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Volume 2: Reactions and Processes
Volume 3: Anthropogenic Compounds
Volume 4: Air Pollution
Volume 5: Water Pollution

The series Volume 1 The Natural Environment and the Biogeochemical Cycles describes the natural environment and gives an account of the global cycles for elements and classes of natural compounds.
The series Volume 2 Reactions and Processes is an account of physical transport, and chemical and biological transformations of chemicals in the environment.
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Preface

Environmental Chemistry is a relatively young science. Interest in this subject, however, is growing very rapidly and, although no agreement has been reached as yet about the exact content and limits of this interdisciplinary discipline, there appears to be increasing interest in seeing environmental topics which are based on chemistry embodied in this subject. One of the first objectives of Environmental Chemistry must be the study of the environment and of natural chemical processes which occur in the environment. A major purpose of this series on Environmental Chemistry, therefore, is to present a reasonably uniform view of various aspects of the chemistry of the environment and chemical reactions occurring in the environment.

The industrial activities of man have given a new dimension to Environmental Chemistry. We have now synthesized and described over five million chemical compounds and chemical industry produces about hundred and fifty million tons of synthetic chemicals annually. We ship billions of tons of oil per year and through mining operations and other geophysical modifications, large quantities of inorganic and organic materials are released from their natural deposits. Cities and metropolitan areas of up to 15 million inhabitants produce large quantities of waste in relatively small and confined areas. Much of the chemical products and waste products of modern society are released into the environment either during production, storage, transport, use or ultimate disposal. These released materials participate in natural cycles and reactions and frequently lead to interference and disturbance of natural systems.

Environmental Chemistry is concerned with reactions in the environment. It is about distribution and equilibria between environmental compartments. It is about reactions, pathways, thermodynamics and kinetics. An important purpose of this Handbook, is to aid understanding of the basic distribution and chemical reaction processes which occur in the environment.

Laws regulating toxic substances in various countries are designed to assess and control risk of chemicals to man and his environment. Science can contribute in two areas to this assessment; firstly in the area of toxicology and secondly in the area of chemical exposure. The available concentration ("environmental exposure concentration") depends on the fate of chemical compounds in the environment and thus their distribution and reaction behaviour in the environment. One very important contribution of Environmental Chemistry to

the above mentioned toxic substances laws is to develop laboratory test methods, or mathematical correlations and models that predict the environmental fate of new chemical compounds. The third purpose of this Handbook is to help in the basic understanding and development of such test methods and models.

The last explicit purpose of the Handbook is to present, in concise form, the most important properties relating to environmental chemistry and hazard assessment for the most important series of chemical compounds.

At the moment three volumes of the Handbook are planned. Volume 1 deals with the natural environment and the biogeochemical cycles therein, including some background information such as energetics and ecology. Volume 2 is concerned with reactions and processes in the environment and deals with physical factors such as transport and adsorption, and chemical, photochemical and biochemical reactions in the environment, as well as some aspects of pharmacokinetics and metabolism within organisms. Volume 3 deals with anthropogenic compounds, their chemical backgrounds, production methods and information about their use, their environmental behaviour, analytical methodology and some important aspects of their toxic effects. The material for volume 1, 2 and 3 was each more than could easily be fitted into a single volume, and for this reason, as well as for the purpose of rapid publication of available manuscripts, all three volumes were divided in the parts A and B. Part A of all three volumes is now being published and the second part of each of these volumes should appear about six months thereafter. Publisher and editor hope to keep materials of the volumes one to three up to date and to extend coverage in the subject areas by publishing further parts in the future. Plans also exist for volumes dealing with different subject matter such as analysis, chemical technology and toxicology, and readers are encouraged to offer suggestions and advice as to future editions of "The Handbook of Environmental Chemistry".

Most chapters in the Handbook are written to a fairly advanced level and should be of interest to the graduate student and practising scientist. I also hope that the subject matter treated will be of interest to people outside chemistry and to scientists in industry as well as government and regulatory bodies. It would be very satisfying for me to see the books used as a basis for developing graduate courses in Environmental Chemistry.

Due to the breadth of the subject matter, it was not easy to edit this Handbook. Specialists had to be found in quite different areas of science who were willing to contribute a chapter within the prescribed schedule. It is with great satisfaction that I thank all 52 authors from 8 countries for their understanding and for devoting their time to this effort. Special thanks are due to Dr. F. Boschke of Springer for his advice and discussions throughout all stages of preparation of the Handbook. Mrs. A. Heinrich of Springer has significantly contributed to the technical development of the book through her conscientious and efficient work. Finally I like to thank my family, students and colleagues for being so patient with me during several critical phases of preparation for the Handbook, and to some colleagues and the secretaries for technical help.

I consider it a privilege to see my chosen subject grow. My interest in Environmental Chemistry dates back to my early college days in Vienna. I received significant impulses during my postdoctoral period at the University of California and my interest slowly developed during my time with the National Research Council of Canada, before I could devote my full time of Environmental Chemistry, here in Amsterdam. I hope this Handbook may help deepen the interest of other scientists in this subject.

Amsterdam, May 1980 *O. Hutzinger*

Twenty-one years have now passed since the appearance of the first volumes of the Handbook. Although the basic concept has remained the same changes and adjustments were necessary.

Some years ago publishers and editors agreed to expand the Handbook by two new open-end volume series: Air Pollution and Water Pollution. These broad topics could not be fitted easily into the headings of the first three volumes. All five volume series are integrated through the choice of topics and by a system of cross referencing.

The outline of the Handbook is thus as follows:

1. The Natural Environment and the Biochemical Cycles,
2. Reaction and Processes,
3. Anthropogenic Compounds,
4. Air Pollution,
5. Water Pollution.

Rapid developments in Environmental Chemistry and the increasing breadth of the subject matter covered made it necessary to establish volume-editors. Each subject is now supervised by specialists in their respective fields.

A recent development is the accessibility of all new volumes of the Handbook from 1990 onwards, available via the Springer Homepage springeronline.com or springerlink.com.

During the last 5 to 10 years there was a growing tendency to include subject matters of societal relevance into a broad view of Environmental Chemistry. Topics include LCA (Life Cycle Analysis), Environmental Management, Sustainable Development and others. Whilst these topics are of great importance for the development and acceptance of Environmental Chemistry Publishers and Editors have decided to keep the Handbook essentially a source of information on "hard sciences".

With books in press and in preparation we have now well over 40 volumes available. Authors, volume-editors and editor-in-chief are rewarded by the broad acceptance of the "Handbook" in the scientific community.

Bayreuth, July 2001 *Otto Hutzinger*

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Foreword

My joy in introducing this book on "Contaminated Sediments" as part of the Handbook of Environmental Chemistry series is for the first time accompanied by a feeling of sadness. The reason for this feeling is that the whole idea and proposal for this book originated from the late Assist. Prof. of Oregon State University Tarek A. Kassim, who is sadly no longer with us to enjoy the final product of his ideas. Everything began as part of my role as co-editor of this series when I received the proposal from Tarek and I was asked to finalize the edition of the book. After receiving the proposal I waited and revised the manuscripts, also adding one from my own research group. So in all honesty the merit of this book belongs to the late Tarek Kassim. I hope Tarek's colleagues and friends can appreciate one of his last projects.

Sediments offer different functions within the environment. Bottom sediments provide a habitat for many aquatic organisms and function as an important component of aquatic ecosystems. Contaminants enter river systems through various pathways. *Point sources* of pollution are identifiable points that are (fairly) steady in flow and quality (over the time scale of years). The magnitude of pollution is not influenced by the magnitude of meteorological factors. Major point sources include: municipal wastewater effluents and industrial wastewater effluents. *Diffuse sources* are highly dynamic, spread-out pollution sources and their magnitude is closely related to meteorological factors such as precipitation. Major diffuse sources of pollution include: surface runoff (load from atmospheric deposition), groundwater, erosion (load from eroded material), and diffuse pollution loads derived from paved urban areas (atmospheric deposition, traffic, corrosion), including combined sewer overflows since these events occur discontinuously over time and are closely related to precipitation (it has to be pointed out that emissions from urban areas are also partly involved in the point source term; so these discharges are not constant in reality). Both point and diffuse sources of pollution contribute to the total contaminant load of rivers. In summary, sediments have been described as a sink or storage place for pollutants and as a source for contaminants to be introduced into the aquatic environment.

