AChE, while plasma was particularly rich in butyrylcholinesterase.

4 Usefulness of Cholinesterase Inhibition in Environmental Monitoring

4.1 Classic Use for Assessing the Environmental Effects of Anticholinesterasic Agents

The classic role attributed to AChE inhibition in environmental analysis has been related to the assessment of effects caused by exposure to the OP and CB pesticides. These classes of compounds share a common mechanism of toxicity, i.e., both classes exert their toxic action directly on the active catalytic site of AChE, thus preventing the in vivo physiological hydrolysis of the neurotransmitter acetylcholine that is needed to terminate the nerve impulse. Some OPs are initially inactive and require in vivo metabolic activation; such activation may dramatically increase toxicity, as observed by Jokanovic (2001). OPs are thus considered to be irreversible AChE inhibitors, requiring a full de novo synthesis of the inhibited enzyme, whereas CBs may be hydrolyzed and consequently removed from the active binding site (Zinkl et al. 1991). The most prominent consequences of exposure to these agents are neurotransmission/neuromuscular impairment. These agents are widely employed to control the unwanted presence of insect pests, and both classes of pesticides are characterized by their propensity to exert deleterious effects on nontarget species. The effects they induce have been extensively analyzed by quantifying the actions of AChE in exposed organisms. Such effects (enzymatic inhibition) explain the significance of the classic role attributed to AChE inhibition as an effect criterion in Ecotoxicology. The need to define a sensitive marker for anticholinesterasic agents led Magnotti et al. (1994) to study the ChE activity of 28 fish species. Such ChE activity in fish may act as a sentinel for the presence of OP compounds in environmental assessment studies. This research team found that, among all tested species, sea bass and flatfish possessed the highest levels of ChEs, and these two species are thus construed to represent prime candidates for environmental assessment of neurotoxic compounds. In this study, the authors proposed the use of the

two mentioned fish species as sentinels to monitor for the presence of anthropogenic agents, such as OP pesticides, hypochlorite-activated organothiophosphates and CB pesticides. Based on the high responsiveness of ChEs from these species, the presence of such compounds can be identified and monitored.

An extensive survey was performed by Chuiko (2000) on the effects of dimethyl 2,2-dichlorovinyl phosphate (DDVP) on ChEs in 11 fish species, (*C. carpio*, *Abramis brama*, *A. ballerus*, *Blicca bjoerkna*, *Rutilus rutilus*, *Alburnus alburnus*, *Leuciscus idus*, *Perca fluviatilis*, *Stizostedion lucioperca*, *Esox lucius,* and *Coregonus albula*). The results of this study indicated that the inhibitory effects caused by DDVP on AChE and BChE activities in brain and serum were rapidly manifested in these species, and brain and serum AChE was promptly inhibited by the insecticide in a manner similar to that already observed for mammals. However, serum BChE was extremely sensitive to DDVP and was more sensitive than were mammalian ChEs.

ChE inhibition has been a widely applied endpoint to assess exposure of OP and related compounds. In addition to the utility of this biomarker in ecotoxicity, human toxicology investigators have quantified red blood cell ChE inhibition to diagnose OP exposure in humans. MacGregor et al. (2005) showed that human sensitivity to the insecticide dichlorvos was similar to that of other test organisms, such as rodents, primates, and dogs. This is important, because it shows that the physiologic role attributed to ChEs is well preserved among vertebrates and is an accurate and reliable marker of neurotoxicity.

Comparing the sensitivity of anticholinesterasic compounds in invertebrates has been a topic of interest to Sánchez-Hernandez (2007). In his review, several invertebrate organisms were defined as possessing high levels of carboxylesterases (CbE), and the sensitivity of these enzymes to OP pesticides is much higher than that of the AChE present in the central nervous system. Therefore, the levels of CbE must be considered when characterizing the toxic response of such organisms to OP pesticides. Therefore, quantifying CbE, rather than AChE inhibition, after OP pesticide exposure (Sánchez-Hernandez 2007) may be equally or more useful.

There are numerous research articles in the field of Ecotoxicology that address the use of AChE as a biomarker. Sancho et al. (2000) exposed eels (*Anguilla anguilla*) to the CB thiobencarb, and monitored the AChE activity in the eyes of this organism for a period of 1 week after exposure. The authors concluded that AChE inhibition could function as a sensitive and satisfactory biomarker for verifying the exposure of this organism to the tested carbamate. Moreover, the ocular tissues showed that sensory organs are also prone to the inhibitory effects of carbamates.

Varó et al. (2002) showed that AChE inhibition occurred after exposure of two species of *Artemia* (namely *A. salina* and *A. parthenogenetica*) to the insecticides chlorpyrifos and dichlorvos. However, AChE inhibition in both of these crustaceans was not indicative of major toxicological implications AChE activity inhibition was not directly correlated with mortality, since death of exposed organisms only occurred at inhibitions exceeding ~80% of the initial enzymatic activity. These results indicate that this enzyme is extremely sensitive as a potential biomarker for pesticides exposure.

The use of OP and CB pesticides in common agricultural processes may have direct consequences on non-target species (Ferrari et al. 2007). These authors studied the inhibitory effects of the OP insecticide azinphos-methyl (AzMe) and the CB

pesticide carbaryl on juveniles of rainbow trout. They concluded that normal patterns of use of both compounds can exert significant real effects on cholinesterasic forms of both head and muscle tissues in fish.

The effects of chlorpyrifos and carbaryl on AChE of hybrid catfish (*Clarias macrocephalus* × *C. gariepinus*) were studied by Somnuek et al. (2007). In this work, test organisms having different body sizes were exposed to sublethal concentrations of the test compounds for a period of 4 days. The endpoint was AChE inhibition and was measured in several tissues: brain, liver, muscle, and gills. The authors observed a marked inhibition of enzyme activity in all tissues. Nevertheless and not surprisingly, AChE inhibition was more prominent in brain tissues. The animals were subjected to a recovery period that was too short to permit a total recovery of the inhibitory effects. These results indicate that deleterious effects caused on non-target species may persist for long periods and may be permanent.

Marine environments are also prone to toxic damage by anti-cholinesterasic agents. Accordingly, Brown et al. (2004) showed that the main cholinesterasic form present in *M. edulis* gill homogenates was responsive to the insecticide azamethiphos (calculated IC_{50} of 100 mM). However, when *M. edulis* was used as a sentinel species in marine environmental assessment, the AChE present in *its* tissues was less susceptible to significant inhibition and was less responsive than in other marine species. The AChE present in eye tissues of *P. serratus* exhibited extreme sensitivity toward OP and CB pesticide exposure (Frasco et al. 2006). The authors tested exposure to the CB carbofuran and the OP chlorpyrifos-oxon, and concluded that this crustacean could be useful for assessing pesticide contamination in saltwater.

The issue of sensitivity of ChEs was also assessed by Jung et al. (2007). They performed a study to quantify the inhibitory effect caused by the insecticide iprobenfos on both acetyl- and butyryl-cholinesterase of *L. yokohamae*. The study results showed that sensitivity may not only relate to the type of ChE involved in the toxic response, but may also derive from a tissue-specific trend. Despite being extremely sensitive when present in muscle tissue of the test organisms, AChE from the nervous system was rather insensitive to the inhibitory effects caused by iprobenfos. Muscle butyrylcholinesterase activity was also sensitive to inhibition by the tested insecticide. These findings suggest that the selection of the most appropriate tissue is mandatory when assessing environmental effects caused by specific pesticidal agents.

The discussion concerning the sensitivity of cholinesterasic forms to be used in quantifying deleterious biological effects was also addressed by Rodríguez-Fuentes et al. (2008). In their study, these authors refer to the presence of atypical cholinesterasic forms in two species of marine fish, namely *Pleuronectes vetulus* and *Pleuronichthys verticalis*. The authors discovered that *P. vetulus* had a predominance of AChE over BChE in muscle tissue, and that the total length of the fish was a critical experimental variable, in that total cholinesterasic activity was negatively correlated with total body length. Furthermore, no significant effects resulted from gender differences or sampling sites. This later observation is extremely important, because, in this study, two quite different priority sampling sites were defined: (1) a sampling site near the wastewater release point coming from a sewage treatment plant and (2) an off-coast location, far from any apparent human influence. The situation

with *P. verticalis* was different, because the majority of the cholinesterasic activity in muscle derived from the presence of butyrylcholinesterase. Furthermore, the sensitivity of ChE to pesticide inhibition was higher in males than was the sensitivity registered for females.

ChEs found in *M. galloprovincialis*, *M. edulis,* and *C. fluminea* exhibited a similar pharmacological behavior, which was characterized as extremely refractory to OP compound exposure (Mora et al. 1999). This finding was significant, because the authors suggested that this species only be used when particularly heavy contamination of OP pesticides existed, rather than being employed in low-level contaminant (chronic) surveys. A different result was obtained by Dauberschmidt et al. (1997) after exposing zebra mussel (*Dreissena polymorpha*) to anti-cholinesterasic agents, such as thiometon, disulfoton, and demeton-S-methyl. Despite having identified the presence of ChEs by radiolabeling of the serine residue in the active site, the authors observed an absolute lack of response of ChE to the tested compounds. Furthermore, the calculated ChE activity was extremely low when compared with cholinesterasic activities already reported for other mollusc species. The range of tested concentrations did not elicit any significant effect in terms of cholinesterasic inhibition, even after the death of exposed organisms. These observations allow one to conclude that interspecific differences must always be taken into account, since similar organisms may retain large differences in the nature and levels of enzymes present.

The effectiveness of OP compounds as anticholinesterasic agents was also assessed in reptiles. Sánchez-Hernandez and Walker (2000) observed the inhibitory effects of the insecticides trichlorphon and parathion on serum and brain ChEs of the lizard *G. galloti*. Besides evaluating the potential inhibitory effects by these OP compounds, the authors also observed that the lizards were less sensitive than were certain other organisms (such as birds or mammals) to the inhibitory effects of the OPs. This same species was again used by Sanchez-Hernandez (2003) to assess the potential impact of agrochemicals on serum BChE activity. The inhibition levels of BChE in animals collected from highly impacted agricultural landscapes were studied in comparison with the results from organisms collected at pesticide-free sampling sites. Results were that the quantification of serum cholinesterase (BChE) in *G. galloti* was a reliable tool to assess the contamination by anticholinesterasic compounds under field conditions. Similarly, Bain et al. (2004) tested the anticholinesterasic effects of fenitrothion on the Australian lizard species *P. vitticeps*. This organism was responsive, in terms of ChE inhibition, to the presence of the insecticide. Other than a significant and dose-dependent ChE inhibition, no other evident symptoms (e.g., changes in diurnal body temperature or alterations in standard metabolic rate) were correlated with exposure to this insecticide.

Effects on ChEs, following accidental exposures of non-target organisms in the wild, may not always be transient. Even if the effects derived from that exposure do not culminate in death, medium to long-term effects are likely to occur and are often not benign. This was shown by Sancho et al. (1998), in a study in which Europeans eels (*A. anguilla*) were acutely exposed to the insecticide fenitrothion. After exposure, a batch of organisms immediately analyzed for plasma AChE activity showed a marked inhibition. A second batch of exposed animals was allowed to

recover; however, recovery was only permitted for a limited period (1 week), after which quantification disclosed AChE activity to be lower than for non-exposed organisms. These results provided evidence that physiological impairment may derive from exposure to anthropogenic chemicals and may be sustained for long periods. These authors suggested that the type of inhibition they observed was not transient, and that a de novo synthesis of the impaired enzyme is required for re-establishing normal levels of activity.

Serum BChE and CbE inhibition were used as effect criteria in a field survey conducted by Sanchez et al. (1997). The authors quantified the levels of these enzymes in plasma of the reptile species *G. galloti*. According to the results obtained, these enzymes could be effectively used as biomarkers to assess pesticidal contamination in reptiles. The authors of this study described the significant inhibitory effects caused by parathion on serum BChE and CbE, for a period of 23 days after the initial spraying and exposure. Consequently, the authors sustained the view that these markers can serve as long-term indicators of chemical contamination if assessed in the serum of the mentioned lizard species.

AChE inhibition was also the criterion adopted by Gao and Zhu (2002), when studying the resistance to OP pesticides exhibited by the insect species *Schizaphis graminum*. The authors concluded that the efficacy of the insecticides (or their active metabolites) chlorpyrifos oxon, paraoxon, methyl paraoxon, malaoxon, demeton-S-methyl, and omethoate was similar in all organisms, but the ones that showed higher resistance had higher levels of AChE. In fact, the authors concluded, resistance came from the increased expression of a ChE gene.

Rahman et al. (2000) showed that OP compounds are unlikely to exert a simple inhibitory effect on the ChEs of exposed organisms. The novel phosphorothionate (2-butenoic acid)-3-(diethoxy phosphinothioyl)-methyl ester was shown to be effective on the AChE activity of rodents but could also affect the activity of several ATPases of the exposed animals. In addition, the authors obtained interesting data that pointed to a gender-selective toxicity (larger susceptibility of females) and a greater sensitivity of cholinesterasic forms to the tested compound. Inhibitory profiles of the effects on different enzymes were created by the authors, but it was clear that recovery of enzymatic activities had not occurred after a period of 28 days. These findings underline the importance of ChEs as putative markers for intoxication by OP compounds.

Hai et al. (1997) showed that the freshwater fish species *C. carpio* was sensitive to the inhibitory effects of the insecticide dichlorvos, with substantial AChE inhibition occurring following exposure. However, this compound not only elicited the common and expected effects related to AChE inhibition but also caused major redox impairment, such as effects on superoxide dismutase, catalase, and lipid peroxidation. It was interesting to note that it was possible to establish a relationship between the two endpoints (neurotoxicity vs. oxidative stress). From these results, it is possible to observe that anthropogenic compounds may exert deleterious effects on biota by various pathways. It is absolutely mandatory to establish comprehensive batteries of biomarkers to fully understand the array of toxic responses that may occur in the wild. Risk assessment studies, based on biomarkers (such as quantification of cholinesterasic activity), may be influenced in unpredictable ways by various confounding factors in the real world.

The use of ChE inhibition as an effect criterion was proposed by Sturm et al. (1999b) for assessing the presence of anti-cholinesterasic agents in streams. This research team selected muscle tissue of the freshwater fish species *G. aculeatus* to assess the combined effects of OP pesticide residues present in the area streams. Their results showed that it was possible to successfully use this biomarker to monitor freshwater environments that are potentially contaminated by OP compounds. The authors reported that the ChE activities measured at several sampling sites varied during the year according to a gradient that corresponded to amounts of organophosphates present. It was concluded that ChE inhibition is a sensitive tool for environmental analysis. The use of fish in biomonitoring programs requires knowledge of the effect of environmental variables on the studied response. For example, cholinesterasic activity in sensitive fish may vary with fluctuations in salinity (Wang et al. 2001). The authors also showed that the neurotoxicity of the CB insecticide aldicarb (evaluated in terms of ChE inhibition) was enhanced in rainbow trout (*O. mykiss*) but not in hybrid striped bass (*Morone saxatilis*×*chrysops*). This finding is important, because during the selection of test organisms for use in biomonitoring, some species possess distinct sensitivities to OPs, and these sensitivities may be affected by the abiotic conditions that exist during toxicant exposure.

The use of living organisms as sentinels of chemical contamination can also be confounded by intrinsic biologic characteristics, such as the age of the test individuals. To study the effects of age on the toxicity of anthropogenic contaminants, Sánchez-Fortún and Barahona (2001) exposed groups of *Artemia salina* of different ages to the insecticide carbophenothion (ChE inhibitor). Increasing age was responsible for enhanced toxicity. Furthermore, the protective role that a specified compound (e.g., ChE-reactivating oxime 2-pyridine aldoxime methochloride; 2-PAM) exerted on carbophenothion toxicity was addressed in the same study. The results showed that by enhancing the regeneration rate of ChEs, 2-PAM significantly reduced the toxicity of this pesticide. The protection offered by compounds such as 2-PAM can also be used as an indirect biomarker of exposure to anticholinesterasic agents. McInnes et al. (1996) observed that wild birds captured in the vicinity of agricultural fields in which the OP insecticide chlorpyrifos was used had lower levels of ChE, AChE, and BChE, when compared with animals from non-contaminated areas. Furthermore, the tested wildfowl required higher amounts of 2-PAM to reactivate AChE activity, which had an unequivocal sign that the birds suffered OP poisoning. A similar study was conducted by Parsons et al. (1999) to assess the presence of anticholinesterasic pesticide residues that could affect bird colonies located mainly in northeast US estuaries. Evaluation of anticholinesterasic effects was again performed through the use of the reactivating oxime, 2-PAM, which gave positive results of the sort mentioned before: birds from agricultural areas were more likely to suffer symptoms of OP poisoning and to require 2-PAM for regenerating serum ChE levels.

Total AChE inhibition and reactivation were the toxicological parameters selected by Maul and Farris (2005) to show the potential impact of anticholinesterasic agrochemicals on *Cardinalis cardinalis*. The authors found that, despite large fluctuations among the groups of tested individuals, the majority of birds captured in OP-use areas had total ChE activities below the diagnostic threshold. Moreover, reactivation rates were also higher, indicating the usefulness of this parameter as an

indicator of OP or CB use. Birds were also the subject of a study undertaken by Iko et al. (2003). These researchers hypothesized that the population decline observed for the mountain plover (*Charadrius montanus*) was possibly associated with a toxic effect caused by agricultural use of OPs. To verify this hypothesis, the work team assessed the serum levels of ChE and found no recent contamination by OPs, as measured by ChE depression; furthermore, potential contamination of wild birds was not confirmed, since no significant differences were observed from animals that came from different areas.

The reactivation of plasma BChE by 2-PAM in *G. galloti* was the best criterion to diagnose exposure to OP and CB pesticides, as shown by Sánchez-Hernández et al. (2004). These authors monitored several populations of this species that existed in specific geographical locations. The organisms captured near agricultural fields had lower serum BChE activities and required higher amounts of 2-PAM.

The Antarctic Ocean is considered to be one of the last pristine environments, and the need to assess anthropogenic pollutant effects of species in it were undertaken by Stefano et al. (2008). They studied the inhibitory effects caused by the pesticides azamethiphos and diisopropylfluorophosphate on the ChEs of *P. jacobaeus*. These authors found that azamethiphos was highly effective in inhibiting cholinesterasic activity, but diisopropylfluorophosphate did not have this effect.

Arufe et al. (2007) studied the effects of azinphosmethyl on the mortality in relation to AChE in *S. aurata* larvae. The study results demonstrated that this insecticide could exert lethal effects in exposed larvae, and the effects were accompanied by a significant AChE inhibition. The relevance of the results obtained by Xuereb et al. (2007) was discussed after they assessed the effects of the OP insecticide chlorpyrifos on ChEs of *G. pulex*. They concluded that the mortality of individuals of this crustacean species occurred at AChE inhibitions of approximately 50% of the initial activity. This means that lethal results can be produced at ecologically relevant concentrations of chlorpyrifos.

The toxic effects of commercial formulations (mixtures), rather than only the technical compounds are important in environmental toxicology. Gambi et al. (2007) showed the differences between the pesticide carbaryl and its commercial formulation on the enzyme kinetics of the earthworm *Eisenia andrei*. The effects of the single compounds and formulated pesticides were somewhat similar in inhibiting AChE. However, when the inhibition was time- and dose-correlated, differences existed in the way the exposure occurred. Notwithstanding, the authors emphasized the importance of AChE inhibition as an effect criterion for pesticide exposure in earthworms. Earthworms are often the unintended targets of pesticides, and when they interact it often culminates in lethal effects. To evaluate the impact of pesticides on earthworms, Panda and Sahu (2004) quantified the extent of AChE inhibition that occurred in tissues of the oligochaete *Drawida willsi*, after exposure to three common insecticides (butachlor, malathion, and carbofuran). These authors observed a significant decrease of AChE activity after exposure to the last two compounds, and this effect persisted for periods ranging from 45 to 75 days. This result indicates the potential deleterious effect that may be derived from pesticide use, even when the use is at levels that give ecologically relevant dosages.

The environmental toxicity of OP pesticides is not limited to immediate or acute effects, as illustrated in a study of the interaction of OPs and ChEs to assess the ecotoxicological impact of pesticides in earthworms (*E. fetida* and *L. terrestris*) (Rodríguez-Castellano and Sanchez-Hernandez 2007b). These authors observed the phenomenon of ChE "aging," which depends upon the release of an alkyl group from the OP–ChE complex, following the inhibition of ChE activity by OPs. This release has a direct consequence: the enzyme can no longer be reactivated, neither spontaneously, nor by the activity of reactivating chemicals, such as pralidoximes. This "aging" of ChEs in earthworms is highly dependent on the chemical structure of the OP pesticide tested, and may possibly be reverted through the use of pralidoxime; consequently, the amount of the antidote used to reactivate inhibited ChEs in earthworms can serve as a contamination index to evaluate OP pesticidal contamination.

Intoxication and inhibition of ChEs by OP compounds is usually followed by a period of recovery. The study by Barata et al. (2004) reported such behavior sensitivity and recovery efficacy of AChE and CbE in juveniles of the freshwater crustacean *D. magna*. In general, CbE was most sensitive to the OPs malathion, and chlorpyrifos; however, both enzymes had equal sensitivity to the CB carbofuran. Recovery rates were similar for all three tested compounds and for both enzymes, since a period of 12–96 h was sufficient to mitigate the inhibitory effects. The environmental significance of this research article was subsequently underscored by other observations that were clear in showing that exposure to OPs and CBs have different consequences. The mortality rates of *D. magna* juveniles increased after OP pesticide inhibition of about 50% of the basal AChE activity; in contrast, carbofuran caused a more pronounced response, since mortality was significantly increased after a very slight AChE inhibition. It is assumed that these results have ecological importance, because they demonstrate that mechanistically similar compounds may have distinctly different outcomes in terms of lethality.

The cholinesterases present in muscle tissue of the crayfish *P. clarkii* were responsive to high concentrations of the insecticide fenitrothion (Escartín and Porte 1996). Both AChE and BChE were significantly inhibited after exposure to a concentration of 20 μg/L. The AChE inhibitory response was dose-dependent; death resulted after reaching AChE inhibition rates of between 39 and 42%. The time course of the intoxication showed that the enzyme inhibition was long lasting. According to the mathematical model that described the recovery kinetics of this intoxication, the predicted time of recovery for muscle AChE was 29 days; therefore, the mode of action of OPs implies a definitive, irreversible inhibition of the affected enzyme, requiring de novo synthesis.

4.2 Use for Diffuse Contamination Sources

Malany et al. (1999) showed that AChE hydrolytic activity was highly dependent upon the electrostatic environment in which hydrolysis takes place. The enzyme itself shows a strong electrostatic dipole that is believed to be important for its

hydrolytic capacity. In most AChE forms, the movement of substrates into the catalytic gorge is favored for cationic molecules, such as acetylcholine (Massoulié et al. 2008). It is thus licit to affirm that AChE activity may be theoretically modulated by many ionic interactions with charged substances that may exist in the environment, namely those deriving from anthropogenic activities. In fact, several studies point to the establishment of an allosteric interaction, not in the active site, but in a distinct segment of the protein, that can explain the inhibition observed for some agents (Kitz et al. 1970). The use of AChE inhibition for environmental analysis has been traditionally linked to pesticide contamination of the aquatic and terrestrial compartments. However, the alleged versatility of AChE inhibition, as an effect criterion after exposure to other non-specific classes of contaminants, has led researchers to employ this biomarker in broad studies for the assessment of effects of diffuse sources of contamination. Kopecka et al. (2004) offers a good example of this trend, since these authors suggested that AChE inhibition be used in selected species to evaluate the anthropogenic contamination status in the area of the Gulf of Gdansk (Baltic Sea, Poland). The authors found that, in general terms, AChE inhibition (measured in gill tissue of the mussel species *Mytilus trossulus*, and also in the muscle tissue of the fish *P. flesus*) followed the patterns and gradients of contamination, mainly resulting from the presence of hydrocarbon runoff (from accidental oil spills), port activities, and domestic and industrial sewage discharges.

4.2.1 Metals

Inhibition of ChEs was reported for a significant number of non-OP pesticides; compounds such as metals or detergents have been found to significantly inhibit in vitro AChE activity in sensitive species, such as *P. reticulata* (Garcia et al. 2000). However, these results seem contradictory with the results published by Romani et al. (2003), who showed that chronic exposure of the fish species *S. aurata* to sublethal concentrations of metallic copper could result in an increase of acetylcholinesterasic activity, both in muscle and brain tissue.

Despite the above-mentioned studies, the point concerning significant interference of metals by AChE (Frasco et al. 2005) suggests that this trend may be an artifact. According to Frasco et al. (2005), AChE inhibition by metals may not be an actual inhibition and may not derive from interference with the catalytic activity of the enzyme. Rather, the inhibition may result from an interference with an important particle present in the reactive medium. The most common methodology employed for determining AChE activity was developed by Ellman et al. (1961). Their method is one in which the enzymatic catalytic activity is responsible for cleavage of the artificial substrate ASCh; this leads to the formation of thiocholine, which in turn reacts with Ellman's reagent (5,5′-dithiobis(2-nitrobenzoic acid; DTNB)) to form a colored complex that can be assessed at a wavelength of 412 nm. Metals, as observed by the authors, can react with all particles present in the reaction media, reducing the formation of the colored complex, and, thereby, leading to the erroneous conclusion that AChE is being inhibited. These observations are only

relevant under in vitro conditions, because exposure in living organisms cannot be measured the same way. This may explain the results obtained by Cunha et al. (2007), when they studied the potential effects of metallic species on ChEs of the gastropods *Monodonta lineata* and *Nucella lapillus*. These authors observed that cadmium increased the ChE activity of *N. lapillus*, whereas no effects were observed on the ChE of *M. lineata* following in vivo exposure. However, in vitro copper exposure decreased the ChE activity of both species, but exposure in vivo was incapable of exerting any effects.

Several examples of published studies indicate the lack of in vivo response of ChEs to metals. Corsi et al. (2004) showed that the cholinesterasic forms present in the scallop *Adamussium colbecki* were refractory to zinc metal exposure, compromising its use in biomonitoring programs for assessing metal contamination in Antarctica. Metallic compounds, such as zinc, copper, and cadmium, were incapable of inducing any sensitive modification in ChE activity from the digestive gland of the snail species, *Helix aspersa* (Dahm et al. 2006). Lead was used to study the influence of metals on several parameters in the grasshopper species *Aiolopus thalassinus* (Schmidt and Ibrahim 1994); these authors observed that lead was capable of significantly inducing ChE activity in this insect. Stefano et al. (2008) reported a high refractory behavior of the ChEs of *P. jacobaeus* after in vitro exposure to zinc; however, the same enzymes were dose-dependently inhibited by cadmium. These apparently contradictory results show that metallic inhibition of ChEs is entirely dependent on the chemical species tested.

The discussion on the feasibility of using ChE inhibition to assess metal toxicity is directly connected to the dose needed to exert a deleterious effect. Sánchez-Hernandez (2001) is quite clear about this point. The concentrations of a metallic species needed to cause a significant ChE inhibition in test organisms is incomparably higher than the amounts of pesticide needed to cause a similar effect. It is not plausible that such high concentrations will ever, in fact, be commonly attained under normal environmentally relevant conditions. Hence, wild organisms are not expected to succumb to effects from metal exposure that results from ChE inhibition.

4.2.2 Surfactants, Detergents, and Other Organic Compounds

Guilhermino et al. (2000) reported a significant inhibition of AChE by surfactant agents in the crustacean species *D. magna*. The authors monitored the influence on these daphnids of the detergents dodecyl benzyl sulfonate, sodium dodecylsulphate (SDS), and a commercial domestic formulation and concluded that all agents were capable of inhibiting AChE, following both in vitro and in vivo exposures. However, Nunes et al. (2005) observed that the alleged inhibitory effect initially reported may derive from the specific physical effect of the detergent activity of such compounds. This work showed that after homogenizing the nervous tissue of a test organism, detergents (such as SDS) tend to form micelles that dissolve portions of cellular membranes. These micelles integrate AChE that is lipid-bound to cellular membranes and prevent the enzyme from interacting with the chemicals used in the

Ellman assay. Such micelles can be reverted by modifying the dieletric constant of buffered media by adding ethanol. Ethanol destabilizes the micelles and results in the release of membrane-bound AChE. By adding ethanol the authors were able to reduce the inhibitory effects of SDS. This suggests that the inhibitory effects caused by exposure to detergents may be a protocol artifact. Li (2008) showed that inhibition of ChE of the planarian species *Dugesia japonica* by detergents or surfactants was clearly dependent on the chemical structure of the compound. This author found that ChE inhibition was possible after exposure of test organisms to Hyamine 1622, pentadecafluorooctanoid acid, perfluooctane sulfonate, and four nonylphenol. In contrast, exposure of planarians to Triton X-100 caused a significant increase in cholinesterasic activity. From these results, no clear pattern arose, indicating that any general conclusion of ChE inhibition by these chemicals is uncertain.

Gonçalves et al. (2010) showed that no effects occurred after acute in vivo exposure of *G. holbrooki* to two types of detergents: anionic (sodium dodecyl sulfate) and cationic (benzalkonium chloride) compounds. Despite the different typology of the tested substances, no evidence existed to show AChE impairment. From these results, again no clear pattern emerged, which leaves doubt that these ChEs are actually inhibited by these chemicals.

The inhibition of AChE by nonspecific agents was referred to by Pham et al. (2010), when they were considering the biological and environmental effects caused by ionic liquids. These authors published an extensive review about the putative effects of ionic liquids, and observed that they could impair a large number of physiologic pathways, such as oxidative homeostasis, AMP deaminase, and most importantly, AChE inhibition.

Solé et al. (2008) showed the utility of AChE inhibition in marine Ecotoxicology. The authors observed a significant inhibition of muscle tissue AChE in the fish *L. pholis*, probably from exposure to neurotoxic compounds present in the marine environment that derive from urban and industrial pollution along the Portuguese Atlantic coast. Klumpp et al. (2007) employed ChE inhibition as a tool to assess anthropogenic effects on the marine fish *Plectropomus leopardus* taken from the Great Barrier Reef. They concluded that ChE was inhibited in fish captured at sampling sites located near pontoons, indicating that man-made chemicals are impacting these areas by exerting deleterious effects on wildlife. The fish species *H. plumieri* can be a sensitive species for assessing the effects of man-made chemicals in reef ecosystems (Leticia and Gerardo 2008). The results of this study showed that the AChE present in the tissues of this species was inhibited by chlorpyrifos and benzo(a)pyrene, which were used as model pollutants. This finding affirmed the potential role for this marine organism as a bioindicator species for monitoring organic chemical contamination.

With the aim of using standardized tools in a future biomonitoring programs adapted to the Antarctic region, Bonacci et al. (2009) studied the potential for using ChE activity in gill from the scallop species, *A. colbecki*. This project allowed the researchers to observe significant cholinesterasic inhibition following in vitro exposure of gill tissue to a combination of Aroclor 1260 (a polychlorinated benzene mixture) and to EPA 610 (a mixture of 16 polyaromatic hydrocarbons).

5 Future Perspectives for the Use of Cholinesterases in Environmental Assessment

From the studies reviewed in this article one can clearly conclude that ChE inhibition may be a valid tool for ecotoxicological assessment of anthropogenic and natural chemicals. However, its valid use requires knowledge about the biological function, forms, and especially, the types of inhibitory interactions of CHEs that may derive from the presence of several chemical classes in the environment. A summary of the main points researchers should remember when employing ChE inhibition assays is as follows:

1. Most important for researchers wanting to rely on ChE-based assessment is to characterize the catalytic properties of the enzyme(s) used; the details on the predominant form of ChEs present in a given tissue of a specific species must be known. As observed for a large number of research articles, ChE characterization is an important underpinning for successful use, particularly when using an unknown species in field studies. Without a positive identification of the type and activity of the cholinesterasic form in tissues of a given species, it will be impossible to express results in terms of a specific cholinesterasic form. Moreover, if one does not know, in detail, the predominant cholinesterasic form present in the evaluated organism, then quantifying the basal levels of enzymatic activities for all isozymes will be rendered impossible. After the forms present are known, one can also successfully test for sensitivity in field monitoring surveys, which may enhance the value of any ecotoxicity assessment.
2. Results show that ChEs are not only different in their substrate affinity/inhibition, but can also be distinct in molecular terms; to differentiate ChE forms, electrophoresis analysis can be a useful tool to discriminate the trends in molecular weight of the enzymatic forms present. Electrophoresis may also be useful for understanding the behavior of carboxylesterases and their isozymes, which are also sensitive to the presence of pesticides in the wild (Kristoff et al. 2010).
3. Researchers must define the mechanisms by which ChE inhibition may occur if at all possible. Without knowing the inhibition mechanism one cannot be certain that results may be affected by artifacts. Indeed, it is possible to overestimate the importance of ChE inhibition as an effect criterion in environmental studies if one does not know the molecular mechanisms that underlie inhibition. What has been found to occur with metallic species and detergents are paradigmatic: some metals and tensioactive compounds are able to significantly inhibit ChE activity in several organisms but only when using in vitro approaches. This means that the enzyme, under in vivo conditions is a actual contaminated field, may or may not be inhibited, because in vitro results may or may not predict those that will occur in vivo. Furthermore, the use of standardized assays that are based on the catalytic activity of an enzyme and a colorimetric reaction (e.g., Ellman method), and occur in a water-based buffered medium, are subject to several confounding factors. When in vitro conditions are used to study the mechanistic aspects of the toxic response, it is common to incubate contaminants with cellular suspensions.

Under such conditions, the contaminant that is being tested may react (e.g., metallic compounds) or otherwise make unviable (e.g., detergents), the chemical reactions that underlie the testing protocol.

4. Researchers who employ cholinesterasic inhibition as an effect criterion must consider the different sensitivities that exist from organism to organism. One can find species that are refractory to common anticholinesterasic agents, whereas others are extremely sensitive.
5. The biological implications of enzymatic inhibition must also be analyzed, because they may represent pronounced behavioral changes that possess ecological relevance or may cause serious ecosystem imbalances in a test system.

The manifold data collected in recent years from the profusion of studies on ChE inhibition as an effect criterion is ample proof of the viability of this monitoring tool. Cholinesterasic inhibition has been used in laboratory-based bioassays, in field monitoring of OP and CB pesticide exposure, and as an indirect measure or diagnostic parameter of contamination. This method has also been used as an analytical tool to improve the understanding of the relationship between chemicals and biological structures present in different species. However, the diversity of ChEs (differentiated by their chemical nature, form, type, hydrolytic activity, inhibition profile, and sensitivity to common pesticides) sometimes confounds the understanding of how contaminants can threaten wildlife. To avoid confounding factors, it is mandatory that critical steps be developed to allow the correct and useful employment of ChE assessment in the environmental sciences.

6 Summary

Cholinesterase (ChE) is one of the most employed biomarkers in environmental analysis. Among ChEs, potentially the most significant in environmental terms is acetylcholinesterase (AChE), an enzymatic form that terminates the nerve impulse. Because of its physiological role, AChE has long been considered a highly specific biomarker for organisms exposed to anticholinesterasic agents, primarily agrochemicals (organophosphate and carbamate pesticides). The effects of these pesticides depend upon their selective inhibition of AChE. Because large amounts of such pesticides are employed, it is plausible that they exert neurotoxic effects on some non-target species. Therefore, AChE is among the most valuable of diagnostic tools that can be used to verify exposure to such chemical agents. It is well known that assays are available for use in quantifying AChE in multiple tissues of several test organisms. Enzymes other than AChE (e.g., butyrylcholinesterase and carboxylesterases) have also been used as putative markers for detecting the environmental presence of contaminating compounds. Researchers must use a step-by-step approach to identify the most prominent cholinesterasic form present in a given species, so that this form can be distinguished from others that may interfere with its use. Such fundamental work must be completed prior to using ChEs for any monitoring to assess for anticholinesterasic effects.

Despite massive employment in environmental analysis, using ChE inhibition as an endpoint or effect criterion has been unsettled by the discovery that ChEs may interact in the environment in previously unknown ways. Several chemicals, in addition to anticholinesterasic pesticides, are now known to inhibit ChE activity. Such chemicals include detergents, metals, and certain organic compounds such as hydrocarbons. The situation is made worse, because the literature is contradictory as to the ability of such chemicals and elements to interact with ChEs. Some results indicate that ChE inhibition by metals, detergents, and complex mixtures do not or are unlikely to occur. These problems and contradictions are addressed in this review.

It is our purpose in this review to address the following practical issues related to the ChEs:

1. The situations and organisms in which ChEs have been employed as biomarkers in laboratory trials, and the need to fully characterize these enzymatic forms before they are used for environmental assessment purposes.
2. The ways in which the ChEs have been used in field monitoring, and the potential for use of other complimentary markers to diagnose organophosphate exposure, and how drawbacks (such as the absence of reference values) can be overcome.
3. What requirements must be satisfied prior to implementing the use of ChEs as biomarkers in species not yet studied.
4. How direct linkages have been established between ChE inhibition and effects from inhibition observed at higher levels of integration (e.g., behavioral changes and population effects, or other indices of ecological relevance).
5. The potential for ChE inhibition to be applied as an effective parameter of toxicity to detect for the environmental presence of compounds other than the organophosphate and carbamate pesticides, and the limitations associated therewith.

Acknowledgments The present work was funded by projects “BiOtoMetal” (PTDC/AMB/70431/2006). We would like to acknowledge the contribution of Sérgio Barreira, for his most appreciated scientific comments and chemical expertise.

References

Aamodt S, Konestabo HS, Sverdrup LE, Gudbrandsen M, Reinecke SA, Reinecke AJ, Stenersen J (2007) Recovery of cholinesterase activity in the earthworm *Eisenia fetida* Savigny following exposure to chlorpyrifos. *Environ Toxicol Chem* **26**(9): 1963–7.

Andersen RA, Aune T, Barstad JAB (1978) Characteristics of cholinesterase of the earthworm *Eisenia foetida*. *Compar Biochem Physiol Part C: Compar Pharmacol* **61**(1): 81–87.

Arufe MI, Arellano JM, García L, Albendín G, Sarasquete C (2007) Cholinesterase activity in gilthead seabream (*Sparus aurata*) larvae: characterization and sensitivity to the organophosphate azinphosmethyl. *Aquat Toxicol* **84**: 328–336.

Bain D, Buttemer WA, Astheimer L, Fildes K, Hooper MJ (2004) Effects of sublethal fenitrothion ingestion on cholinesterase inhibition, standard metabolism, thermal preference, and prey-capture ability in the Australian central bearded dragon (*Pogona vitticeps*, Agamidae) *Environ Toxicol Chem* **23**(1): 109–16.

Barata C, Solayan A, Porte C (2004) Role of B-esterases in assessing toxicity of organophosphorus (chlorpyrifos, malathion) and carbamate (carbofuran) pesticides to *Daphnia magna*. *Aquat Toxicol* **66**: 125–139.

Bonacci S, Corsi I, Focardi S (2009) Cholinesterases in the Antarctic scallop *Adamussium colbecki*: Characterization and sensitivity to pollutants. *Ecotoxicol Environ Saf* **72**: 1481–1488.

Boschetti N, Brodbeck U (1996) The membrane anchor of mammalian brain acetylcholinesterase consists of a single glycosylated protein of 22 kDa. *FEBS Letters* **380**: 133–136.

Brown M, Davies IM, Moffat CF, Redshaw J, Craft JA (2004) Characterisation of choline esterases and their tissue and subcellular distribution in mussel (*Mytilus edulis*) *Mar Environ Res* **57**: 155–169.

