Reviews of Environmental Contamination and Toxicology

Volume 188

Springer

Reviews of Environmental Contamination and Toxicology

VOLUME 188

Reviews of Environmental Contamination and Toxicology

Continuation of Residue Reviews

VOLUME 188

Springer

Coordinating Board of Editors

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Heidelberg: Postfach 10 52 80, 69042 Heidelberg, Germany

Library of Congress Catalog Card Number 62-18595
ISSN 0179-5953

Printed on acid-free paper.

Printed in the United States of America. (BS/SBA)

ISBN-10: 0-387-31911-5 e-ISBN: 0-387-32964-1
ISBN-13: 978-0387-31911-7

springer.com

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 peoples 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 ever-increasing media as well as scientific attention: bioterrorism and global warming. Unfortunately, these very worrisome issues are now super-imposed 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 multi-national consortia to control pollution and the evolution of the environmental ethic. Will the new politics of the 21st 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 either be 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.

Tucson, Arizona G.W.W.

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Marine Halogenated Natural Products of Environmental Relevance

Walter Vetter

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I. Introduction

Polyhalogenated compounds have been used for industrial and agricultural applications for some 50 years. Variations in the degree of halogenation can change their properties in almost any desired direction, so that their application fields were diverse and production rates were high. However, the other side of the coin provided evidence that the polyhalogenated xenobiotics are serious environmental contaminants. Their detection in the environment along with the linking of their presence to adverse affects observed in the living environment was an important step toward the recognition that there is a thorough need of environmental protection.

Communicated by George W. Ware.

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The primary class of contaminants were DDT and its metabolites, as well as polychlorinated biphenyls (PCBs). These "classic" contaminants had a fellowship of related compounds including chloropesticides (HCH/lindane, chlordane, toxaphene, endrin, and related cyclodienes) and industrial chemicals, polychloronapthalenes (PCNs). Their toxic effects and environmental behavior led to their classification as persistent organic pollutants (POPs) and persistent bioaccumulative and toxic chemicals (PBTs). Some PBTs including most just mentioned were ranked as the "dirty dozen" whose production and use have been forbidden in a worldwide act following the Stockholm convention on POPs.

However, new environmental contaminants emerged in recent years including brominated flame retardants [polybrominated diphenyl ethers (PBDEs), polybrominated biphenyls (PBBs), hexabromocyclododecane (HBCD)], polychlorinated paraffins, polychlorinated diphenyl ethers, and the list could be continued. In appears that once a compound or compound class was forbidden or at least "in the news," a substitute with similar properties, good and bad, was created. Examples are polychlorinated terphenyls, which were substitutes for PCBs (without getting the same attention as PCBs), toxaphene, which emerged as a chloropesticide because of restrictions on DDT, and the currently widely discussed PBDEs which are subsequently substituted with more complex fire retardants. These compounds have been unintentionally released into environment in known and traceable manners. Thus, it takes shorter and shorter periods until environmental concerns are developed by researchers.

In addition to this mix of manmade halogenated pollutants, a rather new spectrum of compounds attracts currently more and more attention, i.e., naturally produced organohalogen compounds or halogenated natural products (HNPs). HNPs have a long history, and natural products chemists have thus far identified about 4,000 different compounds (Gribble 2004). Gordon W. Gribble, the late D. John Faulkner, and others have prepared outstanding review articles on this topic (Gribble 1998, 1999, 2000, 2004; Faulkner 1980, 2002; Naumann 1993, 1999; Field et al. 1995). The halogenated secondary metabolites are produced by such diverse organisms as algae, sponges, sea worms, and bacteria, with an increase of ~200 novel HNPs that are discovered annually (Gribble 2004). Relatively new, however, is their link with environmental issues, the topic of this review article. This connection means that the HNPs are detected in higher organisms that were not the natural sources but have accumulated the natural products. Their detection in top predators indicated that HNPs resembled some of the adverse properties of halogenated xenobiotics, i.e., persistency and the bioaccumulative character, and this in turn leads to the question of their (eco)toxicological relevance and thus their role as environmental contaminants.

In the late 1990s, three papers were published that carefully addressed this topic (Haglund et al. 1997; Tittlemier et al. 1999; Vetter et al. 1999a).

The careful announcements that HNPs were probably detected in higher organisms were necessary and justified because it sounded unbelievable. It has to be remembered that a major simplifying argument for the particular toxicity of anthropogenic POPs, that no analogue compounds are found in nature, had to be revised (see following). In the first days, environmental scientists had to face some irrational scepticism of other researchers on their results. Tittlemier et al. (1999) cite in their key article to the field that ". . . some types of synthetic compounds, including halogenated hydrocarbons such as PCB, are not found in nature." When we described a halogenated monoterpene as an abundant contaminant in fish and mammals, one of the anonymous reviewers commented on the chromatogram (Fig. 1) with the remark that the peak of the novel compounds must be an artifact. It was claimed that such an abundant compound would have been detected earlier.

Unexpectedly, the situation has changed within recent years since more and more evidence was provided on the natural origin of some abundant halogenated compounds in the gas chromatograms of various samples. Today, HNPs are recognized as possible contaminants of marine environmental samples and food. Ironically, the situation is now almost the opposite. Residues from unknown compounds in environmental samples are sometimes suggested to arise from HNPs without providing evidence. The natural origin of a halogenated compound is however not always easy to prove. In several cases, the natural producers are still unknown or ambiguous. Nevertheless, it is time for a first review on the environmental issue of the HNPs and a first balance after fewer than 10 years of dedicated research.

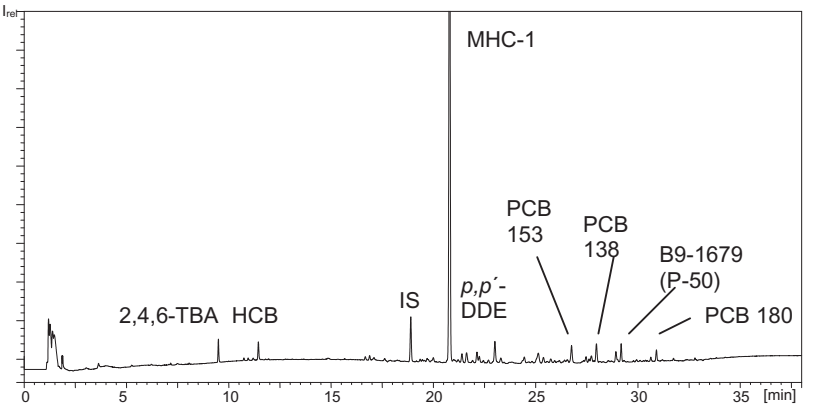


Fig. 1. GC/ECD chromatogram (HP-1) of the purified organohalogen extract of a Norwegian salmon (Adapted from Vetter et al. 2001b with permission from the American Chemical Society).

II. Mass Spectrometric Investigation of Halogenated Natural Products (HNPs)

HNPs elute in the same gas chromatography (GC) retention range and cover the same mass range as anthropogenic halogenated pollutants. The identification of HNPs in an environmental sample is thus not simple. Given the fact that chlorinated anthropogenic compounds are more abundant, more diverse, and more widely distributed in the environment as compared with organobromines, the identification of chlorinated HNPs is more difficult. However, the environmentally relevant HNPs identified to date are mostly brominated or at least contain bromine, whereas exclusively chlorinated, natural products are scarce. In fact, the so-called Q1 (see Section III. B) is currently the only important polychlorinated HNP identified whereas diverse abundant brominated and mixed HNPs have been described (see Section III).

A random worldwide comparision may allow us to estimate that all chlorinated compounds combined are rather two orders than one order of magnitude more abundant than anthropogenic brominated compounds, although there are exceptions. PCBs and chloropesticides are often found in the parts per million (ppm) range in marine mammals, and the detection of traces of HNPs in such samples will not be readily possible. If they occur, they were previously assigned to unknown minor compounds or metabolites of anthropogenic POPs. Consequently, the identification of brominated and mixed brominated-chlorinated HNPs is more likely, whereas chlorinated HNPs can only be identified under particular circumstances. The following scheme thus focuses more on the detection of bromine-containing compounds.

In the 1980s, gas chromatography in combination with electron-capture negative ion mass spectrometry (GC/ECNI-MS) was shown to be a promissing tool for the detection of brominated compounds (Crow et al. 1981). Under these conditions, the bromine atom attached to a carbon has relatively low energetic unoccupied molecular orbitals (LUMO), and the charge is well stabilized by the large bromine atom. Homolytic cleavage of the C-Br bond yields the bromide ion, which then (re-)achieves the Nobel gas configuration for which it strives, whereas a neutral (M-Br) radical is left. Thus, bromine atoms in halogenated compounds are prone to electron capture processes. Due to the equal natural abundance of the bromine isotopes, screening for the bromide ion with virtually equal peak heights of m/z 79 and m/z 81 is a sensitive method for the identification and determination of all organobromine compounds present in environmental and food samples (Buser 1985). Owing to this low selectivity for a particular compound (almost all organobromines respond to the bromide ion), some authors reported coelutions of diverse brominated compounds (Vetter and Jun 2003; Marsh et al. 2004a). It was thus recommended to use further low-mass ions for distinguishing between different classes of dibrominated

to polybrominated compounds. Br^- was found to be typical of all organobromines (Buser 1985), whereas Br_2^- is often found in the GC/ECNI-MS of nonaromatic organobromines with at least two Br substituents. Aromatic organobromines either form no additional low-mass fragments or an intense fragment ion at m/z 159 (HBr_2^-). In several studies, the relevance of this fragment ion was described more precisely (Vetter 2001; Vetter et al. 2002a; Vetter and Janussen 2005). Current knowledge suggests that m/z 161 is only abundant in diphenyl ether derivatives that bear at least one bromine substituent in the ortho position (Melcher et al. 2005a). This condition is fulfilled for anthropogenic BDEs and naturally produced methoxy-BDEs (MeO-BDEs), except the respective non-ortho congeners, which are rarely found. Other compounds forming m/z 159 are diMeO-BDEs.

Likewise, the chloride ion may be used for initial screening on chlorinated and mixed halogenated compounds (Asplund et al. 1999; Vetter et al. 2002a). However, the chloride ion is not necessarily abundant in the mass spectra of mixed halogenated compounds (Buser 1985). Although the electronegativity of chlorine is higher, the larger convalent radius of bromine is obviously favorable and the formation of the heavier halogenide is predominant.

Once all brominated compounds in a sample are detected in the SIM mode, GC/ECNI-MS full-scan analysis may accomplish the initial measurements. Unfortunately, the molecular ion (M^-) can be very low in abundance for polybrominated compounds. For selected compounds only, this can be improved by lowering the ion source temperature. However, the structural information obtained from GC/ECNI-MS full-scan measurements is usually low but often suitable to add the missing piece to a puzzle. Moreover, the information obtained from low-abundance M^- ions is often equivocal. Although brominated isotope patterns are very distinct, as are chlorine isotope patterns, some isotope patterns of mixed brominated and chlorinated/brominated compounds are almost identical (Fig. 2). Excellent mass spectra can be assigned unequivocally to the number and kind of isotopes present in an organohalogen compound, but this is not easy to obtain (compare the isotope patterns of the mass spectra in Section III with those in Fig. 2). Thus, it is unterstandable that misinterpretations may occur (Sinkkonen et al. 2004), and a thorough comparison of the isotope pattern in a sample with the theoretical abundances of the isotopic peaks should be carried out. Attention should be paid particularly to the low-abundance isotopic peaks to overcome erronous assignments of the halogenated patterns. For instance, the major isotopic peaks of heptachloro-, pentabromo-, and dichlorotetrabromo isotope pattern look very similar. However, only the latter two have a low abundant monoisotopic peak. These two can be distinguished by the very low abundant seventh line, which is not present in the pentabromo isotope pattern. Further examples for very similar isotope patterns are shown in Fig. 2.

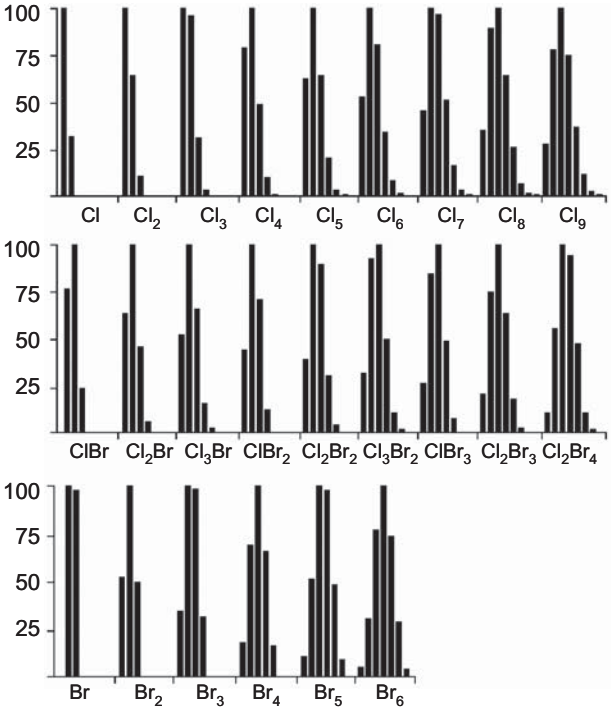


Fig. 2. Halogen isotope patterns.

Gas chromatography in combination with electron ionization (GC/EI-MS) is usually significantly less sensitive for the detection of polybrominated compounds than in GC/ECNI-MS. Another disadvantage of GC/EI-MS is the detection of the background from matrix remainders that are suppressed by GC/ECNI-MS and can reach very high abundance, particularly when GC/EI-MS full-scan analyses require concentrated solutions of sample extracts. Moreover, there are no fragment ions in the GC/EI-MS spectra that directly prove the presence of chlorine and bromine. Thus, distinguishing brominated for mixed halogenated compounds requires high-quality mass spectra (see foregoing). In addition, the more-sensitive SIM technique can hardly be performed in nontarget analysis. However, if high-quality spectra are obtained, GC/EI-MS is the method of choice for studying the fragmentation patterns. For instance, many brominated and mixed halogenated compounds show abundant $[M\text{-}Br]^+$ and $[M\text{-}2Br]^+$ fragment ions that are often very useful for structure information. For instance, the $[M\text{-}2Br]^+$ fragment ions of a pentabromo compound (Br_3 pattern) appear to be more easily distinguished from the respective dichlorotetrabromo compound (Cl_2Br_2 pattern). Furthermore, elimination of chlorine may provide clarity on the exact isotope pattern of a polyhalogenated com-

pound. Table 1 lists the relative abundances within halogen isotope patterns that should be virtually matched by the organohalogen compound being studied.

In many cases, high-resolution mass spectrometry (HRMS; usually in the EI mode) should be used to establish the elemental composition, as was carried out on several occasions (Tittlemier et al. 1999; Vetter et al. 1999a, 2001b; Teuten et al. 2005a). This is, however, much easier if the numbers and types of halogens are known. Given the fact that the hydrogen atom exceeds the nominal value (Table 2), the exact masses of different structural variants are usually the heavier the more hydrogens are found in the molecule. However, the exact masses are usually lower than the nominal masses because the halogens are lighter. The molecular ion can be scanned, or the exact masses of different elemental compositions that can be calculated from the low-resolution mass spectrum can be screened in the SIM

Table 1. Halogen isotope abundances of chlorinated, brominated, and mixed halogenated compounds.[a]

Halogen	X[b]	X + 2	X + 4	X + 6	X + 8	X + 10	X + 12	X + 14
Cl	100	32.0						
Cl_2	100	64.0	10.2					
Cl_3	100	96.0	30.7	3.3				
Cl_4	78.2	100	48.0	10.2	0.8			
Cl_5	62.5	100	64.0	20.5	3.3	0.2		
Cl_6	52.1	100	80.0	34.1	8.2	1.0		
Cl_7	44.7	100	95.9	51.1	16.4	3.1	0.3	
Cl_8	34.9	89.3	100	64.0	25.6	6.5	1.0	0.1
Cl_9	27.2	78.2	100	74.6	35.8	11.4	2.4	0.3
Br	100	97.9						
Br_2	51.1	100	49.0					
Br_3	34.0	100	98.0	32.0				
Br_4	17.4	68.1	100	65.3	16.0			
Br_5	10.4	51.1	100	97.9	47.9	9.4		
Br_6	5.3	31.3	76.6	100	73.4	28.8	4.7	
BrCl	77.0	100	24.1					
$BrCl_2$	61.8	100	45.0	6.2				
$BrCl_3$	51.6	100	64.2	17.2	1.7			
Br_2Cl	43.9	100	69.6	13.5				
Br_2Cl_2	38.5	100	89.0	31.3	3.8			
Br_2Cl_3	31.8	92.8	100	49.4	11.4	1.0		
Br_3Cl	26.2	85.4	100	48.7	7.9			
Br_3Cl_2	20.6	73.7	100	63.4	18.4	2.0		

[a]Only abundances >0.1% are listed.
[b]X = all-^{35}Cl and all-^{79}Br is appropriate.

Table 2. High-resolution mass spectrometry (HRMS) calculation of the monoisotopic peak.[a]

	^{12}C 12.00000	^{1}H 1.007825	^{35}Cl 34.968854	^{79}Br 78.918348	^{16}O 15.994915	^{14}N 14.003074	Calculated exact mass (nominal mass) (u)
DBP-Br_4Cl_2	10	6	2	4	—	2	539.664 (540)
Q1	9	3	7	—	—	2	383.812 (384)
6-MeO-BDE 47	13	8	—	4	2	—	511.726 (512)
MHC-1	10	13	3	2	—	—	395.845 (396)

[a]Other isotopes are ^{13}C (13.003354), ^{37}Cl (36.965896), ^{2}H (2.014012), ^{15}N (15.000108), ^{81}Br (80.916344).

mode. Once the elemental composition is known, the fragmentation pattern can provide valuable information as to the structure of organobromine and mixed halogenated compounds. Currently available data confirm that a wide range of HNPs exist that may end up in food or be accumulated in the environment. These compounds include brominated, mixed halogenated, and to a lesser degree chlorinated compounds with an aromatic, aliphatic, or heterocyclic backbone. Nitrogen and oxygen are frequently found on the HNPs discussed next. In fact, most of the HNPs discussed in this review bear at least one hetero atom in addition to halogens. These different possibilites should be kept in mind when an unknown compound is investigated.

III. Individual HNPs of Environmental Concern

For several decades, the research of environmental chemists on anthropogenic POPs and the research of natural products chemists on HNPs was conducted almost isolated in the respective research discipline. Very little if any overlap was observed at the end of the 20th Century. In retrospect, it is not always clear why there was not more exchange between the two groups. A recent study of sponges led to the detection of >100 HNPs but many of them were in very low abundance and would not have become the focus of natural products chemists (Vetter and Janussen 2005). Some of them could, however, be of environmental concern. Currently, research of natural products chemists is cited by environmental chemists and vice versa, and it appears that interests of both disciplines are becoming more mixed without losing their different directions or intents of research.

It was long thought that HNPs are neither persistent nor lipophilic and thus do not bioaccumulate. A prerequisite for the presence of such halogenated compounds in the lipids of the top predators in food chains is that they are lipophilic ($\log K_{ow} > 5$), persistent (nondegradable in the liver), and bioavailable (able to pass through membranes). Faulkner (1980) predicted that brominated phenols are probably the most stable HNPs and are therefore most likely to appear as contaminants in other analyses.

Although this is generally the case, recent work has identified the natural producer of two compounds previously detected with high concentrations in marine mammals (Vetter et al. 2002b). In addition to the few nonpolar HNPs with known bioproducers (Vetter et al. 2002b; Flodin and Whitfield 1999a; Asplund et al. 2001), the natural origin of several other common organohalogen compounds is no longer debated (Tittlemier et al. 1999; Vetter et al. 1999a, 2001a,b). The classification of compounds described in the following subsections as halogenated natural products is diverse.

A. Halogenated Dimethyl-2,2′-Bipyrroles (HDBPs)

This compound class summarizes halogenated components that share a 1,1′-dimethyl-2,2′-bipyrrole (DBP) spine. Five hexahalogenated congeners with

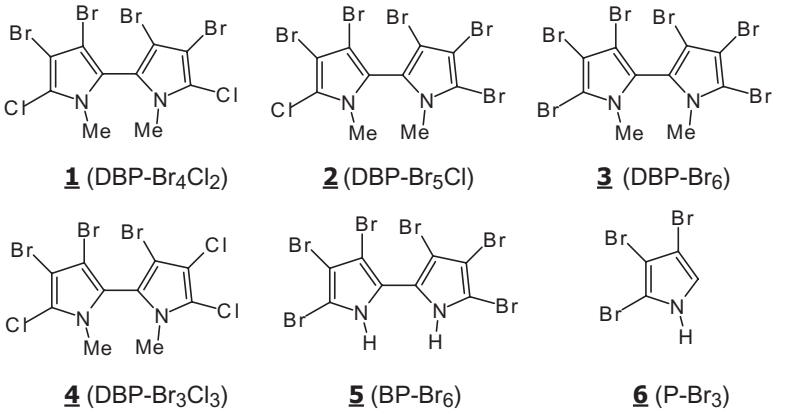


Fig. 3. Structures of halogenated dimethylbipyrroles and related compounds. **1**: 5,5′-dichloro-1,1′-dimethyl-3,3′,4,4′-tetrabromo-2,2′-bipyrrole; **2**: 5′-chloro-1,1′-dimethyl-3,3′,4,4′,5-pentabromo-2,2′-bipyrrole; **3**: 1,1′-dimethyl-3,3′,4,4′,5,5′-hexabromo-2,2′-bipyrrole; **4**: 1,1′-dimethyl-3,3′,4-tribromo-4′,5,5′-trichloro-2,2′-bipyrrole; **5**: 3,3′,4,4′,5,5′-hexabromo-2,2′-bipyrrole; **6**: 2,3,4-tribromopyrrole.

a bromine/chlorine distribution of 3/3 (two isomers), 4/2, 5/1, and 6/0 have been described (Fig. 3) (Tittlemier et al. 1999). Tittlemier et al. designated codes to the compounds based on the abbreviation DBP, separated by a hyphen following Br_x and Cl_y. The short term of the most abundant tetrabromodichloro-1,1′-dimethyl-2,2′-bipyrrole congener (**1**) is thus DBP-Br_4Cl_2. Structure elucidation was performed using isotope exchange, namely N-H→ N-D, and the proposed structures, when synthesized, fully agreed with the MS prediction (Tittlemier et al. 2002c).

Historic Data, Identification, and Linking to Known Natural Sources. In 1992, Elliot et al. (1992) described a relatively abundant compound in bird eggs from both the Canadian Pacific and Atlantic coasts. This compound, labeled UHC, was subsequently isolated from bald eagles (*Haliaeetus leucocephalus*) and studied by GC/MS (Fig. 4) (Tittlemier et al. 1999). Initially suspected to be a pentabromo compound (see Section II and Fig. 2; compare with Fig. 4b), the use of larger amounts (on isolation) and HRMS indicated that this novel compound carried four bromine and two chlorine atoms. Even more surprising, HRMS analysis demonstrated that the compound bore two nitrogens. The molecular formula was established as $C_{10}H_6Br_4Cl_2N_2$, and a possible structure was suggested to be 5,5′-dichloro-1,1′-dimethyl-3,3′,4,4′-tetrabromo-2,2′-bipyrrole (**1**) (Tittlemier et al. 1999). The simultaneous detection of a hexabromo (**3**), a chloropentabromo (**2**), and two tribromotrichloro homologues, one of which was (**4**), along with the related known hexabromo-2,2′-bipyrrole (**5**) previously identified by natural products chemists (Andersen et al. 1974), produced strong evidence

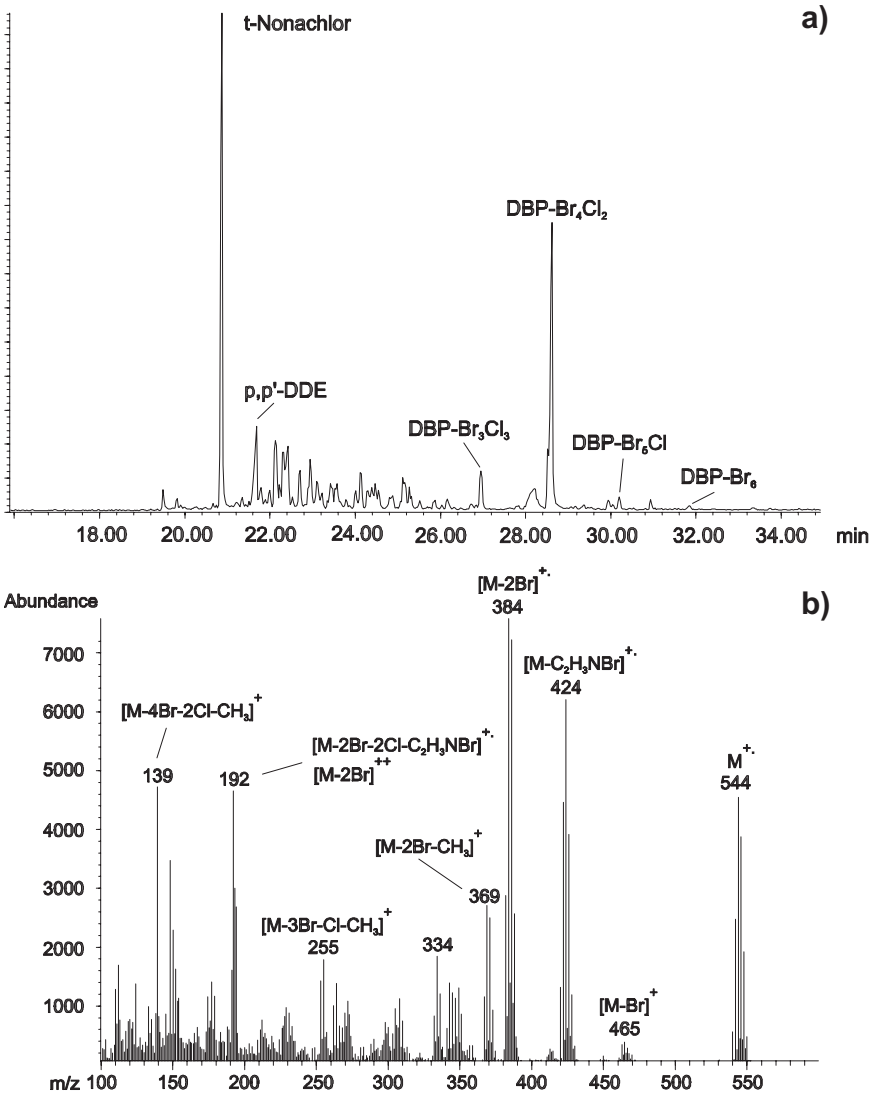


Fig. 4. GC/ECNI-MS total ion chromatogram of bald eagle liver extract (*top*) (a) and EI-MS of DBP-Br_4Cl_2 (*bottom*) (b) (Adapted from Tittlemier et al. 1999 with permission from the American Chemical Society).

for this structure (Tittlemier et al. 1999). In the same year, Gribble et al. (1999) synthesized the key compounds (and the hexabromo congener), and comparison of the synthesized standard and the isolate from bird eggs confirmed that the correct compound was synthesized and that the proposed structure was correct (see Fig. 3). Gribble et al. (1999) based their synthesis on the preparation of the backbone. The subsequent one-pot

halogenation reaction yielded a mixture that proved to be similar to the residue pattern found in seabirds. Thus, it was concluded that the natural halogenation was somewhat random (Gribble et al. 1999). It was suggested that first a chlorination with chloroperoxidase, followed by bromination with bromoperoxidase, had occurred (see Section IV) (Gribble et al. 1999). Monomeric tetrabromopyrrole had also been isolated from the marine bacterium *Chromobacterium* sp. (Andersen et al. 1974). It is noteworthy that tetrabromopyrrole and tribromopyrrole isomers (**6**) were found to be extremely unstable, especially when exposed to light and oxygen (Andersen et al. 1974; John et al. 2004). This finding is in sheer contrast to the recalcitrant HDBPs, the focus of this chapter, which have not been discovered by natural products chemists.

Investigation of samples from Australia led to the detection of several brominated compounds (see Sections III.C, III.D), whereof the one labeled BC-10 turned out to be DBP-Br_4Cl_2 (**1**, Fig. 3) (Vetter 2001; Vetter and Jun 2003). Reddy et al. (2004) isolated DBP-Br_4Cl_2 from marine mammal extracts and determined the $\Delta^{14}C$ value. The ^{14}C radioisotope has a half-life of 5,730 years, which excludes its determination in samples older than ~50,000 years. Because the anthropogenic POPs (exception, toxaphene) are produced from coal or oil sources dating back manifold years more, the detection of ^{14}C is an indirect proof of the natural source. Reddy et al. (2004) indeed determined ^{14}C in the isolate, but the depletion accounted for an age of ~5000 years of the compounds, which is rather unrealistic given the wide distribution in our time and typical half-lives in the environment that do not exceed ~10 years. Reddy et al. (2004) suggested three scenarios that could explain their results, i.e., (i) a mix of anthropogenic and natural sources, (ii) utilization of aged carbon during the biosynthesis, and (iii) biosynthesis in ancient years. The most plausible hypothesis was suggested to be utilization of aged carbon. However, other parameters may apply as well, which are the following: (iv) isotope fractioning during food web enrichment, (v) isotope fractioning during isolation, and (vi) interference from another coisolated compound. The natural producer of DBP-Br_4Cl_2 is unknown (in contrast to **5**), and it appears plausible that methylation of the nitrogens (e.g., the conversion of **5** into **3**) was performed by another organism (see Section III.C). Because radiocarbon measurements only deliver an average value for all carbons, some unequivocal data may arise from this point. Unfortunately, because of the uncertainties addressed in parameters (i) to (vi), the radiocarbon measurements, along with the more striking data obtained for MeO-BDEs, which are different from those determined for HDBPs, provide no unequivocal proof of their natural production; however, they support the HNP theory. Other important issues that clearly point toward a natural source for HDBPs are the high concentrations in marine environments and the virtual absence in industrial regions, as well as a distribution pattern different from that of classic anthropogenic contaminants (Tittlemier et al. 1999, 2002b). In addition, a mixed halogenated

pattern is relatively rare for industrial chemicals except for their formation during incineration (Tittlemier et al. 2002b). In the latter unintended case, however, we would expect a mixture of several DBP-Br_4Cl_2 isomers, which is in contrast to the unique DBP-Br_4Cl_2 isomer found abundantly in the environment.

Tittlemier et al. (2004) determined physicochemical (PC) parameters of five HDBPs (Table 3). As anticipated, the vapor pressures decreased with increasing number of bromine substituents that replaced chlorine atoms [$P°_{L,25}$ (7.55–191) 10^{-6} Pa], but the water solubility and octanol–water coefficient remained untouched from the pattern of halogens (Table 3). These values rank the HDBPs in the range of PCB congeners. For instance, PCB 101 showed comparable water solubility (0.98 10^{-6} g/L), and the log K_{OW} of penta- and hexachloro biphenyls was also comparable to the HDBPs. The PC parameters were used in a distribution model that indicated that >99% of HDBPs are located in sediments and soil (Tittlemier et al. 2004).

It is evident that demethylation of HDBPs will decrease the lipophilic character of the HDBPs, comparable to bromoindols/N-methylindoles (see Section III.G) or halogenated phenols/anisoles (see Section III.E). Monomeric tribromopyrrols, which obviously turned out to be stable, were recently synthesized by John et al. (2004). Pellets spiked with 2,3,4-tribromopyrrole (**6**) administered to predatory fish had a deterrent effect, and only 6 of 14 fish actually consumed the pellets. These fish were significantly larger than those that refused the pellets (John et al. 2004). Attempts to detect the bromopyrroles in fish tissue were not undertaken. Unfortunately, even the major HDBPs, DBP-Br_4Cl_2 and DBP-Br_6, are not commercially available, which hinders a more thorough worldwide study of their relevance.

Analytical Aspects. Standard sample cleanup methods suitable for the determination of POPs (PCBs, chloropesticides, PBDEs) can be applied to HDBPs. The most sensitive and suggested detection method is GC/ECNI-MS (Tittlemier et al. 1999, 2002b). The GC/ECNI-MS of DBP-Br_4Cl_2 is dominated by the molecular and bromide ions, both found in equal amounts (Tittlemier et al. 1999). Therefore, m/z 544 and m/z 546 are recommended for selective determination of DBP-Br_4Cl_2. The higher the degree of bromination, the higher the ratio of M^- to Br^- becomes (SA Tittlemier, personal communication 2005). Limits of detection (S/N > 3) were 0.2 pg (m/z 500/502, DBP-Br_3Cl_3), 0.25 pg (m/z 544/546, DBP-Br_4Cl_2), 0.01 pg (m/z 588/590, DBP-Br_5Cl), and 0.01 pg (m/z 632/634, DBP-Br_6) (SA Tittlemier, personal communication). Given the high response for the bromide ion, m/z 79 and m/z 81 have also been used for DBP-Br_4Cl_2 (Vetter 2001), but coelutions with other brominated compounds of natural or anthropogenic origin may occur. Thus, using the bromide ion is more suitable as a general screening method, whereas quantification should be performed by determining isotope masses of the molecular ion (see Section II); this appears to be

Table 3. Physicochemical parameters of selected halogenated natural products (HNPs).

Compound	Log K_{OW}	Water solubility $S_{W,25}$ (g/L)	Melting point	H_{25} (Pa m^3/mol)
Br_4-Cl_2-DBP (Tittlemier et al. 2004)	6.5 ± 0.3	(0.9 ± 0.1) 10^{-5}	209°–210°C	0.036 ± 0.004
Br_6-DBP	6.7 ± 0.3 (Tittlemier et al. 2004)	(1.4 ± 0.3) 10^{-5}	247°–248°C	0.0020 ± 0.0004
Q1	5.9–6.4 (Hackenberg et al. 2003; Vetter 2000)	0.46 10^{-5} (Vetter et al. 2004)	154°–155.5°C (Jun et al. 2002)	n.d.
TBA	4.44 (Pfeifer et al. 2001) 4.48 (Mackay 1982)	1,220 10^{-5} (Vetter et al. 2004)	87°–89°C (Vetter et al. 2004)	n.d.
2′-MeO-BDE 68	~6.85 (Teuten et al. 2005a)	n.d.	Oil, boiling point 120°C at 0.24 Torr (Vetter and Jun 2003)	n.d.
6-MeO-BDE 47	~6.85 (Teuten et al. 2005a)	n.d.	116.5°–117.5°C (Francesconi and Ghisalberti 1985)	n.d.

particularly necessary for the detection of DBP-Br_6. The major compound DBP-Br_4Cl_2 elutes in the last third between BDE 47 and BDE 100 from DB-5 as stationary phases (Vetter and Jun 2003). A heptachlorobiphenyl congener may coelute with DBP-Br_2Cl_4 on DB-5 columns (data not shown). More comprehensive retention data for all prominent HDBPs on different columns were also published by Tittlemier et al. (2002c).

Distribution and Concentrations of HDBPs in the Environment. In the first study, bird eggs from Pacific offshore surface feeders accumulated more than 10-fold-higher HDBP concentrations than Pacific offshore subsurface feeders and Atlantic bird eggs (see Fig. 4a for an example). By contrast, birds from the Great Lakes did not contain HDBPs, which is an additional clue for HDBPs being natural products (Tittlemier et al. 1999). Marine samples from Canada also confirmed this. Nitrogen stable isotope mass spectrometric (IRMS) analysis of tissue along with the quantification of HDBPs demonstrated that these HNPs biomagnified with trophic level from invertebrate → fish → seabird (Tittlemier et al. 2002a). A global study with marine mammals, which did not include samples from Africa, South America, and the Antarctic, was carried out by Tittlemier et al. (2002b). The highest concentrations of 9.8 mg/kg ΣHDBPs was determined in California sea lions (Table 4) (Tittlemier et al. 2002b). High concentrations (up to 4 ppm) were also found in bottlenose dolphins from Australia. Concentration and distribution of HDBPs did not correlate with PCBs (Tittlemier et al. 2002b). In pinnipeds, HDBPs are less abundant than in cetaceans, and they seem to be less persistent than PCB 153 (Tittlemier et al. 2002b). Surprisingly high concentrations were also determined in selected canned fish samples. DBP-Br_4Cl_2 was also identified in human milk from the Faeroe Islands (Vetter and Jun 2003). Sea eagle eggs from Norway contained traces of DBP-Br_4Cl_2 (Herzke et al. 2005). Pool samples of human milk from southern Canada contained 13–4,480 pg/g lipids, which was low compared to fish and seafood (Tittlemier et al. 2002d). Studies of marine birds of prey demonstrated that they bioaccumulate HDBPs in tissue, plasma, and liver. HDBPs also seem to be transported to yolk during egg development (Tittlemier et al. 2003).

Consequences. Although natural producers of HDBPs have not been identified, their assignment to natural sources is no longer debated. Therefore, the question mark at the end of the initial paper (Tittlemier et al. 1999) is no longer necessary. Marine birds and cetaceans appear to contain the highest burden, but fish are also known to be potentially contaminated with HDBPs. Reference standards are lacking, which hinders a thorough worldwide investigation. Toxicological investigation pointed to dioxin-like effects, albeit the bioactivity was much more moderate (Tittlemier et al. 2003). Even in environmental samples with the highest concentration, no toxic effects could be determined in the respective samples. The relatively high

Table 4. Concentrations (selection) of HDBPs (ng/g lipids) in the marine environment and food.

Species	Location	*N*	sumHDBPs	PCB 153	Source
Beluga	North America		14–18	540–8,000	Tittlemier et al. (2002b)
	Svalbard		2	1,270	Tittlemier et al. (2002b)
Dall's porpoise	NW North Pacific Ocean	5	2,540	1,240	Tittlemier et al. (2002b)
Hector's dolphin	New Zealand	5	48	76	Tittlemier et al. (2002b)
California sealion	California	5	93–9,800	910–90,800	Tittlemier et al. (2002b)
Harbour seals	Different locations	69	0.02–526	65–282,000	Tittlemier et al. (2002b)
Bottlenose dolphin	Australia	4	250–4,150[b]	230–8,800	Vetter et al. (2001a)
Green turtle	Australia	1	26[b]	70	Vetter et al. (2001a)
Marine fish[a]	Canada	62	<0.6–1,100		Tittlemier (2004)
Freshwater fish[a]	Canada	39	<0.6–220		Tittlemier (2004)
Canned fish[a]	Canada	86	25–6,660		Tittlemier (2004)
Shrimp[a]	Canada	33	<0.6–48		Tittlemier (2004)
Sediment	Canadian Arctic	2	~0.03		Tittlemier et al. (2002a)
Arctic cod[a]	Canadian Arctic	5	1.1		Tittlemier et al. (2002a)
Black guillemot[a]	Canadian Arctic	6	9.5		Tittlemier et al. (2002a)
Seal[a]	Canadian Arctic	10	~0.1		Tittlemier et al. (2002a)
Seabird eggs	Pacific, Canada	19	32–140	~70–100	Tittlemier et al. (1999)
Offshore surface feeders + seabird eggs[a]	Atlantic	40	1.7–4.8	>20	Tittlemier et al. (1999)

[a]Wet weight.
[b]Estimated from response factor of 2-MeO-BDE 68.

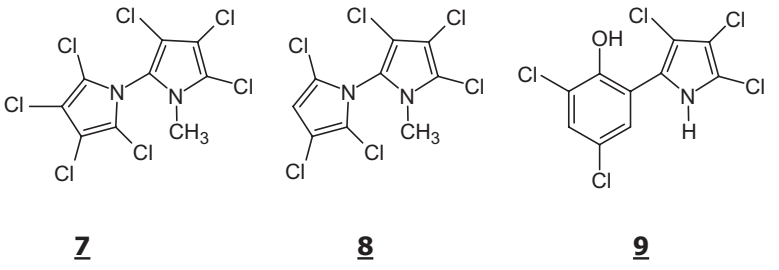
response in the AHR assay is somewhat surprising because it is expected that DBP-Br_4Cl_2 is not planar. In this case, the rotation about the central pyrrole–pyrrole bond would be hindered (this aspect is discussed more detail in Section III.B). An estimate of human exposure to bioaccumulative HNPs was presented by Tittlemier (2004).

B. Heptachloro-1′-Methyl-1,2′-Bipyrrole (Q1)

The trivial name "Q1" (an abbreviation for question 1) was originally assigned to the first prominent unknown compound detected in the GC/MS analysis of Antarctic seals. Unlike all other relevant marine natural products found at elevated concentrations in higher organisms discussed in this article, Q1 is the only exclusively chlorinated HNP, a feature that is obviously more characteristic for terrestrial samples. Of course, there may be many more, but their detection is more complicated than that of brominated natural products (see Section II). Very recently, indications for a chlorohexabromo- and the heptabromo congener of Q1 were presented by Teuten et al. (2005b).

First evidence for a natural origin of Q1 was produced in 1999 when the molecular formula of Q1 was determined by HRMS (Vetter et al. 1999a). The unique composition of $C_9H_3Cl_7N_2$ had never been reported in any scientific paper except as an unstable reaction intermediate that could definitely be ruled out (Findeisen and Wagner 1978; Vetter et al. 1999a). The follow-up synthesis of Q1 led to the chemical structure (**7**) shown in Fig. 5. This structure is spectacular because even the unsubstituted 1,2′-bipyrrole backbone is a chemical feature that was not described in organic chemistry or other disciplines of natural science before the synthesis of Q1 (Jun et al. 2002).

Neither Q1 nor closely related structures have been detected by natural products chemists, and according to present knowledge, no relevant lower chlorinated analogues or chlorinated homologues have been detected in environmental samples. In view of the fact that the chemical synthesis of



7 **8** **9**

Fig. 5. Structures of Q1 (2,3,3′,4,4′,5,5′-1′-methyl-1,2′-bipyrrole, **7**), Q1-hex (2,3′,4,4′,5,5′-1′-methyl-1,2′-bipyrrole, **8**), and pentachloro-2-pyrrolyl-2-phenol, **9**.

Q1 only provided a maximum yield of 3.6% (Jun et al. 2002), the biochemical formation of Q1 without any known by-products appears to be a real masterpiece of nature. In Q1, every third atom is chlorine, which accounts for 64% by weight (Vetter 2002). Moreover, the heptachlorinated natural product Q1 is the compound that brings together the highest number of halogens of all HNPs discussed in this review, and only a very few HNPs top this degree of halogenation.

Several further properties confirmed that Q1 is an HNP, although the natural producer is still unknown. The HNP that most closely resembles the structure Q1 is the pentachloro-2-pyrrolyl-2-phenol (**9**) isolated from the terrestrial bacterium *Actinoplanes* sp. ATCC 33002 (Cavalleri et al. 1978) (see Fig. 5). Pyrrolylphenols appear to be the condensation products of phenyl and pyrrole units and may thus represent the link between the halogenated bipyrroles and diphenyl ethers (see Section III.C) in which two of the same units are dimerized. It is currently unknown whether these compounds can be formed from the same enzymes, and will be a matter of conditions and substrate availibilities, or are products of specific enzymes and organisms.

However, a marine source of Q1 appears to be more plausible. The respective pentabromo isomer of *g*, pentabromopseudilin, also exists. Originally isolated from the marine bacterium *Alteromonas luteoviolaceus*, pentabromopseudilin is both antibiotic and cytotoxic (Burkholder et al. 1966; Laatsch and Pudleiner 1989). Except these examples and the 2,2′-bipyrroles (Section III.A), HNPs from marine bacteria are relatively scarce (Huth 1999). Bacterial HNPs are produced in the late logarithmic phase of growth or in the stationary phase (van Pée 1996). It is largely unknown why these compounds are produced by the bacteria, although they have been mostly discovered upon screening for antibiotics (van Pée 1996).

Historic Data, Identification, and Linking to Known Natural Sources. Given the high abundance of Q1 in various samples from Africa (Vetter et al. 1999b), the Antarctic (Vetter 2000), and Australia (Vetter et al. 2001a), it was likely that other researchers had detected Q1 as well. Using a donated standard of Q1, it was subsequently determined that Q1 is identical with an unknown compound originally labeled U3 in several papers published since the beginning of the 1980s (Hackenberg et al. 2001; Ballschmiter and Zell 1981). However, no attempts to elucidate the structure were made then. In 1996, Weber and Goerke (1996) described an abundant compound in Antarctic samples that showed the molecular ion at *m*/*z* 384. Owing to GC/EI-HRMS investigations the molecular formula $C_{10}H_3Cl_7O$ was assigned to the compound (Weber and Goerke 1996). However, the deviation in the HRMS measurements from the theoretical value of 383.800 of $C_{10}H_3Cl_7O$ was 15 mu (0.015 u) whereas the correct molecular formula of Q1 (383.812; see Table 2) differed only by 3 mu from the value measured by Weber and Goerke. Therefore, this apparent misinterpretation was not

a matter of an inadequacy of the analytical method but that nitrogen-containing compounds were not taken into account at that time. This structural feature would have meant that Q1 is a natural product, which appeared to be unthinkable in those days. Weber and Goerke also mentioned elimination of C_2HOCl and other fragment ions from the molecular ion, which must now be revised. The possibility that there is nitrogen in compounds bioaccumulated in higher organisms was ignored until the discovery of the HDBPs (Tittlemier et al. 1999) and Q1 (Vetter et al. 1999a). In 1997, van den Brink (1997) studied three less commonly known contaminants in Antarctic samples. A figure (7.2b) published in his article shows the GC/MS spectrum of a nonachlor isomer (*trans*-nonachlor or MC6, which was later identified as Q1 (Vetter et al. 2003b). Thus, Q1 was not a new discovery in gas chromatograms but a new interpretation of a compound detected for a long time, i.e., the addressing of its natural origin. Even though Vetter et al. determined the correct molecular formula, several misidentifications were circulated until the structure was determined by synthesis. The unique structural feature of the 1,2′-bipyrrole backbone (see Fig. 5) finally added the missing pieces to the Q1 puzzle (Jun et al. 2002).

PC parameters were estimated or determined for Q1 as well (see Table 3). The log K_{OW} clearly exceeds the target value of 5.0 for bioconcentration but was lower than the value determined for HDBPs while still in the range of pentachlorobiphenyls (Table 3). The lack of any hydrogen directly attached to the aromatic system can be related to some chemical stability. The water solubility is particularly low, and molecular modelling clarified that the Q1 molecule is not planar. The unique appearance of Q1 is striking, because very little evidence was produced for the presence of one of the additional 78 theoretically possible monochloro- to hexachloro homologues in environmental samples (Vetter et al. 2003b). Hackenberg et al. (2001) detected traces of hexachloro isomers whereas Vetter and Jun (2002) isolated and elucidated the structure of one of the hexachloro isomers (see Fig. 5). It is unclear if hexachloro isomers of Q1 would be original HNPs or transformation products of Q1. However, hints on mixed halogenated congeners and the brominated analogue of Q1 (Teuten et al. 2005b) may bring more light into the otherwise mysterious story of Q1.

Reference standards of Q1 are commercially available from LGC Promochem so that researchers from all over the world are able to determine Q1 in food and environmental samples.

Analytical Aspects. As for HDBPs, all standard sample cleanup procedures for POPs can be applied to the analysis of Q1. GC/ECNI-MS is the method that offers the highest selectivity and sensitivity for the detection of Q1 (Vetter et al. 2003b). For this purpose, the most abundant isotope masses of the molecular ion (m/z 386 and m/z 388) are recommended. The limit of detection for Q1 in the GC/ECNI-MS-SIM mode was ~0.13 pg (Vetter et al. 2000). Relative to *trans*-nonachlor, the GC/ECNI-MS response

was 4.5 times higher than the ECD response (Vetter et al. 2000). GC/EI-MS is about 1–2 orders of magnitude less sensitive for Q1 than GC/ECNI-MS. The suggested SIM masses in the GC/EI-MS mode are also *m*/*z* 386 and *m*/*z* 388, but the $[M\text{-}Cl]^+$ (SIM masses *m*/*z* 351 and *m*/*z* 353) and the $[M\text{-}2Cl]^+$ (SIM masses *m*/*z* 316 and *m*/*z* 314) are more abundant (Vetter 2000).

GC/ECD is only suitable for samples in which Q1 is relatively abundant, which appears to be the case only in environmental samples from the Southern Hemisphere because of their lower contamination with man-made POPs. Q1 elutes from DB-5-like columns before *p,p′*-DDE and may coelute with *trans*-nonachlor (Vetter et al. 1999b). Thus, the use of the ECD for Q1 determination is only possible after a thorough testing of the GC oven program with potential interfering compounds. Irrespectively, coelution with Q1 may cause overestimation of nonachlor concentrations in environmental and food samples when GC/ECD is used. This problem can be partly solved by separation of aromatic and aliphatic organochlorine compounds. Under such conditions, Q1 is usually found in the PCB fraction while nonachlor elutes into the chloropesticide fraction (Vetter 2002), although under certain circumstances partial elution into the more-polar chloropesticide fraction has initially been reported (Vetter et al. 1999b). The nonpolar behavior of Q1 during sample cleanup indicates that the nonbinding *n*-electron pair on the nitrogen is fully in possession of the pyrrole ring system to create the aromatic character. Likewise, it was not possible to protonate the pyrrole nitrogen.

A high-selectivity method for Q1 is obtained when GC is used in combination with a phosphorus-nitrogen detector (PND) (Melcher and Vetter 2004). Nitrogen-containing polyhalogenated compounds are hardly found in the anthropogenic "POP"-fraction and Q1 and the HDBPs almost exclusively give response in the PND owing to the two nitrogens (Fig. 6). The detection limit for Q1 in the PND (~20 pg) is relatively high because nitrogen amounts only for ~7.3% of the molecular weight. However, this detector may be useful for the identification of further N-containing HNPs (Melcher and Vetter 2004).

Hackenberg et al. (2003) developed a technique that allows estimation of PC parameters including water solubility and log K_{OW} alone from GC retention data. The suitability was tested with Q1, and this method may be used for other HNPs with unknown structure as well.

Distribution and Concentrations of Q1 In the Environment. Q1 was a very prominent peak in the GC/ECD chromatograms of marine mammals from Africa, the Antarctic, and Australia. The highest Q1 level reported to date (14,000 ng/g) were found in samples from Oceania (Vetter et al. 2003b); this also marks the highest concentration of a HNP determined in environmental samples to date (Table 5). Q1 was detected in the brain of Antarctic skuas (Vetter et al. 2000), in the blubber and brain of Antarctic fur seals

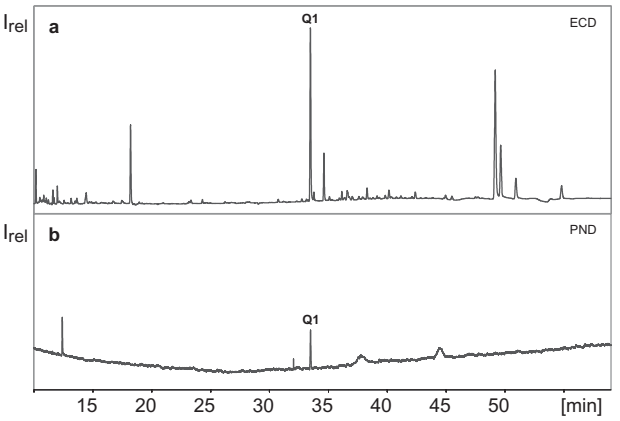


Fig. 6. GC/ECD (*top*) and GC/PND (*bottom*) chromatograms of a dolphin from Australia. The PND chromatogram underscores the high selectivity of this detector (Melcher and Vetter 2004).

(Vetter 2000, 2002), and also in human milk (Vetter et al. 2000). However, Q1 was virtually not present in ringed seals from Spitsbergen or the Canadian Arctic, as well as Baltic seals (Vetter et al. 2000; Vetter 2002). Q1 was detected neither in fish from Hongkong nor in seals from Lake Baikal (Vetter 2002). Low concentrations (~0.1% of *trans*-nonachlor) were determined in beluga from Canada (Vetter 2002). Four tissues of Antarctic fur seal contained Q1 and *p,p′*-DDE in virtually the same amounts, so that a similar body distribution and bioaccumulative behavior was suggested (Vetter 2000). However, cetaceans contained much higher Q1 concentrations than seals from the same habitat (Vetter et al. 2000; Vetter and Jun 2003).