The book contains six chapters covering different aspects of the research on contaminated sediments such as its influence on the sustainable use of the Planet, the fate and behavior of typical sediment pollutants like polycyclic

aromatic hydrocarbons, chlorinated and brominated organic pollutants, the application of sediment toxicity identification evaluation (TIE) protocols and the various ways to degrade toxic pollutants in sediments, for instance by the use of nucleic acid-based techniques for studying the diversity of bacterial communities present in contaminated sediments.

Overall, the present book is certainly timely since the interest in contaminated sediments has never decreased in the EU, where the network SEDNET (European Sediment Research Network) organizes workshops on different sediment issues and in the U.S., Battelle, EPA and The US Army Corps of Engineers are also involved in a sediment symposium that covers different scientific and technical aspects of contaminated sediments.

This book provides new scientific information and critical overviews on how to monitor and remediate contaminated sediments. This book will be of interest to a broader audience of environmental chemists, analytical chemists, microbiologists and specially those that are already working in or planning to enter this field.

Finally, I would like to thank all the contributing authors of this book for their time and effort in preparing this comprehensive compilation of research papers that will make this book on contaminated sediments a reference book in this field.

Barcelona, September 2008 D. Barceló

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The Influence of Contaminated Sediments on Sustainable Use of the Planet

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Abstract Sustainable use of the planet requires systems-level thinking and data management over large temporal and spatial spans. The 20th century was an era of specialization; the 21st century is almost certain to be an era of integration, but this integration will require robust data about contaminated sediments and other components. Moreover, all essential information must be in a form that is understandable to and operational for decision-makers. Sediments are an important part of the physical subsystem that humans propose to use indefinitely (i.e., for an estimated 15 billion years). A living subsystem is closely associated with this physical subsystem. Humans must modify the management of this subsystem to protect the integrity of the geophysical, ecological, climatic, and cyclic (e.g., hydrologic) components of the planet's biospheric life support system if they expect to use it sustainably. Human requirements from natural capital (i.e., living systems) and the services it provides must not exceed the capabilities of the resource. To

this end, human economics and resource use must mesh with the economics of nature. Since resources are finite on a finite planet, human activities must be consistent with the ability of Earth's biospheric life support systems to absorb the effects of human activities. Sediments offer an excellent microcosm to develop this mutualistic relationship. A broad systems-level approach (i.e., the role of the sediment subsystem in the biospheric life support system) is a critical need for decision-makers. Some factors essential to meeting the needs of decision-makers involved with sustainable use of the planet are included.

Keywords Biospheric life support system · Cascading ecological effects · Decision-making · Ecotoxicology · Sustainability · Tipping points

Abbreviations
NRC National Research Council

1 Information Flow Between Different Levels of Organization

Increased ecological damage (e.g., toxic pollution, greenhouse gases, ecosystem breakdown) will result in exceeding one or more ecological "tipping points" [1]. Quite likely, these tipping points are interactive—that is, exceeding one will alter the threshold of others. In addition, societal and ecological tipping points are likely to be interactive, thus increasing both the difficulty of constructing robust predictive models and estimating the likelihood of cascading effects. Significant problems in any part of the system (e.g., contaminated sediments) are likely to result in a cascade of serious problems throughout the rest of the system. All components are important, but no component can be managed in isolation from other components.

Information flow organized in a timely, systematic, and understandable way (understood by all "stake holders") is the best way to keep both societal and ecological systems healthy and functioning. Information flow that keeps all those involved with components of the system informed both of events in the other components and of the long-term role of each component in the system's function is the best way to achieve sustainability. These information flows are now inadequate because: (1) professionals communicate most effectively with those having similar interests, and they are not prepared for communicating with those in "alien" professions, (2) not enough time or money is budgeted for meaningful interactions between and among diverse professions, (3) professional terminology is frequently difficult (hampering communication between disciplines), but individuals fear using simpler terminology because colleagues may accuse them of being simplistic, (4) the 20th century was an era of specialization, but the 21st century is emerging as an era of synthesis and integration of information [2]; both specialization and integrative efforts are essential to resolving systems-level issues, (5) university and government funding is typically allocated to specific disciplines and highly focused issues that are fragments of larger systems; very little time or

money is allocated to information flow among the array of components of the larger system in which the individual components are embedded.

Hardin was fond of saying "you can't do just one thing." Thus, solving contaminated sediments problems may create new problems in other areas if a systems-level perspective is excluded. Systems-level decision-makers are not likely to be highly knowledgeable about the complex information and specialized methodology required for evaluating contaminated sediments. Similarly, those who have such specialized knowledge are not likely to be well acquainted with all the factors a decision-maker must integrate. A primary objective of sustainability is indefinite use without abuse of the entire system, which includes both human society and natural capital plus the ecosystem services it provides. Contaminated sediments are a component of ecosystems that, in turn, are a part of Earth's biospheric life support system, which has maintained the atmosphere, the hydrologic cycle, and so on within limits highly favorable for humans for all of human history.

2 Sustainable Use

Exponential growth of the human population, substantially increased levels of affluence, and rapid advancements in technology have stressed the biospheric life support system to a degree unprecedented in human history. On a finite planet with finite resources, increased attention must be given to systems-level performance than was necessary when humans were spread more thinly over the planet. Successful implementation of this goal will increase the probability of leaving a habitable planet for posterity and providing a quality life for present generations.

If a system is to be used sustainably (i.e., indefinitely), no component can have significant adverse effects upon other components. Contaminated sediments in an aquatic ecosystem must not cause ecological disequilibrium that would disrupt either the quality or quantity of ecosystem services such as: (1) interfering with the flow of energy and materials in food webs, (2) releasing contaminants into the water column at concentrations that would adversely affect both the biota and chemical transformations, (3) changing the quality of water, even episodically, that is used for municipal, industrial, and agricultural purposes, or (4) diminishing natural capital (natural systems and their ecosystem services) by release of toxicants into aquatic systems.

A global systems-level study on sustainable transport has been developed by the World Business Council for Sustainable Development [3] and reports that although sustainable mobility can be achieved, it is beyond the capabilities of any one company, one industry, or one country to resolve. The same statement could be made for contaminated sediments or any other component of the biospheric life support system.

The preliminary statement of goals and conditions for a sustainable world are given elsewhere [4, 5]. A tentative statement of goals and conditions for contaminated sediments follows. Suspended solids can be managed to protect the integrity and health of the biospheric life support system, preserve and accumulate natural capital and enhance the ecosystem services it provides, and establish significant information flow between and among all levels of both biological organization [6] and all levels of societal organization. Whenever possible, contaminated sediments should be managed so that the ecosystems in which they occur are self-regulating [7], since these have lower management costs than do subsidized ecosystems.

Lake Biwa (Japan) has a noteworthy system of coping with contaminated sediments by removing them in such a way that they do not intrude into the water column. The sediments may then be immobilized by incorporating them into material used to pave roads (Lake Biwa Internet site: www.jnto.go.jp/eng/TTP/PTG/PS/pg-502.pdf).

It is regrettable that most publications on sustainable development are not explicit about time frames. About 4.55 billion years were needed for Earth to evolve into the planet of today; it may last another 15 billion years. Living organisms are thought to have appeared approximately 3.5 billion years ago. However, modern *Homo sapiens* has been on the planet about 130 000 years and archaic *H. sapiens* about 200 000 years. The last common ancestor of modern humans and Neanderthals existed about 600 000 years ago (http://www.calvin.linfield.edu/~mrobert/originsfigure17.htm). These numbers are impressive, but they represent a time span when the rates of technological and social change were rather slow. At present, the unprecedented experiment that is putting economics and ecology in conflict will probably continue through at least the first half of the 21st century. McNeill [8] remarks that the massive anthropogenic stress on Earth's biospheric life support system is indeed new. Even if a major shift occurs away from unsustainable practices, the rate of stress will still be felt for a significant period of time. A time management failure in any subsystem will almost certainly cause major problems in reaching sustainability goals. In addition, goals must be more explicitly stated—not only what may be left to posterity but the pre-established quality-control conditions that will verify whether these goals are being met in the time allotted to achieve the stated goals.