Caselli F, Gastaldi L, Gambi N, Fabbri E (2006) In vitro characterization of cholinesterases in the earthworm *Eisenia andrei*. *Comp Biochem Physiol, Part C* **143**: 416–421.

Chebbi SG, David M (2009) Neurobehavioral responses of the freshwater teleost, *Cyprinus carpio* (Linnaeus.) under quinalphos intoxication. *Biotechnol Animal Husband* **25**(3-4): 241–249.

Choi RCY, Yama SCY, Hui B, Wan DCC, Tsim KWK (1998) Over-expression of acetylcholinesterase stimulates the expression of agrin in NG108–15 cells. *Neurosci Lett* 248: 17–20.

Chuiko GM (2000) Comparative study of acetylcholinesterase and butyrylcholinesterase in brain and serum of several freshwater fish: specific activities and in vitro inhibition by DDVP, an organophosphorus pesticide. *Compar Biochem Physiol, part C Toxicol Pharmacol* **127**(3): 233–42.

Corsi I, Bonacci S, Santovito G, Chiantore M, Castagnolo L, Focardi S (2004) Cholinesterase activities in the Antartic scallop *Adamussium colbecki*: tissue expression and effect of $ZnCl_2$ exposure. *Mar Environ Res* **58**: 401–406.

Cunha I, Mangas-Ramirez E, Guilhermino L (2007) Effects of copper and cadmium on cholinesterase and glutathione S-transferase activities of two marine gastropods (*Monodonta lineata* and *Nucella lapillus*). *Compar Biochem Physiol, Part C* **145**: 648–657.

Dahm KCS, Rückert C, Tonial EM, Bonan CD (2006) In vitro exposure of heavy metals on nucleotidase and cholinesterase activities from the digestive gland of *Helix aspersa*. *Compar Biochem Physiol, Part C* **143**: 316–320.

Dauberschmidt C, Dietrich DR, Christian Schlatter C (1997) Esterases in the zebra mussel *Dreissena polymorpha*: activities, inhibition, and binding to organophosphates. *Aquat Toxicol* **37**(4): 295–305.

Denoyelle R, Rault M, Mazzia C, Mascle O, Capowiez Y (2007) Cholinesterase activity as a biomarker of pesticide exposure in *Allolobophora chlorotica* earthworms living in apple orchards under different management strategies. *Environ Toxicol Chem* **26**(12): 2644–9.

Deschênes-Furry J, Bélanger G, Perrone-Bizzozero N, Jasmin BJ (2003) Post-transcriptional Regulation of Acetylcholinesterase mRNAs in Nerve Growth Factor-treated PC12 Cells by the RNA-binding Protein HuD. *J Biolog Chem* **278**(8): 5710–5717.

Duquesne S (2006) Effects of an organophosphate on *Daphnia magna* at suborganismal and organismal levels: Implications for population dynamics. *Ecotoxicol Environ Saf* **65**: 145–150.

Ellman GL, Courtney KD, Andres V, Featherstone RM (1961) A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochem Pharmacol* **7**: 88–95.

Escartín E, Porte C (1996) Acetylcholinesterase Inhibition in the Crayfish *Procambarus clarkii* Exposed to Fenitrothion. *Ecotoxicolo Environ Saf* **34**: 160–164.

Eto M (1984) Organophosphorus Pesticides: Organic and Biological Chemistry. Ohio: CRC Press, Inc. 1974.

Falugi C, Amaroli A, Evangelisti V, Viarengo A, Delmonte Corrado MU (2002) Cholinesterase activity and effects of its inhibition by neurotoxic drugs in *Dictyostelium discoideum*. *Chemosphere* **48**: 407–414.

Ferrari A, Venturino A, Pechén de D'Angelo AM (2007) Muscular and brain cholinesterase sensitivities to azinphos methyl and carbaryl in the juvenile rainbow trout *Oncorhynchus mykiss*. *Compar Biochem Physiol, Part C* **146**: 308–313.

Forget J, Livet S, Leboulenger F (2002) Partial purification and characterization of acetylcholinesterase (AChE) from the estuarine copepod *Eurytemora affinis* (Poppe). *Compar Biochem Physiol Part C* **132**: 85–92.

Forget J, Bocquené G (1999) Partial purification and enzymatic characterization of acetylcholinesterase from the intertidal marine copepod *Tigriopus brevicornis*. *Compar Biochem Physiol Part B* **123**: 345–350.

Fournier D, Bride JM, Karch F, Bergé JB (1988) Acetylcholinesterase from *Drosophila melanogaster* – Identification of two subunits encoded by the same gene. *FEBS Letters* **238**(2): 333–337.

Frasco MF, Fournier D, Carvalho F, Guilhermino L (2006) Cholinesterase from the common prawn (*Palaemon serratus*) eyes: Catalytic properties and sensitivity to organophosphate and carbamate compounds. *Aquat Toxicol* **77**: 412–421.

Frasco MF, Fournier D, Carvalho F, Guilhermino L (2005) Do metals inhibit acetylcholinesterase (AChE)? Implementation of assay conditions for the use of AChE activity as a biomarker of metal toxicity. *Biomarkers* **10**(5): 360–375.

Fulton MH, Key PB (2001) Acetylcholinesterase inhibition in estuarine fish and invertebrates as an indicator of organophosphorus insecticide exposure and effects. *Environ Toxicol Chem* **20**: 37–45.

Gambi N, Pasteris A, Fabbri E (2007) Acetylcholinesterase activity in the earthworm *Eisenia andrei* at different conditions of carbaryl exposure. *Compar Biochem Physiol, Part C* **145**: 678–685.

Gao JR, Zhu KY (2002) Increased expression of an acetylcholinesterase gene may confer organophosphate resistance in the greenbug, *Schizaphis graminum* (Homoptera: Aphididae). *Pesticide Biochem Physiol* **73**: 164–173.

Garcia LM, Castro B, Ribeiro R, Guilhermino L (2000) Characterization of cholinesterase from guppy (*Poecilia reticulata*) muscle and its in vitro inhibition by environmental contaminants. *Biomarkers* **5**(4): 274–284.

Giacobini E (2004) Cholinesterase inhibitors: new roles and therapeutic alternatives. *Pharmacological Res* **50**: 433–440.

Gonçalves A, Padrão J, Gonçalves F, Nunes B (2010) In vivo acute effects of several pharmaceutical drugs (diazepam, clofibrate, clofibric acid) and detergents (sodium dodecyl-sulphate and benzalkonium chloride) on cholinesterases from *Gambusia holbrooki*. *Fresenius Environ Bull* **19**(4): 628–634.

Greene LA, Rukenstein A (1981) Regulation of Acetylcholinesterase Activity by Nerve Growth Factor. *J Biolog Chem* **256**(12): 6363–6367.

Guilhermino L, Lacerda MN, Nogueira AJA, Soares AMVM (2000) In vitro and in vivo inhibition of *Daphnia magna* acetylcholinesterase by surfactant agents: possible implications for contamination biomonitoring. *Sci Tot Env* **247**: 137–141.

Hai DQ, Ilona Varga Sz, Matkovics B (1997) Organophosphate effects on antioxidant system of carp (*Cyprinus carpio*) and catfish (*Ictalarus nebulosus*). *Compar Bioch Physiol* **117C**(1): 83–88.

Iko WM, Archuleta AS, Knopf FL (2003) Plasma cholinesterase levels of mountain plovers (*Charadrius montanus*) wintering in central California, USA. *Environ Toxicol Chem* **22**(1): 119–25.

Jbilo O, Toutant JP, Vatsis KP, Chatonnet A, Lockridge O (1994) Promoter and Transcription Start Site of Human and Rabbit Butyrylcholinesterase Genes. *J Biolog Chem* **269**(33): 20829–20837.

Jokanovic M (2001) Biotransformation of organophosphorus compounds. *Toxicology* **166**: 139–160.

Jung JH, Addison RF, Shim WJ (2007) Characterization of cholinesterases in marbled sole, *Limanda yokohamae*, and their inhibition in vitro by the fungicide iprobenfos. *Mar Environl Res* **63**: 471–478

Key PB, Fulton MH (2002) Characterization of cholinesterase activity in tissues of the grass shrimp (*Palaemonetes pugio*). *Pesticide Biochem Physiol* **72**: 186–192.

Kitz RJ, Braswell LM, Ginsburg S (1970) On the question: is acetylcholinesterase an allosteric protein? *Molecular Pharmacol* **6**: 108–121.

Klumpp D, Humphrey C, King SC (2007) Biomarker responses in coral trout (*Plectropomus leopardus*) as an indicator of exposure to contaminants in a coral reef environment. *Austral J Ecotoxicol* **13**: 9–17.

Kopecka J, Rybakowas A, Barsiené J, Pempkowiak J (2004) AChE levels in mussels and fish collected off Lithuania and Poland (southern Baltic). *Oceanologia* **46**(3): 405–418.

Kozlovskaya VI, Mayer FL, Menzikova OV, Chuyko GM (1993) Cholinesterases of aquatic animals. *Rev Environ Contam Toxicol* **132**: 117–142.

Kristoff G, Guerrero NR, Cochón AC (2010) Inhibition of cholinesterases and carboxylesterases of two invertebrate species, *Biomphalaria glabrata* and *Lumbriculus variegatus*, by the carbamate pesticide carbaryl. *Aquat Toxicol* **96**(2): 115–23.

Laguerre C, Sanchez-Hernandez JC, Köhler HR, Triebskorn R, Capowiez Y, Rault M, Mazzia C (2009) B-type esterases in the snail *Xeropicta derbentina*: An enzymological analysis to evaluate their use as biomarkers of pesticide exposure. *Environ Pollut* **157**: 199–207.

Leticia AG, Gerardo GB (2008) Determination of esterase activity and characterization of cholinesterases in the reef fish *Haemulon plumieri*. *Ecotoxicol Environm Saf* **71**: 787–797.

Li M-H (2008) Effects of nonionic and ionic surfactants on survival, oxidative stress, and cholinesterase activity of planarian. *Chemosphere* **70**: 1796–1803

MacGregor JA, Plunkett LM, Youngren SH, Manley A, Plunkett JB, Starr TB (2005) Humans appear no more sensitive than laboratory animals to the inhibition of red blood cell cholinesterase by dichlorvos. *Regulatory Toxicol Pharmacol* **43**: 150–167.

Magnotti RA, Zaino JP, McConnell RS (1994) Pesticide-sensitive fish muscle cholinesterases. *Compar Biochem Physiol Part C: Pharmaco, Toxicol Endocrinol* **108**(2): 187–194.

Mack A, Robitzki A (2000) The key role of butyrylcholinesterase during neurogenesis and neural disorders: an antisense-5′butyrylcholinesterase-DNA study. *Progress in Neurobiol* **60**: 607–628.

Malany S, Baker N, Verweyst N, Medhekar R, Quinn DM, Velan B, Kronman C, Shafferman A (1999) Theoretical and experimental investigations of electrostatic effects on acetylcholinesterase catalysis and inhibition. *Chemico-Biolog Interact* 119–120: 99–110.

Massoulié J, Perrier N, Noureddine H, Liang D, Bon S (2008) Old and new questions about cholinesterases. *Chemico-Biolog Interact* **175**: 30–44

Massoulié J, Bon S (1982) The molecular forms of cholinesterase and acetylcholinesterase in vertebrates. *Ann Rev Neurosci* **5**: 57–106.

Maul JD, Farris JL (2005) Monitoring exposure of northern cardinals, *Cardinalis cardinalis*, to cholinesterase-inhibiting pesticides: enzyme activity, reactivations, and indicators of environmental stress. *Environ Toxicol Chem* **24**(7): 1721–30.

McInnes PF, Andersen DE, Hoff DJ, Hooper MJ, Kinkell LL (1996) Monitoring exposure of nestling songbirds to agricultural application of an organophosphorus insecticide using cholinesterase activity. *Environ Toxicol Chem* **15**(4): 544–552.

Melo JB, Agostinho P, Oliveira CR (2003) Involvement of oxidative stress in the enhancement of acetylcholinesterase activity induced by amyloid beta-peptide. *Neurosci Res* **45**: 117–127.

Metcalfe M, McWilliams B, Hueth B, Van Steenwyk R, Sunding D, Swoboda A, Zilberman D (2002) Report: The Economic Importance of Organophosphates in California Agriculture. California Department of Food and Agriculture.

Monteiro M, Quintaneiro C, Morgado F, Soares AMVM, Guilhermino L (2005) Characterization of the cholinesterases present in head tissues of the estuarine fish *Pomatoschistus microps*: Application to biomonitoring. *Ecotoxicol Environ Saf* **62**: 341–347

Mora P, Fournier D, Narbonne JF (1999) Cholinesterases from the marine mussels *Mytilus galloprovincialis* Lmk. and *M. edulis* L. and from the freshwater bivalve *Corbicula fluminea* Müller. *Compar Biochem Physiol Part C* **122**: 353–361.

Nunes B, Carvalho F, Guilhermino L (2005) Characterization and use of the total head soluble cholinesterases from mosquitofish (*Gambusia holbrooki*) for screening of anticholinesterase activity. *J Enzym Inhibit Medic Chem* **20**(4): 369–376.

Padilla S (1995) Regulatory and research issues related to cholinesterase inhibition. *Toxicology* **102**: 215–220.

Panda S, Sahu SK (2004) Recovery of acetylcholine esterase activity of *Drawida willsi* (Oligochaeta) following application of three pesticides to soil. *Chemosphere* **55**: 283–290.

Parsons KC, Matz AC, Hooper MJ, Pokras MA (1999) Monitoring wading bird exposure to agricultural chemicals using serum cholinesterase activity. *Environ Toxicol Chem* **19**(5): 1317–1323.

Pham TPT, Cho CW, Yun YS (2010) Environmental fate and toxicity of ionic liquids: A review. *Water Res* **44**: 352–372.

Principato GB, Contenti S, Talesa V, Mangiabene C, Pascolini R, Rosi G (1989) Propionylcholinesterase from *Allolobophora caliginosa*. *Compar Biochem Physiol Part C: Compar Pharmacol* **94**(1): 23–27.

Principato GB, Ambrosini MV, Menghini A, Giovannini E, Dell'Agata M (1978) Multiple forms of acetylcholinesterase in *Allolobophora caliginosa*: Purification and partial characterization. *Compar Biochem Physiol Part C: Compar Pharmacol* **61**(1): 147–151.

Rahman MF, Siddiqui MKJ, Jamil K (2000) Inhibition of Acetylcholinesterase and Different ATPases by a Novel Phosphorothionate (RPR-II) in Rat Brain. *Ecotoxicol Environ Saf* **47**: 125–129.

Rault M, Mazzia C, Capowiez Y (2007) Tissue distribution and characterization of cholinesterase activity in six earthworm species. *Compar Biochem Physiol, Part B* **147**: 340–346.

Rocío Marcos M, Sánchez-Yague J, Herneńdez-Hernández A, Llanillo M (1998) Amphiphilic and hydrophilic forms of acetylcholinesterase from sheep platelets. *Biochimica et Biophysica Acta* **1415**: 163–173.

Rodríguez-Castellano L, Sanchez-Hernandez JC (2007a) Earthworm biomarkers of pesticide contamination: Current status and perspectives. *J Pesticide Sci* **32**: 360–371.

Rodríguez-Castellanos L, Sanchez-Hernandez JC (2007b) Chemical Reactivation and Aging Kinetics of Organophosphorus-Inhibited Cholinesterases from Two Earthworms Species. *Environ Toxicol Chem* **26**(9): 1992–2000.

Rodríguez-Fuentes G, Armstrong J, Schlenk D (2008) Characterization of muscle cholinesterases from two demersal flatfish collected near a municipal wastewater outfall in Southern California. *Ecotoxicol Environ Saf* **69**: 466–471.

Rodríguez-Fuentes G, Gold-Bouchot G (2004) Characterization of cholinesterase activity from different tissues of Nile tilapia (*Oreochromus niloticus*). *Mar Environ Res* **58**: 505–509.

Romani R, Antognelli C, Baldracchini F, De Santis A, Isani G, Giovannini E, Rosi G (2003) Increased acetylcholinesterase activities in specimens of *Sparus auratus* exposed to sublethal copper concentrations. *Chemico-Biological Interact* **145**: 321–329.

Sanchez JC, Fossi MC, Focardi S (1997) Serum "B" Esterases as a Nondestructive Biomarker for Monitoring the Exposure of Reptiles to Organophosphorus Insecticides. *Ecotoxicol Environ Saf* **37**: 45–52.

Sánchez-Fortún S, Barahona V (2001) The use of carbamates, atropine, and 2-pyridine aldoxime methoiodide in the protection of *Artemia salina* against poisoning by carbophenothion. *Environ Toxicol Chem* **20**(9): 2008–2013.

Sánchez-Hernandez JC (2007) Ecotoxicological perspectives of B-esterases in the assessment of pesticide contamination. In: Plattenberg RH (ed.) Environmental Pollution: New Research. Nova Science Publishers, New York, USA, pp. 1–45.

Sánchez-Hernández JC, Carbonell R, Henríquez Pérez A, Montealegre M, Gómez L (2004) Inhibition of plasma butyrylcholinesterase activity in the lizard *Gallotia galloti palmae* by pesticides: a field study. *Environ Pollut* **132**: 479–488.

Sanchez-Hernandez JC (2003) Evaluating reptile exposure to cholinesterase-inhibiting agrochemicals by serum butyrylcholinesterase activity. *Environ Toxicol Chem* **22**(2): 296–301.

Sanchez-Hernandez JC, Sanchez BM (2002) Lizard cholinesterases as biomarkers of pesticide exposure: enzymological characterization. *Environ Toxicol Chem* **21**(11): 2319–25.

Sánchez-Hernandez JC (2001) Wildlife exposure to organophosphorus insecticides. *Rev Environ Contam Toxicol* **172**: 21–63.

Sánchez-Hernandez JC, Walker CH (2000) In vitro and in vivo cholinesterase inhibition in *Lacertides* by phosphonate- and phosphorothioate-type organophosphates. *Pesticide Biochem Physiol* **67**: 1–12.

Sancho E, Fernandez-Vega C, Sanchez M, Ferrando MD, Andreu-Moliner E (2000) Alterations on AChE Activity of the Fish *Anguilla anguilla* as Response to Herbicide-Contaminated Water. *Ecotoxicol Environ Saf* **46**: 57–63.

Sancho E, Ferrando MD, Andreu E (1998) In vivo inhibition of AChE activity in the European eel *Anguilla anguilla* exposed to technical grade fenitrothion. *Comparat Biochem Physiol Part C* **120**: 389–395.

Sandahl JF, Baldwin DH, Jenkins JJ, Scholz NL (2005) Comparative thresholds for acetylcholinesterase inhibition and behavioral impairment in Coho salmon exposed to chlorpyrifos. *Environ Toxicol Chem* **24**(1): 136–145.

Schmidt SR (2003) Reptile Cholinesterase Characterization and Use in Monitoring Anticholinesterases. Thesis in Environmental Toxicology. Texas Tech University.

Schmidt GH, Ibrahim NMM (1994) Heavy metal content (Hg2+, Cd2+, Pb2+) in various body parts: Its impact on cholinesterase activity and binding glycoproteins in the grasshopper *Aiolopus thalassinus* adults. *Ecotoxicol Environ Saf* **29**(2): 148–164.

Scott-Fordsmand JJ, Weeks JM (2000) Biomarkers in earthworms. *Rev Environ Contam Toxicol* **165**:117–59.

Schweitzer ES (1993) Regulated and constitutive secretion of distinct molecular forms of acetylcholinesterase from PC12 cells. *J Cell Sci* **106**: 731–740.

Small DH, Michaelson S, Sberna G (1996) Non-classical actions of cholinesterases: role in cellular differentiation, tumorigenesis and Alzheimer's disease. *Neurochem Internat* **5/6**: 453–483.

Solé M, Lobera G, Lima D, Reis-Henriques MA, Santos MM (2008) Esterases activities and lipid peroxidation levels in muscle tissue of the shanny *Lipophrys pholis* along several sites from the Portuguese Coast. *Mar Pollut Bull* **56**: 999–1007.

Somnuek C, Cheevaporn V, Saengkul C and Beamish FWH (2007) Variability in Acetylcholinesterase upon Exposure to Chlorpyrifos and Carbaryl in Hybrid Catfish. *ScienceAsia* **33**: 301–305.

Stefano B, Ilaria C, Silvano F (2008) Cholinesterase activities in the scallop *Pecten jacobaeus*: Characterization and effects of exposure to aquatic contaminants. *Sci Tot Env* **392**: 99–109.

Stenersen J, Brekke E, Engelstad F (1992) Earthworms for toxicity testing; species differences in response towards cholinesterase inhibiting insecticides. *Soil Biol Biochem* **24**(12): 1761–1764.

Stenersen J (1980) Esterases of earthworms. Part I: Characterisation of the cholinesterases in *Eisenia foetida* (Savigny) by substrates and inhibitors. *Compar Biochem Physiol Part C: Compar Pharmacol* **66**(1): 37–44.

Sturm A, Silva de Assis HC, Hansen PD (1999a) Cholinesterases of marine teleost fish: enzymological characterization and potential use in the monitoring of neurotoxic contamination. *Mar Environ Res* **47**: 389–398.

Sturm A, Wogram J, Hansen PD, Liess M (1999b) Potential use of cholinesterase in monitoring low levels of organophosphates in small streams: natural variability in three-spined stickleback (*Gasterosteus aculeatus*) and relation to pollution. *Environ Toxicol Chem* **18**(2): 194–200.

Talesa V, Romani R, Antognelli C, Giovannini E, Rosi G (2001) Soluble and membrane-bound acetylcholinesterases in *Mytilus galloprovincialis* (Pelecypoda: *Filibranchia*) from the northern Adriatic sea. *Chemico-Biological Interact* **134**: 151–166.

Talesa V, Romani R, Rosi G, Giovannini E (1997) Acetylcholinesterase in *Spirographis spallanzanii* (Polychaeta: *Sedentaria*): Presence of two dimeric membrane-bound forms. *Biochimie* **79**: 397–405.

Talesa V, Grauso M, Giovannini E, Rosi G, Toutant JP (1995a) Acetylcholinesterase in tentacles in *Octopus vulgaris* (cephalopoda). Histochemical localization and characterization of a specific high salt-soluble and heparin-soluble fraction of globular forms. *Neurochem Internat* **27**(2): 201–211.

Talesa V, Grauso M, Giovannini E, Rosi G, Toutant JP (1995b) Solubilization, molecular forms, purification and substrate specificity of two acetylcholinesterases in the medicinal leech (*Hirudo medicinalis*). *Biochem J* **306**: 687–692.

Tsim KWK (1998) The signaling pathway of calcitonin gene-related peptide-induced acetylcholinesterase expression in muscle is mediated by cyclic AMP. Abstract book of the Xth International Symposium on Cholinergic Mechanisms, Arcachon, France September 1–5, 1998: 109.

Valbonesi P, Sartor G, Fabbri E (2003) Characterization of cholinesterase activity in three bivalves inhabiting the North Adriatic sea and their possible use as sentinel organisms for biosurveillance programmes. *Sci To Environ* **312**: 79–88.

Varó I, Navarro JC, Amat F, Guilhermino L (2003) Effect of dichlorvos on cholinesterase activity of the European sea bass (*Dicentrarchus labrax*). *Pesticide Biochem Physiol* **75**: 61–72.

Varó I, Navarro JC, Amat F, Guilhermino L(2002) Characterisation of cholinesterases and evaluation of the inhibitory potential of chlorpyrifos and dichlorvos to *Artemia salina* and *Artemia parthenogenetica*. *Chemosphere* **48**: 563–569.

Vermeire T, McPhail R, Waters M (2001) D. Organophosphorous pesticides in the environment. World Health Organization. IV. Meeting Report of the International Workshop on Approaches to Integrated Risk Assessment: 1–18.

Wan DCC, Choi RCY, Siow NL, Tsim KWK (2000) The promoter of human acetylcholinesterase is activated by a cyclic adenosine 3′, 5′-monophosphate-dependent pathway in cultured NG108-15 neuroblastoma cells. *Neurosc Lett* **288**: 81–85.

Wang J, Grisle S, Schlenk D (2001) Effects of Salinity on Aldicarb Toxicity in Juvenile Rainbow Trout (*Oncorhynchus mykiss*) and Striped Bass (*Morone saxatilis*×*chrysops*) *Toxicolog Sci* **64**: 200–207.

Wogram J, Sturm A, Segner H, Liesse M (2001) Effects of parathion on acetylcholinesterase, butyrilcholinesterase, and carboxylesterase in three-spined stickleback (*Gasterosteus aculeatues*) following short-term exposure. *Environ Toxicol Chem* **20**(7): 1528–1531.

Xuereb B, Noury P, Felten V, Garric J, Geffard O (2007) Cholinesterase activity in *Gammarus pulex* (Crustacea Amphipoda): characterization and effects of chlorpyrifos. *Toxicology* **236**: 178–189.

Yang L, He HY, Zhang XJ (2002) Increased expression of intranuclear AChE involved in apoptosis of SK-N-SH cells. *Neurosci Res* **42**: 261–268.

Zinkl JG, Lochkart WL, Kenny SA, Ward FJ (1991) The effects of cholinesterase inhibiting insecticides on fish. In: Cholinesterase-Inhibiting Insecticides. Their Impact on wildlife and the Environment. Mineau P (ed), pp. 234–243. Elsevier, New York.

Tropospheric Ozone and Plants: Absorption, Responses, and Consequences

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D.M. Whitacre (ed.), *Reviews of Environmental Contamination and Toxicology*, Reviews of Environmental Contamination and Toxicology 212,
DOI 10.1007/978-1-4419-8453-1_3,

1 Introduction

Ozone (O_3) is known to have existed in the atmosphere since ancient times and has played a critical role in the survival of life on the Earth. In the stratosphere, O_3 plays an extremely important and beneficial role in screening the lower layers of the atmosphere and the surface of the Earth from the sun's harmful ultraviolet radiation. In the troposphere, and especially near the surface of the Earth, O_3 is not beneficial. Ozone has been shown to be harmful to human health, vegetation and crop productivity (Dentener et al. 2006). In the past few decades, tropospheric O_3 has emerged as a major secondary pollutant due to increased emissions of its precursors, e.g., nitrogen oxides (NO_x; $x=1$ or 2) and volatile organic compounds (VOCs) (Ashmore 2005). High concentrations of O_3 are associated with hot sunny weather. Such high concentrations of O_3 are frequently observed in tropical areas where conditions are favorable for O_3 formation (Jain et al. 2005; Tiwari et al. 2008). The adverse effects of O_3 were first identified in grapevines (Richards et al. 1958), and it is now recognized as the most important rural air pollutant affecting human health, vegetation, or material that is susceptible to oxidation (Ashmore 2005; Fuhrer and Booker 2003; Karnosky et al. 2007; Karlsson et al. 2003; Laurence and Andersen 2003; Matyssek and Sandermann 2003).

The studies conducted with ozone on different crops to date have used diverse approaches, and results clearly indicate that O_3 is causing a significant reduction in agricultural productivity worldwide; hence, global food security is being affected by the presence of O_3. However, it should be noted that the magnitude of response to O_3 varies significantly from crop to crop (Mills et al. 2007) and even cultivar to cultivar (Sarkar and Agrawal 2010a, b; Singh and Agrawal 2009). O_3 generally enters plants through the stomata where it can be dissolved in the apoplastic fluid and can directly react with the plasma membrane or be converted into different reactive oxygen species (ROS). ROS are capable of altering cellular functions that cause cell death, premature senescence, and up- or down-regulation of specific genes (Long and Naidu 2002). With this in mind, the present review is designed to address all aspects of O_3 and plant interactions, starting from its absorption and continuing to the responses it elicits, and the resulting consequences. We place special emphasis on the mechanism of action by which O_3 induces its effects. We hope that the present review will be useful for students and established researchers alike, in the context of plant responses to environmental atmospheric pollutants. We also hope that the review will be of use to policy makers, who face the task of designing better, and more sustainable approaches for combating O_3 pollution.

2 Current Status and Trends of Worldwide Ozone Levels

2.1 Formation of Tropospheric Ozone

Ozone concentrations build up in the atmosphere as a result of several natural and anthropogenic processes and through several sources. These include (1) downward transport of stratospheric O_3 sources through the free troposphere to near ground level,

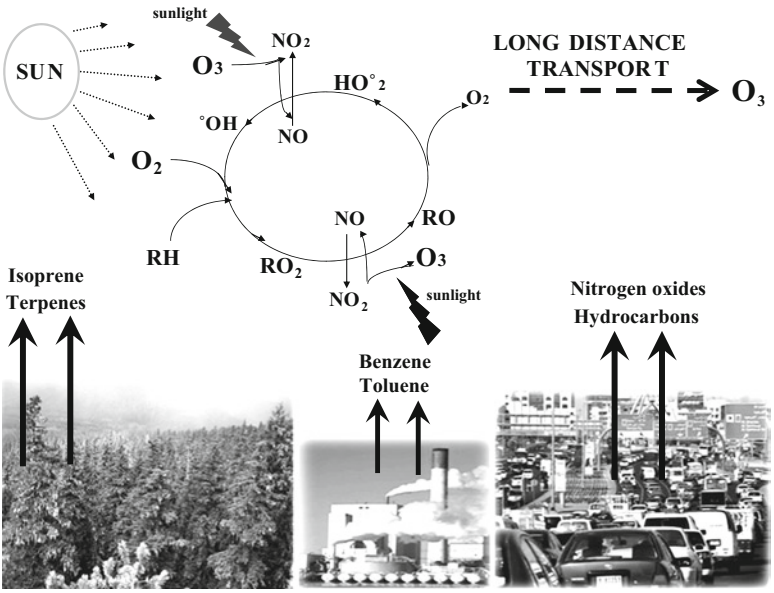

Fig. 1 Cycle showing formation of ground-level ozone (O_3) in the troposphere and the emission sources of different precursor molecules. The sources, which make a major contribution to increasing O_3 concentration in the troposphere, are shown. Abbreviations: *HO·*$_2$ hydroperoxy radical,*·NO* nitrous oxide, NO_2 nitrogen dioxide, *OH* hydroxyl radicals, O_2 oxygen, *RH* organicperoxy radicals, *RO* alcoxy radical, RO_2 reactive oxygen

(2) in situ O_3 production from methane emitted from swamps and wetlands that react with natural NO_x, (3) photochemical production of O_3 from reactions of VOCs with NO_xs (4), and long-range transport of O_3 from distant pollution sources (Kondratyev and Varotsos 2001a, b; Varotsos et al. 2004); these key processes and sources are illustrated in Fig. 1.

In the upper layer of the troposphere, O_3 may persist for a period of many days to 2 weeks. This is because the major loss processes, i.e., scavenging by nitric oxide and dry deposition, occur at or very near the surface of the Earth. This means that O_3 produced in one region, if lifted to higher atmospheric levels, can travel to another region, and then can add to existing background O_3 concentrations there, even if the sources of O_3 precursors are absent. It has been shown that Western North America receives a background O_3 contribution from Asia and Europe (Fiore et al. 2002; Jaffe et al. 2003), while Europe receives O_3 transported from both North America and Asia (Auvray and Bey 2005).

Fusco and Logan (2003) have shown that since 1970, increased surface emissions of NO_xs from fossil fuel combustion have had the largest effect on O_3 levels in the lower troposphere, and have accounted for a 10% increase in the annual O_3 levels over Canada, Europe and Japan. In contrast, hydrocarbon emissions have not significantly contributed to increasing tropospheric O_3 concentrations over the USA in summer time (Fiore et al. 2002). An increment of about 3–4% in worldwide O_3 concentrations can be ascribed to increased methane levels from the late 1970s to 1980s (Fusco and Logan 2003).

2.2 Concentrations and Trends of Ozone in the Troposphere

Ozone occurs naturally at low concentrations ranging from 5 to 15 ppb (Marenco et al. 1994). The earliest O_3 measurements were made in the mid 1800s, when O_3 concentrations were measured at more than 300 recording stations in different parts of Europe and the USA. However, a continuity of O_3 monitoring was maintained only at a few stations, and hence long-term data are limited. These existing data provide a general indication of what the natural background levels of O_3 would be in the absence of significant anthropogenic influences. Re-evaluation of daily O_3 concentrations over Athens (Greece) during the period from 1901 to1940 gave an average concentration of about 20 ppb (Varotsos and Cartalis 1991). Measurements taken from the Great Lakes area of North America yielded an average daily maximum of approximately 19 ppb during the late nineteenth century (Bojkov 1986). European measurements between 1850 and 1900 were found to be in the range of 17–23 ppb (Bojkov 1986). Analysis of O_3 data collected at Montsouris, France between 1876 and 1910 (Volz and Kley 1988) provided an annual (data were collected over a period of only 11 months) average range of values between 5 and 16 ppb.

Background O_3 concentrations have more than doubled in the last century (Meehl et al. 2007), and there is evidence of an increase in annual mean values ranging from 0.1 to 1 ppb $year^{-1}$ (Coyle et al. 2003). In the UK, the annual average O_3 concentrations are predicted to reach 30–40 ppb in rural areas. This will result in a doubling of AOT40 (accumulated exposure over a threshold of 40 ppb) values by 2030 (Coyle et al. 2003). Clean Air Status and Trends Network recorded O_3 concentrations from 11 National Parks in the USA (designated as protected areas), and these showed that annual median values ranged from 13 to 47 ppb, whereas maxima ranged from 49 to 109 ppb (CASTNet 2004). In Canada, the Canadian Air and Precipitation Network (CAPMoN) has recorded annual median O_3 concentrations at Canadian background sites that range between 23 to 34 ppb, while annual maxima ranged from 63 to 108 ppb (Vingarzan 2004). Using a Community Multiscale Air Quality Model researchers have calculated the highest O_3 concentrations (ranging from 55 to 70 ppb) during May and June in the boundary layer over East China and Japan (Yamaji et al. 2006). The rate of increase of tropospheric O_3 concentrations over East Asia is larger than in any other area of the Northern mid latitudes (Akimoto and Narita 1994; Kaneyasu et al. 2000). This unusual increase can be attributed to elevated anthropogenic emissions of O_3 precursors that are released from the rapidly emerging industrialization of the Asian continent (Hoell et al. 1997). Ozone, along with its precursors, is therefore transported over the Pacific Ocean and occasionally reaches North America (Hoell et al. 1997; Derwent et al. 2004; Mauzerall et al. 2000).

Permadi and Oanh (2008) reported high surface O_3 levels in Jakarta during January 2002–March 2004, which frequently exceeded the hourly national ambient air quality standard (120 ppb). They recorded a maximum 1-h O_3 concentration of as high as 243 ppb during the dry season of 2002. Wang et al. (2009) analyzed the variations in the concentrations of tropospheric O_3 from 1994 to 2007 at a coastal site in Hong Kong and reported an increase of 0.87 ppb $year^{-1}$. In the same study, a

linear fit to a 14-year-duration record shows that the O_3 concentration increased by 0.58 ppb year^{-1} (Wang et al. 2009). Authors, who employed global photochemical models, projected that under current legislation emission scenarios, parts of Asia will experience a significant increase in O_3 concentrations by 2030 (Dentener et al. 2006). From these results of monitoring studies it is suggested that a mean monthly O_3 concentration of 50 ppb is of common occurrence in several parts of Asia, especially during the growing seasons of important agricultural crops (EANET 2006).

In India, despite favorable climatic conditions for O_3 formation, very few data from systematic monitoring for O_3 levels are available. Pandey and Agrawal (1992) recorded O_3 concentrations (24 h) ranging from 6.0 to 10.2 ppb during 1989–1990 in the urban areas of Varanasi. During the same period, daytime O_3 concentrations (daytime 9 h mean) were reported to vary from 9.4 to 128.3 ppb at an urban site in Delhi (Varshney and Aggarwal 1992). It was observed that 10-h ground level mean O_3 concentrations in Delhi varied between 34 to 126 ppb, during the winter of 1993 (Singh et al. 1997). An annual average daytime O_3 concentration of 27 ppb and hourly concentration of 2–69 ppb at Pune (India), during August 1991 to July 1992, was reported by Khemani et al. (1995). Lal et al. (2000) studied the pattern of O_3 concentrations from 1991 to 1995 at an urban site at Ahmedabad (India), and reported that daytime mean O_3 concentrations exceeding 80 ppb were rarely observed. Jain et al. (2005) reported that the monthly average O_3 concentration in summer varied between 62 and 95 ppb, whereas in autumn (October to November), it was found to be between 50 and 82 ppb.

Mittal et al. (2007), using the Regional Episodic Chemical Transport Model (HANK), estimated that 8-h daily average O_3 concentrations varied between 33 and 40 ppb in Varanasi during the period February–April 2000. A similar range of variation in O_3 concentration was recorded from an urban and rural site in the state of Maharashtra, India (Debaje and Kakade 2008). Roy et al. (2009) used a Regional Chemistry Transport Model (REMO-CTM) to study the distribution of AOT40 over regions of India (most of the fertile Indo Gangetic Plains of India), and observed high AOT40 values that exceeded the threshold set by the World Health Organization (WHO; 3 ppm h for 3 months) for agricultural crops . Elevated monthly AOT40 values were primarily found between November and May, while the highest value was recorded in March (Roy et al. 2009). Continuous measurements over a period of 6 months (November–April) have shown that AOT40 values exceeded the critical level set for the protection of forests by almost 3.6 times (the actual level was 36 ppm h; Roy et al. 2009).

Monitoring studies performed at a suburban site of Varanasi, India from 2002 to 2006 showed that the daytime 12-hourly mean monthly O_3 concentrations varied from 45.18 to 62.35 ppb during summer, from 28.55 to 44.25 ppb during winter, and from 24 to 43.85 ppb during the rainy season (Tiwari et al. 2008). At the same site, 12-h average O_3 concentrations ranging from 41.65 to 54.2 ppb were recorded during November 2006 to March 2007 (Singh et al. 2009a). Sarkar and Agrawal (2010a) reported that mean monthly ambient O_3 concentrations varied from 41.3 ppb in July 2007 to 59.9 ppb in October 2007 at the same site.

The number of measurement sites in India at which valid and long-term representative measurements of surface O_3 were performed is too small to allow for a

reliable assessment of the regional distribution of AOT40 values. However, recently, a new gridded emission inventory of O_3 precursors over the Indian geographical region have been prepared by Roy et al. (2008). They employed an emission inventory in the regional 3D Chemistry Transport Model for Indian region, but the impact of cumulative O_3 exposure (AOT40) over the entire Indian region has not yet been reported. Rai et al. (2010) have calculated 3-month AOT40 values of 2.1 ppm h for the rice growth period during 2005 at a rural area of Varanasi, India. Sarkar and Agrawal (2010b) have reported 7.9 ppm h and 8.7 ppm h 3-month AOT40 values for the wheat growth period during 2007–2008 and 2008–2009, respectively.

2.3 Ozone Exposure Indices for Vegetation

One of the difficulties in assessing the impact of O_3 on vegetation is the wide range of methods used to describe the effects of O_3 exposure. Concentration, exposure duration, shape of exposure curve, and frequency of peak exposures are all important in determining a plant's response to O_3 (Musselman and Massman 1999). To date, O_3 exposure has been commonly expressed as the accumulated exposure over a threshold of 40 nl L^{-1} (AOT40; Fuhrer et al. 1997). There have also been suggestions that AOT30 be used in some cases (Pleijel et al. 1997). However, taking into account the fact that phytotoxicity will depend on the actual O_3 flux within the mesophyll through the stomata of plants, recent studies have utilized a flux-based concept (Karlsson et al. 2007; Tausz et al. 2007; Weiser and Matyssek 2007). This concept uses a modified version of the Deposition of Ozone and Stomatal Exchange (DO_3SE) model (Emberson et al. 2007; Karlsson et al. 2007). This model, operated at hourly time steps, is used to calculate the rate of O_3 uptake as the product of O_3 concentrations that exist near the leaf surface and the inverse sum of resistances along the transport pathway to the leaf interior; the model also takes the O_3 deposition to external surfaces into account (Emberson et al. 2000).