Three fish species from the Antarctic resulted in lowest concentrations in the bottom invertebrate feeder than in the other fish-feeding species where Q1 was the third most abundant compound, after HCB and *p,p′*-DDE (Weber and Goerke 2003). Thus, it was suggested that the natural source may be in the upper water column and there most likely in the euphotic zone (Weber and Goerke 2003). However, Hackenberg et al. (2001) determined highest concentrations in fish from the South Atlantic and deep-sea fish from the North Atlantic. Moreover, Q1 was detected in Mediterranean deep-sea fish (Vetter 2002). Q1 was detected in a commercial fish-oil capsule and in cod livers canned in 1948 (Vetter and Stoll 2002). In addition, Q1 was detected in fish food and in fish fed with the respective food (Vetter and Stoll 2002). Traces were also detected in an omega-3 egg (Vetter and Stoll 2002).

Concentrations in Antarctic air (Signy Island, 60°72′S, 45°60′W) were relatively low; however, no other compound than Q1 was more abundant

Table 5. Concentrations (selection) of Q1 (ng/g lipids) in the marine environment and food.

Species	Location	Q1	PCB 153	Ratio	Source
South African fur seal (*Arctocephalus pusillus*) ($n = 11$)	Cape Cross (Namibia, Africa)	43–540[a,b]	2–273[a]		Vetter et al. (1999b) Vetter (2000)
South polar and mixed pair Skua eggs ($n = 7$)	Potter Peninsula (Antarctic)	3–110[b,c]	1.5–60		Weichbrodt et al. (1999)
Brown skua ($n = 4$)	Potter Peninsula (Antarctic)	40–194[b,c]	17–147		Weichbrodt et al. (1999)
Antarctic air ($n = 3$)	Signy Island (Antarctic)	1.1–1.4[b] fg/m^3	n.d.		Vetter et al. (2000)
Human milk ($n = 4$)	(Faeroe Islands)	12–230 ng/g			Vetter et al. (2000)
Bottlenose dolphins	Queensland, Australia	690–14,000[b]	230–8,800		Vetter et al. (2001a)
Common dolphin	Queensland, Australia	2,090[b]	175		Vetter et al. (2001a)
Green turtle	Queensland, Australia	15[b]	70		Vetter et al. (2001a)
Dugongs	Queensland	n.d.–246[b]	19–170		Vetter et al. (2001a)
Monk seal ($n = 14$)	Mauretania	9–117[b]			Vetter (2002)
African fur seal ($n = 1$) (blubber/ kidney/liver/lung)	Namibia	323/62/11/2.1)[b]	n.d.		Vetter (2002)
Air	Lista, southern Norway	detected			Vetter et al. (2002a)
White-tailed sea eagle	Norway	3–4 ng[c]			Herzke et al. (2005)
Fish liver *Gobionotothen gibberifrons* (1987/1996)	Antarctic	0.4/0.6 (mean)	1.1/2.1		Weber and Goerke (2003)
Fish liver *Champsocephalus gunnari* (1987/1996)	Antarctic	2.5/3.2	0.4/0.5		Weber and Goerke (2003)
Liver					
Fish liver *Chaenocephalus aceratus* (1987/1996)	Antarctic	4.0/4.9	0.9/1.8		Weber and Goerke (2003)

[a]Highest values in different samples; cocorrelation between the concentrations of Q1 and PCB 153.

[b]Concentrations corrected relative to estimations in the original papers where quantification was carried out using the response factor of *trans*-nonachlor, which turned out to be higher than that of Q1 (Vetter et al. 2003b).

[c]Wet weight.

in Antarctic than in Arctic air (Vetter et al. 2000). It should also be noted that Q1 concentrations, and that of anthropogenic POPs, in the Weddell Sea (which is more than 1,000 km south of Signy Island) were significantly higher than those close to the Antarctic convergence. Thus, it may be possible that the air in the Weddell Sea could contain higher concentrations of Q1 despite the lower air temperatures, which alter the air–water equilibrium in favor of the water phase.

Conclusions (Q1). Molecular modeling led to the observation that the planar pyrrole units have to undergo pyramidal configuration to surmount the barrier of the interannular N-pyrrole–C-pyrrole bond. Thus, Q1 cannot be planar, and this feature was made responsible for the very little or absent activity in the AHR assay (Vetter et al. 2004). Note that this is different from HDBPs, which were moderately toxic in the AHR test although estimated not to be planar as well. Q1 itself is symmetrical and thus a nonchiral molecule so that the hindered rotation about the interannular bond does not lead to atropisomers. However, an isolated Q1-Hex congener (**8**) was nonsymmetrical, and both features, hindered rotation and chirality due to the nonsymmetrical substitution pattern, lead to the formation of atropisomers, a feature than can be investigated by enantioselective gas chromatography. In fact, enantioselective analysis elucidated the structure in this particular case (Vetter and Jun 2002). The question for a natural product is this: Would Q1-Hex be naturally formed enantiopure or as a racemate? Because Q1 is not chiral and Q1-Hex was only chemically synthesized, and thereby of course was formed in racemic composition, this compound cannot be used to be studied in detail. However, several HDBPs are chiral and would be suitable for studying this problem. Unfortunately, any efforts undertaken to date to enantioresolve HDBP astropisomers failed thus far (Vetter et al., unpublished results, 2005). Although Q1 was detected in marine samples from all six continents (see Table 5), the highest concentrations in the ppm range are found in the Southern Hemisphere and particularly in samples from Australia.

C. Brominated Phenoxyanisoles (MeO-BDEs)

Brominated phenoxyanisoles are also named as brominated methoxydiphenyl ethers. The second name refers to the structural similarity with the man-made (poly)brominated diphenyl ethers (PBDEs), which are in wide use as flame retardants (see also Section III.C.). The currently widely applied nomenclature does not use IUPAC rules but "treats" the respective compounds as metabolites of BDEs. The resulting names have their starting point in the plain BDE, without a methoxy group. The IUPAC number valid for the same substitution pattern as for PCBs is assigned to this substructure. In a second step, the additional position of the methoxy group is determined. In this way, 2′-MeO-BDE 68 is the short name for

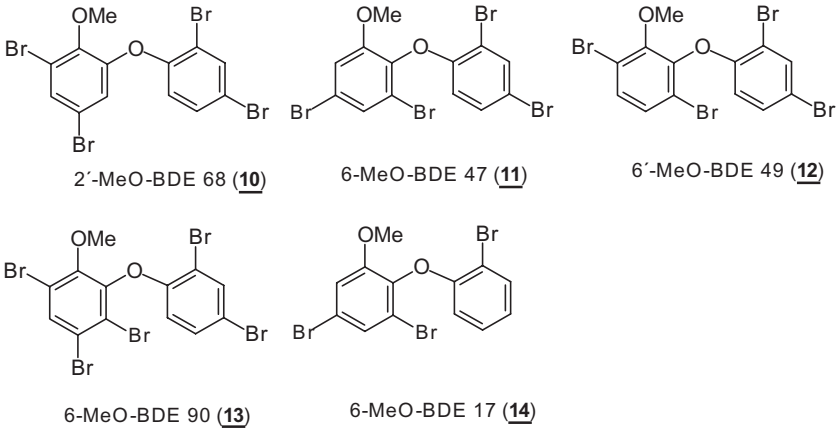


Fig. 7. Structures of relevant tetrabromophenoxyanisoles (**10,11,12**), as well as examples of a penta- and a tribromophenoxyanisole detected in marine fish and mammals (**13,14**).

2′-methoxy-2,3′,4,5′-tetrabromodiphenyl ether or 4,6-dibromo-2-(2′,4′-dibromo)phenoxyanisole (Fig. 7, **10**).

Since the initial paper by Sharma and Vig (1972), natural products chemists have discovered a wide range of tri- to hexabromophenoxyanisoles (or MeO-triBDEs to MeO-hexaBDEs), primarily in sponges but also in algae and cyanobacteria (Carté and Faulkner 1981; Fu et al. 1995; Elyakov et al. 1991; Kuniyoshi et al. 1985; Capon et al. 1981; Asplund et al. 2001; Cameron et al. 2000; Salva and Faulkner 1990; Handayani et al. 1997; Bowden et al. 2000; Agrawal and Bowden 2005; Malmvärn et al. 2005b). It is known that MeO-BDEs exist together with their unmethylated OH-BDE homologues. In the red alga *Ceramium tenuicorne*, for instance, OH-BDEs were about two orders of magnitude more abundant than MeO-BDEs (Asplund et al. 2001). 6-MeO-BDE 47 (**11**) and related compounds were also isolated from the tropical mollusk *Asteronotus cespitosus*, which feeds on sponges (Vetter et al. 2002b). Note, however, that the mollusk accumulated rather than synthesized the MeO-BDEs.

It would be curious if sponges, a species largely without lipids, were to produce highly lipophilic compounds such as MeO-BDEs. In fact, it was shown that cyanobacteria living on the sponge produce brominated secondary metabolites and that *Oscillatoria*, which has a high lipid content, is the original producer of MeO-BDEs (Unson et al. 1994; Moore et al. 2002). It has been suggested that sponges produce the brominated phenoxyphenols and that the cyanobacteria produce the methylether (anisole), which would partly explain the varying proportions between OH- and MeO-BDEs (Vetter and Jun 2003). Malmvärn et al. (2005a) indicated that cyanobacteria may be sources of OH-BDEs, MeO-BDEs, and PBDDs.

These observations may indicate time, or season-dependent variations but also complex varying patterns between individuals. The ecological and biological background on the natural formation of MeO-BDEs is beyond the topic of this article, but it should be borne in mind that variations in the HNP content within one or different populations may be more pronounced than for anthropogenic compounds that have been described with diverse models.

Historical Data, Identification, and Linking to Known Natural Sources. In 1997, Haglund et al. (1997) screened marine samples from the Baltic Sea for brominated aromatic compounds. They detected anthropogenic polybrominated diphenyl ethers (BDEs) including the dominated BDE 47 and three methoxy derivatives of tetrabromo diphenyl ethers (MeO-tetraBDEs), whereof the most abundant accounted for ~50% of the BDEs. Similarly, Asplund et al. (1999) found MeO-BDEs on one level with BDEs in Baltic salmon muscle and egg. The initial dilemma was, as discussed in these papers, that it could not be unequivocally clarified if these compounds were metabolites of BDEs or bioaccumulated HNPs (Haglund et al. 1997; Asplund et al. 1999). However, this uncertainty toward the origin of the MeO-BDEs in both papers stimulated an intense research program in Sweden that soon produced evidence for a natural origin of MeO-BDEs. Today it is known that the initially detected three MeO-tetraBDEs are 2′-MeO-BDE 68 (**10**), 6-MeO-BDE 47 (**11**), and 6′-MeO-BDE 49 (**12**), 6′-MeO-BDE 49 being the first eluting and least relevant congener (Asplund et al. 1999; Malmvärn et al. 2005a). In addition, 2′-MeO-BDE 68 and 6-MeO-BDE 47 were recently isolated from whale blubber and investigated by spectroscopic methods (Teuten et al. 2006).

As seen from the structural code, 6-MeO-BDE 47 differs from BDE 47, a key compound in products used as brominated flame retardants (BFRs), only in the MeO-substituent in the *ortho*-position (Asplund et al. 1999). Reports on residues of BFRs in the environment have increased of late (de Boer et al. 2000), and it seems, for lack of plausible other sources, that the unknown brominated compounds detected in marine organisms may be metabolites of BFRs. A study of the contamination of marine mammals in Australia led to the detection of a series of nonpolar brominated compounds (originally labeled BC-2, BC-1, BC-3, BC-10, and BC-11) including two MeO-tetraBDEs later identified as 2′-MeO-BDE 68 (BC-2) and 6-MeO-BDE 47 (Vetter et al. 2001a; Vetter 2001; Melcher et al. 2004). The concentrations of anthropogenic BFRs were very low, and a natural source was plausible. Shortly after, sponges (*Dysidea* sp.) from the same region were identified as the producers of 2′-MeO-BDE 68 and 2′,6-diMeO-BDE 68 (BC-11, see Section III.D) (Vetter et al. 2002b; Cameron et al. 2000); this was eventually the first direct identification of a bioaccumulated natural organohalogen in wildlife. Teuten et al. isolated 2′-MeO-BDE 68 and 6-MeO-BDE 47 from whale blubber and determined both $\Delta^{14}C$ and $\delta^{13}C$

ratios (Teuten et al. 2005a). Particularly, radiocarbon measurements confirmed the natural origin of these MeO-BDEs because the $\Delta^{14}C$ ratio of about +100 ppm was in range of new inorganic carbon in the Atlantic Ocean whereas technical BDEs, PCBs, and DDT had $\Delta^{14}C$ values of about –998 ppm (Teuten et al. 2005a).

Malmvärn et al. (2005a) identified the red alga *Ceramium tenuicorne* from the Baltic Sea as a producer of phenoxyphenols and phenoxyanisoles. Moreover, fish from the proximity of the algae also contained many of these HNPs. The algae not only contained the major MeO-tetraBDEs but also homologues. However, the MeO-BDE pattern in algae and fish was not identical (Malmvärn et al. 2005a). Varying concentrations and ratios of MeO-BDEs in individuals may be the result of different bioavailability, uptake, elimination, metabolism, and selective retention of the HNPs but also different ages, feeding behavior, and distribution of the investigated species; however, they may also indicate the presence of different natural producers found in different habitats that enter the food web in a different way (Melcher et al. 2005a).

Although different mechanisms may change the ratios of the dominating MeO-BDEs, 2′-MeO-BDE 68, and 6-MeO-BDE 47 on the way from the natural producer to high-trophic biota, it appears that marine mammals that received the MeO-BDEs from sponges, or associated organisms, are more abundant in 2′-MeO-BDE 68, whereas those originating from algae, or associated organisms, are dominated by 6-MeO-BDE 47. However, this hypothesis needs further clarification (see also below). In addition to 2′-MeO-BDE 68 and 6-MeO-BDE 47, several tri- to hexa-MeO-BDEs (see Fig. 7; **13** and **14** for examples) and even mixed halogenated phenoxyphenols and phenoxyanisoles were identified in higher marine biota (Asplund et al. 1999; Sinkkonen et al. 2004; Marsh et al. 2004a; Malmvärn et al. 2005a; Melcher et al. 2005a). Mixed halogenated (one Cl, several Br) phenoxyphenols and phenoxyanisoles point more toward algae being the natural producers, as mixed halogenated compounds in sponges are rare. This observation also produced evidence that OH-BDEs co-occur with MeO-BDEs (Fig. 8).

The biosynthesis of brominated phenoxyanisoles is not known in detail, but the natural MeO-BDEs known to date share the presence of the methoxy group in the *ortho*-position relative to the O-bridge. Thus, it was concluded that the phenol- or methoxy group occurs exclusively in the *ortho*-position. However, natural products chemists have isolated related HNPs with hydroxyl groups in the *para*-position (see Section III.D) (Higa et al. 1979).

Note that both 2′-MeO-BDE 68 and 6-MeO-BDE 47 have previously been mislabeled, and this has caused inaccurate citations in the initial phase of research (Asplund et al. 1999; Vetter et al. 2001a; Sinkkonen et al. 2004). The highest concentrations of MeO-BDEs detected in marine mammals are on one level with the highest concentrations of BFRs determined in environmental samples (Vetter et al. 2002b; de Boer et al. 2000).

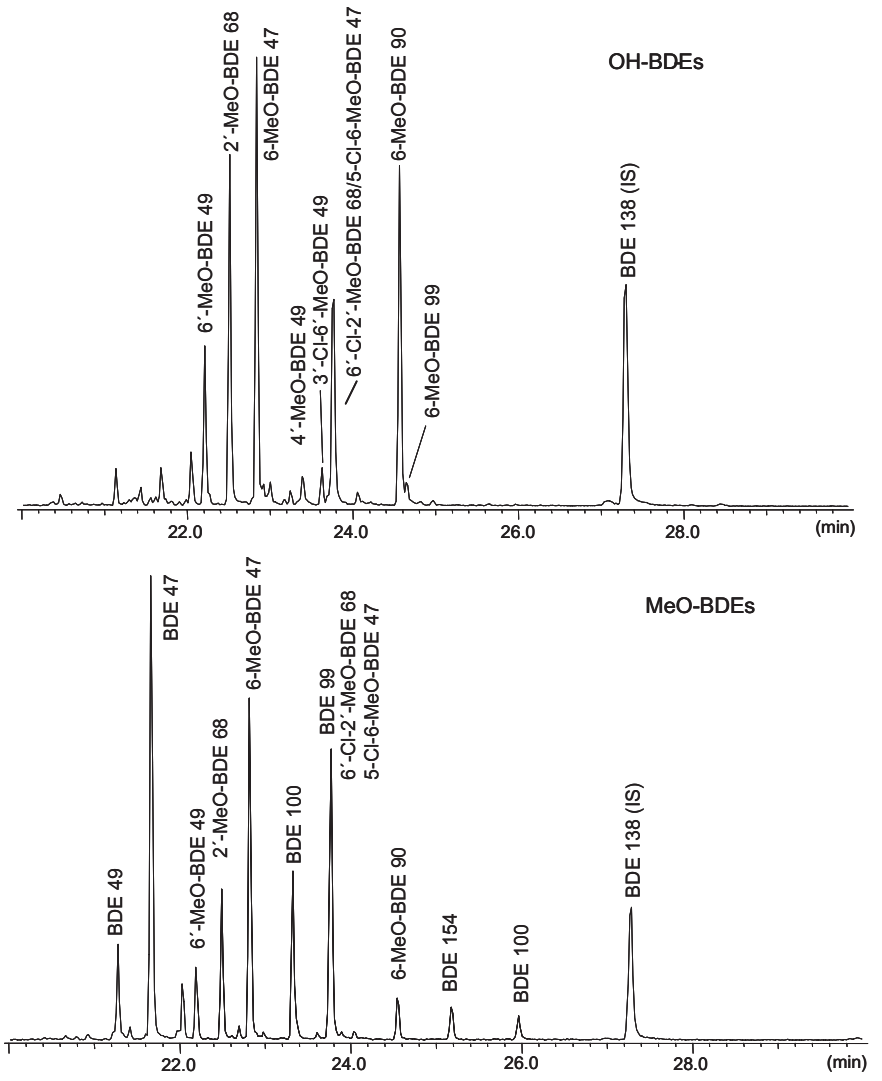


Fig. 8. GC/MS ion chromatogram (CP-Sil 8, *m*/*z* 79 and 81) of Baltic salmon blood. *Top*: OH-BDEs after conversion into the respective anisoles; *bottom*: the MeO-BDE fraction that was separated from the phenols before analysis (*bottom*).
Related MeO-BDEs of most OH-BDEs were detected in the sample (Marsh et al. 2004b).

Authentic MeO-BDE reference standards have been synthesized by several groups (Marsh et al. 1999, 2003, 2004a; Vetter and Jun 2003; Nikiforov et al. 2003), and 6-MeO-BDE 68 is commercially available from LGC Promochem whereas a range of both OH-BDE and MeO-BDE standards is available from CIL.

Analytical Aspects. A sensitive detection for MeO-BDEs is obtained with GC/ECNI-MS in the SIM mode using *m*/*z* 79 and 81 (Haglund et al. 1997; Vetter et al. 2001a). However, several potential coelutions with other brominated compounds in samples have been detected (Marsh et al. 2004a; Vetter et al. 2003b). Therefore, additional confirmation is recommended, which becomes more important if not only the two major MeO-tetraBDEs are analyzed but also tri-, penta-, and hexabromo phenoxyanisoles. Additional confirmation can be obtained by the determination of *m*/*z* 159 and *m*/*z* 161 ($[HBr_2]^-$), which is only abundant in GC/ECNI-MS spectra of BDEs with a bromine subsitutent in the *ortho*-position (see Section II) (Melcher et al. 2005a). This structure is fulfilled for all known naturally produced MeO-BDEs, but reductive debromination may lead to metabolites of natural MeO-BDE that do not contain any Br in the *ortho*-position (Melcher et al. 2005a).

GC/EI-MS is less sensitive, but the molecular ion is abundant and provides a much higher selectivity for MeO-BDEs (Pettersson et al. 2004). GC/EI-MS was shown to distinguish *ortho*- (these form $[M\text{-}94]^+$ fragment ions) from *para*- (these form $[M\text{-}15]^+$ fragment ions) and from *meta*-substituted MeO-BDEs (these do not show the respective fragment ions observed in the EI-MS of the isomers) (Marsh et al. 2003, 2004b). With this information, the unknown MeO-tetraBDE by-product of the synthesis of 2′-MeO-BDE 68 (Vetter and Jun 2003) is also an *ortho*-MeO-tetraBDE.

It became evident that, besides MeO-BDEs, occurrence of OH-BDEs should be explored as well. Although the latter are usually detected in blood but not in blubber, the co-occurrence of both classes is an important feature, and it is still known where the transfer of the phenols into the anisoles actually takes place. Because the halogenated phenoxyphenols have to be methylated before GC analysis, a preseparation step of OH-BDEs and MeO-BDEs is necessary, which allows the individual determination of both classes of compounds (see Fig. 8). Several methods have been developed, for instance, for the determination of OH-PCBs, and are mostly based on KOH partitioning as used by Malmvärn et al. (2005a). Verrevault et al. (2005) reported the presence of 3-MeO-BDE 47 in glaucous gulls (*Larus hyperboreus*) from the Norwegian Arctic, which, owing to the methoxy-group in the *meta*-position, would not support a natural source for this compound. However, it was mentioned by the authors that due to the known coelution with 2′-MeO-BDE 66 (Marsh et al. 2004a), it may also be this congener or a mixture or both (Verreault et al. 2005). In these samples, both MeO-BDEs and OH-BDEs were detected along with high concentrations of anthropogenic BDEs (Verreault et al. 2005). Thus, these samples were suspected of containing MeO-BDEs and OH-BDEs of both natural and anthropogenic origin. In this study, 2′-MeO-BDE 68 was not detected at noticable concentrations; however, 6-MeO-BDE 47 was (Verreault et al. 2005). Moreover, 2′-MeO-BDE 68 was not detected in polar bears from Svalbard (Nikiforov et al. 2003). These examples indicate that determina-

tion of the natural source of MeO-BDEs is complex. Nevertheless, it is unequivocally clear that natural sources do exist (Vetter et al. 2002b; Teuten et al. 2005a). In the laboratory, OH-BDEs can be prepared from MeO-BDEs by treatment with BBr_3 in 1,2-dichloroethane (Francesconi and Ghisalberti 1985; Vetter and Jun 2003).

Distribution and Concentrations of MeO-BDEs in the Environment. Table 6 lists selected concentrations determined in environmental samples. In the first article on MeO-BDEs, they were not detected in human adipose tissue (Haglund et al. 1997). Fish samples from different locations, except Arctic guillemot and freshwater fish, contained MeO-BDEs (Sinkkonen et al. 2004). MeOBDEs up to several mg/kg were detected in dolphins from Australia (Vetter et al. 2001a; Melcher et al. 2005a). Except for the Australian cetaceans, 6-MeO-BDE 47 is usually more abundant than 2′-MeO-BDE 68 (see Table 6).

Concentrations of 2′-MeO-BDE 47 in blood of nestlings of white-tailed sea eagles from the Swedish Baltic coast increased from May to June, i.e., the period at which the potential bioproduction (green algae blooms) occurs. Such an increase was not observed for anthropogenic POPs (Olsson et al. 2000). In four locations from the Baltic Sea, MeO-BDEs were ~20 fold more concentrated than in the Kattegatt (North Sea) (Asplund et al. 2004).

Malmvärn et al. (2005a) investigated the presence of OH-BDEs and MeO-BDEs in algae and fish living in the same habitat. Although many compounds were found in both species, some were not. For instance 6′-OH-BDE 49 and 4′-OH-BDE 49, which was suspected to be a metabolite of anthropogenic PBDEs, were not found in the algae. However, there are too many parameters that remained unknown to put these findings in the right light. For instance, the investigated algae may not necessarily be the only natural producer of OH- and MeO-BDEs in the habitat.

Kierkegaard et al. (1999) studied BDE 47 and 6-MeO-BDE 47 in pike taken between 1968 and 1996 in Lake Bolmen (Sweden). Although the anthropogenic BDE 47 showed an increasing trend, the MeO-BDE decreased in the same period. If 6-MeO-BDE 47, was the metabolite of BDE 47, one could have expected an increasing trend for 6-MeO-BDE 47 as well. This finding supports the thinking that the bulk of the MeO-BDE goes back to natural production (Kierkegaard et al. 1999). However, an increasing trend of euthrophication has been observed in the Lake since the 1970s (Kierkegaard et al. 1999), which suggests that the natural producer of 6-MeO-BDE 47 has been driven out by organisms that found favorable life conditions in the changed ecosystem.

It is noteworthy that Asplund et al. (1999) detected both MeO-BDEs and OH-BDEs in salmon blood. Already in this review it was proposed that both classes of compounds have the same origin. The levels of the OH-BDEs were estimated to account for ~30% of the MeO-BDEs. Hovander

Table 6. Concentrations (selection) of MeO-BDEs (ng/g lipids) in the marine environment and food.

Species	Location	2′-MeO-BDE 68 (ng/g)	6-MeO-BDE 47 (ng/g)	Sum MeO-BDEs (ng/g)	Source
Salmon blood plasma (lw)	Baltic Sea	~60[a]	~170[a]	~270 ng/g	Asplund et al. (1999)
Herring	Baltic Sea			7.4–34	Haglund et al. (1997)
Grey and ringed seal blubber (liver)	Baltic Sea	8.5–40 (0.2–1.0)[a]	95–160 (1.5–1.8)[a]	121.5–220 (2–3.7)	Haglund et al. (1997)
Fish oil		0.1–8.7[a]	0.3–28[a]	0.4–30	Haglund et al. (1997)
Salmon muscle		8[a]	28[a]	30	Haglund et al. (1997)
Dolphins Different species	Mediterrrane an Sea	<1–167[a]	<1–628[a]	<3–808	Petterson et al. (2004)
Arctic cod	Arctic, Norway			0.3–17[a]	Sinkkonen et al. (2004)
Salmon	Atlantic			3.5–6.8	
Salmon	Baltic Sea				
Cetaceans	Australia	1,200–11,200	790–1,910	100–1,530	Melcher et al. (2005a)
Crocodile eggs	Australia	57–69	200–240	Not determined	Melcher et al. (2005a)

[a]Peak assignment by the present author derived from data published in meantime.

et al. (2002) detected 6-OH-BDE 47 in human plasma (peak 39) but its source could not be identified.

Another interesting finding was addressed by Melcher et al. (2005a) on their detection of eight MeO-triBDE congeners structurally related to 2′-MeO-BDE 68 and 6-MeO-BDE 47 in samples from Australia. The lack of identification of several MeO-triBDEs by natural products chemists indi-

cated that, at least to some degree, metabolism of 2′-MeO-BDE 68 and 6-MeO-BDE 47 has played an important role in the formation of MeO-triBDEs. Their formation, however, may not have occurred in dolphins but could also have occurred during transfer from the natural producer to marine mammals via the food chain (Melcher et al. 2005a). This report appears to be one of the first hints in the literature that not only HNPs itself but also their transformation products can be found in the environment.

Conclusions. It has to be repeated that evaluation of the natural contribution to the environmental load of OH- and MeO-BDEs is still not possible. With respect to anthropogenic sources there are open questions as well. For instance, hydroxylation of BDEs is known to occur in a similar manner as hydroxylation of PCBs, for instance, in marine mammals and in fish. However, the methylation step is somewhat strange because MeO-PCBs have not been described in the same context. The ecological role of the natural MeO-BDEs appears to be chemical protection (Gribble 1999). 6-MeO-BDE 47 exhibited antibacterial activity against *Escherichia coli* and other microorganisms (Carté and Faulkner 1981; Kuniyoshi et al. 1985) and acted as enzyme inhibitors (Fu et al. 1995). In another study, 2′-OH-BDE 68 was inactive in two biotests (Lui et al. 2004).

D. Compounds Related to Brominated Phenoxyanisoles

Compounds related to MeO-BDEs are MeO-BDDs, diMeO-BDEs, and diMeO-BBs (Fig. 9, **15**–**19**) all of which have been identified by natural product chemists and/or in higher organisms. These structures are of particular interest because they resemble very closely those of anthropogenic or toxic compounds. In 1981, Carté and Faulkner suggested the presence of brominated dibenzo-*p*-dioxins in sponges but could not confirm the presence of 2,4,7-tribromodibenzo-*p*-dioxin (2,4,7-triBDD) in the poriferans (Carté and Faulkner 1981). However, some 20 yr later Utkina et al. (2001, 2002) identified 4-MeO-1,2,6,8-tetraBDD (**15**), 5-MeO-1,3,6,7-tetraBDD (**16**), and 5-MeO-1,3,7-triBDD (**17**) and the respective OH-PBDDs in sponges (*Dysidea herbacea*) from Northwest Australia. Although the chemical names of the three dibenzo-*p*-dioxin derivatives may give the impression that the compounds have completely different structures, there is actually only one Br substituent that is different between (**15**) and (**16**), and in (**17**) only the varying Br is missing (see Fig. 9). The differences arise due to the chemical naming system based on the unsubstituted PBDD, which receives different labeling for (**15**) and (**16**). So far, the MeO-PBDDs have not yet been found accumulated at a higher trophic level. Vetter and Wu (2003) used charcoal chromatography and found no evidence for the presence of an abundant PBDD derivative in the investigated dolphins. Very recently, however, unsubstituted di- and tribromodibenzo-*p*-dioxins of yet unknown isomer structure were determined by Haglund et al. (2005) in pike

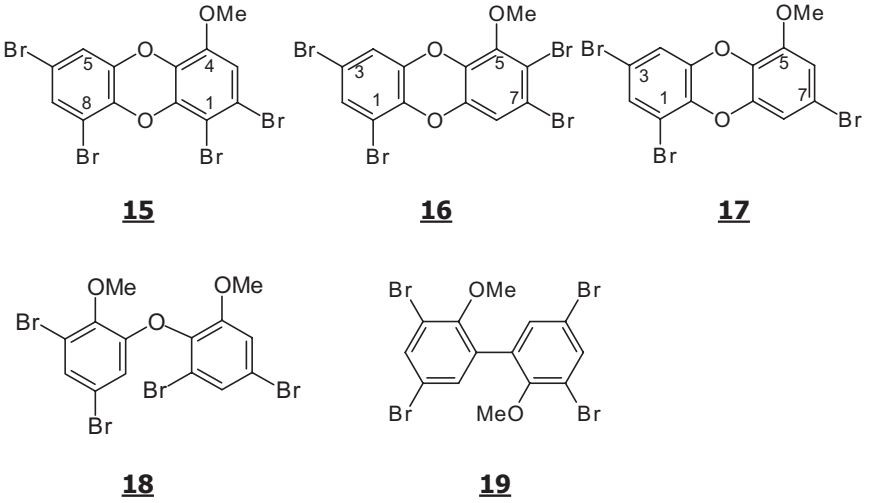


Fig. 9. Structures of methoxylated tri- and tetrabromodibenzo-*p*-dioxins (**15**,**16**,**17**), 2′,6-dimethoxy-2,3′,4,5′-tetrabromodiphenyl ether (BC-10, **18**), and 2,2′-dimethoxy-3,3′,5,5′-tetrabromobiphenyl (2,2′-diMeO-BB 80, **19**).

from Kvädöfjärden (Baltic Sea). Owing to the unique presence at this site, it was proposed that the PBDDs may be of natural origin. However, this hypothesis requires a more thorough investigation, which is surely in process (see also next paragraph).

By contrast, dimethoxylated BDEs were unequivocally identified in marine mammals and food samples (Vetter et al. 2002b). Although diverse congeners including dihydroxy and hydroxylmethoxy derivatives were identified by natural product chemists (Norton and Wells 1980; Carté and Faulkner 1981; Utkina et al. 1987; Elyakov et al. 1991; Fu et al. 1995; Cameron et al. 2000; Voinov et al. 1991; Vetter et al. 2002b; Lui et al. 2004), only 2,6′-diMeO-BDE 68 (the molecular ion is found at *m*/*z* 542) have been described in marine mammals to date (Vetter et al. 2002b; Vetter and Jun 2003). 2,6′-diMeO-BDE 68 concentrations ranging from 0.2 to 49 ng/g were determined in marine mammals from the Pacific Ocean (Marsh et al. 2004b). It was reported that 2,6′-diMeO-BDE 68 and BDE 99 may coelute on DB-5-like columns (Vetter and Jun 2003). Because halogenated MeO-BDEs have also been detected at the retention time of BDE 99 (see Fig. 8b), the peak purity at the respective retention time should be checked, irrespective of the compounds to be analyzed. Fig. 10 illustrates that 6-OH-2′-MeO-BDE 68 (**20**) may be a precursor of both 2,6′-diMeO-BDE 68 (**18**) and the hypothetic 4-MeO-1,3,5,7-tetraBDD (**21**) (Vetter and Jun 2003). Note that reaction of the desmethoxy derivative of **20** to **21** would lead to plain PBDDs, as were descibed by Haglund et al. (2005). 2,6′-diMeO-BDE 68 and isomers did not show inhibitory activity in two bioassays (micro-

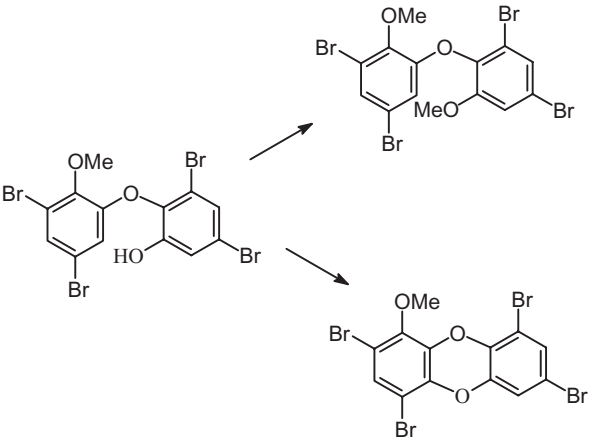


Fig. 10. Proposed mechanism of the formation of 2′,6-dimethoxy-2,3′,4,5′-tetrabromodiphenyl ether (BC-10, **18**) and 4-methoxy-1,3,5,7-tetrabromodibenzo-*p*-dioxin (21) from 6-hydroxy-2′-methoxy-2,3′,4,5′-tetrabromodiphenyl ether (**20**) (Adapted from Vetter and Jun 2003 with permission from Elsevier).

tubule proteins and meiotic maturation of starfish oocytes). The same was found for hydroxymethoxy derivatives whereas diOH-BDEs were active (Lui et al. 2004). Obviously, one hydroxyl group only or two methoxy groups reduced the bioactivity.

Marsh et al. (2004b) synthesized 2,2′-diMeO-BB 80 (**19**) and determined that this compound was identical with one previously detected in marine mammals from Australia (molecular ion *m*/*z* 526) (Vetter et al. 2001a). The abundance in Australian samples along with nondetectable PBBs and the identification of other HNPs in the samples (Fig. 11) clearly supports the natural source of 2,2′-diMeO-BB 80 (Vetter et al. 2001a). However, natural products chemists have not yet detected the compound. Note that peak assignment of BC-1 (2,2′-diMeO-BB 80) and BC-2 (2′-MeO-BDE 68) was switched in the initial work by Vetter et al. (2001a, 2002b) because both compounds eluted in reversed order from DB-5 and β-BSCD GC columns. Thus, the 2,2′-diMeO-BB 80 concentrations in Australian samples (erroneously labeled BC-2 in table 3 of Vetter et al. 2001a) were 250–4.100 ng/g in dolphins, 26 ng/g in a green turtle, and l03 ng/g in a dugong sample (Vetter et al. 2001a), whereas cetacean samples from the Pacific Ocean contained 12–800 ng/g of lipid weight (Marsh et al. 2004b).

Higa et al. (1979) found brominated hydroquinones and substituted diphenylethers and triphenylethers (Fig. 12; **22**–**26**) in worms. These compounds may stem from the dimerization of the respective hydroquinones also detected in the samples. It is noteworthy that these dimers and trimers have hydroxyl groups in the *para*-position (Higa et al. 1979). Therefore, it cannot be excluded that natural brominated 4-phenoxyphenols also exist.

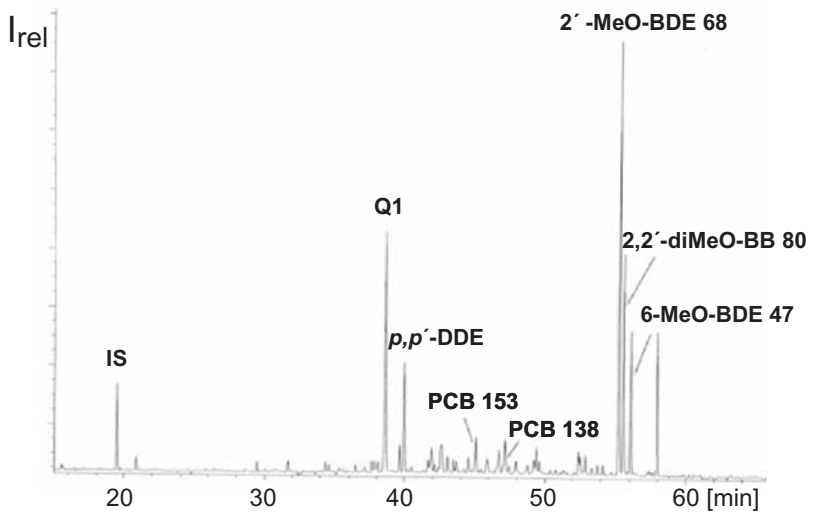


Fig. 11. GC/ECD chromatogram of common dolphin (*Delphinus delphis*) from Australia (Adapted from Vetter et al. 2001a with permission from Springer). Note that the peak assignment was corrected.

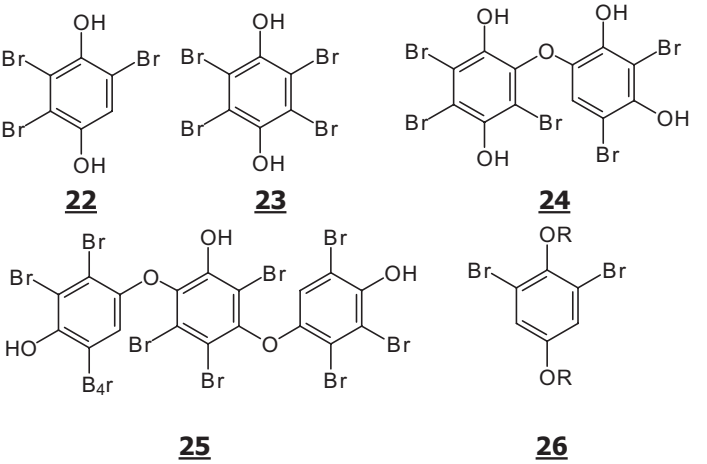
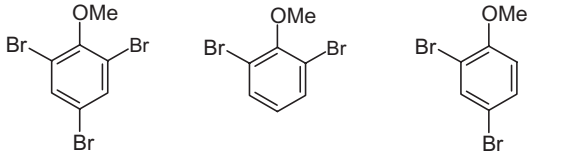


Fig. 12. Structures of bromoquinones and related compounds (**22**–**26**) (Illustrating Higa et al. 1979).

E. Brominated Phenols and Anisoles

2,4,6-Tribromoanisole (Fig. 13; **27**), structurally related bromo- and dibromoanisoles (**28**, **29**), and the corresponding bromophenols are regularly detected in marine fish by food control laboratories. However, publications in this field are relatively scarce (Rimkus and Wolf 1991; Miyazaki et al.



2,4,6-tribromoanisole **(27)** 2,6-dibromoanisole **(28)** 2,4-dibromoanisole **(29)**

Fig. 13. Structures of the most prominent natural bromophenols. 2,4,6-tribromoanisole (2,4,6-TBA or TBA, **27**), 2,6-dibromoanisole (2,6-DBA, **28**), and 2,4-dibromoanisole (2,4-DBA, **29**).

1981; Watanabe et al. 1983; Whitfield et al. 1998; Vetter et al. 2001b; Vetter and Stoll 2002). Bromoanisoles are also known as brominated methoxybenzenes.

As shown in Section III.C, brominated phenols and anisoles are linked to each other, so that when looking for sources, that which is said about anisoles is also valid for phenols and vice versa. Anisoles are readily produced from the respective phenols by microorganisms (Neilson et al. 1983) and fungi (Whitfield et al. 1997c). Brominated phenols and anisoles have both anthropogenic and natural sources. Owing to the low molecular weight and the volatility, dibromo- and tribromoanisoles were made responsible for a musty, corked off-flavor in wine (Chatonnet et al. 2004) and the acknowledged seafood flavor in fish and shrimps (Whitfield et al. 1998; Ma et al. 2005). The first one is most likely going back to anthropogenic sources whereas the second one is attributed to naturally produced compounds. Whitfield et al. (1998) studied a wide range of seafish and found bromophenols abundant in marine fish except for carnivorous pelagic species. Apart from these examples, the differentiation of the two potential sources is often ambiguous, and their abundance in coastal samples may in fact resemble a mixture of both sources. However, the only relevant anthropogenic sources for 2,4,6-TBP are its use (i) as a flame retardant in epoxy, polyurethane, plastics, paper, and textiles and (ii) as an important intermediate for the production of other commercial high-molecular weight flame retardants and fire extinguishing media (Eriksson et al. 2004; Hakk et al. 2004). Furthermore, 2,4,6-TBP is used as an impregnating agent by the wood industry (Mardones et al. 2003). These industrial application rates of TBP could never explain the high amounts and structural diversity of bromophenols and bromoanisoles detected in the environment.

Naturally occurring bromophenols and-anisoles are produced by marine worms (Higa et al. 1979; Gribble 2000), algae (Flodin and Whitfield 1999a), and probably also sponges (Vetter and Janussen 2005). It was demonstrated that the green algae *Ulva lactuca* contains a bromoperoxidase able to

convert phenol, 4-hydroxybenzoic acid, and 4-hydroxybenzylalcohol into bromophenols but failed using L-tyrosine as substrate (Flodin and Whitfield 1999a,b). In this process, only bromophenols with substituents in *ortho*- and *para*-positions were obtained (Flodin and Whitfield 1999a).

Given their relevance as flavor compounds, they are volatile and so it is no surprise that bromoanisoles have been detected in air samples from different locations including the Arctic and the Antarctic (Wittlinger and Ballschmiter 1990; Pfeifer et al. 2001; Führer et al. 1996; Führer and Ballschmiter 1998; Vetter et al. 2002a; Melcher et al. 2005b). Bromoanisoles are readily phototransformed; thus, it was concluded that the occurrence of bromoanisoles in air nearly excludes any long range transport (Wittlinger and Ballschmiter 1990). High concentrations in air from polar regions confirms the prediction that the majority of the compounds found in the environment are of natural origin. It was shown that weekly concentrations of TBA and 2,4-DBA in air do not follow the pattern of HCHs with similar volatility, thus supported their natural origin (Melcher et al. 2005b). The annual profile, low concentrations in spring, and high concentrations in summer that remained high until the end of the year, was virtually identical to the AOBr concentrations determined in surface waters with bioproduction of organobromines (Putschew et al. 2003).

When bromophenol-producing algae were added to fish food, the marine flavor was transferred to the fish by uptake of the bromophenols (Ma et al. 2005). Bromophenols are known compounds in blood of mammals and man, and recently TBP was detected in the blubber of seals, albeit at low concentrations (Vetter and Janussen 2005). Führer et al. (1996) determined that in theory there are 19 bromoanisoles (bromophenols), 19 chlorophenols, and 96 mixed halogenated anisoles (phenols). The PC parameters (see Table 3) indicate that bromoanisoles can evaporate into the atmosphere, are soluble in water, and can be accumulated by organisms in the aquatic environment (Pfeifer et al. 2001).

Historic Data, Identification, and Linking to Known Natural Sources. As mentioned, there are several organisms that can convert halogenated phenols into their respective anisoles. In addition, both compound classes can stem from anthropogenic and natural sources. On a first approximation, 2,4-DBA, 2,6-DBA, 2,4,6-TBA, 2,4-Br-6-Cl-THA, and 2,6-Br-4-Cl-THA originate from natural sources, whereas 2,4,6-TCA, 2,3,4,6-TeCA, and PCA originate mostly from natural sources (Ballschmiter 2003). The most relevant bromophenol and bromoanisole standards (**27**–**29** and others) can be purchased from different suppliers.

Analytical Aspects. The brominated phenols can be analyzed with all common standard cleanup techniques, but solvent concentration steps have to be carried out with care due to the high volatility of bromoanisoles. Therefore, SPME/headspace and stir-bar sorptive extraction techniques

have been developed for the determination of 2,4,6-TBA in aqueous samples and wine (Alzaga et al. 2003; Benanou et al. 2003). Comprehensive data of GC retention times of diverse halogenated anisoles were published by Pfeifer et al. (2001). For instance, bromophenols and bromoanisoles elute before HCH isomers from DB-5-like GC columns (Vetter and Janussen 2005). It is also noteworthy that TBP can be analyzed by GC without derivatization, which is not possible for OH-BDEs. Another peculiarity of bromophenols is the high proportion of the molecular ion (TBP, *m*/*z* 328; DBP, *m*/*z* 250), whereas the bromide ion is lower.

Distribution and Concentrations of Bromoanisoles and Bromophenols in the Environment. In freshwater fish and mussels from the North Sea, 2,4,6-TBA concentrations were usually 10–20 ng/g lw (lipid weight) but could reach up to 300 ng/g lw; under these conditions 2,4,6-TBA was the dominating peak in the chromatograms (Rimkus and Wolf 1991). On the other hand, the absence of 2,4,6-TBA in seal blubber and gull eggs from the same region indicated a low potential for bioaccumulation. It was suggested that the residues were from natural sources (Rimkus and Wolf 1991).

Air samples from the 1980s give rise to the presence of chloroanisoles and 2,4,6-TBA (Atlas et al. 1986; Wittlinger and Ballschmiter 1990). Concentration of 2,4,6-TBA was 18 ± 5 pg/m^3 in air from New Zealand (July–August 1985), 19 ± 10 pg/m^3 in air from American Samoa (August 1981), and 55 ± 24 pg/m^3 in air from the Gulf of Mexico (August 1981) (Atlas et al. 1986). Air from the coast of southern Norway contained ~35 pg/m^3 of 2,4,6-TBA in May 1999, which was on the same level as both α-HCH and lindane (Vetter et al. 2002a). Similar concentrations were determined for these three compounds (Melcher et al. 2005a). However, although the anthropogenic HCH isomers showed a decreasing trend in autumn and winter, 2,4,6-TBA concentrations remained much higher. This trend was even more pronounced for 2,4-DBA, which pointed to different sources for HCH isomers and bromoanisoles, with the latter most likely arising predominantly from natural production (Melcher et al. 2005a). Air from the lower troposphere of the southern Indian Ocean at Réunion (March 1986) contained 8–30 pg/m^3 bromoanisoles (Wittlinger and Ballschmiter 1990), suggesting a widespread distribution in the marine environment. During a cruise through the East Atlantic Ocean, relatively low haloanisole concentrations were detected, except at one site close to Cape Verde Islands, where 17 of the possible bromoanisoles were detected at concentrations that were in the range of 1 ng/m^3 and above (Führer et al. 1997). Halogenated anisoles in real air samples could not be trapped quantitatively on Envi-Carb/silica gel, most likely because of the higher air temperature in comparision to the method development (Führer et al. 1996). Highest concentrations were found for 3,4,5-TCA (99,000 pg/m^3), 2,3,4,5-TeCA (15,100 pg/m^3), and 2,4-DBA (2,310 pg/m^3), whereas 2,4,6-TBA accounted only for 170 pg/m^3 (Führer et al. 1996). The bromoanisole con-

centrations by far exceeded those in terrestrial air above a wastewater treatment plant in Germany (Führer et al. 1996). The high reactivity of bromoanisoles with hydroxyl radicals or transformation by photolysis makes any transport from the Northern to the Southern Hemisphere unlikely (Führer and Ballschmiter 1998). Consequently, the bulk of the marine environmental contamination with bromophenols and bromoanisoles likely originates from biogenic sources (Führer and Ballschmiter 1998). Although chlorinated anisoles in marine air and water more likely stem from anthropogenic processes (Pfeifer and Ballschmiter 2002), mixed halogenated anisoles can also be formed by light-induced Br → Cl exchange (Müller and Crosby 1983).

Pacific salmon from saltwater contained 6–35 ng/g bromophenols whereas freshwater fish from North America contained virtually no bromophenols (Boyle et al. 1992), which illustrates that the natural producers, if this is the source, are not ubiquitously distributed. Bromophenol content (sum of the concentration of 2-BP, 4-BP, 2,4-DBP, 2,6-DBP, and 2,4,6-TBP) in Australian prawns was 10–580 ng/g (*Penaeus plebejus*), 13–166 ng/g (*P. esculentus*), 36–1,100 ng/g (*P. latissulcatus*), and 12–570 ng/g in five other wild-harvested species, whereas commercially pond-raised animals contained no bromophenols (Whitfield et al. 1997a). Heads, including gut, of prawns contained higher bromophenol content than tails (range, 1.3–36 ng/g; mean, 6.8 ng/g) (Whitfield et al. 1997a). Each of the bromophenol congeners was dominant in at least 2 of 30 samples, but in 15 samples 2,4-DBP (**30**) was dominant (Whitfield et al. 1997a).

Owing to their lower bromophenol content (0.3–1.3 ng/g), cultivated prawns lack the desired seafood flavor, which results in a lower taste quality of farmed prawns (Whitfield et al. 1997b). However, a feeding study with cultivated prawns resulted only in a limited uptake of the bromophenols by the prawns, which was attributed to the chemical form of application (Whitfield et al. 2002).

Fish (benthic carnivores) contained up to 2,400 ng/g bromophenols (up to 2,300 ng/g arising from 4-bromophenol). The concentrations in 87 species of algae from East Australia, the potential source for bromophenols, was 0.9–2,590 ng/g ww (Whitfield et al. 1999). In the algae, the highest contribution was from 2,4,6-TBP when the bromophenol content was higher than 250 ng/g ww but could arise from other bromophenols when total bromophenol content was lower (Whitfield et al. 1999).

Recent investigations confirm that sponges and other marine organisms contain a wide range of bromophenols and other brominated compounds, which seem to originate from biogenic sources (Flodin and Whitfield 2000; Kotterman et al. 2003; Whitfield et al. 1997b; Shoeib et al. 2004; Vetter and Janussen 2005).

Conclusions. Despite the varied distribution of bromophenols and bromoanisoles in the marine ecosystem, 2,4,6-TBA appears to be the major

congener that has reached fish. The reason may be a higher lipophilicity and the higher persistence due to the lack of vicinal hydrogens (a feature that simplifies metabolism). Recently, a bacterial strain was isolated from estuarine sediments that is able to dehalogenate 2,4,6-TBA and related substrates into phenol (Boyle et al. 1999). Interestingly, the predominant *ortho*- and *para*-substituted bromoanisoles were all dehalogenated whereas the unusual 3-BP and 2,3-DBP were not transformed by the bacterium (Boyle et al. 1999). Similar results were also found during incubation of bromophenols with anaerobic sediments (Ronen and Abeliovich 2000).

Halogenated phenols and anisoles are widespread in nature. It was suggested that they may be the precursors of more complex HNPs. A plausible formation pathway would be the dimerization of bromophenols (oxidative, **30** + **30**, or via HBr elimination, **30** + **31**) to give the phenoxyphenol **32** followed by methylation to give the phenoxyanisole **10** (Fig. 14). This oxidative reaction scheme is similar to the intramolecular dibenzo-*p*-dioxin formation as discussed for Fig. 10. Similarly, 2,2′-MeO-BB 80 (**19**) may be formed as an artifact from two 2,4-DBP units. This reaction, in turn, may be the same that leads from simple pyrroles to bipyrroles.