If sustainable use of the planet is achieved, it will be a dynamic equilibrium rather than a steady state. Many alternatives are available to present, unsustainable practices. Anderson [9] notes that no industrial company on the planet is sustainable, i.e., industries are meeting present needs but may be depriving future generations of meeting their needs to some degree. To develop sustainable industries, all contributors to the contaminated sediments problem will have to form an alliance to solve the problem. In addition, substantial investment must be made in "cradle to cradle" product management that will involve the participation of manufacturers beyond the life of their

marketable products. Hitachi (Japan) has a deproduction line—a collection and recycling process ensuring that component materials move through multiple "life cycles." When resources are removed from the biosphere, they must be reintroduced into the biosphere so that they do not harm the integrity and health of the system. Ideally, the reintroduction materials should enhance the accumulation of natural capital. This design would reduce harmful wastes that result in negative value for humankind and ecosystems. In a real sense, sustainability would mean replacing the wasteful once-through system with a recycling and less wasteful system. Creative product production might even result in societal wastes that enhance the health and integrity of ecosystems.

3 Connecting the Dots

Much of the planet's surface is a common ground (i.e., the oceans, large lakes, rivers, and land to which the public has access). If this definition is extended to include private property to which individuals and organizations can gain access for extracting resources by means of wealth, a huge part of the planet is common ground. Rivers, lakes, oceans, and the atmosphere into which pollutants are discharged are common grounds. Biomagnification and bioaccumulation of pollutants may both concentrate pollutants and transport them to even remote parts of the globe. As a consequence, the interactions among biospheric components must be considered in the goal of achieving sustainable use of the planet. The information about the components (e.g., sediments) must be available to and understood by those studying and managing the entire system (i.e., the biosphere). In addition, those working at the systems-level must communicate both their needs and their goals to the professionals investigating and managing the components. These are often referred to as top-down and bottom-up strategies [6]. As noted previously, the 20th century was an era of specialization, while the 21st century will probably be an era of integration in order to effectively manage the global system. Both approaches are essential and they are mutualistic rather than competitive.

More important for this discussion, ecosystems are complex systems with self-organized patchiness (i.e., diversity of habitat types) vulnerable to catastrophic shifts between ecosystem states [10]. Such changes can and have resulted in the collapse of powerful human societies [11]. Nineveh was the capital of the Assyrian Empire and was at its peak six centuries before Christ was born [12]. However, archaeologists [13] found only ruins and desolation. Nineveh failed because of a decline in its natural resource base. One underlying cause of the gradual deterioration of the entire region was deforestation in the hills and mountains, the source of the area's water supply. Another cause was environmentally unsustainable irrigation, which was causing salts to build up in the soils. The point is that powerful, rich, and thriving societies

collapsed by not paying sufficient attention to the health of the biospheric life support system upon which they depended.

Regrettably, predictive models for catastrophic ecological shifts are not robust. Some of the turning points towards sustainability (i.e., avoiding catastrophe) focus on transdisciplinary research designs that include both applied and theoretical information from such fields as sociology, ecology, economics, and law [14]. Although achieving a balance between technological and biospheric life support systems is a sine qua non of sustainable use of the planet [15], this concept will be extremely difficult to implement [16–18].

A report of the National Research Council (NRC) [19] has been discussed by Hoagland [20] and criticized for neglecting ecology because degradation of the environment is one of the crucial problems of the century. One anticipated loss is that of "biophilia" (i.e., the love of all life forms). Failure to connect the dots may result in degradation of the planet's biospheric life support system. Decisions about contaminated sediments must routinely include connections to components of the larger systems (ecological and societal) of which they are a part.

4 Carrying Capacity

In an era of exponential environmental change, a balance must be reestablished between anthropogenic environmental stress (e.g., pollutant loading of sediments) and human population size and quality of life. Hardin [21] defines *carrying capacity* as the level of exploitation that will yield the maximum return in the long run. Hardin uses a spaceship metaphor to make this point. A spaceship on a long voyage has a finite carrying capacity that, if exceeded, will cause hardship—even death—to the passengers. Closer to most individuals is the carrying capacity of planes, elevators, and bridges. Problems are avoided if each portion or component of the environment is used at less than its carrying capacity. Sediments have a carrying capacity for a variety of organisms, which will be reduced if that system is stressed by exceeding the carrying capacity. Problems will then arise, including the loss of valuable ecosystem services [22]. Loss of these ecosystem services reduces the carrying capacity for a variety of life forms, including humans.

A major environmental crisis exists at the global level, but the perceived consequences of making the wrong decision are not sufficiently severe to limit the number of bad decisions. In the era of the small family farm, the farmer was the decision-maker who suffered if he made a bad decision, i.e., a violation of natural law (e.g., exceeding carrying capacity) resulted in nature punishing the erring decision-maker. In the present nation/state era, elected or appointed politicians or managers are the designated decision-makers. They may escape the consequences of a bad decision entirely or, at worst,

share the suffering of humankind. If the consequences of a bad decision are distributed over millions or billions of people, the consequences laid on the erring decision-maker are effectively zero, but the aggregate effects may be enormous. In a very real sense, the decision-maker is not held responsible, and as a consequence, he or she will not assume responsibility unless a social contract exists that rewards good decisions and punishes bad ones. Globally, billions of individuals are poorly fed, housed, and educated because economic globalization has resulted in a global commons [23]. The global commons reduces personal responsibility to the point that it has little meaning. In fact, those who exploit the commons reap more short-term benefits that those who attempt to preserve the commons for posterity. The major problems facing humankind at present are associated with the pride and arrogance that led to the decline and collapse of ancient civilizations [11].

Contaminated sediments are one of the factors affecting carrying capacity. Moreover, each decision-maker for each component or factor must accept responsibility for each decision. Good decisions must be appropriately rewarded and bad decisions appropriately punished.

A major aspect of decision-making on carrying capacity is the climate of uncertainty associated with the outcome of stressing complex systems. A superb illustration is the management of lakes and reservoirs during global climate change [24]. Since sediments are an important component of both these aquatic ecosystems, the level of uncertainty of outcome resulting from anthropogenic toxic substances becomes a major issue in decision-making. This factor recently was the subject of a conference involving a number of academic institutions [25]. One response to this situation is precautionary decision-making [26]. Preventing harm to natural systems is superior to responding after harm has been done. Consequently, the basic objective is to determine the potential threats of harm from hazardous materials to both the environment and human health, and decision-makers must routinely cope with uncertainty. The precautionary principle states that when an activity raises threats of harm to human health or the environment, precautionary measures are justified, even if some cause-and-effect relationships are not fully established by science. Since precautionary measures are now generally accepted with regard to human health, it seems justified to assume they will increasingly be accepted for environmental health. After all, human health is strongly influenced by environmental health. Contaminated sediments already demonstrate this connection.

5
Cascading Effects of Toxics in Large, Complex Systems

Persistent toxics are often concentrated in the food chain and, thus, may appear in unexpected places. Elemental mercury may be transformed in anaero-

bic sediments to methyl mercury, which often moves through the food chain and reaches astonishing levels in polar bears and Eskimo mother's milk. As mentioned earlier, these concentrations may occur in places geographically distant from the site of toxic entry.

Robust examples indicate cascading effects of activities in both ecosystems [27] and human society. One of the best examples in the 21st century thus far is the effect of the actions of a few terrorists in the United States, which has had global effects. The use of hijacked planes to crash into the World Trade Center Towers and the Pentagon has affected plane travel and the feelings of vulnerability of the average citizen, has led to major airline security efforts that have increased the time passengers spend in airports, and has led to two major wars (in Afghanistan and in Iraq). Similarly, the postal system and many work places were disrupted by envelopes with a white powder thought to contain anthrax.