3 Plant Responses to Ozone Stress

3.1 Mechanism of Action of Ozone

Chronic long-term exposure to low O_3 concentrations affects the physiological and biochemical processes of plants prior to any visible injury (Heath and Taylor 1997). The degree of sensitivity of the foliar cells depends on the actual amount of pollutant that reaches the target sites, as well as the capacity of the cells to restore homeostatic equilibrium by adapting to metabolic changes. Ozone affects a number of processes in plants through direct or indirect mechanisms, and an illustration of these processes is shown in Fig. 2.

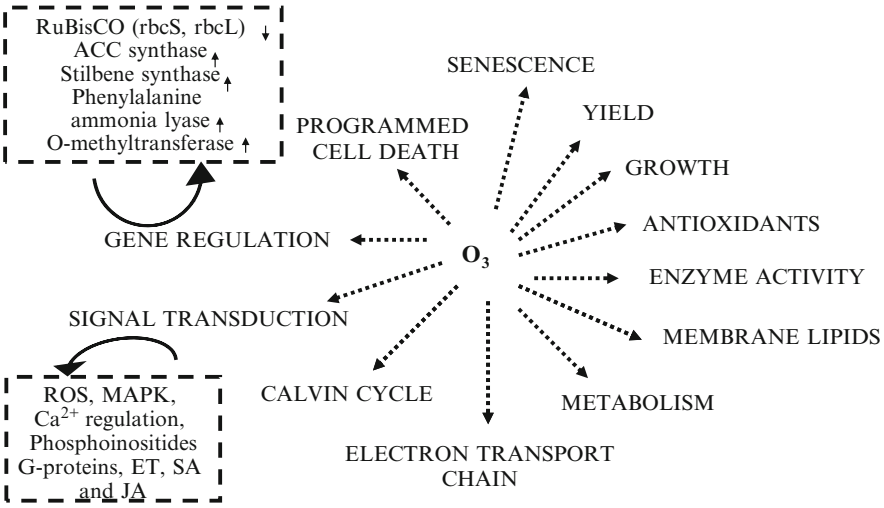

Fig. 2 Effects of O_3 stress on plants. Abbreviations: *ACC synthase* 1-aminocyclopropane-1-carboxylate synthase, *Ca^{2+}* calcium ion, *ET* ethylene, *JA* jasmonic acid, *MAPK* mitogen-activated protein kinase, *ROS* reactive oxygen species, *RuBisCO* Ribulose-1,5-bisphosphate carboxylase oxygenase, *rbcL* RuBisCO large subunit, *rbcS* RuBisCO small subunit, *SA* salicylic acid

3.1.1 Ozone Uptake

Because O_3 entry through the leaf cuticle is negligible, stomata play a fundamental role in determining the flux of O_3 into the apoplastic region of plants (Kersteins and Lendzian 1989; Leitao et al. 2003). The flux of O_3 from the troposphere into plants depends on what resistance (inverse of conductance, 1/[conductance]) exists at different morphological or physiological levels. Conductance is defined as flux per unit driving force (e.g., concentration gradient). The total resistance for the O_3 flux from the atmosphere into plant cells consists of the following: aerodynamic resistance that depends upon atmospheric resistance, the boundary layer resistance caused by a layer of laminar air adjacent to leaves, the stomatal resistance exerted by the stomatal pores, and the internal resistance caused in the leaves after O_3 uptake through stomata (Guderian 1985). It should be noted that internal structures, like the cell wall and inter- and intracellular apparatus, also contribute significantly to the O_3 resistance of any plant.

The sensitivity of plants to O_3 is often correlated with their stomatal response. Because O_3 exposure generally results in a decline in stomatal aperture, plants that show more rapid stomatal closure are reported to be resistant to O_3 in population-level studies (Sarkar et al. 2010; Winner et al. 1991). However, O_3-induced declines in stomatal apertures may be of limited protective value, because stomatal closure is generally a consequence of damage to the photosynthetic apparatus (Farage and Long 1995). Martin et al. (2000) modeled the data from earlier literature and showed that, in most cases, closure of the stomatal aperture that results from acute O_3 exposure can be predicted by the changes occurring in rates of mesophyll photosynthesis.

Joo et al. (2005) have also correlated O_3-induced stomatal response to photosynthesis and showed that the oxidative stress generated by O_3 was first detectable in the chloroplast of guard cells. Stomatal closure, however, is not a universal response to O_3 exposure (Sanders et al. 1992). Reiling and Davison (1995) suggested that the pattern of stomatal response may be considerably more complex on the basis of the experiments they performed on *Plantago major* L.

3.1.2 Ozone-induced Signal Transduction

Ozone appears to act as a gaseous signaling molecule whose effects are similar to those of fungal elicitors (Ebel and Cosio 1994). It is well known that O_3 elicits responses in the cell, which can lead to drastic changes in gene expression (Kubo 2002), even before any visible damage to the plant is evident (Mahalingam and Fedoroff 2003; Mahalingam et al. 2005). Certain transcripts are induced and others are repressed in response to O_3 exposure (Sandermann 1996). These observations were surprising, because nearly all of the O_3 taken up through the stomata is believed to be decomposed in the apoplast (Heath and Taylor 1997). Therefore, the concept of membrane-bound receptors and signal chains to the chloroplast and nucleus was proposed by Sandermann (1996). The exact nature of these receptors is still unknown. Certain plasma membrane lipids may serve as general receptors, since unsaturated lipids could be oxidized by O_3 to initiate lipid signaling (Baier et al. 2005).

3.1.3 Ozone Sensing by the Plant Cell

Earlier studies suggest that O_3 sensitivity is generally correlated with the ascorbate status of leaf tissue (Conklin and Barth 2004). However, recent experimental work strongly indicates that ascorbate is not the only signal initiation point in O_3 sensing (Fig. 3) (Baier et al. 2005). Figure 3 also elaborates on how ROS, generated in the apoplast, can activate calcium (Ca^{2+}) channels and further induce the intracellular O_3-responsive signaling pathways. Clayton et al. (1999) showed a specific increase in the intracellular Ca^{2+} concentration, if atmospheric O_3 reached concentrations above 70 ppb. In terms of signaling, Ca^{2+} channels of the plasma membrane could also function as ROS sensors (Fig. 3). Because ROS and free radicals can affect the ion channel, redox-dependant alterations in ion conductance may be a more general signaling pathway under conditions of oxidative stress. It is tempting to assume that ROS interact directly with plasma membrane-bound receptors and trigger downstream events in the cytoplasm (Dat et al. 2000). Nonenzymatic or lipoxygenase-mediated breakdown of lipids (Munnik and Meijer 2001), ROS (in particular hydrogen peroxide (H_2O_2) as diffusible messenger (Laloi et al. 2004)), and modulation of cytosolic ascorbate and glutathione (Gomez et al. 2004; Noctor and Foyer 1998), are well established regulatory and signaling components that may represent other routes of O_3-triggered signaling from sites of O_3 chemical reaction in the apoplasm or plasma membrane to cytosol (Baier et al. 2005).

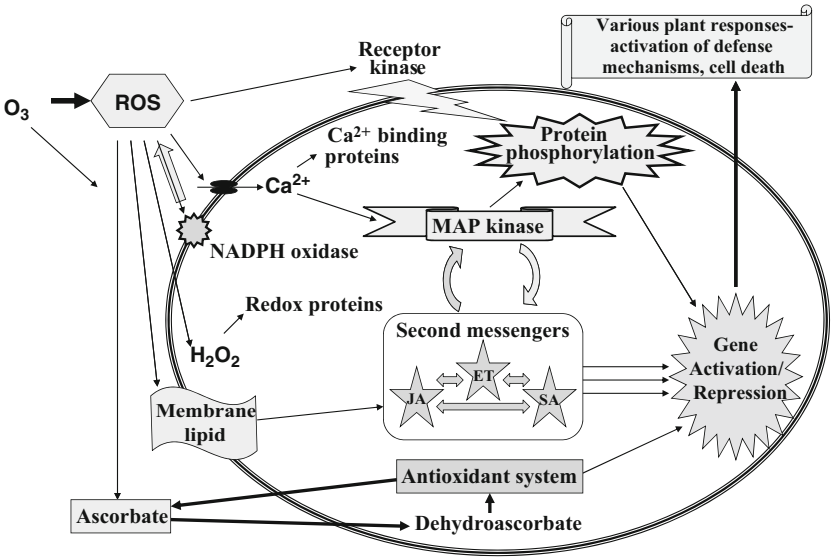

Fig. 3 A simplified representation of the possible O_3-dependant signaling pathways. Reactive Oxygen Species (ROS) generated in the apoplast activate the Ca^{2+} channels, which trigger the Ca^{2+} influx in the cytosol. This leads to the activation of MAPK in the cytosol. MAPK activation, in turn, seems to be involved in the up-regulation of ET, SA, and JA signaling pathways, which are responsible for changes in gene expression. Hydrogen peroxide (H_2O_2) present in the apoplasm, diffuses into the cytosol through the plasma membrane, and leads to the oxidation or modulation of certain cytosolic proteins. Abbreviations: *NADPH oxidase* Nicotinamide adenine dinucleotide phosphate-oxidase

3.1.4 Role of ROS in O_3 Signaling

Studies have shown that ROS can play a role as a second messenger in signal transduction cascades (Apel and Hirt 2004; Dat et al. 2000; Foyer and Noctor 2005; Opden Camp et al. 2003). Dat et al. (2003) have observed reduced levels of antioxidant enzymes like catalase (CAT) and ascorbate peroxidase (APX), along with an accumulation of ROS, in transgenic tobacco plants upon O_3 exposure. Programmed cell death (PCD) was triggered by changes in H_2O_2 homeostasis and a signaling cascade was induced, which led to an NADPH oxidase-dependant burst (Dat et al. 2003). Involvement of plasma membrane NADPH oxidase in O_3-triggered ROS accumulation has been shown in *Arabidopsis thaliana* L. (Heynh) *rcd1* (Overmyer et al. 2000). Overmyer et al. (2000) showed that application of diphenylene iodonium, an inhibitor of NADPH oxidase, inhibited ROS accumulation and reduced leaf damage in *rcd1* mutant, indicating that O_3 initiates active cellular ROS production.

The kinetics of ROS production in sensitive tobacco plants, exposed to O_3, resembled the hypersensitive response (HR) from the oxidative burst that was observed during incompatible pathogen–plant interactions (Schraudner et al. 1997). The O_3-sensitive tobacco cultivar Bel W3 exhibited a strong biphasic oxidative burst in

response to O_3 exposure that was similar to the HR, but the tolerant cultivar Bel B had only a modest rise in the first phase of endogenous radical production (Schraudner et al. 1997). Pasqualini et al. (2002) reported that treatment of O_3-sensitive tobacco (*Nicotiana tabacum* L. cv. Bel W3) with an O_3 pulse (150 ppb for 5 h) triggered a cell death program, which was accompanied by an increase in mRNA level of pathogenesis related (PR) protein 1a, a typical HR marker. Ozone treatment induced a deposition of autofluorescent compounds and callose at 24 h and stimulated the phenolic content at 10 and 72 h after treatment started; concomitantly there was enhanced expression of phenylalanine ammonia lyase (PAL) a and b (Pasqualini et al. 2002). Increased protease activity accompanied by chromatin condensation indicated that oxidative stress induced by ROS initiated PCD (Pasqualini et al. 2002). Vollenweider et al. (2003) performed a study in which 10 of 49 woody plant species displayed typical O_3 injury symptoms at AOT40 exposure levels that varied from 14 to 28 ppm h. Ozone response was characterized by the induction of oxidative stress in the mesophyll resulting in discrete and light-dependant hypersensitivity-like responses and accelerated cell senescence (Vollenweider et al. 2003). The responses included polyphenolic or callose cell wall deposits, starch pattern changes and ultrastructural alterations, especially in the structure of chloroplasts and vacuoles (Günthardt-Goerg et al. 2000; Pääkkönen et al. 1998).

In chloroplast, ROS production has emerged as an important mechanism involved in acclimation of plants to various abiotic stresses (Mullineaux and Karpinski 2002). It was shown that in *Arabidopsis*, O_3-induced ROS production started first in the chloroplasts of the guard cells and then spread to the adjoining cells (Joo et al. 2005). The substance 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), an inhibitor of chloroplast electron transport, suppressed early chloroplastic ROS production, but did not interfere with ROS production in the cytoplasm and membranes of guard cells (Kangasjarvi et al. 2005). The early chloroplastic oxidative burst was a result of signaling through the heteromeric G-protein (Gβγ complex), and was different from the Gα-mediated activation of membrane-bound NADPH oxidase, which was necessary for intercellular signaling during cell death (Joo et al. 2005; Kangasjarvi et al. 2005). The first peak of the biphasic oxidative burst by elevated O_3 in wild-type *Arabidopsis* plants was entirely missing in mutants lacking α and β subunits. The late ROS production in the cells next to guard cells was normal in plants lacking Gβ proteins but was missing in plants lacking Gα proteins (Joo et al. 2005). It has also been shown that H_2O_2 accumulation in tobacco apoplast after O_3 exposure is a result of dismutation of NADPH oxidase-generated superoxide ($^{\cdot}O_2^-$) to H_2O_2 (Langebartels et al. 2002). However, the role of ROS produced by NADPH oxidase is more important for cell-to-cell signaling, which ultimately led to an actual cell death process (Joo et al. 2005).

3.1.5 Membrane-bound Receptors

Miles et al. (2002) have shown that the oxidative signaling in cultured tobacco suspension cells involved membrane receptor-mediated signaling. Use of a membrane nonpermeable reagent that interferes with membrane receptor-mediated signaling

prevented the activation of tobacco Salicylic Acid-induced Protein Kinase (SIPK) (Miles et al. 2002). Although activation of these membrane-associated receptors does not require G proteins, Ca^{2+} influx and ROS may be required (Miles et al. 2004). Ca^{2+} ions released by oxidation of redox-sensitive Ca^{2+} channels (Clayton et al. 1999) cause rapid changes in protein phosphorylation pattern (Agrawal et al. 2002a, b), which is mediated by Mitogen-Activated Protein Kinase (MAPK) (Morris 2001; Samuel et al. 2000).

MAPK cascades are a conserved signal transduction system in all eukaryotes and their importance for plants is well known (Baier et al. 2005; Jonak et al. 2002; Agrawal et al. 2003a). Plant-O_3 sensitivity and the expression of antioxidant genes are affected by these kinase classes, since both suppression and over expression of tobacco SIPK led to increased O_3 sensitivity and changes in expression of APX (Samuel and Ellis 2002). Activation of MAPK by phosphorylation generally leads to nuclear localization, and phosphorylated MAPKs are translocated to nucleus within 30 min of initiating O_3 exposure (Ahlfors et al. 2004). The role and function of these MAPKs in O_3-exposed plants are, however, still unknown, although there is evidence that they are connected to hormonal responses (Kangasjarvi et al. 2005).

3.1.6 Membrane Lipids

Formation of hydroxyl radicals in the apoplast from the presence of H_2O_2 and O_2^- can initiate lipid peroxidation, which results in lipid hydroperoxide (LOOH) formation (Schraudner et al. 1997). Various lipid hydroperoxides and other lipid-based signaling molecules such as jasmonates are biologically active substances that control a variety of downstream processes. Thus, formation of these plasma membrane lipids-based molecules may also be regarded as O_3 perception as a result of O_3 action in the apoplast (Kangasjarvi et al. 2005).

3.1.7 Role of Phytohormones

Plant hormones play an important role in O_3-induced signal transduction. The levels of salicylic acid (SA), ethylene, and jasmonic acid (JA) are important in determining the degree of plant O_3 sensitivity, O_3 lesion initiation, propagation, and containment (Overmyer et al. 2003). This is illustrated by the fact that some mutants of *Arabidopsis* and other species, first described as O_3 sensitive, have turned out to be either partially JA insensitive or ethylene overproducers (Kanna et al. 2003; Koch et al. 2000; Overmyer et al. 2000; Rao et al. 2000). Regulation of the induction and spreading of oxidative stress symptoms by phytohormone ethylene, SA and JA has been reported in several recent studies (Moeder et al. 2002; Vahala et al. 2003a, b). Some of the biosynthetic enzymes for ethylene and their transcripts have been shown to respond to O_3 within a period of less than 60 min (Tuomainen et al. 1997). The hormonal action of ethlylene promotes leaf damage and senescence when exposed to O_3 (Bae et al. 1996; Miller et al. 1999). Under acute conditions, O_3 triggers leaf necrosis, but it induces senescence of leaves under chronic exposure conditions (Nakajina 2002).

Ethylene and SA can thus be considered as second or third messenger molecules for O_3, depending on whether they are induced by O_3 itself or by O_3-derived active oxygen species (Sandermann 2000). In general, ethylene and SA amplify the oxidative signal, while JA constricts O_3-induced damage (Kangasjarvi et al. 2005; Langebartels et al. 2002). It has been hypothesized that early accumulation of ethylene stimulates spreading of cell death and suppresses protection by JA. Late accumulation of JA, however, inhibits the ethylene pathway and propagates cell death (Tuominen et al. 2004). Physiological analysis of *Arabidopsis* mutant *oji1* (O_3 sensitive and JA insensitive) demonstrated that JA signaling antagonizes O_3-induced induction of ACC synthase catalyzed ethylene biosynthesis (Kanna et al. 2003). Rao et al. (2002) observed that *NahG* and *npr1* plants of *Arabidopsis* depend on SA for O_3-induced ethylene production. When potato plants were exposed to 80 ppb O_3 for 5 h day^{-1}, levels of *rbcS* mRNA declined at least five-fold in expanding leaves after 3 days of O_3 exposure, and ethylene levels increased six to ten-fold. The expression of OIP-1, and ACC synthase cDNA from potato, correlated with increased production of ethylene and decreased levels of *rbcS* mRNA in foliage of plants treated with O_3 (Glick et al. 1995). Vahala et al. (2003a) reported that ethylene evolution correlated best with O_3-induced cell death and that a decrease in ethylene sensitivity, together with high ethylene biosynthesis, can potentially cause cell death through a deficient detoxification of cyanide in birch (*Betula pendula* Roth).

3.2 Morphological Responses

Ozone symptoms usually occur between the veins on the upper leaf surface of older and middle-aged leaves but may also involve both leaf surfaces (bifacial) for some species. Schmieden and Wild (1995) have reported that cells damaged from O_3 exposure are often near stomata or stomatal cavities. The palisade parenchyma cells of the leaves are the most sensitive to O_3, and their collapse in the leaves of morning glory, spinach, and radish are associated with transformation of cell wall intercellular spaces (Nouchi 2002). Foliar symptoms of O_3 damage in the graminaceous and leguminous plants, and broadleaf woody species, such as Japanese zelkova (*Zelkova serrata*) and platanus (*Platanus acerifolia*), are numerous reddish-brown stipples. The reddish-brown stipples that develop on the leaves of the above-mentioned plants are the result of the accumulation of black or red pigments in dead cells of the palisade tissue (Nouchi 2002). Sanders et al. (1992) have also reported damaged chloroplast membranes, plasmalemma, and tonoplasts as a result of O_3 exposure. Swelling of thylakoid membranes, leading to breakdown of chloroplast integrity, was shown to be the result of O_3 exposure (Crang and McQuattie 1986). Tonneijk and van Dijk (1997) found that elevated O_3 caused dark brown blemishes mainly on the adaxial leaf surface of *Phaseolus vulgaris* L. cv. Pros. The results of several bioindication/biomonitoring studies involving the use of tobacco cultivars have shown the presence of O_3 injury in Po valley (Mignanego et al. 1992), in Italy and in Eastern Spain (Gimeno et al. 1995). Benton et al. (2000) have reported the presence of O_3-induced visible

injury on watermelon, tomato, and clover. Sarkar and Agrawal (2010a) also observed interveinal yellowing or chlorotic stippling in the leaves of mature rice plants, under both ambient and elevated O_3 exposure conditions in open-top chambers (OTCs), and found that the magnitude of visible foliar injury formation depends on the cumulative effect of both concentration and duration of O_3 exposure.

Tropospheric O_3 stress reduces carbon acquisition by plants and the subsequent allocation of carbon to roots (McCrady and Andersen 2000; Andersen 2003). Decreased carbon assimilation and altered carbon partitioning to O_3-induced metabolic pathways result in altered allocation and lower biomass accumulation (Laurence et al. 1994). Previous studies have shown that chronic O_3 exposure can cause considerable biomass and seed production losses, even at low concentrations, and often in the absence of visible symptoms (Fiscus et al. 1997; Long and Naidu 2002). Pell et al. (1994b) showed that a decrease in above-ground carbon allocation by O_3 is less than below ground, but the magnitude of the decrease varies widely. A meta-analysis study carried out by Morgan et al. (2003) for assessing the response of soybean (*Glycine max* (L.) Merr.) to an average chronic O_3 exposure of 60 ppb showed that reduction in shoot and root dry biomass was approximately equal at 21%. In many tree species, carbon retention is increased in leaves and carbon allocation to roots is reduced after O_3 exposure (Andersen et al. 1997). This higher retention of carbon in leaves is explained by higher carbon demands for repair of damaged foliage by reduced transport in phloem, or by decreased phloem loading (Landolt et al. 1997). Reduced root growth can alter the functioning of rhizospheric organisms and could make trees more susceptible to drought or nutrient deficiency (Woodbury et al. 1994). Andersen et al. (1997) reported lower carbohydrate (starch, sucrose, fructose, and glucose) levels in new roots of Ponderosa pine (*Pinus ponderosa*) seedlings after O_3 exposure, which may result in reduced plant growth over time. In addition, O_3 exposure over a 1-year period resulted in reduced plant growth over time and lower new root growth in the year following exposure (carryover effect). Vorne et al. (2002) reported a decreased concentration of reducing sugars (glucose and fructose) in potato tubers upon exposure to elevated O_3, perhaps as a consequence of decreased assimilate supplies or a reduction in phloem loading, which may be particularly sensitive to O_3 (Grantz et al. 2006). Kollner and Krause (2002) reported that glucose and starch concentration in the leaves of potato increased during the first few weeks of O_3 exposure, suggesting that damage to cellular membranes led to a reduction in phloem loading.

The impact of O_3 on the growth and biomass of plants is variable and this variability results from species or cultivar differences, in nutrient and moisture conditions between experiments, and differences resulting from the developmental stage of the plant (Morgan et al. 2003). For the O_3-tolerant soybean cultivar Essex, elevated O_3 stimulated total dry weight during flowering (Miller et al. 1998; Robinson and Britz 2000), but later in the growing season, total biomass was decreased in the same cultivar (Fiscus et al. 1997; Miller et al. 1998). Meta-analysis performed by Morgan et al. (2003) on soybean showed that the largest response of O_3 exposure was on final plant mass, suggesting that O_3 damage accumulates over the growing season. In fact, the impact of O_3 treatment was not significant in the vegetative stage and was greatest during pod filling, with effects at flowering stage

being intermediate (Morgan et al. 2003). Dermody et al. (2006) found no effect of elevation of O_3 on the leaf area index of soybean during early vegetative growth, but the effect was pronounced during the late grain-filling stage. This increased response with time could result from the compounding of O_3 effects over the life span of the plants (Adams et al. 1996; Miller et al. 1998), or from an increase in susceptibility during seed filling (Tingey et al. 2002). Britz and Robinson (2001) found that leaf growth was affected in different ways in an O_3-tolerant and an O_3-sensitive soybean cultivar. Leaf area was significantly reduced for O_3-sensitive cultivar and specific leaf mass increased significantly in O_3-tolerant cultivar.

Many researchers have reported that O_3 exposure alters a specific measure of carbon allocation, the root shoot biomass ratio (R/S) (Cooley and Manning 1987; Franzaring et al. 2000; Grantz and Yang 1996). The R/S ratio changed predictably with plant development (ontogenetic drift) (Farrar and Gunn 1998), as well as with altered arial or edaphic environmental conditions (van Noordwijk et al. 1998). The ambiguity of R/S as a measure of allocation may be overcome by evaluation of an allometric coefficient *k* (Troughton 1955). This value of *k* compares the relative growth rates of the competing plant parts, and therefore, exhibits a nearly constant magnitude over the period of rapid development in plants exposed to stress condition (Gunn and Farrar 1999). Although O_3 has been reported to alter R/S in many studies (Cooley and Manning 1987), changes in *k* have been documented in only one subset (Reiling and Davison 1995). Grantz et al. (2006) demonstrated that mean *k* declined significantly by 5.6% following O_3 exposure by using meta-analysis. The diversion of biomass to shoot growth in O_3 exposed plants occurs, and acts to facilitate plant defense against ROS generated by O_3 (Sandermann 1996), and ultimately to enable repair of O_3-induced foliar wounding (Grulke and Balduman 1999; Sandermann 1996).

3.3 Physiological Responses

The results from many studies, conducted to evaluate the effects of O_3 on physiological processes, have shown a decrease in the photosynthetic capacity of plants. Reductions in stomatal conductance (gs), net photosynthetic CO_2 assimilation and carboxylation efficiency have all been associated with O_3 exposure (Fiscus et al. 1997; Guidi et al. 2001; Morgan et al. 2003). A decline in the photosynthesis rate of O_3-exposed plants is associated with damage to the photosynthetic machinery that leads to reduced fixation, and increased CO_2 concentration (Ci), resulting in reduced gs (Fiscus et al. 1997; McKee et al. 1997). Elevated O_3 (82 ppb O_3, 7 h day^{-1} for 21 days) depressed the photosynthesis rate and gs by 24 and 8%, respectively, but stimulated dark respiration and intercellular CO_2 concentration by 28 and 39%, respectively, in 20 varieties of wheat released in the last 20 years (Biswas et al. 2008). This study revealed that sensitivity to O_3 in cultivars of wheat progressed with the year of release and was correlated with gs of O_3-exposed plants. Ozone-induced loss in photosynthetic rate was attributed to impaired activity of the mesophyll cells and loss of integrity of the cellular membrane as evidenced by increased CO_2 concentrations and lipid peroxidation (Biswas et al. 2008).

The light reaction system of photosynthesis was found to be more stable under O_3 exposure conditions (Heath 2008). During photosynthesis, ROS are produced and destroyed in such a manner that they contribute to the regulation of electron transport (Asada 1997; Foyer 1997). Operation of the complete pathway involves H_2O oxidation at PS II, accompanied by production of equimolar amounts of H_2O at PS I (Noctor and Foyer 1998). ATP can therefore be formed without concomitant generation of reducing power. The ascorbate pool of the chloroplast plays an important role in maintaining the equilibrium between ROS generation and destruction (Noctor and Foyer 1998). However, when ROS generation exceeds the quenching capacity of ascorbate in plant chloroplasts that were exposed to O_3 or any other stressors, the electron transport chain is also affected. Ozone has been shown to alter photosynthetic electron transport in some plants via reduction in efficiency of excitation capture (Calatayud and Barreno 2001; Guidi et al. 2001). Guidi et al. (2000) exposed 14 Italian cultivars of *P. vulgaris* to a single pulse of O_3 (150 ppb for 3 h) and their sensitivity was assessed on the basis of visible symptoms, effects on chlorophyll content, and changes in chlorophyll *a* fluorescence parameters. Ozone significantly depressed the total chlorophyll content in most of the cultivars and a significant correlation was found between chlorophyll content and visible symptoms. Most cultivars showed significant changes in the Fv/Fm ratio, even when no visible injury symptoms were observed (Guidi et al. 2000). The sensitive cultivars showed visible injury symptoms 2–4 h after the end of fumigation, along with significant alterations in chlorophyll content and chlorophyll *a* fluorescence parameters (Guidi et al. 2000). Guzy and Heath (1993) exposed 12 varieties of *P. vulgaris* to 40–50 ppb O_3 for 75–135 min and reported that sensitive varieties showed higher O_3-induced chlorophyll loss and/or inhibition of photosynthesis as compared to tolerant ones. Calatayud and Barreno (2001) showed that long-term exposure to elevated O_3 caused significant declines in Fm, Fv, and the Fm/Fv ratio in tomato leaves, compared to control plants.

Two poplar clones (*Populus* × *euramericana* I-214 and *P. deltoides* × *maximowiczii* Eridano) were exposed to 60 ppb O_3 (5 h day^{-1} for 15 day), and a reduction in net CO_2 assimilation rate (A), compared to control leaves, was observed (Guidi et al. 2001). In I-214, a decrease in A was accompanied by a strong reduction in gs along with altered mesophyll activity, which was ascribed to the limitation of the dark reaction of photosynthesis. A reduction in the quantum yield of I-214 can be considered to be a mechanism to downregulate photosynthetic electron transport so that the production of ATP and NADPH is maintained in equilibrium with the decreased demand of the Calvin cycle in ozonated leaves (Guidi et al. 2001). In clone Eridano, however, Gw did not change with O_3 exposure, although the internal CO_2 levels increased significantly. This suggests that the avoidance mechanism in response to O_3 stress that was evident in I-214 was absent in Eridano, and reduced A was solely attributed to changes in mesophyll limitations of photosynthesis (Guidi et al. 2001). Similar observations were recorded in two clones (NC-R and NC-S) of clover (*Trifolium repens* L. cv. Regal), wherein a reduction in CO_2 fixing ability was attributed to mesophyllic limitations in NC-S and nonstomatal components in NC-R (Francini et al. 2007).

Disturbances of light reactions can be detected through chlorophyll fluorescence (Fv/Fm) to quantify the impact of stress plants (Buschmann et al. 2000; Chaerle et al. 2004).

The Fv/Fm ratio typically ranges from 0.80 to 0.83 under normal conditions (Kitajima and Butler 1975) and its decrease under stress is indicative of photoinhibition of photosynthesis (Baker and Rosenqvist 2004). In crop plants, O_3 stress has been shown to negatively affect the maximum (Fv/Fm) and effective (Φ_{PSII}) quantum yield of PS II photochemistry. A reduction in the relative fraction of the open PS II reaction center (photochemical quenching coefficient, qP) and an increase in heat dissipation (nonphotochemical quenching, NPQ) have also been observed in plants suffering from O_3 stress (Castagna et al. 2001; Guidi et al. 2001; Rosenqvist and van Kooten 2003). Rai and Agrawal (2008) reported a significant reduction in the photosynthetic rate (i.e., 18 and 28.3%, respectively) in two rice cultivars, NDR 97 and Saurabh 950, grown in nonfiltered chambers compared to filtered chambers at a rural site in India. The mean O_3 concentrations in this study varied between 30.5 and 45.4 ppb. Similar observations have also been made in several tree species (Gielen et al. 2007; Ribas et al. 2005), although a lack of response was also reported (Löw et al. 2007; Maurer et al. 1997). However, the effects of O_3 stress on chlorophyll fluorescence have also been interpreted as a down-regulation of linear electron transport to compensate for O_3-induced reduction in the activity of the Calvin–Benson cycle (Guidi et al. 2001; Heath 2008).

The reductions in net photosynthetic rates and carboxylation efficiency, after exposure to O_3, have been related to reduced levels and activity of RuBisCO (Nouchi 2002). RuBisCO is regarded as a key protein, and it functions to produce negative O_3 effects on plant productivity (Leitao et al. 2008). RuBisCO is made up of two types of subunits: these are designated as the large subunit (LSU) encoded for by chloroplastic DNA and the small subunit (SSU) encoded for by nuclear DNA (Dizengremel 2001). The quantity of LSU was found to be diminished in the foliage of trees that were exposed to O_3 (Pelloux et al. 2001). Decreased levels of *rbcS* (small subunit) and *rbcL* (large subunit) mRNA transcripts were reported in leaves of O_3-treated trees (Pelloux et al. 2001). Transcription of chlorophyll *a*/*b*-binding protein (*cab*) and glyceraldehyde 3-phosphate dehydrogenase (*gapA* and *gapB*) was also reduced in O_3-exposed plants (Glick et al. 1995). Sarkar and Agrawal (2010a) calculated 44.6 and 48.6% foliar injury in two cultivars of rice, Shivani and Malviya Dhan, respectively, when they were exposed to mean O_3 concentration of 69.3 ppb. These authors also observed reductions in the abundance of the LSU and SSU of RuBisCO by using one-dimensional gel electrophoresis (1-DGE or commonly called SDS-PAGE; sodium dodecyl sulfate polyacrylamide gel electrophoresis) protein profiling.

Although O_3 exposure reduced the amount of RuBisCO, it is not clear whether the loss in transcripts is a result of mRNA degradation or from reduced transcription (Pell et al. 1994a). An O_3-induced decrease in the quantity of RuBisCO may involve several mechanisms. These include an increased rate of protease-mediated protein degradation, an alteration of protein through an oxidative process, or an inhibition of protein synthesis (Brendley and Pell 1998; Pell et al. 1994a). These processes could occur simultaneously or separately, depending on the O_3 concentration, exposure time, and leaf age (Junqua et al. 2000).

Another carboxylase involved in CO_2 fixation in plants is phosphoenol pyruvate carboxylase (PEPC). PEPC is not directly implicated in C_3 photosynthesis and is known to be involved in the "anaplerotic pathway" (Jeanneau et al. 2002). Studies in which the impact of O_3 is assessed on both RuBisCO and PEPC are less common and were conducted exclusively on woody plants (Kolb and Matyssek 2001). An increase in PEPC activity, along with a decrease in RuBisCO activity has been evident under O_3 stress conditions in various trees (Gaucher et al. 2003; Landolt et al. 1994; Lutz et al. 2000). Leitao et al. (2008) reported a similar response in *P. vulgaris* exposed to NF+80 ppb O_3 in OTCs. In primary leaves, elevated O_3 reduced RuBisCO activity by 33%, whereas it stimulated PEPC activity by 376% (Leitao et al. 2008). The stimulation of PEPC activity is closely related to an increase in the activities of several enzymes of the glycolysis and pentose phosphate pathways, which would produce greater reducing power (NADPH and NADH) (Dizengremel 2001). Ozone-induced changes in the cell ultrastructure is manifested by several factors: reduction in chloroplast size, disintegration of the thylakoid and cellular organelles (Günthardt-Goerg et al. 2000), increased size and number of plastoglobuli, and appearance of exudates on the walls of mesophyll cells and mesophyll collapse (Britvec et al. 2001; Selldén et al. 1997). Pino et al. (1995) reported that maize (*Zea mays*) leaves, exposed to high O_3 concentrations (360 ppb for 5 h for 3 week), showed increases in several thylakoid membrane-associated proteins, whereas a 32 kDa polypeptide (D1) associated with thylakoid membrane was reduced, along with a reduced incorporation of [^{35}S] methionine into proteins (Pino et al. 1995).

3.4 Cellular Responses

The generation of stress response leads to observable cellular or tissue changes such as water loss (waterlogging); this, in turn, leads to cell death with associated chlorosis and necrosis (Heath 2008). Temporal shifts in development are often observed as an "accelerated senescence" or loss of certain proteins earlier than expected during plant growth (Yang and Poovaiah 2000). In lentil (*Lens culinaris*), it has been shown that O_3 controls the expression of certain enzymes at the transcriptional level, including down-regulating the amine oxidase gene and up-regulating the lipoxygenase gene (Maccarrone et al. 1997). Turcsányi et al. (2000) reported decreased ascorbate concentration, which plays an important role in the interception of O_3 in the leaf cell walls of O_3-treated *Vicia faba*. Ozone produces rapid accumulation of certain specific proteins, such as OsPR5, OsPR10s, APX, MnSOD, and ATP-dependent CLP protease, which could serve as potent markers to monitor O_3-related damage in rice or other plants (Agrawal et al. 2002b, c). The expression of *OsEDR1* varied significantly in vegetative and reproductive tissues of rice after O_3 exposure, suggesting the involvement of this protein in defense/stress signaling pathways and in general plant growth and development (Kim et al. 2003).

Ozone exposure induced increases and decreases in the accumulation of some newly synthesized proteins in the leaves of *Zea mays* L. (Pino et al. 1995). Tang et al. (1999) reported exposure-dependent accumulation of a small group of O_3 responsive proteins (ORPs) in two clones of white clover (*Trifolium repens* L. cv. Regal), NC-R (O_3 resistant), and NC-S (O_3 sensitive) plants. They exposed the plants in the chamber of a continuously stirred tank reactor (CSTR) to a step function O_3 profile for 3 days (8 h day^{-1}) (minimum and maximum concentrations were 50 and 150 ppb, respectively). More ORPs accumulated in NC-R than in NC-S, under similar exposure conditions. There were no detectable OPRs in control plants (Tang et al. 1999). Pääkkönen et al. (1998) exposed saplings of O_3 sensitive and tolerant clones of birch (*Betula pendula* Roth) to 100 ppb O_3 for 43 days and studied the genes that encode the stress-related proteins PR-10, PAL, and late embryogenesis abundant (LEA) group protein BP8. It was observed that the appearance of visible injury (necrotic flecks) and enhanced yellowing of leaves coincided with the induction of PR-10 and PAL genes. Increased PR-10 mRNA levels can also be related to premature senescence in O_3-exposed plants, especially in sensitive clones (Pääkkönen et al. 1998).

In birch (*Betula pendula* Roth), an induction of Mpt1 (mitochondrial phosphate translocator) mRNA was detected during oxidative stress imposed by O_3 on the plants (Kiiskinen et al. 1997). Accumulation of Mpt1 mRNA was first detectable after 2 h of O_3 exposure and its maximum concentration was recorded 12 h after the beginning of an 8 h O_3 exposure (150 ppb) (Kiiskinen et al. 1997). Chiron et al. (2000) observed enhanced levels of transcripts of stilbene synthase (STS) and PMT (*S*-adenosyl-L-methionine: pinosylvin-*O*-methyltransferase) in Scots pine (*Pinus sylvestris*) upon exposure to 300 ppb O_3 (10 h day^{-1} for 2 days), compared to the control. Scots pine stilbene formation appeared to be induced via *STS* and *PMT* gene expression upon O_3 fumigation, fungal stress, and wounding (Chiron et al. 2000). Koch et al. (1998) also reported increased levels of PAL and *O*-methyltransferase in O_3 exposed sensitive and tolerant clones of hybrid poplar (*Populus maximowizii* × *P. trichocarpa*). The levels of these mRNAs, however, were higher in tolerant clones (245) than in O_3-sensitive ones (388).

3.5 Biochemical Responses

Although the stomatal resistance serves as a main obstacle to O_3 flux (Kollist et al. 2000), direct reaction of the pollutant with apoplastic ascorbate is commonly reported (Plochl et al. 2000). Ozone dissolves in the intercellular spaces and gives rise to a series of potentially damaging ROS, triggering an antioxidant response (Baier et al. 2005; Junqua et al. 2000). These oxidative processes also produce H_2O_2 in the humid environment of the leaf interior. H_2O_2 is one of the most important O_3-derived oxidants and may lead to the formation of other ROS such as $^{\cdot}O_2^-$ and hydroxyl ($^{\cdot}OH$) radicals (Langebartels et al. 2002). The ROS oxidize cellular constituents such as lipids, proteins, sulfhydryl groups, and nucleic acids and can

initiate radical chain reactions (Kangasjärvi et al. 1994). Ozone-tolerant varieties appeared to reduce chlorophyll loss, either by blocking O_3 entry through decreased gs, or through the action of the antioxidants ascorbate and nonprotein sulfhydryls (Guzy and Heath 1993). It was suggested that, in sensitive varieties, damage appeared to derive from either comparatively high gs or comparatively low levels of antioxidants in spite of low gs (Guzy and Heath 1993). Other workers have also associated O_3 tolerance of plants to the capability of maintaining high ratios of reduced ascorbate (AA)/total ascorbate (AA + dehydroascorbate DHA) (Burkey et al. 2003; Iriti et al. 2003).