In contrast to marine HNPs, little information on terrestrial HNPs and their environmental relevance is currently available. However, it was noted that the bacterium *Alicyclobacillus acidoterrestris* is able to produce 2,6-DBP and 2,6-DBP in shelf-stable juices (mixed-fruit drinks), adding to them a recognizable disinfectant taint (Jensen and Whitfield 2003). Phenolic compounds in the juices were the substrates. The taste threshold of 2,6-DBP in water is 0.5 ng/L. Particularly when present at lower concentrations, HNPs may reach the consumer in this manner.

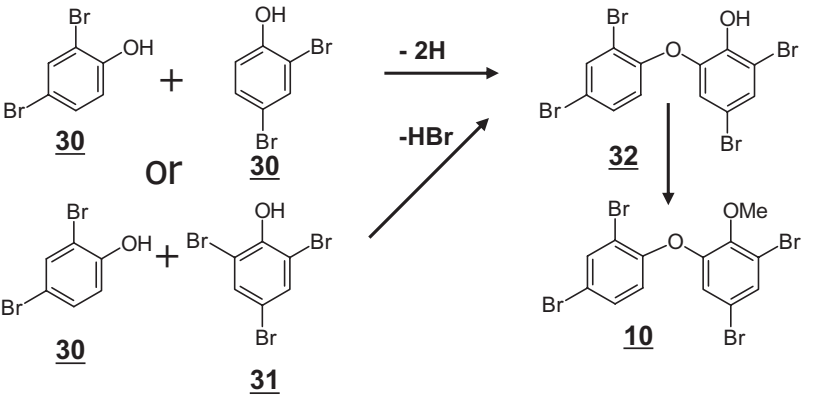
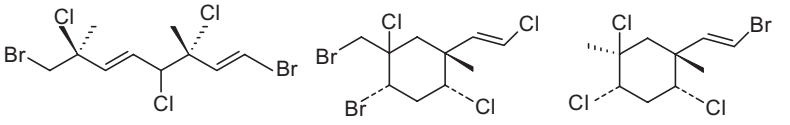


Fig. 14. Suggested formation of phenoxyphenols **32** from 2,4-dibromophenol (2,4-DBP, **30**) and 2,4,6-tribromophenol (2,4,6-TBP, **31**).

F. Mixed Halogenated Monoterpenes (MHC-1)

The key chromatogram that led to the investigation of the so-called mixed halogenated compound 1 (MHC-1) has already been shown (see Fig. 1). The elemental composition was determined using HRMS to be $C_{10}H_{13}Br_2Cl_3$ (Vetter et al. 2001b), which is met by halogenated monoterpenes with two double bond equivalents. Interestingly, MHC-1 is the only HNP discussed in this review without hetero atoms other than halogens. Gribble's very valuable review articles on halogenated natural products indicate that this composition equals that of two secondary metabolites **33** and **34** isolated from marine algae (Gribble 2000; Higgs et al. 1977; Stierle and Sims 1979; Jongaramruong and Blackman 2000). These algae are widespread so that bioproduction of MHC-1 is likely to occur at different places (Vetter et al. 2001b). The detection of MHC-1 correlated with the habitats of the red alga *Plocamium cartilagineum*, a known producer of related halogenated monoterpenes (Higgs et al. 1977; Stierle and Sims 1979; Vetter and Jun 2003). Thus, analysis of this seaweed may be suitable for identifying the natural producer and subsequently the exact isomer structure of MHC-1. One of the potential structures of MHC-1 (**34**) is similar to telfairine (**35**), which is 100% lethal to mosquito larvae at 10 ppm (Higgs et al. 1977; Stierle and Sims 1979). In addition, (**33**) and four other structurally similar halogenated monoterpenes from *Plocamium cartilagineum* were mutagenic in the Ames test (Leary et al. 1979) (Fig. 15).

Historical Data, Identification, and Linking to Known Natural Sources. MHC-1 was initially identified as an abundant peak in the gas chromatograms obtained from commercial fish samples under food control routine inspection. Fish is regularly controlled by food laboratories, and under such a study, a compound previously not detected in the GC/ECD chromatograms of comparable samples was detected (J. Hiebl, personal communication, 1998). Owing to similar GC retention times, it was first suspected to be Q1 (see Section III.B) but GC/MS analysis confirmed the presence of both Br and Cl. A mass spectrometric study gave evidence of a mixed halogenated monoterpene (Vetter et al. 2001b). The exact isomeric structure of MHC-1 is still unknown. Given the several asymmetrical



33 **34** **35**

Fig. 15. Halogenated natural products that are isomeric to MHC-1 (**33**,**34**) as well as the related monoterpene telfarine (**35**), a known natural pesticide.

carbons on halogenated monoterpenes, a total synthesis of MHC-1 cannot be expected in the near future. Therefore, efforts should be undertaken to isolate sufficient amounts from the natural producer.

Analytical Aspects. MHC-1 eluted from DB-1 and DB-5 columns between *trans*- and *cis*-chlordane and slightly before Q1 (Vetter et al. 2001b). Abundant fragment ions are present neither in the GC/EI-MS nor in the GC/ECNI-MS spectra (Vetter et al. 2001b). The molecular ion at *m*/*z* 396 is very small in GC/ECNI-MS but is detectable in GC/EI-MS and displays a dibromo-trichloro isotope pattern.

GC/ECNI-MS identification of MHC-1 in sample extracts is possible using *m*/*z* 158/160 (95%) and *m*/*z* 114/116 (50%), along with *m*/*z* 79/81 (100%) (Vetter 2001). Screening for both the bromide ion and the $[BrCl]^-$ ion together with the retention time range above enables an unequivocal determination of MHC-1 (Vetter et al. 2001b; Vetter 2001; Vetter and Janussen 2005). MHC-1 is stable against H_2SO_4 and is usually found in one fraction together with the chloropesticides (hexachlorocyclohexanes, chlordane, toxaphene) (Vetter et al. 2001b). Because no reference standard is available, *trans*-chlordane has been used for semiquantitative purposes (Vetter et al. 2001b).

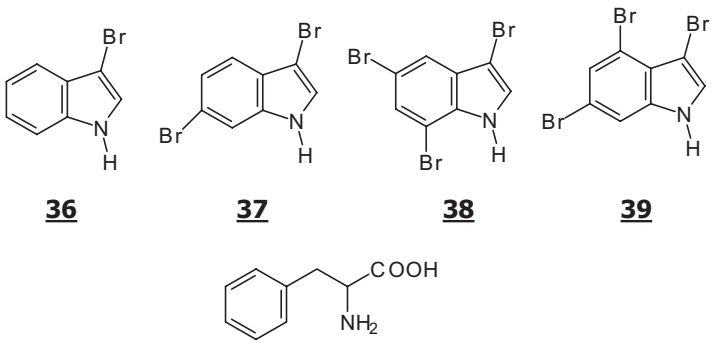
Distribution and Concentrations of MHC-1 in the Environment. MHC-1 was abundant in pollack from Denmark (940 ng/g lw) but also in selected samples of farmed salmon from Norway (7–700 ng/g). In the latter samples, MHC-1 at least accounted for 20% of PCB 153 but could surmount this most prominent PCB congener in fish as well (Vetter et al. 2001b). In the pollack sample, MHC-1 was the dominant peak (see Fig. 1). In seals, highest concentrations were determined in hooded seals from Jan Mayen (58 and 59 ng/g) (Vetter et al. 2001b). MHC-1 was also detected in freshwater fish fed with food produced with marine fish (Vetter et al. 2001b; Vetter and Stoll 2002). The commercial fish food contained MHC-1 half as abundant as PCB 153 (Vetter et al. 2001b; Vetter and Stoll 2002). Fish fed 3 mon with MHC-1-containing food contained MHC-1, but the concentrations were relatively constant. The increase in concentrations was virtually balanced out by the increase in size of the fish (Vetter and Stoll 2002).

MHC-1 was also detected in air samples from the North Sea coast in the south of Norway (Vetter et al. 2002a) as well as in a fish oil capsule and cod livers canned in 1948 (Vetter and Stoll 2002). Human milk samples from the Faeroe Islands that contained Q1, 2′-MeO-BDE 68, 6-MeO-BDE 47, Br_4Cl_2-DBP, 2′,6-diMeo-BDE 47, and diMeO-BB 80 also contained MHC-1 (Vetter and Jun 2003). Finally, MHC-1 was detected in a sponge sample collected in the Antarctic (Vetter and Janussen 2005). It could not be established if this sponge was the natural producer or only accumulated MHC-1 (Vetter and Janussen 2005).

Conclusions. MHC-1 is regularly detected in fish analyzed by official German food control authorities, albeit the concentrations are relatively low (J. Hiebl, personal communication, 2005). The high concentrations in the original paper on MHC-1 (Vetter et al. 2001b) might thus be a consequence of a particular ecological or environmental condition. Many related compounds have shown bioactivity, and a more thorough investigation of MHC-1 should follow in the future.

G. Bromoindoles

The most famous bromoindole derivative is surely tyrian purple, which is produced by marine mollusks. However, simple bromoindoles are biosynthesized by acorn worms and add an iodoformic flavor to these species (Higa et al. 1979). Several mono- to tribromoindoles were determined in such worms from the tropical Indo-Pacific (Fig. 16; **36**–**39**) (Higa et al. 1979). However, due to the N–H bond, the polarity is increased. Consequently, the log K_{OW} of 5-bromoindole was reported to be 2.97 (Mackay 1982), so that bioaccumulation cannot be expected. Nevertheless, Maruya identified three dominating peaks in the GC/ECD chromatograms of oyster from Georgia (USA) (Fig. 17) that did not match the retention times of known anthropogenic contaminants. GC/MS analysis led to the discovery of three bromoindoles (Maruya 2003). The molecular ion provides the known feature of brominated compounds, i.e., an odd molecular mass (dibromoindole m/z 273, tribromoindole m/z 351 (Maruya 2003)). Samples collected in November had much higher bromoindole content (sum of the three bromoindoles was estimated at ~120 ng/g) than those in March and August (estimated concentrations, 1–10 ng/g) (Maruya 2003). The sources are not fully understood. 2,6-Dibromoindole was detected in marine infauna samples from South Carolina (Fielman et al. 1999) whereas 3,6-dibromindole was detected in ascidians from Palau (Qureshi and Faulkner 1999). Others have detected more complex bromoindoles including brominated indole aldehydes.



36 **37** **38** **39**

Fig. 16. Structures of known natural bromoindoles (**36**–**39**) and the known precursor phenylalanin (**40**). Tyrosin may be a substate as well.

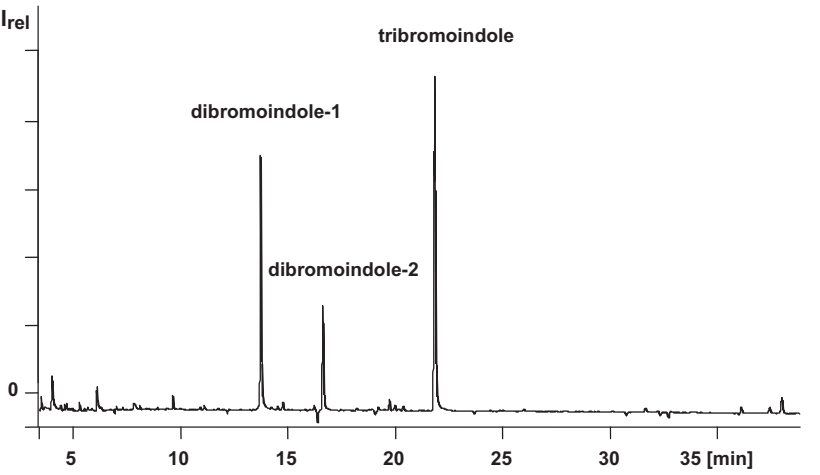


Fig. 17. GC/ECD chromatogram of the detection of three bromoindoles in common oyster (Adapted from Maruya 2003 with permission from Elsevier).

The oyster species feeds exclusively on suspended particulates, which may be the source for the bromoindoles. Another option would be that they are transfromation products of more complex bromoindoles synthesized by algae or other organisms (Maruya 2003). Liu and Gribble (2002) have synthesized the most relevant *N*-methyl-indoles (2,3,6-tribromo- and 2,3,5,6-tetrabromo-1-methylindole) detected in algae and brittle star, which are now available for toxicological testing. Surprisingly, only the bromoindoles and not the more lipophilic *N*-methyl-bromoindoles have been detected in biota samples.

H. HNPs That Resemble Structures of Chlorinated Anthropogenic POPs

Recent research has also elucidated the occurrence of naturally produced chlorinated dioxins (Gaus et al. 2000, 2001; Hoekstra et al. 2000), which may be formed in a similar way as shown in Fig. 10. In addition to being present in sediments, the PCDDs are also found in relatively higher concentrations in higher organisms (Moore et al. 2002; Jiminez et al. 2000; McLachlan et al. 2001).

New cytotoxins were isolated from toxic mussels from the Adriatic Sea. These compounds are most likely produced by dinoflagellates and taken up by the mussels (*Mytilus galloprovincialis*) via filtration of contaminated water. The compounds related to the diarrhetic shellfish poisoning (DSP) toxins to date have only been described in edible shellfish from the Mediterranean Sea. Interestingly, one of the isolates, 2,3,5,6,7,15-hexachloro-4-sulfoxy-14-pentadecen (Ciminiello et al. 2001), is closely related to the anthropogenic medium-chain chloroparaffins.

I. Unknown Compounds and Potential HNPs

In the early work of Ballschmiter and coworkers, several abundant unknown compounds were labeled. U3 was shown to be Q1 (Section III.B), but the structures of the other "U's" suggest some of these may be HNPs of unknown structure and origin. Kuehl et al. (1991) discovered an abundant major mixed halogenated compound in the blubber of dolphins collected during a mass mortality along the U.S. Atlantic coast in 1987–1988. The structure of the compound remains unknown.

Pettersson et al. (2004) detected several brominated compounds by using GC/ECNI-MS in the SIM mode. One unknown organobromine was coeluting with BDE 138. It was reported that this and other unknown compounds could lead to an overestimation of BDEs when GC/ECNI-MS determination is based on the bromide ion only (Pettersson et al. 2004).

A compound labeled UBC-1 has been described in the European Arctic by Vetter (2001). The molecule formed no molecular ion in GC/ECNI-MS. m/z 160 suggested, at least partly, a saturated backbone with a molecular mass of 526 u. UBC-1 eluted slightly ahead of BDE 47 (Vetter 2001). The same retention range was recently described for pentabromophenylpropylether (Hackenberg et al. 2003), which indeed has a molecular mass of 526 Da, which makes it possible that UBC-1 is no HNP but an anthropogenic flame retardant.

A series of brominated compounds was detected in Arctic and Antarctic air and Antarctic sponges (Vetter and Janussen 2005; Vetter et al. 2002a). Some of them were related to TBA but the structure of others, partly mixed halogenated compounds, is still unknown (Vetter 2002). However, the compounds described in this section do not necessarily originate from HNPs, but a careful study of their origin and relevance appears to be warranted.

IV. Biosynthesis of HNPs

An extensive discussion of this topic is beyond the scope of this review. Many papers have been published in this field that have been summarized in many valuable review articles which should be consulted in case of more interest in this field (van Pée 1996; Butler and Walker 1993; Moore 1999).

Ocean water contains ~0.5 mol/L chloride and 0.001 mol/L bromide. This rich source is utilized by many marine organisms in the formation of metabolites (Butler and Walker 1993). A major pathway toward HNPs is the reaction of activated hydrogens by haloperoxidases. These enzymes occur widely in nature, including bacteria (Neidleman and Geigert 1986; Jensen and Whitfield 2003). Interestingly, microbial haloperoxidases differ from similar enzymes in animals and plants in that they need neither metal ions nor cofactors to catalyze reactions (Picard et al. 1997; Jensen and Whitfield 2003).

The principal reaction of organic substrates with haloperoxidases is the following (Butler and Walker 1993):

$$\text{Org-H} + \text{Hal}^- + \text{H}_2\text{O}_2 + \text{H}^+ \rightarrow \text{Org-Hal} + 2\ \text{H}_2\text{O} \quad (1)$$

Haloperoxidases are classified in the following way: chloroperoxidases utilize Cl^-, Br^-, and I^-, bromoperoxidases utilize bromide and iodide, and iodoperoxidases utilize iodide to produce HNPs (Ballschmiter 2003). If chloride is added to a bromoperoxidase, no chlorinated products are formed (Flodin et al. 1999). A common bromoperoxidase contains vanadium (V) as a prosthetic group. The vanadium bromoperoxidases (V-BrPO) are acidic proteins. Other important peroxidases are Fe-Heme containing (protoporphyrin as the prosthetic group) (van Pée 1996). Because peroxidases are commercially available or can be easily gained from natural sources, the halogenation can be reproduced in the lab.

In this way, Walter and Ballschmiter (1991) showed that incubation of anisole, H_2O_2, bromide, or chloride with different peroxidases yielded a wide range of halogenated anisoles. When only 200 ppm bromide was present in the chloride source (NaCl), mixed halogenated compounds were produced (Walter and Ballschmiter 1991). Flodin et al. isolated a bromoperoxidase from the green algae *U. lactuca* and studied the production of bromophenols. It is noteworthy that both bromophenol content and bromoperoxidase activity underlay extreme seasonal variation, with high values in summer and low values in winter (Fig. 18) (Flodin et al. 1999).

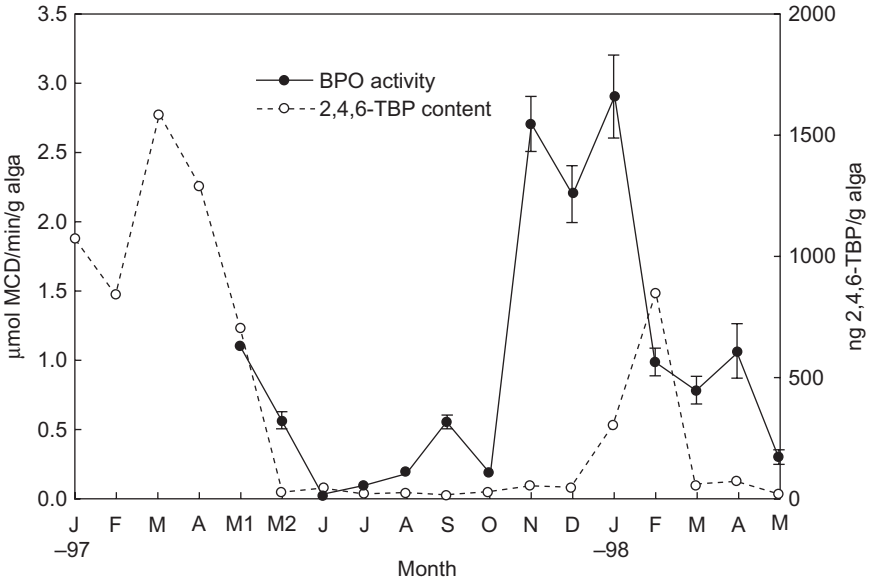


Fig. 18. Annual cycle (January 1997–May 1998) of 2,4,6-tribromophenol content and bromoperoxydase activity in algae from Australia (Adapted from Flodin et al. 1999 with permission from Elsevier).

Highest concentrations in algae were determined in late spring/early summer (in the Southern Hemisphere in November to February) (Flodin et al. 1999). During winter, the bromoperoxidase activity was very low. Therefore, the date of harvesting algae and sponges for the isolation of HNPs is of great importance (Flodin et al. 1999).

V. Perspectives

Given the emerging importance of HNPs, these compounds have to be considered as environmental chemicals once they reach higher organisms. This case applies when the halogenated secondary metabolites, whose natural functions are, among others, chemical defense against a direct predator, are reaching higher organisms that may even be found in different habitats. In line with this description, Ballschmiter distinguished five classes of polyhalogenated compounds (Ballschmiter 2003): (i) biogenic polyhalogenated compounds (including chloromethane and the HNPs described in this work), (ii) natural/geogenic (probably specific halogenated dioxins in clay), (iii) nonhalogenated precursors (e.g., phenols that are halogenated in the environment), (iv) halogenated precursors (halophenols that are converted into haloanisoles), and (v) anthropogenic polyhalogenated compounds (chloropesticides, PCBs).

As pointed out previously, we operate in a "data-poor" environment when dealing with our understanding of HNPs (Moore et al. 2002). Furthermore, there is a need for the development of models that adequately describe the transfer of HNPs to specific environmental compartments (Moore et al. 2002). Current distribution models for HNPs are based on anthropogenic pollutants (Tittlemier et al. 2004; Hackenberg et al. 2003; Vetter et al. 2004), but because of seasonal variations in their production, a less predictable distribution pattern may exist.

It should be noted that HNPs might have a more important impact on anthropogenic compounds than currently understood. For instance, it was concluded that the same microorganisms that transformed 2,4,6-TBA were also able to transform the anthropogenic fire-retardant tetrabromobisphenol A (TBBPA) (Ronen and Abeliovich 2000). Thus, evolutionary pollution of sediments with natural bromophenols and other HNPs may have caused development of specialized microorganisms that now are able to transform these compounds. Without such breakdown mechanisms for HNPs, the environment would have become contaminated with HNPs steadily produced for millions of years.

Given the structural similarity of some anthropogenic POPs with the HNPs, it is not surprising that microorganisms can use some anthropogenic POPs as substrates. In this context, the selective halogenation pattern of anthopogenic POPs appears to be important. Because not all substitution patterns on aromatic compounds are naturally produced, some anthro-

pogenic POPs with unique isomer structures may be transformed to a lesser degree whereas others that closely resemble the structure and pattern of HNPs may be metabolized more easily. Consequently, differences in the transformation rate may go back to the limited feasibility of the natural enzymes for the POPs. Therefore, a thorough knowledge of HNPs will support the understanding of the environmental fate of man-made POPs. This knowledge requires a closer inspection of the structure-dependent transformation of HNPs, which may be a key in the understanding of transformation of anthropogenic POPs.

In other studies it was shown that AOBr formation in sediments that received wastewater was biotic. Indeed, a wide range of HNPs are found in marine infauna (Fielman et al. 1999). It was also found that a low content of nutrients favors formation of organobromine compounds (Putschew et al. 2003). In such media, however, the co-occurrence of natural and anthropogenic compounds does not always allow assigning the proportions of the respective sources. For instance, a high number of brominated compounds, partly of unknown sources, was recently detected in sediments of River Havel and Spree (Berlin, Germany) (Schwarzbauer et al. 2001).

In the 1970s, chemical stress by anthropogenic POPs (PCBs and DDT) was made responsible for such threatening effects as eggshell thinning and reproductive failure in seals. In connection with the latter, 50–70 ppm PCBs has been defined as the critical concentration where reproductive failure begins (Helle et al. 1980). The highest concentration of all HNPs determined in a single sample was ~25 ppm. Given the low number of samples analyzed for HNPs to date, this seems to be remarkable.

Summary

A wide range and steadily increasing number of halogenated natural products (HNPs) is detected in marine organisms that are not the natural source of these compounds but which have accumulated these HNPs in a similar way as known to occur with anthropogenic halogenated pollutants such as PCBs and DDT. The HNPs have aromatic, aliphatic, and heterocyclic spines and are brominated, chlorinated, or mixed halogenated (Cl and Br). The exact isomer structures of HNPs are often closely related to the anthropogenic POPs, and for some compounds both natural and anthropogenic sources are likely to exist. Some of the HNPs are nonpolar, persistent, and can thus be found even in marine mammals and birds of prey. The most important HNPs detected in top predators are halogenated 1,1′-dimethyl-2,2′-bipyrroles (HDBPs), the heptachloro-1′-methyl-1,2′-bipyrrole Q1, the tetrabromophenoxyanisole isomers 6-MeO-BDE 47 and 2′-MeO-BDE 68, and related compounds. Each of these compounds has been detected in higher trophic biota with concentrations exceeding 1 mg/kg.

Acknowledgments

I particularly wish to thank Gordon W. Gribble for his outstanding review articles on naturally produced organohalogen compounds, which delivered a steadily inspiring source of information. I am also grateful to my coworkers and my research partners for their valuable input.

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Manuscript received August 16; accepted September 16, 2005.

Rev Environ Contam Toxicol 188:59–84

Heavy Metals Remediation of Water Using Plants and Lignocellulosic Agrowastes

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Contents

I. Introduction

Metals in the environment arise from natural sources or directly or indirectly from human activities such as rapid industrialization, urbanization, and anthropogenic sources, threatening the environment and human health (Nriagu 1979). Mining and metallurgical activities produce wastewaters that can be considered as the major source of heavy metal contamination of natural waters (Schalcsha and Ahumada 1998; Reddad et al. 2002a). In the United States alone, more than 50,000 metal-contaminated sites await remediation, many of them Superfund sites (Ensley 2000). They are potential hazards to aquatic, animal, and human life because of their toxicity and bioaccumulative and nonbiodegradable nature (Zuane 1990). Nonessential metals such as Hg, Cd, Cr, Pb, As, and Sb are toxic in their chemically combined forms as well as the elemental form (Manahan 1993). Acute metal poisoning in humans causes severe dysfunction in the renal, reproductive, and nervous systems, and chronic exposures even at low concentrations in the

Communicated by George W. Ware.

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environment can prove to be harmful to human health (Wyatt et al. 1998). In addition, heavy metals that are discharged from a wide variety of industries such as electroplating, metal finishing, leather tanning, chrome preparation, production of batteries, phosphate fertilizers, pigments, stabilizers, and alloys to the aquatic environment have adverse impacts on aquatic species because they are conserved pollutants that are not subject to bacterial attack or other breakdown and remain as permanent additions to the marine environment (MacCarthy et al. 1995; El-Nady and Atta 1996). They are dangerous to aquatic animals because they tend to bioaccumulate and cause physiological defects and histopathological manifestations in tissues, resulting in reduced reproduction (Gardner 1975; Cutter 1991; Joseph et al. 2002; Krishnani et al. 2003a). Once mobile in the environment in ionic form, they find their way into the human body through drinking water, food, and air. There is a reasonable chance of having a fair amount of toxic metals in the body if a person has eaten fish regularly, has amalgam fillings, has received vaccinations, has drunk contaminated water, or been involved in industrial or agricultural work or pharmaceutical manufacturing.

II. Heavy Metals as Health Hazard to the Aquatic Environment

Arsenic is both ubiquitous in the environment and potentially toxic to humans. It was ranked first on the Comprehensive Environmental Response, Compensation and Liability Act priority list of hazardous substances in the U.S. in 1999 and 2001. Arsenic can occur in water in organic or inorganic forms but the inorganic form is more common (Fuhrman et al. 2004). Inorganic As may exist in –3, +3, and +5 oxidation states, with As(III) and As(V) being the dominant species in natural waters. There is evidence that As(III), once absorbed, may be oxidized to As(V) and/or methylated in humans. Methylation of inorganic arsenic in the human body is a detoxification process that occurs in the kidney and reduces its affinity for that tissue (Das et al. 1995). Arsenic is classified as a human carcinogen based on sufficient epidemiological evidence (USEPA 2002). Drinking water with high arsenic concentrations is of particular concern, because studies of chronic arsenic exposure have shown that even small amounts of arsenic in drinking water can cause cancer if ingested over a long period (Karim 2000). The high concentration of arsenic found in drinking water sources has raised concern in many parts of the world including the Bengal Delta (Bangladesh and West Bengal, India) (Nickson et al. 1998), the Red River Delta (Vietnam) (Berg et al. 2001), and the western U.S. (Reid 1994). Occurrence of arsenic in the groundwater of Bangladesh and West Bangal has been described as the greatest mass poisoning in human history (Smith et al. 2000; Rahman et al. 2005), with 36 million people exposed to elevated arsenic in their drinking water (Nordstrum 2002). Today, 97% of Bangladeshi drink well water, and unfortunately vast areas of Bangladesh contain groundwater with arsenic concentrations above the World Health

Organization (WHO 1993) and U.S. Environmental Protection Agency (USEPA 2001, 2002) water guidelines of 10 μg/L. However, some experts believe that an even tougher standard of 3 μg/L should be adopted. The severity of chronic arsenic poisoning in Bangladesh suggests that other metals such as Mn, Pb, Ni, and Cr in groundwater maybe magnifying As toxicity (Frisbie et al. 1999, 2002), which raises serious concerns relating to environmental health issues caused by multimetal effects. Thus, the development of more efficient arsenic treatment technologies is still urgently required.

Certain organic metal compounds are much more harmful to living organisms than the elements themselves. Examples include methyl and ethyl mercury and tributyl tin used as pesticides. Contamination of aquatic ecosystems with mercury creates health concerns because consumption of fish is the primary means by which humans are exposed to the neurotoxic, methylated form of mercury (Hightower and Moore 2003). Reduction, methylation, and demethylation are important mechanisms of Hg speciation in both the water column and the benthic sediment (Kim et al. 2004). When mercury enters water, it is often transformed primarily by sulfate-reducing bacteria, by microorganisms at the water – sediment interface, or by bacteria associated with the fish gut into the toxic methylmercury, which bioaccumulates and concentrates in living organisms (Compeau and Bartha 1985; Bodaly et al. 1997; King et al. 2000). Field measurements found that almost all Hg (>90%) in fish muscle was in methylated forms (Bloom 1992). In 1932, sewage containing mercury was released into Minamata Bay in Japan. The mercury accumulated in sea creatures, leading eventually to mercury poisoning in man from the consumption of contaminated fish (Kudo and Miyahara 1991). In the U.S., some 60,000 babies per year are born with neurological damage caused by mercury poisoning of their mothers from consuming large amounts of fish from polluted locations during pregnancy (Schrope 2001). Pregnant women and women breast-feeding their children are advised never to eat this kind of fish.

Cadmium is biopersistent and may interfere with the ability of metallothionein to regulate zinc and copper concentrations in the body. Metallothionein is a protein that binds to excess essential metals to render them unavailable. When cadmium induces metallothionein activity, it binds to copper and zinc, disrupting homeostasis levels (Kennish 1992).

Antimony is a metal used in the compound antimony trioxide, a flame retardant; this is a suspected human carcinogen. Previous studies suggest that the severity of chronic arsenic poisoning in Bangladesh might be magnified by the presence of antimony in the drinking water (Gebel 1999). Most antimony compounds do not bioaccumulate in aquatic life.

Small amounts of nickel are needed by the human body to produce red blood cells. Nickel can accumulate in aquatic life, but its presence is not magnified along the food chain. Nickel is a probable human carcinogen. Similarly, selenium and zinc are also needed by humans and other animals

in small amounts. Selenium prevents the cytotoxic effects of arsenic (Biswas et al. 1999). Zinc promotes the repair of tissues damaged by arsenic (Engel et al. 1994). The apparent absence of these essential nutritive elements in drinking water and possibly in food may cause a magnification of As toxicity in Bangladesh water (Frisbie et al. 1999, 2002). However, these metals also accumulate in living tissues of fish and other organisms, causing greater health problems in humans over a lifetime of overexposure.

Because of size and charge similarities, lead can substitute for calcium and be included in bone. Children are especially susceptible to lead because developing skeletal systems require high calcium levels. Lead that is stored in bone is not harmful, but if high levels of calcium are ingested later, the lead in the bone may be replaced by calcium and mobilized in the body.

In the U.S., chromium is the second most common inorganic contaminant in waters after lead (Wielinga et al. 2001). Chromium usually exists in both trivalent and hexavalent oxidation states in soils and aqueous systems. The hexavalent form is of particular concern because of its great toxicity, resulting from its powerful oxidation properties. Based on chronic effects, the USEPA (Nkhalambayausi-Chirwa and Wang 2001) and the International Agency for Research on Cancer (IARC 1987) categorize Cr(VI) as a carcinogen, whereas the trivalent form of chromium Cr(III) is not classifiable as it is about 300 times less toxic than Cr(VI). Furthermore, it has limited hydroxide solubility, making it relatively immobile and less available for biological uptake. As a result, Cr(VI) toxicity could be reduced and then become less bioavailable when reduced to Cr(III). Because of these differences, the discharge of Cr(VI) to surface water is regulated at 50 μg/L by the USEPA while total chromium is regulated at 2000 μg/L.

Applications, sources of contamination, and potential health effects of heavy metals are given in Table 1. Various agencies have recommended safe levels for heavy metals for the protection of drinking water, fish, and other aquatic life, which are given in Table 2. Although the content of metals in aquatic the environment has been rising in recent years, there is still a lack of effective means for the removal of metals. The increased concern about metal poisoning and stricter regulations for metal pollution have accelerated many efforts in developing cost-effective methods for removing metals from contaminated water for preserving the quality of aquatic systems, streams, and groundwater. Contaminated waters are generally cleaned by currently used water treatment technologies involving chemical precipitation, adsorption, evaporation, electrochemical treatment, and the use of ion-exchange resins (Leppert 1990; Ouki et al. 1997; Yang and Lin 1998). However, these technologies have been found to be limited because they often involve high operational costs and are sometimes ineffective, especially when metals are present in solutions at very low concentrations. They may also be insufficient to satisfy strict regulatory requirements for chemical precipitation. Among these methods, adsorption is by far the most

Table 1. Applications, sources of contamination, and potential health effects of heavy metals.

Metal	Applications	Sources of contaminant in drinking water	Potential health effects
As	Pesticides, wood preservatives	Erosion of natural deposits, runoff from glass and electronics production wastes	Nausea, vomiting, damage to skin and blood vessels, circulatory problems, cancer
Hg	Batteries, lamps, thermometers, as amalgam in dentistry, pharmaceutical	Erosion of natural deposits, discharge from refineries and factories, runoff from landfills and croplands	Abdominal pain, headache, diarrhea, hemolysis, chest pain, kidney damage, neurotoxicological disorders
Pb	Batteries, petrol additives, alloys, pigments	Corrosion of household plumbing systems; erosion of natural deposits	Anemia, vomiting, loss of appetite, convulsions, damage of brain, liver and kidney, high blood pressure, delays in physical or mental development in children
Cd	Nickel cadmium battery, pigments anticorrosive agent, stabilizers for PVC	Corrosion of galvanized pipes; erosion of natural deposits; discharge from metal refineries; runoff from waste batteries and paints	Diarrhea, growth retardation, bone deformation, kidney and lung damage, testicular atrophy, anemia, injury of central nervous system and liver, hypertension, cancer
Cr	Metal alloys, paints, cement, paper, rubber	Discharge from steel and pulp mills; erosion of natural deposits	Nephritis, gastrointestinal ulceration, diseases in central nervous system, cancer, allergic dermatitis
Cu	Additives to control fungal growth, electrical pipes	Corrosion of household plumbing systems, erosion of natural deposits	Hypertension, uremia, anemia, coma, sporadic fever, gastrointestinal distress, liver or kidney damage
Sb	Flame retardant, battery, pigments ceramics, glass	Discharge from petroleum refineries, fire retardants, ceramic, electronics; solder	Nausea, vomiting, diarrhea; increase in blood cholesterol, decrease in blood sugar, suspected human carcinogens
Se	Photoelectric cells, TV cameras, glass industry	Discharge from petroleum refineries: erosion of natural deposits, discharge from mines	Hair or fingernail loss, numbness in fingers or toes, damage to kidney, nervous system and circulatory tissues, irritability

Table 2. General Water Quality Criteria Recommended by Tennessee Department of Environment and Conservation (Tennessee Water Quality Control Board), USEPA, and WHO.

	Tennessee Water Quality Standards (μg/l)[a]				USEPA (μg/l)[b]	WHO (μg/l)[c]
Heavy metals	Domestic Water Supply	For protection of fish and aquatic life	For general -use ground water	For recreation	Groundwater and drinking water standards	Groundwater and drinking water standards
As	10	340[d]	50	10	10	10
Cd	5	2	5	—	5	3
Cr (Total)	100	16[e]	100	—	100	50
Pb	5	65	50	—	0	10
Hg	2	1.4	2	0.05	2	—
Ni	100	470	—	610	100	20
Sb	6		—	5.6	6	5
Se	50	20	50	—	50	10
Cu	—	13	1,000	—	1,300	2,000
Zn	—	120	5,000	—	—	—
Mn	—		500	—	—	500

[a]Rules of Tennessee Department of Environment and Conservation (Tennessee Water Quality Control Board), January 2004 (revised).

[b]USEPA (1996) USEPA drinking water regulations and health advisories. EPA 822-B-96-002. USEPA, Washington, DC.

[c]WHO (1997) Guidelines for Drinking Water Quality. Health Criteria and Other Supporting Information, 2nd ed, vol 1. World Health Organization, Geneva, Switzerland.

[d]As(III).

[e]Cr(VI).

versatile and widely used method for the removal of toxic metals (Mattson and Mark 1971; Cheremisinoff and Ellerbush 1979; Gupta et al. 2003; Gupta and Ali 2004). Activated carbon has been used very frequently for the removal of various metal ions from wastewater for more than three decades (Huang and Wu 1977; Lalwani et al. 1998), but the high cost of activated carbon restricts its large-scale use for the abatement of heavy metal pollution, and in recent years the search for an low-cost adsorbent has grown (Reddad et al. 2002a–d, 2003; Dupont et al. 2003; Dupont and Guillon 2003; Krishnani et al. 2004; Parimala et al. 2004).

III. Plant Remediation as Alternative to Chemical Technologies

With the high costs of site remediation, it is important that we continue to develop and refine innovative low-cost methods for cleaning the environment. Advances in groundwater and soil remediation continue to lead to a

better understanding of the many processes by which plants can have a positive impact on contamination in the environment. This realization has provided impetus to studies in an emerging field of interest, which employs certain plants possessing the natural ability to take up heavy metals for an inexpensive means of environmental cleanup. This method is referred to as plant-assisted remediation or phytoremediation, and it also has the benefit of contributing to site restoration when remedial action is ongoing. The action of plants can include enzymatic degradation, also called phytotransformation or phytodegradation, immobilization by chemical compounds produced by the plants (adsorption or phytostabilization), accumulation (phytoextraction or phytoaccumulation), volatilization (phytovolatilization), and the enhancement of bacterial activity (phytostimulation or plant-assisted bioremediation) (Lee and Charles 2004; Anton and Mathe-Gaspar 2005; Chaney et al. 1997; Davis et al. 2003; Krishnani et al. 2004). To date there are approximately 400 known metal hyperaccumulators in the world (Reeves and Baker 2000), and the number is increasing. However, the remediation potential of many of these plants is limited because of their slow growth and low biomass. A plant suitable for phytoremediation should possess high biomass with high tolerance and metal accumulation in the shoot tissues (Chaney et al. 1997; Eapen and D'Souza 2005). Many hyperaccumulator plants excrete organic acids such as citric, malic, malonic, and oxalic acids (Ma et al. 2001), phytosiderophores that act as metal chelators and decrease the rhizosphere pH, thus making metal cations bioavailable (Kinnersely 1993).

The phytoremediation of heavy metals has been reviewed by previous researchers (Moffat 1995; Salt et al. 1996; Bailey et al. 1999; Eapen and D'Souza 2005; Chuah et al. 2005). However, they highlighted mainly plant genetic engineering and reported only maximum adsorption capacity of some selected sorbents with very little information on removal mechanisms. This review describes the current technologies prevalent for plant-assisted remediation using live and dead biomass from plentiful natural sources and lignocellulosic wastes, with the major emphasis on removal mechanisms.

A. Using Living Biomass

In recent years biosorption research, which focuses on using readily available biomass that can passively accumulate heavy metals, has received growing attention (Davis et al. 2003). This approach involves the use of biological materials that form complexes with metal ions using their ligands or functional groups. This process can be applied as a cost-effective way of purifying industrial wastewater whereby drinking water quality can be attained. Marine brown algae have been the focus of numerous biosorption studies, and their excellent metal-binding capacity has been well documented (Ramelow et al. 1992; Holan et al. 1993; Leusch et al. 1995; Davis

et al. 2003). The main components of the brown algal cell wall are cellulose as the fibrous skeleton, alginate and fucoidan, which constitute the amorphous matrix, and extracellular mucilage (South and Whittick 1987). Of these compounds, alginate contains carboxyl groups and fucoidan has sulfate groups, both of which are known to form complexes with metals (Buffle 1988; Davis et al. 2003). Kuyucak and Volesky (1989) speculated that alginate may be one of the main compounds involved in brown algae metal accumulation, and Fourest and Volesky (1996) have confirmed the importance of the alginate carboxylic groups; after blocking the weakly acidic carboxylic groups with propylene oxide, metal binding was reduced by 80%–95%.

Arsenic uptake by plants is associated with the phosphate uptake mechanism, where presumably arsenate is taken up as a phosphate analogue (Mkandawire et al. 2004; Khattak et al. 1991; Meharg and Macnair 1990; Pickering et al. 2000). To date, there is only one report (Ma et al. 2001) of a terrestrial plant, the Fern *Pteris vittata*, that hyperaccumulates arsenic, and it has been suggested that it could be used for the phytoremediation of arsenic-contaminated sites. However, Caille et al. (2004) reported that *P. vittata* may be suitable for phytoremediation only in moderately contaminated soils. The ability of *Lepidium sativum*, a watercress, to take up large amounts of arsenic from substrates containing relatively low concentration of this element indicate the plant may have potential for phytoremediation (Robinson et al. 2003).

Hyperaccumulating plants have been identified for a number of metals (Chaney et al. 1997; Lombi et al. 2001; Leduce and Terry 2005); however, the phytoremediation efficiency of most metal hyperaccumulators is limited by their slow growth rate and low biomass. For example, *Thlaspi caerulescens* a Penny-cress, is a Cd and Zn hyperaccumulator, and successfully removed 43% Cd and 7% Zn from an industrially contaminated soil, but this required 391 d (Lombi et al. 2001). The use of microorganisms such as bacteria (Texier et al. 1999), fungi, and algae (Kratochvil and Volesky 2000; Schiewer and Wong 1999; Yang and Volesky 1999) in treating waste effluents containing toxic metal ions is today an attractive technique but not yet suitable for application on a large scale (Reddad et al. 2002b).

B. Using Nonliving Biomass

In addition to live plants, studies have demonstrated that nonviable plant biomass can effectively bind toxic metals and as such can be used to remove metals from solution (Seki et al. 1998). The unique ability of these plants to bind metals has been attributed to the presence of various functional groups that attract and sequester metal ions (Baig et al. 1999). This technology is attractive mainly because it is environmentally friendly and inexpensive. Baig et al. (1999) examined the binding of Pb(II), Cu(II), Ni(II), Cd(II), Zn(II), Cr(III), and Cr(VI) to the inactivated biomass of *Solanum*

elaeagnifolium (silverleaf nightshade) and reported that all showed binding to the biomass with optimum binding occurring between pH 5.0 and 6.0. Previous studies have reported the binding of metals to some organic acids that contain carboxyl ligands (Korshin et al. 1998). Work done by Baig et al. (1999) suggested that to some extent carboxyl groups (–COOH) are responsible for the binding of metal ions, and at lower pHs the carboxyl groups retain their protons, reducing the probability of their binding to any positively charged ions, whereas at higher pHs (above 4.0), the carboxyl groups are deprotonated and, as such, are negatively charged ligands ($–COO^-$), which attract the positively charged metal ions resulting in binding.

Thus, metal ion binding to the biomass is in essence an ion-exchange mechanism that involves electrostatic interaction between the negatively charged groups in the cell walls and metallic cations (Wase and Forster 1997), which means that metal binding can be enhanced by increasing the number of carboxylate ligands in the biomass. Interestingly, cellulose, hemicellulose, and lignin, major constituents of most plant tissues, contain methyl esters that do not bind metal ions significantly. However, these methyl esters can be modified to carboxylate ligands by treating the biomass, thereby increasing the metal-binding ability of the biomass. Baig et al. (1999) also observed that the biomass binds more than 80% of Pb^{2+} at pH 3.0 and about 50% at pH 2.0, suggesting that besides carboxyl groups, other groups may also be involved in Pb(II) binding. Conversely, the biomass binds more Cr(VI) at pH 2.0 compared to that bound at pH 5.0, for which two processes have been hypothesized (Baig et al. 1999). First, because Cr(VI) occurs as an oxoanion such as CrO_4^{-2}, $HCrO_4^{-1}$, or $Cr_2O_7^{-1}$, binding at higher pH where negatively charged carboxylate ions prevail is highly unlikely. Second, it has been reported that at lower pH, Cr(VI) is reduced to Cr(III) (Kratochvil et al. 1998). However, no significant difference in Cd(II) binding is observed upon modification, which suggests that probably other ligands are involved in the binding of Cd(II) to the biomass. Also as expected, the amount of Cr(VI) bound by the biomass is not enhanced by modification, because Cr(VI) exists as an oxoanion and therefore cannot bind to the negatively charged carboxylate ligands.

C. Using Lignocellulosic Agrowastes

Life on earth can exist only because of the cycling of matter, which is therefore of utmost importance to all living systems. For humans, biomass in the form of lignocellulosics provides a means of harnessing and storing solar energy and hence represents an important energy and material resource. But, before this renewable Carbon source can be used, its conversion to applicable form is necessary. As such, recycling of organic matter has assumed great significance from the point of view of resource utilization and pollution abatement. Among the chemical constituents in plant

biomass, cellulose, hemicellulose, and lignin occupy the major portion (Mani et al. 1998). The number of glucosidic bonds available for enzymatic action depends to a large extent on the degree of swelling of cellulose, which can be achieved by physical or chemical methods. Mild pretreatment of lignocellulosics with steam, acid, or alkali helps loosen the crystalline structures of cellulose.

Agricultural countries generate considerable amounts of lignocellulosic agrowastes and by-products such as sugar-cane bagasse, rice straw, rice husks, ground nut husks, crop wastes, peanut hulls, and animal manure. Many of these fibrous by-products are generally used as an ingredient of formulated feed (Miltner et al. 1983) and fertilizer to enhance the natural productivity of ponds, especially in freshwater aquaculture systems (Hepher and Pruginin 1981). These materials have the advantage of being readily available and could provide value-added products that otherwise would be considered as a waste. Today, the world's industry is utilizing less than 10% of the biomass of raw materials from plantations (Pauli and Gravitis 1997). In the U.S., approximately 350 million t of agricultural residues (AR) are currently disposed of every year, and many types of these abundant residues from tropical plantations are waiting for effective utilization. AR are the most abundant renewable organic resources of energy and production of a diversity of chemicals, including ethanol production (Ikeuchi et al. 1999), activated carbon (Namasivayan and Kadirvelu 1999; Bailey et al. 1999; Bansode et al. 2003), and ion exchangers (Simkovic and Laszlo 1997). Agricultural wastes have been reported to be efficient in removing toxic metals from aqueous solutions (Low et al. 2000; Cimino et al. 2000; Ho and McKay 2000; Vaughan et al. 2001). Many other applications for these residues are being developed. Development of new economically feasible ecofriendly products from agricultural wastes/by-products and natural plants for the treatment of shrimp culture water is the objective of continued research of the Central Institute of Brackishwater Aquaculture, Chennai (Krishnani et al. 2002, 2003b, 2004, 2006).

Lignocellulosic residues are composed mainly of cellulose, hemicellulose, and lignin. Lignin is the main component, which adds to the lack of efficacy of these materials, the result of mainly covalent bonds between lignin and carbohydrates. Lignin shields the carbohydrate (cellulose) from any kind of microbial attack. Hence, for incorporation in the aquatic system, the lignocellulosics need prior processing to increase efficacy to the maximum possible extent and to render them manually more efficient. The differences in the chemical composition of the lignocellulosics also affect their degradation in aquatic environments. Another factor that plays a significant role during decomposition is the C/N ratio. In the past few years, continuous efforts have been made to process the lignocelluloses through physical, chemical, and biological treatment. These treatments increase the accessibility for degradation due to partial removal of hemicellulose

(Woodford 1984). These wastes have potential manurial values in different farming practices, including freshwater aquaculture (Ayyappan et al. 1992; Barik et al. 2002), and have been used as substrates for a periphyton food source in aquaculture (Bombeo-Tuburan et al. 1993; Azim et al. 2002; Keshawanath et al. 2001).

Numerous by-products of agroindustrial production have been studied for potential use as inexpensive biosorbents (Laszlo and Dintzis 1994; Marshall and Champagne 1995; Basso et al. 2002). One of these low-cost sorbents particularly suited to biosorption is bagasse, a complex native lignocellulosic fibrous waste remaining after extraction of juice from sugar cane. This by-product amounts to 25%–30% of the cane weight and contains about 50% cellulose, 27.9% hemicellulose, 9.8% lignin, and 11.3% cell contents (Kewalramani et al. 1988). Sugar-cane mills produce more bagasse than can be utilized as a fuel source for sugar processing; few commercial uses for the excess bagasse have been developed, and its accumulation presents a waste problem for the sugar industry. It is reported that about 8 million t dry bagasse was produced in India in 2001 (Khan et al. 2004). One potential use of bagasse is as a feedstuff for shrimp (Freeman et al. 1992), as this is an attractive agricultural by-product for a pond supplement due to its low cost and general availability without any adverse impact on water quality in shrimp-growing latitudes (Visscher et al. 1991).

Among available conventional processes used to remove Cr(VI), the most commonly used are precipitation as chromium hydroxide or ion exchange using macroporous resins (Jianlong et al. 2000). However, these methods suffer from disadvantages due to their relatively high operational costs. Conversely, in recent years, a promising alternative method for removal of Cr(VI) uses the sorption by lignocellulosic solid wastes such as bagasse (Krishnani et al. 2004), sugar-beet pulp (Reddad et al. 2003), wheat bran (Dupont and Guillon 2003), and sawdust (Raji and Anirudhan 1998). Bagasse has been found to be effective in removing chromium from coastal waters by ion-exchange and adsorption mechanisms (Krishnani et al. 2004). Krishnani et al. (2004) and Parimala et al. (2004) studied the efficacy of five different types of materials prepared from bagasse and coconut husk for detoxification of Cr(VI) from coastal waters, which is the source of brackish-water aquaculture. They found that acid-treated materials are the most effective materials for detoxification of Cr(VI) in the acidic medium, which can be attributed to the reduction of Cr(VI) into Cr(III), whereas the removal of Cr(VI) in treatments with other material prepared from bagasse and coconut husk in an alkaline medium has been attributed to the reduction by increase in the native microbial community in the coastal waters. Furthermore, bagasse is a biodegradable substrate that harbors a higher periphytic biomass than nondegradable materials, possibly because biodegradable substrates provide a better surface structure to which periphytic species can attach, or they may leach nutrients beneficial for the growth of periphyton, which has more than one role in aquaculture

(Azim et al. 2002; Keshavanath et al. 2001). It improves fish and shrimp production and water quality, thus enhancing the efficiency of aquaculture systems.

Sugar-beet pulp is a low-cost, unconventional sorbent that exhibits a large capacity to bind metals (Dronnet et al. 1997; Gerente et al. 2000). Actually, about 14×10^6 t sugar-beet pulp are produced every year in Western Europe, where it is used mainly as animal feed (Micard et al. 1997). Sugar-beet pulp, a common waste from the sugar-refining industry, was used for the removal of metal ions from aqueous solutions by Reddad et al. (2002a); they found that it has great potential for the removal of heavy metals from aqueous solutions with the affinity order $Pb^{2+} > Cu^{2+} > Zn^{2+} > Cd^{2+} > Ni^{2+}$. They identified the predominant ion-exchange mechanism involving numerous carboxylic groups of the galactouronic acid residues in the pectins.

Previous studies (Gerente et al. 2000; Reddad et al. 2002c,d) have revealed that the lignocellulosic substrate has lignin and cellulose as its major constituents. Lignins bear functional groups such as alcohols, ketones, and carboxylic groups that may be involved in complexation reactions with metallic cations. The removal of Cr(III) and Cr(VI) from aqueous solutions using sugar-beet pulp as a biosorbent substrate was performed by Reddad et al. (2003) under various experimental conditions. They found that Cr(VI) removal was largely involved in a reduction mechanism with the appearance of Cr(III) ions in the solution and that the carboxylic groups of the biosorbent are the main reduction sites of the Cr(VI) species. They also found that Cr(III) ions are adsorbed onto the biosorbent by an ion-exchange mechanism with Ca^{2+} cations neutralizing the carboxyl groups of the material. The influence of solution pH greatly affected the adsorption efficiency of Cr(VI), and the optimum removal resulting from the reduction mechanism was achieved at acidic pH values (Reddad et al. 2003).