Another example of cascading ecological and social effects is anthropogenic gases that exacerbate global climate change. Increased hurricane intensity will probably have a major effect on the redistribution of contaminated sediments in both salt and freshwater ecosystems. Global climate change is expected to produce a significant increase in both the rainfall and intensity of hurricanes [28]. Persuasive evidence exists that monsoon rains in India, which results in both flooding and landslides, should change the distribution of contaminated sediments and increase sedimentation.

Many of the dams of the world contain contaminated sediments in the upstream pool of water. A team of engineers from the Nature Conservancy and the US Army Corps of Engineers plan to remove the major parts of the Cuddeback Dam on the Neversink River [29]. The unique feature of this project is the upstream diversion of water by means of a cofferdam, which will enable workers to move backhoes and large hydraulic hammers into the area to chip away at concrete. This activity will also permit the removal of contaminated sediments, although the news releases made no mention of this.

6
Multiple-Response Thresholds and Tipping Points

A toxicological or ecological threshold is one below which no observable deleterious effects occur. A variety of dose/response curves exist [30], and means are available for extrapolating from micro- or macroscale tests to natural systems [31]. Tipping points occur in situations in which the factors that create stability are overcome by the factors that create instability and the ship, vehicle, or system tips over into disequilibrium [32, 33]. If present trends continue, such factors as biotic impoverishment, depletion of natural capital, fragmentation of natural systems, and the increasing probability of major

global climate change may cause one or more tipping points to be reached in the 21st century.

Malfunction of any critical component of a complex system (the human body or social, economic, and ecological systems) is likely to result in malfunction of other components. Cascading effects are possible, and remedial measures may be too little and too late. The Kyoto Protocol on global warming illustrates this point quite well. This protocol requires collective emissions reduction of six greenhouse gases to greater than 5% below 1990 levels by 36 countries. The US was responsible for 36.1% of emissions from industrialized countries in 1990, but rejected the protocol. China, as a developing country, is not restricted as are the industrialized nations, even though China is a major polluter of the environment. More than 40 times the emissions reduction called for by the protocol would be required to prevent a doubling of the pre-industrial concentration of carbon dioxide in this century [34]. Global warming could easily produce cascading effects.

7
Ecotoxicology, Ecosystem Services, and Resilience

Ecotoxicology is the science of determining the effects of toxicants upon the biosphere or some complex subcomponent of it [35]. The use of the prefix *eco* affirms that these toxicity tests will involve endpoints characteristic of levels of biological organization higher than single species. Ecotoxicologists bear a number of responsibilities in projects related to sustainable use of the planet, as follows:

- Shift goals and endpoints from an absence of observable harm to persuasive evidence of ecosystem health;
- Increase both temporal and spatial scales of ecotoxicological studies;
- Achieve a critical mass of qualified personnel;
- Include demographic change in the ecotoxicological analysis;
- Develop new ecological thresholds;
- Be prepared for environmental surprises;
- Focus on design for a quality environment;
- Develop ecosystem services as endpoints in ecotoxicological studies;
- Be prepared for climate change and other events that might destabilize the biosphere and require major adjustment in the process of ecotoxicological testing [36].

Sediments in aquatic ecosystems provide a variety of services (e.g., transformation of complex organic substances). Regrettably, not all, perhaps even most, ecosystem functions have been identified and even those that have been identified generally do not have an information base adequate for estimating

the stress conditions (e.g., toxic substances) that would impair either quality or magnitude of these services. Anthropogenic wastes affect ecosystem health and integrity and, thus, affect the delivery of ecosystem services [37]. Sustainable use of the planet is based on the assumption that a sustainable, harmonious relationship between technological and ecosystem services can be achieved [15]. Cairns [38] has provided the rationale for using ecosystem services as endpoints for toxicity tests. Arguably, the most significant difference between present toxicity tests based on no-observable-deleterious effects and those based on ecosystem services is that the latter involve ecosystem health and integrity [37]. The importance of this distinction is highlighted by evidence that climate change is increasingly viewed as a security threat to human society [39].

The quest for sustainable use of the planet is not hopeless, despite the degradation of the biosphere, since humankind does have the means to halt and even partially reverse the many distressing trends. Guidance is available for establishing a suitable relationship between human society and natural systems [9, 26, 40–43]. Contaminated sediments are an important part of the quest for sustainable use of the planet, but they are just one component of a vast array of components. Information from all components must be integrated. All components of an ecosystem affect its resilience (the ability to return to its original form after being stressed). As a consequence, it is important to determine how sediments can be utilized without affecting ecosystem resilience. Ideally, microscale toxicity tests should be used, but much judgment is necessary in determining the specific attributes of microcosms and/or mesocosms used for this purpose. Ecosystem monitoring to provide an early warning of threats to ecosystem integrity and resilience should also serve to validate or confirm that predictive models based on microscale testing are valid or need to be redesigned. Sustainability will not be possible if significant portions of the planet's biospheric life support system lose resilience. Regrettably, ecosystem resiliency is poorly understood, and reducing ecosystem resilience is a very real possibility. Fortunately, an environmental catastrophe does not commonly occur in a sudden cataclysmic moment, although it is possible. Generally, such events occur from a succession of incremental changes that individually seem unimportant. For example, global warming, caused by anthropogenic activities, is literally the result of many billions of small decisions. Yet, these small decisions could and may cause major societal and ecological upheavals. Contaminated sediments usually are not the result of a single inappropriate decision, although such cases exist.

A single component, such as contaminated sediments, may have an episodic release of toxics that could push an entire system beyond its resiliency. Ecotoxicological tests in microcosms and/or mesocosms can reduce uncertainty about this threshold.

8
Restoring Damaged and Aquatic Ecosystems—The Role of Sediments

A number of economic issues are involved in river restoration [44]. Since Wackernagel et al. [45] provided preliminary but persuasive evidence that humankind's load is in excess of the planet's carrying capacity, ecological restoration has become extremely important. Ecological restoration would replace some, but probably not all, of the reduced carrying capacity, but cannot counter decreased per-capita carrying capacity due to increased human population growth and increased per-capita use of natural resources. Capital value of the industries that depend on Australia's Murry River is over $1.6 Australia billion. Dams, irrigation, and drought have dried the river, which has increased salinity. Restoration of water flows is essential for improving the health and integrity of the Murry River. The Norwegian Nobel Committee stated that "Peace on earth depends on our ability to secure our living environment" when it awarded Wangari Maathai the Nobel Peace Prize [46]. This statement represents the first time that the Nobel Committee has publicly acknowledged the crucial link between environmental health and peace.

However, restoring ecosystems to their pre-damaged condition may not be possible. One reason is that a complete and precise ecological inventory of an ecosystem is rarely available before it is damaged. A second reason is that many crucial species may be unavailable for recolonization if they are lost in a damaged ecosystem or if removing such species from other ecosystems would weaken the donor ecosystems and decrease their resilience. Berger [47] stated:

"It is axiomatic that no restoration can ever be perfect: it is impossible to replicate the biogeochemical and climatological sequence of events over geological time that led to the creation and placement of even one particle of soil, much less to exactly reproduce an entire ecosystem. Therefore, all restorations are exercises in approximation and in the reconstruction of naturalistic rather than natural assemblages of plants and animals with their physical environments."

Many toxic substances partition into sediments so that removal of contaminated sediments may be necessary as a major component of the ecological restoration process. The NRC [48] states:

"Often, natural resource restoration requires one or more of the following processes: reconstruction of antecedent physical hydrologic and morphologic conditions; chemical cleanup or adjustment of the environment; and biological manipulation, including revegetation and the reintroduction of absent or currently nonviable native species."

The NRC [48] also calls attention to the excavation marsh where bulldozers unearthed an old landfill that contained toxic materials. The sediment was sufficiently contaminated with lead that large quantities had to be trucked to a toxic waste dump at a large and unanticipated cost. Wetlands can both remove and transform organic and inorganic substances from inflow-

ing waters [49], but contaminants should not be so large and numerous that this function becomes unsustainable. Zedler and Langis [50] have developed a functional equivalency index based on 11 marsh attributes that would serve to determine how much of the functional attributes of a marsh (mostly sediments) are lost to contaminants. Hydrological needs and requirements of wetland plants and animals should be met [48]. Contaminated sediments are only one part of the problem in maintaining ecosystem services.