The apoplastic antioxidants scavenge O_3 and its derivatives, and serve as the first detoxifying barrier of the cells (Fig. 4); presumably, this protects the next organizational level (membranes) from injury (Heath 2008). The level of ascorbate, especially apoplastic ascorbate, is considered to be a good indicator of O_3 tolerance

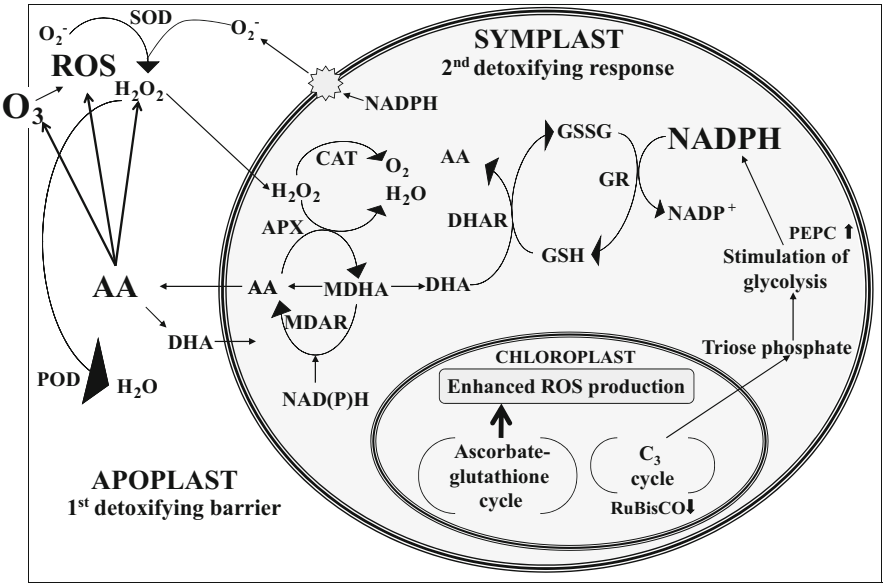

Fig. 4 A model that shows the antioxidant- and carbon metabolism-dependant detoxification process in cells. Antioxidants, such as ascorbic acid (AA), present in the apoplasm serves as the first detoxification barrier against ROS generated under O_3 stress. The ascorbate-glutathione cycle, which takes place in the cytosol and chloroplasts, serves as a second detoxification barrier. Oxidized glutathione that is generated through this cycle is reduced by glutathione reductase (GR) using the reduced form NADPH. Excess ROS affects the tricarboxylic acid cycle (C3 cycle), wherein the activity of RuBisCO is depressed and that of phosphoenolpyruvate carboxylase (PEPC) is stimulated. These series of events stimulate the enzymes of glycolysis, which eventually leads to more production of NADPH and acceleration of the ascorbate-glutathione cycle. Abbreviations: *APX* ascorbate peroxidase, *CAT* catalase, *DHA* dehydroascorbate, *DHAR* reduced dehydroascorbate; *GSH* reduced glutathione, *GSSG* oxidized glutathione, H_2O water, *MDAR* reduced monodehydroascorbate radical, *MDHA* monodehydroascorbate radical, *POD* peroxidase, *SOD* superoxide dismutase

(Tausz et al. 2007; Turcsányi et al. 2000). The antioxidant ascorbate accumulates in millimolar concentrations in leaf apoplasts and may scavenge significant amounts of O_3 (Plochl et al. 2000). Study results have shown that tolerance to O_3 was increased in an O_3-sensitive genotype of *Raphanus sativus* L., when its L-ascorbate content was increased by feeding hydroponically cultivated plants the biosynthetic precursor, L-galactono-1,4-lactone (Maddison et al. 2002). Van Hove et al. (2001) exposed sensitive (*Populus nigra* "Brandaris") and tolerant (*P. euramericana* "Robusta") species to filtered and ambient air for 30 days in fumigation cabinets and observed a reduction in gs and an increase in apoplastic water volume (V_{apo}). They also observed reduced ascorbate levels from O_3 treatment. However, these responses could not prevent a reduction in photosynthesis or early senescence in leaves that had prolonged exposures. These results suggest that the increase in apoplastic ascorbate concentration was a general stress reaction of the affected poplar leaves, rather than a defense reaction induced by O_3 (van Hove et al. 2001).

Depletion of antioxidants from the apoplastic region of cells, or a rapid entry of O_3, reduces protection and leads to the membrane injury (Conklin and Barth 2004; Zheng et al. 2000). Membranes are believed to be the initial sites of O_3 injury (Heath and Taylor 1997). Any change in the membrane leads to some membrane leakage (Marre et al. 1998), or shifts in signal transduction proteins within the membrane (Cerana et al. 2006; Rossard et al. 2006). These are initial causes of changes that can lead to internal cellular events. Nouchi and Toyama (1998) observed that O_3 exposure affected the lipid metabolic processes in morning glory during the initial exposure stages. Metabolic decomposition of glycolipids, increased amounts of triacylglycerol (TG) being synthesized from acyl moieties of monogalactosylglycerol (MGDG), and increased amounts of free fatty acid, produced by decomposition of MGDG, were observed in O_3-exposed plants (Sakaki et al. 1990a, b, c, 2008). Francini et al. (2007) studied the metabolic response of two clover clones (NC-S and NC-R) at 200 ppb O_3 for 5 h in the form of a square wave. NC-S exhibited a significant increase in membrane permeability, whereas no alteration was observed in NC-R. Barth and Conklin (2003) reported that the recessive O_3-sensitive *Arabidopsis* mutant *lcd-1* exhibited an increased amount of the lipid peroxidation product malondialdehyde (MDA) in leaves, compared to the wild-type, when exposed to O_3.

ROS trigger the exchange of antioxidants between the symplastic detoxification system and the apoplasm (Dizengremel et al. 2008). Luwe et al. (1993) observed that, when subjected to fumigation at high concentrations of O_3 (300 ppb), the cellular ascorbate redox state was unaltered. Ascorbate regeneration was tightly coupled to GSH (reduced glutathione) within the cell and transport activity was induced to replenish the reduced ascorbate pool in the apoplast (Baier et al. 2005). The capacity of the plants to regenerate the antioxidants within the cell serves as the second detoxification barrier (Fig. 4), which depends on carbon metabolism changes and alteration in gene expression (Foyer and Noctor 2005). The reducing power required to drive this process is supplied by NAD (P) H (Dizengremel et al. 2008).

Efficient destruction of $^{\cdot}O_2^-$ and H_2O_2 requires the action of several antioxidant enzymes. Superoxide radicals ($^{\cdot}O_2^-$) that are produced in different compartments of the plant cells are rapidly converted to H_2O_2 by the action of superoxide dismutase

(SOD) (Bowler et al. 1991). H_2O_2 is a strong oxidant that rapidly oxidizes thiol groups, and its accumulation in the chloroplast can be harmful, because photosynthesis depends on many thiol-regulated enzymes (Kaiser 1979). Catalase converts H_2O_2 to water and molecular oxygen. The absence of this enzyme in the chloroplast precludes protection to the thiol-regulated enzymes of the Calvin cycle. An alternative mode of H_2O_2 destruction is via peroxidases (POXs), which include either the specific APX or the unspecified POX. Glutathione present in the symplast also plays an important role in this protective mechanism (Noctor and Foyer 1998). The reduction of GSSH (oxidized glutathione) to GSH occurs through the functioning of glutathione reductase. The GSH/GSSH couple plays a redox sensor role (Foyer and Noctor 2005), allowing further reduction of ascorbate to occur. GSH not only serves as a reducing cofactor for several enzymes involved in ROS detoxification but could also conjugate to proteins to prevent their oxidation (Rouhier et al. 2004). Ranieri et al. (2000) have shown an enhancement in ascorbate-dependent POX activity at both the apoplastic and symplastic levels and extracellular syringaldazine-dependent POX activity. These changes occurred despite the fact that both extracellular and intracellular guaiacol-dependent and stromal and thylakoid-bound ascorbate-dependent POX activities remained unchanged in the sunflower plants exposed to O_3. Dizengremel et al. (2008) proposed reducing power [NAD(P)H] as one of the additional indicators for O_3 tolerance in plants. Ruzsa et al. (1999) studied the response of maize seedlings to a single, acute 6 h exposure to O_3, and observed that CAT (*Cat1 and Cat3*), glutathione *S*-transferase (*Gst1*), SOD (*Sod3*, *Sod4,* and *Sod4A*) transcript levels generally increased, whereas *Cat2*, D-ribulose-1,5-bisphosphate carboxylase/oxygenase (*RbcS*), and *Sod1* levels decreased. Transgenic tobacco (*N. tabacum* L. cv. Xanthi) expressing for cucumber ascorbate oxidase (AO) revealed up to a 380-fold increase in AO activity, with expression predominantly associated with leaf cell walls in response to 100 ppb O_3 (7 h day^{-1} for 28 days) (Sanmartin et al. 2003). Levels of ascorbic acid and glutathione in the symplast were not affected by the AO overexpression, but the redox state of ascorbate was reduced and that of glutathione increased (Sanmartin et al. 2003). Biswas et al. (2008) exposed 20 varieties of wheat plants, released over the last 20 years in China, to elevated O_3 (82 ppb O_3, 7 h day^{-1}) levels for 21 days in OTCs. The results observed were that O_3 significantly reduced foliar ascorbate (14%) and soluble proteins contents (27%) but increased POX activity (46%) and the malondialdehyde content (38%). Variations in the responses of both enzymatic as well as nonenzymatic antioxidants are shown in Table 1.

3.6 High-throughput Analyses of Ozone Responses at the Omics Level

Many studies of O_3 stress responses have been reported in several plant species during the last decade, and such studies have used typical research approaches. Although the demonstration of complicated mechanisms of O_3 response in plants has been attempted, much work still remains to be done in this area. In recent years, the decoding of the genomic sequences of several plant species has opened the functional

Table 1 Variations in the enzymatic and nonenzymatic antioxidative responses in selected plants under ozone (O_3) stress

Name of the plant	O_3 concentration	Parameter assessed		Variations recorded	References
Pinus canariensis L.	O_3-FACE, 67 ppb	AA		Not significant	Then et al. (2009)
		GR		Reduction (19%)	
Populus deltoids×*P. cv. caudina*	Fumigation, 180 ppb for 3 h	Total glutathione		Increment (significant)	Sen Gupta et al. (1991)
		GSH/GSSG		Reduction by 0.8	
		SOD		Increment (two-folds)	
Picea rubens Sarg.	Fumigation, 2× ambient	Total glutathione		Increment ($p<0.02$)	Hausladen et al. (1990)
		SOD		Reduction ($p<0.03$)	
Picea abies L.	Fumigation, 60–79.8 ppb				Polle et al. (1995)
	5.5 h	Apoplastic AA		Reduction (30%)	
	17 h			Increment (three-folds)	
Plantago major L.	Fumigation, 70 ppb (7 h d^{-1} for 42 d)	AWF	POD	Increment ($p<0.01$)	Lyons et al. (1999)
			SOD	Reduction ($p<0.01$)	
			APX	Not significant	
			AA	Not significant	
		RLE	POX	Increment ($p<0.05$)	
			SOD	Not significant	
			APX	Not significant	
			AA	Increment ($p<0.01$)	
Citrus clementina Hort. ex Tan. cv. Marisol	Filtration, 30 ppb	AA		Reduction (80%)	Iglesias et al. (2006)
		AA		Reduction (75%)	
Phaseolus vulgaris L.	Fumigation, 77 ppb (12 h)	AA			Burkey (1999)
cv. Oregon 91				Increment (80.5%)	
cv. S248				Increment (151%)	
cv. Strike				Increment (116%)	
cv. Tenderette				Reduction (53.6%)	
Triticum aestivum L. cv. Turbo	Fumigation, 90 ppb	APX		Increment (significant)	Bender et al. (1994)
		Total glutathione		Increment (significant)	
		AA		Not significant	
		GR		Not significant	

Oryza sativa L.	Filtration				Rai and Agrawal (2008)
cv. Saurabh 950		POD		Increment (36.5%)	
		SOD		Increment (43.6%)	
		AA		Increment (6.4%)	
cv. NDR 97		POD		Increment (74.8%)	
		SOD		Increment (43.3%)	
		AA		Increment (16.8%)	
Fragaria x *ananassa* Duch.	Fumigation				Keutgen and Pawelzik (2008)
cv. Korona	73 ppb (7 d)	SOD		Not significant	
		POD		Increment (420%)	
	86 ppb (8 h d^{-1}, 5 d $week^{-1}$, for 2 months)	SOD		Increment (significant)	
		POD		Increment (significant)	
cv. Elsanta	Fumigation				Keutgen and Pawelzik (2008)
	73 ppb (7 d)	SOD		Reduction (significant)	
		POD		Reduction (60%)	
	86 ppb (8 h d^{-1}, 5 d $week^{-1}$, for 2 months)	SOD		Reduction (significant)	
		POD		Not significant	
Nicotiana tabacum L. cv. Bel W3	Field study, 21.1–60.5 ppb	AA		Increment (40%)	Esposito et al. (2009)
		SOD		Increment (48%)	
		POD		Not significant	
Helianthus annuus L.	Fumigation, 150 ppb (4 h d^{-1}, for 4 d)	Symplast	AA	Increment (68.6%)	Ranieri et al. (2000)
			Guaicol POD	Not significant	
		Apoplast	AA	Increment (112%)	
			Guaicol POD	Not significant	
		Cell wall bound POD		Increment (82%)	
Lycopersicon esculentus Mill. cv. Tiny Tim	Filtration, 9.9 ppm h (AOT40)	APX		Not significant	Calatayud and Barreno (2001)
		GR		Not significant	
		SOD		Reduction (significant)	

(continued)

Table 1 (continued)

Name of the plant	O_3 concentration	Parameter assessed	Variations recorded	References
Lactuca sativa L. cv. Candela RZ	Filteration, 34 ppb (12 h)	APX	Not significant	Calatayud et al. (2002)
		GR	Not significant	
	Fumigation, 80 ppb (12 h)	SOD	Increment (23%)	
		AA	Not significant	
		APX	Reduction (significant)	
		GR	Not significant	
Spinacia oleracea L. cv. Avanti	Filtration, 2.4 ppm h (AOT40)	AA	Not significant	Calatayud et al. (2003)
		APX	Not significant	
		GR	Not significant	
		SOD	Increment (49.2%)	
	Fumigation, 15.08 ppm h (AOT40)	AA	Reduction (15%)	
		APX	Increment (16%)	
		GR	Reduction (12%)	
		SOD	Increment (67%)	
Glycine max L.	Fumigation			Singh et al. (2010)
cv. PK 472	70 ppb (4 h)	CAT	Reduction (16.3%)	
		POX	Increment (34%)	
		AA	Reduction (18%)	
	100 ppb (4 h)	CAT	Reduction (21.5%)	
		POD	Increment (47%)	
		AA	Reduction (18.9%)	
cv. Bragg	70 ppb (4 h)	CAT	Reduction (18%)	
		POD	Increment (29%)	
	100 ppb (4 h)	AA	Not significant	
		CAT	Reduction (23%)	
		POD	Increment (41%)	
		AA	Reduction (significant)	

Abbreviations: *AA* total ascorbic acid content, *APX* ascorbate peroxidase, *AWF* apoplastic washing fluid, *CAT* catalase, *FACE* Free Air Concentration Enrichment, *GR* glutathione reductase, *GSH* reduced glutathione, *GSSG* oxidized glutathione, *h* hour, *ppb* parts per billion, *ppm* parts per million, *POD* unspecified peroxidase, *RLE* residual leaf extract, *SOD* superoxide dismutase

genomics era, and new integrative and systemic analytical approaches have emerged; such approaches permit the rapid analysis of gene (one or many) function, and analysis at the level of the transcript (transcriptomics), the protein (proteomics) and the metabolite (metabolomics). In recent years, analyses have been performed to obtain information on O_3-triggered responses in plants; to this end, many high-throughput omics approaches were performed in *Arabidopsis* (Mahalingam et al. 2006; Tamaoki et al. 2003), aspen (Bohler et al. 2007; Gupta et al. 2005), bean (Torres et al. 2007), birch (Kontunen-Soppela et al. 2007; Ossipov et al. 2008), maize (Torres et al. 2007), pepper (Lee and Yun 2006) and rice (Agrawal et al. 2002c; Cho et al. 2008). In the model tree plant, poplar, the contribution of transcriptomics, proteomics, and metabolomics, in enhancing the understanding of mechanistic cellular responses to O_3, has been recently reviewed (Renaut et al. 2009). Some examples are presented below of the O_3-responsive gene, protein, or metabolite components.

3.6.1 Transcriptomics Approach

Tamaoki and coworkers found that 157 and 48 genes were induced and suppressed, respectively, in *Arabidopsis* wild-type (Col-0) plants exposed to 200 ppb O_3 for 12 h, using cDNA macroarray filters containing 12,028 nonredundant EST clones (Tamaoki et al. 2003). Furthermore, analysis of gene expression in O_3-exposed ethylene-, JA-, and SA-signaling mutants was performed using a subset macroarray containing the 157 induced genes, and the results revealed interaction among ethylene, JA, and SA-signaling pathways in the induction of gene expression in response to O_3 exposure (Tamaoki et al. 2003).

Studies were performed on the response to O_3 (using DNA microarrays) of aspen (Gupta et al. 2005), pepper (Lee and Yun 2006), *Arabidopsis* (Mahalingam et al. 2006), and rice (Cho et al. 2008). In these experiments, microarray analyses were used to collect new information on the O_3 response of these species to gene expression changes among different species. Moreover, identification of cellular responses between cultivars with different O_3 sensitivity was also examined in these studies.

The analysis of the trembling aspen tree (using a 4,608-element nylon cDNA array) after long-term O_3 exposure indicated that 88 and 7 genes, respectively, are up- and down-regulated (Gupta et al. 2005). Their functional categorization showed that O_3 exposure induces expression of genes involved in signaling, defense, senescence and the flavanoid pathway, and represses expression of genes related to photosynthesis and energy.

The comparative analysis of O_3 transcriptomes between O_3-sensitive and -tolerant peppers, using a 5 K pepper cDNA microarray, showed that 180 genes are O_3 responsive and most of them are involved in transcription, defense and ROS-scavenging, photosynthesis, and metabolism (Lee and Yun 2006).

Moreover, the study on oxidative signaling induced by O_3, in the O_3-sensitive Wassilewskija (Ws-0) ecotype of *Arabidopsis*, was reported using an *Arabidopsis* 70-mer oligonucleotide microarray (Mahalingam et al. 2006). A total of 371 genes were differentially expressed at 1, 4, 8, 12, and 24 h after O_3 exposure for 6 h.

The observations of early induction of proteolysis and hormone-responsive genes and down-regulation of carbon utilization, energy, and signaling-related genes indicated that O_3 rapidly triggers oxidative cell death and induces an inefficient defense response in the Ws-0 ecotype. Comparative analyses with other microarray data showed some overlap between O_3-responsive genes and ethylene-induced genes, or between O_3-responsive genes and methyl jasmonate-repressed genes, indicating a relevance of ethylene and jasmonate in O_3 response.

Recently, a transcriptomics approach was applied to investigate the molecular responses to O_3 (0.2 ppm) of 2-week-old rice leaves (Cho et al. 2008). Expression profiles of genes on a 22 K rice DNA microarray chip were evaluated in O_3-exposed rice leaves for 1, 12, and 24 h. In total, 1,535 genes were differentially expressed by a factor of more than five-fold vs. control. The results suggested that the following genes played a crucial role in the response to O_3: transcription, pentose phosphate pathway and signal transduction at 1 h, and genes related to antioxidant enzymes, ribosomal protein, posttranslational modification, signal transduction, jasmonate, ethylene and secondary metabolism at 12 and 24 h. In Fig. 5, the responses of rice-ozone omics are presented as an example.

3.6.2 Proteomics Approach

The first study performed on O_3 responses in rice were conducted in 2-week-old rice seedling, and used typical two-dimensional gel electrophoresis (2-DGE), amino acid sequencing and immunoblot analysis to characterize the effects on proteins. The results were that O_3 caused drastic changes of 37 proteins, including a decrease in RuBisCO and increases in various defense/stress-related proteins such as a PR class 5 protein, three PR 10 class proteins, APX(s), SOD, calcium-binding protein, calreticulin, and a novel ATP-dependent CLP protease (Agrawal et al. 2002c). In a later study, integrative and systematic analyses of molecular responses at the gene, protein, and metabolite levels in O_3-exposed rice (2-week-old) leaves for 24 h (using parallel transcriptomics, proteomics, and metabolomics approaches) were undertaken to obtain more information about responses to O_3 (Cho et al. 2008) (Fig. 5). This integrated analysis resulted in the identification of 1,535 genes, 21 proteins, and 24 metabolites. The identified proteins were mainly related to cellular processing, signaling, defense, and photosynthesis. Sarkar and Agrawal (2010a) also demonstrated a change in the mature leaf proteome of two Indian rice cultivars grown in OTCs (Malviya Dhan 36 and Shivani) after elevated O_3 exposure (ambient +20 ppb).

Sarkar et al. (2010) employed in-depth proteomics approaches (1-/2-DGE coupled with protein sequencing through MS and western blotting) to reveal the effect of O_3 on mature wheat plants under near natural field conditions. Results showed a significant decrease in some major photosynthetic (i.e., RuBisCO, RuBisCO activase, etc.) and carbon metabolism-related proteins, and an increase in some defense/stress-related proteins. However, the study also pointed toward a differential cultivar response between two wheat cultivars at the proteome level, under elevated O_3 stress conditions.

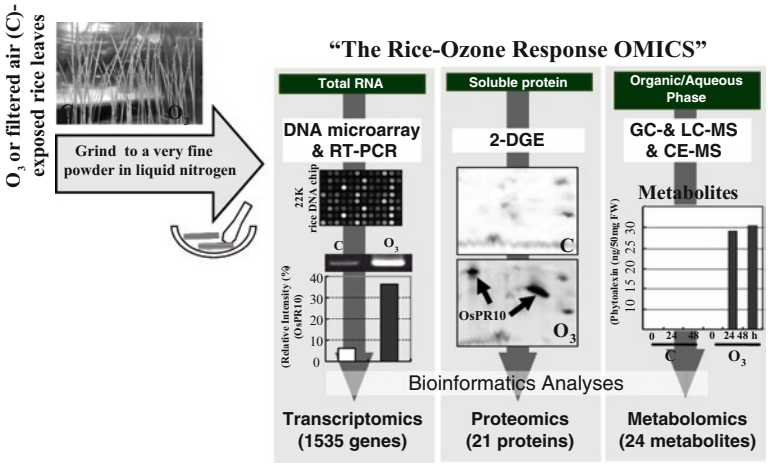

Fig. 5 Integrated transcriptomics, proteomics, and metabolomics approaches to investigate the effect of O_3 in rice. Essentially, starting from a single sample (powdered rice leaf), our approach is to study in parallel the changes in expression of genes (at mRNA level), proteins, and metabolites, after exposure occurs. More details are presented in Cho et al. (2008). Abbreviations: *C* control, *CE* capillary electrophoresis, *GC* gas chromatography, *LC* liquid chromatography, *MS* mass spectrometry, *OsPR10 Oryza sativa* pathogenesis related protein 10, *RT-PCR* reverse transcriptase-polymerase chain reaction, *2-DGE* two-dimensional gel electrophoresis

One-DGE was used in conjunction with western blot analysis to identify changes in proteins upon exposure to O_3 in beans (*P. vulgaris* L. cv. IDIAP R-3) and maize (*Zea mays* L. cv. Guarare 8128) (Torres et al. 2007). In this study, 2-DGE was also used in combination with mass spectrometry (MS) to identify leaf proteome changes. Western blot analysis in O_3-exposed bean and maize leaves showed dramatic changes in expression of SOD, APX, small heat-shock protein and naringenin 7-*O*-methyltransferase. Furthermore, 2-DGE and electrospray ionization-tandem mass spectrometry (ESI-MS/MS) analysis revealed that the decreased protein spots are mainly involved in glycolysis and photosynthesis, implying inefficiency of energy supply and photosynthesis; many of the increased protein spots are implicated as antioxidant enzymes and PR proteins.

Bohler et al. (2007) investigated the response to ozone in poplar with 2-DGE. Approximately 50% of O_3-responsive 144 protein spots were identified and most of these proteins were involved in carbon metabolism.

3.6.3 Metabolomics Approach

Recently, the alterations in plant metabolites from O_3 stress were evaluated in different birch tree genotypes (Kontunen-Soppela et al. 2007; Ossipov et al. 2008). Birch trees were subjected to long-term O_3 exposure (for 7 years) under open-field condition. Changes in metabolites such as phenolics, polar, and lipophilic compounds,

and pigments were analyzed using HPLC and GC/MS. The results showed that O_3 causes increases in quercetin–phenolic compounds and leaf cuticular wax layer-related compounds, whereas decreases occurred in several compounds involved in carbohydrate metabolism, chloroplast membranes, and pigments.

Quantitative changes of amino acids, glutathione, gamma-aminobutyric acid (GABA), and a phytoalexin were investigated in O_3-exposed rice leaves (Cho et al. 2008) (Fig. 5). Integrative analysis between these metabolites and the transcriptome of O_3-exposed leaves indicated that glycolysis, the citric acid cycle, the skimate pathway, and biosynthesis of phenolic secondary metabolites and glutathione were activated in rice leaves exposed to O_3 (0.2 ppm) for 12 and 24 h. Moreover, these studies demonstrated the potential suitability of glutamate and GABA as biomarkers for indexing damage to rice from O_3 exposure.

3.7 Yield

The productivity of several agricultural and horticultural crops in many regions of the world is adversely affected by current and anticipated atmospheric concentrations of O_3. The results of using various approaches to evaluate O_3 effects concur on the point that current yield losses range from 5 to 15% for sensitive crops. The use of emission models of the O_3 precursor, NO_x, in eastern USA, Europe, and East Asia imply that 9–35% of the world's cereal crops are exposed to seasonal O_3 concentrations that reduce yields by at least a few percent (Chameides et al. 1994). In 2002, more than 20% of Europe's crop production land was estimated to be at risk for yield losses of 5% or more from O_3 pollution, and this does not consider effects on grasslands or effects on forage nutritive value (Mills et al. 2007). Modeled ground-level O_3 concentrations combined with an experimentally derived yield loss function indicated that ambient O_3 levels reduced US soybean (*Glycine max* (L.) Merr.) production by 10% in 2005 (Tong et al. 2007). Simulations of cumulative O_3 concentrations in China suggested that soybean and wheat (*Triticum aestivum* L.) yields were suppressed by 12–19%, respectively, in 1990 (Wang and Mauzerall 2004). Climate models were used to forecast that the areas with the greatest production of peanuts (*Arachis hypogaea* L.), rice and soybeans, namely China, Japan, India, central Africa, the USA, and Indonesia, will continue to experience phytotoxic concentrations of ground-level O_3 in the coming 50 years (Emberson et al. 2009; Dentener et al. 2006; Wang and Mauzerall 2004).

Forty-one studies were conducted during the National Crop Loss Assessment Network (NCLAN) involved 14 crop species grown across the USA during a 7-year period; results indicated an approximate yield loss of 5%, equaling a value loss of $1 billion in the annual national economy (Heagle 1989; Murphy et al. 1999). The total production loss from ambient O_3 concentrations in the Kanto district of Japan ranged from 16,000 metric t in 1981, to 78,500 metric t in 1985, corresponding to 1.1–4.6% declines, respectively (Kobayashi and Okada 1995). Combining NCLAN data obtained from 12 species comprising 38 cultivars, it was found that cultivars of seven species [cotton, peanut, spinach, soybean, tomato, turnip

(*Brassica rapa rapa* L.) and wheat] would exhibit 10% yield reductions when exposed to an average O_3 concentration below 50 ppb (7 h day^{-1}) (US EPA 1996). Species such as maize, sorghum, barley, and some wheat cultivars showed a 10% yield loss when average O_3 concentration (7 h day^{-1}) exceeded 80 ppb (Booker et al. 2009). An extensive survey of season-long field studies conducted in OTCs found that O_3-sensitive crops, such as bean, cotton, lettuce, onion, soybean, tomato, turnip, watermelon, and wheat, suffered 5% yield losses at seasonal AOT40 values of 6 ppm h or less (Mills et al. 2007). Yields of moderately O_3-sensitive crops such as broccoli, grape, maize, potato, rape, rice, sugar beet, and tobacco were suppressed by 5% at seasonal AOT40s of 8.6–20 ppm h (Mills et al. 2007).

Ambient O_3 concentrations in the Mediterranean region induced yield losses on crops such as watermelon (19–39%) and tomatoes (17–24%) (Fumagalli et al. 2001a, b). Mills et al. (2000) have reported reductions of 20–40% in total dry weight of clover under ambient O_3 conditions. Velissariou (1999) reported a 100% crop loss for lettuce and chicory in Greece following an O_3 exposure episode. Ozone-induced losses on horticultural crops can be greater than expected as O_3 effects may enhance the sensitivity of plants to pathogens (Gimeno et al. 1999). Gimeno et al. (1999) reported that watermelon (*Citrullus lanatus*) exposed to O_3 concentrations (10 h) of 45 and 61 ppb in OTCs showed significant reductions of 19 and 39%, respectively, as compared to charcoal-filtered air. Reduction in fruit yield predominantly resulted from a decline in fruit number rather than average fruit weight (Gimeno et al. 1999). Heagle et al. (2003) reported significant reductions in both total tuber yield and number in two pot grown potato cultivars, Dark Red Norland, and Superior.

Asian countries have experienced tremendous increases in O_3 concentrations in the last decade and the potential impact of elevated O_3 on agricultural productivity of this region is important, because it is the home to approximately 60% of the world's undernourished people (MEA 2005), and Asia will experience a further population increase by 2030 (FAO 2003). Several studies conducted in OTCs have shown adverse effects of O_3 on productivity of crops in different areas such as East Asia (Jin et al. 2001; Zheng et al. 1998), India (Rai et al. 2007; Rai and Agrawal 2008; Singh et al. 2009a; Singh and Agrawal 2009; Tiwari et al. 2005), China (Zeng et al. 2008) and Pakistan (Wahid 2006a, b) (Table 2). Wang and Mauzerall (2004) have indicated that crop losses from elevated O_3 would increase by 2–16% for wheat, rice, and corn and to 28–35% for soybeans in China, Japan, and South Korea. Aunan et al. (2000), in a study on the impact of O_3 on agriculture in China, utilized a global three-dimensional chemical tracer model to calculate the surface O_3 concentration, and then applied the NCLAN or European OTC Programme (EOTCP) dose–response data to Chinese crops. Reduction in crop yields were less than 3% for most of the test plants, except soybean (Aunan et al. 2000). It has been suggested that crop losses for soybean and spring wheat may reach up to 20 and 30%, respectively, by 2020 (Aunan et al. 2000). In the Yangtze River Delta (YRD) region of China, yield losses from O_3 pollution in 1999 were estimated from dose–response functions and were based on local OTC studies and monitoring data in that region; 10 and 2.8% losses in yield for wheat and rice, respectively, were reported (Feng et al. 2003). A study that was based on dose–response functions in China disclosed that the Relative

Table 2 Reduction in the yield of selected agricultural and horticultural plants grown under O_3 stress

Plant species	Cultivar	Experimental type	O_3 concentration	Yield reduction (%)	References
Triticum. aestivum L.	Dragon	Fumigation	57 ppb (8 h d^{-1})	40	Pleijel et al. (2006)
	Lantvete	Fumigation	52 ppb (8 h d^{-1})	21	Pleijel et al. (2006)
	M 234	Filteration	40 ppb (8 h d^{-1})	20.5	Rai et al. (2007)
	Blueboy II	Fumigation (potted medium) (ground medium)	130 ppb (7 h d^{-1})	20.3 31.16	Heagle et al. (1979)
	Coker 42-72	Fumigation (potted medium) (ground medium)	130 ppb (7 h d^{-1})	22.5 42.82	Heagle et al. (1979)
	Holly	Fumigation (potted medium) (ground medium)	130 ppb (7 h d^{-1})	47 33.3	Heagle et al. (1979)
	Oasis	Fumigation (potted medium) (ground medium)	130 ppb (7 h d^{-1})	14.64 26.29	Heagle et al. (1979)
	Albis	Fumigation	57–58 ppb (8 h d^{-1})	37.4; 30.9[a]	Fuhrer et al. (1989)
			78–90 ppb (8 h d^{-1})	6.4; 65.4; 65[a]	Fuhrer et al. (1989)
	Riband	Fumigation	80 ppb	13	Ollerenshaw and Lyons (1999)
	Pak-81	Filteration	35.6 ppb (6 h d^{-1})	46.7	Wahid et al. (1995)
	Chakwal-86	Filteration	35.6 ppb (6 h d^{-1})	34.8	Wahid et al. (1995)
	Inqilab-91	Filteration	72 ppb (8 h d^{-1})	18	Wahid (2006a)
	Punjab-96	Filteration	72 ppb (8 h d^{-1})	39	Wahid (2006a)
	Pasban-90	Filteration	71 ppb (8 h d^{-1})	43	Wahid (2006a)
	Gemini	Filteration	43 ppb (7 h d^{-1}) 7.6 ppm h[b]	21.2	Fumagalli et al. (2001a, b)
			49 ppb (7 h d^{-1}) 6.7 ppm h[b]	17.9	
			54 ppb (7 h d^{-1}) 11.1 ppm h[b]	22.8	
			51 ppb (7 h d^{-1}) 8.3 ppm h[b]	27.4	
			46 ppb (7 h d^{-1}) 7.9 ppm h[b]	21.7	
			53 ppb (7 h d^{-1}) 9.6 ppm h[b]	20	

Oryza sativa L.	Saurabh 950	Filteration	30.5–45.4 ppb (8 h d^{-1})	11.5	Rai and Agrawal (2008)
	NDR 97	Filteration	30.5–45.4 ppb (8 h d^{-1})	15.9	Rai and Agrawal (2008)
	SY63	O_3 FACE	250 ppb (7 h d^{-1})	17.5	Shi et al. (2009)
	LYPJ	O_3 FACE	250 ppb (7 h d^{-1})	15	Shi et al. (2009)
	SY63	Filteration	13.8–74.2 ppb (7 h d^{-1})	20.7	Pang et al. (2009)
	Pathumthani	Fumigation	150 ppb (7 h d^{-1})	78	Ariyaphanphitak et al. (2005)
Hordeum vulgare L.	Haider-93	Filteration	71 ppb (8 h d^{-1})	13	Wahid (2006b)
	Haider-91	Filteration	71 ppb (8 h d^{-1})	30	Wahid (2006b)
	Jou-87	Filteration	71 ppb (8 h d^{-1})	34	Wahid (2006b)
	Jou- 85	Filteration	71 ppb (8 h d^{-1})	44	Wahid (2006b)
	Barberousse	Filteration	49 ppb (7 h d^{-1}) 6.7 ppm h[b]	11.2	Fumagalli et al. (2001a, b)
		Filteration	43 ppb (7 h d^{-1}) 7.6 ppm h[b]	15.5	
		Filteration	51 ppb (7 h d^{-1}) 7.9 ppm h[b]	20.1	
Phaseolus vulgaris L.	BBL-290	Filteration	40 ppb (12 h d^{-1})	21.8	Burkey et al. (2005)
	S156	Filteration	40 ppb (12 h d^{-1})	36	
	Taylor's Horticultural	Filteration	49 ppb (7 h d^{-1}) 6.8 ppm h[b]	24.3	Fumagalli et al. (2001a, b)
			43 ppb (7 h d^{-1}) 6.6 ppm h[b]	26.4	
			51 ppb (7 h d^{-1}) 6.4 ppm h[b]	31.5	
			46 ppb (7 h d^{-1}) 7.3 ppm h[b]	21.8	
	Lingua di fuco	Filteration	49 ppb (7 h d^{-1}) 6.8 ppm h[b]	16.9	Fumagalli et al. (2001a, b)
			51 ppb (7 h d^{-1}) 6.4 ppm h[b]	28.6	
	Saluggia	Filteration	49 ppb (7 h d^{-1}) 6.8 ppm h[b]	19.1	Fumagalli et al. (2001a, b)
			51 ppb (7 h d^{-1}) 6.4 ppm h[b]	30.8	
	Lit	Filteration	59 ppb (7 h d^{-1}) 20.1 ppm h[b]	28.5	Fumagalli et al. (2001a, b)
Vigna radiata L.	M-Jyoti	Transect study	58.5 ppb	20.45	Agrawal et al. (2003b)
	M-28	Filteration	61.6 ppb	47.06	Ahmed 2009
	M-6601	Filteration	61.6 ppb	51.12	

(continued)

Table 2 (continued)

Plant species	Cultivar	Experimental type	O_3 concentration	Yield reduction (%)	References
Glycine max L.	Ceresia	Filteration	50 ppb (8.7 ppm h)[b]	5.6	Fumagalli et al. (2001a, b)
Brassica compestris L.	Pusa Jaikisan	Transect study	58.5 ppb	20.45	Agrawal et al. (2003b)
	Kranti	Filteration	41.6–54.2 ppb (12 h d^{-1})	16.4	Singh et al. (2009a)
Brassica napus ssp. *oleifera*	Biennis	Fumigation	75 ppb (6.6 h d^{-1})	14	Ollerenshaw et al. (1999)
Trifolium subterraneum L.	Woogenellup	Fumigation	90 ppb (4 h d^{-1})	37	Horsman et al. (1981)
Trifolium repens L.	*N.Z.* White	Fumigation	90 ppb (4 h d^{-1})	28	
Lycopersicon esculentus L.	Nikita	Fumigation	(35.9+70) ppb (8 h d^{-1})	48	Calvo et al. (2007)
	Alisa Craig	Fumigation	(35.9+70) ppb (8 h d^{-1})	57	
	Money Maker	Fumigation	(35.9+70) ppb (8 h d^{-1})	63	
Lactuca sativa L.	Valladolid	Fumigation	83 ppb (12 h d^{-1})	54	Calatayud and Barreno (2001)
	Morella	Fumigation	83 ppb (12 h d^{-1})	33	
Solanum tuberosum L.	Dark Red Norland	Filteration	45 ppb (12 h d^{-1})	14	Heagle et al. (2003)
		Fumigation	80 ppb (12 h d^{-1})	31	

Abbreviations: *AOT40* Accumulated ozone over a threshold concentration of 40 ppb, d^{-1} per day, *h* hour, *ppb* parts per billion, *ppm* parts per million

[a]Yield reduction in second year

[b]AOT40 values

Yield Loss (RYL) for rice in the Chongqing region from 1990 to 1995 was 1.1–5.8%, and would reach 10.8% in 2020; in winter wheat, losses were estimated to be 0.2–9.8% in 1990, and are expected to reach ~12% in 2020 (Liu et al. 2009). In this same study, the RYL in the YRD region for rice was estimated at 2.5–6.6% from 1990 to 1999, and was projected to reach 9.2% in 2020. Similarly in winter wheat, the RYL was estimated to be 7.1–8.4% (based on simulated O_3 doses), whereas, when based on monitored O_3 doses, it was ~12% in 1999 (Liu et al. 2009).