Studies conducted by Gerente et al. (2000) on the removal of Cu^{2+}, Pb^{2+}, and Ni^{2+} using sugar-beet pulp revealed that a key part of the mode of fixation is attributed to ion exchange. They found that the movements of Na and K ions are in the same order of magnitude and seem to be independent of the Cu^{2+} concentration, and thus their role in copper removal would be low. On the other hand, the release into solution of Ca^{2+} is correlated with the fixation of Cu^{2+}. As far as lead is concerned, adsorption seems to play an important role in fixation, and 25% would be fixed by adsorption (Gerente et al. 2000). In contrast, nickel seems to be fixed completely by ion exchange, and as for copper, the major part of fixation is attributed to ion exchange, only 5% being adsorbed.

Lignocellulosic substrates (LCS) such as rice hull (RH), sugar-cane bagasse (BG), and wheat straw are now regarded as abundant, inexpensive, and readily available natural resources for the chemical and paper indus-

tries. Dupont and Guillon (2003) studied the adsorption mechanism of Cr(VI) onto the LCS and showed that the adsorption reaction consumes a large amount of protons that go with reduction of Cr(VI) into Cr(III). The oxidation of lignin moieties takes place concurrently with chromium reduction and leads to the formation of hydroxyl and carboxyl groups. The latter contribute to an increase in the number of ion-exchange sites for the reduced chromium. They reported maximum adsorption capacity for Cr(VI) of about 35 mg/g in an acidic medium. Also, they used X-ray photo electron spectroscopy (XPS) to characterize the surface chemistry of LCS and Cr speciation adsorbed onto the LCS. Sorption capacity of the LCS could be related to the abundance of lignin and fatty acid moieties, which allow the reduction of hexavalent chromium into the trivalent form as well as fixation of Cr(III) on carboxylic moieties. The increase of carboxyl groups was estimated by XPS, and potentiometric titrations can be easily related to the increase in the retention capacity of LCS.

Lignocellulosic substrates isolated from wheat straw and bran exhibited high complexing capacities (Gauthier et al. 2002). A large difference in composition was observed between bran and straw LCS due to a much higher contribution of alkyl moieties in the former. These moieties correspond to fatty acids esterified to the lignocellulosic macromolecular structure, and such carboxyl groups play an important role in metal complexion and hence in applications for metal removal from industrial effluents.

Reddad et al. (2002b) conducted studies on the Ni(II)- and Cu(II)-binding properties of native and modified sugar beet and found that the chemical modifications applied to the native material resulted in an improvement of the cation exchange capacities. Because of the loss of all methoxy groups from the carboxyl moieties, base-extracted pulp and saponified pulp exhibited the highest Ni(II) and Cu(II) ion uptake among the materials tested.

IV. Adsorption Capacities of Sorbents

The reported capacities of various sorbents such as live and dead biomass from natural sources and lignocellulosic wastes are given in Table 3, and adsorption capacities of various other sorbents such as activated carbon, chitosan, lignin, clay, xanthate, peat moss, and bark are given in Table 4. These data show that sorbents prepared from lignocellulosic wastes are comparable to ordinary adsorbents and sorbents from other natural sources except chitosan, which have comparatively high adsorption capacity. However, lignocelluloses have an edge on other sorbents because of their great availability, very low cost, and simple operational process. In general, a sorbent can be assumed as low cost if it requires little processing, is abundant in nature, or is a by-product or waste material from another industry (Bailey et al. 1999).

Table 3. Adsorption capacities (mg/g) for various biomass and lignocellulosic materials in the literature.

Type	Material	Source	Cd	Cr(VI)	Pb	Zn	Cu	Ni	As(V)
Dead biomass	*Chlorella minutissima*	Roy et al. (1993)	11.14	162.23	9.74	—	—	—	—
	Solanum elaeagnifolium	Baig et al. (1999)	18.94	2.16	20.6	6.96	13.14	6.5	—
	Penicillium chrysogenum	Niu et al. (1993)	—	—	116	—	—	—	—
	Strptomyces griseus	Matis and Zouboulis (1994)	28	—	—	—	—	—	—
Living biomass	Seaweed	Leusch et al. (1995)	215	—	344	—	—	—	—
Lignocellulosic substrates	Sugar-beet pulp	Reddad et al. (2003)	—	10[a]	—	—	—	—	—
	Sugar-beet pulp	Reddad et al. (2002d)	24.4	—	73.76	17.78	21.16	11.85	—
	Wheat bran	Dupont et al. (2003)	—	35	—	—	—	—	—
	Sugar-beet pulp	Gerente et al. 2000	—	—	60	—	30	12	—
	Rice husk	Wong et al. (2003)	—	—	108	—	29	—	—
	Rice husk	Tarley et al. (2004)	4	—	45	—	—	—	—
	Rice husk	Roy et al. (1993)	21.36	164.31	11.4	—	—	—	615.1
	Rice husk	Lee et al. (1999)	—	—	—	—	—	—	18.98
	Sawdust	Sharma and Forster (1994)	—	39.7	—	—	—	—	—
	Bagasse	Sharma and Forster (1994)	—	13.4	—	—	—	—	—
	Sugar-beet pulp	Sharma and Forster (1994)	—	17.2	—	—	—	—	—
	Coconut husk fibers	Tan et al. (1993)	—	29	—	—	—	—	—
	Palm pressed fibers	Tan et al. (1993)	—	15	—	—	—	—	—

[a]Cr(III).

Table 4. Adsorption capacities (mg/g) for various other sorbents in the literature.

Type	Material	Source	Hg	Cd	Cr(VI)	Pb	Zn	Cu	Ni
Lignin	Lignin	Srivastava et al. (1994)	—	—	—	1,865	95	—	—
	Sulfuric acid lignin	Masri et al. (1974)	150	—	—	—	—	—	—
Xanthate	Cellulose xanthate	Bricka and Hill (1989)	0.64	19.88	19.67	—	—	—	—
Alginate	Sodium alginate	Jang et al. (1995)	—	—	—	—	—	138.3	—
Chitosan	Composite chitosan	Boddu et al. (2003)	—	153.8	—	—	—	—	—
	Chitosan from lobster shells	Peniche-Covas et al. (1992)	430	—	—	—	—	—	—
	Chitin	Masri et al. (1974)	100	—	—	—	—	—	—
	Chitosan	Manuel et al. (1995)	—	—	78	—	—	—	—
Bark	Black oak	Masri et al. (1974)	400	25.9	—	153.3	—	—	—
	Douglas-fir	Masri et al. (1974)	100	—	—	—	—	—	—
	Pinus sylvestris	Alves et al. (1993)	—	—	19.5	—	—	—	—
Activated carbon	Filtrosorb 400	Huang and Wu (1977)	—	—	125.5	—	—	—	—
	Coconut shell	Alaerts et al. (1989)	—	—	20	—	—	—	—
Peat moss	Rastunsuo peat	Tummavuori and Aho (1980a,b)	16.2	5.058	4.63	20.038	—	—	—
Clays	Wallastonite	Yadava et al. (1991)	—	—	—	0.217	—	—	—
	Heat-treated bentonite	Pradas et al. (1994)	—	16.5	—	—	—	—	—
Zeolite	Zeolite	Leppert (1990)	150.4	84.3	26.0	155.4	—	—	—
Fly ash	Bagasse	Gupta et al. (2003)	—	1.4	—	—	—	—	1.2
Fly ash	Bagasse	Gupta and Ali 2004	—	—	20	30	—	—	—

V. Langmuir or Freundlich Isotherms Versus NICA–Donnan Model

Although numerous authors have reported on screening of biosorptive properties of different biomass types (Volesky and Holan 1995), most biosorption data are interpreted using simple Langmuir or Freundlich isotherms that can only reflect the influence of metal concentration on the uptake of that one particular metal. Few studies have focused on different conditions (e.g., metal concentrations, pH, ionic strength), whereas the Donnan model has been used to interpret ionic strength effects in polyelectrolytes (Marinsky 1987). For biosorption, ionic strength effects have only recently been taken into account using the Donnan model (Schiewer and Volesky 1997; Schiewer 1999; Bouanda et al. 2002). It has long been recognized that the binding of most divalent metals increases with increasing pH (Ferguson and Bubela 1974; Tsezos and Volesky 1981; Ramelow et al. 1992; Holan et al. 1993), and this is explained as an effect of decreasing competition with protons for the same binding sites (Greene et al. 1987; Crist et al. 1994). The Donnan model has been used successfully to determine the effect of ionic strength on biosorption by *Sargassus* seaweed biomass (Schiewer 1999). The binding of protons and metal ions is reduced with increasing ionic strength because Na^+, H^+ and Mg^{2+} compete for electrostatic binding, all acting as counterions for the negatively charged binding sites in the biomass.

Currently, various kinds of unconventional substrates generated from agricultural and forest by-products are also being tested to evaluate their efficiency in the removal of toxic metal ions. Dupont et al. (2003) demonstrated the value of natural organic matter in the retention of heavy metal ions and has extracted a lignocellulosic substrate from wheat bran, which is able to fix these three metal ions efficiently in this order: $H^+ \approx$ copper $\approx$ lead and cadmium ions. Greater affinity of H^+ ions has also been observed by Ravat et al. (2000) with a lignocellulosic substrate, and it is classic in the case of humic substances (Kinniburgh et al. 1996; Benedetti et al. 1995); this represents a very simple model of natural organic matter derived from lignin and cellulose. The total metal binding is composed of three contributions, the Donnan phase and the carboxylic- and phenolic-type sites. Whatever the pH, indeed, the implication of lignin and cellulose in the retention and transport of metal ions is now more and more assumed (Guillon et al. 2001). Metal ion binding to natural organic matter is assumed to occur through specific interactions between cations and surface functional groups and by nonspecific binding to any residual negative charge. Proton binding involves two major contributions from weak and high-affinity site types; the former can be identified with carboxyl sites and the latter with the phenol sites. Dupont et al. (2003) described the acid–base properties of LCS using NICA–Donnan formalism, where electrostatic interactions are taken into account. The affinity of metal ions or high-

affinity sites increases in the order Cd < Pb ≈ Cu ≈ H. The same sequence is observed for the low-affinity site except that H^+ has a greater affinity than Cu^{2+} and Pb^{2+}.

Summary

Toxic heavy metals and metalloids are constantly released into the environment, and their removal is a very difficult task because of the high cost of treatment methods. Various methods exist for the removal of toxic metal ions from aqueous solutions. Among these are adsorption using activated carbon, by far the most versatile and widely used method for the removal of toxic metals; however, it is relatively expensive and less feasible to use in developing countries. Furthermore, activated carbon loaded with toxicants is generally incinerated or disposed of on land, thereby causing environmental pollution through different routes. There is an urgent need to develop low-cost, effective, and sustainable methods for their removal or detoxification. The use of lignocellulosic agrowastes is a very useful approach, because of their high adsorption properties, which results from their ion-exchange capabilities. Agricultural wastes can be made into good sorbents for the removal of many metals, which would add to their value, help reduce the cost of waste disposal, and provide a potentially cheap alternative to existing commercial carbons. Although the abundance and very low cost of lignocellulosic wastes from agricultural operations are real advantages that render them suitable alternatives for the remediation of heavy metals, further successful studies on these materials are essential to demonstrate the efficacy of this technology.

Acknowledgments

The Department of Biotechnology, Ministry of Science and Technology, Government of India, is gratefully acknowledged for awarding a Biotechnology Overseas Associateship to Dr. K.K. Krishnani.

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Manuscript received September 17; accepted September 23, 2005.

Rev Environ Contam Toxicol 188:85–126

Earthworm Biomarkers in Ecological Risk Assessment

J.C. Sanchez-Hernandez

Contents

I. Introduction

Earthworms are important components of the soil system, mainly because of their favorable effects on soil structure and function (Paoletti 1999; Jongmans et al. 2003). Their burrowing and feeding activities contribute notably to increased water infiltration, soil aeration, and the stabilization of soil aggregates. In addition, earthworms help to increase soil fertility by formation of an organic matter layer in topsoil. These features, among others, have led to the popularity of earthworms as excellent bioindicators of soil pollution (Cortet et al. 1999; Lanno et al. 2004). These organisms ingest large amounts of soil, or specific fractions of soil (i.e., organic matter), thereby being continuously exposed to contaminants through their alimentary surfaces (Morgan et al. 2004). Moreover, several studies have shown that earthworm skin is a significant route of contaminant uptake as well (Saxe et al. 2001; Jager et al. 2003; Vijver et al. 2005).

Communicated by George W. Ware.

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Several earthworm species (e.g., *Eisenia fetida* and *E. andrei*) have occupied an important place in toxicity testing (OECD 1984). The primary goals of these tests have been (i) the assessment of potential toxicity of new chemicals to be introduced into the environment, and (ii) the risk assessment for toxic effects from historically contaminated soils. Earthworms have also been used as bioindicators in the field monitoring of soil pollution. Changes in abundance, biomass, or species richness of natural populations have been common ecological endpoints to identify point-sources of pollution (Spurgeon and Hopkin 1999; Nahmani and Lavelle 2002; Dunger and Voigtländer 2005; Vandecasteele et al. 2004). Their tolerance to highly metal-contaminated soils and capacity to accumulate elevated concentrations of heavy metals in their tissues have led to the use of earthworms as sentinel species (Lukkari et al. 2004a; Carpené et al. 2006).

Earthworm biomarkers have scarcely been investigated, particularly under field conditions. Some of them (e.g., lysosomal fragility) have received particular attention in recent years. Generally, the term "biomarker" is easily exchangeable by "bioindicator" in the ecotoxicological literature and can lead to the reader's confusion. In this review, a biomarker or biological marker refers to any biological response (from molecular to behavioral changes) to one or more contaminants (Peakall 1992; Lagadic et al. 2000; Walker et al. 2001; Handy et al. 2003; Vasseur and Cossu-Leguille 2003). The term bioindicator, however, defines an organism that gives information on the environmental conditions of its habitat by its presence or absence (van Gestel and van Brummelen 1996). Most authors agree that biomarkers are sensitive indicators of contaminant exposure, whose main goal is to serve as early warning signs of predictive adverse effects at higher biological organization levels (population or community). To date, however, biomarkers provide an indication of exposure only. Thus, the determination of multiple biomarkers across different levels of biological organization is recommended to provide a better assessment of ecological consequences of contamination (Spurgeon et al. 2005a). Recently, biomarkers have gained ecotoxicological meaning when they have been integrated in an ecological weight-of-evidence (WOE) framework (Neuparth et al. 2005).

Two international meetings held in Denmark (3rd International Workshop on Earthworm Ecotoxicology; special issue of *Ecotoxicology and Environmental Safety*, vol. 57, 2004) and UK (7th International Symposium on Earthworm Ecology; special issue of *Pedobiology*, vol. 47, 2003) have examined the current knowledge of earthworm ecotoxicology. Previously, two exhaustive reviews summarized the available information on the most common earthworm biomarkers (Kammenga et al. 2000; Scott-Fordsmand and Weeks 2000). Some remarkable conclusions can be drawn from these reviews. Although a broad group of molecular biomarkers such as cholinesterases (ChEs), cytochrome P450-dependet monooxygenases, DNA breakage, or enzymes of oxidative stress have been traditionally measured in earthworms, they have been mainly studied in response to

heavy metal exposure (Cd, Cu, Pb, Zn). Therefore, there is a need for developing biomarkers of exposure/effects to organic contaminants of current concern (e.g., polycyclic aromatic hydrocarbons, pesticides, polybrominated flame retardants) or even other metals such as mercury. Furthermore, some of the features that define an ideal biomarker have yet not been investigated in the earthworm. For example, impact of confounding factors (environmental and biological) on biomarker responses and their normal variations need to be investigated (Scott-Fordsmand and Weeks 2000). A set of recommendations drawn from the 3rd International Workshop on Earthworm Ecotoxicology (van Gestel and Weeks 2004) can be summarized in the following points:

It is necessary to investigate the toxicodynamic (i.e., mechanism of toxicity at the target site) of chemicals to develop new, sensitive, and reliable biomarkers.

Biomarkers should be examined under field conditions to validate them as early warning indicators of negative ecological consequences.

Biomarker responses must be linked to adverse effects on life cycle traits (cocoon production rate or changes in body weight) under laboratory bioassays.

It is necessary to assess the impact of environmental factors (e.g., temperature, pH, osmotic stress, organic matter content, or photoperiod) and biological variables (e.g., reproductive cycle, nutritional status) on the biomarkers.

Most of the research on earthworm biomarkers involves the effects of certain heavy metals only (e.g., Cu, Cd, Zn or Pb), and investigations on biomarker responses to organic pollutant exposure are rather scarce.

The purpose of this review is to examine the current knowledge on earthworm biomarkers, as well as the application of biomarkers in ecological risk assessment (ERA) of contaminated soils. A critical discussion, organized in three sections, undertakes (1) the potential use of earthworm biomarkers as sublethal endpoints in standardized toxicity tests, (2) the main drawbacks in the assessment procedures of contaminated soils, and (3) the use of earthworm biomarkers for assessing the effectiveness of two procedures currently applied for recovering/protecting the environment: the soil bioremediation and the agrienvironment schemes, implemented in many countries of the European Union. Finally, future lines of research are suggested to increase the understanding of earthworm biomarkers.

II. Earthworm Biomarkers

A. Ecotoxicological Tests

Toxicity tests constitute an essential element of the ERA scheme (exposure and effect assessment). They are used to predict acute and/or chronic effects of new chemicals before release into the environment or to assess the

ecological impact of a new aqueous or atmospheric emission sources (predictive ERA). Similarly, ecotoxicity assays are also used in a retrospective approach of ERA to assess the historical contamination with possible ongoing ecological consequences. In general terms, toxicity testing has been the main instrument for legal requirements and environmental management decisions, which has led to the development of multiple standardized protocols depending mainly on the ambient media or test organism. An extensive description of toxicity tests used for aquatic environment assessments is compiled in the textbook *Fundamentals of Aquatic Toxicology* (Rand 2003). A guideline for conducting soil toxicity tests has been reported by the Organisation for Economic Co-operation and Development (OECD 1984, 2004) or by the International Standard Organization (ISO 1993, 1998, 2004). A description of the most common soil toxicity tests is available in van Straalen and van Gestel (1998) or Jänsch et al. (2005).

The typical endpoints in any standardized acute or chronic toxicity test are survival, reproduction rate, growth, or immobilization (e.g., daphnids). When field-contaminated soils or sediments are used to assess their toxicity (retrospective ERA), uncertainties in the test results can be associated to factors other than the contaminant burden present in the environmental media. The application of the appropriate biomarkers could provide further information about the active bioavailable fraction of the contaminant (Lanno et al. 2004). Moreover, biomarkers can give clear evidence of a cause–effect relationship between the contaminant in the environmental media and the occurrence of adverse effects at the individual level. Sediment toxicology, for instance, has been initiated to integrate certain molecular biomarkers in acute toxicity tests to assess sublethal toxic effects at multiple levels of biological organization (Neuparth et al. 2005). This current tendency is also becoming a common practice in soil toxicity tests using earthworms. This review does not attempt to give an exhaustive compilation of the earthworm toxicity assays but describes only those studies in which biomarkers have been integrated in the suite of toxicological endpoints.

The measurement of lysosomal membrane stability through the neutral red retention (NRR) assay, which combines analytical simplicity and ecological realism (complexity), has become one of the most popular earthworm biomarkers. The NRR assay in earthworms was first described by Weeks and Svendsen (1996); a review of their qualities was published by Svendsen et al. (2004). The NRR assay is determined in coelomocytes collected from the coelomic fluid. The quantification of this biomarker response implies the measurement of the time required to achieve 50% stained cells of the total cells counted periodically under a light microscope during a fixed time period. Lysosomal membrane stability can decrease in response to stress, and this is manifested in the NRR assay as a gradual leak of the neutral red from the lysosomes into the surrounding cytoplasm.

Damage in the lysosomal membrane caused by contaminant exposure is associated, therefore, with a decrease in the NRR time with respect to that in intact lysosomes.

Some studies have demonstrated that this biomarker is a useful predictor of adverse effects on life cycle traits (e.g., survival, growth, or reproduction). For example, Svendsen and Weeks (1997a) found that NRR times in *E. andrei* exposed to Cu were significantly reduced when metal concentration in soil was 20 mg kg^{-1}, whereas survival or changes in body weight were significantly affected at Cu concentrations as high as 320 mg kg^{-1} (Table 1). Similarly, Booth and O'Halloran (2001) reported that the NRR assay in adult earthworms (*Aporrectodea caliginosa*) exposed for 28 d to sublethal concentrations of the organophosphate insecticides diazinon and chlorpyrifos was a more-sensitive indicator than growth rate or cocoon production. Exposure to Pb also caused a significant and concentration-dependent reduction in the NRR time of *E. fetida* after 4 wk of metal exposure (Booth et al. 2003). A negative linear correlation was found between the logarithmic-transformed Pb concentrations in the earthworm body and the NRR times. This earthworm species also showed a substantial decrease of the NRR time up to 4 min (NRR times were ~50 min in control group) after exposure to Cu concentrations higher than 300 mg kg^{-1} (Scott-Fordsmand et al. 2000). In this study, the reduction in NRR time corresponded to an earthworm body Cu concentration of about 50 mg kg^{-1}. This is a clear example of why internal metal concentration is a more reliable endpoint than traditional external metal concentration, especially when parameters such as EC_{50} are estimated (Escher and Hermens 2004). Nevertheless, the internal metal concentration does not reflect the bioactive fraction (internal effect concentration). The toxicant concentration or dose at target site (bioactive fraction) can be estimated from models based on simple partitioning or more complex kinetics (Escher and Hermens 2004). Biomarkers such as the NRR assay might be a useful tool for estimating the internal effect concentration because they reflect the bioactive contaminant fraction.

The historical use of earthworms as biomonitors of metal soil pollution has contributed notably to the characterization of metallothioneins (MTs) in these organisms. These low molecular weight and cysteine-rich proteins have been isolated and fully characterized in *Lumbricus rubellus* (Stürzenbaum et al. 1998) and *E. fetida* (Gruber et al. 2000). In the case of *L. rubellus*, two MT isoforms (i.e., wMT-1 and wMT-2) have been isolated and seem to have different physiological functions and responses to metal exposure (Stürzenbaum et al. 1998; Morgan et al. 2004). wMT-2 has been the MT isoform more studied in relation to metal exposure because of its role in heavy metal sequestration. It shows a marked induction in *L. rubellus* exposed to increasing Cd or Cu concentrations in soil (Burgos et al. 2005; Spurgeon et al. 2005b).

Table 1. Biomarker responses in earthworms experimentally exposed to organic pollutants or heavy metals.

Species	Test substance: chemical form	Concentration and time of exposure	Test conditions	Biomarkers	Main remarks	Reference[a]
Eisenia fetida andrei (clitellate adults)	Carbaryl	12, 25, and 50 mg kg^{-1} 2, 7, and 14 d	Artificial soil (OECD) Soil moisture = 35% Temp. = 20° ± 1°C pH = 6.5 ± 0.5 Continuous light	AChE MROD NADH Red NADPH Red Catalase GR GST LP and LPI Total GSH % GSSG	Significant depression of enzymes involved in biotransformation of xenobiotics (MROD, NADH Red, and NADPH Red), but lack of a consistent concentration–response relationship. Significant inhibition of AChE activity in all treatments and time of exposure.	(1)
E. fetida andrei (clitellate adults)	Pb: lead acetate	30, 60, 120, and 250 mg kg^{-1} 2, 7, 14, and 28 d	Artificial soil (OECD) Soil moisture = 35% Temp. = 20° ± 1°C pH = 5.8–6.20 Continuous light	AChE MROD NADH Red NADPH Red Catalase GR GST LP and LPI Total GSH % GSSG	Induction of lipid peroxidation but related to neither Pb concentration nor time of exposure. Depression of GST at all treatments after 2 d of exposure. Significant variations of enzymes involved in biotransformation process but no clear relationships with Pb concentrations were observed. Reactive oxygen species (ROS) probably generated after Pb exposure.	(2)
E. fetida andrei (clitellate adults)	Benzo(*a*)pyrene	0.05, 1, 100, and 1,000 mg kg^{-1} 1, 2, 7, and 14 d	Artificial soil (OECD) Soil moisture = 35% Temp. = 20° ± 1°C pH = 6.5 ± 0.5 Continuous light	AChE MROD NADH Red NADPH Red Catalase GR GST LP and LPI Total GSH % GSSG	Induction of MROD activity at low B(a)P concentrations, but inhibition at high (>100 mg kg^{-1}) concentrations. Induction of lipid peroxidation and increase of several enzyme activities (AChE, catalase), but no evident relationship with B(a)P concentrations or time of exposure. ROS probably involved in B(a)P exposure.	(3)

Table 1. *Continued*

Species	Test substance: chemical form	Concentration and time of exposure	Test conditions	Biomarkers	Main remarks	Reference[a]
E. foetida	Chlorpyrifos	2.96 ± 0.39, and 2.33 ± 0.39 mg ml^{-1} 12, 24, 36, and 48 hr	Filter paper contact test (OECD, method 207)	AChE	Significant inhibition (>60%) of AChE activity at the two pesticide concentrations. Morphological abnormalities (necrosis and damage of the structure of muscles, bloody lesions, rectal areas detached).	(4)
Drawida willsi (juveniles)	Butachlor, Malathion and Carbofuran	1.1. and 2.2 mg kg^{-1} (butachlor and carbofuran) 2.2 and 4.4 mg kg^{-1} (malathion) Perodically from 1 to 105 d	Natural soil Soil moisture = 20% Temp. = 25° ± 2°C pH = 6.8	AChE	No variations of AChE activity in earthworms exposed to butachlor. Maximum AChE inhibition (41% and 46%) after 9 d of malathion exposure, and after 12 d (54% and 62.9%) of carbofuran exposure. AChE recovered its normal activity after 45 d of malathion exposure, while it needed 75 d to full activity recovery under carbofuran exposure.	(5)
Lumbricus rubellus (adults)	Pyrene	10, 40, 160, 640, and 2,560 mg kg^{-1} 42 d	Artificial sterilized loam soil Soil moisture = 80% Temp. = 15° ± 1.5°C 16 : 8 hr, L : D cycle	EROD Catalase	No EROD activity was detected. Catalase activity was lower in earthworms exposed to 160 and 640 mg kg^{-1} respect to control group.	(6)
Aporrectodea tuberculata (clitellate adults)	Cu : $CuCl_2 \cdot 2H_2O$ Zn: $ZnCl_2$	Cu/Zn = 100/175, 200/350, and 400/700 mg kg^{-1} 2, 7, and 14 d	Natural soil (collected from Jyväskylä, a nonpolluted area in Finland) Soil moisture = 26% ± 4% pH = 6.7 ± 0.1 Temp. = 15°C Darkness	CYP1A MT GST	Concentration-dependent induction of MT in earthworms exposed to Cu and Zn after 2 and 7 d of exposure. Earthworm populations naturally exposed to metal-contaminated soils showed a higher MT induction than not naturally metal-exposed population after 2 and 7 d of exposure to Cu/Zn-spiked soils. Induction of CYP1A activity after Cu and Zn exposure.	(7)

Table 1. *Continued*

Species	Test substance: chemical form	Concentration and time of exposure	Test conditions	Biomarkers	Main remarks	Reference[a]
E. fetida (clitellate adults)	Zn and Pb	Reference soil: Zn = 1.75 ± 0.25 μg g^{-1} Pb = 4.30 ± 0.99 μg g^{-1} Metalliferous soil: Zn > 1,500 μg g^{-1} Pb > 500 μg g^{-1} 20 d	Reference soil: commercial Kettering loam pH = 6.1 Organic matter = 7.0% Metalliferous soil: collected at an abandoned Zn/Pb mine pH = 6.4 Organic matter = 35% Temp. = 15° ± 1°C Soil moisture = 75% 12 : 12 hr, L : D cycle	Annetocin	A 20-fold reduction in the annetocin expression was found in earthworms exposed to the metalliferous soil respect to those exposed to the reference soil.	(8)
E. andrei (clitellate adults)	Cu: $CuCl_2 \cdot 2H_2O$	Cu = 20, 40, 80, 160, and 320 mg kg^{-1} 28 d	Field soils Soil moisture = 50% pH = 5.6 Organic matter <1% Temp. = 15°C Constant light	NRR assay	Significant dose-related decrease in the NRR time with soil and body Cu concentrations. Suggested toxic threshold for the Cu of 40–80 mg kg^{-1}. NRR assay becomes a more sensitive indicator than mortality or individual growth.	(9)
E. fetida (clitellate adults)	Cu: $CuCl_2 \cdot 2H_2O$	Cu = 50, 100, 300, 700, and 1400 mg kg^{-1} 21 d	Natural soil. pH = 6.5–7.0 Temp. = 20° ± 1°C 12 : 12 h, L : D cycle	NRR assay	Earthworms exposed to soils spiked with 300 mg kg^{-1} showed a NRR time <4 min, indicating a significant toxic effect. A range of NRR times between 4 and 6 min corresponded to an internal Cu concentration >50 mg kg^{-1}.	(10)

Table 1. *Continued*

Species	Test substance: chemical form	Concentration and time of exposure	Test conditions	Biomarkers	Main remarks	Reference[a]
E. fetida (clitellate adults)	Pb: $Pb(NO_3)_2$	Pb = 20, 40, 80, 160, and 320 mg kg^{-1} 28 d	Sieved cattle manure Soil moisture = 75% pH = 7.0 Temp. = 25°C	NRR assay	Decrease of NRR time with increasing of Pb body burden.	(11)
Aporrectodea caliginosa (adults and juveniles)	Diazinon: Basudin 600EW, 60% a.i. Chlorpyrifos: Lorsban 40EC, 40% a.i.	Diazinon = 12 and 60 mg kg^{-1} Chlorpyrifos = 4 and 28 mg kg^{-1} 28 d	Natural soil. Soil moisture = 25% pH = 6.5–7.0 Temp. = 20°C Constant light	NRR assay ChE GST	ChE activity was drastically inhibited in juvenile earthworms exposed to 12 (75% inhibition) and 60 (90%) mg kg^{-1} of diazinon. Chlorpyrifos caused a ChE depression of 35% and 70% respect to controls in earthworms exposed to 4 and 28 mg kg^{-1}, respectively. GST activity was inhibited by both diazinon treatments. NRR time was significantly decreased in adult earthworms exposed to diazinon (>64% reduction) and chlorpyrifos (>53% reduction). ChE activity and NRR assay resulted suitable biomarkers of pesticide suitable exposure, and it was linked to suitable changes in growth or fecundity.	(12)
E. fetida (adults)	Pb: lead acetate	Pb = 102, 221, 283, 588, and 1,233 mg kg^{-1} 28 d	Natural soils spiked with Pb Soil moisture = 20% Organic matter = 5.6% pH = 7.6 Temp. = 18°C 12 : 12 hr L : D cycle	NRR assay	NRR times showed a significant negative relationship with body Pb concentration. All treatments caused a reduction (>50% reduction) respect to the control group.	(13)

Table 1. *Continued*

Species	Test substance: chemical form	Concentration and time of exposure	Test conditions	Biomarkers	Main remarks	Reference[a]
A. caliginosa *E. fetida* *L. terrestris* *L. rubellus* (clitellate adults)	Zn: $Zn(NO_3)_2 \cdot 6H_2O$	Zn = 190, 350, 620, 1,200, 2,000, and 3,600 mg kg^{-1} 42 d	Natural soil (with 10% sphagnum peat) Soil moisture = 60% Temp. = 15°C Constant light	NRR assay	NRR assay reveals as a good biomarker for predicting adverse effects at life cycle level. NRR assay showed significant species-specific differences.	(14)
L. rubellus (clitellate adults)	Cu: $CuCl_2 \cdot 2H_2O$ Cd: $CdCl_2 \cdot 2\frac{1}{2}H_2O$	5, 25, 125, and 200 µg g^{-1} dry wt, each metal 21 d	Artificial soil (OECD) Soil moisture: 35%–40% pH = 6.0 ± 0.5 Temp. = 17°C 12 : 12 hr L : D cycle	wMT-1 wMT-2 AOX LYS	Exposure to Cu-spiked soils caused an induction of the biomarkers at the lowest Cu concentrations only. wMT-2 isoform showed a concentration–response relationship with increasing Cd concentrations in the soil. These molecular genetic biomarkers were more sensitive to Cu exposure than mortality, growth, or cocoon production.	(15)

AChE, acetylcholinesterase; MROD, methoxyresorufin-*O*-deethlylase; NADH Red, NADH cytochrome reductase; NADPH Red, NADPH cytochrome reductase; GR, glutathione reductase; GST, glutathione-*S*-transferase; LP, lipid peroxides; LPI, peroxidizable lipids; total GSH, total glutathione; % GSSG, percentage of oxidized glutathione; EROD, ethoxyresorufin-*O*-deethylase; ChE, cholinesterase; CYP1A, cytochrome P4501A isoenzyme; MT, metallothionein; NRR, neutral red retention; wMT-1, metallothionein isoform 1; wMT-2, metallothionein isoform 2; AOX, amine oxidase; LYS, lysosomal glycoprotein.

[a]References: (1) Ribera et al. 2001; (2) Saint-Denis et al. 2001; (3) Saint-Denis et al. 1999; (4) Rao et al. 2003; (5) Panda and Sahu 2004; (6) Brown et al. 2004; (7) Lukkari et al. 2004a; (8) Ricketts et al. 2004; (9) Svendsen and Weeks 1997a; (10) Scott-Fordsmand et al. 2000; (11) Reinecke and Reinecke 2003; (12) Booth and O'Halloran 2001; (13) Booth et al. 2003; (14) Spurgeon et al. 2000; (15) Burgos et al. 2005.

Earthworm biomarkers related to the detoxification systems have become of increasing concern. Using a similar exposure protocol, Ribera and coworkers examined the effects of Pb (Saint-Denis et al. 2001), carbaryl (Ribera et al. 2001), and benzo(*a*)pyrene (Saint-Denis et al. 1999) in a suite of biochemical biomarkers in *E. fetida andrei* (see Table 1). In general, a nonclear concentration–response relationship was observed for most of the biomarkers. However, factorial discriminant analysis of all biomarker responses enabled them to establish differences related to the toxicant concentration in soil. The use of multivariate statistics has been applied and suggested by others (Burgos et al. 2005) when concentration(dose)–response relationships are not clearly defined. The results by Ribera's group showed that the three assayed contaminants caused biomarker responses comparable to those found in other organisms such as fish (van der Oost et al. 2003). Thus, carbaryl drastically inhibited the acetylcholinesterase (AChE) activity, whereas Pb increased lipid peroxidation and caused inhibition of enzyme activities involved in xenobiotic metabolism such as glutathione-*S*-transferase (GST) or methoxyresorufin-*O*-deethylase (MROD). Similarly, benzo(a)pyrene caused an induction of the MROD and catalase activities and lipid peroxidation. The authors suggested that the formation of reactive oxygen species (ROS) accounts for the response of certain biomarkers such as catalase or GST or the increase in lipid peroxidation. The mechanism causing the lysosomal membrane fragility in earthworm coelomocytes is not yet well understood (Svendsen et al. 2004), although the participation of ROS should not be totally excluded. One of the effects of these highly reactive chemical species is the formation of lipid hydroperoxides from the polyunsaturated fatty acids, leading to altering membrane integrity and function (Abuja and Albertini 2001); this could be one of the mechanisms of toxic action leading to lysosomal membrane damage (Pellerin-Massicotte and Tremblay 2000).

Earthworms are important members in the agroecosystem because of their beneficial contribution to soil structure and function. Despite this, laboratory and field studies involving biomarkers for assessing pesticide impact on earthworms are still scarce in comparison to other organisms (Scott-Fordsmand and Weeks 2000). Organophosphorus (OP) and carbamates (CB), commonly named anticholinesterase (anti-ChE) pesticides, are an important group of agrochemicals widely used in modern agriculture. More than two decades of ecotoxicological research on ChEs have demonstrated that these enzymes are suitable biomarkers of pesticide exposure and toxic effects, and they continue to be an important component in the biomonitoring programs of pesticide contamination. In a standardized toxicity test (paper contact assay; OECD 1984), Rao et al. (2003) measured variations of AChE activity in *E. foetida* exposed to the median lethal concentration (LC_{50}) of chlorpyrifos. They found AChE inhibition above 60% after 12 hr exposure, which increased up to 91% after 48 hr OP exposure. Simultaneously, a gradual morphological damage in the animals (rupture of

the cuticle, bloody lesions, or fragmentation of posterior parts) was observed in relation to the chlorpyrifos concentration and time of exposure (24 or 48 hr).

Toxic effects of anti-ChE pesticides on the earthworm reproduction system have been described in *E. fetida*. In a histological study, Sorour and Larink (2001) showed that the fungicide benomyl caused gradual damage on the male reproduction system (abnormal cytophores and malformed spermatides) in individuals exposed for a week to sublethal concentrations (8.3–112 mg kg^{-1}). Likewise, Espinoza-Navarro and Bustos-Obregon (2005) also observed alterations in the male reproduction system in specimens exposed to the OP malathion (80–600 mg kg^{-1}). Besides a loss of body weight up to 50% in the treated groups compared to nonexposed, they also found vacuolization of spermatheca and fragmentation of DNA in a high proportion of spermatogonia. All these toxic effects probably cause alterations in the reproductive performance of earthworms. In this sense, the biomarker responses to this class of pesticides should be investigated in detail in future research. In their review, Scott-Fordsmand and Weeks (2000) showed that a considerable number of ChE-inhibiting pesticides have been assayed in earthworms but that the potential use of ChEs as biomarkers of pesticide exposure has not been sufficiently explored. For example, very few data exist on the recovery rate of phosphorylated or carbamylated ChE activity of earthworms. Indeed, one of the most important features in a good biomarker is the stability of its response, especially when it is used in the field. As an example, OP-inhibited ChE of birds take from hours to a few days for full recovery, whereas phosphorylated ChE in aquatic invertebrates, fish, or reptiles recovers its normal activity more slowly, taking several weeks for full recovery (Fulton and Key 2001; Sanchez-Hernandez 2001).

This slow recovery rate enables the detection of OP impact over a longer period after OP applications, a desirable feature when these types of pesticides show a low persistence in the environment (Racke 1992). Panda and Sahu (2004) determined the time to full recovery of AChE activity in the tropical earthworm *Drawida willsi* after exposure to butachlor (a herbicide), malathion, and carbofuran. Although butachlor did not cause any variation in AChE activity, maximum inhibition of AChE activity was found after 9 d exposure to malathion (2.2 and 4.4 mg kg^{-1}) and after 12 d exposure to carbofuran (1.1 and 2.2 mg kg^{-1}). The recovery of AChE activity of *D. willsi* was found to be extremely slow (45–75 d). Moreover, the recovery rate of the phosphorylated (or carbamylated) AChE activity did not appear to be related to the pesticide concentration. However, in that study earthworms were continuously exposed to the OP- or CB-contaminated soils, and it is difficult therefore to draw any conclusion about AChE recovery. To investigate the recovery rate of ChE activity, it would be ideal to transfer earthworms to clean soil when ChE activity is inhibited. This approach would be more environmentally realistic than keeping the earthworms

continuously in the contaminated soils for a long time, especially if earthworms tend to avoid contaminated soils (Schaefer 2003). Natural variability and impact of ambient variables on earthworm ChE activity need to be studied, as well as the ecological meaning of ChE inhibition (e.g., alterations of burrowing or feeding activities). On the other hand, there exist two main groups of esterases that participate in the manifestation of tolerance and resistance to ChE-inhibiting pesticides: fosfotriesterases and carboxylesterases (Jokanovic 2001; Sogorb and Vilanova 2002). To date, one study has reported the existence of fosfotriesterases in the earthworm *E. andrei*; these appear to be primarily localized in the intestinal tissues (Lee et al. 2001), but the implication in OP tolerance still needs to be explored.

Earthworms avoid contaminated soils. Several studies have demonstrated that the avoidance response of earthworms often occurs at low levels of metal concentration at which survival and reproduction are not affected (Schaefer 2005; Loureiro et al. 2005; Lukkari et al. 2005; Lukkari and Haimi 2005). van Gestel and Weeks (2004) reported that the earthworm behavior of avoiding contaminated soils should be among the aspects of earthworm ecotoxicology to be investigated. Indeed, there is a growing interest in the use of earthworm behavior in soil ERAs (Table 2). Different designs have been used for the avoidance behavior test. Schaefer (2003) compared test results from the most common test chambers, i.e., two- and six-chamber test systems. Although both systems gave similar results, the two-chamber system was recommended for future avoidance behavior tests mainly by its simplicity. This chamber consists of a rectangular container divided in two equal compartments by a removable plastic separator (Fig. 1). Control soil is placed in one compartment and the contaminated soil is placed in the other. A number of earthworms are then released in the middle of the rectangular container after removing the partition. The test starts when earthworms enter the soil, and 48 hr later, the partition is inserted again in the middle of the rectangular container. Individuals are counted in each soil compartment, and an avoidance response is judged as positive when more than 80% live earthworms is found in the compartment containing the control soil.

The two-chamber system is gaining acceptance in soil toxicology. Lukkari et al. (2005) used the avoidance test to examine whether the earthworm *Aporrectodea tuberculata* showed a positive response to Cu/Zn-contaminated soils. They exposed two natural populations of earthworms, with and without earlier wildlife exposure to metal-contaminated soils, to field soils spiked with seven Cu/Zn concentration pairings ranging from 23/41 to 267/467 mg kg^{-1}. Earthworms avoided the contaminated soils with Cu and Zn concentrations higher than 53 and 92 mg kg^{-1}, respectively. In this study, the avoidance response was a more-sensitive index than the standardized acute toxicity and reproductive tests. The avoidance behavior has also been applied to the toxicity assessment of field soils. Loureiro et al. (2005) tested soil samples collected from the abandoned mine Mina de Jales

Table 2. Avoidance Behavior Test in Earthworms Experimentally Exposed to Contaminated Soils.

Species	Test substance: chemical form	Concentrations	Test conditions	Test chamber	Main remarks	Reference[a]
Aporrectodea tuberculata (clitellate adults)	Cu: $CuCl_2 \cdot 2H_2O$ Zn: $ZnCl_2$	Cu/Zn = 23/41 to 267/467 mg kg^{-1}	Natural soil. Temp. = 18°C Soil moisture = 26%–27% pH = 6.0–5.8 Organic matter = 6.5%–7.5% Darkness 48-hr exposure	Cylindrical chamber (17 cm diameter × 11 cm depth)	Significant response (>80% individuals in control soils) was found in earthworms exposed to soils with Cu and Zn concentration pairings higher than 53 and 92 mg kg^{-1}, respectively. Soils contaminated with Cu and Zn concentration pairings higher than 79 and 138 mg kg^{-1}, respectively, caused an avoidance response in 80% of earthworms. Avoidance response test resulted to be a more sensitive endpoint than standardized toxicity tests (acute or reproduction tests).	(1)
Eisenia andrei (clitellate adults)	Carbendazim Benomyl Dimethoate Cu: $CuSO_4 \cdot 5H_2O$	Carbendazim and benomyl = 1, 10, and 100 mg kg^{-1} Dimethoate = 2.5, 5, 10, and 20 mg kg^{-1} Cu = 40, 80, 160, and 320 mg kg^{-1}	Artificial Lufa 2.2 soil Soil moisture = 60% pH = 5.03 Organic matter = 1.28% Temp. = 20° ± 2°C 16:8 hr L:D cycle 48-hr exposure	Rectangular chamber (21 × 12.3 cm)	Earthworms avoided the contaminated soils (>80% individuals in the control soil) at 320 mg kg^{-1} of Cu, >10 mg kg^{-1} of benomyl and carbendazim, and 40 mg kg^{-1} of dimethoate.	(2)
E. fetida (clitellate adults)	Total petroleum hydrocarbons (TPH) and 2,4,6-Trinitotoluene (TNT)	TNT = 2, 7, 29, and 1,142 mg kg^{-1}. TPH = 200, 316, and 1,047 mg kg^{-1}.	Natural contaminated soils (mixed with Lufa 2.2 soil to obtain different contaminant concentrations) Soil moisture = 60% pH = 6.0 ± 0.5 Temp. = 20° ± 2°C Darkness 48-hr exposure	Square chamber (20 × 20 × 10 cm)	Significant avoidance behaviour response (>90%) of the soil contaminated with 1,047 mg kg^{-1} of TPH and >29 mg kg^{-1} of TNT. Avoidance behavior response was a more sensitive endpoint than mortality.	(3)

Table 2. *Continued*

Species	Test substance: chemical form	Concentrations	Test conditions	Test chamber	Main remarks	Reference[a]
E. fetida (clitellate adults)	2,4,6-Trinitotoluene (TNT)	TNT = 2, 7, 29, and 1,142 mg kg^{-1}	Natural contaminated soils Soil moisture = 60% pH = 6.0 ± 0.5 Temp. = 20°C Darkness 48-hr exposure	Round container (28 cm diameter × 10 cm high) with six different chambers connected to a central chamber	Earthworm avoided the soils contaminated with TNT concentration higher than 29 mg kg^{-1}. Avoidance response of earthworm was a more sensitive indicator of TNT exposure than mortality or reproduction rate.	(4)
E. andrei, *L. rubellus*, and *A. caliginosa* (clitellate adults)	Pb: $Pb(NO_3)_2$	350–15,000 mg Pb kg^{-1}	Kettering loam soil. Soil moisture = 50% pH = 4.56 ± 0.01–5.84 ± 0.01 Temp. = 15°C (*A. caliginosa*) and 20°C (*E. andrei* and *L. rubellus*) Darkness 3-hr exposure	Petri dishes (210-mm diameter)	Avoidance response increased with Pb concentration for the three species. Almost all test individuals avoided the contaminated soil at Pb concentrations higher than 5,000 mg kg^{-1}. There was no significant difference between the avoidance behaviour of the three species.	(5)
Dendrobaena octaedra, *L. rubellus*, and *A. tuberculata* (clitellate adults)	Cu: $CuCl_2 \cdot 2H_2O$ Zn: $ZnCl_2$	Cu/Zn = 19/32 to 300/500 mg kg^{-1}	Field soil Temp. = 18°C Soil moisture = 27%–29% pH = 5.8–6.6 Organic matter = 13%–14% Darkness 48-hr exposure	Cylindrical chamber (17 cm diameter × 11 cm depth)	There was a species-specific response to the metal-contaminated soils. *D. octaedra* was the most sensitive species, whereas *L. rubellus* avoided the contaminated soil at the highest metal concentrations.	(6)

Table 2. *Continued*

Species	Test substance: chemical form	Concentrations	Test conditions	Test chamber	Main remarks	Reference[a]
E. fetida (clitellate adults)	Cu: $CuSO_4$, $Cu(NO_3)_2$, $Cu_2(OH)_2(CO_3)$	110–1,750 µg Cu g^{-1} using $Cu(NO_3)_2$ 600–5,000 µg Cu g^{-1} using $CuSO_4$ 1,100–17,500 µg Cu g^{-1} using $Cu_2(OH)_2(CO_3)$	Kettering loam soil. Soil moisture = 50% pH = 6.85 ± 0.05 Temp. = 20°C Darkness 24-hr exposure	Petri dishes (210-mm diameter)	Earthworms avoided contaminated soils at all the Cu concentrations assayed using $Cu(NO_3)_2$ and $CuSO_4$. No positive avoidance behaviour responses was observed when soil was spiked with $Cu_2(OH)_3(CO_3)$ concentrations ≤3,500 µg Cu g^{-1}. Avoidance behaviour response was more a sensitive index to assess Cu exposure than mortality or weight changes.	(7)
Aporrectodea nocturna and *Allolobophora icterica*	Imidacloprid: Confidor	1, 0.1, and 0.01 mg kg^{-1}	Field soil pH = 8.3 Temp. = 12° ± 1°C Organic matter = 28.3 g kg^{-1} Darkness 48-hr exposure	Plastic chambers (8-cm depth) 25 × 25 cm for *A. nocturna*, 15 × 25 cm for *A. icterica*	Both species avoided the contaminated soil at pesticide concentrations ≥0.1 mg kg^{-1}, being the avoidance response more evident for *A. icterica*.	(8)

References: (1) Lukkari et al. 2005; (2) Loureiro et al. 2005; (3) Schaefer 2003; (4) Schaefer 2005; (5) Langdon et al. 2005; (6) Lukkari and Haimi 2005; (7) Arnold et al. 2003; (8) Capowiez and Bérard (2006).

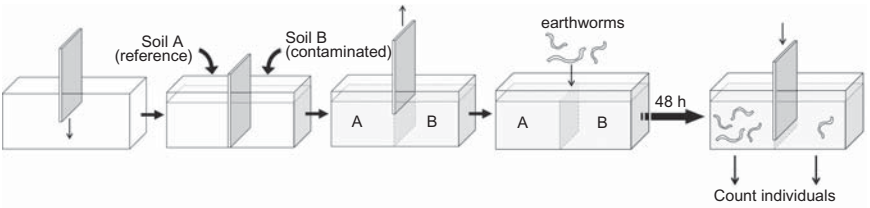

Fig. 1. Scheme of avoidance behavior response test.

(Portugal) with the avoidance response assay. The most contaminated soils (As = 251, Cd = 8.2, Cr = 15, Cu = 24, Mn = 255, Ni = 9, Pb = 209, Zn = 97 mg kg^{-1}) showed a significant behavior response in *E. andrei* when these soils were mixed (75% w/w) with an artificial control soil. In a similar study, Lukkari and Haimi (2005) exposed individuals of a natural earthworm population (*A. tuberculata*) to soils sampled close to a mining area in Finland. Metal-contaminated soils were first mixed with uncontaminated natural soil to obtain contaminated soil proportions of 25%, 50%, 75%, and 100%. Earthworms showed a significant avoidance response when they were exposed for 24 hr to soil containing 25% of the metal-contaminated soil. Although no biomarkers were involved in these studies, it would be attractive to establish a relationship between molecular biomarkers and avoidance behavior responses, especially when the earthworm behavior has direct ecological implications.

B. Field Studies

In a retrospective ERA, four types of approaches can be performed: (1) biological surveys, (2) laboratory tests of ambient media (e.g., soil, water, or sediment), (3) simulated field studies, and (4) *in situ* exposure bioassays. These approaches have used earthworms to assess toxicity of contaminated soils. Summarized next are those studies in which biomarkers were measured in combination with other toxicological endpoints (body residues, growth, survival, or reproduction rate).

Biological Surveys. A few studies have documented body contaminant residues and biomarker fluctuations in relation to soil contamination. Induction of the cytochrome P4501A (CYP1A) and GST activities and MT levels were examined in the earthworm *A. tuberculata* collected along a 4-km transect from an area contaminated by a steel smelter in Finland (Lukkari et al. 2004b). An increase in the response of the three biomarkers was positively correlated with decreasing distance from the steel smelter, which was accompanied by a progressive increase of metal concentrations in soils. Increase of MT levels and GST activity were not related to body metal concentration. Conversely, an induction of CYP1A,

measured by ethoxyresorufin-*O*-deethylase (EROD) activity, positively correlated with metal (Cu, Zn, Fe, and Al) concentrations in the earthworm tissues. Although CYP1A induction is generally attributed to organic contaminant exposure (Whyte et al. 2000), enzyme induction observed in *A. tuberculata* seemed due to metal exposure only. This unexpected finding was corroborated by the authors in a parallel laboratory experiment using natural populations of *A. tuberculata* exposed to a field soil spiked with Cu and Zn (Lukkari et al. 2004a; see Table 1). Laszczyca et al. (2004) also documented spatial and temporal variations of selected biomarkers (CbE, AChE, and antioxidant enzyme activities) in three natural earthworm populations (*A. caliginosa*, *L. terrestris*, and *E. fetida*) collected from meadow sites situated along a 32-km-long transect from a Zn/Pb ore mine and a smelter metallurgic complex. Although body metal (Zn, Pb, Cd, and Cu) concentrations increased in earthworms with decreasing distance from the point-source of pollution, biomarkers showed peak responses at the middle of the transect (4–8 km from the point-source of metal pollution). The authors attributed these biomarker responses to a hormetic-like effect, and suggested that this type of response could be useful in identifying areas where soil contaminants cause adverse effects on organisms in contrast to those areas where toxic effects are balanced by compensatory responses. Hormesis is defined as overcompensation to alterations of homeostasis (Chapman 2001). However, although hormesis is a phenomenon observed generally in the laboratory, its occurrence under field conditions is difficult to assess, mainly because many environmental factors can affect biomarker responses.