Since sediments function as both a sink and a source of organic and inorganic chemicals [51], they are a dynamic component of a larger dynamic ecosystem. Griffiths [52] discussed microbial enzyme functions, which are almost certainly also ecosystem services. Most ecological restoration projects focus on structural attributes, assuming that restoring structure will also restore function. However, if ecosystem services are regarded as important endpoints, it is essential to have robust evidence verifying this hypothesis. It is a sine qua non that this information should be integrated [53].

There are a number of important issues on restoring damaged ecosystems, as follows:

- Wackernagel et al. [45] provide evidence that human society has exceeded Earth's biospheric regenerative capacity. Where contaminated sediments contribute to this problem, restoration must include return to a close approximation of antecedent conditions.
- If even one component, such as contaminated sediments, is malfunctioning, the entire system may go into disequilibrium.
- There is inadequate robust evidence on ecosystem resilience, indicating that precautions are justified in avoiding both ecological and societal tipping points.
- If the human population continues to increase, so would the pressure on natural resources. The only major way to counter these trends is to restore as many damaged ecosystems as possible to a significant replica of their pre-damaged conditions.

9
Ecological Monitoring of Sediments for Sustainable Use

Monitoring is an activity common in human society. Intensive care patients in hospitals, production of pharmaceuticals and food supplies, industrial processes, and economic health are but a few examples. The purpose of monitoring is to confirm that previously established quality control conditions are being met. Monitoring for sustainable use of the planet is far more complex than monitoring for any other purpose, but the principle remains the same. All monitoring involves feedback loops that provide information about the system of concern. Expectations are that the system is functioning within ac-

ceptable limits. If it is not, corrective actions are initiated at once and are continued until normality returns.

Systems-level monitoring is already in the preliminary stages of development. The National Oceanic and Atmospheric Administration, the National Aeronautics and Space Administration, and the US Navy are developing an advanced imaging instrument (the hyperspectral environment suite) to provide critical data to resource managers who study coastal ecology and oceanography [54]. Basically, the instrument provides a shared collection of data designed to monitor coastal waters. Endpoints compatible with this systems-level endeavor from component subsystems, such as contaminated and uncontaminated sediments, will almost certainly get more attention from decision-makers than endpoints whose connections to the system are not clear.

All important components, such as sediments, should be monitored to provide an early warning of threats to the entire system. It is equally important to monitor entire systems because attributes occur at more complex levels of organization that are not exhibited at lower levels. These attributes could provide verification that monitoring at lower levels of organization is functioning as expected or that it is not. Very often, this information will be helpful in determining which components should have a reevaluation of their monitoring endpoints.

When using any monitoring system, precautionary measures should be taken to minimize the number of false-negatives and false-positives. A false-negative indicates that previously established quality control conditions are being met when they are not being met. As a consequence, the system is at risk, but managers have no information to indicate this risk. A false-positive indicates the system is at risk when it is not. False-negatives and false-positives are most common in the early developmental stages of a monitoring system. Multiple lines of monitoring evidence provide considerable protection against both false-negatives and false-positives. Clearly, the number of attributes monitored should be largest at the outset of the monitoring program. Then, a determination can be made of the value of the various types of information. A certain level of information redundancy is useful, but such judgments should be appropriate for each situation. Regrettably, there is a paucity of monitoring efforts for most ecosystem components, and sediments are no exception.

10
Natural Capital, Industrial Ecology, and Ecosystem Services—The Role of Sediments

Natural capital consists of living systems, the ecosystems services they provide, and other natural resources. Human capital is both natural resources and living systems, plus the social and cultural systems. The value of hu-

man capital is usually underestimated. For example, the *Wealth Index* of the World Bank [55] found the sum value of human capital to be three times greater than all the financial and manufactured capital on the global balance sheets. Attempts to place a value on natural capital is both difficult as well as imprecise, but Costanza et al. [56] have made a persuasive effort to do so. Anderson [9], a successful businessman, provided leadership in the movement to persuade corporations to function more sustainably. Anderson's approach diminishes the probability of releasing toxic chemicals that contaminate sediments.

Natural systems are dynamic, so natural capital and the services it provides are not constant [57]. Brunckhorst [14] remarks that, over major time spans, both social and ecological systems reach crisis points at which future options are no longer available because of damage to natural resources. Those decision-makers who are prepared to make mid-course corrections and that have a high adaptive capacity possess a higher probability of maintaining sustainability. Natural disasters, resulting from natural episodic events, may block or impair sustainability [58].

A good way to monitor ecological stress caused by human activities is the ecological footprint [42]. Wackernagel et al. [45] have reported that the human economy has overshot the regenerative capacity of the biosphere, which, if it persists, could result in global ecological disequilibrium, making sustainability even more difficult, even impossible. Information on contaminated sediments must be integrated into the larger body of information essential to first achieve and then maintain sustainability.

11
Meeting the Requirements of Decision-Makers

No single approach is appropriate for all decision-makers. Ecosystem information must often meet both scientific and regulatory requirements [59]. Frameworks are available to provide guidance for types of information suitable for various types of decisions [60]. This undertaking is relatively new and is in the early stages of development [38]. Since the decisions involve dynamic systems, approaches must be continually adaptable to new conditions. However, much can be learned from ecological history [61].

The World Wildlife Fund has asserted that humankind is now consuming Earth's resources at a rate that is overwhelming the planet's capacity to support life [54]. The average ecological footprint is, at present, 2.2 ha per person, although only 1.8 ha of land per-capita is available to provide natural capital and the ecosystem services it provides [62]. An ecological overshoot will require vastly different decisions than merely not exceeding Earth's carrying capacity for humans. An overshoot will require restoring natural capital until it is adequate for long-term sustainable use.

Many nation-states are demonstrating increased interest in the precautionary principle as a means of regulating risk, especially if the decision must be made while there is still significant uncertainty about the evidence. The precautionary principle connects science and policy to protect human health and the environment. The 1998 Wingspread Statement states: "When an activity raises threats of harm to human health or the environment, precautionary measures should be taken even if some cause and effect relationships are not fully established scientifically" [26]. Often, additional protection is provided for human health before it is for environmental health. Ellman and Sunstein [63] caution that the precautionary principle is designed to give safety the benefit of the doubt and create margins of safety that may or may not materialize. The precautionary principle runs into serious difficulty when risks appear on all sides of the relevant situation [63]. Ricci et al. [64] remark that the principles of precaution do not explain how to make choices or how to identify what is protective when incomplete and inconsistent scientific evidence of causation characterizes potential hazards. Some discussion has emerged on climate decisions in a context of uncertainty [44]. All professionals working with contaminated sediments and all decision-makers would benefit from researching decision analysis [65]. Cairns et al. [66] have provided a sequential hazard-assessment procedure that results in increasingly narrow confidence limits for both estimates of a no-biological-effect concentration and expected environmental concentration of potentially hazardous substances.

Contaminated sediments are embedded in a much larger system with which all component decisions must be congruent. Risks in one component (e.g., contaminated sediments) must be balanced with risks in other components (e.g., water column). All risks and hazard evaluations are probabilistic and require value judgments.

12 Epilogue

Sustainable use of the planet requires systems-level thinking and planning over large temporal and spatial spans. If any component (e.g., sediments) malfunctions, the entire system is at risk. Decision-makers must balance risks to one component against risks to the other components and to the larger system. Significant depletion of natural capital and its ecosystem services endangers the quest for sustainability. If ecosystem resilience is not impaired, restoration of natural capital and ecosystem services is possible. Sustainable use of the planet requires continuous quality-control monitoring of a variety of metrics. A synthesis of this information is essential to effective decision-making.