Results of a meta-analysis assessment indicated that elevated O_3 levels, in the range of 31–200 ppb, decreased wheat grain yields by 29%, and the harvest index (HI) by 9% (Feng et al. 2008). The large yield loss was caused by a combination of decreases in individual grain weight (18%), ear number plant^{-1} (6%) and grain number ear^{-1} (11%) (Feng et al. 2008). In the same meta-analysis study, reductions of 19 and 22%, respectively, were recorded in RuBisCO activity and gs, which led to a decrease of 20% in leaf photosynthetic rate (Feng et al. 2008). Based on 22 independent measurements across 10 cultivars and 7 countries, it was concluded that, under current ambient O_3 level scenarios, wheat grain yield was depressed by 17.5% (Feng et al. 2008). Wahid et al. (2001) reported a yield reduction of 53% at 75 ppb O_3 concentration in soybean at a remote rural site in Pakistan. Mulchi et al. (1988) exposed 12 cultivars of soybean to charcoal-filtered and -nonfiltered air plus 40 ppb O_3 for 6 h day^{-1}, 5 days week^{-1} for 13 weeks, using OTCs, and reported that plant growth rates and relative growth rates were reduced by 17 and 14.4%, respectively (based on the average over cultivars). Christ et al. (2006), however, found that increased assimilate partitioning to the pods prevented any yield loss when soybean was exposed to O_3, although at the cost of decreased leaf growth. Dermody et al. (2006) hypothesized that exposure to elevated O_3 levels of 1.2 times normal during the seed-filling stage of soybean growth would lead to reduced growth and photosynthesis in the upper leaf canopy and would eventually reduce yield. Morgan et al. (2003) reported a mean yield loss of about 10% in soybean exposed to 30–60 ppb O_3, through a meta-analysis study. Free air gas concentration enrichment (FACE) experiments result in a yield loss in soybean by 20%, when a 23% enriched O_3 concentration was used (Morgan et al. 2006). Significant yield reductions of 20 and 33.6% and 12 and 30% were recorded in two varieties of soybean grown in chambers fumigated with 70 and 100 ppb O_3 for 4 h day^{-1}, respectively (Singh et al. 2010). Feng and Kobayashi (2009), through their meta-analytical approach, compared yield losses of several crops by exposing them to O_3 concentrations at existing (31–50 ppb) or future levels (51–75 ppb). They found that current O_3 concentrations reduced the yield of potato, soybean, barley, wheat, rice, and bean by 5.3, 7.7, 8.9, 9.7, 17.5, and 19.0%, respectively. However, future levels of O_3 resulted in further yield loss by 10%, in soybean, wheat and rice, and by 20% in bean.

Ainsworth (2008) reported that, compared to charcoal-filtered air, elevated O_3 decreased rice yield by 14%, which involved a 5% decrease in individual grain weight and 20% decrease in grain number, without significantly affecting the panicle number. Zeng et al. (2008) showed reductions in rice yield per square meter of 14.3% and 20.2%, for plants exposed to lower or higher O_3 concentrations, respectively, in OTCs. According to these authors, a decrease in secondary rachis was the main cause

that led to yield loss from O_3 exposure. Shi et al. (2009) used a FACE system to investigate the yield loss of rice from O_3 exposure, and found that two cultivars showed yield losses of 17.5 and 15% (Table 2), whereas the other two cultivars showed no yield reduction. Pang et al. (2009) reported that photosynthesis of flag leaves in an O_3-sensitive rice cultivar, grown in a FACE field, was inhibited much larger than that of a tolerant one. Sawada and Kohno (2009) investigated the difference in O_3 sensitivity among many rice cultivars using OTCs, and found that the sensitivity, as evaluated by visible injury, did not coincide with the injury evaluated by yield loss. In two recent studies, Rai et al. (2010) and Sarkar and Agrawal (2010b) also showed significant negative effects on rice and wheat plants from ambient O_3 level exposure in a Northern Indian region, wherein near natural conditions existed.

4 Amelioration of Ozone Effects on Plants

4.1 Chemical Protectants

A diverse group of chemical compounds are known to provide various degrees of short-term protection to plants from O_3 injury. These include antioxidants, antisenescent agents, growth regulators, growth retardants, pesticides, among others. Results from various studies have proved the usefulness of these chemicals in preventing effects from acute exposure to O_3 (Agrawal et al. 2005; Manning 2000; Singh and Agrawal 2009; Tiwari and Agrawal 2009). Use of protective chemicals to ameliorate the harmful effects of O_3 has several advantages over other techniques. Establishing the experimental setup is simple and uncomplicated, and requires little equipment. These experiments can be done under ambient field conditions, wherein plant number, plot size, and replication can be varied according to experimental requirements. This technique also has certain disadvantages, such as the fact that the O_3 dose response cannot be evaluated, unless it is coupled with the use of OTCs. Moreover, repeated applications of the chemical could cause phytotoxicity, especially under dry conditions.

The antiozonant, ethylenediurea (N-[2-(2-oxo-1-imidazolinidyl) ethyl]-N′ phenylurea), abbreviated as EDU, has been extensively used in the recent years to ameliorate the negative effects of O_3. It is evident from the exhaustive literature that EDU, if properly used, can serve as a very powerful tool for verifying the incidence of phytotoxic concentrations of ambient O_3, and detecting yield and growth losses under field conditions (Bortier et al. 2001; Paoletti et al. 2008; Singh and Agrawal 2009; Tiwari et al. 2005; Tonneijk and van Dijk 1997; Wang et al. 2007).

Carnahan et al. (1978) reported that EDU was effective in protecting *P. vulgaris* L. from acute foliar O_3 injury. In an EDU dose–response study, it was found that increasing EDU concentrations resulted in increasing protection against O_3. Weidensaul (1980) reported safe use of EDU up to 5,000 ppm as a foliar spray. In recent years, EDU has also been applied as a soil drench or potting medium, as stem injections or gravitational infusion (Bortier et al. 2001; Manning 2000; Paoletti et al. 2008).

To be effective, EDU must be applied on a regular basis (Manning 2000). EDU is known to be rapidly transported acropetally, probably via the xylem stream, and accumulates in the apoplastic space of the leaves (Gatta et al. 1997; Weidensaul 1980). No mobilization of EDU from old to new leaves has been detected, so repeated EDU applications are needed at 7–14 days intervals (Weidensaul 1980).

The mode of action of EDU is not completely understood. Bennett et al. (1978) found that EDU does not affect stomatal behavior of plants, suggesting that the anti-ozonant activity of EDU is of biochemical rather than biophysical in nature. Agrawal and Agrawal (1999), however, found that stomatal resistance increased in snap bean plants treated with EDU + O_3, compared to plants treated with O_3 alone. In a recent study, it has been shown that both biochemical and biophysical processes may be modified under EDU treatment (Paoletti et al. 2008).

EDU does not act directly as an antioxidant, but helps to maintain cellular antioxidants during O_3 stress (Hassan 2006; Lee et al. 1997). Lee and Bennett (1982) reported enhancement of SOD activity after EDU treatment. However, no significant variation in SOD activity was observed by other workers (Lee et al. 1997; Pitcher et al. 1992). Lee et al. (1997) have reported that total POX activity was not affected by EDU, while others have indicated an increase in apoplastic APX activity (Batini et al. 1995). Hassan (2006) applied 300 mg L^{-1} EDU to potato plants growing at suburban and rural sites. These plants were exposed to O_3 concentrations of 78 and 95.5 ppb, respectively, for 10 h. Results were that the activities of certain enzymes (CAT, guaiacol POX, and APX) did not vary significantly upon EDU treatment. However, SOD and glutathione POX activities increased by 58 and 55%, respectively, at the suburban site, and by 100 and 54%, respectively, at the rural site (Hassan 2006). In a recent study, Singh et al. (2009b) found significant increments in the activities of protective enzymes that included SOD, POX, and APX, in different cultivars of wheat plants treated with 400 ppm EDU.

The use of protective chemicals allows the treated as well as control plants to grow under natural conditions. The advantage of this technique is that no chambers or artificial enclosures are necessary to evaluate O_3 effects. Therefore, EDU may also serve as a potential research tool in remote areas where electricity is not available and is especially recommended for use in developing countries (Bytnerowicz et al. 1993; Tiwari et al. 2005). However, Singh and Agrawal (2010) have shown that an optimized concentration of EDU (400 ppm) can most efficiently ameliorate the effect of O_3. Results of this study revealed that application of 400 ppm EDU increased the yield in wheat by 27% under ambient O_3 concentrations (Singh and Agrawal 2010).

4.2 CO_2 Amelioration of O_3 Impact

In the past few years, elevated CO_2 concentrations have been utilized to ameliorate the adverse effects of tropospheric O_3 in plants (Fiscus et al. 2002; Olszyk et al. 2002). Elevated CO_2 concentrations in the troposphere are known to increase plant growth and yield (Franzaring et al. 2008; Ramo et al. 2006). Tropospheric O_3, on

the other hand, is high enough to suppress plant growth and yield in several parts of the world (Emberson et al. 2009; Liu et al. 2009). Because O_3 and CO_2 cause opposite plant responses, numerous studies have been performed over the past decade to address the effects of O_3–CO_2 mixtures. Most of these studies have revealed that apparent stimulation caused by CO_2 enrichment is much greater when O_3 concentrations are also high (Heagle et al. 2000; Idso and Idso 1994; Olszyk et al. 2002; Poorter and Perez-Soba 2001). Further, at a given O_3 level, O_3-sensitive plants may be more responsive to CO_2 enrichment than O_3-tolerant ones (Heagle et al. 2003). Booker et al. (2007) have also demonstrated that elevated CO_2 suppressed the inhibitory effects of O_3 on photosynthesis and biomass of peanuts. Cardoso-Vilhena et al. (2004) demonstrated that ameliorative effects afforded by CO_2 enrichment mainly results from the exclusion of O_3 from the leaf interior, which is caused by the decline in gs of the plants upon CO_2 enrichment.

5 Summary

Ozone is now considered to be the second most important gaseous pollutant in our environment. The phytotoxic potential of O_3 was first observed on grape foliage by B. L. Richards and coworkers in 1958 (Richards et al. 1958). To date, unsustainable resource utilization has turned this secondary pollutant into a major component of global climate change and a prime threat to agricultural production. The projected levels to which O_3 will increase are critically alarming and have become a major issue of concern for agriculturalists, biologists, environmentalists, and others. Plants are "soft targets" for O_3. Ozone enters plants through stomata, where it dissolves in the apoplastic fluid. O_3 has several potential effects on plants: direct reaction with cell membranes; conversion into ROS and H_2O_2 (which alters cellular function by causing cell death); induction of premature senescence; and induction of and up- or down-regulation of responsive components such as genes, proteins, and metabolites. In this review, we attempt to present an overview picture of plant–O_3 interactions. We summarize the vast number of available reports on plant responses to O_3 at the morphological, physiological, cellular, biochemical levels, and address effects on crop yield, and on genes, proteins, and metabolites.

It is now clear that the machinery of photosynthesis and its associated components are the primary target of O_3. Ultimately, O_3 affects photosynthesis, thereby decreasing the economic yield of most plants and inducing a common morphological symptom, called the "foliar injury." The "foliar injury" symptom can be authentically utilized for biomonitoring of O_3 under natural conditions. Elevated O_3 stress has been convincingly demonstrated to trigger an antioxidative defense system in plants. The past several years have seen the development and application of high-throughput omics technologies (transcriptomics, proteomics, and metabolomics) that are capable of identifying and profiling the O_3-responsive components in model and nonmodel plants. Such studies have been carried out and have generated an inventory of O_3-responsive components – a great resource to the scientific community.

Recently, it has been shown that certain organic chemicals and elevated CO_2 levels are effective in ameliorating O_3-generated stress. Both targeted and high-throughput approaches have advanced our knowledge concerning what O_3-triggerred signaling and metabolic pathways exist in plants. Moreover, recently generated information, and several biomarkers for O_3, may, in the future, be exploited to better screen and develop O_3-tolerant plants.

Acknowledgments ST, SBA, MA, and AS are thankful to CSIR, DST, MOEn, and UGC, Government of India for the financial support. KC, JS, and AK of NIES also appreciate the support by the Global Environment Research Fund (A-0806) of the Ministry of the Environment, Japan. RR also acknowledges the support of Professors Seiji Shioda (Showa University) and Yoshinori Masuo (Toho University) in promoting interdisciplinary research and unselfish encouragement.

References

Adams RM, Andersen C, Garner JHB (1996) Environmental effects of ozone and related photochemical oxidants. In: Environmental Protection Agency (ed) Air quality criteria for ozone and related photochemical oxidants. National Centre for Environmental Assessment Office of Research and Development, US Environmental Protection Agency, Research Triangle Park, NC, USA, pp. 1.

Agrawal GK, Iwahashi H, Rakwal R (2003a) Rice MAPKs. Biochem Biophys Res Commun 302: 171–180.

Agrawal GK, Rakwal R, Iwahashi H (2002a) Isolation of novel rice (*Oryza sativa* L.) multiple stress responsive MAP kinase gene, OsMSRMK2, whose mRNA accumulates rapidly in response to environmental cues. Biochem Biophys Res Commun 294: 1009–1016.

Agrawal GK, Rakwal R, Yonekura M, Saji H (2002b) Rapid induction of defense/stress related proteins in leaves of rice (*Oryza sativa*) seedlings exposed to ozone is preceeded by newly phosphorylated proteins and changes in 66 K-Da ERK-type MAPK. J Plant Phys 159: 361–369.

Agrawal GK, Rakwal R, Yonekura M, Kubo A, Saji H (2002c) Proteome analysis of differentially displayed proteins as a tool for investigating ozone stress in rice (*Oryza sativa* L.) seedlings. Proteomics 2: 947–959.

Agrawal M, Singh B, Rajput M, Marshall F, Bell JNB (2003b) Effect of air pollution on periurban agriculture: a case study. Environ Pollut 126: 323–329.

Agrawal SB, Agrawal M (1999) Low temperature scanning electron microscope studies of stomatal response in snap bean plants treated with ozone and ethylenediurea. Biotronics 28: 45–53.

Agrawal SB, Singh A, Rathore D (2005) Role of ethylenediurea (EDU) in assessing impact of ozone on *Vigna radiata* L. plants in suburban areas of Allahabad (India). Chemosphere 61: 218–228.

Ahlfors R, Macioszek V, Rudd J, Brosché M, Schlichting R, Scheel D, Kangasjärvi J (2004) Stress hormone-independent activation and nuclear translocation of mitogen-activated protein kinases in *Arabidopsis thaliana* plants during ozone exposure. Plant J 40: 512–522.

Ahmed S (2009) Effects of air pollution on yield of mungbean in Lahore, Pakistan. Pak J Bot 41: 1013–1021.

Ainsworth EA (2008) Rice production in changing climate: a meta-analysis of responses to elevated CO_2 and elevated ozone concentration. Global Change Biol 14: 1642–1650.

Akimoto H, Narita H (1994) Distribution of SO_2, NO_x and CO_2 emissions from fuel combustion and industrial activities in Asia with 1 deg_1 deg resolution. Atmos Environ 28: 213–225.

Andersen CP (2003) Source-sink balance and carbon allocation below ground in plants exposed to ozone. New Phytol 157: 213–228.

Andersen CP, Wilson R, Plocher M, Hogsett WE (1997) Carry-over effects of ozone on root growth and carbohydrate concentrations of ponderosa pine seedlings. Tree Physiol 17: 805–811.

Apel K, Hirt H (2004) Reactive oxygen species: oxidative stress and signal transduction. Annu Rev Plant Biol 53: 373–399.

Ariyaphanphitak W, Chidthaisong A, Sarobol E, Bashkin VN, Towprayoon S (2005) Effects of elevated ozone concentrations on Thai jasmine rice cultivars (*Oryza sativa L.*). Water Air Soil Pollut 167: 179–200.

Asada K (1997) The role of ascorbate peroxidase and mono-dehydroascorbate reductase in H_2O_2 scavenging in plants. In: Scandalios JG (ed.) Oxidative stress and the molecular biology of antioxidant defences, New York: Cold Spring Harbor Laboratory Press, pp. 527.

Ashmore MR (2005) Assessing the future global impacts of ozone on vegetation. Plant Cell Environ 28: 949–964.

Aunan K, Berntsen TK, Seip HM (2000) Surface ozone in China and its possible impact on agricultural crop yields. Ambio J Human Environ 29: 294–301.

Auvray M, Bey I (2005) Long-range transport to Europe: Seasonal variations and implications for the European ozone budget. J Geophys Res 110: D11303.

Bae GY, Nakajima N, Ishizuka K, Kondo N (1996) The role in ozone phytotoxicity of the evolution of ethylene upon induction of 1-aminocyclopropane-1-carboxylic acid synthase by ozone fumigation in tomato plants. Plant Cell Physiol 37: 129–134.

Baier M, Kandlbinder A, Golldack D, Dietz K-J (2005) Oxidative stress and ozone: perception, signaling and response. Plant Cell Environ 28: 1012–1020.

Baker NR, Rosenqvist E (2004) Applications of chlorophyll fluorescence can improve crop production strategies: an examination of future possibilities. J Exp Bot 55: 1607–1621.

Barth C, Conklin PL (2003) The lower cell density of leaf parenchyma in the *Arabidopsis thaliana* mutant *lcd1-1* is associated with increased sensitivity to ozone and virulent *Pseudomonas syringae*. Plant J 35: 206–218.

Batini P, Ederli L, Pasqualini S, Antoneilli M, Valeniti V (1995) Effects of ethylenediurea and ozone in detoxificant ascorbic-ascorbate peroxidase system in tobacco. Plant Physiol Biochem 33: 717–723.

Bender J, Weigel HJ, Wegner U, Jager HJ (1994) Response of cellular antioxidants to ozone in wheat flag leaves at different stages of plant development. Environ Pollut 84: 15–21.

Bennett JH, Lee EH, Heggestad HE (1978) Apparent photosynthesis and leaf stomatal diffusion in EDU treated ozone sensitive bean plants. In: Abdel-Rahman M (ed.) Proceedings of Plant Growth Regulator Working Group Fifth Annual Meeting, Virginia Polytechnique Institute and State University, Blacksburg, VA, Longmont, CO, USA.

Benton J, Fuhrer J, Gimeno BS, Skarby L, Palmer- Brown D, Ball G, Roadknight C, Mills G (2000) An international cooperative programme indicates the widespread occurrence of ozone injury in crops. Agri Ecosys Environ 78: 19–30.

Biswas DK, Xu H, Li YG, Sun JZ, Wang XZ, Han XG, Jiang GM (2008) Genotypic differences in leaf biochemical, physiological and growth responses to ozone in 20 winter wheat cultivars released over the past 60 years. Global Change Biol 14: 46–59.

Bohler S, Bagard M, Oufir M, Planchon S, Hoffmann L, Jolivet Y, Hausman J-F, Dizengremel P, Renaut J (2007) A DIGE analysis of developing poplar leaves subjected to ozone reveals major changes in carbon metabolism. Proteomics 7: 1584–1599.

Bojkov RD (1986) Surface ozone during the second half of the nineteenth century. J Climate Appl Met 25: 343–352.

Booker F, Muntifering R, McGrath M, Burkey K, Decoteau D, Fiscus E, Manning W, Krupa S, Chappelka A, Grantz D (2009) The ozone component of global change: Potential effects on agricultural and horticultural plant yield, product quality and interactions with invasive species. J Integ Plant Biol 51: 337–351.

Booker FL, Burkey KO, Pursley WA, Heagle AS (2007) Elevated carbon dioxide and ozone effects on peanut. I. Gas-exchange, biomass, and leaf chemistry. Crop Sci 47: 1475–1487.

Bortier K, Dekelver G, De Temmermenn L, Ceulemans R (2001) Stem injection of *Populus nigra* with EDU to study ozone effects under field conditions. Environ Pollut 111: 199–208.

Bowler C, Slooten L, Vanderbranden S, De Rycke R, Botterman J, Sybesma C, Van Montagu M, Inze D (1991) Manganese SOD can reduce cellular damage mediated by oxygen radicals in transgenic plants. EMBO J 10: 1723–1734.

Brendley BW, Pell EJ (1998) Ozone-induced changes in biosynthesis of Rubisco and associated compensation to stress in foliage of hybrid poplar. Tree Physiol 18: 81–90.

Britvec M, Reichenauer T, Soja G, Ljubeši´c N, Eid M, Pecina M (2001) Ultrastructure changes in grapevine chloroplasts caused by increased tropospheric ozone concentrations. Biologia, Bratislava 56: 417–424.

Britz SJ, Robinson JM (2001) Chronic ozone exposure and photosynthate partitioning into starch in soybean leaves. Inter J Plant Sci 162: 111–117.

Burkey KO (1999) Effects of ozone on apoplast/cytoplasm partitioning of ascorbic acid in snap bean. Physiol Plantarum 107: 188–193.

Burkey KO, Eason G, Fiscus EL (2003) Factors that affect leaf extracellular ascorbic acid content and redox status. Physiol Plantarum 117: 51–57.

Burkey KO, Miller JE, Fiscus EL (2005) Assessment of ambient ozone effects on vegetation using snap bean as bioindicator species. J Environ Quality 34: 1081–1086.

Buschmann C, Langsdorf G, Lichtenthaler HK (2000) Imaging of the blue, green and red fluorescence emission of plants: an overview. Photosynthetica 38: 483–491.

Bytnerowicz A, Manning WJ, Grosjean D, Chamiclswki W, Dmuchowski W, Grodzinska K, Godzik B (1993) Detecting ozone and demonstrating its phytotoxicity in forested areas in Poland: a pilot study. Environ Pollut 80: 301–306.

Calatayud A, Barreno E (2001) Chlorophyll fluorescence, antioxidant enzymes and lipid peroxidation in tomato in response to ozone and benomyl. Environ Pollut 115: 283–289.

Calatayud A, Ramirez JW, Iglesias DJ, Barreno E (2002) Effects of ozone on photosynthetic CO_2 exchange, chlorophyll a fluorescence and antioxidant systems in lettuce leaves. Physiol Plantarum 116: 308–316.

Calatayud A, Iglesias D, Talon M, Barreno E (2003) Effects of 2 months ozone exposure in spinach leaves on photosynthesis, antioxidant systems and lipid peroxidation. Plant Physiol Bioch 41: 839–845.

Calvo E, Martin C, Sanz M (2007) Ozone sensitivity differences in five tomato cultivars: visible injury and effects on biomass and fruits. Water Air Soil Pollut 186: 167–181.

Cardoso-Vilhena J, Balaguer L, Eamus D, Ollerenshaw J, Barnes J (2004) Mechanisms underlying the amelioration of O_3-induced damage by elevated atmospheric concentrations of CO_2. J Exp Bot 55: 771–781.

Carnahan JE, Jenner EL, Wat EKW (1978) Prevention of ozone injury to plants by a new protectant chemical. Phytopathology 68: 1222–1229.

Castagna A, Nali C, Ciompi G, Lorenzini G, Soldatini GF, Ranieri A (2001) O_3 exposure effects photosynthesis of pumpkin (*Cucurbita pepo*) plants. New Phytol 152: 223–229.

CASTNet (Clean Air Status and Trends Network) (2004) http://www.epa.gov/castnet/.

Cerana M, Bonza MC, Harris R, Sanders D, Michelis MID (2006) Abscisic acid stimulates the expression of two isoforms of plasma membrane Ca2+-ATPase in *Arabidopsis thaliana* seedlings. Plant Biol 8: 572–578.

Chaerle L, Hagenbeek D, De BruyneValce R, Van Der Straeten D (2004) Thermal and chlorophyll fluorescence imagimg distinguish plant pathogen interactions at an early stage. Plant Cell Physiol 45: 887–896.

Chameides WL, Kasibhatla PS, Yienger J, Levy H (1994) Growth of continental-scale metro-agro-plexes. Regional ozone pollution and world food production. Science 264: 74.

Chiron H, Drouet A, Lieutier F, Payer H-D, Ernest D, Sandermann H Jr (2000) Gene induction of stilbene biosynthesis in scots pine in response to ozone treatment, wounding, and fungal infection. Plant Physiol 124: 865–872.

Cho K, Shibato J, Agrawal GK, Jung YH, Kubo A, Jwa NS, Tamogami S, Satoh K, Kikuchi S, Higashi T, Kimura S, Saji H, TanakaY, Iwahashi H, Masuo Y, Rakwal R (2008) Integrated transcriptomics, proteomics, and metabolomics analyses to survey ozone responses in the leaves of rice seedling. J Proteome Res 7: 2980–2998.

Christ MM, Elizabeth AA, Nelson R, Schurr U, Walter A (2006) Anticipated yield loss in field grown soybean under elevated ozone can be avoided at the expense of leaf growth during early reproductive growth stages in favourable environmental conditions. J Exp Bot 57: 2267–2275.
Clayton H, Knight MR, Knight H, McAinsh MR, Hetherington AM (1999) Dissection of ozone induced calcium signature. Plant J 17: 575–579.
Conklin PL, Barth C (2004) Ascorbic acid, a familiar small molecule intertwined in the response of plants to ozone, pathogens, and the onset of senescence. Plant Cell Environ 27: 959–970.
Cooley DR, Manning WJ (1987) The impact of ozone on assimilated partitioning in plants: a review. Environ Pollut 47: 95–113.
Coyle M, Flower D, Ashmore MR (2003) New directions: implications of increasing tropospheric background ozone concentrations for vegetation. Atmos Environ 37: 153–154.
Crang RE, McQuattie CJ (1986) Qualitative and quantitative effects of acid misting and two air pollutants on foliar structures of *Liriodendron tulipifera*. Can J Bot 64: 1237–1243.
Dat J, Vandenabeele S, Vranova E, Van Montagu M, Inze D, Van Breusegem F (2000) Dual action of the active oxygen species during plant cell responses. Cell Mol Life Sci 57: 779–795.
Dat JF, Pellinen R, Beeckman T, Van De Cotte B, Langebartels C, Kangsjarvi J, Inze D, Van Breusegem F (2003) Changes in hydrogen peroxide homeostasis trigger an active cell death process in tobacco. Plant J 33: 621–632.
Debaje SB, Kakade AD (2008) Surface ozone variability over western Maharashtra, India. J Haz Mat 161: 686–700.
Dentener F, Stevenson D, Ellingsen K, Van Noije T, Schultz M, Amann M, Atherton C, Bell N, Bergmann D, Bey I, Bouwman L, Butler T, Cofala J, Collins B, Drevet J, Doherty R, Eickhout B, Eskes H, Fiore A, Gauss M, Hauglustaine D, Horowitz L, Isaken ISA, Josse B, Lawrence M, Krol M, Lamarque JF, Montanaro V, Muller JF, Peuch VH, Pitari G, Pyle J, Rast S, Rodriguez J, Sanderson M, Savage NH, Shindell D, Stahan S, Szopa S, Sudo K, Van Dingenen R, Wild O, Zeng G (2006) The global atmospheric environment for the next generation. Environ Sci Tech 40: 3586–3594.
Dermody O, Long SP, DeLucia EH (2006) How does elevated CO_2 or ozone affect the leaf-area index of soybean when applied independently? New Phytol 169: 145–155.
Derwent RG, Stevenson DS, Collins WJ, Johnson CE (2004) Intercontinental transport and the origins of the ozone observed at surface sites in Europe. Atmos Environ 38(13): 1891–1901.
Dizengremel P (2001) Effects of ozone on the carbon metabolism of forest trees. Plant Physiol Biochem 39: 729–742.
Dizengremel P, Thiec D Le, Bagard M, Jolviet Y (2008) Ozone risk assessment for plants: Central role of metabolism dependant changes in reducing power. Environ Pollut 156: 11–15.
EANET (2006) Data report on acid deposition on East Asia region 2005. Network Centre of EANET, Japan (http://www.eanet.cc/).
Ebel J, Cosio EG (1994) Elicitors of plant defense responses. Int Rev Cytol 148: 1–36.
Emberson LD, Ashmore MR, Cambridge HM, Simpson D, Tuovinen JP (2000) Modelling stomatal ozone flux across Europe. Environ Pollut 109: 403–413.
Emberson LD, Buker P, Ashmore MR (2007) Assessing the risk caused by ground level ozone to European forest trees: a case study in pine, beech and oak across different climate regions. Environ Pollut 147: 454–466.
Emberson LD, Buker P, Ashmore MR, Mills G, Jackson LS, Agrawal M, Atikuzzaman MD, Cinderby S, Engardt M, Jamir C, Kobayashi K, Oanh NTK, Quadir QF, Wahid A (2009) A comparison of North American and Asian exposure–response data for ozone effects on crop yields. Atmos Environ 43: 1945–1953.
Esposito MP, Ferreira ML, Sant'Anna SM, Domingos M, Souza SR (2009) Relationship between leaf antioxidants and ozone injury in *Nicotiana tabacum* 'Bel-W3' under environmental conditions in Sa˜o Paulo, SE-Brazil. Atmos Environ 43: 619–623.
FAO-UN (2003) FAO trade yearbook, Publising Management Service, Information Division, FAO, volume 165.
Farage PK, Long SP (1995) An in vivo analusis of photosynthesis during short term ozone exposure in three contrasting species. Photosyn Res 43: 11–21.

Farrar JF, Gunn S (1998) Allocation: allometry, acclimation-and alchemy. In: Lambers H, Poorter H, Van Vuren MMI (eds.) Inherent Variation in Plant Growth. Physiological Mechanisms and Ecological Consequences, Backhuys Publishers, Leiden, The Netherlands, pp. 183.

Feng ZW, Jin MH, Zhang FZ, Huang YZ (2003) Effects of ground-level ozone (O_3) pollution on the yields of rice and winter wheat in the Yangtze River Delta. J Environ Sci (China) 15: 360–362.

Feng ZZ, Kobayashi K (2009) Assessing the impacts of current and future concentrations of surface ozone on crop yield with meta-analysis. Atmos Environ 43: 1510–1519.

Feng ZZ, Kobayashi K, Ainsworth EA (2008) Impact of elevated ozone concentration on growth, physiology and yield of wheat (*Triticum aestivum* L.): a meta-analysis. Global Change Biol 14: 2696–2708.

Fiore AM, Jacob DJ, Bey I, Yantosca RM, Field BD, Wilkinson JG (2002) Backgroundozone over the United States in summer: origin and contribution to pollution episodes. J Geophys Res 107: ACH11-1-ACH11–25.

Fiscus EL, Miller JE, Booker FL, Heagle AS, Reid CD (2002) The impact of ozone and other limitations on the crop productivity to CO_2. Technology 8: 181–192.

Fiscus EL, Reid CD, Miller JE, Heagle AS (1997) Elevated CO_2 reduces O_3 flux and O_3-induced yield losses in soybeans: possible implications for elevated CO_2 studies. J Exp Bot 48: 307–313.

Foyer CH, Noctor G (2005) Oxidant and antioxidant signaling in plants: a re-evaluation of the concept of oxidative stress in a physiological context. Plant Cell Environ 28: 1056–1071.

Foyer CH (1997) Oxygen metabolism and electron transport in photosynthesis. In: Scandalios J (ed.), Molecular biology of free radical scavenging systems. Cold Spring Harbor Laboratory Press, NY, USA, pp. 587.

Francini A, Nali C, Picchi V, Lorenzini G (2007) Metabolic changes in white clover exposed to ozone. Environ Exp Bot 60: 11–19.

Franzaring J, Hogy P, Fangmeier A (2008) Effects of free air CO_2 enrichment on growth of summer oilseed rape (*Brassica napus* cv. Campino). Agri Ecosys Environ 128: 127–134.

Franzaring J, Tonneijck AEG, Kooijman AWN, Dueck TA (2000) Growth response to ozone in plant species from wetlands. Environ Exp Bot 44: 39–48.

Fuhrer J, Booker F (2003) Ecological issues related to ozone: agricultural issues. Environ Inter 29: 141–154.

Fuhrer J, Egger A, Lehnherr B, Grandjean A, Tschannen W (1989) Effects of ozone on yield of spring wheat (*Triticum aestivum* L. cv Albis) grown in open top field chambers. Environ Pollut 60: 273–289.

Fuhrer J, Skarby L, Ashmore MR (1997) Critical levels for ozone effects on vegetation in Europe. Environ Pollut 97: 91–106.

Fumagalli I, Ambrogi R, Mignanego L (2001a) Yield responses of plants exposed to ambient ozone in the river Po valley (Italy). Agronomie 21: 227–233.

Fumagalli I, Gimeno BS, Velissario D, De Temmermen L, Mills G (2001b) Evidence of ozone induced adverse effects on crops in the Mediterranean region. Atmos Environ 35: 2583–2587.

Fusco AC, Logan JA (2003) Analysis of 1970-1995 trends in tropospheric ozone at Northern Hemisphere midlatitudes with the GEOS-CHEM model. J Geophys Res 108: ACH4-1–ACH4–25.

Gatta L, Mancino L, Fedrico R (1997) Translocation and persistence of EDU (Ethylenediurea) in plants: the relationship with its role in ozone damage. Environ Pollut 96: 445–448.

Gaucher C, Costanzo N, Afif D, Mauffette Y, Chevrier N, Dizengremael P (2003) The impact of elevated ozone and carbon di oxide on young *Acer saccharum* seedlings. Physiol Plantarum 117: 392–402.

Gielen B, Low M, Deckmyn G, Metzger U, Franck F, Heerdt C, Matyssek R, Valcke R, Ceulemans R (2007) Chronic ozone exposure affects leaf senescence of adult beech trees: a chlorophyll fluorescence approach. J Exp Bot 58: 785–795.

Gimeno BS, Bermejo V, Reinert RA, Zheng Y, Barnes JD (1999) Adverse effects of ambient ozone on watermelon yield and physiology at a rural site in Eastern Spain. New Phytol 144: 245–260.

Gimeno BS, Penuelas J, Porcuna JL, Reinert RA (1995) Biomonitoring ozone phytotoxicity in eastern Spain. Water Air Soil Pollut 85: 1521–1526.

Glick RE, Schlagnhaufer CD, Arteca RN, Pell EJ (1995) Ozone-induced ethlylene emission accelerates the loss of ribulose-1, 5-bisphosphate carboxylase/oxygenase and nuclear-encoded mRNAs in senescing potato leaves. Plant Physiol 109: 891–898.

Gomez LD, Noctor G, Knight MR, Foyer CH (2004) Regulation of calcium signaling and gene expression by glutathione. J Exp Bot 55: 1851–1859.

Grantz DA, Yang S (1996) Effect of O_3 on hydraulic architecture in Pima cotton: Biomass allocation and water transport capacity of roots and shoots. Plant Physiol 112: 1649–1657.

Grantz DA, Gunn S, Vu H-B (2006) O_3 impacts on plant development: a meta-analysis of root/shoot allocation and growth. Plant Cell Environ 29: 1193–1209.

Grulke NE, Balduman L (1999) Deciduous conifers: high N deposition and O_3 exposure effects on growth and biomass allocation in ponderosa pine. Water Air Soil Pollut 116: 235–48.

Guderian R (1985) Effects of pollutans combination. In: Guderian R (ed.) Air pollution photochemical oxidants, Sring-Verlag, Berlin, pp. 246.

Guidi L, Di Cagno R, Soldatini GF (2000) Screening of beans cultivars for their response to ozone as evaluated by visible symptoms and leaf chlorophyll fluorescence. Environ Pollut 107: 349–355.

Guidi L, Nali C, Lorenzini G, Filppi F, Soladatini GF (2001) Effect of chronic ozone fumigation on the photosynthetic process of poplar clones showing different sensitivity. Environ Pollut 113: 245–254.

Gunn S, Farrar JF (1999) Effects of a 4°C increase in temperature on partitioning of leaf area and dry mass, root respiration and carbohydrates. Funct Ecol 13: 12–20.

Günthardt-Goerg MS, McQuattie CJ, Maurer S, Frey B (2000) Visible and microscopy injury in leaves of five deciduous tree species related to current critical ozone levels. Environ Pollut 109: 489–500.

Gupta P, Duplessis S, White H, Karnosky DF, Martin F, Podila GK (2005) Gene expression patterns of trembling aspen trees following long-term exposure to interacting elevated CO_2 and tropospheric O_3. New Phytol 167: 129–142.

Guzy MR, Heath RL (1993) Response to ozone of varieties of common bean (*Phaseolus vulgaris* L.). New Phytol 124: 617–625.

Hassan IA (2006) Physiological and biochemical responses of potato (*Solanum tuberosum* L. cv. Kara) to ozone and antioxidant enzymes. Annals App Biol 148: 197–206.

Hausladen A, Madamanchp NR, Fellows S, Alscher RG, Amundson RG (1990) Seasonal changes in antioxidants in red spruce as affected by ozone. New Phytol 115: 447–458.

Heagle AS (1989) Ozone and crop yield. Ann Rev Phytopathol 27: 397–423.

Heagle AS, Miller JE, Pursley WA (2000) Growth and yield responses of winter wheat to mixtures of ozone and cvaebon dioxide. Crop Sci 40: 1656–1664.

Heagle AS, Miller JE, Pursley WA (2003) Atmospheric pollutants and trace gases: Growth and yield responses of potato to mixtures of CO_2 and ozone. J Environ Qual 32: 1603–1610.

Heagle AS, Philbeck RB, Rogers HH, Letchworth MB (1979) Dispensing and monitoring ozone in open top field chambers for plant effect studies. Phytopathol 69: 15–20.

Heath RL, Taylor GE (1997) Physiological processes and plant responses to ozone exposure. In: Sanderman H, Welburn AR, Heath RL (eds.) Forest decline and ozone, Spring-Verlag, Berlin, pp. 317 .

Heath RL (2008) Modification of the biochemical pathways of plants induced by ozone: What are the varied routes to changes? Environ Pollut 155: 453–463.

Hoell JM, Davis DD, Liu SC, Newell R, Akimoto H, McNeal RJ, Bendula R (1997) The Pacific Exploratory Mission–West Phase B: February–March 1994. J Geophys Res 102: 28223–28239.

Horsman DC, Nicholls AO, Calder DM (1981) Effects of chronic ozone exposure on the growth of *Trifolium subterterraneum* and *Trifolium repens*. Austra J Plant Physiol 8: 405–408.

Idso KE, Idso SB (1994) Plant responses to atmospheric CO_2 enrichment in the face of environmental constraints: A review of the past 10 years research. Agri Forest Met 69, 153–203.

Iglesias D J, Calatayud A, Barreno E, Primo-Milloa E, Talon M (2006) Responses of citrus plants to ozone: leaf biochemistry, antioxidant mechanisms and lipid peroxidation. Plant Physiol Biochem 44: 125–131.