The high sensitivity of the NRR assay widely demonstrated in laboratory experiments has been also observed in field studies. A temporal study was carried out to assess the negative impact on the indigenous earthworm *Microchaetus* sp. of copper oxychloride applications (Maboeta et al. 2002). After simulated applications (at $4.25\,g\,L^{-1}$) of the fungicide on a demarcated area, earthworms were periodically sampled to complete a 6-mon survey, and NRR times were recorded. The NRR assay in *Microchaetus* sp. was a more sensitive indicator of pesticide exposure than earthworm biomass or abundance, a finding that agrees with the observations reported in laboratory experiments using other earthworm species and toxicants (see Table 1).

Laboratory Tests of the Soil. The biological survey approach presents a set of drawbacks such as lack of information about exposure history, difficulties in species identification and specimen collection in the sites of interest, the impact of environmental stressors other than the contaminants, and other sources of uncertainty. These limitations can be resolved, in part, when field soils are tested under stable laboratory conditions. The use of a model earthworm species (e.g., *E. fetida*) and controlled conditions (soil moisture,

pH, temperature, organic matter content, photoperiod, etc.) help to link biomarker responses to bioavailable contaminants in soil.

Similar to spiked soil experiments, the NRR assay has proved to be a highly sensitive biomarker of metal exposure when earthworms are exposed to field-contaminated soils. Scott-Fordsmand et al. (2000) found a significant relationship between NRR times measured in *E. fetida* and Cu concentrations in soils collected from a Cu-contaminated site in Denmark. Besides noting that the NRR assay was more sensitive to Cu exposure than reproduction rate, they found that field soils with 70 yr contamination history were less toxic than Cu-spiked soils. This observation suggests that results from standardized toxicity tests using spiked soils should be taken with serious reservations, and they should not be considered alone for decision making related to ecosystem management. In a similar study, Booth et al. (2003) exposed *E. fetida* to soils collected from prairie skeet ranges in Canada. The authors also found a rapid response of NRR assay compared to growth rate, cocoon production or cocoon viability. The highly significant correlations between NRR times and soil Pb concentrations, or concentrations of $Ca(NO_3)_2$-extractable Pb, demonstrated that the NRR assay can be a sensitive and predictive biomarker of earthworm Pb body burdens (or bioavailable Pb).

Simulated Field Studies. In general terms, these studies can be defined as artificially bounded systems that represent specific ecosystems or fractions of these. Their main application is to investigate the contaminant effects on organisms under the influence of multiple environmental fluctuating variables. Depending on the dimensions, it is possible to distinguish two types of artificial ecosystems: microcosm and mesocosm. A soil microcosm consisting of a cylinder (7.5 cm inside diameter × 15 cm high) made from high-density polypropylene pipe was used by Burrows and Edwards (2002) to assess the effects of the fungicide carbendazim on a representative group of soil organisms including plants, earthworms, and nematodes. This approach not only examines the toxic effects on each organism but also investigates the alterations on ecosystem processes such as nitrogen mineralization, nutrient transformation, or ecological interactions between organisms. Generally, soil microcosm experiments are carried out indoors under stable ambient conditions [temperature, light/dark (L : D) cycles, artificial rainwater, etc.].

An alternative man-made ecosystem segment of higher dimensions is the mesocosm, which is structurally and functionally closer to the "real world" than the microcosm. The mesocosm is generally constructed as an outdoor system, and environmental variables (pH, temperature, humidity, organic matter, etc.) are routinely recorded to help in the data interpretation. Mesocosms were employed by Svendsen and Weeks (1997b) and Spurgeon et al. (2005b) to study the effects of Cu and Cd on the earthworm *L. rubellus*;

they concluded that seasonal changes or fluctuating environmental conditions typical of northern temperate regions did not appear to affect significantly the toxicity of these heavy metals.

In Situ Exposure Bioassays. The least used approach in ERA, probably for logistic reasons, *in situ* exposure bioassays are generally performed in the site of interest when minimal alteration of soil (e.g., mix of horizons) and more realistic exposure conditions are required. An example of an *in situ* exposure assay is the study by Hankard et al. (2004), who used caged earthworms (*L. terrestris*) to assess the suitability of NRR assay and total immune activity (TIA) to soils contaminated by both heavy metals and the 16 priority pollutant PAHs. Although percent of survival was high, a significant reduction in the NRR time (<10 min) was found in earthworms caged for 12 d in the contaminated sites compared to NRR times (20–27 min) measured in worms deployed in the control sites. The TIA test was a less sensitive biomarker than the NRR assay after 12 d exposure. Exposure to heavy metals (Cu, Zn, Pb) and PAHs accounted for biomarker responses in *L. terrestris* because of the positive relationship found between the body residues and soil concentrations.

The main advantages and limitations of these four approaches of the retrospective ERA are summarized in Fig. 2. Factors such as the objectives of the ERA, the physical features of the site under study, the resources available for conducting the ERA, and the nature of the contamination are determinants in the selection of the best approach. Nevertheless, it is recommended to use more than one methodology integrated in a WOE framework to obtain a more reliable ERA of a contaminated site.

III. Discussion

A. Biomarkers in Standardized Toxicity Tests

In general, standardized toxicity tests are characterized by their simplicity, rapidity, and low cost. However, these attributes could lead to erroneous conclusions in environmental management decisions or bioremediation procedures. Four important aspects are frequently ignored when running toxicity testing, or when ecological consequences are forecast from the test results: (1) low contaminant concentrations in the field, (2) long-term exposure to sublethal concentrations of contaminants, (3) toxic effects from contaminant mixtures, and (4) fluctuating environmental factors affecting toxicity.

Intuitively, one would think that the levels of certain universal contaminants (e.g., organochlorine pesticides, polychlorinated biphenyls) in the environment have decreased in the past two decades due to measures such as the application of remediation technologies, improvement in the

	Laboratory test of field soils	*In situ* exposure bioassay
Advantages	Earthworms are exposed under stable conditions (e.g., soil temperature and moisture). Biomarker responses are easily related to the bioavailable fraction of soil contaminants. Low cost, rapid, and relatively low effort. Standardized protocols (e.g., OECD or ISO toxicity tests).	Influence of environmental variables on earthworm sensitivity to contaminants can be assessed. Homogeneous earthworms (e.g., weight, sexual maturation) can be used to minimize variations of biomarker responses due to biological variables. Impact of fluctuating environmental variables on biomarkers (natural variation of biomarker responses) can be assessed in the control (nonexposed) group. Possibility of assessing impact of earthworm activity (bioturbation) on contaminant distribution along the soil profile. Realistic exposure conditions. Earthworm movements are limited and exposure is better defined.
Limitations	Field soils are generally sieved <5 mm before testing, and the influence of soil structure on bioavailability and toxicity of contaminants are not considered. Lack of ecological meaning: impact of fluctuating environmental variables is not assessed. Risk for soil disturbation and contaminant alteration during transport and storage. Risk for erroneous conclusions related to ecosystem management based on a suitable (sensitive?) species. Soil conditions (moisture, temperature, pH) must be periodically controlled.	Vandalism and damage by adverse atmospheric conditions. Locating of suitable reference sites. Time consuming and relatively high effort (e.g., cage construction). Indigenous juvenile earthworms can be caged with sentinel earthworms if soil cores are not exhaustively examined before placed into the mesh bag. Biomarker responses highly influenced by environmental factors other than the contaminants. Effects from the deployment system.

Fig. 2. Advantages and limitations of ecological risk assessment (ERA) approaches used to assess toxic effects of contaminated soils.

	Ecological survey	Simulated field study(mesocosm)
Advantages	Indigenous earthworm population are sampled (ecological realism). Earthworms show integrated responses to accumulation and toxic effects. Relatively easy and low cost. Biomarker responses can be complemented by ecological index such as biomass or species richness. Sampling limited to wet seasons in temperate areas.	Used for assessing the impact of contaminated-spiked soils under natural conditions. Toxic effects and biomarker responses are environmentally realistic. Impact of fluctuating environmental variables on biomarkers can be assessed before add the contaminant (natural variation of biomarkers). Biomarker responses and toxic effects can be investigated in a considerable number of soil organisms other than earthworms. Indirect effects of contaminant can be investigated. Homogeneous earthworms (e.g., weight, sexual maturation) can be used. Earthworm movements are limited and exposure is better defined.
Limitations	Unknown exposure history, and tolerance or resistance to pollution could have developed. Difficulties in sampling and species identification. Biomarker responses are difficult to interpret (contaminant mixture effects, impact of biological variables). Relatively high costs of field surveys.	Time consuming and a relatively high effort is required. Vandalism and damage by adverse atmospheric conditions. Natural sieved soils are generally used, and extrapolation to field soil must be carefully performed.

Fig. 2. *(Continued)*

treatment of liquid or solid wastes, the forbidden manufacture and use of persistent organic pollutants in many countries, the use of low-persistent pesticides (OPs, CBs, or pyrethroids), and the shutdown of mining activities, among others. Under this hypothetical scenario of low contaminant levels, which is probably true for many environments suspected of being contaminated, the current purpose of ecotoxicity testing is questioned. Similar to sediment, soil is an environmental medium in which many types of contaminants accumulate up to concentrations potentially toxic to biota. A contaminant generally coexists with its metabolites, other types of contaminants, or different chemical forms (i.e., metal speciation). This chemical cocktail can be harmful to organisms as a result of synergism, potentiation, or antagonism interactions between toxicants. Eggen et al. (2004) stressed these aspects of the ecotoxicology and suggested focusing efforts on organism responses at molecular level (e.g., genomic and proteomic responses) using simple biological systems such as cells, subcellular systems, or unicellular organisms. However, predictions of adverse effects at population or community levels from molecular biomarker responses continue to be a challenge in ecotoxicology.

Appropriate biomarkers may be applied in standardized bioassays to provide evidence of the cause–effect relationship between soil contaminants and toxic effects in the individuals. In aquatic toxicity testing, the biomarker approach has brought about promising results. For example, Neuparth et al. (2005) included certain biomarkers (MT induction, DNA strand breakage, and lipid peroxidation) in a standardized sediment toxicity test to assess toxic effects at multiple biological organization levels. They found that several estuarine sediments affected the survival and reproduction of the amphipod *Gammarus locusta*. In addition, a positive response in the MT induction and the frequency of DNA strand breakage was found in the organisms, and they concluded that the use of biomarkers in these ecotoxicity tests can help to distinguish biological responses to contaminant exposure from those originating from physicochemical variables of the sediment.

Biomarkers have also been applied in standardized toxicity tests using earthworms. Most of these studies have tried to link biomarker responses to adverse effects on life cycle traits. Ideally, the biomarker should show a concentration-dependent response to pollutants, particularly under stable experimental conditions. However, many laboratory studies involving earthworm biomarkers do not show a straightforward dose–response relationship (see Table 1). For example, many studies have reported that the NRR response linearly correlates with heavy metal concentrations in soil, or its bioavailability fraction, as well as with the metal body burden. However, this consistent cause–effect relationship needs to be validated for other classes of contaminants (PAHs, pesticides, PCBs, Hg) before making conclusions about its potential as predictor of deleterious effects at individual or population levels.

B. Toward an Environmentally Realistic Assessment of Contaminated Soils

Environmental processes influencing the contaminant toxicity in the nature are difficult to replicate in the laboratory. Consequently, a direct relationship between laboratory toxicity test results and ecological consequences could be extremely risky. Although this statement is well accepted by most ecotoxicologists, the results from standardized toxicity tests are generally used to identify a chemical as slightly or highly toxic and for environmental management decisions.

In general, soil toxicity tests with earthworms are typically performed using the OECD artificial soil or the LUFA 2.2 standard soil (see Jänsch et al. 2005 for soil characteristics). The general procedure involves the mixture of the test substance, using aqueous solutions for heavy metals or solvent solutions in the case of hydrophobic organic contaminants, with the artificial soil. After a few days of equilibration, earthworms are released into the spiked soils and the test is started. A more environmental realistic approach is to perform laboratory toxicity tests with field soils (see Fig. 2). Nevertheless, some limitations of these standard procedures should be taken into account. In a comparative context, Chapman et al. (2002) examined the ecological meaning of sediment toxicity tests and provided a number of issues and recommendations to be considered in future sediment ERA. Some of them are cited here to compare with soil toxicity testing:

The test organism is generally a species relatively easy to culture under laboratory conditions; however, it is sometimes more resistant than the native related organism.

The test organism is not often the best species for assessing toxicity or bioaccumulation in sediment toxicity testing. For example, the amphipod *Hyalella azteca* is a common organism in sediment toxicology. However, the natural behavior and food habits of this aquatic species are not simulated in the test chambers used in the standardized tests. The use of this species as a suitable test organism is thereby questioned (Wang et al. 2004).

The most common endpoints in sediment toxicity assays are survival, reproduction, and immobilization. Sometimes these do not define the potential toxicity of sediment contaminants, and the biomarker approach may be an important line of evidence (Neuparth et al. 2005; Costa et al. 2005).

Natural populations can develop tolerance or resistance to pollution by acclimatization or genetic selection. These aspects should be considered in ecotoxicity testing with native organisms (Chapman et al. 2002).

In addition, one of the main problems in sediment toxicity tests is the alteration of the sample during collection, handling, and storage, which can chemically transform the contaminants and consequently their bioavailability and toxicity (Ingersoll 2003). In view of these limitations existing in

sediment toxicology, should we consider related issues, and others, when running toxicity tests using field or contaminant-spiked soils? A large base of evidence suggests that some modifications should be included in the current soil toxicity protocols.

The Test Earthworm. For decades, standardized soil toxicity tests have been carried out using primarily two earthworm species: *E. fetida* and *E. andrei*, which were regarded as one species, termed indiscriminately as *E. fetida* or *E. foetida*. Currently, *E. fetida* and *E. andrei* are two different species (Dominguez et al. 2005) with differences in biological features (growth rate or cocoon production) of ecotoxicological concern (Jänsch et al. 2005). As for *H. azteca*, the ecological relevance of using these two species is also under current discussion. Biological and ecological aspects of these two *Eisenia* species, as well as the toxic effects of many classes of chemicals, are well known. Therefore, their use in soil toxicity testing could be justified. However, exposure of these species to soil contaminants is sometimes questioned, mainly because of the natural habits of these earthworms. *E. fetida* and *E. andrei* are epigeic earthworms that live in the soil surface, forming no permanent burrows, and feed on decaying organic matter. They require a high content of organic matter in soil (Jänsch et al. 2005), which explains why they are commonly found in compost heaps, manure piles, or sewage sludge. The question could be: Are these species suitable bioindicators when contaminants occur at soil depths where these earthworms are rarely found? When deep soil layers are tested for toxicity, are the test results ecologically realistic when using *Eisenia*?

Again, the example of the amphipod *H. azteca* examined in Wang et al. (2004) is useful to call into question the use of an inappropriate organism to extrapolate laboratory results to the field. In nature, this aquatic invertebrate is always found grazing on macrophytes, and contact with sediment is minimal or nonexistent; however, it is used for assessing sediment toxicity. Standardized test guidelines force *H. azteca* to burrow into sediment because assays are generally run without macrophytes and under constant light or L:D cycles (*H. azteca* is negatively phototactic). Laboratory soil testing conditions with *Eisenia* generally involve continuous light to force the earthworms to stay in the soil throughout the test (see Table 1). However, *E. fetida* and *E. andrei* are litter dwellers on the soil surface and generally do not ingest large amount of soils. Despite this, *Eisenia* is compelled to behave like an endogeic earthworm during the test. It is likely that we are making the same experimental error with *Eisenia* in soil toxicity testing as for *H. azteca* (Wang et al. 2004). From the ecotoxicological aspect, it would be desirable to use indigenous nonexposed earthworms as test organisms to achieve ecological realism.

Metal speciation is a determinant factor in the bioavailability of the heavy metals, which is, in turn, highly dependent on physicochemical features of the soil (e.g., pH, moisture, and organic matter). However, recent

studies have demonstrated that earthworms are able to alter the chemical forms of the metals in soil. Wen et al. (2004) found significant variations in heavy metal concentrations in three fractions extracted according to the Community Bureau of Reference's protocol (1, water soluble, exchangeable, and carbonate bound; 2, Fe- and Mn oxides bound; and 3, organic matter and sulfide bound). After incubation of soils in the presence of *E. fetida*, the metal concentrations associated with the bioavailability fraction increased. Changes in metal availability seem to be dependent on earthworm habits. In a laboratory experiment, Zorn et al. (2005a) found that the epigeic earthworm *L. terrestris* contributed to the increased availability of Zn ($CaCl_2$-exchangeable Zn) after 80 d. In contrast, the endogeic earthworm *A. caliginosa* was able to decrease Zn availability after 175 d (Zorn et al. 2005b). Modification of metal availability by earthworm activity is a matter of increasing concern in earthworm ecotoxicology and could have a notable application in the phytoremediation of contaminated soils.

The Test Substance. Davies et al. (2003a) demonstrated that the chemical form of the test substance significantly affects the test results. They exposed *E. fetida* to three chemical forms of Pb [$Pb(NO_3)_2$, $PbCO_3$, and PbS] following the OECD guideline for acute and chronic toxicity testing. In their experiments, the solid salts were added directly to the soil to attain the desirable Pb concentrations. The results revealed differences in cocoon production in relation to the chemical form of Pb. Toxic effects of Pb salts were related to their water solubility; the most toxic Pb salt was the most water soluble, i.e., $Pb(NO_3)_2$. This result could be explained because Pb uptake (dermal and gut exposure) requires the metal to be in solution. Similar results were obtained by Arnold et al. (2003), who exposed *E. fetida* to both aqueous and solid forms of several Cu salts [$CuSO_4$, $Cu(NO_3)_2$ and $Cu_2(OH_2)CO_3$]. The more water-soluble salt, i.e., $Cu(NO_3)_2$, was the most toxic Cu form. In addition, they found that the form in which Cu was applied to soils (aqueous or solid) did not significantly affect the results of acute and sublethal tests as well as the avoidance behavior response.

In these two related studies, it was also demonstrated that the conventional extractable procedures for measuring the metal fraction available to plants (water, $CaCl_2$, or diethylenetriaminepentacetic acid) are indicative of low metal availability to earthworms. As an alternative approach, the use of selected biomarkers (e.g., inhibition of delta-aminolevulinic acid dehydratase (ALAD) activity, MT induction) together to metal body burdens could help to determine the bioavailable, and bioactive, fraction of the metal. For example, highly significant correlations have been reported between the response of the NRR assay and the body Pb concentrations (Reinecke and Reinecke 2003).

The study by Davies et al. (2003b) also demonstrated that the bioavailability of Pb, added to test substrate as a $Pb(NO_3)_2$ solution, decreased over time probably because Pb did not rapidly reach equilibrium with soil. In fact, acute toxicity was higher when earthworms were immediately released

after soil spiking with $Pb(NO_3)_2$ than when added after soil–Pb equilibrium. As suggested by the authors, the equilibrium concept between soil and metal has serious implications in the laboratory-to-field extrapolations. A field study by Scott-Fordsmand et al. (2000) also illustrates the importance of considering the contamination history of the soil. They collected soils in a metal-polluted area with more than 70 yr Cu contamination. These soils were less toxic to *E. fetida* than soils spiked with the chloride salt of Cu. They concluded that differences for Cu toxicity could be explained by variations in Cu speciation, a result of Cu equilibration with soil. The time for equilibrium depends on the toxic substance and soil type. In a speculative context, the equilibrium phase for phophorothioate types of OP insecticides could lead to an increase of their toxicity because these compounds need to be transformed to the highly toxic "oxon" form by soil microorganisms or physicochemical factors, but simultaneously OP degradation can also occur. Thus, the time for equilibrium between soil and OP pesticides can be a critical parameter in ecotoxicity tests.

The Exposure Conditions. In a conventional acute or chronic toxicity test with earthworms, factors such as temperature, soil moisture, or photoperiod are kept at stable optimal values so that the only stress factor is the test substance or the contaminant mixture in the field soil. Obviously, this is not the only stress factor in the field, and many fluctuating environmental variables contribute to change earthworm sensitivity to pollutants (van Straalen 2003). One study shows clearly how toxicity is strongly influenced by environmental variables and therefore should be considered in future toxicity testing schemes. Bindesbøl et al. (2005) exposed the freeze-tolerant earthworm *Dendrobaena octaedra* to a range of Cu concentrations and different temperature regimens to investigate possible interactions between these two stress factors. Two important findings were reported: (i) acute Cu toxicity was affected by ambient temperature and metal toxicity increased with decreasing temperature, and (ii) there was a negative relationship between frost tolerance of the earthworm and Cu concentration in soil. In a comparative study, Spurgeon et al. (2005b) evaluated the impact of environmental factors on Cd and Cu toxicities in both adult and juvenile specimens of *L. rubellus* exposed to the metals for 70 d using a mesocosm system. Results were then compared with analogous experiments carried out under laboratory conditions (Spurgeon et al. 2003, 2004). They found no substantial differences in biomarker responses (metal-binding protein MT-2 or NRR assay) or life cycle traits (survival, growth, and reproduction) between those exposed in the mesocosm and those exposed under laboratory conditions. It was concluded that climatic conditions such as temperature (ranging from 15°–20°C to 5°C) or soil moisture (rainfall up to 20–25 mm resulting waterlogging) did not alter the sensitivity of *L. rubellus* to Cu or Cd. The results of this study and those by Bindesbøl et al. (2005) seem to draw contradictory conclusions, which encourages future investigations

aimed to demonstrate if fluctuating environmental variables such as temperature, soil moisture, pH, or organic matter content have a significant influence on earthworm sensitivity to pollutants.

In aquatic toxicology, *in situ* exposure using caging systems has gained acceptance, and a more realistic picture about ecological consequences from sediment contamination is often obtained. Suitable organisms can be exposed to water column, surface sediment, or sediment pore water using appropriate caging systems (Burton et al. 2005). Surprisingly, caged earthworms have rarely been used for assessing soil toxicity *in situ*. Several phenomena are not generally replicated in the laboratory, mostly for logistic reasons. For example, it has been demonstrated that some epigeic (*L. terrestris*) and endogeic (*A. caliginosa*) earthworms are able to transport contaminated soil from deeper layers to the soil surface, contributing to increased risk of adverse effects to other surface soil organisms. Thus, *in situ* exposure bioassays become a suitable approach for investigating the impact of this bioturbation process on soil toxicity.

C. Biomonitoring the Effectiveness of Bioremediation and Agrienvironment Schemes

Mining is among the main human activities causing metal pollution of soils. Although many mines have stopped their activity in numerous countries, they have contributed to greatly increased metal concentrations in soils. As an example, the Almadén mining district in Central Spain is one of the largest mercury mineral deposits in the world (Rytuba 2003), and it has been intensively mined since Roman times. A hazardous legacy was left inevitably: it is one of the most Hg-contaminated places on the Earth (Higueras et al. 2006). Here, although mining activity has ceased entirely, Hg concentrations up to 8,890 $mg\,kg^{-1}$ dw are commonly measured in soil. In an attempt to recover these heavily contaminated sites, a number of remediation processes have been, and continue to be, developed. Among them, phytoremediation, i.e., use of plants for environmental restoration, is of particular concern because heavy metals cannot be degraded, and their removal by plants seems to be an effective and environmentally friendly method (Lasat 2002).

One of the main limitations of phytoremediation is metal bioavailability. It has been demonstrated that earthworms are able to increase metal uptake by plants, thereby increasing the efficiency of phytoextraction (Wen et al. 2004). This beneficial "cooperation" has also been used to recover contaminated soils containing harmful organic chemicals such as PCBs or petroleum hydrocarbons; however, in these cases plants are substituted by microorganisms. Singer et al. (2001) used the anecic earthworm *Pheretima hawayana* to increase the degradation rate of arochlor 1242 by the bacteria *Rhodococcus* sp. ACS and *Ralstonia eutrophus* H850. In a similar study, Schaefer et al. (2005) investigated the effects of three species (*E. fetida, A.*

chlorotica, and *L. terrestris*) on soils spiked with petroleum hydrocarbons [10,000 mg kg^{-1} total petroleum hydrocarbons (TPHs)]. The authors concluded that earthworms increased the degradation rate of hydrocarbons after 28 d incubation, probably as a result of stimulation of microbial activity. Furthermore, such an increase in TPH degradation was species dependent with the following order: *L. terrestris* (30%–42% TPH decrease) > *E. fetida* (31%–37%) > *A. chlorotica* (17%–18%).

On the other hand, earthworms have been used to assess the effectiveness of soil bioremediation procedures. In a laboratory experiment, Morgan et al. (2002) determined body metal concentrations in the earthworm *L. rubellus* after 4 wk exposure to metal-contaminated soils that were previously treated with several chemical ameliorants (montmorillonite, hydroxylapatite, or ferrous oxide). They concluded that the use of earthworms as sentinel species can be a suitable approach for screening remediation effectiveness. In a related study, Davies et al. (2002) evaluated the efficacy of bone meal (phosphorus source) treatment in Pb-contaminated soils through ecotoxicological tests using *E. fetida*. Treatment of soils with bone meal (1:20) resulted in an increase of earthworm survival (7, 14, and 28 d exposure), growth, and cocoon production, and a decrease of Pb bioavailability. Lock and Janssen (2003) used adults of *E. fetida*, among other soil invertebrates, to determine the capacity of metal-immobilizing agents (called by the authors type I and type II) to reduce bioavailabity of Zn in contaminated soils from Belgium. The addition of these agents (5% w/w) to the soils, allowing 1 yr for equilibration before starting toxicity testing, resulted in a total elimination of soil acute toxicity (100% survival of *E. fetida* after 21 d exposure). The effectiveness of chemical immobilization amendments to metal-contaminated soils was also assessed through a 14-d toxicity test using *E. fetida* following the American Society for Testing Materials (ASTM) guideline (Conder et al. 2001). Toxicity of metal-contaminated smelter soils was significantly reduced when soils were treated with municipal sewage sludge biosolids stabilized with lime.

In these bioremediation studies, determination of biomarkers was not included despite that they are an indirect biological measure of contaminant available and toxic fraction. On the other hand, conclusions about remediation effectiveness are based on acute toxicity test results using a single earthworm species (*E. fetida*), which is not necessarily the most sensitive. In addition, acute bioassays do not show sublethal toxic effects, and chronic bioassays are required to provide long-term ecological impacts from contamination. Monitoring methods for assessing the progress of remediation actions in contaminated soils are traditionally based in chemical analysis of soil, employing sophisticated and high-cost instrumental analysis. Maila and Cloete (2005) reviewed the biomonitoring tools most used for evaluating effectiveness of the bioremediation for restoration of hydrocarbon-contaminated soils. Soil enzyme activities (lipase, dehydrogenase, urease, catalase), microbial biomass, microbial bioluminescence, seed

germination, and earthworm survival tests are among the main biological indicators for assessing soil remediation procedures. Maila and Cloete (2005), in line with other authors, concluded that it is necessary to develop new biomonitoring methods of soil remediation based on the use of ecologically relevant species. Biomarkers were not mentioned among these recommendations. In light of the literature discussed in this review, it can be concluded that certain earthworm biomarkers, such as the NRR assay or the avoidance behavioral response, can be useful indicators of sublethal effects during a soil remediation procedure.

Currently, it is widely accepted that modern agriculture represents a serious threat to wildlife. In the European Union, the increasing concern in developing environmentally-friendly agriculture has led to the introduction of the agrienvironment schemes (AES) in many Member States (Council Regulation No. EEC 2078/92). Reduction of fertilizer and pesticide inputs are among the most important measures. However, there exists a lack of information about the real effectiveness of European AES. An exhaustive review examined the most relevant ecological studies on the efficacy of the AES, measured in terms of changes in biodiversity (Kleijn and Sutherland 2003). It was concluded that the implementation of these schemes increased the biodiversity of several zoological groups such as insects or birds. Hole et al. (2005) also reviewed a considerable number of studies that compared the impacts of organic (no use of synthetic chemicals) and modern farming systems on biodiversity. From the 76 studies reviewed, only 13 involved comparisons of earthworm abundance and activity between organic and modern agriculture. In line with the results for other taxa (birds, soil microbes, spiders, butterflies, and others), most of the earthworm studies indicated that organic farming contributed to a higher abundance and species richness of earthworms compared to modern agriculture fields.

Kleijn and Sutherland (2003), however, called into question the use of comparative biodiversity studies between AES-implemented fields and control areas (modern agriculture) to assess the success of these schemes. They suggested that ecological evaluations must be initiated at the time that AES are implemented, comparing control and AES spots randomly selected in the same study area where AES began to be introduced. Therefore, this approach would allow a more reliable assessment of the effectiveness of AES.

Most of the investigations on the AES effectiveness are based in abundance and/or species richness studies. Unquestionably, these studies respond to one of the objectives of the schemes: protection of biodiversity. However, short-time responses to the AES introduction can be required in many cases so that remedial procedures can be included in time. The abusive use of pesticides and fertilizers in modern agriculture is a current practice that the AES implementation tries to reduce. In general, current insecticides and herbicides have a low persistence in the environment; however, many of them show high acute toxicity (OP and CB), which could

justify the inclusion of biomarkers in future biological surveys of pesticide applications. The use of *in situ* exposure bioassays using earthworms in the agricultural field with and without AES implementation could be a complementary approach to assess the impact of AES in the agrienvironment with consequence for the local earthworm biodiversity (Sepp et al. 2005).

IV. Perspectives in Earthworm Biomarkers

Most biomarkers provide an indication of pollutant exposure only. Under this consideration, the general strategy is to assay a suite of biomarkers covering molecular to whole-organism endpoints to obtain clear evidence of individual health deterioration (Beliaeff and Burgeot 2002; Handy et al. 2003). In the past 5 years, significant progress has been achieved regarding certain earthworm biomarkers such as MT induction or the NRR assay. In addition, new and promising biomarkers have been explored such as the induction of annetocin, a neuropeptide involved in the induction of egg-laying behavior in earthworms (Ricketts et al. 2004). Traditionally, earthworms have been used as bioindicators of metal pollution. Thus, biomarkers related to metal exposure (MT induction) have been extensively investigated (Kammenga et al. 2000; Scott-Fordsmand and Weeks 2000; Burgos et al. 2005). Other biomarkers (e.g., ChE, CbEs, or CYP1A) commonly used in biomonitoring programs with vertebrates have received little attention in earthworm studies. These organisms are considered suitable indicators of environmental change in agricultural environments (Paoletti 1999). Paradoxically, very few studies have involved the impact of pesticides on earthworms through the use of biomarkers of pesticide exposure. For example, earthworm ChE activity is sensitive to OP or CB pesticide contamination, and a slow recovery rate is frequently observed after ChE inhibition (Booth and O'Halloran 2001; Panda and Sahu 2004). However, the use of this well-known biomarker under field conditions has scarcely been investigated. Moreover, measurements of earthworm ChE activity levels together with the chemical reactivation of the enzyme in the presence of pralidoxime (McInnes et al. 1996; Sanchez-Hernandez 2003) could be a suitable methodology for identifying exposure to OP and CB pesticides in field.

Behavioral responses are included in the biomarker definition by several authors (Lagadic et al. 2000; Walker et al. 2001); nevertheless, they have had low consideration in ecotoxicological research compared molecular biomarkers. The behavior of an organism is defined as the final integrated result of a diversity of physiological processes interacting with the abiotic and biotic components of the environment (Fig. 3). Sensory, hormonal, neurological, and metabolic systems are the main physiological systems involved in behavior performance, and in turn, they represent the primary target systems of many contaminants.

Behavioral responses to pollution are becoming a matter of increasing concern in ecotoxicology. A substantial volume of literature describing

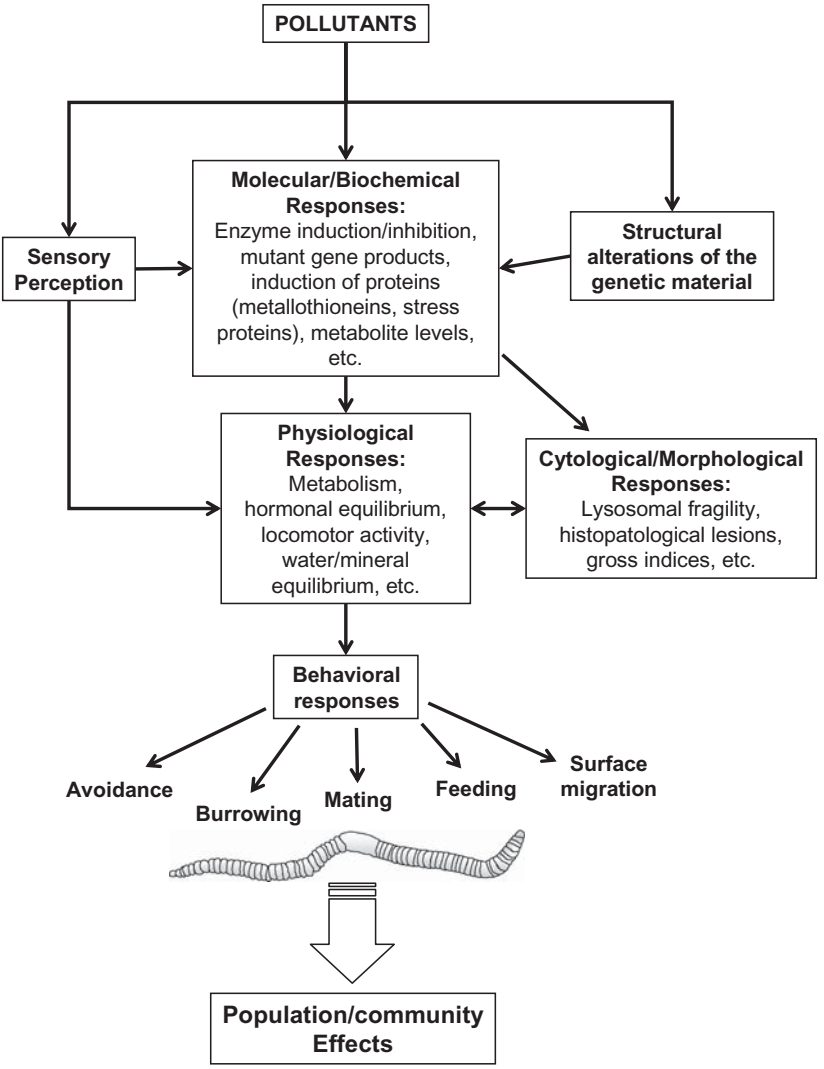

Fig. 3. Scheme of earthworm biological responses to pollutants. Behavioral changes are the result of the integration of several physiological systems affected by pollutants.

perturbation or disruption of physiological systems directly involved in fish behavior has been reviewed by Scott and Sloman (2004). According to the concept of a hierarchical cascade of biological responses to pollution occurring at different levels of biological organization, behavioral responses could be the key biomarkers for making predictive assessments of pollution at population or community levels. Efforts to correlate molecular

biomarkers to behavioral changes, with direct ecological implications, could be one of the future challenges in earthworm ecotoxicology. A well-known example is the relationship between brain AChE inhibition by OP/CB insecticides and behavioral disturbances in vertebrates (Peakall 1992; Sanchez-Hernandez 2001; Hill 2003; Bain et al. 2004). However, the absence of studies on disturbance of earthworm behavior by pesticides does not permit drawing any conclusions about this well-established relationship. Capowiez et al. (2003) examined the response of two common biochemical biomarkers (AChE and GST) and the burrowing behavior of two earthworm species exposed to the chloronicotinyl insecticide imidacloprid. Burrowing behavior was a more sensitive endpoint than biochemical biomarkers, which did not respond to the insecticide. However, behavior is the final product of many interacting physiological systems, and pollutants can interact with many of these systems. Thus, the identification of involved biochemical biomarkers becomes a difficult task.

Earthworm biomarkers need still to be investigated extensively to use them for predictive assessments of ecological consequences from pollution. In line with the main recommendations from van Gestel and Weeks (2004), it is opportune to add other lines of future research:

Biomarkers are sensitive indicators of exposure and should be included in the standardized toxicity tests under a well-developed and defined WOE framework. Biomarkers will make a significant contribution in acute bioassays as a measurement of the bioavailable and bioactive fraction of contaminants and in chronic bioassays as sublethal endpoints. The promising results obtained in sediment toxicology (Neuparth et al. 2005) encourage the application of biomarkers in soil bioassays.

The knowledge gained on certain earthworm biomarkers such as the MTs or the lysosomal membrane stability stimulates the development of standardized earthworm biomarker assays. This is an important step in applying biomarkers in a regulatory context. However, international agreement for developing a standard operating procedure for biomarker determination could become a difficult task with several biomarkers such as MTs, which can be measured by multiple analytical techniques (e.g., spectrophotometric, chromatographic, polarographic, or inmunodetection assays).

The main ecotoxicological meaning of the biomarker approach is to make predictions on changes in populations or communities from subcellular or individual responses. However, very little research has demonstrated such a relationship. Biochemical or physiological biomarkers could have an ecological meaning when they can be related directly to behavioral responses with significant ecological impact. The most common behavior response measured in earthworms is the avoidance of contaminated soil. However, Capowiez and Bérard (2006) pointed out that "avoidance is not a measure of toxicity but rather a measure of repellence". In

agreement with this assumption, the impact of contaminants on other behavioral responses such as burrowing, feeding, or surface migration must be studied together as biomarker responses.

To date, most of the earthworm biomarker investigations have been performed in a heavy metal pollution scenario. There is a need for increasing the knowledge of biomarkers of exposure to organic contaminants of current concern, i.e., anti-ChE insecticides, pyrethroids, brominated flame retardants, and PAHs. Biomarkers related to insecticide toxicity (e.g., AChEs) and detoxification (CbEs, phosphotriesterases, or CYP450-dependent monooxygenases), or biomarkers of oxidative stress requires further exploration to obtain a better understanding of the negative impact of organic pollutants on earthworms.

New biomarkers need to be investigated, especially when they could be directly involved in earthworm survival. For example, Na^+/K^+-ATPase is an important electrogenic component in the contraction mechanism of longitudinal muscle fibres of *L. terrestris* (Volkov et al. 2000), and it has been demonstrated in fish and aquatic invertebrates that this adenosine triphosphatase is inhibited by a wide range of heavy metals and pesticides leading to osmoregulation impairment.

Summary

Earthworms have had a notable contribution in terrestrial ecotoxicology. They have been broadly used to assess environmental impact from metal pollution, and they are typical test organisms (e.g., *Eisenia*) in standardized toxicity tests. Several reviews and international workshops have stressed the need for increasing the understanding and applicability of earthworm biomarkers in the ecological risk assessment (ERA) process. This review summarizes recent available information concerning the most investigated earthworm biomarkers. In earthworms, the use of biomarkers has been focused on assessing metal pollution, and available data on biomarker responses to organic contaminants are rather limited. The potential for applying earthworm biomarkers in the standardized toxicity tests is suggested in view of their significant contribution to the risk assessment of contaminated soils (e.g., estimation of bioavailable and bioactive fraction or sublethal effects). Field studies involving earthworm biomarkers are still scarce and are summarized according to their main practical approaches in retrospective ERA: biological surveys, laboratory tests of the soil, simulated field studies, and *in situ* exposure bioassays.

Despite the great volume of laboratory studies on earthworm biomarkers, future lines of research are suggested besides the recommendations made by others: (1) the potential and limitations of the inclusion of biomarkers in the standardized toxicity tests should be examined under a well-defined weight-of-evidence framework; (2) it is necessary to develop operating guidelines to standardize earthworm biomarker assays, an impor-

tant step to apply biomarkers in a regulatory context; (3) molecular and physiological biomarkers should be directly linked to behavioral changes with significant ecological implications, an important step in considering them as ecotoxicological biomarkers; and (4) biomarkers to organic pollutants of current concern (e.g., polycyclic aromatic hydrocarbons, anti-ChE and pyrethroid insecticides, polybrominated flame retardants, etc.) need to be developed and validated in the field. Also, an increase in the knowledge of earthworm biomarkers is undoubtedly useful in assessing the effectiveness of procedures for recovering/protecting the environment (e.g., phytoremediation or agrienvironment schemes) besides its potential use in the ERA framework.

Acknowledgments

This study was funded by the Consejería de Educación y Ciencia (Junta de Castilla-La Mancha, Spain). The author thanks Ximena Andrade for her dedicated assistance in the manuscript preparation.

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Manuscript received October 13; accepted October 21, 2005.

Rev Environ Contam Toxicol 188:127–148

Using Soil Health to Assess Ecotoxicological Impacts of Pollutants on Soil Microflora

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Contents

I. Introduction

The ecotoxicity testing approach allows for an overall evaluation in the measurement of pollutant impacts on soil life. Ecotoxicological evaluation takes into consideration the complexity of the contaminant mixture as well as the multiple interactions that condition bioavailability and the level of exposure to living species. The measurement of ecotoxicity is generally performed using single organisms or species. The observed toxic effects can vary in gravity and extent depending on whether they affect survival, growth, or reproduction. However, this standard method cannot easily be used to evaluate pollutant ecotoxicological impacts on a soil microbial ecosystem. Indeed, soil is a complex living system in which a microbial community is intricately related with the chemical, and physical soil components (Avidano et al. 2005). Moreover, the soil biological content is in itself a relatively unknown elaborate structure. Its ecotoxicological evaluation goes beyond the individuals or species responses to the presence of pollutants (Kools et al. 2005).

Soil microorganisms play an essential role in the cycling of elements and the stabilization of soil structure. They are responsible for essential parts of

Communicated by George W. Ware.

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the global C, N, P, and S cycles and soil organic matter is a major terrestrial source of C, N, P, and S. In fact, the essential functions of soil are all related to the role that soil plays in biogeochemical cycles (Seybold et al. 1998). The health of this dynamic living resource is vital to the global balance of ecosystem function (Doran et al. 1996). Thus, it seems logical that when evaluating the toxic impacts of pollutants on soil, the vital functions of soil should be of significant concern. Moreover, because the soil microbial community is a complex ecosystem essential to the continuation of the global life cycle, terrestrial ecotoxicity measurements should include this trophic level. The proper functioning of the ecosystem is usually qualified as its "healthy" state (Doran and Zeiss 2000), and ecosystem health is a concept that relates to the vital functions of this system.

In the evaluation of the ecotoxicological impact of a pollutant on the soil microbial trophic level, the soil health concept is an approach that would allow a global vision of the effect of a contaminant on the soil ecosystem and would widen the measure of bioavailability and toxicity to soil functions.

This review presents the evolution of the concept of soil health, initially developed to address issues related to durable husbandries, and its possible use as an ecotoxicological tool for the soil microbial trophic level. Soil health indicators are also reviewed. Several indicators that could be useful for ecotoxicological evaluation are proposed and discussed.

II. Concept of Soil Health

The concepts of soil quality and soil health appeared in early civilizations and were considered more or less as synonyms (Doran et al. 1996). Their purpose was to describe in general terms the state of a soil. The evaluation of soil health, or quality, took on significance with population growth, combined with a badly controlled use of agricultural resources. Indeed, it is the desire to produce more food and fiber from the soil that justified the study of soil health or quality (Bezdicek et al. 1996). Soil is a living resource whose health is vital to the production of food and to the global balance of ecosystem functions (Doran and Safley 1997).

To be useful as an indicator of the impact of contamination, a unified concept of soil health is required. Several authors have pondered the definitions of soil health and quality, whether it was in the field of agriculture or forestry. Some authors separate the concepts of soil health and soil quality, and others consider the limits of the two concepts as not particularly clear (Gil-Sotres et al. 2005). Several definitions for soil health or quality were suggested in the last decade. A few were developed solely for agricultural purposes as reported by Acton and Padbury (1993), who defined soil quality characteristics as measurable soil properties that influence the capacity of soil to perform crop production or environmental functions. A subsequent Agriculture Canada report (Acton and Gregorich 1995)

used the terms "quality" and "health" indifferently and defined them as the soil fitness to support crop growth without resulting in soil degradation or otherwise harming the environment.

In the same line of thought, in a reflection on the ambiguity of the environmental terms and the need to standardize their meaning, Johnson et al. (1997) defined soil quality as being "the measurement of the condition of a soil relative to the requirements of one or more species and/or to any human use." Likewise, Mausbach and Tugel (1995) defined soil health in a broad sense as the ability of soil to perform or function according to its potential, and changes over time due to human use and management or to unusual events. The limitation in using this definition of soil health is that it is not broad enough to include contaminated or remediated soils.

Interestingly, quite a few authors defined soil as an ecosystem, including van Straalen (2002), who considered that "an ecosystem is healthy and free of distress syndrome if it is stable and sustainable, that is if it is active and maintains its organization and autonomy over time and is resilient to stress." Filip (2002) simplified this definition by stating that a healthy soil is a biologically active soil. van Bruggen and Semenov (2000) believed that soil health is primarily an ecological characteristic and can be considered a subset of ecosystem health. Rapport (1997) addressed soil ecological health and mentioned the relationship between soil health and ecosystem health, positioning the soil in the Earth global cycle of life (nutriments and energy). According to this approach, soil health can be defined as "the state of the soil in which it can perform its vital functions." According to these authors, soil health seems therefore directly related to the correct functioning of its biological fraction.

The most cited work, however, is that of the S-5518 committee set up in 1997 by the Soil Science Society of America (SSSA), charged with defining soil quality as well as finding ways to evaluate it, who opposed the simultaneous use of the terms quality and health (Kirchmann and Andersson 2001). According to this committee, soil quality should be evaluated based solely on the use of the soil, while soil health still remains for them a nebulous term requiring specification. The SSSA proposed in 1997 to define soil quality as being "the capacity of a soil to function within the limits of an ecosystem, to support biological production, to maintain environmental quality and to support fauna and flora health" (Doran et al. 1996). Karlen et al. (1997) described soil quality in simpler terms: "capacity of a soil to function." In other words, according to these authors, soil quality is the combination of the physical, chemical and biological properties contributing to soil functions. Oddly, in spite of the committee's recommendation of separating the two concepts, several authors continue using indifferently the terms soil health and soil quality although they base their work on the definition published by the SSSA committee in 1997 (Alkorta et al. 2003a,b; Avidano et al. 2005; Doran 2002; Doran and Safley 1997; Doran et al. 1996; Gil-Sotres et al. 2005; Jackson et al. 2003;

Kirchmann and Andersson 2001; Seybold et al. 1998, 1999; van Bruggen and Semenov 2000).

In general, the term "quality" is used in reference to a particular need or function of the soil, while "health" is associated rather with the concepts of durability and stability of the soil. Several authors prefer to use the term health because it relates to the living and essential portion of soil. On the other hand, Sutter (1993), for example, criticized the use of the term health in the context of ecosystem evaluation. He considered that it is a mistake for environmental scientists to treat the metaphor as a reality and preferred the terms quality or sustainability. However, according to van Straalen (2002), the main advantage of the soil health concept is that even if its definition is somewhat unclear for the moment, it still is a comprehensible and accessible concept for everybody, therefore easier to communicate. In this review, the term health is used combined with the SSSA definition because it relates well to sustainability and durability of the soil functions. The ecological role of the soil remains important throughout the text because the objective of this chapter is to consider the concept of soil health as an ecotoxicological tool.

III. Soil Health Measurement

For the past 20 years, researchers in the agriculture and forestry sectors have been working on the determination of indicators to quantify soil health. Soils can perform a given function because they possess certain attributes: by evaluating the condition of these attributes, one can judge soil health or its ability to function. If soil attributes cannot be measured directly, then surrogate properties are used. The latter are called soil health indicators (Alkorta et al. 2003a). Table 1 lists studies or reviews based on the SSSA definition of soil quality given in the previous section. The indicators studied or chosen by the authors as well as the principal conclusions of their work are also presented.

Several physical, chemical, and/or biological indicators are currently used in agriculture or forestry to evaluate soil health. These indicators describe the state of certain soil functions of interest for their use in agriculture or their productivity in forestry. The most often considered functions are (1) soil structure and development, (2) nutrient storage, and (3) biological activity (Doran and Zeiss 2000).

The classification of soils according to their state of health differs depending on the indicator used. Thus, the origin of the soil studied determines the type of indicator most appropriate to evaluate its health. This is why the soil type (agricultural, forest, or polluted soils) in the articles listed in Table 1 is indicated in parentheses in the first column so as to relate it to the proper indicators. Authors often, seek an indicator that can describe the optimal conditions for a good agricultural or forest production (Kirchmann and Andersson 2001; Knoepp et al. 2000; van Diepeneningen et al. 2005).

Table 1. Studies or Reviews Using Soil Health/Quality Indicators According to the Soil Science Society of America (SSSA) Definition of Soil Health/Quality.

Author (soil type)	Tested indicators	Main conclusions
Avinado et al. (2005) (polluted soils)	Bacterial density Biolog GN MicroPlates Enzyme activity	The presence of different pollutants did not affect the bacterial density but had an influence on community structure. Characterization of soil chemical properties, metabolic fingerprinting, enzyme activity, and bacterial community structure were all considered useful to soil health evaluation.
Kools et al. (2005) (heavy metal pollution)	Glyphosate (herbicide) degradation	Average degradation rates showed no difference between the soils compared. A positive correlation between glyphosate degradation rates and metal pollution was observed.
van Diepeneningen et al. (2005) (agricultural soils)	Physical and chemical properties (soil texture, NO_3, NH_4, total soluble N, PO_4, Norg, pH) Basal respiration resilience following drying-rewetting disturbance Copiotrophic and oligotrophic bacterial densities Microbial diversity Nematode population	The effect of organic versus conventional management in agricultural soils was studied. Organic management resulted in lower levels of NO_3, total N, higher number and diversity of bacteria and nematodes, and more resilience to dry-rewetting disturbance. Other indicators did not show significant results.

Table 1. *Continued*

Author (soil type)	Tested indicators	Main conclusions
Alkorta et al. (2003a) (agricultural and polluted soils)	Enzyme activity	Enzymes are central to the functions that soils perform which concern biogeochemical cycling of C, N, P and S. However, they are substrate specific; therefore, measuring a group of enzymes is necessary. Enzyme assays measure potential activities and not *in situ* activities.
Alkorta et al. (2003b) (agricultural and polluted soils)	Molecular microbial biodiversity	The assessment of soil microbial biodiversity was shown to have the potential to be a good biological indicator of soil health. Knowledge in the field of microbial ecology needs further advancement. Molecular methods are promising.
Jackson et al. (2003) (agricultural soils)	C and N dynamics Microbial biomass Microbial community structure (PLFA)	The authors concluded that tillage events contribute to decreasing soil quality by increasing CO_2 emissions and, in turn, increasing the potential for nitrate leaching. Microbial biomass was not influenced by tillage, although community structure was.
Schloter et al. (2003) (agricultural soils)	Microbial activity (N mineralization, respiration and enzyme activities) Microbial biomass Structural microbial diversity Nitrogen turnover Nematode community	The authors concluded that microorganisms are growth limited in soils and they may not reach their full capacities. However, they respond rapidly to disturbance or stress by adjusting their activity rates, biomass, and community structure.