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Chlorinated and Brominated Organic Pollutants in Contaminated River Sediments

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Abstract Sediments are the main abiotic reservoirs where POPs from different emission sources are accumulated. In the last few decades, a great deal of emphasis has been placed on evaluating the contamination of "classical" POPs such as PCBs, PCDDs and PCDFs. More recently, scientific interest has been focused on other groups of POPs, the so-called "emerging" POPs, such as PCAs and brominated POPs. These emerging POPs are of concern due to their toxicological properties, their capability to bioaccumulate and

their widespread and unrestricted use. Knowledge of the environmental impact of these emerging POPs is based on previous analytical work that focused on the development of sensitive and selective methodologies. Herein, an overview of current analytical methods, including different sample preparation techniques as well as the different instrumental approaches, is presented. Finally, a review of the available data concerning the occurrence of chlorinated and brominated POPs in sediments is also reported. Conclusions and future perspectives are also outlined.

Keywords Brominated flame retardants · Chlorinated paraffins · Persistent organic pollutants · Polychlorinated naphthalenes

Abbreviations

ASE	Accelerated solvent extraction
BFR	Brominated flame retardant
BSEF	Bromine Science Environmental Forum
BTIE	Bioavailable toxicity identification evaluation
CALUX	Chemically activated luciferase expression
CP	Chlorinated paraffin
DLC	Dioxin-like compound
DDT	Dichlorodiphenyltrichloroethane
dw	Dry weight
ECNI	Electron capture negative ionization
EI	Electron ionization
EROD	7-Ethoxyresorufin *O*-deethylase
GC	Gas chromatography
GCxGC	Two-dimensional gas chromatography
HBCD	Hexabromocyclododecane
HRMS	High-resolution mass spectrometry
IAC	Immunoaffinity chromatography
IT	Ion trap
K_{OW}	Octanol/water partition coefficient
LC	Liquid chromatography
LCCP	Long-chain chlorinated paraffin
LOD	Limit of Detection
LRMS	Low-resolution mass spectrometry
MAE	Microwave-assisted extraction
MCCP	Medium-chain chlorinated paraffin
MS	Mass spectrometry
MS-MS	Tandem mass spectrometry
NCI	Negative chemical ionization
PBB	Polybrominated biphenyl
PBDD	Polybrominated dibenzo-*p*-dioxin
PBDE	Polybrominated diphenyl ether
PBDF	Polybrominated dibenzofuran
PCA	Polychlorinated *n*-alkane
PCB	Polychlorinated biphenyl
PCDD	Polychlorinated dibenzo-*p*-dioxin
PCDF	Polychlorinated dibenzofuran
PCN	Polychlorinated naphthalene

PLE	Pressurized liquid extraction
POP	Persistent organic pollutant
ppb	Parts per billion
ppq	Parts per quadrillion
ppt	Parts per trillion
QqLIT	Quadrupole linear ion trap
QqQ	Triple quadrupole
SCCP	Short-chain chlorinated paraffin
SIM	Selected ion monitoring
TBBPA	Tetrabromobisphenol A
TEF	Toxic equivalent factor
TIE	Toxicity identification evaluation
ToF	Time-of-flight
WHO	World Health Organization
2,3,7,8-TCDD	2,3,7,8-Tetrachlorodibenzo-*p*-dioxin

1 Introduction

Persistent organic pollutants (POPs) have been shown to exhibit potentially harmful effects in man and the environment. In addition to being persistent, POPs are typically lipophilic (therefore, bioaccumulative), semivolatile and toxic. Some of these POPs have been deliberately produced by the industry for a wide variety of applications [i.e., pesticides, polychlorinated biphenyls (PCBs), polychlorinated naphthalenes (PCNs)]. Other chemicals, such as brominated flame retardants (BFRs) are still produced in large quantities for use in electric equipment, plastics and building materials. Others are accidentally formed or eventually released as a byproduct from various activities, such as industrial or combustion processes [i.e., polychlorinated dibenzo-*p*-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs)]. Since 1995, the international community has been working on a legally binding instrument to eliminate POPs. Different organizations initiated an assessment process, which, in December 2000, resulted in the conclusion of the text for the POPs convention. Initial action is directed at 12 POPs, including aldrin, chlordane, dichlorodiphenyltrichloroethane (DDT), dieldrin, endrin, heptachlor, hexachlorobenzene, mirex, toxaphene, PCBs and PCDDs/PCDFs. However, future obligation will be to add other POPs as new evidence becomes available.

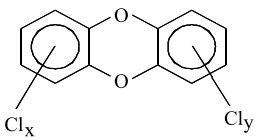
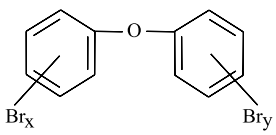
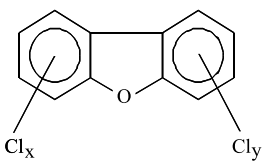
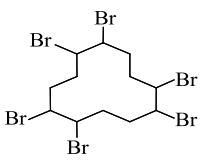
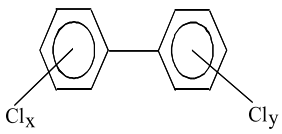
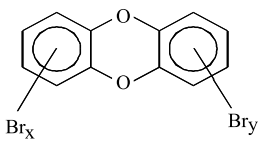
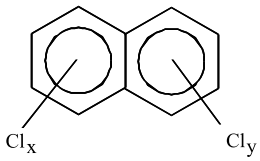
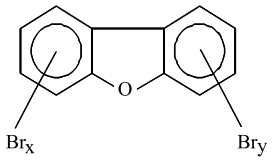
Within classical POPs, chlorinated compounds are the most relevant, standing out are PCBs, PCDDs and PCDFs. However, among the emerging POPs, different brominated compounds have attracted the most scientific interest during the last decade. This work will focus on the most relevant chlorinated and brominated organic pollutants in river sediments.

2
Chlorinated Organic Pollutants

2.1
Polychlorinated Dibenzo-*p*-dioxins (PCDDs) and Dibenzofurans (PCDFs)

PCDDs and PCDFs constitute a class of ubiquitous pollutants with aromatic structure, high chemical stability and extremely poor water solubility. They can occur in the form of 75 PCDD congeners and 135 PCDF congeners (Table 1). At present, most PCDD and PCDF sources are well characterized. These sources include chemical, thermal, photochemical and enzymatic reactions. Combustion processes, mainly incineration plants such as municipal solid waste incinerators, clinical waste incinerators and industrial waste incinerators are known to be some of the most important sources responsible for the presence of these contaminants in the environment. Soils and sedi-

Table 1 Structure of selected chlorinated and brominated POPs

Chlorinated POPs		Brominated POPs	
PCDDs $x + y = 1–8$ 75 congeners		PBDEs $x + y = 1–10$ 209 congeners	
PCDFs $x + y = 1–8$ 135 congeners		HBCDs 3 isomers	
PCBs $x + y = 1–10$ 209 congeners		PBDDs $x + y = 1–8$ 75 congeners	
PCNs $x + y = 1–8$ 75 congeners		PBDFs $x + y = 1–8$ 135 congeners	

ments are the main abiotic reservoirs where PCDDs and PCDFs from different emission sources are accumulated.

It is well known that dioxins constitute a group of lipophilic, persistent, ubiquitous, and bioaccumulative environmental chemicals exhibiting a broad spectrum of biological (high toxicity) and chemical (long-range transport) effects. Dioxins have been referred to as "the most toxic man-made compounds". The International Agency for Research on Cancer named 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (2,3,7,8-TCDD) a known human carcinogen [1]. Although 210 different PCDDs and PCDFs are possible with one to eight chlorines, only 17 of these congeners are considered to be toxic. The most toxic molecules are those that contain from four to eight chlorine atoms and, in particular, those in which positions 2, 3, 7 and 8 are chlorinated. Since the individual toxicity of these compounds is different, the real toxicity of a mixture is best assessed bearing in mind the relative toxicity of the isomers with respect to the most toxic isomer, the 2,3,7,8-TCDD. There are a number of toxic equivalent factor (TEF) tables proposed by different organizations. Today, it is well accepted to use the TEFs proposed by the World Health Organization (WHO) in 1998, called WHO-TEFs [2].