Iriti M, Rabotti G, De Ascensao A, Faoro F (2003) Benzothidiazole-induced resistance modulates ozone tolerance. J Agri Food Chem 51: 4308–4315.
Jaffe DA, Parrish D, Goldstein A, Price H, Harris J (2003) Increasing backgroundozone during spring on the west coast of North America. J Geophys Res 30: D1613.
Jain SL, Arya BC, Kumar A, Ghude SD, Kulkarni PS (2005) Observational study of surface ozone at New Delhi, India. Int J Remote Sens 26: 3515–3524.
Jeanneau M, Vidal J, Gousset-Dupont A, Lebouteillor B, Hodges M, Gerentes D, Perez P (2002) Manipulating PEPc levels in plants. J Exp Bot 53: 1837–1845.
Jin M, Feng Z, Zhang F (2001) Impacts of ozone on the biomass and yield of rice in open-top chamber. J Environ Sci (China) 13: 233–236.
Jonak C, Okresz L, Bogre L, Hirt H (2002) Complexity, cross talk and integration of plant MAP kinase signalling. Cur Opi Plant Biol 5: 415–424.
Joo JH, Wang S, Chen JG, Jones AM, Fedoroff N (2005) Different signaling and cell death roles of heterotrimeric G protein a and b subunits in the *Arabidopsis* oxidative stress response to ozone. Plant Cell 17: 957–970.
Junqua M, Biolley JP, Pié S, Kanoun M, Duran R, Goulas P (2000) In vivo occurrence of carbonyl residues in Phaseolus vulgaris proteins as a direct consequences of a chronic ozone stress. Plant Physiol Biochem 38: 853–861.
Kaiser WM (1979) Reversible inhibition of the Calvin cycle and activation of oxidative pentose phosphate cycle in isolated intact chloroplasts by hydrogen peroxide. Planta 145: 377–382.
Kaneyasu N, Takeuchi K, Hayashi M, Fujita SI, Uno I, Sasaki H (2000) Outflow patterns of pollutants from east Asia to the north Pacific in the winter monsoon. J Geophys Res 105: 17361–17377.
Kangasjarvi J, Jaspers P, Kollist H (2005) Signalling and cell death in ozone-exposed plants. Plant Cell Environ 28: 1021–1036.
Kangasjärvi J, Talvinen J, Utriainen M, Karjalainen R (1994) Plant defence systems induced by ozone. Plant Cell Environ 17: 783–794.
Kanna M, Tamaoki M, Kubo A, Nakajima N, Rakwal R, Agrawal GK, Tamogami S, Ioki M, Ogawa D, Saji H, Aono M (2003) Isolation of an ozone-sensitive and jasmonate-semi-insensitive *Arabidopsis* mutant (*oji1*). Plant Cell Physiol 44: 1301–10.
Karlsson PE, Uddling J, Skarby L, Wallin G, Sellden G (2003) Impact of ozone on the growth of birch (*Betula pendula*) seedlings. Environ Pollut 124: 485–495.
Karlsson PE, Braun S, Broadmeadow M, Elvira S, Emberson L, Gimeno BS, Le Thiec D, Novak K, Oksanen E, Schaub M, Uddling J, Wilkinson M (2007) Risk assessments for forest trees: The performance of the ozone flux versus the AOT concepts. Environ Pollut 146: 608–616.
Karnosky D, Skelly J, Percy K, Chappelka A (2007) Prospectives regarding 50 years of research on effects of tropospheric ozone air pollution on US forests. Environ Pollut 147: 489–506.
Kersteins G, Lendzian KJ (1989) Interactions between ozone and plant cuticles. 1. Ozone deposition and permeability. New Phytol 112: 13–19.
Keutgen AJ, Pawelzik E (2008) Apoplastic antioxidative system responses to ozone stress in strawberry leaves. J Plant Physiol 165: 868–875.
Khemani LT, Momin GA, Rao PSP, Vijaykumar R, Safai PD (1995) Study of surface ozone behaviour at urban and forested sites in India. Atmos Environ 29: 2021–2024.
Kiiskinen M, Korhonen M, Kangasjärvi J (1997) Isolation and characterization of cDNA for a plant mitochondrial phosphate translocator (Mpt1): Ozone stress induces Mpt 1 mRNA accumulation in birch (*Betula pendula* Roth). Plant Mol Biol 35: 271–279.
Kim JA, Agrawal GK, Rakwal R, Han KS, Kim KN, Yun CH, Heu S, Park SY, Lee YH, Jwa NS (2003) Molecular cloning and mRNA expression analysis of a novel rice (*Oryza sativa* L.) MAPK kinase kinase, OsEDR1, an ortholog of *Arabidopsis* AtEDR1, reveal its role in defense/stress signalling pathways and development. Biochem Biophys Res Commun 300: 868–876.
Kitajima H, Butler WL (1975) Quenching of chlorophyll fluorescence and primary photochemistry in chloroplasts by dibromothynoquinone. Biochimica et Biophysica Acta 376: 105–115.
Kobayashi K, Okada M (1995) Effects of ozone on the ligh use of rice (*Oryza sativa* L.) plants. Agriculture, Ecosys Environ 53: 1–12.

Koch J, Scherzer A, Eshita S, Davis K (1998) Ozone sensitivity in hybrid poplar is correlated with a lack of defense-gene activation. Plant Physiol 118: 1243–1252.

Koch JR, Creelman RA, Eshita SM, Seskar M, Mullet JE, Davis KR (2000) Ozone sensitivity in hybrid poplar correlates with insensitivity to both salicylic acid and jasmonic acid: The role of programmed cell death in lesion formation. Plant Physiol 123: 1–10.

Kolb TE, Matyssek R (2001) Limitations and perspectives about scaling ozone impacts in trees. Environ Pollut 115: 373–393.

Kollist H, Moldau H, Mortensen L, Rasmussen SK, Jorgensen LB (2000) Ozone flux to plasmalemma in barley and wheat is controlled rather by stomata than by direct reaction of ozone with apoplastic ascorbate. J Plant Physiol 156: 645–651.

Kollner B, Krause GHM (2002) Assessment of the response of the NC-S/NC-R clover clone system to ambient ozone levels at the Ruhr Valley. Water Air Soil Pollut 137: 63–79.

Kondratyev KY, Varotsos C (2001a) Global tropospheric ozone dynamics. Part I: Troposopheric ozone precursors. Environ Sci Pollut Res 8: 57–62.

Kondratyev KY, Varotsos C (2001b) Global tropospheric ozone dynamics. Part II: Numerical modelling of tropospheric ozone variability. Environ Sci Pollut Res 8: 113–119.

Kontunen-Soppela S, Ossipov V, Ossipova S, Oksanen E (2007) Shift in birch leaf metabolome and carbon allocation during long-term open-field ozone exposure. Global Change Biol 13: 1053–1067.

Kubo A (2002) Effects of air pollutans on gene expression in plants. in: Omasa, K., Saji, H., Youssefian, S., Kondo, N. (Eds.) Air pollution and plant biotechnology, Chapter 6, Springer, Tokyo, Japan, 121pp.

Lal S, Naja M, Subbaraya BH (2000) Seasonal variations in surface ozone and its recursors over an urban site in India. Atmos Environ 34: 2713–2724.

Laloi C, Mestres-Ortega D, Marco Y, Meyer Y, Reichheld JP (2004) The *Arabidopsis* cytosolic thioredoxin h5 gene induction by oxidative stress and itsW-Box mediated response to pathogen elicitor. Plant Physiol 134: 1006–1016.

Landolt W, Gunthardt-Georg MS, Pfenninger I, Scheidegger C (1994) Ozone induced changes and quantitative carbohydrate contents of hybrid poplar (*Populous* × *eurramericana*). Trees 8: 183–190.

Landolt W, Gunthardt-Georg MS, Pfenninger I, Eining W, Hampp R, Maurer S, Matyssek R (1997) Effect of fertilization on ozone induced changes in the metabolism of birch leaves (*Betula pendula*). New Phytol 137: 389–397.

Langebartels C, Wohlgemuth H, Kschieschan S, Grün S, Sandermann H (2002) Oxidative burst and cell death in ozoneexposed plants. Plant Physiol Biochem 40: 567–575.

Laurence JA, Amundson RG, Friend AL, Pell EJ, Temple PJ (1994) Allocation of carbon in plants under stress: An analysis of the ROPIS experiments. J Environ Qual 23: 412–417.

Laurence JA, Andersen CP (2003) Ozone and natural systems: understanding exposure, response, and risk. Environ Int 29: 155–160.

Lee EH, Bennett JH (1982) Superoxide dismutase [A possible protective enzyme against ozone injury in snap bean (*Phaseolus vulgaris* L.)]. Plant Physiol 69: 1444–1449.

Lee EH, Upadhyaya A, Agrawal M, Rowland RA (1997) Mechanism of ethyledene diurea (EDU) induced ozone protection: reexamination of free radical scavenger system in snap bean exposed to ozone. Environ Exp Bot 38: 199–209.

Lee S, Yun SC (2006) The ozone stress transcriptome of pepper (*Capsicum annuum* L.). Mol Cells 21: 197–205.

Leitao L, Dizengremel P, Biolley J-P (2008) Foliar CO_2 fixation in bean (*Phaseolus vulgaris* L.) submitted to elevated ozone: Distinct changes in Rubisco and PEPc activities in relation to pigment content. Ecotoxicol Environ Saf 69: 531–540.

Leitao L, Goulas P, Biolley J-P (2003) Time-course of Rubisco oxidation in beans (*Phaseolus vulgaris* L.) subjected to a lon-term ozone stress. Plant Sci 165: 613–620.

Liu F, Wang X, Zhu Y (2009) Assessing current and future ozone induced yield reductions for rice and winter wheat in Chongqing and Yangtze river delta of China. Environ Pollut 157: 707–709.

Long SP, Naidu SL (2002) Effects of oxidants at biochemical, cell and physiological levels with particular reference to ozone. In: Bell JNB, Treshow M (eds.) Air Pollution and Plant Life, John Wiley & Sons Ltd., West Sussex, England, pp. 69

Löw M, Häberle KH, Warren CR, Matyssek R (2007) O_3 flux-related responsiveness of photosynthesis, respiration, and stomatal conductance of adult *Fagus sylvatica* to experimentally enhanced free-air O_3 exposure. Plant Biol 9: 197–206.

Lutz C, Anegg S, Gerant D, Alaoui-Sosse B, Gerard J, Dizengremel P (2000) Beech trees expose to high CO_2 and to simulated summer ozone levels: effects on photosynthesis, chloroplast components and leaf enzyme activity. Physiol Plantarum 109: 252–259.

Luwe MWF, Takahama U, Heber U (1993) Role of ascorbate in detoxifying ozone in the apoplast of spinach (*Spinacia oleraceae* L.) leaves. Plant Physiol 101: 967–976.

Lyons T, Ollerenshaw JH, Barnes JD (1999) Impact of ozone on *Plantago major*: apoplastic and symplastic antioxidant status. New Phytol 141: 253–263.

Maccarrone M, Veldink GA, Vliegenthart FG, Finazzi Agró A (1997) Ozone stress modulates amine oxidase and lipoxygenase expression in (*Lens culinaris*) seedlings. FEBS Letters 408: 241–244.

Maddison J, Lyons T, Plochl M, Barnes J (2002) Hydroponically cultivated radish fed L-galactono-1, 4-lactone exhibit increased tolerance to ozone. Planta 214: 383–391.

Mahalingam R, Fedoroff N (2003) Stress response, cell death and signalling: the many faces of reactive oxygen species. Physiol Plantarum 119: 56–68.

Mahalingam R, Jambunathan N, Gunjan SK, Faustin E, Weng H, Ayoubi P (2006) Analysis of oxidative signaling induced by ozone in *Arabidopsis thaliana*. Plant Cell Environ 29: 1357–1371.

Mahalingam R, Shah N, Scrymgeour A, Fedorof N (2005) Temporal evolution of the *Arabidopsis* oxidative stress response. Plant Mol Biol 57: 709–730.

Manning WJ (2000) Use of protective chemicals to assess the effects of ambient ozone on plants. in: Agrawal, S.B., Agrawal, M. (Eds.) Environmental pollution and plant responses, Lewis Publishers, Boca Raton, FL, USA, 24pp.

Marenco AH, Gouget PN, Pages JP (1994) Evidence of a long term increase in tropospheric ozone from Pic Du Midi data series, consequences, positive radiative forcing. J Geophys Res 99: 166–177.

Marre MT, Amicucci E, Zingarelli L, Albergoni F, Marre E (1998) The respiratory burst and electrolyte leakage induced by sulfhydryl blockers in Egeria densa leaves are associated with H_2O_2 production and are dependent on Ca^{2+} influx. Plant Physiol 118: 1379–1387.

Martin MJ, Farage PK, Humphries SW, Long SP (2000) Can the stomatal changes caused by acute ozone exposure be predicted by changes occurring in the mesophyll? A simplification for models of vegetation response in tropospheric elevated ozone episodes. Austr J Plant Physiol 27: 211–219.

Matyssek R, Sandermann H (2003) Impact of ozone on trees: an ecophysiological perspective. In: Progress in Botany 64, Springer-Verlag, Heidelberg, Germany, pp. 349.

Maurer S, Matyssek R, Gunthardt-Georg MS, Landolt W, Eining W (1997) Nutrition and ozone sensitivity of birch (Betula pendula). I. Responses at the leaf level. Trees 12: 1–10.

Mauzerall DL, Narita D, Akimoto H, Horowitz L, Walters S, Hauglustaine DA, Brasseur G (2000) Seasonal characteristics of tropospheric ozone production and mixing ratios over East Asia: a global three-dimensional chemical transport model analysis. J Geophys Res 105: 17895–17910.

McCrady JK, Andersen CP (2000) The effect of ozone on below ground carbon allocation in wheat. Environ Pollut 197: 465–472.

Mckee IF, Bullimore JF, Long SP (1997) Will elevated CO_2 protect the yield of wheat from O_3 damage? Plant Cell Environ 20: 77–84.

MEA (2005) Millennium ecosystem assessment. Chapter 8: food ecosystem services. Available on: http://www.millenniumassessment.org/documents/document.277.aspx.pdf.

Meehl GA, Stocker TF, Collins WD, Friedlingstein P, Gaye AT, Gregory JM, Kitoh A, Knutti R, Murphy JM, Noda A, Raper SCB, Watterson IG, Weaver AJ, Zhao ZC (2007) Global Climate Projections. in: Solomon, S., Qin, D., Manning, M., Chen, Z., Marquis, M., Averyt, K.B., Tignor, M., Miller, H.L. (Eds.), Climate Change 2007: The physical basis. Contribution of working group I to fourth assessment report of IPCC on climate change. Cambridge University Press, Cambridge, UK, NY, USA.

Mignanego L, Biondi F, Schenone G (1992) Ozone biomonitoring in northern Italy. Environ Monit Assess 141: 141–159.
Miles GP, Samuel MA, Ellis BE (2002) Suramin inhibits oxidant signaling in tobacco suspension-cultured cells. Plant Cell Environ 25: 521–527.
Miles GP, Samuel MA, Jones AM, Ellis BE (2004) Mastoparan rapidly activates plant MAP kinase signaling independent of heterotrimeric G proteins. Plant Physiol 134: 1332–1336.
Miller JD, Arteca RN, Pell EJ (1999) Senescence-associated gene expression during ozone-induced leaf senescence in *Arabidopsis*. Plant Physiol 120: 1015–1023.
Miller JE, Heagle AS, Pursley WA (1998) Influence of ozone stress on soybean response to carbon dioxide enrichment. II. Biomass and development. Crop Sci 38: 122–128.
Mills G, Buse A, Gimeno B, Bermejo V, Holland M, Emberson L, Pleijel H (2007) A synthesis of AOT40-based response functions and critical levels of ozone for agricultural and horticultural crops. Atmos Environ 41: 2630–2643.
Mills G, Hayes F, Buse A, Reynolds B (2000) Air pollution and vegetation. In: Annual Report 1999/2000 of UN/ECE ICP Vegetation. Centre for Ecology and Hydrology, Bangor, UK.
Mittal ML, Hess PG, Jain SL, Arya BC, Sharma C (2007) Surface ozone in the Indian region. Atmos Environ 41: 6572–6584.
Moeder W, Barry CS, Taurianin AA, Betz C, Tuomainen J, Utrianen M, Grierson D, Sandermann H, Langebartals C, Kangsjarvi J (2002) Ethylene synthesis regulated by biphasic induction of 1 aminocyclopropane-1-carboxylic acid synthase and 1-aminocyclopropane-1-carboxylic acid oxidase genes is required for hydrogen peroxide accumulation and cell death in ozone exposed tomato. Plant Physiol 130: 1918–1926.
Morgan PB, Ainsworth EA, Long SP (2003) How does elevated ozone impact soybean? A meta-analysis of photosynthesis, growth and yield. Plant Cell Environ 26: 1317–1328.
Morgan PB, Mies TA, Bollero GA, Nelson RL, Long SP (2006) Season-long elevation of ozone concentration to projected 2050 levels under fully open-air conditions substantially decreases the growth and production of soybean. New Phytol 170: 333–343.
Morris PC (2001) MAP kinase signal transduction pathways in plants. New Phytol 151: 67–89.
Mulchi CL, Lee E, Tuthill K, Olinick EV (1988) Influence of ozone stress on growth processes, yields and grain quality characteristics among soybean cultivars. Environ Pollut 53: 151–169.
Mullineaux P, Karpinski S (2002) Signal transduction in response to excess light: getting out of the chloroplast. Cur Opi Plant Biol 5: 43–48.
Munnik T, Meijer HJG (2001) Osmotic stress activates distinct lipid and MAPK signaling pathways in plants. FEBS Letters 498: 172–178.
Murphy JJ, Delucchi MA, McCubbin DR, Kim HJ (1999) The cost of crop damage caused by ozone air pollution from motor vehicles. J Environ Manag 55: 273–289.
Musselman RC, Massman WJ (1999) Ozone flux to vegetation and its relationship to plant response and ambient air quality standards. Atmos Environ 33: 65–73.
Nakajina N (2002) Effects of ethylene on plant responses to air pollutants. In: Omasa K, Saji H, Youssefian S, Kondo N (eds.), Air pollution and plant biotechnology, Chapter 5, Springer, Tokyo, Japan, pp. 111.
Noctor G, Foyer CH (1998) Ascorbate and Glutathione: Keeping active oxygen under control. Ann Rev Plant Physiol Plant Mol Biol 49: 249–279.
Nouchi I, Toyama S (1998) Effects of ozone and peroxyacetyl nitrate on poplar lipids and fatty acids in leaves of morning glory and kidney bean. Plant Physiol 87: 638–646.
Nouchi I (2002) Responses of whole plant to air pollutants. In: Omasa K, Saji H, Youssefian S, Kondo N (eds.) Air pollution and plant biotechnology, Chapter 1, Springer, Tokyo, Japan, pp. 3.
Ollerenshaw JH, Lyons T (1999) Impacts of ozone on growth field grown winter wheat. Environ Pollut 106: 67–72.
Ollerenshaw JH, Lyons T, Barnes JD (1999) Impacts of ozone on the growth and yield of field-grown winter oilseed rape. Environ Pollut 104: 171–179.
Olszyk DM, Tingly DT, Wise C, Davis S (2002) CO_2 and O_3 alter photosynthesis and water vapor exchange for *Pinus ponderosa* leaves. Phyton 42: 121–134.
Op den Camp RLG, Przybyla D, Ochsenbein C (2003) Rapid induction of distinct cell responses after the release of singlet oxygen in *Arabidopsis*. Plant Cell 15: 2320–2332.

Ossipov V, Ossipova S, Bykov V, Oksanen E, Koricheva J, Haukioja E (2008) Application of metabolomics to genotype and phenotype discrimination of birch trees grown in a long-term open-field experiment. Metabolomics 4: 39–51.

Overmyer K, Brosché M, Kangasjärvi J (2003) Reactive oxygen species and hormonal control of cell death. Trends Plant Sci 8: 335–342.

Overmyer K, Tuominen H, Kettunen R, Betz C, Langebartels C, Sandermann H Jr, Kangasjärvi J (2000) The ozone sensitive *Arabidopsis rcd1* mutant reveals opposite roles for ethylene and jasmonate signaling pathways in regulating superoxide-dependent cell death. Plant Cell 12: 1849–1862.

Pääkkönen E, Seppänen S, Holopainen T, Kokko H, Kärenlampi S, Kärenlampi L, Kangasjärvi J (1998) Induction of genes for the stress proteins PR-10 and PAL in relation to growth, visible injuries and stomatal conductance in birch (*Betula pendula*) clones exposed to ozone and/or drought. New Phytol 138: 295–305.

Pandey J, Agrawal M (1992) Ozone: concentration variabilities in a seasonally dry tropical climate. Environ Inter 18: 515–520.

Pang J, Kobayashi K, Zhu J (2009) Yield and photosynthetic characteristics of flag leaves in Chinese rice (*Oryza sativa* L.) varieties subjected to free-air release of ozone. Agri Ecosys Environ 132: 203–211.

Paoletti E, Contran N, Manning WJ, Castagna A, Ranieri A, Tagliaferro F (2008) Protection of ash (*Fraxinus excelsior*) trees from ozone injury by ethylenediurea (EDU): roles of biochemical changes and Decreased stomatal conductance in enhancement of growth. Environ Pollut 155: 464–472.

Pasqualini S, Della Torre G, Ferranti F, Ederli L, Piccioni C, Reale L, Antonielli M (2002) Salicylic acid modulates ozone induced hypersensitive cell death in tobacco plants. Physiol Plantarum 115: 204–212.

Pell EJ, Landry LG, Eckardt NA, Glick RE (1994a) Air pollution and Rubisco: Effects and implications. In: Alscher RG, Wellburn AR (eds.) Plant responses to the gaseous environment, Chapman & Hall, London, UK, pp. 239.

Pell EJ, Temple PJ, Friend AL, Mooney HA, Winner WE (1994b) Compensation as a plant response to ozone and associated stresses: an analysis of ROPIS experiments. J Environ Quality 23: 429–436.

Pelloux J, Jolivet Y, Fontaine V, Banvoy J, Dizengremel P (2001) Changes in Rubisco and Rubisco activase gene expression and polypeptide content in *Pinus halepensis* M. Plant Cell Environ 24: 123–131.

Permadi DA, Oanh NTK (2008) Episodic ozone air quality in Jakarta in relation to meteorological conditions. Atmos Environ 42: 6806–6815.

Pino ME, Mudd JB, Bailey-Serres J (1995) Ozone-induced alterations in the accumulation of newly synthesized proteins in leaves of maize. Plant Physiol 108: 777–785.

Pitcher LH, Brennan E, Zilinskas BA (1992) The antiozonant ethylenediurea does not act via superoxide dismutase induction in bean. Plant Physiol 99: 1388–1392.

Pleijel H, Eriksen AB, Danielsson H, Bondesson N, Sellde´n G (2006) Differential ozone sensitivity in an old and a modern Swedish wheat cultivar-grain yield and quality, leaf chlorophyll and stomatal conductance. Environ Exp Bot 56: 63–71.

Pleijel H, Ojenpara K, Mortensen L (1997) Effects of tropospheric ozone on yield and grain protein content of spring wheat (*Triticum aestivum* L.) in the Nordic countries. Acta Agriculturae Scandinavica Section B – Soil & Plant Science 47: 20–25.

Plochl M, Lyons T, Ollerenshaw J, Barnes J (2000) Simulating ozone detoxification in the leaf apoplast through the direct reaction with ascorbate. Planta 210: 454–467.

Polle A, Weiser G, Havernak WM (1995) Quantification of ozone influx and apoplastic ascorbate content in needles of Norway spruce trees (*Picea abies* L. Krast) at high altitude. Plant Cell Environ 18: 681–688.

Poorter H, Perez-Soba M (2001) The growth response of plants to elevated CO_2 under non optimal environmental conditions. Oecologia 129: 1–20.

Rai R, Agrawal M (2008) Evaluation of physiological and biochemical responses of two rice (*Oryza sativa* L.) cultivars to ambient air pollution using open top chambers at rural site in India. Sci Total Environ 407: 679–691.

Rai R, Agrawal M, Agrawal SB (2007) Assessment of yield losses in tropical wheat using open top chambers. Atmos Environ 41: 9543–9554.

Rai R, Agrawal M, Agrawal SB (2010) Threat to food security under current levels of ground level ozone: A case study for Indian cultivars of rice. Atmos Environ (doi: 1011016/j.atmosenv.2010.06.022) (in press).

Ramo K, Slotte H, Kanvera T, Ojanpera K, Manninen S (2006) Growth and visible injuries on four *Centaurea jacea* L. ecotypes exposed to elevated ozone and carbon dioxide. Environ Exp Bot 58: 287–298.

Ranieri A, Petacco F, Castagna A, Soldatini GF (2000) Redox state and peroxidase system in sunflower plants exposed to ozone. Plant Sci 159: 159–167.

Rao MV, Jennifer RK, Davis KR (2000) Ozone: a tool for probing programmed cell death in plants. Plant Mol Biol 44: 345–358.

Rao MV, Lee H, Davis KR (2002) Ozone-induced ethylene production is dependent on salicylic acid, and both salicylic acid and ethylene act in concert to regulate ozone-induced cell death. Plant J 32: 447–456.

Reiling K, Davison AW (1995) Effect of ozone on stomatal conductance and photosynthesis in populations of *Plantago major* L. New Phytol 129: 587–592.

Renaut J, Bohler S, Hausman JF, Hoffmann L, Sergeant K, Ahsan N, Jolivet Y, Dizengremel P (2009) The impact of atmospheric composition on plants: A case study of ozone and poplar. Mass Spec Rev 28: 495–516.

Ribas A, Penuelas J, Elvira S, Gimeno BS (2005) Ozone exposure induces the activation of leaf senescence related processes and morphological and growth changes in seedlings of Mediterranean tree species. Environ Pollut 134: 291–300.

Richards BL, Middleton JT, Hewitt WB (1958) Air pollution with reference to agronomic crops. Agron J 50: 559–561.

Robinson JM, Britz SJ (2000) Tolerance of a field grown soybean cultivar to elevated ozone level is concurrent with higher leaflet ascorbic level, higher ascorbate-dehydroascorbate redox status, and long term photosynthetic productivity. Photosyn Res 64: 77–87.

Rosenqvist E, van Kooten O (2003) Chlorophyll Fluorescence: a general description and nomenclature. In: DeEll JR, Toivonen PMA (eds.) Practical applications of chlorophyll fluorescence in plant biology, Kluwer Academic Publishers, Norewell, MA, USA, pp. 31.

Rossard S, Luini E, Perault JM, Bonmort J, Roblin G (2006) Early changes in membrane permeability, production of oxidative burst and modification of PAL activity induced by ergosterol in cotyledons of *Mimosa pudica*. J Exp Bot 57: 1245–1252.

Rouhier N, Gelhaye E, Jacquot JP (2004) Plant glutaredoxin: still mysterious reducing systems. Cellu Mol Life Sci 61: 1266–1277.

Roy SD, Beig G, Ghude SD (2009) Exposure-plant response of ambient ozone over the tropical Indian Region. Atmos Chem Phys 9: 5253–5260.

Roy S, Beig G, Jacob D (2008) Seasonal distribution of ozone and its precursors over the tropical Indian region using regional chemistry-transport model. J Geophys Res 113: D21307.

Ruzsa SM, Mylona P, Scandalios JG (1999) Differential response of antioxidant genes in maize leaves exposed to ozone. Redox Rep 4: 95–103.

Sakaki T, Kondo N, Sugahara K (2008) Breakdown of photosynthetic pigments and lipids in spinach leaves with ozone fumigation: Role of active oxygens. Physiol Plantarum 59: 28–34.

Sakaki T, Kondo N, Yamada M (1990a) Pathway for the synthesis of triacylglycerol from monogalactosyldiacylglycerols in ozone-fumigated spinach leaves. Plant Physiol 94: 773–780.

Sakaki T, Kondo N, Yamada M (1990b) Free fatty acids regulate two galactosyltransferases in chloroplast envelope mambranes isolated from spinach leaves. Plant Physiol 94: 781–787.

Sakaki T, Saito K, Kawaguchi A, Kondo N, Yamada M (1990c) Conversion of monogalactosyldiacylglycerols to triacylglycerol in ozone-fumigated spinach leaves. Plant Physiol 94: 766–772.

Samuel MA, Ellis BE (2002) Double jeopardy: both overexpression and suppression of a redox-activated plant mitogenactivated protein kinase render tobacco plants ozone sensitive. Plant Cell 14: 2059–2069.

Samuel MA, Miles GP, Ellis BE (2000) Ozone treatment rapidly activates MAP kinase signaling in plants. Plant J 22: 367–376.

Sandermann H Jr. (1996) Ozone and plant health. Annu Rev Phytopathol 34: 347–366.
Sandermann H Jr. (2000) Ozone/biotic disease interactions: molecular biomarkers as a new experimental tool. Environ Pollut 108: 327–332.
Sanders GE, Robinson AD, Geissler PA, Colls JJ (1992) Yield stimulation of a commonly grown cultivar of *Phaseolus vulgaris* L. at near ambient ozone concentrations. New Phytol 122: 63–70.
Sanmartin M, Drogoudi PD, Lyons T, Pateraki I, Barnes J, Kanellis AK (2003) Over-expression of ascorbate oxidase in the apoplast of transgenic tobacco results in altered ascorbate and glutathione redox states and increased sensitivity to ozone. Planta 216: 918–928.
Sarkar A, Agrawal SB (2010a) Identification of ozone stress in Indian rice through foliar injury and differential protein profile. Environ Monit Assess 161: 205–215.
Sarkar A, Agrawal SB (2010b) Elevated ozone and two modern wheat cultivars: an assessment of dose dependent sensitivity with respect to growth, reproductive and yield parameter. Environ Exp Bot 69: 328–337.
Sarkar A, Rakwal R, Agrawal SB, Shibato J, Ogawa Y, Yoshida Y, Agrawal GK, Agrawal M (2010) Investigating the impact of elevated levels of ozone on tropical wheat using integrated phenotypical, physiological, biochemical and proteomics approaches. J Proteome Res 9: 4565–4584.
Sawada H, Kohno Y (2009) Differential ozone sensitivity of rice cultivars as indicated by visible injury and grain yield. Plant Biol 11: 70–75.
Schmieden U, Wild A (1995) The contribution of ozone to forest decline. Physiol Plantarum 94: 371–378.
Schraudner M, Langebartels C, Sandermann H (1997) Changes in the biochemical status of plant cells induced by the environmental pollutant ozone. Physiol Plantarum 100: 274–280.
Selldén G, Sutinen S, Skärby L (1997) Controlled ozone exposures and field observations in Fennoscandia. In: Sandermann H, Wellburn AR, Heath RL (eds.) Forest Decline and Ozone, Vol. 127. Ecological Studies, pp. 249.
Sen Gupta A, Alscher RG, McCune D (1991) Response of Photosynthesis and Cellular Antioxidants to Ozone in Populus Leaves. Plant Physiol 96: 650–655.
Shi G, Yang L, Wang Y, Kobayashi K, Zhu J, Tang H, Pan S, Chen T, Liu G, Wang Y (2009) Impact of elevated ozone concentration on yield of four Chinese rice cultivars under fully open-air field conditions. Agri Ecosys Environ 131: 178–184.
Singh A, Sarin SM, Shanmugam P, Sharma N, Attri AK, Jain VK (1997) Ozone distribution in the urban environment of Delhi during winter months. Atmos Environ 31: 3421–3427.
Singh E, Tiwari S, Agrawal M (2010) Variability in antioxidant and metabolite levels, growth and yield of two soybean varieties: An assessment of anticipated yield losses under projected elevation of ozone. Agri Ecosys Environ 135: 168–177.
Singh P, Agrawal M, Agrawal SB (2009a) Evaluation of physiological, growth and yield responses of a tropical oil crop (*Brassica campestris* L. var. Kranti) under ambient ozone pollution at varying NPK levels. Environ Pollut 157: 871–880.
Singh S, Agrawal SB (2009) Use of ethylenediurea (EDU) in assessing the impact of ozone on growth and productivity of five cultivars of Indian wheat (*Triticum aestivum* L.). Environ Monit Assess 159: 125–141.
Singh S, Agrawal SB (2010) Impact of tropospheric ozone on wheat (*Triticum aestivum* L.) in the eastern gangetic plains of India as assessed by ethylene diurea (EDU) application during developmental stages. Agri Ecosys Environ 138: 214–221.
Singh S, Agrawal SB, Agrawal, M (2009b) Differential protection of ethylenediurea (EDU) against ambient ozone for five cultivars of tropical wheat. Environ Pollut 157: 2359–2367.
Tamaoki M, Nakajima N, Kubo A, Aono M, Matsuyama T, Saji H (2003) Transcriptome analysis of O_3-exposed *Arabidopsis* reveals that multiple signal pathways act mutually antagonistically to induce gene expression. Plant Mol Biol 53: 443–456.
Tang Y, Chevone BI, Hess JL (1999) Ozone-responsive proteins in a tolerant and sensitive clone of white clover (*Trifolium repens*). Environ Pollut 104: 89–98.
Tausz M, Grulke NE, Wieser G (2007) Defense and avoidance of ozone under global change. Environ Pollut 147: 525–531.

Then C, Herbinger K, Luis VS, Heerdt C, Matyssek R, Weiser G (2009) Photosynthesis, chloroplast pigments and antioxidants in *Pinus canariensis* under free air ozone fumigation. Environ Pollut 157: 392–395.

Tingey DT, Rodecap KD, Lee EH, Hogsett WE, Gregg JW (2002) Pod development increases the ozone sensitivity of *Phaseolus vulgaris*. Water Air Soil Pollut 139: 325–341.

Tiwari S, Agrawal M (2009) Protection of palak (*Beta vulgaris* L. var Allgreen) plants from ozone injury by ethylenediurea (EDU): Roles of biochemical and physiological variations in alleviating the adverse impacts. Chemosphere 75: 1492–1499.

Tiwari S, Agrawal M, Manning WJ (2005) Assessing the impacts of ambient ozone on growth and productivity of two cultivars of wheat in India using three rates of applications of ethylenediurea (EDU). Environ Pollut 138: 153–160.

Tiwari S, Rai R, Agrawal M (2008) Annual and seasonal variations in tropospheric ozone concentrations around Varanasi. Inter J Remote Sens 29: 4499–4514.

Tong D, Mathur R, Schere K, Kang D, Yu S (2007) The use of air quality forecasts to assess impacts of air pollution on crops: Methodology and case study. Atmos Environ 41: 8772–8784.

Tonneijk AEG, van Dijk CJ (1997) Effects of ambient ozone on injury and yield of *Phaseolus vulgaris* at 4 rural sites inNetherlands as assessed by using ethylenediurea (EDU). New Phytol 135: 93–100.

Torres NL, Cho K, Shibato J, Kubo A, Iwahashi H, Jwa NS, Agrawal GK, Rakwal R (2007) Gel-based proteomics reveals potential novel protein markers of ozone stress in leaves of cultivated bean and maize species of Panama. Electrophoresis 28: 4369–4381.

Troughton A (1955) The application of the allometric formula to the study of the relationship between roots and shoots of the young grass plants. Agricultural Progress 30: 59–65.

Tuomainen J, Betz C, Kangasjärvi J, Ernst D, Yin ZH, Langebartels C, Sandermann H Jr. (1997) Ozone induction of ethylene emission in tomato plants: regulation by differential transcript accumulation for the biosynthetic enzymes. Plant J 12: 1151–1162.

Tuominen H, Overmeyer K, Keinänen M, Kollist H, Kangasjärvi J (2004) Mutual antagonism of ethylene and jasmonic acid regulates ozone induced spreading cell death in *Arabidopsis*. Plant J 39: 59–69.

Turcsányi E, Lyons T, Plöchl M, Barnes J (2000) Does ascorbate in the mesophyll cell walls form the first line of defence against ozone? Testing the concept using broad bean (*Vicia faba* L.). J Exp Bot 51: 901–910.

US EPA (1996) Air Quality Criteria for Ozone and Related Photochemical Oxidants. Research Triangle Park, NC: Office of Health and Environmental Assessment, Environmental Criteria and Assessment Office; Report no. EPA-600/p-93/004aF-cF.

Vahala J, Ruonala R, Keinäne M, Tuominen H, Kangasjärvi J (2003a) Ethylene insensitivity modulates ozone-induced cell death in Birch. Plant Physiol 132: 185–195.

Vahala J, Keinänen M, Schützendübel A, Polle A, Kangasjärvi J (2003b) Differential effects of elevated ozone on two hybrid aspen genotypes predisposed to chronic ozone fumigation. Role of ethylene and salicylic acid. Plant Physiol 132: 196–205.

Van Hove LW, Bossen ME, San Gabino BG, Sgreva C (2001) The ability of apoplastic ascorbate to protect poplar leaves against ambient ozone concentration: a quantitative approach. Environ Pollut 114: 371–382.

Van Noordwijk M, Martikainen P, Bottner P, Cuevas E, Rouland C, Dhillion SS (1998) Global change and root function. Global Change Biol 4: 759–772.

Varotsos C, Cartalis C (1991) Re-evaluation of surface ozone over Athens, Greece, for the period 1901–1940. Atmos Res 26: 303–310.

Varotsos C, Cartalis C, Vlamakis A, Tzanis C, Keramitsoglou I (2004) The long term coupling between column ozone and tropopause properties. J Climate 17: 3843–3854.

Varshney CK, Aggarwal M (1992) Ozone pollution in the urban atmosphere of Delhi. Atmos Environ 26: 291–294.

Velissariou D (1999) Toxic effects and losses of commercial value of lettuce and other vegetables due to photochemical air pollution in agricultural areas of Attica, Greece. In: Fuhrer J, Achermann B (eds.) Critical Levels for Ozone – Level II, Swiss Agency for Environment, Forest and Landscape, Bern, Switzerland, pp. 253.

Vingarzan R (2004) A review of surface ozone background levels and trends. Atmos Environ 38: 3431–3442.

Vollenweider P, Woodcock H, Kelty MJ, Hofer RM (2003) Reduction of stem growth and site dependency of leaf injury in Massachusetts black cherries exhibiting ozone symptoms. Environ Pollut 125: 467–480.

Volz A, Kley D (1988) Evaluation of the montsouris series of ozone measurements made in the 19th-century. Nature 332: 240–242.

Vorne V, Ojenpera K, De Temmermen L, Bindi M, Hogy P, Jones MB, Lawson T, Persson K (2002) Effects of elevated CO_2 and ozone on potato tuber quality in European multiple site experiment 'CHIP project'. Euro J Agron 17: 369–381.

Wahid A (2006a) Influence of atmospheric pollutants on agriculture in developing countries: a case study with three new wheat varieties in Pakistan. Sci Total Environ 371: 304–313.

Wahid A (2006b) Productivity losses in barley attributable to ambient atmospheric pollutants in Pakistan. Atmos Environ 40: 5342–5354.

Wahid A, Maggs R, Shamasi SRM, Bell JNB, Ashmore MR (1995) Effects of air pollution on rice yield in the Pakistan Punjab. Environ Pollut 90: 323–329.

Wahid A, Milne E, Shamsi SRA, Ashmore MR, Marshall FM (2001) Effects of oxidants on soybean growth and yield in Pakistan Punjab. Environ Pollut 113: 271–280.

Wang T, Wei XL, Ding AJ, Poon CN, Lam KS, Li YS, Chan LY, Anson M (2009) Increasing surface ozone concentrations in the background atmosphere of Southern China, 1994–2007. Atmos Chem Phys 9: 6217–6227.

Wang X, Mauzerall DL (2004) Characterizing distribution of tropospheric ozone and its impacts on grain production in China, Japan and South Korea: 1990 and 2000. Atmos Environ 38: 4348–4402.

Wang X, Zheng Q, Yao F, Chen Z, Feng Z, Manning WJ (2007) Assessing the impact of ambient ozone on growth and yield of a rice (*Oryza sativa* L.) and wheat (*Triticum aestivum* L.) cultivar grown in Yangtze Delta, China, using three rates of application of Ethylenediurea (EDU). Environ Pollut 148: 390–395.