Table 1. *Continued*

Author (soil type)	Tested indicators	Main conclusions
Filip (2002) (agricultural and polluted soils)	N_2-fixing bacteria Total microbial biomass Soil respiration Dehydrogenase activity Humification activity of soil microorganisms	The authors used the terms soil quality, sustainability, and ecocologically related parameters. They tested 20 individual parameters and concluded on the sensitivity of the indicators listed on the left. All parameters were related to microbial activity and were selected because of their importance in preserving ecological balance.
Griffiths et al. (2001) (agricultural and polluted soils)	Functional stability, resistance and resilience to disturbance Protozoan populations Substrate utilization profiles	Protozoan populations might be potentially useful for detecting differences within soil types, but require greater taxonomic input to be of interest. Functional stability, especially resistance, was able to differentiate between and within soils. Substrate utilization profiles did not provide a representative measure of soil health.
Kirchmann and Anderson (2001) (agricultural soils)	Crop production (acid–base conditions, soil fertility level, soil structure, crop cadmium content) Biological decomposition (organic carbon, trace metals) Matter exchange with atmosphere and groundwater (phosphorus leakage)	The authors presented the Swedish system for quality assessment of agricultural soils based on the concept of soil functions. The objective of the system is to allow relative comparison between soils that may be helpful in an environmental monitoring program. Although the system has been in official use since 1999, the authors consider it needs more development.
Badiane et al. (2001) (agricultural soils)	Enzyme activities (β-glucosidase, amylase, chitinase, xylanase)	Significant correlation was found between enzyme activities and age as well as enzyme activities and agricultural soil management.
Knoepp et al. (2000) Soil quality (forest soils)	Nitrogen availability Litter decomposition and forest floor characteristics Fauna population Carbon availability	Several soils were ranked according to soil quality based on chemical and physical properties. The ranking was then compared with the indicators listed on the left and it was shown to vary with the indicator. Emphasis was placed on the difficulty of determining appropriate indicators and their values for multiple use sites.

Table 1. *Continued*

Author (soil type)	Tested indicators	Main conclusions
Schoenholtz et al. (2000) (forest soils)	Organic matter content Nutrient-supplying capacity Acidity Bulk density Porosity and available water holding capacity	Indicators were chosen according to a literature review on forest soil quality and sustainability evaluation. The chosen soil properties or processes are sensitive to management perturbation and relate to forest productivity and health. One indicator may be appropriate for several soil functions simultaneously.
van Bruggen et al. (2000) (agricultural soils)	Response of microbial measurements to application of stress Copiotrophic and oligotrophic organisms	Soil health was related to ecosystem stability and disease suppression.
Karlen et al. (1997) (agricultural soils)	Organic matter Infiltration Aggregation (soil structure) pH Microbial biomass Forms of N Bulk density Topsoil depth (for water and nutrient availability Salinity Available nutrients	Each measurement was assigned to one of the following functions: accommodating water entry, retaining and supplying water to plants, resisting degradation, supporting plant growth. The evaluation did not include the assessment of the state of the soil ecosystem. It is a traditional evaluation of soil quality for crop production.

However, in the sustainable development perspective, the concepts of soil productivity and protection must converge. Moreover, if the measurement of soil health must be used as an ecotoxicological tool, the chosen indicators must be related to how the microbial flora functions or its state.

Several authors have defined the characteristics of the ideal indicator of soil health. According to the literature review conducted by Doran and Parkin (1996), the perfect indicator should (1) describe main soil functions, (2) integrate physical, chemical, and biological soil properties and processes, (3) be sensitive to variations in management and climate, and (4) be accessible to specialists, policy makers, conservationists, and producers and be easy to use. Hence, the main functions of the soil must be defined to determine which indicators should be used. Doran and Parkin (1994) identified three principal soil functions related to soil health: (1) to act as a matrix and growth medium for plants, (2) to control water mobility and distribution, and (3) to be an environmental buffer. Moreover, indicator results must be expressed in appropriate units and be transparent. As pointed out by Sutter (1993), when indexes of heterogeneous variables are used, low values of one component can be overshadowed by high values of another.

For now, there is no consensus on what soil properties or functions can be used as universal indicators. However, the current trend is to select the indicators according to soil use. For example, in agriculture, the production of harvests, the biological decomposition, and the matter exchange are usually the selected functions (Kirchmann and Andersson 2001). The characteristics of soil health indicators are mainly related to their capacity to describe the processes of the soil ecosystem and to integrate physical chemical and biological properties. They are also connected to their sensitivity, their accessibility and their usefulness to agricultural or forest producers (Doran and Safley 1997).

A. Chemical and Physical Properties as Soil Health Indicators

Chemical and physical soil properties such as pH, soil texture, nutrient content, phosphorus leakage, nitrogen availability, or organic matter content are often used as soil health indicators because they can be measured easily and provide information on the biological fraction of the soil. These properties can be separated into two categories: (1) static parameters (a "point in time" parameter, such as pH and soil texture) and (2) the dynamic parameters (related to a process, such as N turnover and matter exchange). Several indicators used are associated with the soil organic matter cycle, which is, according to Gregorich et al. (1997), a key component of soil quality. Soil organic matter is critical for the continued availability of nutrients, soil structure, air and water infiltration, retention of water, immobilization of contaminants, etc. Most importantly, the organic matter fraction of soil is directly related to soil life.

Although these parameters represent a specific soil function, they do not represent its state of health. Parameters must therefore be aggregated to translate their meaning into a soil health indicator. Schoenholtz et al. (2000) presented a synthesis of various studies undertaken in the field of quality evaluation of a forest soil. The authors considered that the selection of a standard set of specific properties for the definition of a quality index is complex because it can vary from one forest system to another. However, they were able to identify certain properties that are sensitive to disturbances brought on by management practices while remaining applicable to forest productivity (see Table 1).

Most authors include a biological indicator in their study. For example, Knoepp et al. (2000) extended the concept of soil quality to include sustainability and added soil fauna to the traditional chemical and physical parameters. The authors stressed that the presence of certain organisms or populations, such as protozoa or nematodes, are invaluable indicators of soil quality. Similarly, Doran and Zeiss (2000) considered that soil health evaluation and soil sustainability are both related to soil organisms and discussed this subject during the "Soil Health: Managing the Biological Component of Soil Quality" conference held in 1998 that joined together producers and scientists concerned with these issues.

B. Microbial Indicators of Soil Health

The concept of soil health requires the inclusion of soil ecological attributes. These attributes are mainly those associated to soil biota, its biodiversity, the structure of its trophic network, its activity, and the range of its functions (Pankhurst 1997). Microbial indicators were defined by Avinado et al. (2005) as microbial parameters that represent properties of the environment or environmental impacts which can be interpreted beyond the information of the measured or observed parameter itself. As compared to chemical and physical monitoring, biological indicators provide information that is integrative of several environmental factors. According to Elliott (1997), useful biological indicators should (1) be measured easily, (2) work equally well in all environments, and (3) reliably reveal what problems existed where. These criteria are compatible with the ones proposed by Hinojosa et al. (2004): (1) sensitivity to perturbation or contamination, (2) relation to soil function, (3) reproducibility and low temporal and spatial variability, and (4) simple sampling and analytical methods.

To illustrate the integrative character of microbial indicators, Pankurst (1997) used the example of microbial biodiversity; although it is not directly crucial to the production of cereals, it is nevertheless a property that can be important to the continued capacity of the soil to produce crop. A loss of biodiversity can indeed be translated into a reduction of the soil capacity to recover from natural or anthropogenic disturbances (Degens et al. 2001).

Dalal (1998) added another requirement to this list: the indicator should provide reference, critical, or threshold values. This requirement may not be easy to fulfill. It is difficult to compare indicator values, either because the analytical methods are not standardized or because of the differences in soil sample pretreatment and storage conditions (Gil-Sotres et al. 2005). Moreover, the natural variability of biological indicators complicates the interpretation of results (Alkorta et al. 2003a). For crop soils, it has been suggested to use threshold soils instead of threshold values, such as climax soil (Fedoroff 1987), developed under climax vegetation, or central USA Mollisols (Gil-Sotres et al. 2005). Once again, these choices are not valid for all soil types and soil use.

Often, knowledge of physical and chemical characteristics allows the accurate interpretation of biological characteristics. It is essential therefore to consider a set of abiotic and biotic properties and processes as soil indicators in an ecosystem (Schloter et al. 2003). Table 2 presents the most accepted categories of biological indicators of soil health with their advantages and limitations. All these indicators have the advantage of integrating the chemical, physical, and ecological aspects of soil life, but their quantification remains controversial. Following Table 2 is a more detailed description and discussion of soil health microbial indicators and their application to the ecotoxicological impact evaluation of pollutants on the soil microbial trophic level.

Soil Microbial Biomass Versus Biodiversity. Soil microbial biomass is believed to be a useful indicator of soil health since pollution may reduce this pool. However, several studies have concluded that bacterial density was not influenced by contamination or physical perturbation (Alkorta et al. 2003b; Avidano et al. 2005; Baath 1989). Falih and Wainwright (1996) found that numbers of microorganisms do not always reflect rates of measured activity. On the other hand, the abundance of microbial species, or biodiversity, is useful in revealing overall trends as well as specific changes in particular classes of species. Because of the general local irreversibility of species extinction, the relation between species richness (or biodiversity) and ecosystem functions has attracted considerable attention (Alkorta et al. 2003b). Moreover, biodiversity is said to beget ecosystem stability. A healthy soil microbial community should be able to cope with changes in its environment and continue to function properly. Sustainability of the soil ecosystem was evaluated by Holling (1986) in terms of a system's ability to withstand external perturbation. He also believes that a minimum number of species is necessary to carry out essential tasks. It is hypothesized that high biodiversity amplifies functional redundancy, which leads to a higher soil functional stability and, thereby, a greater capacity to recover from perturbation (Pankhurst 1997).

A certain degree of reduction in the diversity of soil organisms will cause declines in the soil resistance to stress or perturbation (Griffiths et al. 2000).

Table 2. Principal Categories of Microbial Indicators of Soil Health [Inspired by Schloter et al. (2003) and Pankhurst (1997)].

Categories	Indicators	Advantages and limitations
Microbial biomass	Viable or total bacterial density NPP Fungi counts Algae	+ "Microbial biomass is the eye of the needle through which all organic matter needs to pass" (Jenkinson et al. 1987). – Great heterogeneity, black box approach, lack of baseline for comparison between soils.
Microbial diversity	GC-Fame BIOLOG DNA-based methods (DGGE, SSCP)	+ Allows observation of the adapting capacity of the microbial community. – The connection between biodiversity and soil functions is not well understood. There are no reliable quantitative measurements.
Microbial activity	Respiration N mineralization Denitrification Nitrification Enzyme activity.	+ Microbial activity is directly related to nutrient release, mineralization, and xenobiotics immobilization. – Methods using incubation approaches can modify microbial structure and alter microbial responses.
Soil microfauna	Nematode diversity Protozoan population.	+ Protozoa have a rapid growth rate and are pollution sensitive. Nematodes are diversified and show different degrees of tolerance to pollution. – A special expertise is necessary for the identification of trophic groups and species. – Does not apply to the microbial trophic level.

For this reason, there is a growing interest in the study of the relationship between soil microbial diversity and how a soil functions. This is a great challenge considering a significant level of diversity in soil microbes can be found in just a few grams of soil (Degens et al. 2001). According to Westergaard et al. (2001), biodiversity is a reservoir of variety that allows adaptation to changing conditions, which is essential to soil health. Several

authors consider, however, that species richness is not an issue, because the diversity of the microbial gene bank in soil is generally so high that microorganisms cannot always play their full part in ecosystem functions (Ekschmitt et al. 2001; Finlay et al. 1997). These authors further suggest that it is very likely that only highly specialized functions are sensitive to lower diversity because they are dependent on the presence of particular species (Ekschmitt et al. 2001). Nevertheless, changes in biodiversity of soil microorganisms can be a good indicator of changes in soil health. The downside of this category of indicator is that the available techniques (DGGE Biolog, GC-Fame) require specialized equipment and technicians. Moreover, the traditional biodiversity indexes, such as the Shannon–Weaver index or the Simpson index, are not adapted well to microbial ecosystems, as pointed out by Hill et al. (2003). Further research is needed to develop useful quantitative biodiversity indicators that could be applied to the development of a soil health indicator.

Microbial Activity. Soil microbial activity leads to the liberation of nutrients available for plants and microorganisms but also to the mineralization and mobilization of pollutants. To evaluate soil microbial activity, two types of microbial approaches can be used. The first requires long incubation periods, such as respiration, nitrification, and denitrification rates, and the second short incubation periods, such as enzyme activities. Usually, shorter incubation period parameters are more sensitive to environmental or management changes because there is no time for the microbial community to adapt to the experimental conditions (van Beelen and Doelman 1997).

Soil respiration is a well-accepted parameter for monitoring decomposition (Sparling 1997), but it is highly variable and can show wide fluctuations (Brookes 1995). Carbon mineralization and soil respiration are relatively resistant to xenobiotics, whereas nitrification appears to be sensitive (Kostov and Van Cleemput 2001). This sensitivity is attributed to the fact that all heterotrophic organisms contribute to CO_2 production whereas only a few bacteria genera are responsible for nitrification (van Straalen 2002). Nitrogen mineralization is a very useful indicator of microbial processes. High N mineralization rates indicate a rapid decomposition of soil organic N and active microbial population (Schloter et al. 2003). However, a major limitation to the application of microbial activity to assess soil health is that we do not know the ideal values for a healthy soil (Sparling 1997). Soil enzyme activities are often used as indicators of microbial activity and soil fertility because of their central and crucial role in how a soil ecosystem functions (Ajwa et al. 1999; Dick et al. 1996; Nannipieri et al. 2002). Soil enzyme activities are considered indicative of specific biochemical reactions of the entire microbial community in soil. Several hydrolases are involved in the N, P, S, and C cycles, and they play an important role in the biochemistry of soils (Nannipieri et al. 2002).

Because microorganisms participate in several soil functions, it is important to determine a number of enzyme activities representative of a wide range of microbial functions in order to reliably represent a soil system and, ergo, soil health (Rao et al. 2003). Avinado et al. (2005) consider that a modification in the pattern of soil enzyme activities reflects changes in the microbial activity, microbial community structure, and environmental conditions. On the other hand, Gianfreda et al. (1994) believed that it is almost impossible to explain a change in enzyme activity in response to soil contamination by a pollutant because the methods used to measure enzyme activities do not discriminate between the various components contributing to the overall activity. According to several authors, individual enzyme activities are of very limited value as soil health indicators, the parameters influencing their activities being too numerous and difficult to control (Dick et al. 1996; Kandeler et al. 1999; Trasar-Cepeda et al. 2000). Enzyme activities must be associated with complementary parameters, such as organic matter content or bacterial densities, to be useful as soil health indicators.

Microbial Functional Stability. The current capacity of a system to perform key processes is not necessarily a good indication of its capacity to maintain functional integrity over time and seasons (Herrick and Wander 1998). To evaluate its capacity to maintain functional integrity, it is therefore essential to monitor the system over time and following environmental disturbances. The capacity of a system to withstand environmental disturbance is called stability.

Several authors considered using the evaluation of soil microbial functional stability to measure soil health (Bécaert et al., 2006; Griffiths et al. 2001; Lynch 2002; Müller et al. 2002; Orwin and Wardle 2004; Pimm 1984; Schmitt et al. 2005). They applied various types of disturbances to soils and then measured the change in functional capacity of the soil microflora. The type of disturbance used varied from one study to another. For example, Griffiths et al. (2001, 2004) and Müller et al. (2002) used heat and copper contamination to disturb the soil microbial community. Orwin and Wardle (2004) subjected soils to a series of drying and humidification cycles. Degens et al. (2000) tested the effect of three stresses (pH change, salinity change, and copper contamination) and two disturbances (cycles of freeze-thawing and drying-humidification). The monitored function was carbon mineralization in almost all studies. Bécaert et al. (2006) monitored the stability of several soil enzyme activities, in this way evaluating the impact of perturbations on several nutrient cycles. In general, the very polluted soils, as well as soils whose microbial diversity was lowered by the addition of antibiotics or fumigants, showed lower stability than the clean or untouched soils. The study of functional stability as a tool for soil health evaluation is increasingly attracting the interest of researchers. Standardized methods and definitions are, however, needed to quantify stability in the context of soil health evaluation.

IV. Impact of Pollutants on Soil Health

Soil health indicators are required, not only as surrogates for reflecting the functionality of soils, but also to guide remediation actions. Gil-Sotres et al. (2005) recorded that of all the papers listed in the Soil Science CAB Abstract Database that used the keyword "soil quality" (1,500 publications), less than 20% evaluated the quality of degraded soils during the process of recovery or dealt with the loss of soil quality induced by contaminants such as pesticides or heavy metals. The choice of a sensitive and relevant indicator is crucial in evaluating the impact of pollutant on soil health because not all pollutants act similarly on soil functions and a pollutant does not impact all soil functions equally.

Even if pesticides and herbicides are not designed to inhibit soil enzyme activities or microbial processes (Speir and Ross 2002), many of them can disrupt the soil food web or organic matter breakdown (Edwards 2002). For example, Gianfreda et al. (1994) observed inhibition of free and immobilized urease following pesticide applications. Conversely, Klose and Ajwa (2004) observed that microbial respiration was not sensitive to repeated applications of fumigant pesticide. It should also be noted that ecotoxicological responses of the microbial community are easier to detect in a metabolically activated soil. For instance, Hinojosa et al. (2004) found that rewetting soil samples generally increased enzyme activities of nonpolluted and reclaimed soils, which improved differentiation between these soils and polluted soils.

Kools et al. (2005) studied the impact of Cu, Pb and Zn contamination on the degradation rate of the herbicide glyphosate. They introduced the concept of using glyphosate degradation rate as an indicator of soil ecosystem health. Their results were inconclusive, however, because no comparison could be made between the soils. Hinojosa et al. (2004) used enzyme activities as an indicator of heavy-metal contamination and evaluation of the level of soil remediation. They found that reclaimed soils still showed significantly lower enzyme activities than nonpolluted soils, indicating that these soils had not been fully restored. The microbial population was still affected by the contamination. Remediation did not restore the soil to its initial healthy state. The use of microbial activities to monitor metal pollution is not always this simple, however. Several studies have shown a negative relationship between heavy-metal concentration and microbial activities (Baath 1989). Nevertheless, there have also been cases where microbial parameters were not correlated with increasing heavy-metal pollution (Stuczynski et al. 2003; Trasar-Cepeda et al. 2000). To investigate this problem, Castaldi et al. (2004) studied the impact of heavy-metal pollution on microbial parameters (microbial biomass, respiration, dehydrogenase, phosphatase, sulfatase, glucosidase protease, and urease activities) and compared results obtained using a single microbial parameter with results given by an index expressing the average microbial response of the

microbial community. They found that the variability in responses was more pronounced in soils with lower levels of pollution than in highly polluted soils but stopped short of providing an explanation for the contradictory results found in literature.

To easily quantify the influence of pollutants on microbial-mediated processes in soil, Moreno et al. (2001) suggested using the ecological dose concept (ED_{50}). This dose corresponds to the pollutant concentration at which 50% of the process is inhibited. They chose to monitor and to model the dose–response curve of the soil ATP content and enzyme activities (urease and dehydrogenase) to evaluate the impact of cadmium toxicity on several soils. Renella et al. (2003) used the ED_{50} of cadmium on alkaline and acid phosphates activity and the ATP content of three forest soils to study the additive toxic effects of copper and zinc. It was concluded that the ED_{50} may be a sensitive tool for assessing toxic effects on soil biochemical parameters. Such indices would have the advantage of being easily comparable and incorporable in soil health criteria. Representative parameters for the soil microbial trophic level must be chosen by identifying those that best describe soil health or soil ecosystem health. The latter can be used and transformed into ecotoxicological data.

V. Conclusions

Several ecotoxicological tests have been developed and used to evaluate the impact of pollutants on individual soil animals or populations. However, few, if any, can evaluate the damage that contamination can cause to the soil microbial community (Breyer and Linder 1995). Soil is an ecosystem with complex and numerous interactions among its components (biota, minerals, organic matter, etc.). Therefore, pollutant toxicity must be evaluated by the way the microbial community functions in its environment and not only on the basis of the response of a single species. The soil health concept meets this need. Indicators or indices of soil health could be used to assess the ecotoxicological impact of pollutant on the soil microbial community and be incorporated in a risk assessment study or into the impact assessment phase of a Life Cycle Assessment (LCA) study.

Research in environmental sciences must be able to draw public attention, and also government attention, in order to build on its progress. Everyone can relate to the concept of health. “Our soils are in poor health” says much more, in fewer words, than “Our soils are less resistant to parasites and heat waves, their biomass and capacity to produce crop are declining, etc.” The evolution of soil health indicators relate to the evolution of our knowledge and understanding of their relation to soil vital functions. Several limitations still exist in the applicability of the concept: lack of a baseline, lack of consistency in bioindicator responses, and lack of standardized methods.

The determination of appropriate indicators and their values for multiple use sites is still considered to be very difficult (Knoepp et al. 2000). Accordingly, Schoenholtz et al. (2000) concluded in their review on soil forest quality evaluation that the choice of a standard set of specific properties as indicators of soil quality can be complex and will vary among forest systems and management objectives. A realistic assessment of soil health requires consideration of several soil functions and their relative importance. To overcome limitations, some authors have proposed the use of climax soil or Mollisol as a maximum benchmark of soil quality. However, this approach takes into account neither ecosystem limits nor soil use. The nutrient-poor soil of the arctic tundra could very well be as healthy as the rich brown soils of grasslands, if they are both functioning as they should. Hence, the question scientists must now address to determine what could be a proper soil health indicator is "What are the vital soil functions and what influences them?" Although each soil will respond differently to pollution, a common ground must be found or soil health and sensitive indicators must be defined according to the soil type and use.

Summary

Microorganisms are essential for a properly functioning soil ecosystem. However, few methods allow an ecotoxicological evaluation of pollutant impact on the soil microbial community. This review proposes the use of the concept of soil health as an ecotoxicological evaluation tool for soil microflora. Initially limited to sustainable agriculture, the concept of soil health is now being applied to novel situations including contaminated and remediated soils. A large amount of work has been published in the last few decades on soil health indicators, and a review of the most relevant studies is presented here. The most cited work is that of the S-5518 committee set up in 1997 by the Soil Science Society of America (SSSA), which proposed to define soil quality as being "the capacity of a soil to function within the limits of an ecosystem, to support biological production, to maintain environmental quality and to support fauna and flora health." The soil health indicators reviewed here are the ones based on this definition because it relates well to sustainability and durability of the soil functions. Several indicators proposed in these studies could be employed in the evaluation of the ecotoxicological impact of pollutants on the soil microbial community, including microbial diversity, microbial activity, and functional stability. However, research is still required to unify the concept, to set threshold values, and to standardize methodologies.

Acknowledgment

This manuscript was realized in the framework of a French-Canadian (Quebec) cooperation program "Development of tools for assessing the

bioavailability of contaminants and their environmental impacts / Part 4: Microbial tools and health ecosystem assessment."

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Manuscript received October 31; accepted November 7, 2005

Rev Environ Contam Toxicol 188:149–217

Adsorption of Ionisable Pesticides in Soils

M. Kah and C.D. Brown

Contents

I. Introduction

Pesticides are intensively used in agriculture, and much effort is expended to manage and reduce possible deleterious effects on the environment. The soil compartment has a major influence on the fate and behaviour of pesticides applied to crops preemergence or early postemergence or chemicals subject to washoff from crop surfaces. Once in the soil, pesticide molecules partition between the aqueous and solid phases, which affects many other aspects of their behaviour: sorption can be rate limiting to volatilization, bioavailability (and thus efficacy and biodegradation rate), and subsurface transport. Understanding the fate of a pesticide in soil is fundamental to the accurate assessment of its environmental behaviour and vital in ensuring the safe use of new and existing products. It is also necessary to develop and validate computer simulation models for use as predictive tools in future environmental fate assessments.

Communicated by Professor C.D. Brown.

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It is estimated that ionisable compounds comprise 25% of the existing active substances currently undergoing review for reregistration by the European Union (EU 2002). Also, a significant and increasing proportion of new actives proposed for registration are ionisable, including most sulfonylureas, and the formation of acidic metabolites is common during degradation processes (EU 2002). Ionisable herbicides (e.g., phenoxy acids, triazines, sulfonylureas, imidazolinones) are particularly common and represent the largest major group of soil-applied herbicides (Harper 1994). This group includes chemicals that are frequently found in groundwater and surface waters worldwide. Among the 15 main molecules quantified in surface and groundwater in France in 2002, 8 were ionisable compounds (IFEN 2002). This category represented up to half of the pesticides detected in surface and drinking water samples in Hungary (Győrfi et al. 1998).

Among the 9 pesticides most frequently exceeding 0.1 µg/L in surface fresh waters in the UK between 1998 and 2003, 6 were ionisable (mecoprop, MCPA, 2,4-D, dichlorprop, simazine, and atrazine). Similarly, 7 of the 10 pesticides exceeding threshold concentrations in UK groundwater in 2003 were ionisable (Environment Agency 2003). Atrazine and simazine were among the three most frequently detected pesticides in groundwater collected from wells of agricultural areas in the US (USEPA 1990) and Portugal (Cerejeira et al. 2003).

Ionisable compounds possess either weak acidic and/or basic functional group(s). As a consequence, they may be partially ionised within the range of normal soil pH, which strongly affects their soil reactivity. The adsorption of neutral organic compounds in soils occurs mainly by hydrophobic partitioning, whereas a number of additional mechanisms are postulated for the adsorption of ionisable pesticides. It is essential that this specific behaviour is recognised within risk assessment procedures to obtain a robust analysis of likely behaviour.

Several reviews are available on the adsorption of organic chemicals in soils (Calvet 1989; Harper 1994; Von Oepen et al. 1991; Wauchope et al. 2002). These reviews mainly covered the behaviour of hydrophobic compounds in soils, which is now relatively well understood. Relatively less information was available concerning ionisable pesticides. Although similar levels of information are available concerning the sorption of ionisable pesticides, there is still much debate regarding the underlying mechanisms and the approaches to describe and predict variation in sorption with properties of the pesticide and of the soil. Numerous articles reported results of adsorption of ionisable pesticides in soils in the past 15 years. The purpose of this review is to present the state of knowledge on the particular behaviour of ionisable pesticides in soils.

The review first introduces the issues concerning adsorption and the characteristics of this particular kind of pesticide. The mechanisms postulated for their adsorption are described. Subsequently, it focuses on the

influence of soil properties on adsorption and on the potential to predict the behaviour of ionisable pesticides in soils. We concentrate particularly here on those soil factors that do not particularly influence the adsorption of neutral compounds but which often have a great importance for the sorption of ionisable pesticides (soil pH, clay and oxide contents). Finally, it briefly reviews degradation of ionisable compounds in soil and evidence for its dependence on the adsorption process.

II. Background

A. Ionisation

A weakly acidic compound dissociates in water to produce protons. Thus, it exists in both anionic and neutral forms in aqueous solutions. The relative amounts of each form are determined by the acid equilibrium constant, K_a, and the pH of the aqueous solution. Assuming activity coefficients to be near unity, this equilibrium may be represented as:

$$[HA] + [H_2O] \rightleftharpoons [A^-] + [H_3O^+] \quad \text{with } K_a = [H_3O^+] \cdot [A^-]/[HA]$$

where $[H_3O^+]$, $[A^-]$, and $[HA]$ are defined as the aqueous concentration of hydronium ion (or proton), anionic species, and neutral species, respectively (all in mole L^{-1}).

In addition,

$$pK_a = -\log_{10} K_a \quad \text{and} \quad pH = -\log_{10} [H_3O^+]$$

which gives

$$[HA]/[A^-] = 10^{(pK_a - pH)}$$

This gives the ratio of the neutral species to the anion as a function of pH and shows the increasing dominance of the anion at higher pHs (Fig. 1).

A weakly basic compound dissociates in water to produce OH^- or is a compound that can accept a proton (Brønsted definition). Thus, it exists both in cationic and neutral form in solution. As for acidic compounds, a basic equilibrium constant, K_b, can be defined:

$$[B] + [H_2O] \rightleftharpoons [BH^+] + [OH^-] \quad \text{with } K_b = [OH^-] \cdot [BH^+]/[B]$$

where $[OH^-]$, $[BH^+]$, and $[B]$ are defined as the aqueous concentration of the hydroxide ions and positive and neutral species, respectively (all in mole L^{-1}). The ratio of cationic to neutral species in solution can also be calculated according to the pH of the solution. However, it is now more usual to describe the strength of bases also in terms of K_a and pK_a, thereby establishing a single continuous scale for both acids and bases. To make this possible, our reference reaction for bases becomes the equilibrium:

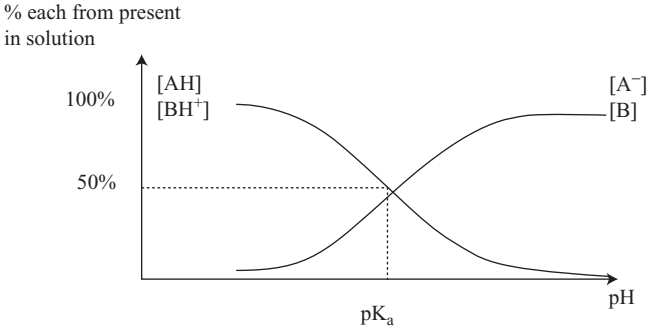


Fig. 1. Dominance of neutral or ionic form in solution according to the pH, assuming that the activity coefficient is near unity. [AH] and [BH^+] are the protonated form, [A^-] and [B] the dissociated form, of the acidic and basic compounds, respectively. With [AH] the concentration of the protonated form of an acid and pK_a, its dissociation constant: $[AH] = \frac{1}{1+10^{pH-pK_A}}$.

$$[BH^+] + [H_2O] \rightleftharpoons [B] + [H_3O^+] \quad \text{with } K_a = [B] \cdot [H_3O^+]/[BH^+]$$

Here, K_a is a measure of the acid strength of the conjugate acid BH^+ of the base B. The stronger BH^+ is as an acid, the weaker B will be as a base (pK_a + pK_b = 14).

A zwitterion is an ion that has a positive and negative charge on the same group of atoms. Zwitterions can be formed from compounds that contain both acidic and basic groups in their molecules. For example, imazethapyr is an ampholytic compound due to the presence of both carboxyl (pK_a = 3.9) and basic quinoline groups (pK_a = 2.1). As pH decreases, the imazethapyr molecule will be alternatively negatively charged (COO^-; N), neutral (COOH; N), and then positive (COOH; NH^+). As for acidic and basic compounds, it is possible to determine the ratio of each form at a given pH. See Table 1 for examples of ionisable pesticides and their main characteristics.

It is important to notice that compounds with a very low/high pK_a dissociate at pH not relevant to the soil environment. Therefore, only one type of species is present in the soil solution for the range of natural soil pH. The behaviour of this kind of ionisable compound is unlikely to be sensitive to soil pH. Ionic pesticides (e.g., diquat, paraquat) whose charge is not dependent on pH shifts are not considered in this review.

B. Measurement of Soil pH

Soil pH values are usually determined in 1:5 soil:liquid suspension (in water, 0.01 M $CaCl_2$ or 1 M KCl according to ISO 10390; 1994), but it is known that the pH at soil surfaces may be lower than in the bulk solution.

Indeed, according to electrical double-layer theory, the net negative charge at soil surfaces is compensated by cations held in a diffuse layer close to the surface. Some of the excess of cations in the diffuse layer over those in bulk solution will be hydrogen ions, and so pH close to soil surfaces is lower than that in bulk solution (Talibudeen 1981).

Hayes (1970) assumed that the pH at the surface of humic substances might be 0.5 to 2 units lower than that of the liquid phase, or that localised areas of low pH could exist within soil organic matter (OM). Bailey et al. (1968) reported that the pH at a montmorillonite surface appears to be 3 to 4 units lower than the pH of the bulk solution. Moreover, decreasing water content increases the conversion of NH_3 to NH_4^+ on the surface of clay minerals (Raman and Mortland 1969); this is caused by the enhanced ionization of water molecules in the solvation spheres of adsorbed inorganic cations at lower water contents (greater Brønsted acidity). Therefore, Che et al. (1992) proposed that the protonation of imazaquin and imazethapyr by clay mineral surfaces would also be greatly enhanced at lower water content, which implies that dissociation could occur in the field at higher pH than in batch conditions and that pH effects could thus be stronger under field conditions. Thus, significant surface protonation of a basic or acidic molecule may occur even though the measured pH is greater than the pK_a of the compound.

This phenomenon complicates the examination of pH effects on the retention of ionisable compounds on soil surfaces. A consequence is that although sorption versus pH curves for ionisable pesticides resemble the sigmoidal shape of acid dissociation curves, they are often positioned about 1.8 pH units more alkaline than the pK_a curve (Nicholls and Evans 1991a). Another way to interpret this phenomenon has been given by Feldkamp and White (1979), who concluded that ionization of weak bases such as triazines can be modified by an adsorbent phase, or as a consequence of adsorption. The equilibrium is displaced toward the formation of BH^+, and thus the amount adsorbed is greater than the amount deduced from the pK_a value. This explanation was also proposed for adsorption of atrazine and simazine on clay by Celis et al. (1997a) (cf. III. C).

The difference in pH between soil particle surfaces and soil solution is mediated by soil characteristics such as the charge of soil particles and the type and quantity of cations present in solution. There is thus no general rule on the relative difference. For instance, Regitano et al. (1997) obtained a reasonable agreement between a model and measured sorption data, and concluded that the pH measured in the bulk soil solution was representative of the pH encountered by the herbicide imazaquin at the sorbent surface. Current techniques for measuring pH do not allow the observation of these specific phenomena at sorbent surfaces. Thus, further research is needed to better understand and determine the pH at soil interfaces.

Table 1. Molecular Structures, Uses, and Properties for Examples of Ionisable Pesticides (Source: Tomlin 1997; www.inra.fr/agritox).

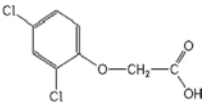
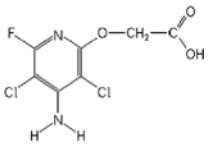
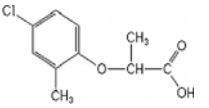
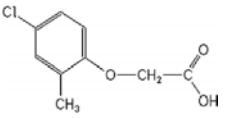
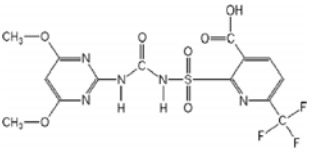
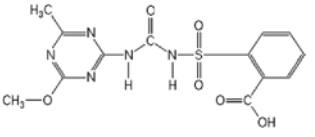
Pesticide name [CAS RN]	Formula	Use and application rate ($g\,ha^{-1}$)
Acidic compounds		
Carboxylic acids		
2.4-D [94-75-7]		Herbicide on cereals, maize, sorghum, grassland, orchards, sugar cane, rice, noncrop lands (280–2300)
Fluroxypyr [69377-81-7]		Control of a range of broad-leaved weeds in all small grain crops and pasture (200)
Mecoprop [7085-19-0]		Hormone-type herbicide for the control of broad-leaved weeds in wheat, barley, oats, grassland, and pasture (1200–1500)
MCPA [94-74-6]		Hormone-type herbicide for the control of broad-leaved weeds in cereals, rice, peas, potatoes, grassland, turf, roadside, and embankments (3000)
$NHSO_2$ acids		
Flupysulfuron-methyl-sodium [144740-54-5]		Selective control of black grass and other weeds in cereals (10)
Metsulfuron-methyl [74223-64-6]		Control of a wide range of annual and perennial broad-leaved weeds in wheat, barley, rice, and oats (4–7.5)

Table 1. *Continued*

pK_a	K_{oc} (mL g^{-1})	DT_{50} (d)	Solubility (in water, g L^{-1})	Log P
2.97	5–212	5–59	0.6	–1 (pH9) 2.7 (pH1)
2.94	51–81	5–68	0.091	156 (pH4) 0.23 (pH10)
3.11	5–43	7–13	>250	–0.19
3.73	10–157	7–79	294	2.7 (pH1) –1.07 (pH9)
4.94	15–47	6–26	0.063 (pH5) 0.600 (pH6)	0.06 (pH6)
3.75	4–60	4–100	0.548 (pH7); 213 (pH9)	–1.7 (pH7)

Table 1. *Continued*

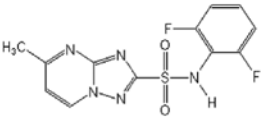
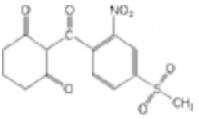
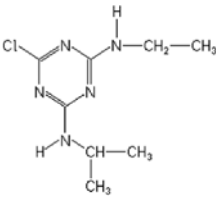
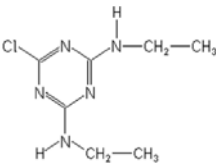
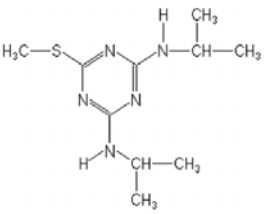
Pesticide name [CAS RN]	Formula	Use and application rate ($g\,ha^{-1}$)
Flumetsulam [98967-40-9]		Control of broad-leaved weeds and grasses in soya beans, field peas, and maize (10–20)
Other acids		
Mesotrione [104206-82-8]		Control of broad-leaved and some grass weeds in maize (70–225)
Basic compounds		
Triazines	***Chlorotriazines***	
Atrazine [1912-24-9]		Control of annual broad-leaved weeds and annual grasses in maize, sorghum, sugar cane, grassland, conifers, industrial weed control (750–1000)
Simazine [122-34-9]		Control of most germinating annual grasses and broad-leaved weeds in pome fruit, stone fruit, bush and cane fruit, citrus fruit, vines, strawberries, nuts, olives, pineapples, cocoa, coffee (1500 in EU to 3000 in tropics and subtropics)
	Methylthiotriazines	
Prometryn [7287-19-6]		Selective systemic herbicide on cotton, sunflowers, peanuts, potatoes, carrots, peas, and beans (800–2500)

Table 1. *Continued*

pK_a	K_{oc} (mL g^{-1})	DT_{50} (d)	Solubility (in water, g L^{-1})	Log P
4.6	5–182	30–60	0.049 (pH2.5)	–0.68
3.12	19–387	4–31.5	2.2 (pH5) 22 (pH9)	0.9 (pH5) –1 (pH7)
1.7	39–173	166–77	0.033 (pH7)	2.5
1.62	103–277	27–102	0.062 (pH7)	2.1 (unionised)
4.1	185–575	14–150	0.033	3.1 (unionised)

Table 1. *Continued*

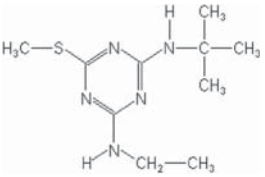
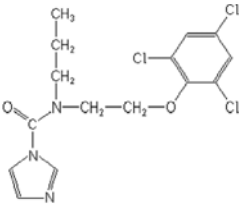
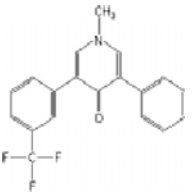
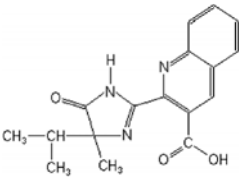
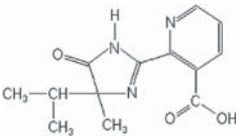
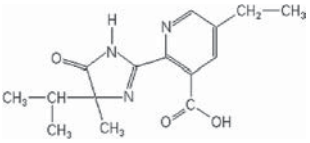
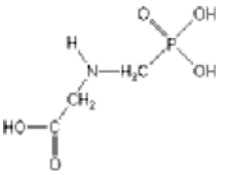
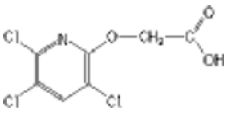
Pesticide name [CAS RN]	Formula	Use and application rate ($g\,ha^{-1}$)
Terbutryn [886-50-0]		Control of black grass and annual meadow grass in winter cereals; also in mixture on sugar cane, sunflower, beans, peas potatoes, cotton, and peanut (200–2000)
Other basic compounds		
Prochloraz [67747-09-5]		Protectant and eradicant fungicide effective against a wide range of diseases affecting field crops, fruit, turf, and vegetables (400–600)
Fluridone [59756-60-4]		Aquatic herbicide for control of most submerged and emerged aquatic plants in ponds, reservoirs, irrigation ditches (10^{-6}–$10^{-3}\,g\,L^{-1}$)
Amphoteric compounds		
Imidazolinones		
Imazaquin [81335-37-7]		Preplanting or preemergence control of broad-leaved weeds in soya beans (70–140)
Imazapyr [81334-34-1]		Control of annual and perennial grasses, sedges, and broad-leaved weeds in noncrop areas, forestry management, and plantation of rubber trees and oil palms (100–1700)

Table 1. *Continued*

pK_a	K_{oc} (mL g^{-1})	DT_{50} (d)	Solubility (in water, g L^{-1})	Log P
4.3	2000	14–50	0.022	3.65 (unionised)
3.8	1222–5818	5–37	0.034	4.12 (unionised)
1.7	350–1100	>343	0.012 (pH7)	1.87 (pH7)
3.8	13–40	60	0.060–0.120	0.34
1.9 3.6 11	4–170	30–150	9.74	0.11

Table 1. *Continued*

Pesticide name [CAS RN]	Formula	Use and application rate ($g\,ha^{-1}$)
Imazethapyr [81335-77-5]		Control of many major annual and perennial grass and broad-leaved weeds in soya and other leguminous crops (70–200)
Others		
Glyphosate [1071-83-6]		Control of annual and broad-leaved weeds in cereals, peas, beans, oilseed rape, vines, olives, orchards, pasture, forestry and industrial weed control; inactivated on contact with soils (1500–4300)
Triclopyr [55335-06-3]		Control of woody plants and many broad-leaved weeds in grassland, uncultivated land, industrial areas, coniferous forest, rice field, and plantation crops (100–8000)

pK_a, dissociation constant; K_{oc}, distribution coefficient in soils normalised by the organic carbon content; DT_{50}, half-life in soil, time required for 50% of the initial dose to be degraded; log P, hydrophobicity of the compound.

III. Adsorption of Ionisable Compounds

A. Measurement of Sorption

Adsorption refers to the attraction and accumulation of molecules at the soil–water or soil–air interface, resulting in molecular layers on the surface of soil particles (Harper 1994). Soil sorption is characterized by a partition constant K, conventionally written with a subscript d ("distribution"), defined as the ratio of the quantity of molecules adsorbed to the quantity of molecules in solution at equilibrium. For direct measurement of the distribution coefficient (K_d), the batch slurry method is generally used (OECD 1997). However, the soil–solution ratios required to reach equilibrium

Table 1. *Continued*

pK_a	K_{oc} (mL g^{-1})	DT_{50} (d)	Solubility (in water, g L^{-1})	Log P
2.1 3.9	75–173	30–90	1.4	1.04 (pH 5) 1.49 (pH 7) 1.2 (pH 9)
2.3 5.7 10.2	1,000–59,000	3–174	11.6	–3.2 (pH 2–5)
2.7 3.97	41–59	8–156	7.9 (pH 5) 8.22 (pH 9)	0.42 (pH 5) –0.45 (pH 7) –0.96 (pH 9)

(from 1:1 to 1:100) are atypical of field soil moisture conditions, and the results may not adequately reflect sorption processes in field-moist or unsaturated soil.

Recovery of soil solution from field-moist soil provides a more realistic representation of field situations because the soil is wetted to field capacity and is not reduced to slurry as in a batch equilibrium experiment. However, Wehtje et al. (1987) found good agreement in sorption of both sulfometuron and imazapyr as determined by batch equilibrium and solution recovery protocols. Goetz et al. (1986) pointed out that differences in sorption across soils were much more apparent with the soil solution recovery than with the batch technique.

Walker and Jurado-Exposito (1998) compared the adsorption data obtained from standard batch measurements and those obtained using a

centrifugation technique for isoproturon, diuron and metsulfuron-methyl. Although the results were not fully consistent, they generally indicate lower K_d, less adsorption, and lower n values (greater curvature) of the isotherms than in the standard batch system, implying that batch experiments might overestimate K_d in some cases.

Johnson and Sims (1998) compared soil thin-layer chromatograpy (TLC) and batch equilibrium results for the sorption of atrazine and dicamba on six soils. Agreement between the two methods was good for some horizons but differed significantly for others. It was suggested that the soil TLC gives results under nonequilibrium conditions whereas the batch procedure, by definition, measures quasiequilibrium. The authors concluded that the soil TLC procedure could provide additional information relevant to pesticide partitioning in the field environment.

Gel filtration chromatography was found useful for the study of ionic as well as nonionic pesticides (Madhun et al. 1986) but only gives a relative evaluation of the strength of sorption. Another procedure to measure sorption is to estimate sorption based on retardation of the solute during its transport through a column of soil (Heistermann et al. 2003; Shaw and Burns 1998b; Tuxen et al. 2000). This method has the advantage of maintaining soil structure during measurements and thus incorporating the importance of water flow through soil pores and the accessibility of soil particles within aggregates at a realistic soil to solution ratio. The use of intact soil cores provides the greatest similarity to natural soil. However, this method is more complex than those already described, and degradation also becomes a factor in reducing the accuracy of the results (Harper 1994).

Berglöf et al. (2003) proposed the use of low-density supercritical fluid extraction ($0.3\,g\,mL^{-1}$) to remove metsulfuron-methyl from the soil water phase of three soils at 11% water content. The authors were able to predict K_d values obtained using the batch slurry technique with a combination of the results, the pK_a value, and the pH of the soil. Although this could provide an easy method to predict sorption in soil at different pH levels, it still must be validated with other compounds.

Finally, the partition coefficient (K_d) could be calculated indirectly from regression with other partition coefficients (solubility, K_{ow}, HPLC capacity factor) or estimated using quantitative structure–activity relationships (QSAR). However, no satisfactory model has yet been proposed for ionisable pesticides.

B. Factors Influencing Adsorption of Ionisable Compounds in Soils

Soil Properties. Once the organic molecule reaches the soil, its partition between the solid and liquid phase depends to a large extent on soil properties. Nonpolar compounds mainly adsorb by hydrophobic partitioning, so OM content is generally the dominant soil parameter determining their

adsorption. Ionic compounds also sorb on organic matter, but also can bind to clay and Fe/Al (hydr)oxides. These two components seem to play a significant role in certain cases. Last, in contrast to hydrophobic compounds, ionisable pesticide adsorption is highly sensitive to pH variation. The importance of pH influence depends on the molecule and on the other soil properties. The influence of soil properties on sorption is considered in detail in Section III.D.

Climatic Factors: Temperature, Water Content. The main climatic factors that influence adsorption of organic compounds in soils are the temperature and moisture content of the soil. It is often assumed that adsorption is an exothermic process, whereby an increase in temperature leads to decreased adsorption and increased desorption rates (Calvet 1989; Harper 1994). However, thermodynamic studies have shown a highly variable relationship to temperature due to the complexicity of the soil environment (Harper 1994) and a variable influence of temperature on the different binding mechanisms (Hayes 1970). Di Vicenzo and Sparks (2001) explore the differences in the sorption mechanisms of the protonated and ionized forms of pentachlorophenol (PCP) by measuring their sorption coefficients at different temperatures (4°, 25°, and 55°C). Although no apparent trend was observed for the neutral form, a clear decrease in K_d with increasing temperature was observed for the ionised form (suggesting more-specific adsorption processes). Similarly, Thirunarayanan et al. (1985) observed an increase in K_d values for chlorsulfuron with a decrease in temperature from 30° to 8°C. Temperature affected the amount adsorbed with the smallest effect at the lowest pH, where the neutral form dominates. The same inverse relationship was observed with glyphosate (Eberbach 1998) and atrazine on clays (Fruhstorfer et al. 1993). In practice, temperature seems to have only a minor effect on sorption. Ukrainczyk and Ajwa (1996) found no significant effect of temperature, between 10° and 35°C, on primisulfuron adsorption to 23 soils, and a study carried out in three Norwegian reference soils indicated that the effect of a colder climate on the soil formation does not affect sorption of bentazone, dichlorprop, and MCPA (Thorstensen et al. 2001).

It has often been reported that adsorption coefficients increase as water content decreases. This effect can be attributed to reduced competition by water for sorption sites and an influence of solubility as the herbicide solution becomes more concentrated (Harper 1994). Indeed, Goetz et al. (1986) observed that temporarily drying and returning to field capacity generally increased sorption of imazaquin; this was attributed to a reduction in thickness of the water film coating the soil minerals, which serves to concentrate the imazaquin near the sorption surface or facilitate precipitation. Wehtje et al. (1987) confirmed that desiccation apparently concentrates sulfometuron and imazethapyr near the sorptive surface. Roy et al. (2000) have

shown that weakly basic compounds such as prochloraz may partition rapidly into the liquid-like interior of humus at low soil moisture contents. However, increased diffusion at high soil moisture content may cause additional sorption by ion exchange at colloid surfaces. Stronger basic compounds (e.g., fenpropimorph, $pK_a = 6.98$) may essentially adsorb due to ionic interactions, and their sorption is enhanced at high soil moisture content due to diffusion. Increased sorption with increased water content has been observed with atrazine (Koskinen and Rochette 1996; Rochette and Koskinen 1996) and metsulfuron methyl (Berglöf et al. 2003). Thus, effects of moisture content on sorption seem to be more complex when compounds are likely to be protonated in soil.

Pesticide Properties. Several chemical characteristics have been correlated successfully to sorption of neutral compounds onto soil. However, broad-spectrum applicability to include ionisable compounds has not been achieved (Harper 1994). For hydrophobic compounds, sorption to soil OM can be described predominantly as a partitioning process between a polar aqueous phase and a nonpolar organic phase (soil OM). Significant correlations have been published between the sorption coefficient (K_d) and water solubility or K_{ow} (octanol–water partition coefficient evaluating the hydrophobicity of the compound) (Karickhoff et al. 1979; Karickhoff 1981; Gerstl 1990; Nicholls and Evans 1991a; Schwarzenbach and Westall 1981; Von Oepen et al. 1991). For hydrophobic ionisable compounds, the solvophobic mechanism alone is not sufficient for estimating soil–water distribution coefficients as the sorption mechanism depends on the degree of dissociation, which is itself a function of the dissociation constant and the pH of the soil solution. Riise and Salbu (1992) showed that K_{ow} for dichlorprop was inversely related to pH and that the relationship was similar to that between K_{oc} (K_d normalised by the organic carbon content) and pH. In the pH range 4–7, the K_{ow} value changes from 114 to 0.6. Thus, the relationship between K_{oc} and K_{ow} for dichlorprop corresponds to that previously reported for neutral organic chemicals.

Experimental Factor: Importance Of Ionic Strength. Different salt solutions, including $CaCl_2$ or KCl, NH_4Cl, HCl, NaCl, $Ca(H_2PO_4)_2$, $Na_4P_2O_7$, and KH_2PO_4 have been used to assess the influence of ionic strength and ionic composition of the soil solution on pesticide sorption. Solution concentration varied usually between 0 and 1 M, although the strength of natural soil solution rarely exceeds 10^{-3} M. Results demonstrate that this variation can strongly influence the sorption of ionic molecules, either positively or negatively, according to the electrolyte composition and concentration and characteristics of the pesticide and sorbent. Uncharged molecules seem to be much less sensitive to variation in ionic strength (Alva and Singh 1991; Clausen et al. 2001; de Jonge and de Jonge 1999).

A positive influence of ionic strength on adsorption is often observed. For instance, Clausen et al. (2001) observed increasing adsorption of mecoprop and 2,4-D on kaolinite with increasing $CaCl_2$ concentration and increasing mecoprop adsorption on quartz. Increased sorption of PCP (Lee et al. 1990), imazaquin (Regitano et al. 1997), 2,4,5-T (Koskinen and Cheng 1983), silvex and DNOC (Jafvert 1990), 2,4-DNP, DNOC, dinoseb, and dinoterb (Martins and Mermoud 1998), and glyphosate (de Jonge and de Jonge 1999) were also observed with increasing ionic strength. The positive influence of ionic strength on sorption results in part from a replacement of protons from the soil surface as ionic strength increases, causing a slight decrease in pH and shifting acidic compounds toward neutral forms that are more strongly sorbed than the anionic forms (de Jonge and de Jonge 1999; Regitano et al. 1997). Complexation of the pesticide molecule with surface-exchanged multivalent cations could also contribute to stronger sorption at higher ionic strengths, as the diffuse double layer is compressed and Ca^{2+} becomes more strongly attached to the clay surfaces (Clausen et al. 2001; de Jonge and de Jonge 1999). Ion pairing between the anionic form of the pesticide and cations in the solution could occur, and sorption of neutral ion pairs would be possible. This process depends on the availability of the "complementary cations" in solution, either due to high salt concentrations or near negatively charged colloid surfaces (Spadotto and Hornsby 2003). Colloidal stability may influence sorption processes as fine colloids and dissolved OM coagulate at higher ionic strength; this would lead to an increase in the measured K_f value (de Jonge and de Jonge 1999). Lower solubility of 2,4-D in 1 M NaCl compared to 0.01 and 0.1 M NaCl, could explain the increasing sorption of 2,4-D on goethite with increasing ionic strength observed by Watson et al. (1973). The salting-out effect can vary directly or inversely with salt concentration, depending on the salt of interest, but an increase in sorption with increasing salt concentrations occurs for most common salts (e.g., NaCl, $CaCl_2$, and KCl) (Lee et al. 1990).