2.2 Polychlorinated Biphenyls (PCBs)

PCBs are industrial contaminants formed as a result of human activities. They were widely used as industrial chemicals, particularly as dielectric fluids in electrical transformers and capacitors, hydraulic fluids, lubricating and cutting oils, and as additives in sealants, plastics, paints, copying paper, adhesives and casting agents. PCBs were commercially produced as complex mixtures from 1929 onwards under various trade names (e.g., Acelor, Aroclor, Clophen, Montar, PCBs, Sobol, Turbinol, etc.). Although uses of these ubiquitous contaminants have been banned in industrialized countries since the late 1970s, their continued presence in the environment poses considerable hazards. Their physical and chemical properties vary widely across the class, but all PCB congeners have low water solubility and low vapour pressure. They are highly soluble in non-polar solvents, oils and fats. Because of their high thermodynamic stability, all degradation mechanisms are difficult, and environmental and metabolic degradation is generally very slow. Their thermal stability and resistance to degradation contributed to their commercial usefulness, but also to their long-term environmental effects. Similar to dioxins, PCBs have accumulated in soils and sediments.

By varying the number and the position of the chlorine atoms, there are 209 theoretical PCB congeners (Table 1). Most of these have been shown to be present in the PCB mixtures that have been available on the market. Recently, interest has been focused on 12 congeners (four non-*ortho* and ten mono-*ortho*) due to their similar toxicological properties to 2,3,7,8-TCDD.

2.3 Polychlorinated Naphthalenes (PCNs)

PCNs are a group of compounds composed of two fused benzene rings (naphthalene) with one to eight chlorine substitutions. There are a total of 75 possible PCN congeners (Table 1), which have physical and chemical properties such as melting point, volatility, water solubility, octanol/water partition coefficients and bioconcentration factors, favouring their environmental persistency and bioaccumulation. These products have properties and uses similar to those of PCBs. PCN formulations have been used in industry as dielectric fluids in capacitors, transformers and cables. The production of technical PCN mixtures has ceased in many countries, but they are still found, for example, in electrical equipment. In addition, PCNs are formed and released into the environment via other processes. There are three main sources of these contaminants: (a) The use of technical PCN products manufactured in different countries under various trade-names such as Halowax (Koopers Company, USA), Seekay Wax (Imperial Chemical Industries, UK) and Nibren Wax (Bayer, Germany) accounts for most of the direct or indirect input into the global environment. (b) Commercial PCB products, such as Aroclor or Clophen, which have, at the ppm-level, PCNs as by-products. (c) PCN formation during high-temperature processes. The total world-wide production of PCNs from the above-mentioned sources has been roughly assessed at 150 000 tons (technical PCNs), plus 100 tons (technical PCBs), plus 1–10 tons (thermal formation in this century) [3].

2.4 Polychlorinated *n*-Alkanes (PCAs)

Polychlorinated *n*-alkanes (PCAs), also known as chlorinated paraffins (CPs), are a class of industrially prepared mixtures of the general formula $C_nH_{2n+2-z}Cl_z$. These mixtures have a chlorination degree between 30 and 70 wt. %, and a linear alkane chain with length of C_{10}–C_{13} (short-chain chlorinated paraffins, SCCPs), C_{14}–C_{17} (medium-chain chlorinated paraffins, MCCPs) or $C_{>17}$ (long-chain chlorinated paraffins, LCCPs). The number of theoretically possible congeners, homologues, diastereomers and enantiomers is unknown, but by far exceeds 10 000 compounds [4]. PCAs have been produced in technical formulations since the early 1930s. Because they are produced with free radical chlorination, a single PCA formulation consists of thousands of different compounds with a range of physical-chemical properties. They are used for a variety of industrial applications, including lubricating additives in plastics, adhesives, sealants, paints, cutting oil additives and flame retardants [5]. The world production of PCAs has shown a slow

growth over the last decades from ca. 230 000 tons per year in 1977–1979 [6] to ca. 300 000 tons per year in 1997 [7].

PCAs have physical and chemical properties that are similar to other high molecular weight organochlorine pollutants, such as PCBs and DDT. Water solubility was estimated based on octanol/water partition coefficient (K_{OW}) correlations, and an apparent inverse relationship between carbon chain length and water solubility was noticed with values ranging from 0.49 to 1260 μg/l for SCCPs, from 0.029 to 14 μg/l for MCCPs, and from 1.6×10^{-6} to 0.086 μg/l for LCCPs [8]. The Cl substitution pattern had also significant effects on water solubility, and in contrast with known trends for chlorinated aromatic compounds, an increasing water solubility with increasing degree of chlorination up to 5 chlorines was reported [9].

Toxicity of PCAs appears to be inversely related to carbon chain length and because of this much attention has been given to SCCPs. Although PCAs generally have shown low toxicity to mammals, SCCPs have a carcinogenic potential in rats and mice [10]. However, no evidence of carcinogenicity was found for MCCPs and LCCPs. In addition, dose-response studies have shown that oral intake of SCCPs by mice results in an increase in liver weight. Moreover, in some studies, C_{10}–C_{12} CPs with 58% chlorine content caused growth inhibition and reproductive effects. Bioconcentration factors are high, reaching values of nearly 1.4×10^5 in mussels with polychlorinated dodecane with 69% chlorine content [11]. Greater bioconcentration factors were found for SCCPs, probably due to their greater water solubility. Moreover, highly chlorinated SCCPs are predicted to have the greatest bioconcentration factors because they are more hydrophobic and resistant to biotransformation than lower chlorinated PCAs, and their accumulation is not hindered by a large molecular size or extremely high K_{OW}, as observed for MCCPs and LCCPs [12].

3 Brominated Organic Pollutants

In many materials, brominated compounds have proved more suitable as flame retardants than chlorinated ones. Brominated flame retardants (BFRs) sometimes make up as much as 10–30% of the plastics used for example in the printed circuit board and housings of computers and other electrical and electronic equipment. Large-scale computerization in the 1970s and 1980s, combined with more stringent fire safety standards, resulted in rapidly growing use of these chemicals. The first BFR to be introduced included polybrominated biphenyls (PBBs), a group of compounds with the same structure as PCBs. Following an accident in the United States in 1973 (PBBs had mistakenly been sold as a feed supplement, resulting in large-scale poisoning of cattle and chickens) polybrominated

diphenyl ethers (PBDEs) assumed the position of most important category of BFRs.

3.1 Polybrominated Diphenyl Ethers (PBDEs)

Like PBBs and PCBs, this group comprises a total of 209 theoretically possible congeners (Table 1). The commercial PBDE mixtures are nominally deca-, octa-, and pentabrominated. Penta-BDE formulation consists of 41–42% tetra-BDEs (mainly BDE-47) and 44–45% penta-BDEs (predominantly BDE-99 and BDE-100), whereas deca-BDE formulation consists mainly of BDE-209 (97–98%), with a small amount of nona-BDES (0.3–3%) [13]. Some 67 000 tons were manufactured in 1999 [14], an amount rivalling PCBs at the height of their production.

Structural similarity to other environmental chemicals with known toxic effects (PCBs, PCDDs, PCDFs) could indicate that also PBDEs could be harmful to health. The acute toxicity of PBDEs is low. However, there is concern for its long-term effects on the endocrine system. Since 1994, PBDEs have been listed as compounds that can affect the regulation of thyroid and steroid hormones. Several studies indicate that commercially obtained tetra- and penta-BDE are endocrine disrupters, which can exert effects on the thyroid system. The effects of penta-BDE on thyroxine and the thyroid gland are considered to be principally due to the induction of liver enzymes, although several mechanisms may operate. The liver appears to be sensitive, and for penta-BDE, a no-observed-adverse-effect level of 1 mg/(kg bw day) has been determined, with effects evident at 2 mg/(kg bw day). Meerts et al. [15] have reported on estrogenic activities on PBDEs and hydroxylated PBDEs as determined in the human T47D breast tumor cell line stably transfected with an estrogen-responsive luciferase reporter gene construct. A commercial penta-PBDE mixture has been reported to reduce circulating thyroxine and to induce rat liver 7-ethoxyresorufin *O*-deethylase (EROD) activity in the parent animal as well as in the offspring [16].