Weidensaul TC (1980) N-[2-(2-oxo-1-imidizolidiny1) ethyl-]-N-phenylurea as a protectant against ozone injury to laboratory fumigated pinto bean plants. Phytopathology 70: 42–45.

Weiser G, Matyssek R (2007) Linking ozone uptake and defense towards a mechanistic risk assessment for forest trees. New Phytol 174: 7–9.

Winner EW, Coleman JS, Gillespie C, Mooney HA, Pell EJ (1991) Consequences of evolving resistance to air pollution. In: Taylor GE, Pitelka LF, Clegg M (eds.) Ecological genetics and air pollution, Springer-Verlag, New York, USA, pp. 177.

Woodbury PB, Laurence JA, Hudler GW (1994). Chronic ozone exposure alters the growth of leaves, stems and roots of hybrid Populus. Environ Pollut 85: 103–108.

Yamaji K, Ohara T, Uno I, Tanimoto H, Kurokawa J, Akimoto H (2006) Analysis of seasonal variation of ozone in the boundary layer in East Asia using the Community Multiscale Air Quality model: what controls surface ozone level over Japan? Atmos Environ 40: 1856–1868.

Yang T, Poovaiah BW (2000) An early ethylene up-regulated gene encoding a calmodulin-binding protein involved in plant senescence and death. J Biol Chem 275: 38467–38473.

Zeng G, Pyle JA, Young PJ (2008) Impact of climate change on tropospheric ozone and its global budgets. Atmos Chem Phys 8: 369–387.

Zheng Y, Lyons T, Ollerenshaw JH, Barnes JD (2000) Ascorbate in the leaf apoplast is a factor mediating ozone resistance in Plantago major. Plant Physiol Biochem 38: 403–411.

Zheng Y, Stevenson KJ, Barrowcliffe R, Chen S, Wang H, Barnes JD (1998) Ozone levels in Chongqing: a potential threat to crop plant commonly grown in the region? Environ Pollut 99: 299–308.

Strategies for Decolorization and Detoxification of Pulp and Paper Mill Effluent

Satyendra K. Garg and Manikant Tripathi

Contents

1 Introduction

The manufacture of pulp and paper is a resource-intensive industry that requires a large quantity of water, energy, and lignocellulosic materials of plant origin. Presently, the high cost of energy inputs, and realization of increased environmental concerns are the driving forces behind the need for the pulp and paper industry to seek more cost-effective and environmental-friendly alternatives for pollution abatement. This industry currently constitutes one of the largest contributors to air and water pollution.

The three main component groups of lignocellulosic plant materials are cellulose, hemicelluloses, and lignin. Pulp and paper mill effluent is highly colored and imparts a dark brown/black appearance to the receiving waterbody. The color of these wastewaters primarily results from the presence of lignin and its derivatives that are released from the substrate, and discharged in such effluents. The color mainly derives from various processing steps undertaken during paper manufacture (Prouty 1990; Esposito

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D.M. Whitacre (ed.), *Reviews of Environmental Contamination and Toxicology*,
Reviews of Environmental Contamination and Toxicology 212,
DOI 10.1007/978-1-4419-8453-1_4, © Springer Science+Business Media, LLC 2011

et al. 1991; Bergbauer and Eggert 1992). The major source of phenolic compounds in pulp and paper mill wastewater is lignin (Amat et al. 2005), whereas pentachlorophenol is generated in the effluent as a by-product from chlorine bleaching of pulp (Steinle et al. 1998; Vallecillo et al. 1999). The chlorinated phenols, chlorinated dibenzo-*p*-dioxin and dibenzofuran, chlorinated hydrocarbon, etc., that are released from lignin during chlorine bleaching of pulp are largely responsible for the toxic compounds released in pulp mill effluents. In addition, extractives are also present in such effluents. Although the concentration of extractives is lower than that of the phenolic compounds, some are relatively more toxic, and significantly reduce water quality and degrade the aquatic habitat of lakes and rivers receiving paper mill effluents.

In general, colored effluents are not only aesthetically unacceptable, but also reduce the transmission of light in contaminated waterways, thereby reducing aquatic plant photosynthesis. This, in turn, reduces the dissolved oxygen content and ultimately causes the death and putrefaction of aquatic fauna (Sahoo and Gupta 2005).

The decolorization and detoxification of paper mill effluents has been the subject of several studies performed in recent years (Raj et al. 2007). Several physical and chemical methods (e.g., precipitation, sorption, ozonation, ultrafiltration, reverse osmosis, and electrochemical treatment) have been attempted to decolorize pulp and paper mill effluents. Other physicochemical treatment methods, viz., incineration of black liquor lignin (Harila and Kivilinna 1999), titanium oxide oxidation systems (Chang et al. 2004; Yeber et al. 2007), Fenton and photo-Fenton reactions (Perez et al. 2002; Kazmi and Thul 2007; Zahrim et al. 2007), and chemical coagulation of lignin (Ganjidoust et al. 1997) have also been reported to be effective for reducing color in, and toxicity of paper mill wastewaters.

Other treatment procedures have also been evaluated. Garg et al. (2004) studied the removal of nonbiodegradable and toxic compounds in pulp and paper mill effluent by wet air oxidation (WAO), using a heterogeneous catalyst (CuO–ZnO) supported on alumina and ceria. The authors recorded a maximum chemical oxygen demand (COD) reduction of 83% for this (CuO–ZnO)/CeO_2 catalyst; the concentration of the pulp–paper mill wastewater was 5 kg/m^3 (2-h retention time), and the initial pH was 3.0. Recently, Mishra et al. (2009) compared the efficacy of activated charcoal and heat-treated coal for decolorizing pulp and paper mill effluent.

However, these latter methods have not been implemented at an industrial scale, mainly because they are energy intensive, and too expensive per unit volume of the effluent. Therefore, alternative biotreatment processes are being considered (Boman et al. 1988). Physical and chemical processes are capable of removing only coloring agents, toxicants, suspended solids, COD, and high molecular weight lignins. But biological oxygen demand (BOD) is not reduced and low molecular weight compounds are not efficiently removed by such physical and chemical treatments (Singh and Singh 2004). The biological color-removal processes are particularly efficient, attractive, and cost and energy effective, because they reduce color, COD, BOD, and toxic low molecular weight chlorinated lignin derivatives (Barton et al. 1996; Nagarathnamma et al. 1999).

At present, conventional biological treatment methods employed in the industry include use of aerated lagoons and activated sludge processes. These biological treatment methods are employed to ensure that effluents meet the BOD discharge limits, and pass fish toxicity tests. Such treatment processes usually remove 85–90% of the readily

biodegradable fraction of the BOD from these effluents. However, such systems are generally less effective in removing color, COD and chlorinated phenolic compounds (Saunamaki 1989; Raj et al. 2007). Therefore, more advanced alternative biological wastewater treatment strategies will be required to meet the new and more stringent discharge limits set for absorbable organic halogens (AOX). Such new biological treatment technologies must be designed to degrade halogenated chemicals in effluents that pose the greatest threat to human health. Dealing effectively with the AOX chemicals is important, because they are toxic and/or mutagenic, can bioaccumulate in primary and secondary consumers of the food chain, and are difficult to degrade.

Microorganisms, such as fungi, bacteria, and algae, are suitable biological candidates for treating wastewaters. The role of white-rot (Basidiomycetes) and other fungi in lignin/phenolics-laden wastewater treatment is gaining momentum, because of their potential for degrading lignin and its derivatives (Eriksson et al. 1980; Eaton et al. 1981; Driessel and Christov 2001; Christian et al. 2005; Saritha et al. 2010). Biodegradation of lignin is of ecological significance and also has wide industrial application (Bhoominathan and Reddy 1992). Since fungi cannot utilize lignin either as a carbon or energy source, basic nutrients (i.e., carbon and nitrogen) are added to the medium to stimulate fungal growth and to advance the breakdown of lignin in the effluent (Kirk et al. 1976; Keyser et al. 1978; Archibald et al. 1990; Wang et al. 2003; Sahoo and Gupta 2005; Sukumar et al. 2006; Jaganathan et al. 2009). However, an increased carbon supply stimulates, whereas increased nitrogen inhibits, lignin degradation. Therefore, the C:N ratio is considered to be a better predictor of lignin degradation than are the absolute levels of carbohydrates and nitrogen (Reid 1979). However, fungi are able to remove chromophoric constituents and lignin compounds during aerobic treatment but are not efficient in degrading chloroorganic compounds. Vora et al. (1988) have reported that many bacteria, viz., *Pseudomonas*, *Flavobacterium*, *Xanthomonas*, *Nocardia*, *Aeromonas*, and *Arthrobacter* are able to utilize several lignocellulosic compounds of the bleached plant effluent, including organochlorine constituents.

Anaerobic biological treatment of effluents also plays a pivotal role in cleaning wastewaters. Anaerobic treatment efficiently destroys chlorophenolic compounds, and reduces mutagenicity and toxicity caused by the effluents (Hickey et al. 1995). Therefore, the development of advanced biological treatment processes, particularly hybrid or dual systems may offer advantages, wherein both anaerobic and aerobic digestion approaches are used.

In the near future, the paper industry will face more severe legal restrictions, having mainly to do with the toxicity and level of environmental pollution caused by their effluents. New regulations are expected that will improve the environmental quality, and will reduce human health associated with the pulp and paper industry. The present laws around the world have already forced paper manufacturing companies to implement relevant manufacturing improvements and end-of-pipe biological treatment processes.

It is the aim of this paper to present an overview of the advanced technologies that are used by the pulp and paper mill industries, in some developed countries, and are suggested for use by other nations, to decolorize and remediate the toxic pollutants that exist in mill effluents. In addition, we endeavor to summarize and consolidate the scattered literature on removing harmful constituents and color from paper mill effluents.

2 The Structure of Woody Materials and Other Lignocellulosics

Insights into the primary substrate from which pulp and paper are made will help to understand the sources of the resulting manufacturing pollutants. Paper is made from waste paper, agricultural residues, and wood. The respective amounts of paper made from each of these is 29, 28, and 43%. In earlier times, small paper mills were dependent upon linen and cotton rags and recycled rope, jute cloth, flax wastes, hemp, ramie, kenaf, straw, bagasse, etc., for their raw feedstocks in paper making. All such substrates either had the advantage of being already processed cellulosic materials, or were raw substrates from which cellulosic fibers could be easily extracted (Van Roekel 1994). The mills of yesteryear were not equipped with the machines, or economically viable alternative technology, for grinding wood, and therefore wood was not preferred as the substrate for paper manufacturing. The use of wood as a cellulosic feedstock for paper making was therefore a technological breakthrough. This occurred in 1850 by Don Valley Paper Mill, Toronto, Canada, which almost completely transformed the paper making industry. However, the use of wood-based paper manufacture has gradually declined, particularly in India, from the constraints of cost and raw material availability. The share of waste paper and agro-residue-based technology will increase as future feedstocks in paper manufacturing, because they require less energy input.

Although the proportion of the three main components of wood, cellulose, hemicellulose, and lignin is about 50, 25, and 25%, respectively, the content of these constituents varies among wood species. Cellulose is a very long molecule and is composed of repeating D-glucose units joined through a linear linkage of β, 1-4 glycosidic units. The extent of polymerization varies from species to species. This long natural polymer is variously folded, bundled, and stabilized by hydrogen bonding (Ranby 1969). The degree of order created within the bundle determines whether it is in a crystalline or amorphous cellulosic form. The cellulose units are assembled to first form microfibrils, then fibrils, and finally through hydrogen bonding between linear molecules produces a strong microcrystalline structure. This structure for cellulose is amenable to biodegradation by a variety of microorganisms. However, in their natural state, cellulose fibrils are associated with lignin, hemicellulose, and other materials in a complex and heterogeneous structure. Therefore, many bacteria and mold species are generally discouraged by the presence of the hemicellulose and lignin, which effectively provide a protective covering to cellulosic fibers.

Hemicellulose is composed of five-carbon sugars, which are randomly arranged to form long branched molecules. These hemicellulosic molecules cover the cellulosic fibers and also enter the pores of cellulose. This layer acts as an anchoring agent between cellulose and lignin, because hemicellulose forms chemical bonds with the adjoining layer of lignin. Lignin is both the most abundant aromatic (phenolic) polymer and the second most abundant raw material (Li et al. 2009). The lignin layer is a complex phenylpropanoid polymer responsible for imparting

strength to cellulose–hemicellulosic structures and is also described as a heterogeneous polymer comprising substituted cinnamyl alcohols.

A large portion of the lignin in woody plants is always associated with cellulose and hemicellulose, not only in intimate physical admixture, but through ether, ester, acetyl, ketal, and hydrogen bonds. Lignin forms a matrix that surrounds cellulose, the most abundant natural polymer. Lignin is basically composed of three randomly polymerized phenol-based building blocks, which is very difficult to degrade because of its random arrangement. The biodegradative recalcitrance of lignin is believed to result from its high molecular weight and three dimensional structure (Zeikus et al. 1982). The presence of this intractable polymer creates an obstacle to the efficient use of cellulose for a wide range of industrial applications. However, lignin may be slowly degraded to release toxic phenolic compounds. The aromatic content of lignin, expressed as monomeric phenol, is ~51%. It is the phenolic compounds released from lignin during the chlorine bleaching of pulp that are responsible for a large percentage of the toxic compounds released into pulp mill effluents. Only a limited number of microorganisms derive benefit from the biological breakdown of lignin.

In addition to cellulose, hemicellulose, and lignin, which constitute the major components, wood contains approximately 1.5–5.0% of compounds that are extractable with organic solvents. Some of these extractable compounds include resin acids, fats, waxes, terpenoids, tannins, flavanoids, stilbenes, and troppolines.

3 Hazardous Colored and Toxic Compounds Released During Pulping and Bleaching

The sole purpose of pulping is to separate cellulose fibers from other hemicellulosic and lignin components that comprises the lignocellulosic substrate. Certain pulping procedures, such as mechanical stone-grinding and chemical sulphite pulping, were employed until the Kraft chemical pulping process was introduced. Kraft pulping involves cooking woodchips in a high concentration of sodium hydroxide and sodium sulfide for 2–4 h at 170°C (Gellerstedt 2001). The advantage of Kraft pulping is to produce a high quality cellulose fiber. In addition, fatty and resin acids, turpentine, bioenergy, etc., collectively called tall oil, can be recovered from the spent-liquors. The flow chart for a Kraft pulping process is illustrated in Fig. 1.

Approximately 55% of the original wood is dissolved in the Kraft pulping process and is released as “black liquor.” The black liquor contains 90–95% lignin, nearly all hemicellulose, wood extractives, and a small quantity of soluble cellulosic oligo- and polysaccharides, derived from the original wood. In most developed countries that have stringent emission standards, by-products are recovered from the black liquor, remaining amounts of the black liquor is evapoconcentrated, and the solids are burned for energy, although some inorganic chemicals are sometimes also recovered from the solids (Fig. 1).

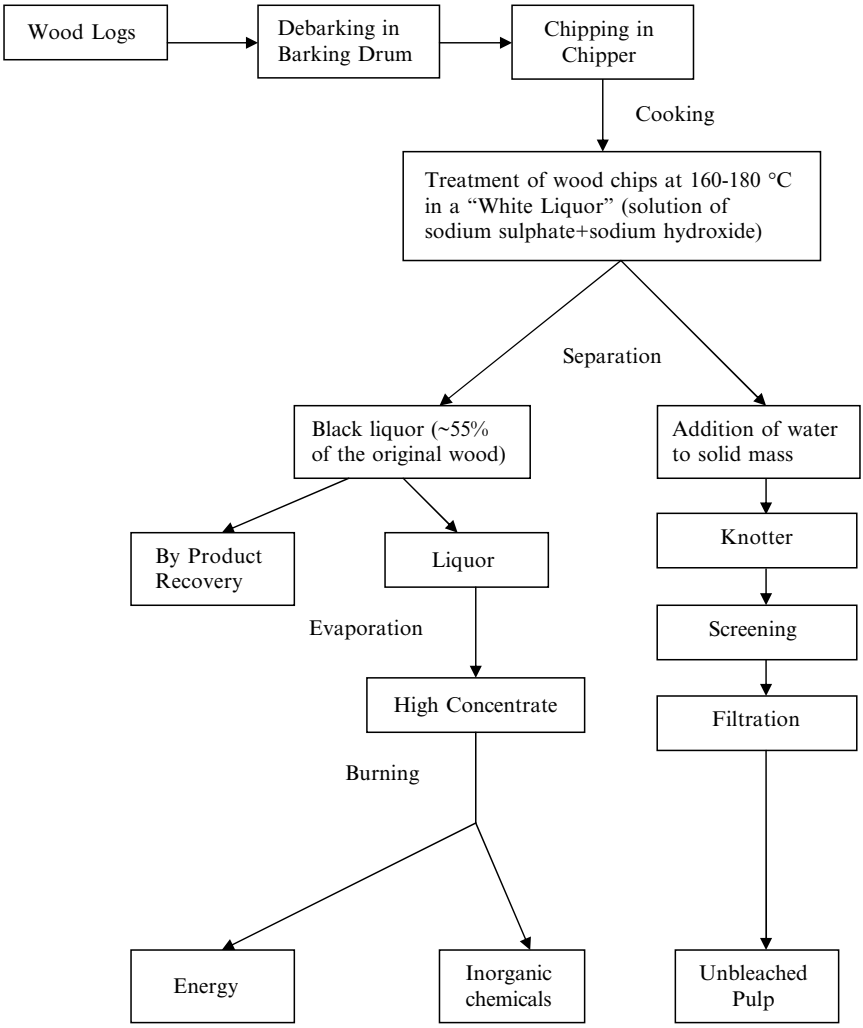

Fig. 1 Flow chart depicting the (Kraft) pulping process

The pulping process is terminated when about 5–10% lignin remains in the pulp. Further delignification at this stage is avoided because it damages fiber quality (Kringstad and Lindstorm 1984). The process is often performed differently in some underdeveloped and developing countries. In these cases, the black liquor is indiscriminately mixed with bleachery effluent (pooled wastewater released from various bleaching steps) and is discharged into nearby streams or other waterbodies. Such release of untreated material threatens aquatic organisms and depending on how local water supplies for human use are handled, may threaten human health as well. Some lignin-degradates that are released in the black liquor during the pulping stage are toxic, and more specifically may be mutagenic; moreover, these compounds may be bioaccumulated in the tissues of animals, particularly fish, and

in humans (Bajpai et al. 2000). Currently, many mills do not opt to utilize recovery plants, and thereby release a load of lignin waste directly to local streams or water bodies. The rivers or streams that receive pulp and paper mill wastes usually acquire a blackish or coffee color, and display a considerable amount of surface foam (Bharati et al. 1992).

In the bleaching process, a slurry (3%) of pulp is made and is usually serially treated with chlorine, alkali, hypochlorite, chlorine dioxide, alkali, and finally, with chlorine dioxide (Fig. 2). Each treatment process is followed by filtration, and

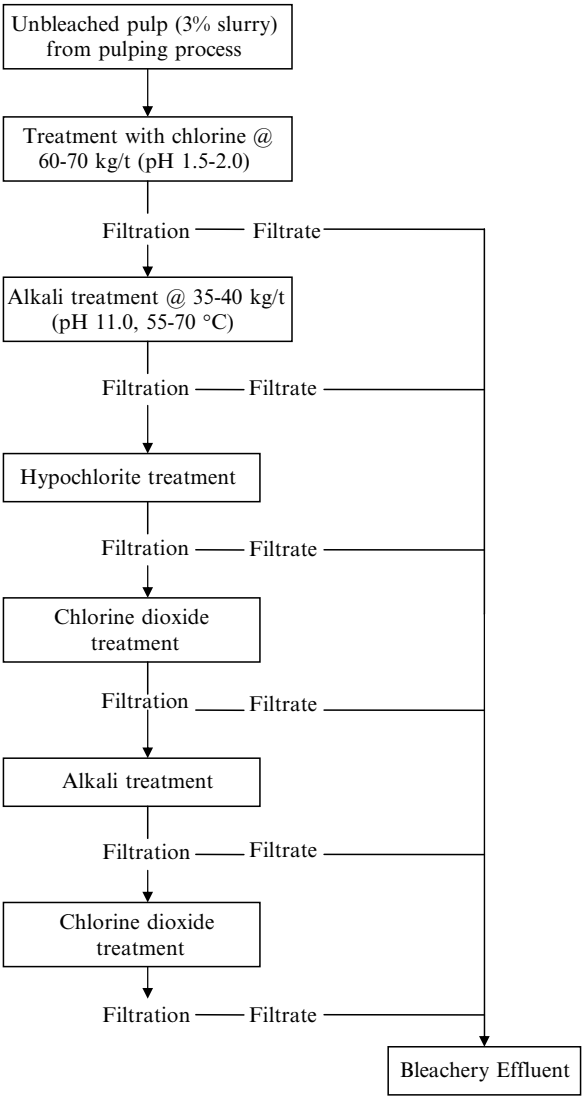

Fig. 2 Flow chart depicting the bleaching process

ultimately all filtrates are combined and discharged as bleachery effluent (Kringstad and Lindstorm 1984). The chlorine present during the pulp bleaching process reacts with organic wastes (wood extractives, polysaccharides and remaining amounts of lignin), to form several organochlorine compounds. Such compounds are toxic and include simple monoaromatic lignin derivatives (e.g., chlorinated phenol, quaiacols, catechols, and chlorolignins). The effect of chlorolignins in receiving water systems is not well understood. Therefore, chlorolignins are considered not to be acutely toxic, because they are of large molecular size and do not penetrate cytoplasmic membranes. However, it is possible that they are degraded to release smaller compounds that are toxic. There is a real danger to human health from the presence in drinking water of chlorinated organic compounds that come from pulp–paper mill effluents. Such compounds not only pollute drinking water but are bioaccumulated by and contaminate fish, which, if consumed, could be another source of human exposure (Neilson et al. 1983; Eriksson et al. 1985).

4 Pulp and Paper Mill Effluent

Pulp and paper mills are one of the five major contributors to environmental pollution by industrial wastewaters. Approximately 160 million t of wood pulp was produced worldwide in 2006 (Metso Corp. 2006); production was expected to increase to ~260 million t for the current year 2010. For every t of paper produced, the mills are estimated to generate ~220–380 m^3 of highly colored and potentially toxic wastewater (Eriksson and Kohler 1985; Chuphal et al. 2005). In the year 1951, there were only 17 paper mills in India, and they had a combined production capacity of ~0.13 million t paper per annum. The number of paper mills has increased to 406 in 2002, and their annual combined capacities now equal ~1.9 million t of paper (Singh and Thakur 2006). The world's population now uses a minimum of 300 million t of paper annually, and this is likely to increase in the coming years.

In the pulping process, the debarked wood chips are treated at 160–180°C in a "white liquor" solution of sodium sulfide (Na_2S) and sodium hydroxide (NaOH), which dissolves 90–95% of lignin, nearly all hemicellulose and wood extractives and a small amount of cellulose-derived polysaccharides (Garg et al. 2004). Approximately 55% of the original wood is dissolved in the form of "black liquor." The pulp is then mixed with water and is filtered. The resultant unbleached pulp is made into a slurry, which is then treated with chlorine at the charging rate of 60–70 kg/t, and at a pH of 1.5–2.0. After filtration, the bleached pulp is treated with alkali @ 35–40 kg/t, at 55–70°C, followed by series of bleaching treatments with hypochlorite, chlorine dioxide, alkali, and chlorine dioxide. After each treatment step, the pulp is filtered and the resultant liquids are pooled as bleachery effluent. During the bleaching process, ~50 kg of lignin, ~19 kg of polysaccharides, and ~1 kg of extractives are dissolved, and thereby released, from 1 t of the softwood pulp. Chlorine reacts with these organic wastes to produce structurally

diverse organochlorine compounds. Chlorinated lignin is formed during the bleaching of pulp with chlorine chemicals. Most of the remaining lignin in pulp is dissolved during the first two chlorine and alkali extraction steps. Reactions at these stages lead to the formation of chlorolignin (as mentioned above) that has a high content of carboxyl, hydroxyl, and conjugated carbonyl groups, and a low content of aromatic moieties (Lindstorm and Osterberg 1984; Osterberg and Lindstrom 1985). Such effluents impose coloration and toxicity problems in the receiving waters that can cause serious environmental hazards. This problem of toxicity and color, and its removal from pulp–paper mill effluents, has been a subject of much study during the last few decades. Although the pollution load derived from other factors can be satisfactorily reduced, color removal is more difficult and has been a matter of great concern.

Most of the chlorinated organics that are randomly synthesized during pulp bleaching are toxic xenobiotics and tend to persist. The reason is that many native microorganisms have not evolved the enzymatic machinery to rapidly degrade these chlorinated organics. If they did, or do develop such capacity, the conditions favoring degradation of lignin and its chlorinated derivatives would also be suitable to decolorize the lignin-based effluents.

Several color-removal strategies have been developed which rely on either physicochemical or biological techniques (Fig. 3). The former includes ultrafiltration, lime coagulation, rapid land filtration, adsorption on activated carbon and

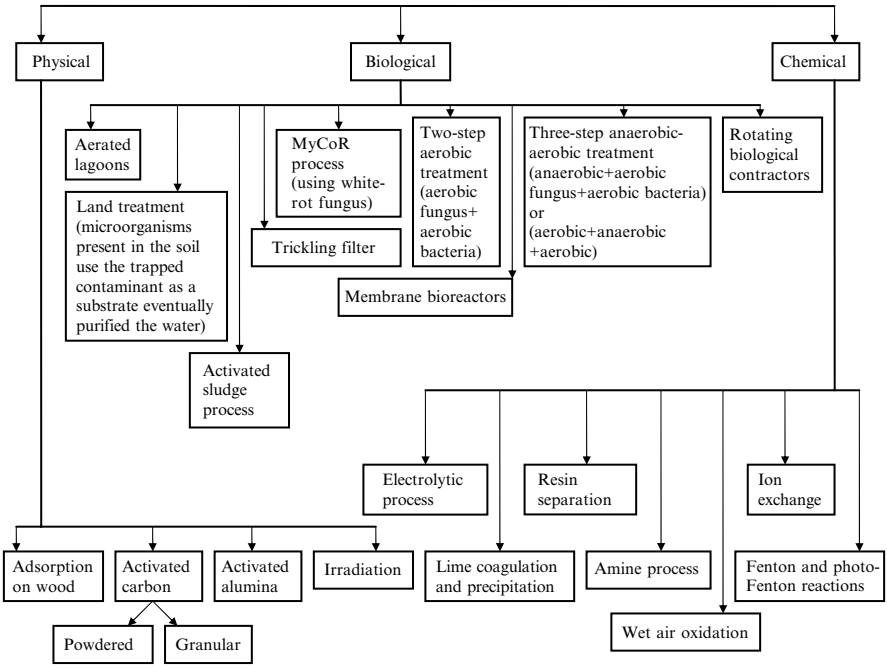

Fig. 3 Different strategies for pulp and paper mill effluent treatment

polymeric adsorbants (Springer 1985). However, such methods have operational difficulties and are prohibitively expensive for application on an industrial scale (Royer et al. 1991). Further, the conventional biological methods employed in paper industries, such as use of aerated lagoons, and activated sludge process are usually able to remove 85–90% of the readily biodegradable fraction that contributes to BOD. However, these biological systems are generally less efficient and scarcely effective in removing color, COD, and chlorinated phenolic compounds (Saunamaki 1989). This may be attributed to the low content of nutrients (nitrogen and phosphorus) in these wastewaters, and to the toxicity caused by the phenolic compounds present. It is, therefore, necessary to develop new, more advanced treatment technologies that degrade such chemicals.

The colored and potentially toxic chemicals are also known to possess mutagenic and carcinogenic characteristics, have a tendency to bioaccumulate, and are difficult to degrade. Therefore, the focus of future treatment processes should also effectively address the degradation of the mutagenic/carcinogenic compounds, resin acids and chlorinated phenols, guaiacols, catechols, and chlorinated aliphatic hydrocarbons.

Saravanan and Sreekrishnan (2005) suggested that the problem of sludge disposal, cost, and toxicity can be reduced if a biological treatment step is combined with appropriate physicochemical treatment. The authors reported that, when pulp and paper mill effluent was treated with *Trichoderma* sp. in batch studies, 72% color reduction was achieved within 24 h. When the effluent was treated in continuous mode by this fungus in a fluidized bed reactor, ~27% color reduction was achieved. Color reduction further increased to 81%, when the biological and physicochemical treatments (using poly-electrolyte (potash alum)) were combined.

5 Microbial Decolorization of Effluent

Attempts have been made to decolorize or degrade toxicants in pulp and paper mill effluents with variety of microorganisms, including bacteria, fungi and algae (Table 1). Wood degrading white-rot fungi have been found to be effective in treating effluent. Their effectiveness derives from their ability to degrade lignin and its chlorinated compounds, which are mainly responsible for the color and toxicity of pulp and paper mill effluent (Prouty 1990; Bergbauer et al. 1991; Sayadi and Ellouz 1992; Bajpai et al. 1993; Martin and Manzanares 1994; Prasongsuk et al. 2009; Saritha et al. 2010). Garg and Modi (1999) and Tripathi et al. (2007) have reviewed various aspects of papermill wastewaters, including decolorization by white-rot and other fungi. Fukuzumi and coworkers (1977) were first to use white-rot fungi for wastewater treatment. They inoculated effluent with a variety of fungi, and discovered that *Tinctoporia* sp. was the best fungus for wastewater treatment. Eaton et al. (1982) performed similar experiments using *Phlebia brevispora*, *P. subserialis*, *Poria cinerascens,* and *Tremetes versicolor*. All of these were capable of efficiently

Table 1 Microorganisms involved in pulp and paper mill effluent decolorization and detoxification

Microorganism	System	% Reduction					Day(s)[a]	References
		COD	BOD	color	Phenols	Lignin		
Trametes versicolor	Shake flask	–	–	60	–	–	4	Modi et al. (1998)
Trametes versicolor	Shake flask	–	–	34	–	–	3	Garg et al. (1999)
Pseudomonas fluorescens	Fixed-film bioreactor	79	–	75	66	45	15	Chauhan and Thakur (2002)
Paecilomyces sp.	Sequential bioreactor	86	–	86	53	82	7	Singh and Thakur (2004)
Paecilomyces sp. + *Microbrevis luteum*		86	–	78	89	85		
Paecilomyces sp. + *Pseudomonas syringae* pv *myricae*	Three-step sequential bioreactor	83.9	–	87.7	87.2	76.5	7	Chuphal et al. (2005)
Ceriporiopsis subvermispora CZ-3	Shake flask	89.4	–	78.6	–	79.0		Sahoo and Gupta (2005)
Trichoderma sp.	Fluidized bed bioeactor	–	–	72–73	–	–	1	Saravanan and Sreekrishnan (2005)
Paenibacillus sp.	Shake flask	52	65	39	64–77	28	6	Raj et al. (2007)
Aneurinibacillus aneurinilyticus		~65	~70	45		38		
Bacillus sp.		78	82	61		53		
Penicillium camemberti	Shake flask	–	–	84	–	–	10	Taseli (2007)
Bacillus cereus (ITRC-S6)	Shake flask	60	40	45	32	30	7	Chandra et al. (2009)
Serratia marcescens (ITRC-S7)		50	70	52	40	42		
Bacillus cereus (ITRC-S6) + *Serratia marcescens* (ITRC-S7)		90	70	62	90	54		
Phanerochaete chrysosporium	Fluidized bed bioreactor	78.8	70.5	86.4	–	–	3	Jaganathan et al. (2009)
Bacillus pumilus	Shake flask	22.08	–	41.87	–	–	2	Oliveira et al. (2009)
Paenibacillus sp.		22.89	–	42.30	–	–		
Cryptococcus sp.	Shake flask	–	–	50–53	–	35–40	1	Singhal and Thakur (2009)
Phanerochaete chrysosporium	Shake flask	73.5	65.3	67.7	–	60	2	Saritha et al. (2010)
Trametes hirsute		84	–	55.8	–	78.4	1	

COD chemical oxygen demand, *BOD* biological oxygen demand

[a] Treatment or incubation time in days

decolorizing the effluent. Livernoche et al. (1983) isolated and screened 15 strains of white-rot fungi and reported that several of them, viz., *T. versicolor*, *Phanerochaete chrysosporium*, *Pleurotus ostreatus, Polyporus versicolor,* and one unidentified strain were capable of decolorizing pulp–paper mill effluent.

White-rot fungi, such as *P. chrysosporium* (Pellinen et al. 1988; Mittar et al. 1992; Celal and Filiz 1994; Sukumar et al. 2006), *Trametes versicolor* (Manzanares et al. 1995; Pallerla and Chambers 1995; Modi et al. 1998; Garg et al. 1999; Srinivasan and Murthy 1999), *P. radiata* (Lankinen et al. 1991; Hatakka et al. 1992), *Marulius tremellosus* (Lankinen et al. 1991), were found to have the ability to decolorize and detoxify effluent from the bleaching stage of pulp and paper mills. Moreover, the white-rot fungus *P. chrysosporium* was shown to degrade a wide variety of xenobiotic compounds, in addition to its natural substrate, lignin. Recalcitrant xenobiotics that *P. chrysosporium* can degrade include aromatic compounds, polycyclic aromatics, chlorinated aromatics, polycyclic chlorinated aromatics and nonaromatic chlorinated compounds, as well as some naturally occurring biopolymers (Bumpus and Aust 1987; Mileski et al. 1988; Bumpus 1989; Aust 1990; Fernando et al. 1990; Yadav and Reddy 1993). The ability of white-rot fungi to detoxify and decolorize bleach plant effluents is believed to derive from their production of ligninolytic enzymes. These exoenzymes exhibit substrate specificity for solubilized lignin and chlorinated lignin derivatives in the effluent (Nagarathnamma and Bajpai 1999). The greater emphasis for use in biological treatment processes has been laid on the use of *P. chrysosporium* and *T. versicolor* (Chang et al. 1987; Archibald et al. 1990, Eriksson 1990; Terron et al. 1991, 1992).

Sahoo and Gupta (2005) isolated several ligninolytic microorganisms from the environment of small pulp and paper mills and evaluated their ability to decolorize effluents of an agriresidue-based effluent that were dark brown in color. The promising *Aspergillus fumigatus* and *A. flavus* isolates were more efficient in color removal than was the lignin-degrading *T. versicolor*. Taseli (2007) studied the capability of *Penicillium camemberti* to dechlorinate and decolorize wheat straw-based pulping and bleaching effluents. They reported that the rates of highest color removal for AOX were 65% and, under no-shaking conditions 84%; these values compare to 60% or 79% color removal for AOX in shake-flask experiments. The fungus was effective in treating softwood pulping and bleaching effluents (Taseli and Gokcay 2006; Taseli 2007) and chlorinated model compounds like pentachlorophenol and 2-chlorophenol (Taseli and Gokcay 2005). Similarly, Apiwattanapiwat et al. (2005) screened 64 fungal strains for their ability to decolorize pulp and paper mill effluent. Only three strains, identified as *Trichoderma* sp., *Datronia* sp., and *Tremetes* sp., decreased the effluent color (by 54.4, 54.9, and 53.7%, respectively).

Malaviya and Rathore (2007) used a novel consortium of white-rot and soft-rot fungi for bioremediation of pollutants from pulp–paper mill effluent, and reported reduction in color, lignin, and COD by 78.6, 79.0, and 89.4%, respectively, within 4 days. Jaganathan et al. (2009) used ligninolytic fungus *P. chrysosporium* for aerobic pollution abatement of pulp–paper mill effluent. The average removal of color,

COD, and BOD was 86.4, 78.8, and 70.5%, respectively, after 3 days in the shake-flask batch experiments. The entire fungal mycelial mass was fragmented from the shaking action. The fragmentation of mycelial mass, which increased the contact area, was probably responsible for better performance in batch experiments. Prasongsuk et al. (2009) isolated three thermotolerant ligninolytic enzyme-producing fungi that were capable of paper mill effluent decolorization. *Daedaleopsis* sp., a new fungal isolate, exhibited the highest effluent decolorization efficiencies of 52 and 86%, respectively, in wastewaters from pulping, and from combined pulping and paper recycling processes. The thermotolerant fungi are particularly suitable for decolorizing wastewater in tropical environments.

The main constraint in using a fungal degrading system is the requirement to maintain growth and/or enzyme (ligninases) activity at the prevailing low pH (4–5). However, at low pH the solubility of high molecular weight fragments that are derived from lignin is reduced. Furthermore, the natural pH of pulp and paper mill effluents generally remains alkaline (in a range of pH 8–9). Therefore, any requirement to reduce the pH to the acidic range of 4–5 prior to fungal augmentation would be uneconomical (Raj et al. 2007). Further, Garg et al. (1999) have reported that a pH adjustment of paper mill effluent to lower values, and/or sterilization of effluent, are unnecessary, because such treatments resulted in sedimentation of the chromophoric compounds, rendering the effluent light colored. Readjustment of effluent pH to normal (pH 8–9) redissolved the sediments and restored the effluent color. Livernoche et al. (1983) also reported similar findings, in which the color of effluent was pH sensitive, and the pH effect on the color of effluent was reversible. In view of these observations, Garg et al. (1999) suggested that effluent decolorization studies should be performed that employ unsterilized and unfiltered effluent without pH adjustment. Considering the foregoing, bacterial treatment systems that have an optimum pH range of 7–9 may play a pivotal role in decolorizing pulp and paper mill effluents, without any requirement for pH adjustment.

Lignin is decomposed by bacteria in the natural environment. Because of their immense environmental adaptability and biochemical versatility, both anaerobic and aerobic bacteria have been studied for their ligninolytic potential (Chandra et al. 2007). Several bacterial species capable of metabolizing various industrial pollutants have been isolated from the natural environment, viz., *Bacillus* sp., *B. subtilis* (IS13) (Niazi et al. 2001; Andretta et al. 2004), *Pseudomonas* sp., *P. veronii*, *P. fluorescens*, *P. aeruginosa* (Premlatha and Rajkumar 1994; Thakur et al. 2002; Nam et al. 2003; Shah and Thakur 2003), *Flavobacterium* (Saber and Crawford 1985), *Sphingomonas chlorophenolicum* ATCC 39723 (Xun et al. 1999), *Desulfomonile tiejei* DCB-1 (Mohn and Kennedy 1992), *Arthrobacter chlorophenolicus* A6 (Agneta et al. 2004). Many researchers have evaluated *Pseudomonas* sp., *P. putida*, *Flavobacterium* sp., *Xanthomonas* sp., *Nocardia* sp., *Aeromonas* sp., *Arthrobacter* sp., *Ancylobacter* sp., *Methylobacterium* sp., *Acinetobacter calcoaceticus*, etc., for effluent color and toxicity reduction (Vora et al. 1988; Fulthrope and Allen 1995; Jain et al. 1996). Blair and Davis (1980) treated Kraft mill effluent with *P. aeruginosa* under aerobic conditions, and reported a color reduction of 26–54%. A mixed population of bacteria and yeast, including *P. putida*, *Nocardia coralina,*

and *Torula* sp., were employed to degrade lignin (Bajpai and Bajpai 1997). Chauhan and Thakur (2002) treated pulp–paper mill effluent in a fixed-film bioreactor by *P. fluorescens*, and noted reductions of color (75%), phenol (66%), COD (79%), and lignin (45%) within 15 days. Raj et al. (2007) examined three lignin-degrading bacterial isolates, viz., *Paenibacillus* sp., *Aneurinibacillus aneurinilyticus,* and *Bacillus* sp. for the treatment of effluent, and observed reduction in color (39–61%), lignin (28–53%), BOD (65–82%), COD (52–78%), and total phenols (64–77%) within 6 days, with highest reduction achieved by *Bacillus* sp.