A negative relationship between adsorption and ionic strength has been reported as well, especially for variably charged sorbents. For instance, Hyun and Lee (2004) observed a fivefold decrease in prosulfuron sorption as the solution changed from 0.0015 to 1.5 M $CaCl_2$ in a variably charged soil with a high contribution of hydrophilic processes (high anion-exchange capacity, AEC). In contrast, no difference was observed for a soil with an AEC approaching zero. Clausen et al. (2001) noted that the adsorption of ionic pesticides on calcite and alpha-alumina decreases with increasing $CaCl_2$ concentration. The authors proposed several effects that might oppose that resulting from an increasing positive charge at the surface with increasing ionic strength: (i) enhanced competition with the chloride anion that is known to adsorb on iron oxides (owing to its relative larger size and lower concentration, the anionic pesticide is not able to compete effectively for anion exchange sites); (ii) possible complexation between the anionic

pesticides and Ca^{2+}, which results in nonsorbing solution complexes; or (iii) a decrease in the activity of the charged ions caused by the increasing electrolyte concentration. The addition of $Ca(H_2PO_4)_2$ and KH_2PO_4 resulted in significantly less adsorption of imazaquin and glyphosate, respectively (de Jonge and de Jonge 1999; Regitano et al. 1997), probably because of competition effects. This result suggests that the application of large amounts of phosporus and lime to agricultural fields could reduce pesticide sorption and enhance pesticide concentration in solution, especially in weathered soils.

Finally, variation in sorption does not necessarily vary linearly with ionic strength. For instance, in batch experiments involving 2,4-D, mecoprop, bentazone, and iron oxides, Clausen and Fabricius (2001) observed that the addition of $CaCl_2$ at concentrations between 0 and 0.01 M caused adsorption capacity to diminish, with the greatest effect between 0 and 0.0025 M. The effect seemed to arise from the type of binding mechanism, with outer-sphere complexation being more strongly affected by the electrolyte concentration than inner-sphere complexation. Similarly, sorption values for picolinic acid measured in distilled water by Nicholls and Evans (1991b) were almost the same as those measured in 0.01 M $CaCl_2$, but strength of sorption decreased about fivefold when $CaCl_2$ increased from 0.01 to 1 M, probably because the protonated form of picolinic acid was displaced by calcium ions.

Ionic composition has been shown to play a role in ionic pesticide sorption, but there is some conflict in results, and further research is needed to better understand the complex interaction of mechanisms involved. The ionic strength of natural soil solution does not normally exceed 10^{-3} M, so that effects of ionic strength on sorption can usually be neglected (Lee et al. 1990). Nevertheless, the choice to use 0.01 M $CaCl_2$ in standardized soil sorption experiments (OECD 1997) will affect the sorption coefficients of ionisable pesticides, and this places a constraint on the use of results from these standardized tests to predict sorption behaviour of ionisable compounds in the field.

C. Adsorption Mechanisms

Adsorption–desorption is a dynamic process in which molecules are continually transferred between the bulk liquid and solid surface. A number of mechanisms have been postulated to be involved in the retention of pesticides. However, it is difficult to isolate a definitive mechanism because most retention arises from an interaction of a variety of forces and factors. In addition, direct experimental evidence for a particular mechanism is quite rare, and one is often confined to propose a hypothesis (Calvet 1989). Only kinetic, thermodynamic, and spectroscopic studies can truly lead to mechanistic interpretations (Di Vincenzo and Sparks 2001), and spectroscopic

studies are often impractical because of the heterogeneous nature of soil. Several reviews are available on the retention mechanisms of pesticides in soils (Calvet et al. 1980a,b; Calvet 1989; Harper 1994; Koskinen and Harper 1990; Senesi 1992; Von Oepen et al. 1991), and we concentrate here on the current state of knowledge for ionic compounds.

Most organic compounds of interest as environmental contaminants are hydrophobic. Thus, they have low polarity and solubilities in the mg L^{-1} (ppm) range or less. The driving force for their adsorption consists mainly of entropy changes (solvent-motivated adsorption: hydrophobic interaction) and relatively weak enthalpic forces (sorbent-motivated adsorption: van der Waals and hydrogen bonding). The combined effect of these two mechanisms is often referred to as hydrophobic sorption (Hamaker and Thomson 1972; Pignatello 1989). Other sorption mechanisms can occur for more polar solutes, including ionic exchange, charge transfer, ligand exchange, and cation or water bridging. Furthermore, decreased extractability of organic chemicals with increased incubation time may be due to the formation of covalent bonds or the physical trapping of the chemical in the soil matrix (Koskinen and Harper 1990). Advanced techniques such as Fourier transform infrared (FT-IR), X-ray diffraction, or electron spin resonance (ESR) spectroscopy have been applied in some studies to prove or disprove the existence of some retention mechanisms in soils. The results are summarized in Table 2. Seven mechanisms have been identified of which hydrogen bonding is the most frequently inferred. Evidence is usually cited to support the operation of one or more mechanisms. It is rare to find studies that have demonstrated that any particular mechanism is not operating.

Hydrophobic Sorption. Hydrophobic adsorption is proposed as the main mechanism for the retention of nonpolar pesticides by hydrophobic active sites of humic substances (HS) or clay. The hydrophobic solute is expelled from the water (solvent-motivated adsorption), and this mechanism can also be regarded as a partitioning between a solvent and a non-specific surface. These sites include aliphatic side chains or lipid portions and lignin-derived moieties with high carbon content of the HS macromolecules (Senesi 1992). Hydrophobic adsorption to soil has been suggested as an important mechanism for some ionisable pesticides in their molecular form, including some weakly basic sterol fungicides (Roy et al. 2000), prometryn (Khan 1982), 2,4-D and triclopyr (Johnson et al. 1995), PCP (Lee et al. 1990), primisulfuron (Ukrainczyk and Ajwa 1996), imazaquin (Ferreira et al. 2002), and atrazine and simazine (Herwig et al. 2001). Celis et al. (1997a) suggested that *s*-triazine sorption on montmorillonite, as the protonated species (cationic form), must be preceded by sorption as the molecular species on hydrophobic microsites of the clay. However, cation exchange would also be operative if the pH of the bulk solution were close to the pK_a of the herbicide. The authors demonstrated that the protonation of atrazine

Table 2. Experimental Evidence of Adsorption Mechanisms.

Pesticide	Sorbent	Hydrophobic	van der Waals	H-bonding	Ionic exchange
Acidic pesticides					
Mecoprop, 2,4-D; bentazone	Iron oxides				x
2,4-D	Organoclays			x	
Acifluorfen	Cu (II)				
Fluazifop-butyl	Homoionic clays			x	
Fluazifop-butyl, fluazifop	Smectites				
Pentachlorophenol	Variable charge soils	x			x
Azimsulfuron	Iron oxides				
Primisulfuron	Oxides and soils	x		x	x
Ethametsulfuron-methyl	Acidic soil			x	
Basic pesticides					
Atrazine	HA			x	x
Atrazine	HA			x	
Atrazine	HA	x		x	
Hydroxyatrazine	HA	x		x	
Atrazine	OM			x	
Zwitterionic pesticides					
Imazaquin	Soils, HA	x		x	x
Imazethapyr	Soils, HA		x	x	x
Glyphosate	Clays and oxides			x	x
Glyphosate	Goethite				

FT-IR, Fourier transform infrared spectroscopy; ESR, electron spin resonance spectroscopy; TLC, thin-layer chromatography; EPR, electron paramagnetic resonance; NMR, nuclear magnetic resonance.

and simazine at clay interfaces would involve a movement from hydrophobic to hydrophilic sites on the clay surface, so new hydrophobic sites would become available for the molecular species in solution.

Hydrophobic partitioning is usually regarded as a pH-independent mechanism. However, the dissociation of some humic acid (HA) functional groups at low pH might reduce the potential of OM for hydrophobic adsorption. Conversely, Ferreira et al. (2001) propose that consequent conformation changes might create water protected sites at pH < 5 and thus create some very hydrophobic adsorption sites at low pH.

van der Waals Interactions. Particular adsorption on hydrophobic constituents of OM can be explained either in terms of solute partition between water and organic matter (solvent-motivated sorption, entropy-driven) or

Table 2. *Continued*

Charge transfer	Ligand exchange	Cation bridging	Methods	Reference
	x		Interpretation of isothems	Clausen and Fabricius 2001
		x	FT-IR, X-ray diffraction	Hermosin and Comejo 1993
	x		Polarographic techniques, X-ray diffraction	Kozlowski et al. 1990
x			IR and X-ray diffraction	Gessa et al. 1987
x	x	x	IR spectroscopy, X-ray diffraction, and TLC	Fusi et al. 1988
			Interpretation of isotherms	Hyun et al. 2003
	x	x	IR	Pinna et al. 2004
			Interpretation of isotherms	Ukrainczyk and Ajwa 1996
			FT-IR	Si et al. 2005
x			FT-IR and ESR	Senesi et al. 1995
no			UV-visible, FT-IR and ESR	Martin-Neto et al. 1994
no			UV-visible, FT-IR and ESR	Martin-Neto et al. 2001
x			UV-visible, FT-IR and ESR	Martin-Neto et al. 2001
			NMR spectroscopy	Welhouse and Bleam 1993a,b
no		x	FT-IR and EPR	Ferreira et al. 2002
x			FT-IR and EPR	Senesi et al. 1997
		x	IR and X-ray diffraction	McConnell and Hossner 1989
	x		FT-IR	Sheals et al. 2002

in terms of solute adsorption (sorbent-motivated, enthalpy-driven). Physical adsorption on OM by van der Waals interactions is probably the more satisfactory explanation, according to Calvet (1989). Such interactions are usually weak (2–4 kJ mol^{-1}), constituting short-range dipolar or induced-dipolar attractions, but may be magnified by the hydrophobic effect. Because these forces are additive, their contribution increases with an increasing area of contact. Bonding by van der Waals forces has not been proved or disproved (Koskinen and Harper 1990) because scarce experimental evidence is available. However, Barriuso et al. (1994) suggested that atrazine is primarly retained on surfaces of smectites with low surface charge density through relatively weak van der Waals forces or H bonds. This mechanism was also proposed as contributing to sorption of imazethapyr (Senesi et al. 1997) and fluridone (Weber et al. 1986).

H-Bonding. H-bonding is an intra- or intermolecular dipole–dipole interaction that is stronger than van der Waals bonds. The energy of this binding amounts to about 2–40 kJ mole^{-1}. It is caused by the electron-withdrawing properties of an electronegative atom (F, N, O) on the electropositive hydrogen nucleus of functional groups such as —OH and —NH. The presence of numerous oxygen and hydroxyl-containing functional groups on HS renders the formation of H-bonding highly probable for pesticides containing suitable complementary groups, although a strong competition with water molecules may be expected for such sites (Senesi 1992). Martin-Neto et al. (1994) applied UV-visible, FT-IR, and ESR spectroscopy to HA samples reacted with atrazine and found evidence for weak adsorption involving H-bonding. Moreover, Welhouse and Bleam (1993b) observed the formation of weak to moderately strong complexes between atrazine and amine, and hydroxyl and carbonyl functional groups. The strong complexation observed with carboxylic acid and amide functional groups was the result of cooperative interactions (multifunctional H-bonds) in which both partners in the complex donate and accept hydrogen bonds (Welhouse and Bleam 1993b). H-bonding has also been proposed as a binding mechanism for primisulfuron (Ukrainczyk and Ajwa 1996), metribuzin (Ladlie et al. 1976c), and 2,4-D and triclopyr (Johnson et al. 1995) on soils. It has been implicated in the adsorption of fluazifop-butyl on homoionic clays (Gessa et al. 1987), atrazine on smectite (Barriuso et al. 1994), and atrazine (Senesi et al. 1995; Piccolo et al. 1998), imazethapyr (Senesi et al. 1997), ethametsulfuron-methyl (Si et al. 2006), and imazaquin (Ferreira et al. 2002) on soil OM.

Ionic Exchange. Ionic exchange is a nonspecific electrostatic interaction (>20 kcal mole^{-1}) that can involve either anionic or cationic pesticide forms.

Anion exchange is the attraction of an anion to a positively charged site on the soil surface and involves the exchange of one anion for another at the binding site. Adsorption of organic anions by soils via anion exchange is not likely in temperate climates as clays and organic matter are generally either noncharged or negatively charged. Moreover, direct sorption involving the few positive charges at the edge of sheets in clays or protonated amine groups within the organic matter is an insignificant mechanism for weak acids (Stevenson 1972). Anion exchange is more likely to occur in tropical soils that contain significant quantities of positively charged adsorption surfaces in the form of aluminium and iron (hydr)oxides. For instance, pentachlorophenol was readily desorbed on addition of phosphate with no apparent hysteresis, suggesting that pentachlorophenol sorption on variably charged soils is primarily through nonspecific ion-exchange reactions (Hyun et al. 2003). Hyun and Lee (2004) demonstrated that anion exchange of prosulfuron accounted for up to 82% of overall sorption in the pH range 3–7, and that its relative importance was positively correlated to the ratio of anion and cation exchange capacities of the 10 variably charged

soils studied. Similarly, anion exchange was implicated in the adsorption of the dissociated form of chlorsulfuron (Shea 1986), 2,4-D (Celis et al. 1999; Watson et al. 1973), mecoprop and bentazone (Clausen and Fabricius 2001), and clofenset, salicylic acid, and 2,4-D (Dubus et al. 2001). However, Ukrainczyk and Ajwa (1996) did not observe any correlation between the anionic-exchange capacity (AEC) of minerals and primisulfuron adsorption and concluded that anion exchange is not an important mechanism for primisulfuron sorption on mineral surfaces. Because anion exchange is affected by the presence of other anions, Hyun et al. (2003) suggest that sorption of acidic pesticides could be better predicted by considering the electrolyte composition.

Cation exchange is relevant to those pesticides that are in the cationic form in solution or can accept a proton and become cationic (e.g., basic compounds at $pH < pK_a$). For these pesticides, it is among the most prevalent sorption mechanism due to the large proportion of negatively charged sites associated with clay and organic matter in soils (Harper 1994). For instance, there is abundant evidence for cation exchange involving triazines (Herwig et al. 2001; Ladlie et al. 1976a; Piccolo et al. 1998; Roy et al. 2000), even though their pK_a ($1.7 < pK_a < 4.3$) is lower than the pH of most common soils. Cation exchange can occur at negatively charged sites on clay mineral surfaces occupied by a metal cation. According to Sannimo et al. (1999), simazine arrived at a montmorillonite interface mostly as the molecular species, where the compound was protonated by the microenvironmental pH (lower than the bulk solution pH), and eventually adsorbed by cation exchange. Cation exchange can also occur between the protonated triazines or the positively charged bipyridylium compounds (e.g., diquat or paraquat) and the negatively charged sites of HS (carboxylate, phenolate groups) (Senesi et al. 1995). However, not all negative sites on OM seem to be positionally available to bind large organic cations, probably because of steric hindrance. For instance, the higher reactivity of simazine relative to atrazine and prometryn may be related to the smaller steric hindrance of the reactive N–H group of the former herbicide (Senesi 1992).

Charge Transfer. The presence in humic substances of both electron-deficient structures, such as quinones, and electron-rich moieties, such as diphenols, suggests the possible formation of charge-transfer complexes via electron donor-acceptor mechanisms (π–π reaction). Pesticides can act as electron donors (amine and/or heterocyclic nitrogen atoms of the s-triazines, pyridines, imidazolinones) or electron acceptors (e.g., deactivated bypyridilium ring of atrazine) (Senesi 1992). Charge transfer involves the overlapping of the respective molecular orbitals and a partial exchange of electron density (Von Oepen et al. 1991).

The interaction between atrazine and soil OM has been widely studied, but the mechanisms are still a topic of considerable controversy. Martin-Neto et al. (1994) concluded, in agreement with theoretical studies by

Welhouse and Bleam (1993a,b), that the electron-donating capability of atrazine was usually not sufficient to allow an electron-transfer complexation with HA. In contrast, the results of Piccolo et al. (1992) indicate that atrazine is mainly adsorbed through a charge-transfer mechanism. FT-IR and ESR spectroscopic results suggested charge-transfer bonds between the electron-donor triazine ring or the electron-acceptor deactivated bipyridylium ring and complementary electron-donor or -acceptor structural moieties of HA (Senesi et al. 1995). Nevertheless, Martin-Neto et al. (2001) confirmed their previous results indicating that atrazine does not readily undergo electron-transfer reactions with humic substances. However, they demonstrated that hydroxyatrazine reacts through an electron-transfer mechanism with HA and FA.

This behaviour is similar to other *s*-triazine herbicides, such as prometon, which has a significant basicity ($pK_a = 4.28$) that renders it highly effective in engaging electron-transfer mechanisms to complex HA (Senesi and Testini 1982). Atrazine readily converts to hydroxyatrazine, even in laboratory samples at low water content, and this may explain some of the electron-transfer product detected in studies of atrazine–HA interactions (Celis et al. 1997a). Senesi et al. (1997) suggest a charge transfer between the electron-donating pyridine ring and/or imidazolinone ring of imazethapyr and the electron-acceptor structural units of HA (e.g., the quinone groups). In contrast, Ferreira et al. (2002) observed no change in the semiquinone-type free radical contents between HA and HA–imazaquin complexes; this indicated that imazaquin did not undergo charge-transfer reactions with HA.

Although charge transfer seems to be most likely for sorption to humic acids (Pignatello 1989), some authors also infer this mechanism for interactions between acidic pesticides and clays. Indeed, the polarizing power of a cation determines the degree of acidity of the coordinated water molecules and therefore the tendency to protonate an organic molecule according to the strength of its basic character. Fusi et al. (1988) have shown that fluazifop-butyl could apparently adsorb to Al- and Fe-homoionic clays by protonation of the pyridine nitrogen, but this was not the case with other exchangeable cations. Similar results were obtained for fluazifop-butyl (Gessa et al. 1987) and azimsulfuron (Pinna et al. 2004).

Ligand Exchange. Adsorption by a ligand-exchange mechanism involves the replacement, by suitable adsorbent molecules such as *s*-triazines and anionic pesticides, of hydration water or other weak ligands that partially hold polyvalent cations associated to soil OM or hydrous oxide surface (Senesi 1992). Ainsworth et al. (1993) proposed a two-step reaction: the first reaction represents the rapid formation of an ion-pair complex on the protonated surface site (outer-sphere complex; 4–16 kJ mol^{-1}); the second reaction, much slower and thus rate limiting, involves the breaking and forming of bonds and results in the formation of an inner-sphere complex (>20 kJ mol^{-1}) that may be bidentate or binuclear. A study involving several dif-

ferent iron oxides suggested that mecoprop adsorbs by outer- and inner-sphere complexes, whereas 2,4-D and bentazone are only weakly adsorbed through outer-sphere complexes (Clausen et al. 2001).

The ligand-exchange mechanism is implicated in the retention of many organic acids to oxide surfaces: an organic functional group, such as carboxylate or hydroxyl, displaces a surface coordinated —OH or water molecule of a metal ion (Fe, Al) at the surface of a soil mineral. For instance, sorption of clofenset and salicylic acid onto oxide surfaces is achieved predominantly through ligand exchange (Dubus et al. 2001). Moreover, salicylic acid and clofenset have both —COOH and —OH groups close to one another, making possible the formation of surface bidendate complexes with metals. The chemical structure of 2,4-D does not seem to allow the formation of these complexes with metals (Dubus et al. 2001). IR spectra of azimsulfuron sorbed to iron oxide indicate a Fe^{3+} coordination to the azimsulfuron sulfonylurea group acting as a bidentate bridging ligand through sulfonyl and carbonyl oxygen atoms (Pinna et al. 2004). This binding mode, giving rise to a six-membered chelated ring, explains the unusual IR spectra of the azimsulfron–iron oxide complex. Similarly, Nicholls and Evans (1991b) explained the difference in sorption between the two weak bases methyl-nicotinate and methyl-picolinate by the capacity of the latter to form a weak bidentate ligand to an acceptor atom.

Ligand exchange has also been proposed as a mechanism of retention for zwitterionic compounds such as imazaquin on highly weathered tropical soils (Regitano et al. 2000) and glyphosate on goethite (Sheals et al. 2002). While the phosphonate group of glyphosate binds directly to goethite by formation of inner-sphere complexes, predominantly as a monodentate complex, the carboxylate group remains relatively free from complexation, leaving it subject to degradation and/or complexation with metal ions present in the environment (Sheals et al. 2002).

Cation (or Water) Bridging. Cation bridging arises from the formation of an inner-sphere complex between an exchangeable cation, at a clay or OM surface, and an anionic or polar functional group on a pesticide. Because cations are normally surrounded by hydrating water molecules, the organic functional group must be able to either displace the water or it must react in the presence of a dry surface to form an inner-sphere complex. Water bridging occurs when the organic functional group is unable to displace the solvating water molecule: it is an outer-sphere interaction between a proton in a hydrating water molecule of an exchangeable cation and an organic functional group (Koskinen and Harper 1990). Water molecules participate in H-bonding if they are involved in bonds between organic molecules and cations.

Water bridging is more likely to occur with the larger, higher-valency cations such as Fe^{3+}, Al^{3+}, and Mg^{2+} because they have large negative enthalpy of hydration so that water molecules are more difficult to displace

(Harper 1994). A measurable adsorption of mecoprop and 2,4-D on kaolinite, which exhibits a negative surface charge, was only found when $CaCl_2$ was added as an electrolyte; this probably resulted from the formation of Ca–pesticide–surface complexes (Clausen et al. 2001). Complexation with surface-exchanged multivalent cations has been suggested as a possible sorption mechanism for glyphosate, clofenset, and salicylic acid onto oxide surfaces (de Jonge and de Jonge 1999; Dubus et al. 2001; McDonnell and Hossner 1985, 1989; Sheals et al. 2002). Fusi et al. (1988) concluded that fluazifop-butyl and fluazifop are adsorbed to homoionic smectites through both a water bridge and a direct coordination between their C═O group and an exchangeable cation. The extent and strength of this coordination depended on the nature of the cation that saturated the clays.

Bound Residues. For most pesticides, it is often assumed that a rapid and reversible equilibrium is established between the chemical in solution and the chemical adsorbed onto the soil surface. However, once adsorbed, many organic chemicals react further to become covalently and irreversibly bound while others may become physically trapped in the soil matrix (Koskinen and Harper 1990). These mechanisms lead to stable, mostly irreversible incorporation of the molecule, mainly into humic substances (Harper 1984; Scribner et al. 1992; Senesi 1992).

Bound residues are common for pesticides and for their intermediates and degradation products (Koskinen and Harper 1990). For instance, 9 yr after application of atrazine to a soil under field condition, the soil contained about 50% ^{14}C residues in the bound (nonextractable) form, distributed among the various soil humic fractions (Capriel et al. 1985). Trapping of molecules by humic materials acting as a molecular sieve form has been hypothesized as a retention mechanism for prometryn (Khan 1982) and simazine (Scribner et al. 1992). Moreover, X-ray diffraction has shown that prometon (Weber et al. 1965), fluridone (Weber et al. 1986), and fluazifop (Fusi et al. 1988; Gessa et al. 1987) can penetrate into interlamellar spaces of smectites.

The proportion and distribution of bound residues depends on properties of the herbicide and the soil (Barriuso et al. 1997; Hang et al. 2003; Weber et al. 1993; Yutai et al. 1999). Von Oepen et al. (1991) showed that the higher the lipophilicity of a substance, the lower its tendency to form nonextractable residues. This mechanism occurs because polar compounds, those that contain OH^- or NH_2 groups, similar to those in humic substances, are more easily incorporated into humic substances. Thus, the formation of nonextractable residues may require particular attention when assessing the behaviour and mobility of polar compounds in soil.

Conclusion. Soil constituents have a complex chemistry, and a multitude of functional groups have the potential to react with polar organic xenobiotics. Many retention mechanisms have been postulated to be responsible

for the adsorption of ionisable pesticides in soils, even if relatively little experimental evidence is available. The relative importance of one mechanism over another depends on the soil constituents, the molecule, and the chemical environment of the soil (Table 3), and several mechanisms are often found to be operating in combination. Nearly 15 years after the original assertion of Von Oepen et al. (1991), we are still unable to determine the quantitative contribution of each sorption mechanism in a particular situation.

Johnson et al. (1995) observed that the amount of 2,4-D and triclopyr desorbed increased with initial concentration, suggesting that specific binding sites became saturated at higher concentrations and that weaker sites were then responsible for retaining excess herbicide. Moreover, the capacity to form specific bonds, for instance, the formation of a bidentate complex with metal by the ligand-exchange mechanism, depends on the molecular structure of the pesticide and might explain the different sorption behaviour of some compounds having similar pK_a (Dubus et al. 2001).

Phosphate is applied as a fertilizer to agricultural soils and adsorbs mainly by ligand exchange. Several recent articles reported a likely reduction in the adsorption of some ionisable pesticides with increasing phosphate application (de Jonge and de Jonge 1999; de Jonge et al. 2001; Regitano et al. 1997). This phenomenon depends on the adsorbent (Gimsing and Borggaard 2002) and seems more likely on mineral surface sites such as Fe and Al (hydr)oxides (Nearpass 1976).

Our understanding of soil constituent chemistry, particularly that of humic substances and their modes of interaction with pesticides, deserves further research with a more extended application of advanced techniques such as NMR, ESR, FT-IR, and fluorescence spectroscopies. Finally, the formation of bound residues seems more likely for polar than for neutral compounds and also needs to be taken into account.

D. Prediction of the Adsorption Behaviour of Ionisable Pesticides in Soils

Influence of Soil pH. Soil pH has been shown to influence the sorption of many ionisable pesticides. Several strategies can be followed to obtain a range of pH and study its influence on pesticide behaviour, but each has some disadvantages.

Artificial Modification. Experiments in which the pH of a soil is adjusted artificially are useful with respect to experimental design and control. In some experiments, only the pH of the soil suspension is modified before the K_d measurement (Barriusso et al. 1992; Weber et al. 1986; Berglöf et al. 2002). In others, the pH of the soil has been modified and equilibrates for a long period (de Jonge et al. 2001; Loux and Reese 1992), or soil samples taken from different depths or with different pretreatment histories (tillage, crop) are compared (Barriusso et al. 1992; Harper 1988; Reddy et al. 1995;

Table 3. Potential Mechanisms for the Adsorption of Ionisable Compounds and How These are Influenced by Properties of the Compound and the Soil.

	pH dependent?	Type	Energy
Hydrophobic partition	To some extent	Partitioning	Low
van der Waals	No	Short-range induced dipolar attractions	2–4 kJ mol^{-1} (Koskinen and Harper 1990)
H-bonding	To some extent	Dipole–dipole interaction	2–110 kJ mol^{-1} (Haberhauer et al. 2001; Koskinen and Harper, 1990)
Anion exchange	Yes	Nonspecific electrostatic interaction	>80 kJ mol^{-1}
Cation exchange	Yes	Nonspecific electrostatic interaction	>80 kJ mol^{-1}
Charge transfer	Yes	Electron donor-acceptor mechanisms (π–π reaction)	12 kJ mol^{-1} (Haberhauer et al. 2001)
Ligand exchange	Yes	Inner-sphere complex, may be multidentate or multinuclear	>20 kJ mol^{-1}
Water bridging	Yes	Outer-sphere complex	4–16 kJ mol^{-1}
Cation bridging	Yes	Inner-sphere complex	150–330 kJ mol^{-1} on clays 140 kJ mol^{-1} on OM (Haberhauer et al. 2001)

OM, organic matter.

Table 3. *Continued*

Compound		Soil	
Positive influence	Negative influence	Positive influence	Negative influence
Hydrophobicity (high K_{ow})		High OM (with high carbon content) and/or clay content, low pH: creation of water protected site at pH < 5 by (Ferreira et al. 2001)	low pH: OM dissociation
		OM and clay content	
Nonionic but polar molecule capable of cooperative (or multifunctional) interactions		OM and clay content	Competition with water molecules
Anion low pK_a	Steric hindrance	Aluminium and iron (oxi)hydroxides	OM (coating), ionic strength (competition)
Cation high pK_a	Steric hindrance	High OM (functional acidity) and/or clay content	Ionic strength (competition)
Basicity of compound (ability to give electrons$^-$) or acidity (ability to accept electrons$^-$)	Very low or high pK_a	OM: capacity to give or accept electron (aromaticity) Clay: type of exchangeable cation (different acidity of water molecule surrounding)	
Chemical structure allowing the formation of multidentate/nuclear complexes.		High aluminium and iron (oxi)hydroxides and/or clay content (but less hydroxyl group at the edges than oxides)	OM (coating the oxides)
Anionic or polar functional group		Large, high-valency exchangeable cations such as Fe^{3+}, Al^{3+}, and Mg^{2+}	
Anionic or polar functional group able to displace the water surrounding the cation		Small, low-valency exchangeable cations	

Walker et al. 1989). However, such experiments have been deemed unsatisfactory because changes in soil characteristics other than pH can occur during pH adjustments. For instance, liming causes an increase in concentrations of amorphous aluminium and iron (hydr)oxides and a reduction in concentrations of Olsen-P (de Jonge et al. 2001). These factors might have opposing effects on the sorption or degradation characteristics of the pesticide, and this may obstruct interpretation of the results (Koskinen and Harper 1990; Singh et al. 1989; Walker and Thomson 1977).

Range of Native pH. The comparison of soils representing a range of native pH is expected to provide more realistic information on the behaviour of a compound but also gives results with multiple, often conflicting influences. Furthermore, relationships between sorption and pH that have been demonstrated in a soil adjusted to different pH level are often not confirmed by regression analyses involving different soils. For instance, Weber et al. (1986) did not observe a significant correlation between pH and the adsorption of fluridone in 18 soils studied at their native pH (4.4–8.1; $r^2 = 0.10$). However, fluridone adsorption increased by between 38% and 42% when solution pH was decreased artificially from 6.4 to 3.5. Fontaine et al. (1991) obtained similar results with fluridone, which can partly be attributed to the generally narrower range and higher level of native pH values compared to those considered in adjusted soils. Moreover, surface acidity (exchangeable acidity) is probably the real operative, and it may not be appropriate to compare apparent acidity (pH of a soil suspension) for a wide range of soil types (see section on pH measurements).

The determination of the effect of a single soil variable on sorption is always difficult because soil properties are often correlated with each other. Nevertheless, experiments dealing with a natural pH range or soil equilibrated for a long period are preferred because they are more prone to give realistic results.

Theory. The effect of pH on the adsorption of ionisable pesticides has been investigated in many studies and depends on soil composition and the characteristics of the compound. The pH dependence of sorption derives mainly from the different proportions of ionic and neutral forms of the pesticide present at each pH level and from differences in their strength of sorption. As described above, these effects are already relevant at pH above the pK_a. Studies into the effects of pH on adsorption are complicated by the influence of varying pH on the electrostatic charge of soil colloids (OM and (hydr)oxides). Indeed, as organic colloids have strongly pH-dependent charge, the solution pH also governs the degree of ionisation of humic acid groups. At neutral pH, the phenolic and alcoholic groups with pK_a of about 8 are assumed to be nonionised, whereas uncharged and ionized forms of carboxylic groups with pK_a of about 5.2 are assumed to coexist (Moreau-Kervevan and Mouvet 1998; Stevenson 1972).

The dissociation constant descibes the sensitivity of ionisable pesticides to soil pH (Table 4), and four types of pH influence have been recorded (Fig. 2). These are discussed in turn next.

Curve A. The most common case when sorption is negatively related to pH is represented by curve A. A greater change in sorption coefficient is generally observed at lower pH (because pK_a are generally low). Weak acids (e.g., carboxylic acids, sulfonylureas, phenols) exist predominantly in the anionic form at pH values greater than their pK_a. With decreasing pH, the proportion of the protonated fraction increases. This neutral form is much more strongly sorbed in soils than the anion for several reasons.

Some are direct consequences of the molecular dissociation:

i. The neutral molecule does not undergo repulsion by the negatively charged surfaces of soil particles.
ii. The hydrophobicity of the neutral form is greater than that of the ionic form (Hyun et al. 2003; Lee et al. 1990; Ukrainczyk and Ajwa 1996). For instance, Hyun et al. (2003) showed that hydrophobic sorption of neutral PCP is two orders of magnitude greater than that of the anion.
iii. The solubility in water of the anionic form is greater than that of the neutral form. For instance, Mersie and Foy (1985) showed that solubility of chlorsulfuron is higher at pH 7 than in acidic solutions. However, this should not have a significant effect in the field as pesticide concentrations in soil solution rarely approach the solubility limit (Nicholls 1988), except perhaps immediately after application.

Others are consequences of pH-dependent characteristics of the soil:

iv. In variable-charge soils (mainly tropical and subtropical soils with significant quantities of iron and aluminium (hydr)oxides), the anionic-exchange capacity increases at lower pH values (or the surface charge becomes more positive as pH decreases). Thus, while pH decreases, sorption of the anion increases by ionic interactions (Hyun et al. 2003).
v. Conformational changes due to OM dissociation could further account for the low adsorption under alkaline pH (Martin-Neto et al. 2001; Spadotto and Hornsby 2003). Indeed, some molecular environments, including protected sites of significant hydrophobicity, could disappear at high pH because of conformational changes induced by acidic functional group deprotonation (Ferreira et al. 2001; Martin-Neto et al. 2001).
vi. With increasing pH, more hydroxyl ions are present to outcompete other anions for any remaining positively charged sites (Hyun et al. 2003).

A decrease of adsorption with increasing pH is also observed with some basic pesticides. This time, the explanation lies simply in the effect of pH on protonation of the molecule. Weak bases (e.g., triazines) are mainly

Table 4. Changes with pH in Form, Behaviour, and Adsorption Mechanism Operating for Acidic and Basic Compounds.

pH →			
Acidic compounds:			
pK_a	$pK_a > 10$	$3 < pK_a < 10$	$pK_a < 3$
Dominant form	AH	Ratio A^-/AH	A^-
General behaviour	Like neutral compounds, except under extremely alkaline conditions	Mobility, solubility, and volatility sensitive to pH	Highly mobile in soils unless chemical complexes are formed
			Temperate soils
Adsorptionmechanisms	hydrophobic interactions (OM, clay) van der Waals (OM, clay) H-bonding (OM, clay)		Anion repulsion by negatively charged adsorbents Cation (or water) bridging (OM, clay) H-bonding Charge transfer (OM) van der Waals (OM)
			Soil with pH-dependent charge
			Anion exchange (Al, Fe (hydr)oxides) Ligand exchange (protonated (oxi)hydroxides, OM) Cation bridging (through ligand exchange: H_2O–metal)
Basic compounds:			
pK_a	$pK_a > 10$ ($pK_b < 4$)	$3 < pK_a < 10$ ($4 < pK_b < 11$)	$pK_a < 3$ ($pK_b > 11$)
Dominant form	BH^+ or B^+	ratio BH^+/B or B^+/B(OH)	B or B(OH)
Generalbehaviour	Extreme soil sorption and generally slow degradation	Sensitive to pH: extreme sorption of cation produces an apparent soil surface acidity effect	Like neutral compounds, except at extremely acid conditions
Adsorptionmechanisms	Cation exchange (OM, clay) Charge transfert (OM)		Hydrophobic partitioning (OM, clay) van der Waals (OM, clay) H-bonding (OM, clay) Ligand exchange (OM) Charge transfer: pesticide e-donor (OM)

AH and A^-, BH^+ and B, are the protonated and dissociated form of weakly acidic or basic pesticides, respectively.
Adapted from Wauchope et al. (2002).

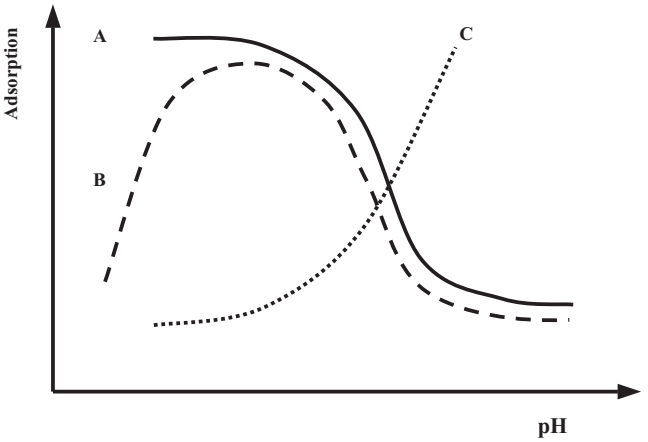


Fig. 2. Three adsorption behaviours have often been recorded for ionisable compounds as a function of soil pH. A pH-independent sorption can also be observed in some cases. Reproduced from Calvet 1989 with permission from Environmental Health Perspectives.

present as neutral molecules under alkaline conditions and as cations at pH values below their pK_a. The cationic form is much more strongly retained than the dissociated form because of attraction by the negatively charged soils particles (cation exchange). Also, a likely solubilization of dissolved organic matter (DOM) at high pH levels that can complex with the neutral form could lead to a reduction in the measured sorption coefficient making the K_d observed at high pH lower still (Ben Hur et al. 2003; Celis et al. 1998a; de Jonge and de Jonge 1999).

Curve B. This type of curve is generally observed with weak bases. Adsorption increases with decreasing pH until a maximum is achieved and decreases thereafter. The pH corresponding to the adsorption maximum is sometimes close to the pK_a of the molecule, but it is not a general rule (Calvet et al. 1980a). The decrease in sorption at more acidic pH is generally attributed to:

Competition for anionic adsorption sites between the cationic form and other cations (H^+ and Al^{3+}) present in the solution (postulated for atrazine by Martin-Neto et al. 2001).

Increase in the cationic form that reduces the hydrophobic interaction between the pesticide and humic acid (also postulated for atrazine by Martin-Neto et al. 2001).

Ionization of acidic functional groups on OM that influences the nature of the adsorption mechanisms and could reduce the relative importance of hydrogen bonding (proposed for triazines by Moreau-Kervevan and Mouvet 1998; Wang et al. 1992).

Decrease in the concentration of anionic forms when the adsorption of an acidic compound is studied on some oxides where the surface is positively charged (Watson et al. 1973).

A bell-shaped curve has been observed in experiments in which the pH was modified artificially for terbutryn (Barrriuso and Calvet 1992; Barriuso et al. 1992), atrazine on humic substances (Martin-Neto et al. 2001), several dibasic carboxylic acids (Nicholls and Evans 1991a), and 2,4-D on goethite (Watson et al. 1973). It was also observed for a weak acid (salicylic acid) studied in soils with a range of natural pHs (Dubus et al. 2001).

Curve C. The last curve corresponds to an increase in adsorption with increasing pH. The behaviour may occur for some weak bases that are mainly adsorbed as neutral molecules (hydrophobic effect), and this has been observed for simazine and atrazine on active charcoal (Yamane and Green 1972). The behaviour can also result for molecules that are bonded by complexation with a metallic cation as for terbutryn on Al-montmorillonite (Calvet 1989) or carbendazim in a Vietnamese soil (Berglöf et al. 2002). Fruhstorfer et al. (1993) also observed a higher adsorption of atrazine on montmorillonite at pH 9.5 than at pH 4.5. The only explanation lies in the fact that cation-exchange capacity is usually saturated by hydrogen ions in solution at pH < 8, but remains unsaturated in alkaline solution.

de Jonge et al. (2001) observed a significant positive correlation between pH and adsorption of glyphosate ($p < 0.001$). The soils were from long-term field experiments that received different additions of phosphorus and lime over at least 60 yr. The authors explained this behaviour by two liming responses (increase of Al and Fe oxides and reduction of Olsen-P concentrations) that counteract the effect of molecular charge on the strength of sorption of glyphosate.

It is unlikely that curve C will be observed in soils as the protonated form of ionisable compounds always has a larger propensity for sorption than the dissociated form.

No pH Influence. In some cases, no influence of pH on sorption is found. Different strategies can be applied to obtain a pH range and their consequences on the other soil properties may complicate the interpretation of the results in some cases. The pH range studied may sometimes be too narrow or too wide to underscore any influence of pH. Finally, the difference between the pH at the surface of soil particles and in the soil solution might also differ according to the measurement technique used and the characteristics of the soil.

Observations. Many articles report results concerning the influence of pH on the adsorption of ionisable pesticides in soils. However, differences in the experimental methods used (e.g., ionic strength, soil to solution ratio,

method to measure and modify pH, use of the formulation or technical grade) and in the range of soils considered sometimes make their interpretation and comparison difficult. Theoretical behaviour is sometimes observed, but conflicting results are also obtained. To highlight any specific behaviour that might be related to the chemical structure of the pesticide, references identifying relationships between sorption and pH are listed below, sorted by pesticide "families" (according to their ionisable functional group). The main correlations obtained between adsorption coefficients and soil properties (pH, OM, and clay contents) are summarised in Table 5.

Weak Acids: Carboxylic Acids. The herbicide 2,4-D has often been taken as an example for the study of acidic pesticides in soils. Barriuso and Calvet (1992) studied its adsorption on 58 soils. The results of a principal-component analysis indicated a strong inverse correlation between K_d and soil pH. In the same study, the pH of three ferrasols was artificially increased and the authors observed a decrease in the K_{oc} value, confirming the importance of pH for the adsorption of 2,4-D. Similarly, the K_d value of 2,4-D decreased when the pH of some oxisols was increased from 3.5 to 7 (Barriuso et al. 1992). In this latter study, 2,4-D adsorption seemed to be dependent on pH and mineral type but independent of the OM content, whereas terbutryn adsorption was pH- and OM dependent (Barriuso et al. 1992). Johnson et al. (1995) also observed that sorption of 2,4-D was lower in slurries adjusted to pH 7 than to pH 5. In the same study, an inverse relationship between sorption and native pH of four soils was also obtained (pH between 4.2 and 5.9). However, Dubus et al. (2001) could not find any clear relationship between the adsorption of 2,4-D and the pH of 10 cambisols and eight ferralsols (pH from 4.6 to 8.3). Sorption of clofenset (pK_a = 2.8) and salicylic acid (pK_a = 2.8) (two other carboxylic acids) decreased exponentially with increasing solution pH in the 10 cambisols whereas a bell-shaped curve was obtained for the sorption of salicylic acid in the ferrasols studied (Dubus et al. 2001).

Carrizosa et al. (2001) studied adsorption of dicamba on organoclays and found that pH had a negative effect on sorption, especially at high pesticide concentration. Greatest sorption of dichlorprop (pK_a = 3) and MCPA (pK_a = 3.7) was observed in the soil with highest organic carbon content and lowest pH (Thorstensen et al. 2001). Finally, increasing the pH (2–10) caused a fivefold decrease in the adsorption of fluroxypyr (Gao et al. 1998).

Phenols. Hyun et al. (2003) studied adsorption of pentachlorophenol (pK_a = 4.71) in several variable-charge soils. Sorption decreased with increasing pH as the fraction of pentachlorophenolate (anionic form) increased and anion-exchange capacity decreased.

The Aminosulfonyl ($NHSO_2$): Sulfonylureas. Sorption of the weakly acidic sulfonylurea herbicides generally increases with decreasing pH, as was

Table 5. Details of Experiments Investigating Correlation Between the Adsorption Coefficient of Ionisable Pesticides and Selected Soil Properties: OM, Clay, and pH.

Acidic pesticides (pK_a)	Adsorbent	Native pH	pH range	Presence of correlation and r^2 value[a]			References
				pH	OM	Clay	
Carboxylic acids	Database			(–) 0.16**	(+) 0.006	(+) 0.01	Weber et al. 2004
Salicylic acid (2.8)	10 cambisols	x[b]	6.2–8.3	(–) 0.76 Exponential fitting ($r^2 = 0.97$)	(+)(–)	nc	Dubus et al. 2001
	8 ferralsols	x	4.6–7.2	Bell-shaped curve	(+)(–)	nc	Dubus et al. 2001
Clofencet (2.83)	10 cambisols	x	6.2–8.3	(–) 0.72 Exponential fitting ($r^2 = 0.98$)	(+)(–)	nc	Dubus et al. 2001
	8 ferralsols	x	4.6–7.2	(–)	(+)(–)	nc	Dubus et al. 2001
Haloxifop (2.9)	15 soils, sediments	x	4.2–8.3	(–)	nc	nc	Rick et al. 1987
2.4-D (2.97)	2 oxisols	x	4.1–5.5	(–)	nc		Barriuso et al. 1992
	2 oxisols	0[c]	3.5–7	(–)	nc		Barriuso et al. 1992
	58 soils (9 soil types)	x	4.2–7.8	(–)	nc		Barriuso and Calvet 1992
	10 soils	x	5.3–6.1	nc	(+) 0.96	(+)	Bolan and Bascaran 1996
	10 cambisols	x	6.2–8.3	(–) 0.18	(+)(–)	nc	Dubus et al. 2001
	8 ferralsols	x	4.6–7.2	nc	(+)(–)	nc	Dubus et al. 2001
	4 soils	x	5–6.9	(–) 0.93 Regression with pH, OM, clay	(+)		Johnson et al. 1995
	5 soils	x	5.1–7.3	nc	(+) 0.85	nc	Picton and Farenhorst 2004
Fluazifop (3.2)	15 soils, sediments	x	4.2–8.3	(–)	nc	nc	Rick et al. 1987

Table 5. *Continued*

Acidic pesticides (pK_a)	Adsorbent	Native pH	pH range	Presence of correlation and r^2 value[a]			References
				pH	OM	Clay	
$NHSO_2$ acids	Database			(–) 0.16**	(+) 0.15**	(+) 0.0025	Weber et al. 2004
Sulfonylureas							
Metsulfuron-methyl (3.3)	24 soils	x	3.9–7.9	(–) 0.62***	(+) 0.88**	nc	Walker et al. 1989
Primisulfuron (3.47)	23 soils	x	4.6–8	(–) 0.56*** Exponential fitting ($r^2 = 0.85$)	(+) 0.05	(+) 0.43***	Ukrainczyk and Ajwa 1996
	6 Colorado soils	x	5.6–7.8	(–) 0.31	(+) 0.81*	(+) 0.82*	Vicari et al. 1996
Chlorsulfuron (3.6)	1 soil	2wk.[d]	4.2–7.8	(–) (*)	Phytotoxicity; (+) 0.87**	nc	Mersie and Foy 1985
	24 soils	x	3.9–7.9	(–) 0.87***	(+) 0.74**	nc	Walker et al. 1989
Rimsulfuron (4)	6 Colorado soils	x	5.6–7.8	(–) 0.37	(+) 0.59	(+) 0.74*	Vicari et al. 1996
Nicosulfuron (4.3)	10 Iowa soils	x	6–8.2	(–) 0.12	(+) 0.75*	(+) 0.92***	Gonzalez and Ukrainczyk 1996
	4 tropical soils	x	4.6–5.2	(–) 0.88*	(+) 0.94*	(–) 0.27	Gonzalez and Ukrainczyk 1996
Sulfometuron (5.2)	5 Alabama soils	3mon.	5.1–6.7	(–) ***	nc	nc	Wehtje et al. 1987
Other $NHSO_2$							
Flumetsulam (4.6)	14 surface soils	x	4.6–7.9	(–) 0.09	(+) 0.49**	(–) 0.001	Strebe and Talbert 2001
	14 subsurface soils	x	4.6–7.9	(–) 0.15	(+) 0.02	(+) 0.03	Strebe and Talbert 2001

Table 5. *Continued*

Acidic pesticides (pK_a)	Adsorbent	Native pH	pH range	Presence of correlation and r^2 value[a]			References
				pH	OM	Clay	
Other acids							
Mesotrione (3.12)	15 soils	x	4.6–7.7	(–) 0.66	(+)		Dyson et al. 2002
	Database			(–) 0.18	(+) 0.27**	(+) 0.14**	Weber et al. 2004
Triazines							
Metribuzin (0.99)	Silty clay loam profile	x	6.5–7.2	(–) 0.86 Regression with clay, pH	(+) 0.01	(+) 0.75	Harper 1988
	9 soils	x	5.1–6.8	(–) 0.19	(+) 0.94**	(+) 0.45	Peter and Weber 1985
Atrazine (1.7)	58 soils (9 soil types)	x	4.2–7.8	nc	(+) 0.81		Barriuso and Calvet 1992
	241 samples (1 field)	x	5.5–7.3	(–) 0.76*** Regression with CO, pH, clay	(+)	(+)	Novak et al. 1997
Terbutryn (4.3)	2 oxisols	0	3.5–7	Bell-shaped curve (max. at pH 5)	(+) 0.90		Barriuso et al. 1992
	Oxisols	x	4.1–5.5	(–)	(+) 0.64		Barriuso et al. 1992
	58 soils (9 soil types)	x	4.2–7.8	(–) some correlations	(+) 0.36		Barriuso and Calvet 1992

Table 5. *Continued*

Acidic pesticides (pK_a)	Adsorbent	Native pH	pH range	Presence of correlation and r^2 value[a]			References
				pH	OM	Clay	
Others							
Fluridone (1.7)	18 soils	x	4.4–8.1	(–) 0.10	(+) 0.40** OM; (+) 0.69** OC	(+) 0.56** 0.72** with smectite	Weber et al. 1986
	2 soils	0	3.5–6.4	(–)			Weber et al. 1986
Carbendazim (4.2)	4 soils	x	2.9–5.4	(–) 0.35	(+) 0.99**	(+) 0.73*	Berglöf et al. 2002
	2 soils	0	3–7	(+)			Berglöf et al. 2002
Imidazolinones							
Imazaquin (2; 3.8)	10 soils	x	4.5–8.3	(–) <0.15	(+) 0.55 If two classes of pH 0.89 < r^2 < 0.99	(+) <0.03	Gennari et al., 1998
	22 soils	x	4.2–8.3	(–) 0.35**	(+) 0.20*	(+) 0.04	Loux et al. 1989a
	2 soils	10 yr.	4.5–6.7	(–) 0.98 Quadratic relation	(–)	—	Loux and Reese 1992
	9 tropical soils	x	3.9–5.9	(–) 0.38*	(+) 0.83**	nc	Regitano et al. 2000
	3 tropical soils	3 mon.	3–8	(–) 0.74**		nc	Rocha et al. 2002
Imazapyr (1.9; 3.6; 11)	10 soils	x	4.5–8.3	(–) 0.15	(+) 0.77 If two classes of pH 0.89 < r^2 < 0.99	(+) <0.03	Gennari et al. 1998
	5 Alabama soils	3 mon.	5.1–6.7	(–) ***	nc	nc	Wehtje et al. 1987

Table 5. *Continued*

Acidic pesticides (pK_a)	Adsorbent	Native pH	pH range	Presence of correlation and r^2 value[a]			References
				pH	OM	Clay	
Imazethapyr (2.1; 3.9)	10 soils	x	4.5–8.3	(–) <0.15	(+) 0.61 If two classes of pH 0.89 < r^2 < 0.99	(+) <0.03	Gennari et al. 1998
	4 Minesota soils	x	4.8–7.1	nc	nc	nc	Gan et al. 1994
	22 soils	x	4.2–8.3	(–) 0.13	(+) 0.02	(+) 0.27**	Loux et al. 1989a
Others							
Glyphosate (2.6; 5.6; 10.6)	2 soils	60 yr.	3.5–6.5	(+) 0.51***	nc	nc	de Jonge et al. 2001
Triclopyr (2.28; 3.35)	7 soils	x	4.4–7.7	(–) 0.37 Regression with CEC+pH (r^2 = 0.98)	(+) 0.87**	(+) 0.06	Pusino et al. 1994
	4 soils	x	5–6.9	(–) 0.96 Regression with pH and OM	(+)	nc	Johnson et al. 1995

[a](–) and (+) indicate a negative and positive correlation (r^2 value); *, **, and *** indicate a significance at $p < 0.05$, 0.01, and 0.001 levels, respectively; nc: no correlation was observed.

[b]Studies involving soils representing a range of native pH.

[c]pH was modified by adding the buffer agent into the batch soil suspension, just before K_d measurement.

[d]wk., mon., yr: the time the soil was left to equilibrate after addition of a buffer agent (in weeks, months, or years, respectively).

observed for soils with different native pH values (Gonzalez and Ukrainczyk 1996; Reddy et al. 1995; Shea 1986) and for soils adjusted to different pH levels before the sorption experiment (Mersie and Foy 1985; Shea 1986; Wehtje et al. 1987). In experiments by Walker et al. (1989), sorption of chlorsulfuron and metsulfuron-methyl was significantly negatively correlated with pH and positively correlated with the organic matter content of 23 soil samples from eight sites and three depths. Soil pH was found to be the most important variable controlling sorption. The relationship between sorption coefficients and pH was exponential; i.e., a stronger change in sorption occurred at lower pH. Ukrainczyk and Ajwa (1996) studied primisulfuron sorption on eight minerals and 23 soils and noted a great decrease of sorption with increasing pH on both adsorbents (significant negative correlation with pH with $r^2 = 0.55$). The same trend was observed for prosulfuron in 10 variable-charge soils (Hyun and Lee 2004). Vicary et al. (1996) observed maximum adsorption of rimsulfuron and primisulfuron on the soil that had the lowest pH (pH 5.6–7.8), but K_d and soil pH were not significantly correlated. Finally, Gonzalez and Ukrainczyk (1996) observed a strong negative correlation between the adsorption of nicosulfuron and the pH of 4 Brazilian soils, whereas no correlation was found for 10 Iowa soils. The explanation might lie in the lower range of pH represented by the Brazilian soils (4.6–5.2) compared to the Iowa soils (6.0–8.2).

Other Pesticides with a $NHSO_2$ Functional Group. In 21 soils with pH ranging from 5.9 to 7.9, Fontaine et al. (1991) observed no relationship between K_d values of the weak acid flumetsulam and pH. However, a nonlinear relationship between K_{oc} and pH was obtained with a marked decrease in K_{oc} values up to pH 6–6.5 and a lesser change thereafter. This result was attributed to a strong influence of organic matter on the sorption of the neutral form of flumetsulam, which is the dominant form at low pH values. Flumetsulam sorption decreased as pH increased in four soils in which the solution pH was adjusted to 1.3–7.1. An equation was proposed to calculate the net K_{oc} value as a function of K_{oc} for the neutral and anionic form, pH, and pK_a. Strebe and Talbert (2001) also studied the adsorption of flumetsulam in 14 soils. K_d and K_{oc} were correlated with OM in surface soils while K_d was correlated with extractable Fe and inversely correlated to pH in subsurface soils. The mobility of flumetsulam (TLC study) was negatively correlated with K_d values at both soil depths and with K_{oc} in the subsurface soils. However, multiple linear regressions suggested that no soil property was an adequate predictor for mobility. For bentazone (p$K_a = 3.3$), the highest Freundlich coefficient (K_f) values were in the soil with highest organic carbon content and lowest pH (Thorstensen et al. 2001).

Other Acidic Compounds. Mesotrione (p$K_a = 3.1$) adsorption was negatively related to pH and to a lesser extent to organic carbon content in a study carried out on 15 different soils (pH 4.6–7.7) (Dyson et al. 2002).

Overall, the results are consistent with soil pH having a major influence on the amount of mesotrione adsorbed, accounting for more than half the variation present in the data.

Weak Bases: Triazines, Triazinones. Gao et al. (1998) studied the adsorption of seven pesticides and metabolites on sediments with different physicochemical properties, and only the nonionisable pesticide was not greatly influenced by pH. The adsorption of desethylatrazine and atrazine decreased with increasing pH (2–10), while a bell-shaped curve was obtained for terbutylazine and anilazine. The K_d of terbutryn also presented a bell-shaped curve between pH 3.5 and 7 and reached a maximum between pH 4.5 and 5.5 on oxisols with artificially stabilized pH conditions (Barriuso et al. 1992). Decrease in adsorption of terbutryn with pH < 5 could be explained by its protonation ($pK_a = 4.3$) as the soil colloids become positively charged.

Novak et al. (1997) used multiple regression analyses with data from batch experiments carried out on 241 surface soil samples from a 6.25-ha field (pH 5.5–7.3). Results revealed that atrazine sorption was positively influenced by soil organic carbon content and negatively by pH and, to a lesser extent, soil clay content. A negative influence of pH on the adsorption of simazine was also demonstrated in soils adjusted to different pH by Singh et al. (1989). Metribuzin has a very low pK_a (0.99), and pH should therefore be less important to its sorption than to the binding of other basic compounds. Nevertheless, sorption of metribuzin increased as pH decreased in soils pretreated for 15 yr with ammonium sulfate or calcium nitrate to achieve different pH values (Ladlie et al. 1976a,b) and in soils allowed to equilibrate for 3 mon after addition of HCl or NaOH (Wehtje et al. 1987).

Other Basic Compounds. de Jonge and de Jonge (1999) observed that the pH rise (from 7.7 to 10.4) after addition of NH_4OH and NaP_2O_7 reduced the adsorption coefficient of prochloraz ($pK_a = 3.8$) by nearly 50%. As prochloraz is a neutral compound in this range of pH, the solution chemistry does not directly influence the sorption mechanism. The authors explained the observation by the solubilization of DOM at high pH levels, subsequently allowing formation complexes with prochloraz and leading to a reduction in the measured sorption coefficient. Similar behaviour has been reported for atrazine (Ben Hur et al. 2003; Celis et al. 1998a). Malik and Drennan (1990) observed that sorption of the weak base fluridone was inversely related to pH with a stronger decrease in sorption as pH increased from 2 to 5 than within the range from pH 5 to 9. Similarly, the highest Freundlich coefficient (K_f) values for propiconazole ($pK_a = 1.07$) were in the soil with highest organic carbon content and lowest pH (Thorstensen et al. 2001). The influence of pH on sorption of carbendazim ($pK_a = 4.2$) was studied on two soils that differed with respect to pH, clay, and OC. Sorption by the sandy soil (pH, 5.4; clay, 26.3%; OC, 0.3%) increased as the

pH decreased, while sorption on the second soil (pH, 2.9; clay, 49.8%; OC, 9.8%) decreased as pH decreased. One explanation may be that the solubility of carbendazim decreases with increased pH (Berglöf et al. 2002).

Zwitterionic Compounds: Imidazolinones. Besides these results for weak acids and bases, evidence of the influence of pH on sorption exists for other ionisable compounds. The imidazolinone herbicides imazaquin, imazapyr, and imazethapyr are amphoteric compounds with acidic and basic functional groups (Stougaard et al. 1990). Their sorption was found to increase with decreasing pH in the pH range 3–8, probably due to effects on ionisation of the different ionisable functional groups (Goetz et al. 1986; Renner et al. 1988; Stougaard et al. 1990; Wehtje et al. 1987). However, in common pH ranges of agricultural soils, ionisation of the acidic groups should have a greater effect on sorption because pK_a values are very low (e.g., 1.8 for imazaquin, 1.2 for imazethapyr). For instance, Loux and Reese (1992) found a considerable decrease in imazaquin sorption when pH increased from 4.5 to below 6, while sorption varied only slightly above pH 6. In the afore mentioned studies on imidazolinones, soil pH was adjusted to different levels, although soils were allowed to equilibrate in the field for at least 10 yr in the experiments carried out by Loux and Reese (1992).

In contrast, Loux et al. (1989a) studied imazaquin and imazethapyr sorption in 22 soils and six sediment samples with a range of native pH values from 4.2 to 8.3 and a considerable variation in other soil properties. Linear regression analyses revealed a positive correlation between imazaquin sorption and organic carbon content and a negative relationship with pH. Imazethapyr sorption was positively correlated to clay content and cation-exchange capacity (CEC). In multiple regressions involving linear and quadratic terms, pH was found to be an important variable determining sorption of both compounds, but its effect on imazaquin sorption was the more significant. The authors included a quadratic term to account for the greater effect of pH in the range 4–6 as compared to that above pH 6. Imazethapyr seems to be less sensitive to soil pH than imazaquin or imazapyr. Indeed, correlation coefficients are usually very low (Gennari et al. 1998; Loux et al. 1989a), and Gan et al. (1994) could not observe any clear relationship with soil pH.

For soils relatively rich in aluminium and iron (hydr)oxides, pH-dependent charges of the adsorbents were considered to have an additional effect on imazaquin sorption (Goetz et al. 1986). Sorption of imazaquin decreased as pH values were increased from 2 to 6 for both HA and oxisol suspensions (Ferreira et al. 2002). Rocha et al. (2002) also observed a negative correlation between imazaquin sorption and artificially modified pH (from 3 to 8; $r^2 = 0.55^{**}$) in highly weathered soils. Regitano et al. (1997) studied sorption of imazaquin on 18 soils (6 with pH-dependent charges) and observed an increase in K_{oc} with decreasing native pH (from 4.8 to 8). The K_{oc} values obtained with artificially reduced pH (to pH 3.1) show a very strong increase of adsorption at low pH level. Similarly, Regitano

et al. (2000) observed a low adsorption of imazaquin in 9 highly weathered tropical soils, with the exception being a soil with high organic carbon content and low soil-solution pH. In this article, the authors combined the results obtained in Loux et al. (1989a) and Regitano et al. (1997) and proposed a model that allowed a good prediction of imazaquin sorption in surface soils but not in subsurface samples.

Other Zwitterionic Compounds. Although Torstensson (1985) reported that sorption of glyphosate was not strongly dependent on soil pH, other studies have shown a strong dependence of sorption on pH. This effect was explained by the reduction in net charge of glyphosate as pH increases (McConnell and Hossner 1985; Nicholls and Evans 1991b) and possibly by the amount of dissolved organic matter (DOM) that went into solution at higher pH values (de Jonge and de Jonge 1999). Similarly, the relationship between triclopyr (amphoteric) sorption and the native pH of different soils was weak in the study of Pusino et al. (1994), perhaps as a result of the limited pH range. However, a combination of CEC and pH accounted for 98% of the variance in triclopyr sorption. In contrast, Johnson et al. (1995) found a strong inverse relationship between triclopyr sorption and native pH of four soils from two sites and two depths.

This listing demonstrates the great variability in the results obtained in various experiments, and highlights the difficulty in interpreting and comparing them. Although significant correlations between sorption and pH have been observed for all categories of ionisable pesticide, some sulfonylureas and imidazolinones seem to be particularly sensitive to changes in soil pH, even if they do not necessarily have higher dissociation constants. This difference might be related to their mechanism of adsorption and could be linked to some particular chemical properties of these pesticides. In some cases, pH has been shown to strongly influence sorption of a compound, while other studies cannot determine a relationship. In these cases, the influence of some experimental parameters and/or other soil properties might mask the influence of pH. The standardization of experimental settings (e.g., ionic strength, soil-to-solution ratio, method to modify pH), the inclusion of the methods used to determine soil properties, especially OM and pH, and a judicious choice of the range of soil studied would allow an easier comparison between studies and a clearer understanding of that part of variability in sorption that is attributable to variations in pH.

Attempts to Model the Influence of pH on Sorption. Bailey et al. (1968) noted early on the difficulty in predicting the sorptive behaviour of pesticides that dissociate to form ions. Many factors, including the dissociation constant (pK_a), soil solution pH, ionic strength and ionic composition, and the type and charge of soil components may have to be considered to successfully predict sorption of ionisable compounds in soils (Koskinen and Harper 1990). Furthermore, as already described, sorption of these com-

pounds can occur through various mechanisms that depend on both the molecule and the soil properties, making any generalisation difficult.

Several authors developed equations to predict the sorption of ionisable compounds in soils or sediments (Fontaine et al. 1991; Jafvert 1990; Lee et al. 1990; Regitano et al. 1997; Shimizu et al. 1992). Different assumptions were made regarding the relationship between pH and the adsorption of the neutral and ionic forms and the pH-dependent changes to consider in the surface charges or soil components. Adsorption in the system studied could usually be predicted from the combination of two partition coefficients (one for each form coexisting in solution), with the pK_a, the soil pH, and OM content. Unfortunately, the applicability of these models to other systems in which other factors can become more important was rarely demonstrated. More recently, Spadotto and Hornsby (2003) developed a model from theoretical modelling and experimental data, initially based on the adsorption of 2,4-D in a variable-charge soil. Although the adsorption of the anion was considered to be negligible, the accessibility of OM (as a consequence of OM dissociation with increasing pH) was considered to explain the observed differences in sorption. Experimental data for sorption of 2.4-D and K_{oc} values from the literature for flumetsulam and sulfentrazone in several soils fitted the model.

Among the pesticide fate models commonly used to predict the behaviour of pesticides in the environment, only PEARL (Tiktak et al. 2000), PELMO (Jene 1998), and RZWQM (Wauchope 1992) have an option for ionisable pesticides. The parameters needed to model pH influence are generally not available and need to be determined for each soil–pesticide combination. Further experimentation should be considered to test the robustness of the equations proposed and to select the assumptions to take into account to develop a unique or specific approach if necessary, able to describe the complexity of interactions among ionisable molecules.

Influence of Soil Components. There have been many attempts to develop a universally applicable sorption constant, or regression equations able to predict adsorption of organic contaminants in soils based on soil properties, without need for time- and cost-consuming experiments in every case. Organic carbon content has been shown to be the single most important soil property for predicting the sorption of neutral organic compounds. That is why Hamaker and Thomson (1972) proposed to refer the adsorption coefficient (K_d) to the soil organic carbon content using a normalised coefficient (K_{oc}) that appears to be much less variable for adsorption of a given hydrophobic molecule (Karickhoff 1981). It has now become a widely used parameter for comparing pesticide binding in soil. However, this approach is not suitable for ionisable compounds (Von Oepen et al. 1991) as their adsorption depends to a greater or lesser extent on soil pH and because they can interact strongly with the other soil fractions such as clay and Al, Fe (hydr)oxides.

Soil components function more as a unit than as separate entities, and the relative contributions of organic and inorganic surfaces to sorption depend on the extent to which the minerals are coated with organic substances (Stevenson 1972). The consequences of these interactions on pesticide sorption are not fully understood. Some authors have reported that the interassociation processes may block functional groups for sorption on mineral and organic surfaces (Pusino et al. 1994). This is supported by soil experiments that show that the contribution of the clay fraction to adsorption is generally much smaller than studies with the pure minerals would predict. Similarly, better fits are generally obtained with regression analysis with CEC instead of OM content because the CEC considers the sorptive capacity from both OM and clays and the likely reduction in sorption due to their interactions (Pusino et al. 1994).

Nevertheless, the different soil constituents may also complement one another, leading to enhanced sorption on the resultant aggregate. For instance, Fe coatings on montmorillonite surfaces decreased sorption of the polar uncharged herbicide thiazafluron (Celis et al. 1997b), but promoted sorption of the basic herbicides atrazine and simazine on the clay surfaces (Celis et al. 1998b). The results of Celis et al. (1999) showed how the complexity of the surface of a natural particle was far from the sum of its individual components (i.e., humic acid, clay mineral and (hydr)oxides), for the sorption fate of 2,4-D. As a consequence, the use in modelling of sorption parameters (K_{oc}, K_{ow}, $K_{mineral}$, or K_{Fe}) estimated assuming sorption on a single soil component alone may result in serious deviation from reality (Celis et al. 1999).

Soil Organic Matter. Many studies show that adsorption of organic chemicals in soils is mainly to organic matter, even though structure and properties of organic constituents are not yet clearly understood. For instance, Stephenson et al. (1990) showed that, throughout a 4-mon experiment, 90% or more of triclopyr was recovered in the soil organic layer. Consequently, published results on pesticide adsorption frequently report some positive correlation between distribution coefficient values and soil OC content or OM content (see Table 5).

Both the type of material being decomposed and stage of decomposition are important in this process. The major HA groups include carboxylic, phenolic, hydroxyl, carbonyl, amine, amide, and aliphatic moieties. Due to this polyfunctionality, HA are one of the most powerful chelating agents among natural organic substances. The prominent role of HA, compared to other organic fractions, has been highlighted for four *s*-triazines (Stevenson 1972), imazethapyr (Senesi et al. 1997), and MCPA (Haberhauer et al. 2001).

The molecular structure of HA will also influence the adsorption of pesticide on the soil organic fraction. For instance, Gennari et al. (1998) reported that the higher the content of carboxyl groups, the higher the amount of imidazolinones adsorbed. Piccolo et al. (1992) observed a higher adsorption of atrazine on HA with higher aromaticity, polycondensation,

and molecular size. However, Piccolo et al. (1998) suggest that atrazine retention in soils might be controlled by specific molecular structure of OM rather than by its acidic functionality or aromaticity, which would indicate ionic bonding and charge transfer reaction, respectively. The aliphatic carbon content of soil OM may be a more important parameter controlling atrazine adsorption to soils because the conformational rigidity conferred to humic fractions with a large content of aromatic moieties appeared conducive only to surface adsorption and thus to easier desorption of herbicide (Piccolo et al. 1998).

Even though OM generally provides most of the adsorption sites in soils, the correlation between adsorption and OM depends more or less on the nature of both the herbicide molecule and the soil, and the positive influence of OM on the adsorption of ionisable compounds is not always obvious. For instance, Barriuso et al. (1992) did not find any relationship between the K_d value for 2,4-D and the soil OC content of two oxisols, whereas the relationship was very strong in the case of atrazine ($r^2 = 0.86$) and terbutryn ($r^2 = 0.64$). The study was carried out on two sites with plots with different crop histories, resulting in a difference in OC content, and at a pH at which the 2,4-D molecule was anionic and thus less subject to hydrophobic partitioning on the OM than the two bases. Another example was observed in three ferralsols samples, where sorption of clofenset and salicylic acid was found to increase with depth in a soil profile where organic matter decreased (Dubus et al. 2001). Enhanced sorption of weak organic acids in subsurface layers is not uncommon in soils with pH-dependent charges and has been reported in several other studies (Goetz et al. 1986; Loux et al. 1989a; Mersie and Foy 1985; Regitano et al. 2000).

In these examples, adsorption of acidic pesticides mainly involved ionic interactions with positive charges in soil, generated by iron and aluminium (hydr)oxides. The OM can adsorb some 2,4-D through weak interactions (van der Waals forces and charge transfer), but more often its overall negative charge causes charge repulsion for anionic compounds (Stevenson 1972). Coating of the mineral surfaces by soil OM might block specific sorption sites on oxide surfaces and might also explain the negative influence of OM content on adsorption observed in some cases (Dubus et al. 2001). The negative relationship between sorption and OM content observed for these profiles confirms that sorption mechanisms for ionisable compounds are different from those involved in the sorption of nonionisable compounds. However, this behaviour would never be expected for subsurface horizons of soils with permanent negative charges (temperate soils), where the higher soil-solution pH in the subsurface would enhance repulsion between anionic pesticide and the negatively charged soil sites, and where the lower OM content would provide less hydrophobic sites for sorption (Regitano et al. 2000).

Soil organic matter can also be divided into solid (SOM) and water-dissolved (DOM) fractions, both of which can associate with herbicides. The latter has been the subject of several recent studies that investigated how

organic amendments, producing soluble OM, affect pesticide adsorption. Formation of a complex between the pesticide and DOM decreases pesticide adsorption if DOM is not adsorbed to the soil, and vice versa (Barriuso et al. 1997; Ben-Hur et al. 2003; Said-Pullicino et al. 2004; Si et al. 2006). The latter case occurs where complexes adsorb in lower soil horizons that have typically a smaller OM content. In soils with large SOM contents (7% or greater), the contribution of atrazine–DOM complexes to total sorption appeared to be negligible (Ben-Hur et al. 2003; Spark and Swift 2002).

Clay. The clay fraction of the soil is composed of both crystalline and amorphous minerals. Most of the charged and polar sorption sites are on the secondary minerals, the layer silicates. Amorphous minerals can also provide some hydrophobic sorption sites. In contrast, crystalline minerals such as quartz and feldspar typically contribute little to the sorption capacity of a soil (Harper 1994). Strong correlations have sometimes been found between the clay content and the adsorption of certain ionisable pesticides, especially some sulfonylureas and basic compounds (see Table 5). For instance, Harper (1988) studied the behaviour of metribuzin down a silty clay loam profile and observed that clay content was the single best predictor of its adsorption. Indeed, in low organic matter soils, the contribution of inorganic constituents to pesticide retention can be dominant (Barriuso et al. 1994). The results of Ben-Hur et al. (2003) indicate that clay plays a significant role in atrazine adsorption when the clay/soil organic C ratio is >30, as may occur in cultivated soils with low OM content (Barriuso et al. 1992), in deep soil horizons, and in some sediments.

It is known that *s*-triazines can be adsorbed on clay minerals as both protonated and neutral species, depending on the pH of the soil solution. The neutral form is adsorbed by relatively weak physical forces (hydrophobic partitioning, van der Waals forces, H-bonds), whereas the positively charged molecule is mostly adsorbed by cation exchange (Fruhstorfer et al. 1993). The type of surface cation seems to play a key role in the adsorption process at low pH (Herwig et al. 2001). Indeed, cations in solution may compete for negatively charged adsorption sites in cation exchange. They may also behave as adsorption sites in cation or water bridging.

Nonionic or anionic herbicides sometimes sorb to clay surfaces through the formation of a complex between the herbicide, an exchangeable cation, and the soil surface. These complexes have been found for acifluorfen, glyphosate, and some *s*-triazines (Harper 1994); they can lead to immobilization and inactivation when metal concentrations are high (Harper 1994; Kozlowski et al. 1990).

Gonzalez and Ukrainczyk (1996) observed that the sorption of nicosulfuron on Iowa soils was most correlated with clay content while in Brazilian soils it was most correlated with OC content. These differences were interpreted in terms of different clay mineralogy of Iowa (expandable 2:1 clay minerals) and Brazilian soils (kaolinite, Al and Fe oxides). Results of X-ray

diffraction analysis showed that, in some cases, sorption was not limited to the external surface of clays. Indeed, it has been demonstrated that fluazifop-butyl (Fusi et al. 1988) and fluridone (Weber et al. 1986) could enter into the interlayer space of montmorillonte. Although excluded from this review of ionisable pesticides, cationic bipyridylium herbicides (e.g., diquat and paraquat) adsorb in the internal surfaces of clays, and this process is not fully reversible (Hayes et al. 1975). Herwig et al. (2001) found atrazine adsorption to be proportional to the external surface area in Na^+ layer silicates such as kaolinite, illite, and montmorillonite, and concluded that atrazine molecules do not intercalate even in swelling Na^+ clay minerals.

Aluminium and Iron (Hydr)oxides. Positively charged oxide surfaces have been shown to play a significant role in the sorption of clofenset and salicylic acid (Dubus et al. 2001), primisulfuron (Ukrainczyk and Ajwa 1996), 2,4-D (Barriusso et al. 1992), imazaquin (Goetz et al. 1986; Regitano et al. 1997, 2000), 2,4-D and dicamba (Stolpe and Kuzila 2002), and mecoprop, 2,4-D, and bentazone (Clausen and Fabricius 2001). This sorption behaviour is probably more common in tropical and semitropical soils due to the greater prevalence of Al and Fe (hydr)oxides. In temperate areas, the relatively high concentration of organic compounds in the soil serves to complex with the Al and Fe as it is released by the weathering of soil parent material, thereby preventing the formation of their respective (oxi)hydroxides. Their role is particularly important when OM and clay content are low and at pH values where acidic compounds exist almost exclusively as an anion (Goetz et al. 1986). Adsorption of weak bases onto iron oxides is insignificant (Clausen and Fabricius 2001; Stolpe and Kuzila 2002).

The net charge of these surfaces varies with pH. At pH values above the point of zero charge (PZC) of the minerals, the surfaces have a net negative charge. Thus, adsorption of anions is restricted due to electrostatic repulsion. At pH values lower than PZC, adsorption of anions is promoted due to electrostatic attraction to the positively charged surfaces (Dubus et al. 2001). In fact, anionic moieties can interact not only with the positive $Al(OH)^{2+}$ and $Fe(OH)^{2+}$ groups on the clay surface through electrostatic interactions (anion exchange, cation bridging), but also exchange with –OH or OH_2 and create a bridge with one or two adjacent Fe or Al atoms through a ligand-exchange mechanism (Regitano et al. 2000).

Hyun and Lee (2004) observed that the fraction of hydrophilic sorption of prosulfuron correlated well with the ratio of the AEC to CEC, whereas the correlation with only AEC led to poor fits. The authors concluded that normalizing AEC by CEC accounted for repulsion by negatively charged sites on the soil surface, which may attenuate the potential for organic anions to interact with the positively charged sites.

Conclusion. Weber et al. (2000) correlated K_d values and soil properties reported in the literature for 28 herbicides including acidic and basic

compounds. The results show that OM is the soil constituent most highly correlated with binding of most organic herbicides; clay content is correlated with retention of all cationic and many weakly basic compounds; and pH is inversely correlated with retention of many weakly acidic herbicides. A few years later, Weber et al. (2004) repeated the procedure for an assortment of 57 pesticides. K_d values for all pesticides were correlated with the OM content and a combination of OM and clay contents, but correlation coefficients were very small, and soil properties were often correlated with one another. However, after separating pesticides into chemical classes, some trends could be discerned (see Table 5). K_d values were not related to soil pH for any of the 6 nonionisable pesticide families, whereas sorption of weakly acidic pesticides in soils was most strongly related to OM content and/or inversely related to pH. Soil pH was inversely correlated with K_d for carboxylic acids, and inclusion of OM or clay content did not improve the relationship. Sorption of weakly basic pesticides was most strongly related to soil OM and clay contents and inversely related to pH. OM and clay were correlated, but pH was not related to either parameter confirming the importance of pH for the binding of ionisable pesticides.

These results, in association with the numerous studies showing a significant influence of pH on K_d or K_{oc}, confirm that the use of a unique K_{oc} is not suitable to predict the behaviour of ionisable pesticides in soils (Wauchope et al. 2002). However, it seems difficult to define a modelling approach applicable to all ionisable pesticides in all situations. The standardization of experimental settings, the inclusion of the methods used to determine soil properties, especially OM and pH, and progress in the determination of the pH at the surface of soil particles should help in comparing results of different studies and support identification of the parameters that should or should not be taken into account for a specific type of soil or compound. Better results might also be achieved by considering different categories of clay and OM rather than their total content in soil. Some fractions can adsorb ionisable pesticides very efficiently (e.g., montmorillonite clays for bases, iron and aluminium oxides for acids) while others might be essentially inert.

Until we have a better understanding and prediction of the phenomenon specific to ionisable compounds, it is advised to base assessment of the fate of ionisable pesticides on, instead of a unique K_{oc}: (i) a K_d (or K_{oc}) determined at a standardized pH, with its decrease calculated as a function of pH; (ii) multiple regression equations defined for each ionisable pesticide family (Weber et al. 2004); or (iii) models that take into account the influence of pH and oxides on adsorption.

IV. Degradation of Ionisable Compounds and Soil pH

After partitioning between the liquid and solid phases, molecules present in soil solution and molecules adsorbed on soil particles often have different potential to undergo additional processes such as volatilization, leaching,

chemical hydrolysis, or biodegradation. As a consequence, adsorption is often shown to influence the rate at which pesticides degrade in soil.

Along with sorption, degradation is the second important process used to predict the fate of organic compounds in soils (Boesten and van der Linden 1991). Standard laboratory and field dissipation studies are performed to assess the rate of degradation, often expressed as a first-order half-life or DT_{50}, the time required for 50% of the initial dose to be degraded. A greater understanding of the factors that influence biodegradation rate is required to allow prediction for soils where experimental data are not available. The rate of degradation is influenced by chemical properties of the soil (such as pH and OM content), biological properties (activity and distribution of microorganisms), and environmental conditions that control soil temperature and moisture content. Both route and rate of degradation also depend on properties of the chemical. Degradation of pesticides in soils usually involves the activities of soil microorganisms, although abiotic transformations can become dominant in some cases. In addition, soil properties are often interrelated and may influence these processes in opposite directions, thereby exhibiting a stimulating and restricting effect on the overall degradation process. We have seen how pH influences the adsorption of ionisable pesticides in soils. Because adsorption is often important for controlling the rate of degradation, an additional pH effect on the degradation rate of ionisable pesticides might be expected. Consequently, we only focus here on the influence of soil pH on degradation rates.

Influence of Soil pH on Degradation. A relationship between soil pH and rate of degradation has been demonstrated for many ionisable pesticides, although there are exceptions. No influence of pH on degradation was found for atrazine (Hance 1979), 2,4-D (Picton and Farenhorst 2004), and rimsulfuron (Vicari et al. 1996). Soil pH may influence the degradation of a pesticide directly if its stability is pH dependent (chemical hydrolysis) or indirectly via changes in soil microbial biomass/activity, or pesticide sorption. If degradation is influenced indirectly by pH, it tends to proceed faster at high pH.

Positive Influence: Indirect Effects via Changes to Microbial Activity and Sorption. Soil pH significantly influences the structure of the microbial community. Many studies have demonstrated a positive influence of pH on total microbial biomass and activity (Price et al. 2001; Walker et al. 2001), although microbial degradation seems to be restricted when pH becomes greater than 8–8.5 (Awasthi et al. 2000; Fredrickson and Shea 1986; Thirunarayanan et al. 1985; Walker et al. 1989). Consequently, degradation of many neutral compounds has been shown to be faster at high pH. Moreover, we have seen that adsorption of ionisable pesticides generally decreases as pH increases. Therefore, molecules are generally more available for biodegradation under alkaline conditions, and the positive influence of pH on degradation rate is, consequently, more obvious for ionisable

compounds. A positive influence of pH on degradation has been shown for imazaquin (Loux and Reese 1992), metribuzin (Ladlie et al. 1976b), dichlorprop (Hance 1979), prochloraz (Höllrigl-Rosta et al. 1999), mesotrione (Dyson et al. 2002), dicamba (Voos and Groffman 1997), and flumetsulam (Lehmann et al. 1992).

Negative Influence: Specific Case of Sulfonylureas. Transformation of organic compounds in soils occurs through both microbial breakdown and chemical degradation. Abiotic degradation can be the dominant reaction in soils for many sulfonylureas. For instance, Hultgren et al. (2002) studied prosulfuron degradation and observed that pH-dependent hydrolysis of the sulfonylurea bridge was the primary transformation process. Significant microbial degradation occurred in only 2 of the 10 soils. Microbial reaction tends to be faster under alkaline conditions (up to a maximum value), whereas abiotic hydrolysis of sulfonylureas is generally more favoured under acidic conditions (Sabadie 1990; Said-Pullicino et al. 2004; Sarmah and Sabadie 2002; Sarmah et al. 2000; Vicari et al. 1996). For instance, Sarmah et al. (2000) observed that the hydrolysis of triasulfuron, metsulfuron-methyl, and chlorsulfuron involving attack by neutral water was at least 100 fold faster when the molecule was undissociated (acidic conditions) than when present as the anion at near-neutral pH. Chlorimuron hydrolysis also increased by 150 fold as the pH decreased from 7 to 4 in buffered aqueous solution (Brown 1990). The dominance of acidic hydrolysis explains the negative relationship between degradation and pH often reported for sulfonylureas including chlorsulfuron, metsulfuron-methyl, prosulfuron, primisulfuron methyl, rimsulfuron, and thifensulfuron methyl (Sarmah and Sabadie 2002), chlorsulfuron (Fredrickson and Shea 1986; Thirunarayanan et al. 1985; Walker et al. 1989), prosulfuron (positive correlation between DT_{50} and soil pH: $r^2 = 0.86$) (Hultgren et al. 2002), metsulfuron-methyl (Pons and Barriuso 1998; Walker et al. 1989), and chlorsulfuron and triasulfuron (Sarmah et al. 1999). Similar results have been obtained for the weak bases atrazine and simazine (Walker and Blacklow 1994; Walker and Thompson 1977).

Conclusion. Considering the complexity of interactions between the different processes, it seems to be more difficult to prove a link between degradation and pH than to demonstrate the influence of pH on sorption. However, the influence of pH on degradation seems to be more apparent for ionisable compounds. For nonionisable compounds, pH primarily controls the microbial activity of the soil, leading to a positive influence of pH on degradation rates. In the case of ionisable compounds, strength of sorption decreases and availability for degradation generally increases with increasing pH. There are thus both biological and physical processes underpinning an increase in rate of degradation with pH for ionisable compounds subject to microbial degradation. However, when abiotic degradation is dominant (e.g., sulfonylureas), pH generally has a negative influence on

degradation rates. In this case, the increase in bioactivity at high pH is less significant than the decrease in rate of chemical hydrolysis.

V. Link Between Sorption and Degradation Processes

It is quite well recognised that sorption processes may affect biodegradation mainly by modifying chemical bioavailability. Evidence that degradation can be restricted to the soil solution and that sorbed molecules may be protected from microbial attack has been provided by several studies (Ogram et al. 1985; Radosevish et al. 1996; Smith et al. 1992). The protection of a sorbed compound may arise from (i) the reduction of soil solution concentrations to levels below that necessary for microbial utilization, (ii) surface stabilisation against desorption of the compound (formation of bound residues), and (iii) inaccessibility of the micropores to microbes (Ainsworth et al. 1993).

A positive relationship between adsorption coefficient (K_d) and half-life has been reported for diallate (Anderson 1981), simazine (Walker and Blacklow 1994), 2,4-D (Bolan and Baskaran 1996), flumetsulam ($r^2 = 0.85$; Lehmann et al. 1992), several imidazolinones (Basham et al. 1987; Cantwell et al. 1989; Loux et al. 1989b; Loux and Reese 1992, 1993); metribuzin (Ladlie et al. 1976b); 2,4-D and trichlopyr (Johnson et al. 1995), mesotrione ($r^2 = 0.45$; Dyson et al. 2002), and 2,4-D (empirical power equation, $r^2 = 0.99$) (Guo et al. 2000).

Factors other than sorption also influence degradation rates, and the link between sorption and degradation is not always obvious (Barriuso et al. 1997; Radosevish et al. 1996; Shaw and Burns 1998a). A negative relationship between K_d and DT_{50} can even be observed in some cases (Walker and Thomson 1977). Several factors might counterbalance the influence of sorption on degradation:

i. Biodegradation might not always be restricted to chemical in solution (Eberbach 1998; Guo et al. 2000; Khan and Ivarson 1981; Park et al. 2001).
ii. Microorganisms are generally more abundant at or near soil particle surfaces (Stotzky 1986). Sorption may thus concentrate the pesticide in regions of greatest microbial activity, thereby facilitating degradation.
iii. Adsorption to OM can facilitate the abiotic transformation of the molecule as shown for metribuzin and its metabolites using infrared spectroscopy (Henriksen et al. 2004), for azimsulfuron (Pinna et al. 2004), and for triasulfuron (Said-Pullicino et al. 2004). This process seems to operate especially at low pH and to be related to the mechanisms of sorption.
iv. OM content can have opposing effects on degradation, either via an increase in sorption or via an increase in microbial activity.

The positive or negative correlation between OM and degradation should indicate the strength and sign of the correlation between sorption

and degradation. Bolan and Bascaran (1996) observed a bell-shaped relationship between K_d and DT_{50} of 2,4-D measured in 10 soils. The increase of degradation rate for the highest K_d values was explained by (i) the tendency of these soils to have a higher microbial activity and (ii) an inhibitory effect of 2,4-D on activity that decreased with an increase in OM content. The decrease of pesticide concentration in solution with an increasing K_d was thus compensated by an increased microbial activity, thereby increasing the rate of degradation.

More experiments coupling measurement of adsorption and degradation under differing conditions would help us to better understand the extent and mechanisms of interactions between the various processes. Nevertheless, we have seen that pH generally decreases sorption of ionisable pesticides and that pH can also influence their degradation to a great extent via changes in microbiological activity and sorption. If a negative influence of sorption on degradation can be demonstrated, it should be stronger in the case of ionisable compounds, especially for basic compounds that often show a high adsorption potential. This effect has implications, for example, in risk assessment where associating the lowest K_d with the highest DT_{50} may constitute an unrealistic and thus overly protective assumption (Dyson et al. 2002).

VI. Conclusions

Ionisable pesticides comprise a significant proportion of the existing and new active substances currently undergoing review for registration by the European Union (EU 2002). This group of pesticides includes chemicals that are frequently found in groundwater and surface waters worldwide. It is thus essential to understand their fate in the environment, and the specifics of their behaviour need to be recognised within risk assessment procedures. A great deal of work has been undertaken concerning the adsorption of ionisable pesticides in soils, but generalised conclusions cannot be made, and significant open questions remain.

Many retention mechanisms in addition to hydrophobic partitioning have been postulated to be responsible for the adsorption of ionisable pesticides in soils (e.g., ionic exchange, charge transfer, ligand exchange, and cation or water bridging). However, relatively little experimental evidence is available, and we are still unable to determine the quantitative contribution of each sorption mechanism in a particular situation. Further research using techniques such as nuclear magnetic resonance, electron spin resonance, Fourier transform infrared, and fluorescence spectroscopies, and including measurement of soil and pesticide properties, should help to better understand and predict the adsorption mechanisms that operate.

More generally, knowledge is still lacking concerning the phenomena occuring at the surface of soil particles. It is difficult to assess likely competition effects with the other ions present in soil solution, and consequently the complex effect of ionic strength, or moisture content effects, on the

adsorption of ionisable compounds. Measurements of pH do not allow the determination of the operative pH at the surface of soil particles or in microenvironments, although this is often assumed to be one to four units lower than the pH measured in the bulk solution.

The adsorption of ionisable compounds in soils is strongly influenced by pH, and this effect depends on soil composition and the characteristics of the compound. This pH dependence derives mainly from the different proportions of ionic and neutral forms of the pesticide present at each pH level and from differences in their strength of sorption. The varying pH on the charge at the surface of soil particles also plays a role in some cases. A decrease in adsorption with increasing pH is often observed. However, bell-shaped curves, increases in adsorption, and pH-independent behaviour have also been reported. Experiments in which the pH of a soil is adjusted artificially are useful with respect to experimental design and control, but experiments dealing with a natural pH range or soils equilibrated for a long period will give more realistic results. The two methods have generated conflicting results because the influence of some experimental factors and/or soil properties have superposed and often masked the influence of pH. The standardisation of experimental settings (e.g., ionic strength, soil-to-solution ratio, method to modify pH) would allow an easier determination of that part of the variance trully attributable to the influence of pH. Soil OM generally promotes the adsorption of ionisable pesticides in soils, although its negative influence has sometimes been observed as well, which confirms that sorption of ionisable compounds in different soils cannot be assessed simply by normalising to organic carbon content. Clay and Al or Fe (oxi)hydroxides can also play a significant role and might have to be considered in some situations.

So far, no modelling approach has been applied successfully to a range of ionisable pesticides to predict their adsorption in soils. Further experimental data are required to test the robustness of equations proposed and to select the necessary assumptions. Approaches specific to a particular class of pesticide, with the inclusion of QSAR for instance, and/or soil type might be necessary to describe the complexity of interactions among ionisable molecules.

Degradation of ionisable pesticides is influenced by soil pH in a particular way that relates to changes in sorption, changes in composition and activity of the microbial community, and to shifts in the balance between different degradative mechanisms. Degradation tends to proceed faster at high pH for compounds mainly degraded by microorganisms while degradation of sulfonylureas, particularly sensitive to chemical hydrolysis, is generally faster under acidic conditions.

Questions remain concerning the link between the processes of adsorption and degradation. Experiments measuring these two parameters under standardized conditions could help to better understand their relationship and their dependence regarding soil and chemical properties and could

support the choice of more realistic input parameters in risk assessment procedures.

Summary

Understanding the fate of a pesticide in soil is fundamental to the accurate assessment of its environmental behaviour and vital in ensuring the safe use of new and existing products. Ionisable pesticides comprise a significant proportion of both existing and new active substances registered for use in agriculture worldwide. This group of pesticides includes chemicals that are frequently found in groundwater and surface waters in many different countries. Despite this, approaches to predict the influence of soil properties on the behaviour of ionisable pesticides in soils are poorly developed. Current regulatory assessments frequently default to methods developed for nonionic chemicals, although it is evident that ionisable compounds do not often react like neutral molecules.

This review presents the state of knowledge on the adsorption of ionisable pesticides in soils. It first introduces the issues concerning adsorption and the characteristics of this particular kind of chemical. The mechanisms postulated for the adsorption of ionisable pesticides are then described: these are hydrophobic partitioning, ionic exchange, charge transfer, ligand exchange, cation or water bridging, and the formation of bound residues. Relatively little experimental evidence is available, and we are still unable to determine the quantitative contribution of each process in a particular situation. Knowledge is still lacking concerning phenomena occurring at the surfaces of soil particles. Measurements do not allow determination of the operative pH at the surface of soil particles or in microenvironments, and the influence of ionic strength or competition effects is difficult to assess.

Subsequently, the review focuses on the influence of soil properties on adsorption and on potential to predict the behaviour of ionisable pesticides in soils. Unlike hydrophobic compounds, adsorption of ionisable pesticides is highly sensitive to variation in pH. This relationship mainly derives from the different proportion of ionic and neutral forms of the pesticide present at each pH level but also from the presence of surfaces with pH-dependent charges in soils. Soil organic matter generally promotes adsorption, although a negative influence has sometimes been reported. Clay and oxides can also play a significant role in some cases. So far, no modelling approach has been applied successfully to a range of ionisable pesticides to predict their adsorption in soils. The standardization of experimental settings and the application of approaches specific to a particular class of pesticide or different type of soil might be necessary to describe the complexity of interactions among ionisable molecules. Degradation of ionisable pesticides is influenced by soil pH in a particular way that relates to changes in sorption, changes in composition and activity of the microbial community, and to shifts in the balance between different degradative mechanisms.

Appendix. CAS numbers and chemical names of pesticides reported.

Compound	CAS	Chemical name
Acifluorfen	50594-66-6	5-[2-Chloro-4-(trifluoromethyl)phenoxy]-2-nitrobenzoic acid
Ametryne	834-12-8	*N*-Ethyl-*N*?-(1-methylethyl)-6-(methylthio)-1,3,5-triazine-2,4-diamine
Anilazine	101-05-3	4,6-Dichloro-*N*-(2-chlorophenyl)-1,3,5-triazin-2-amine
Atrazine	1912-24-9	6-Chloro-*N*-ethyl-*N*?-(1-methylethyl)-1,3,5-triazine-2,4-diamine
Azimsulfuron	120162-55-2	*N*-[[(4,6-dimethoxy-2-pyrimidinyl)amino]carbonyl]-1-methyl-4-(2-methyl-2*H*-tetrazol-5-yl)-1*H*-pyrazole-5-sulfonamid
Bentazon	25057-89-0	3-(1-Methylethyl)-1*H*-2,1,3-benzothiadiazin-4(3*H*)-one 2,2-dioxide
Carbendazim	10605-21-7	methyl 1*H*-benzimidazol-2-ylcarbamate
Chlorimuron	99283-00-8	2-[[[[(4-Chloro-6-methoxy-2-pyrimidinyl)amino]carbonyl]amino]sulfonyl]benzoic acid
Chlorsulfuron	64902-72-3	2-Chloro-*N*-[[(4-methoxy-6-methyl-1,3,5-triazin-2-yl)amino]carbonyl]benzenesulfonamide
Clofencet	129025-54-3	2-(4-Chlorophenyl)-3-ethyl-2,5-dihydro-5-oxo-4-pyridazinecarboxylic acid
Desethylatrazine	6190-65-4	2-Chloro-4-amino-6-isopropylamino-1,3,5-triazine
Diallate	2303-16-4	*S*-(2,3-Dichloro-2-propenyl) bis(1-methylethyl)carbamothioate
Dicamba	1918-00-9	3,6-Dichloro-2-methoxybenzoic acid
Dichlorprop	120-36-5	2-(2,4-Dichlorophenoxy)propanoic acid
Dinoseb	88-85-7	2-(1-Methylpropyl)-4,6-dinitrophenol
Dinoterb	1420-07-1	2-(1,1-Dimethylethyl)-4,6-dinitrophenol
Diquat	2764-72-9	6,7-Dihydrodipyrido[1,2-*a*:2?,1?-*c*]pyrazinediium
Diuron	330-54-1	*N*?-(3,4-Dichlorophenyl)-*N*,*N*-dimethylurea
DNOC	534-52-1	2-Methyl-4,6-dinitrophenol
Ethametsulfuron	111353-84-5	2-[[[[[4-Ethoxy-6-(methylamino)-1,3,5-triazin-2-yl]amino]carbonyl]amino]sulfonyl]benzoic acid
Ethametsulfuron-methyl	97780-06-8	Methyl-2-[[[[[4-ethoxy-6-(methylamino)-1,3,5-triazin-2-yl]amino]carbonyl]amino]sulfonyl] benzoic acid
Fenpropimorph	67564-91-4	*rel*-(2*R*,6*S*)-4-[3-[4-(1,1-Dimethylethyl)phenyl]-2-methylpropyl]-2,6-dimethylmorpholine

Appendix. *Continued*

Compound	CAS	Chemical name
Flumetsulam	98967-40-9	*N*-(2,6-Difluorophenyl)-5-methyl[1,2,4]triazolo[1,5-*a*]pyrimidine-2-sulfonamide
Fluazifop	69335-91-7	2-[4-[[5-(Trifluoromethyl)-2-pyridinyl]oxy]phenoxy]propanoic acid
Fluazifop-P	83066-88-0	(2*R*)-2-[4-[[5-(Trifluoromethyl)-2-pyridinyl]oxy]phenoxy]propanoic acid
Fluazifop butyl	69806-50-4	Butyl-2-[4-[[5-(trifluoromethyl)-2-pyridinyl]oxy]phenoxy]propanoic acid
Fluridone	59756-60-4	1-Methyl-3-phenyl-5-[3-(trifluoromethyl)phenyl]-4(1*H*)-pyridinone
Fluroxypyr	69377-81-7	[(4-Amino-3,5-dichloro-6-fluoro-2-pyridinyl)oxy]acetic acid
Glyphosate	1071-83-6	*N*-(Phosphonomethyl)glycine
Haloxyfop	69806-34-4	2-[4-[[3-Chloro-5-(trifluoromethyl)-2-pyridinyl]oxy]phenoxy]propanoic acid
Hydroxyatrazine	2163-68-0	2-Hydroxy-4-(ethylamino)-6-(isopropylamino)-s-triazine
Imazapyr	81334-34-1	2-[4,5-Dihydro-4-methyl-4-(1-methylethyl)-5-oxo-1*H*-imidazol-2-yl]-3-pyridinecarboxylic acid
Imazaquin	81335-37-7	2-[4,5-Dihydro-4-methyl-4-(1-methylethyl)-5-oxo-1*H*-imidazol-2-yl]-3-quinolinecarboxylic acid
Imazethapyr	81335-77-5	2-[4,5-Dihydro-4-methyl-4-(1-methylethyl)-5-oxo-1*H*-imidazol-2-yl]-5-ethyl-3-pyridinecarboxylic acid
Isoproturon	34123-59-6	*N*,*N*-Dimethyl-N′-[4-(1-methylethyl)phenyl]urea
Mesotrione	104206-82-8	2-[4-(Methylsulfonyl)-2-nitrobenzoyl]-1,3-cyclohexanedione
Metsulfuron	79510-48-8	2-[[[[(4-Methoxy-6-methyl-1,3,5-triazin-2-yl)amino]carbonyl]amino]sulfonyl]benzoic acid
Metsulfuron-methyl	74223-64-6	Methyl-2-[[[[(4-methoxy-6-methyl-1,3,5-triazin-2-yl)amino]carbonyl]amino]sulfonyl]benzoic acid
MCPA	94-74-6	(4-Chloro-2-methylphenoxy)acetic acid
Mecoprop	7085-19-0	2-(4-Chloro-2-methylphenoxy)propanoic acid
Metribuzin	21087-64-9	4-Amino-6-(1,1-dimethylethyl)-3-(methylthio)-1,2,4-triazin-5(4*H*)-one
Nicosulfuron	111991-09-4	2-[[[[(4,6-Dimethoxy-2-pyrimidinyl)amino]carbonyl]amino]sulfonyl]-*N*,*N*-dimethyl-3-pyridinecarboxamide
Paraquat	4685-14-7	1,1′-Dimethyl-4,4?-bipyridinium

Appendix. *Continued*

Compound	CAS	Chemical name
Pentachlorophenol (PCP)	87-86-5	Pentachlorophenol
Primisulfuron	113036-87-6	2-[[[[[4,6-bis(Difluoromethoxy)-2-pyrimidinyl]amino]carbonyl]amino]sulfonyl]benzoic acid
Primisulfuron-methyl	86209-51-0	2-[[[[[4,6-bis(Difluoromethoxy)-2-pyrimidinyl]amino]carbonyl]amino]sulfonyl]benzoic acid
Prochloraz	67747-09-5	*N*-Propyl-*N*-[2-(2,4,6-trichlorophenoxy)ethyl]-1*H*-imidazole-1-carboxamide
Prometon	1610-18-0	6-Methoxy-N,N′-bis(1-methylethyl)-1,3,5-triazine-2,4-diamine
Prometryn	7287-19-6	*N*,*N*′-bis(1-Methylethyl)-6-(methylthio)-1,3,5-triazine-2,4-diamine
Propiconazole	60207-90-1	1-[[2-(2,4-Dichlorophenyl)-4-propyl-1,3-dioxolan-2-yl]methyl]-1*H*-1,2,4-triazole
Prosulfuron	94125-34-5	*N*-[[(4-Methoxy-6-methyl-1,3,5-triazin-2-yl)amino]carbonyl]-2-(3,3,3-trifluoropropyl)benzenesulfonamide
Rimsulfuron	122931-48-0	*N*-[[(4,6-Dimethoxy-2-pyrimidinyl)amino]carbonyl]-3-(ethylsulfonyl)-2-pyridinesulfonamide
Salicylic acid	69-72-7	2-Hydroxybenzoic acid
Silvex	93-72-1	2-(2,4,5-Trichlorophenoxy)propanoic acid
Simazine	122-34-9	6-Chloro-*N*,*N*?-diethyl-1,3,5-triazine-2,4-diamine
Sulfentrazone	122836-35-5	*N*-[2,4-Dichloro-5-[4-(difluoromethyl)-4,5-dihydro-3-methyl-5-oxo-1*H*-1,2,4-triazol-1-yl]phenyl]methanesulfonamide
Sulfometuron	74223-56-6	2-[[[[(4,6-Dimethyl-2-pyrimidinyl)amino]carbonyl]amino]sulfonyl]benzoic acid
Terbutryn	886-50-0	*N*-(1,1-Dimethylethyl)-*N*?-ethyl-6-(methylthio)-1,3,5-triazine-2,4-diamine
Terbutylazine	5915-41-3	6-Chloro-N-(1,1-dimethylethyl)-N′-ethyl-1,3,5-triazine-2,4-diamine
Thiazafluron	25366-23-8	*N*,*N*′-Dimethyl-*N*-[5-(trifluoromethyl)-1,3,4-thiadiazol-2-yl]urea
Thifensulfuron-methyl	79277-27-3	Methyl-3-[[[[(4-methoxy-6-methyl-1,3,5-triazin-2-yl)amino]carbonyl]amino]sulfonyl]-2-thiophenecarboxylic acid
Triasulfuron	82097-50-5	2-(2-Chloroethoxy)-*N*-[[(4-methoxy-6-methyl-1,3,5-triazin-2-yl)amino]carbonyl]benzenesulfonamide
Triclopyr	55335-06-3	[(3,5,6-Trichloro-2-pyridinyl)oxy]acetic acid
2,4-D	94-75-7	(2,4-Dichlorophenoxy)acetic acid
2,4-DNP	51-28-5	2,4-Dinitrophenol
2,4,5-T	93-76-5	(2,4,5-Trichlorophenoxy)acetic acid

Acknowledgment

This work was funded by the Department for Environment, Food and Rural Affairs and the Pesticides Safety Directorate as Project PS2203.

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Manuscript received November 12; accepted November 22, 2005.

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