In this same experiment, the maximum reduction in total phenol (77%) was recorded with *Paenibacillus* sp. (Raj et al. 2007). The authors asserted that a significant reduction in color and lignin by the three bacterial strains was noted at the second day of incubation. This indicated that bacterial strains initially utilized growth supportive substrates that consequently cometabolized chromophoric compounds, ultimately reducing lignin and the color of the effluent. Chandra et al. (2009) employed two pentachlorophenol-degrading bacterial strains *B. cereus* ITRC-S6 and *Serratia marcescens* ITRC S-7 for pulp–paper mill effluent treatment. The effective reduction in color (45–52%), lignin (30–42%), BOD (40–70%), COD (50–60%), total phenol (32–40%), and PCP (85–90%) was noted. However, maximum reduction in various study parameters, viz., color (62%), lignin (54%), BOD (70%), COD (90%), total phenol (90%), and PCP (100%) was recorded by a mixed culture of the above two bacterial strains (Table 1). The reduction of COD and total phenol may have resulted from the degradation of lignin and chlorinated organic compounds (Singh and Thakur 2006; Latorre et al. 2007). Further, the bacterial degradative capability of lignin and PCP was apparent, because the aromatic compounds (e.g., 2-chlorophenol, 2,4,6-trichlorophenol, tetrahydroquinone, 6-chlorohydroxyquinol, and tetrachlorohydroxyquinone) that were absent in untreated effluent were detected in the treated effluent.

Singhal and Thakur (2009) studied the decolorization and detoxification of pulp–paper mill effluent by *Cryptococcus* sp. This bacterial isolate, designated as PF7, reduced the color (27%) and lignin content (24%) of the effluent on the fifth day under unoptimized conditions. However, enhanced reduction in color (50–53%) and lignin (35–40%) were noted to occur after optimum treatment conditions were reached during the 24 h incubation: temperature (35–40°C), shaking (125 rpm), dextrose (1.0% w/v), tryptone (0.1% w/v), inoculum size (7.5% v/v), and pH (5.0).

The biological treatment strategy was slightly changed by Singh and Thakur (2004) to improve the efficiency of effluent decolorization and detoxification by microorganisms in a two-step aerobic bioreactor. In the first step, the effluent was treated by eight individual fungal isolates, among which *Paecilomyces* sp. exhibited reduction in phenol (40%), lignin (66%), COD (81%) (performed in a 100 L sequential bioreactor at 6 h retention time). When this fungus-treated effluent was subsequently treated by the bacterial isolate *Microbrevis luteum*, the reduction in phenol (77%), color (84%), COD (83%), and lignin (72%) further increased. The authors concluded that, although *Paecilomyces* sp. was more efficient than was *M. luteum* in removing color, lignin, and COD, the bacterial strain *M. luteum* was capable of removing significant amounts of chlorinated phenols and their metabolites.

In another study, Chuphal et al. (2005) applied fungal (*Paecilomyces* sp.) and bacterial (*P. syringae* pv *myricae*) isolates to treat pulp–paper mill effluent in a two- and three-step fixed-film sequential bioreactor. The two-step aerobic treatment (aerobic fungus + aerobic bacteria) was slightly better in decreasing color (88.5%), lignin (79.5%), COD (87.2%), and phenol (87.5%), than was the three-step anaerobic–aerobic treatment (anaerobic + aerobic fungus + aerobic bacteria), in which color was reduced by 87.7%, lignin by 76.5%, COD by 83.9%, and phenol by 87.2%. The advantage of anaerobic treatment, in the three-step process, is that biogas is produced that is utilized for energy generation. Singh and Thakur (2006) performed sequential anaerobic and aerobic treatment in a two-step bioreactor to remove color in pulp and paper mill effluent. All pollution parameters such as color (70%), lignin (25%), COD (42%), AOX (15%), and phenol (39%) decreased slowly over 15 days, without appreciable increase in biomass. To further reduce pollutants, the 7 days anaerobically treated effluent was separately applied to a bioreactor, in the presence of the fungus *Paecilomyces* sp. and the bacterium *M. luteus*, in step two and three. The reduction in color (76%), lignin (69%), COD (75%), AOX (82 %), and phenol (93%) by day 3 was achieved. Thus, degradation of the pollutants and associated parameters was relatively faster under aerobic than under anaerobic treatment conditions. This was attributed to the unique ability of aerobic microbes to secrete enzymes that efficiently degraded chromophoric compounds and toxic chlorinated phenols from the effluent (Livernoche et al. 1983; Pokhrel and Viraraghavan 2004).

6 Anaerobic vs. Aerobic Biological Treatment Strategies

Many highly chlorinated compounds are known to be quite stable and difficult to degrade. However, anaerobes can sometimes catalyze biotransformation reactions in which chloride ions (Cl^-) of the chlorinated compounds are displaced by protons (H^+). The more chloride ions that are thus removed, the more reactive the resultant compounds become, thereby rendering them susceptible to conventional activated sewage sludge treatment (Mikesell and Boyd 1986; Haggblom 1990).

Anaerobic treatment is cost effective and has become the most commonly used method for treating medium and high strength effluents. Different anaerobic technologies have been applied for treating less concentrated effluents, such as domestic wastewater and some industrial effluents. Such anaerobic technologies provide good treatment efficiencies at low hydraulic retention times (Hickey et al. 1995). Accordingly, the establishment of sequential anaerobic–aerobic two-step wastewater treatment facilities at Kraft pulp–paper mills would probably help to reduce color and toxic contaminants. Haggblom and Salonen (1992) studied the biodegradability of chlorinated organics and conventional pollutants in Kraft bleaching effluents, using a two-stage anaerobic fluidized bed/aerobic trickling filter treatment system. The anaerobic stage removed >65% of AOX, and chlorinated phenolics removed >75%. COD and BOD reduction was greatest in the aerobic process,

whereas dechlorination was significant in the anaerobic process. The concept of sequential treatment is very important, because both anaerobic and aerobic fungi and bacteria can be used to treat effluent at different stages in the bioreactor. However, Thakur (2004) reported that bacteria are more potent for degrading aromatic compounds. Furthermore, two or more types of microbes may be attempted sequentially, in which one organism may transform the original organic pollutant by initial catabolic reactions to products that are then mineralized by (an) other organism(s). Pokhrel and Viraraghavan (2004) and Thakur (2004) have developed such consortia for mineralizing bicyclic aromatics, viz., chlorinated biphenyls, chlorinated dibenzofurans, and naphthalene sulphonates. Singh and Thakur (2006) indicated that sequential anaerobic and aerobic treatment was more efficient in removing color and chlorinated compounds, because anaerobic microorganisms degraded highly chlorinated organics more efficiently than did aerobic microbes, although the latter microbes removed the last remaining chlorine atom. The combined treatment typically removed 82% of AOX, COD, and chlorinated phenolics and completely eliminated chlorate.

Mutagenic and chlorinated aliphatic compounds in effluent, many of which are known mammalian carcinogens, are also effectively degraded in sequential anaerobic–aerobic treatment processes. However, resin acids, which are excessively present in certain wastewaters, such as chemothermo-mechanical pulping (CTMP), are highly toxic to anaerobic bacteria, ultimately causing them to fail (Sierra-Alvarez and Lettinga 1990). But, resin acids can be degraded by aerobic microorganisms. Therefore, a three-step process of aerobic–anaerobic–aerobic degradation of effluent pollutants has been suggested as a means to successfully degrade both resin acids and organochlorine compounds (Welander 1988).

7 Other Strategies for Reducing Effluent Color and Toxicity

The major constraints of usual chemical pulping and the bleaching process are (1) excessive use of chemicals and electrical energy and (2) generation and release of enormous colored and toxic wastewater, thus contributing to environmental pollution. These shortcomings can be largely overcome by using a pulping process, in which cooking time is reduced. The biopulping process removes lignin, along with some wood extractives, thereby reducing effluent toxicity (Ali and Sreekrishnan 2001). Moreover, biopulping and biobleaching hold enormous potential in the pulp–paper industry for rendering it more energy efficient, more economical, and more environmentally friendly. The exposure of wood chips to steam treatment prior to biopulping also eliminates another risk, to wit, that it destroys certain indigenous sporulating fungi that are capable of causing respiratory problems in mill workers (Agbiotech Bulletin 2003). Biopulping is often followed by biobleaching. Biobleaching employs white-rot fungi and/or ligninolytic enzymes and is effective in removing unwanted constituents in the pulp, including residual lignin. One new

strategy that could add to pollutant removal efficiency in the industry is to use a new pulping/bleaching process, in which delignification by oxygen, hydrogen peroxide, and ozone is undertaken.

7.1 Biopulping and Biobleaching

The fungal and/or enzymatic treatment of woodchips and pulps, respectively referred to as biopulping and biobleaching, offer promising alternatives to alkali and chemical treatments (Keller et al. 2003; Camarero et al. 2007). Such new environment-friendly technologies with elemental chlorine-free (ECF) and totally chlorine-free (TCF) bleaching strategies are necessary to achieve the following: (1) minimize hemicellulose in the pulp, (2) achieve a high level of paper brightness, and (3) improve effluent quality in terms of reduced toxicity and AOX. In biobleaching, pulp can be bleached with white-rot fungi and their ligninolytic enzymes, to reduce chemical use, and to establish a chlorine-free bleaching process (Shukla et al. 2004).

Biopulping with the use of various species of white-rot fungi have been reported, and *Ceriporiopsis subvermispora* has proved suitable both for soft and hard woods (Milagres et al. 2005; Ferraz et al. 2007). Yaghoubi et al. (2008) employed *C. subvermispora* for biochemical pulping of agriresidues, and the results were comparable to those achieved with the chemical pulping process. Moreover, the paper quality produced by biochemical pulping of straws was excellent. Singh and Chen (2008) reported that pretreatment with *P. chrysosporium* could reduce fiberizing and refining energy costs by 30% (Singh et al. 2010). Arias et al. (2009) studied the suitability of *Streptomyces* strains for biochemical pulping of spruce wood (*Picea abies*). The engineering and economic analyses indicate that the biopulping process is technologically feasible and economically beneficial. However, the major constraints of the process are that the biological pretreatment step has a slow reaction rate, and the process control is complex (Chen et al. 2010).

7.2 In Plant Processing

Another strategy is to develop new pulping processes that emphasize improved delignification and complete or partial replacement of chlorine in bleaching processes. The use of elemental chlorine for bleaching causes environmental problems by releasing toxic and recalcitrant chlorinated aromatic compounds. In recent years, TCF pulp production is gaining momentum. Alternative chemicals, e.g., oxygen, hydrogen peroxide, and ozone for bleaching, have been successfully used. The new process, called oxygen delignification, has proved quite effective in removing lignin and hemicellulose fractions from cellulose, without damaging the fibers. This process releases less lignin than does traditional chlorine bleaching. Moreover, partial replacement of elemental chlorine with chlorine dioxide would greatly reduce the

amount of organochlorine compounds in the effluent, while complete substitution with H_2O_2, would totally eliminate these toxic compounds. H_2O_2 also assists in oxidizing other organic contaminants in the effluent. Unfortunately, these alternative compounds are not free from shortcomings, and have the disadvantage of producing lower quality paper, during the bleaching process.

8 Summary

The potential hazards associated with industrial effluents, coupled with increasing awareness of environmental problems, have prompted many countries to limit the indiscriminate discharge of untreated wastewaters. The pulp and paper industry has been among the most significant of industrial polluters of the waterways, and therefore has been one of the industries of concern.

The pulp and paper industry produces large quantities of brown/black effluent that primarily result from the pulping, bleaching, and paper-making production stages. The dark color and toxicity of pulp–paper mill effluent comes primarily from lignin and its chlorinated derivatives (e.g., lignosulphonic acid, resins, phenols, and hydrocarbons) that are released during various processing steps of lignocellulosic materials. The color originates from pulping and pulp bleaching stages, while adsorbable organic halides (AOX) originate exclusively from chlorine bleaching. Discharge of untreated effluent results in increased BOD/COD, slime growth, thermal problems, scum formation, discoloration, loss of aesthetic quality and toxicity to the aquatic life, in the receiving waterbodies.

The dark brown color of pulp–paper effluent is not only responsible for aesthetic unacceptability, but also prevents the passage of sunlight through colored waterbodies. This reduces the photosynthetic activity of aquatic flora, ultimately causing depletion of dissolved oxygen. The pulp–paper organic waste, coupled with the presence of chlorine, results in the generation of highly chlorinated organic compounds. These toxic constituents of wastewater pose a human health risk through long-term exposure via drinking water and/or through consumption of fish that can bioaccumulate certain pollutants from the food chain. Therefore, considerable attention has been focused by many countries on decolorization of paper mill effluents, along with reduction in the contaminants that pose human health or other environmental hazards.

Various physicochemical remediation treatments in the pulp–paper industry are now used, or have been suggested, but often are not implemented, because of the high costs involved. More recently, the paper and pulp industry has been investigating the use of biological remediation steps to replace or augment current treatment strategies. Certain biological treatments offer opportunities to reduce cost (both capital and operating), reduce energy consumption, and minimize environmental impact. Two primary approaches may be effective to curtail release of toxic effluents: first, development of pulping and bleaching processes that emphasize improved oxygen delignification or biopulping, plus partial or complete replacement of chlorine treatment with hydrogen peroxide or with biobleaching; second, implementation of biological processing that involves sequential two-step anaerobic–aerobic or

three-step aerobic–anaerobic–aerobic treatment technologies at end of pipe. The selection of the specific process will depend upon the type of pollutants/toxicants/mutagens present in the effluent.

The use of environmental-friendly technologies in the pulp and paper industry is becoming more popular, partly because of increasing regulation, and partly because of the availability of new techniques that can be used to economically deal with pollutants in the effluents. Moreover, biotechnology research methods are offering promise for even greater improvements in the future. The obvious ultimate goal of the industry and the regulators should be zero emission through recycling of industrial wastewater, or discharge of the bare minimum amount of toxicants or color.

References

AgBiotech Bulletin (2003) Biopulping: technology learned from nature that gives back to nature. 11(1) Ag-West Biotech Inc.

Agneta B, Maraha N, Jansson JK (2004) Impact of temperature on the physiological status of potential bioremediation inoculants, *Arthrobacter chlorophenolicus* A6. Appl Environ Microbiol 5: 2952–2958.

Ali M, Sreekrishnan TR (2001) Aquatic toxicity from pulp and paper mill effluents: a review. Adv Environ Res 5(2): 175–196.

Amat AM, Arques A, Miranda MA, Lopez F (2005) Use of ozone and/or UV in the treatment of effluents from board paper industry. Chemosphere 60(8): 1111–1117.

Andretta CWS, Rosa RM, Tondo EC, Gaylarde CC, Henriques JAP (2004) Identification and molecular characterization of a *Bacillus subtilis* IS13 strain involved in the biodegradation of 4,5,6-trichloroguaiacol. Chemosphere 55: 631–639.

Apiwattanapiwat W, Siriacha P, Vaithanomsat P (2005) Screening of fungi for decolorization of wastewater from pulp and paper industry. Kasetsart J: Natural Sci 40: 215–221.

Archibald F, Paice MG, Jurasek L (1990) Decolourization of kraft bleachery effluent chromophores by *Coriolus (Trametes) versicolor*. Enzyme Microb Technol 12: 846–853.

Arias ME, Rodriguez J, Perez MI, Hernandez M, Polvillo O, Gonzalez-Vila Francisco J (2009) Analysis of chemical changes in *Picea abies* wood decayed by different *Streptomyces* strains showing evidence for biopulping procedures. Wood Sci Technol 44(2): 179–188.

Aust S (1990) Degradation of environmental pollutants by *Phanerochaete chrysosporium.* Microb Ecol 20: 197–209.

Bajpai P, Bajpai PK (1997) Reduction of organochlorine compounds in bleach plant effluent. Adv Biochem Engg Biotechnol 57: 213–259.

Bajpai P, Bajpai PK, Kondo R (2000) Biotechnology for environmental protection in the pulp and paper industry. Springer-Verlag Berlin Heidelberg, New York, pp 65–85.

Bajpai P, Mehna A, Bajpai PK (1993) Decolorization of kraft bleach plant effluent with the white-rot fungus *Trametes versicolor*. Process Biochem 28: 377–384.

Barton DA, Lee JW, Buckley DB, Jett SW (1996) Biotreatment of kraft mill condensates for reuse. Proc. Tappi Minimum Effluent Mills Symp, Atlanta, GA., USA, pp 270–288.

Bergbauer M, Eggert C (1992) Differences in the persistence of various bleachery effluent lignins against attack by white-rot fungi. Biotechnol Lett 14: 869–874.

Bergbauer M, Eggert C, Kraepelin G (1991) Degradation of chlorinated lignin compounds in a bleach plant effluent by the white rot fungus *Trametes versicolor.* Appl Microbiol Biotechnol 35: 105–107.

Bharati SG, Salanki AS, Taranath TC, Acharyulu MVRN (1992) Role of cyanobacteria in the removal of lignin from the paper mill waste waters. Bull Environ Contam Toxicol 49: 738–742.

Bhoominathan K, Reddy CA (1992) Fungal degradation of lignin: biotechnological applications. In: Arora DK, Elander RP, Mukherjee KG (eds) Handbook of Applied Mycology, Fungal Biotechnology. Marcel Dekker, New York, pp 763–822.

Blair JE, Davis LT (1980) Process for decolorizing pulp and paper mill wastewater. U.S. Patent 4199444.

Boman B, Ek M, Eriksson KE, Frostell B (1988) Some aspects on biological treatment of bleached plant effluents. Nordic Pulp Paper Res 51: 1089–1098.

Bumpus J (1989) Biodegradation of polycyclic aromatic hydrocarbons by *Phanerochaete chrysosporium*. Appl Environ Microbiol 55: 154–158.

Bumpus J, Aust S (1987) Biodegradation of environmental pollutants by the white rot fungus *Phanerochaete chrysosporium*: involvement of the lignin degrading system. Bioessays 6: 166–170.

Camarero S, Ibarra D, Martinez AT, Romero J, Gutierrez A, Rio JCD (2007) Paper pulp delignification using laccase and natural mediators. Enz Microb Technol 40: 1264–1271.

Celal FG, Filiz BD (1994) Treatment of effluents from hemp-based pulp and paper industry. II Biological treatability of pulping effluents. Water Sci Tech 29: 165–168.

Chandra R, Raj A, Purohit HJ, Kapley A (2007) Characterization and optimization of three potential aerobic bacterial strains for kraft lignin degradation from pulp-paper waste. Chemosphere 67: 839–846.

Chandra R, Raj A, Yadav S, Patel DK (2009) Reduction of pollutants in pulp paper mill effluent treated by PCP-degrading bacterial strains. Environ Monit Assess 155: 1–11.

Chang CN, Ma YS, Fang GC, Chao AC, Tsai MC, Sung HF (2004) Decolourization of lignin wastewater using the photochemical UV/TiO_2 process. Chemosphere 56: 1011–1017.

Chang H-M, Joyce TW, Vasudevan B, Kirk TK (1987) Process of treating effluent from a pulp or paper-making operation. U.S. Patent No. 4655926.

Chauhan N, Thakur IS (2002) Treatment of pulp and paper mill effluent by *Pseudomonas fluorescens* in fixed film bioreactor. Poll Res 21: 429–434.

Chen S, Zhang X, Singh D, Yu H, Yang X (2010) Biological pretreatment of lignocellulosics: potential, progress and challenges. Biofuels 1: 177–199.

Christian V, Shrivastava R, Shukla D, Modi HA, Vyas BRM (2005) Degradation of xenobiotic compounds by lignin-degrading white-rot fungi: enzymology and mechanisms involved. Indian J Exp Biol 43: 301–312.

Chuphal Y, Kumar V, Thakur IS (2005) Biodegradation and decolorization of pulp and paper mill effluent by anaerobic and aerobic microorganisms in a sequential bioreactor. World J Microbiol Biotechnol 21: 1439–1445.

Driessel BR, Christov L (2001) Decolorization of bleach plant effluent by mucoralean and white-rot fungi in a rotating biological contactor reactor. J Biosci Bioeng 92: 271–276.

Eaton DC, Chang H-M, Joyce TW, Jeffries TW, Kirk TK (1981) The FPL/NCSU MyCoR process for treatment of bleach plant effluents. Proc. TAPPI Annual Meeting, TAPPI Press, Atlanta, Chicago, pp 157–161.

Eaton DC, Chang H-M, Joyce TW, Jeffries TW, Kirk TK (1982) Methods obtains fungal reduction of the colour of extraction stage kraft bleach effluents. Tappi J 65: 89–92.

Eriksson KE, Grunewald A, Vallander L (1980) Studies of growth conditions for three white-rot fungi and their cellulaseless mutants. Biotechnol Bioeng 22: 363–376.

Eriksson KE, Kohler KC (1985) Microbial degradation of chlorolignins. Environ Sci Technol 19: 1086–1089.

Eriksson KE, Kolar MC, Ljungquist PO, Kringstad KP (1985) Studies on microbial and chemical conversions of chlorolignins. Environ Sci Technol 19: 1219–1224.

Eriksson KEL (1990) Biotechnology in the pulp and paper industry. Wood Sci Technol 29: 79–101.

Esposito E, Canhos V, Durn N (1991) Screening of lignin-degrading fungi for removal of color from kraft mill wastewater with no additional extra carbon-source. Biotechnol Lett 33 (8): 571–576.

Fernando T, Bumpus J, Aust S (1990) Biodegradation of TNT (2,4,6-trinitrotoluene) by *Phanerochaete chrysosporium.* Appl Environ Microbiol 56: 1666–1671.

Ferraz A, Guerra A, Mendonca R, Vicentim MP, Aguiar A, Masarin F, Seabra GG, Pavan PC (2007) Mill evaluation of wood chips biotreated on a 50 ton biopulping pilot plant and advances on understanding biopulping mechanisms. 10th International Congress on Biotechnology in the Pulp and Paper Industry, June 10-15, 2007, United States Book of Abstracts, Madison, WI, pp 23–24.

Fukuzumi K, Nishida A, Aoshima K, Minami K (1977) Mokuzai Garraishi 23 (6): 290.

Fulthrope RR, Allen DG (1995) A comparison of organochlorine removal from bleached kraft pulp and paper mill effluents by dehalogenating *Pseudomonas, Ancylobacter* and *Methylobacterium* strains. Appl Microbiol Biotechnol 42: 782–789.

Ganjidoust H, Tatsumi K, Yamagishi T, Gholian RN (1997) Effect of synthetic and natural coagulant on lignin removal from pulp and paper wastewater. Water Sci Technol 35: 291–296.

Garg A, Narayana VVVSS, Chaudhary P, Chand S (2004) Treatment of pulp and paper mill effluent. J Sci Ind Res 63: 667–671.

Garg SK, Chandra H, Modi DR (1999) Effect of glucose and urea supplements on decolourization of bagasse and gunny bag-based pulp-paper mill effluent by *Trametes versicolor.* Indian J Exp Biol 37: 302–304.

Garg SK, Modi DR (1999) Decolorization of pulp-paper mill effluents by white-rot fungi. Crit Rev Biotechnol 19(2): 85–112.

Gellerstedt G (2001) Pulping Chemistry. In: Hon DN-S, Shiraishi N (eds) Wood and Cellulosic Chemistry, 2nd Ed. Marcel Dekker, New York, pp 859–905.

Haggblom M (1990) Mechanisms of bacterial degradation and transformation of chlorinated monoaromatic compounds. J Basic Microbiol 30: 115–141.

Haggblom MM, Salonen MS (1992) Biodegradability of chlorinated organic compounds in pulp bleaching effluents. Water Sci Technol 24: 161–170.

Harila P, Kivilinna VA (1999) Biosludge incineration in a recovery boiler. Water Sci Technol 40: 195–200.

Hatakka A, Valo M, Lankinen P (1992) Relationships between Lignin-modifying enzymes and the removal of AOX and color by white-rot grown on pulp-mill effluents. Proc. 5th International Conference on Biotechnology in Pulp and Paper Industry, Kyoto Japan, pp 69–74.

Hickey RF, Jones WW, Veiga MC (1995) The start-up operation, monitoring and control of high-rate anaerobic treatment system. Water Sci Technol 24: 207–255.

Jaganathan B, Hossain SKM, Begum KMMS, Anantharaman N (2009) Aerobic pollution abatement of pulp mill effluent with the white rot fungus *Phanerochaete chrysosporium* in three-phase fluidized bed bioreactor. Chem Eng Res Bull 13: 13–16.

Jain N, Shrivastava AK, Srivastava SK (1996) Treatment of black liquor by *Pseudomonas putida* and *Acinetobacter calcoaceticus* in continuous reactor. Environ Technol 17: 903–907.

Kazmi AA, Thul R (2007) Colour and COD removal from pulp and paper mill effluent by q Fenton's oxidation. J Environ Sci Engg 49: 189–194.

Keller FA, Hamilton JE, Nguyen QA (2003) Microbial pretreatment of biomass potential for reducing severity of thermo-chemical biomass pretreatment. Appl Biochem Biotechnol 105: 27–41.

Keyser P, Kirk TK, Zeikus JG (1978) Ligninolytic enzyme system of *Phanerochaete chrysosporium*: synthesized in the absence of lignin in response to nitrogen starvation. J Bacteriol 135: 790–797.

Kirk TK, Connors WJ, Zeikus JG (1976) Requirement for a growth substrate during lignin decomposition by wood-rotting fungi. Appl Environ Microbiol 32: 192–194.

Kringstad KP, Lindstorm K (1984) Spent liquors from pulp bleaching. Environ Sci Technol 18: 236–248.

Lankinen VP, Pellinen I, Hatakka J (1991) The onset of lignin-modifying enzymes, decrease of AOX and color removal by white-rot fungi grown on bleach plant effluents. Water Sci Technol 24: 189–198.

Latorre A, Malmqvist A, Lacorte S, Welander T, Barcelo D (2007) Evaluation of treatment efficiencies of paper mill whitewater in terms of organic composition and toxicity. Environ Poll 147: 648–655.

Li J, Yuan H, Yang J (2009) Bacteria and lignin degradation. Front Biol China 4: 29–38.

Lindstorm K, Osterberg F (1984) Characterization of the high molecular mass chlorinated matter in spent bleaching liquors (SBL). Part I Alkali SBL Holzforschung 38: 201–212.
Livernoche D, Jurasek L, Desrochers M, Dorica J (1983) Removal of colour from kraft mill waste waters with cultures of white rot fungi and with immobilized mycelium of *Coriolus versicolor*. Biotechnol Bioengg 25: 2055–2065.
Malaviya P, Rathore VS (2007) Bioremediation of pulp and paper mill effluent by a novel fungal consortium isolated from polluted soil. Bioresour Technol 98: 3647–3651.
Manzanares P, Fajardo S, Martin C (1995) Production of ligninolytic activities when treating paper pulp effluents by *Trametes versicolor*. J Biotechnol 43: 125–132.
Martin C, Manzanares P (1994) A study of the decolorization of straw soda-pulping effluents by *Trametes versicolor*. Bioresour Technol 47: 209–214.
Metso Corporation (2006) Pulp production growing in new areas (Global production). Archived from the original on 2007-10-23. Retrieved 2007-10-13. http://www.wikipedia.com/pulp (paper).
Mikesell MD, Boyd SA (1986) Complete reductive dechlorination and mineralization of pentachlorophenol by anaerobic microorganism. Appl Environ Microbiol 52: 861–865.
Milagres AMF, Magalhaes PO, Ferraz A (2005) Purification and properties of a xylanase from *Ceriporiopsis subvermispora* cultivated on *Pinus taeda*. FEMS Microbiol Lett 253: 267–272.
Mileski G, Bumpus J, Jurek M, Aust S (1988) Biodegradation of pentachlorophenol by white rot fungus *Phanerochaete chrysosporium*. Appl Environ Microbiol 54: 2885–2889.
Mishra SB, Mishra AK, Khan MA (2009) Decolourization of pulp and paper mill effluents using heat-treated coal: a comparison with activated charcoal. Environ Chem Lett. doi: 10.1007/s10311-009-0211-4.
Mittar D, Khanna PK, Marawaha SS, Kennedy JF (1992) Biobleaching of pulp and paper mill effluents by *Phanerochaete chrysosporium*. J Chem Technol Biotechnol 53: 81–92.
Modi DR, Chandra H, Garg SK (1998) Decolourization of bagasse-based paper mill effluent by the white-rot fungus *Trametes versicolor*. Bioresour Technol 66: 79–81.
Mohn WW, Kennedy KJ (1992) Reductive dehalogenation of chlorophenol by *Desulfomonite tiedjei* DCB-1. Appl Environ Microbiol 58: 1367–1370.
Nagarathnamma R, Bajpai P (1999) Decolorization and detoxification of extraction-stage effluent from chlorine bleaching of kraft pulp by *Rhizopus oryzae*. Appl Environ Microbiol 57: 1078–1082.
Nagarathnamma R, Bajpai P, Bajpai PK (1999) Studies on decolourization, degradation and detoxification of chlorinated lignin compounds in kraft bleaching effluents by *Ceriporiopsis subvermispora*. Process Biochem 34: 939–948.
Nam IH, Chang YS, Hong HB, Lee YE (2003) A novel catabolic activity of *Pseudomonas veronii* in biotransformation of pentachlorophenol. Appl Microbiol Biotechnol 62: 284–290.
Neilson AH, Allard AS, Hynning PA, Remberger M, Landner L (1983) Bacterial methylation of chlorinated phenols and guaiacols: formation of veratroles from guaiacols and high-molecular weight lignins. Appl Environ Microbiol 45: 774–783.
Niazi JH, Prasad DT, Karegoudar TB (2001) Initial degradation of dimethylphthalate by esterases from *Bacillus* species. FEMS Microbiol Lett 196(2): 201–205.
Oliveira PL, Duarte MCT, Ponezi AN, Durrant LR (2009) Use of *Bacillus pumilus* CBMAI 0008 and *Paenibacillus* sp. CBMAI 868 for colour removal from pulp mill effluent. Braz J Microbiol 40: 354–357.
Osterberg F, Lindstrom K (1985) Characterization of the high molecular mass chlorinated matter in spent bleaching liquors (SBL). Part II Acidic SBL Holzforchung 39: 149–158.
Pallerla S, Chambers RP (1995) Continuous decolorization and APX reduction of bleach plant effluents by free and immobilized *Trametes versicolor*. J Environ Sci Health A30: 423–437.
Pellinen J, Joyce TW, Chang H-M (1988) Dechlorination of high molecular weight chlorolignin by the white rot fungus *Phanerochaete chrysosporium.* Tappi J 71: 191–194.
Perez M, Torrades F, Gracia-Hortal JA, Domenech X, Peral J (2002) Removal of organic contaminants in paper pulp treatment effluents under Fenton and photo-Fenton conditions. Appl Catal 36: 63–74.

Pokhrel D, Viraraghavan T (2004) Treatment of pulp and paper mill effluent wastewater-a review. Sci Total Environ 333: 37–58.

Prasongsuk S, Lotrakul P, Imai T, Punnapayak H (2009) Decolourization of pulp mill wastewater using thermotolerant white-rot fungi. Science Asia 35: 37–41.

Premlatha A, Rajkumar GS (1994) Pentachlorophenol degradation by *Pseudomonas aeruginosa*. World J Microbiol Biotechnol 10: 334–337.

Prouty AL (1990) Bench-scale development and evaluation of a fungal bioreactor for color removal from bleach effluents. Appl Microbiol Biotechnol 32: 490–493.

Raj A, Reddy MMK, Chandra R (2007) Decolourisation and treatment of pulp and paper mill effluent by lignin-degrading *Bacillus* sp. J Chem Technol Biotechnol 82: 399–406.

Ranby B (1969) Recent progress on the structure and morphology of cellulose. Adv Chem Ser 95*:*139–151.

Reid ID (1979) The influence of nutrient balance on lignin degradation by the white-rot fungus *Phanerochaete chrysosporium.* Can J Bot 57: 2050–2058.

Royer G, Yerushalmi L, Rouleau D, Desrochers M (1991) Continuous decolorization of bleached kraft effluents by *Coriolus versicolor* in the form of pellets. J Ind Microbiol 7: 269–278.

Saber DL, Crawford RL (1985) Isolation and characterization of *Flavobacterium* strains that degrade pentachlorophenol. Appl Environ Microbiol 50: 1512–1518.

Sahoo DK, Gupta R (2005) Evaluation of ligninolytic microorganisms for efficient decolorization of a small pulp and paper mill effluent. Process Biochem 40: 1573–1578.

Saravanan V, Sreekrishnan TR (2005) Bio-physico-chemical treatment for removal of color from pulp and paper mill effluents. J Sci Ind Res 64: 61–64.

Saritha V, Maruthi YA, Mukkanti K (2010) Potential fungi for bioremediation of industrial effluents. BioResources.com 5 (1): 8–22.

Saunamaki R (1989) Biological wastewater treatment in the finish pulp and paper industry. Paper Timber 2: 158–164.

Sayadi S, Ellouz R (1992) Decolourization of olive mill waste-waters by the white-rot fungus *Phanerochaete chrysosporium*: involvement of lignin degrading system. Appl Microbiol Biotechnol 37: 813–817.

Shah S, Thakur IS (2003) Enzymatic dehalogenation of pentachlorophenol by *Pseudomonas fluorescens* of the microbial community from tannery effluent. Curr Microbiol 47: 65–70.

Shukla OP, Rai UN, Subramanyam SV (2004) Biopulping and biobleaching: An energy and environment saving technology for Indian pulp and paper industry. EnviroNews 10: 1–2.

Sierra-Alvarez R, Lettinga G (1990) The methanogenic toxicity of wood resin constituents. Biol Wastes 33: 211–226.

Singh D, Chen S (2008) The white-rot fungus *Phanerochaete chrysosporium* conditions for the production of lignin degrading enzymes. Appl Microbiol Biotechnol 81: 399–417.

Singh P, Singh A (2004) Physico-chemical characteristics of the distillery effluent and its chemical treatment. J Nature Sci Technol 3 (2): 205–208.

Singh P, Sulaiman O, Hashim R, Rupani PF, Peng LC (2010) Biopulping of lignocellulosic material using different fungal species: a review. Rev Environ Sci Biotechnol 9: 141–151.

Singh P, Thakur IS (2004) Removal of colour and detoxification of pulp and paper mill effluent by microorganisms in two step bioreactor. J Sci Ind Res 63: 944–948.

Singh P, Thakur IS (2006) Colour removal of anaerobically treated pulp and paper mill effluent by microorganisms in two steps bioreactor. Bioresour Technol 97(2): 218–23.

Singhal A, Thakur IS (2009) Decolourization and detoxification of pulp and paper mill effluent by *Cryptococcus* sp. Biochem Engg 46 (1): 21–27.

Springer A (1985) Industrial Environmental Control, Pulp and Paper Industry. Wiley, New York, pp 181–194.

Srinivasan SV, Murthy DVS (1999) Colour removal from bagasse-based pulp mill effluent using a white rot fungus. Bioprocess Engg 21: 561–564.

Steinle P, Stucki G, Stettler R, Hanselmann KW (1998) Aerobic mineralization of 2,6 diphenol by *Ralstonia* sp. strain RK1. Appl Environ Microbiol 64: 2566–2571.

Sukumar M, Sivasamy A, Swaminathan G (2006) Influence of co-substrates for the decolorization of dye house effluent by *Phanerochaete chrysosporium* and *Trametes versicolor*. Poll Res 25 (3): 557–562.

Taseli BK (2007) Dehalogenation and decolorization of wheat straw-based bleachery effluents by *Penicillium camemberti*. African J Biotechnol 6 (3): 304–306.

Taseli BK, Gokcay CF (2005) Degradation of chlorinated compounds by *Penicillium camemberti* in batch and up-flow column reactors. Process Biochem 40: 917–923.

Taseli BK, Gokcay CF (2006) Degradation of low and high molecular weight fractions of softwood bleachery effluents by *Penicillium camemberti* in up-flow column reactor. Bull Environ Contam Toxicol 76: 481–489.

Terron MC, Calvo AM, Fidalgo ML, Manzanares P, Ballesteros M, Martinez AT, Martin C, Gonzalez AE (1992) Chemical characterization and fungal decolorization of straw soda pulping effluents. In: Kuwahara M, Shimada M (eds), Proc. 5th International Conference on Biotechnology in Pulp and Paper Industry, Uni Publishers, Tokyo, Japan, pp 51–56.

Terron MC, Fidalgo ML, Manzanares P, Ballesteros M, Saez F, Martin C and Gonzalez AE (1991) Chemical characterization and biological decolorization by fungi of effluents from wheat straw alkaline pulping. Med Fac Landbouww Rijksuniv Gent 56: 1569–1572.

Thakur IS (2004) Screening and identification of microbial strains for removal of colour and adsorbable organic halogens in pulp and paper mill effluent. Process Biochem 39: 1693–169.

Thakur IS, Verma PK, Upadhaya CK (2002) Molecular cloning and characterization of pentachlorophenl-degrading Monooxygenase genes of *Pseudomonas* sp. from a chemostat. Biochem Biophy Res Comm 290: 770–774.

Tripathi AK, Harsh NSK, Gupta N (2007) Fungal treatment of industrial effluents: a mini-review. Life Sci 4 (2): 78–81.

Vallecillo A, Garcia-Encia PA, Peana M (1999) Anaerobic biodegradability and toxicity of chlorophenols. Water Sci Technol 40: 161–168.

Van Roekel GJ (1994) Hemp pulp and paper production. J Inter Hemp Assoc 3: 112–114.

Vora KA, Singh C, Modi VV (1988) Degradation of 2-fluorobenzoate by a *Pseudomonad*. Curr Microbiol 17: 249–254.

Wang T, Wang J, Yang W, Jin Y (2003) Experimental study on bubble behaviour in gas-liquid-solid three phase circulating fluidized beds. Powder Technol 137 (1): 83–90.

Welander T (1988) An anaerobic process for treatment of CTMP effluent. Water Sci Technol 20: 143–147.

Xun L, Bohuslavek J, Cai M (1999) Characterization of 2,6-dichloro-p-hydroquinone 1,2-dioxygenase (PcpA) of Sphingomonas chlorophenolica ATCC 39723. Biochem Biophy Res Comm 266: 322–325.

Yadav J, Reddy C (1993) Biodegradation of benzene, toluene, ethyl-benzene, and xylenes (BTEX) by lignin-degrading basidiomycete *Phanerochaete chrysosporium.* Appl Environ Microbiol 59: 756–762.

Yaghoubi K, Mohammad P, Seyed AS (2008) Variable optimization for biopulping of agricultural residues by *Ceriporiopsis subvermispora*. Bioresour Technol 99: 4321–4328.

Yeber MC, Onate KP, Vidal G (2007) Decolorization of kraft bleaching effluent by advanced oxidation processes using copper (II) as electron acceptor. Environ Sci Technol 41(7): 2510–2514.

Zahrim AY, Gilbert ML, Janaun J (2007) Treatment of pulp and paper mill effluent using photo-Fenton's process. J Appl Sci 7: 2164–2167.

Zeikus JG, Wellstein AL, Kirk TK (1982) Molecular basis for the biodegradative recalcitrance of lignin in anaerobic environments. FEMS Lett 15: 193–197

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D.M. Whitacre (ed.), *Reviews of Environmental Contamination and Toxicology*, Reviews of Environmental Contamination and Toxicology 212,
DOI 10.1007/978-1-4419-8453-1, © Springer Science+Business Media, LLC 2011