Of the three main technical mixtures in use, the penta-BDE and octa-BDE mixtures are currently being phased out in Europe. Consequently, a shift in production of deca-BDE mixtures for these lower brominated PBDE mixtures has taken place. A recent report of The Bromine Science Environmental Forum (BSEF) estimated the total market demand for the major commercial BFRs in 2001 [17]. This shows the dominance of tetrabromobisphenol-A (TBBPA) (59% of total world usage) and the deca-mix PBDE formulation (27% of total world usage) in volume terms.

3.2
Hexabromocyclododecane (HBCD)

Recent reports also suggest that usage of hexabromocyclododecane (HBCD) is increasing [18] and that because attention is now switching to this compound which is widely used. The physical-chemical properties of HBCD (Table 1) are similar to PBDEs and other persistent organic pollutants, in fact the $\log K_{ow}$ of HBCD is 5.6 and that value places it in the optimum range for bioaccumulation [19]. Thus, HBCD is not covalently bonded to the material leading to the risk of migration out of the product during use or disposal [20]. On the basis of these properties, there is a high potential for this material to absorb to soil and sediments.

Technical 1,2,5,6,9,10-HBCD is produced industrially by addition of bromine to *cis-trans-trans*-1,5,9-cyclododecatriene, with the resulting mixture containing three predominant diastereoisomers α-, β- and γ-HBCD. Normally, the γ-isomer is the most dominant in the commercial mixtures (ranging between 75 and 89%), followed by α- and β-isomer (10–13% and 1–12%, respectively) [21, 22].

The dissimilarities in the structure of the α-, β- and γ-isomer might raise differences in polarity, dipole moment and in solubility in water; for example the solubility of α-, β- and γ-HBCD in water was 48.8, 14.7 and 2.1 μg/l, respectively. These different properties may explain the differences observed in their environmental behaviour [21]. In sediments, the stereoisomeric profile of HBCD is similar to that on commercial HBCD formulations, with γ-isomer being the most abundant stereoisomer. In contrast to sediments, the α-isomer is the most prominent stereoisomer in the majority of aquatic invertebrate and fish samples [23, 24].

3.3
Polybrominated Dibenzo-*p*-dioxins (PBDDs) and Dibenzofurans (PBDFs)

Polybrominated dibenzo-*p*-dioxins (PBDDs) and dibenzofurans (PBDFs) are the bromine homologues of PCDDs and PCDFs (Table 1). Thus, depending on the number and position of bromine atoms, there are 75 PBBD and 135 PBDF congeners. PBDDs and PBDFs occurred as trace contaminants in BFRs (e.g., PBDE or PBB) and were produced during combustion of these chemicals. They are formed when organics are incinerated in the presence of bromine. Combustion of organics in the presence of both bromine and chlorine results in the formation of mixed (i.e., bromo-, bromo/chloro- and chloro-) halogenated dibenzo-*p*-dioxins and dibenzofurans. There are 4,600 potential mixed congeners.

Incineration at sufficiently high temperatures under well-controlled conditions will destroy the BFRs, but less well-controlled incineration may result in the formation of limited quantities of PBDDs, PBDFs and bromochlorodi-

benzo-*p*-dioxins and furans [25, 26]. Different studies showed that bromine substitution appears to have a stronger toxic effect than chlorine substitution. In the chemically activated luciferase expression (CALUX) bioassay the brominated analogue of 2,3,7,8-TCDD showed equivalent activity to their chlorinated analogue [27]. Behnisch et al. [28] published a comparative study of the activity of PBDD/F congeners and their chlorinated homologues (PCDD/F). In the case of dioxins, similar potencies were obtained for the chlorinated and brominated congeners. However, significant differences were detected for the furans, with greatest activities for brominated furans.

4
Analytical Methodologies

The detection and quantification of chlorinated and brominated POPs in environmental samples requires sensitive analysis techniques. There are two main approaches for determinations, the first one focused on the congener specific analysis (chemical analysis), and bioanalytical methods that directly provide a total toxicity value. Chemical analytical methods are expensive and require sophisticated instrumentation and considerable effort in the sample preparation. However, the main advantage of chemical analysis is that it provides information on congener distributions and levels, which is very useful in identifying sources of the contaminants responsible for increased levels and risk management. Unlike chemical analysis, bioanalysis provides information on the total activity of the samples under study, without providing information on the levels of individual congeners. The main advantage of these methods is their high throughput rate and low cost. These characteristics make bioassays valuable screening techniques.

4.1
Chemical Analysis

The majority of POPs occur in very limited quantities in the natural environment [from parts per billion (ppb) to parts per quadrillion (ppq)]. To be able to detect such pollutants, therefore, it is first of all necessary to increase their concentration in a small portion of the sample collected. This process of sample preparation is often appreciably time-consuming. However, another property of pollutants of this type is more helpful to the analyst: being persistent, they are not destroyed even if the sample is subjected to fairly rough treatment. Moreover, the analytical methodologies are especially difficult due to the complexity of the mixtures of congeners: 75 PCDDs, 135 PCDFs, 209 PCBs, 75 PCNs, 209 PBDEs, etc. The different toxicity of each congener requires the development of congener-specific methods. Overcoming all these analytical problems has only been possible with the application of rigorous

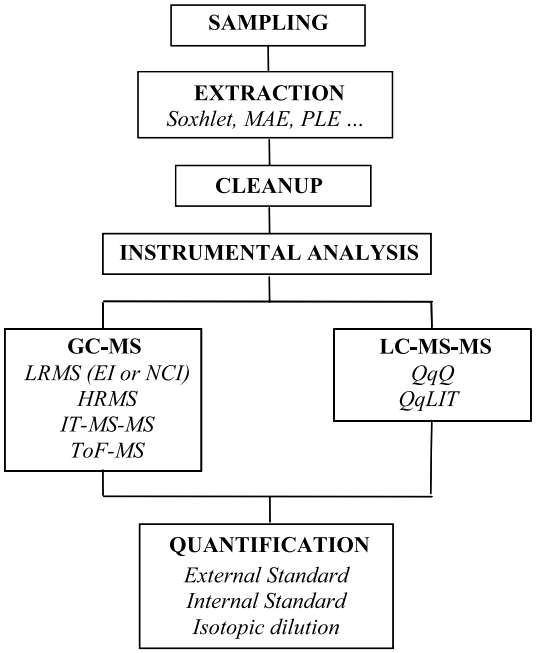


Fig. 1 Principal steps of the analytical methods used for the analysis of chlorinated and brominated POPs

cleanup schemes and by using gas chromatography (GC) coupled to mass spectrometry (MS). The cleanup steps provide a suitable removal of the bulk matrix and some interfering compounds; the GC allows an appropriate separation between the different congeners, and MS affords a sensitive and selective method of detection. An analytical protocol to determine chlorinated and brominated POPs includes the steps shown in Fig. 1. As can be seen, instrumental analysis is divided into two different options: GC-MS and liquid chromatography (LC)-MS. Whereas POPs were classically analyzed using gas chromatographic techniques, recently some liquid chromatographic methods have been introduced due to the occurrence of some emerging POPs such as HBCD, for which isomeric determination must be carried out by LC-MS.

4.1.1 Extraction

In the environmental analysis of chlorinated and brominated compounds, substantial analyte enrichment is necessary to isolate the target compounds from the matrix and to achieve the detection limits required. For trace analysis of these contaminants, Soxhlet extraction is widely accepted as a robust liquid–solid extraction technique. However, the main drawback of this tech-

nique is the fact that refluxing with cold solvent is time consuming (up to 48 hours). Furthermore, the solvent consumption is considerable ($\sim$ 300 ml), demanding evaporation of a large amount of solvent before subsequent cleanup. Over the past few years, efforts were made on the development of extraction techniques that allow efficient extraction along with reduced solvent volumes in shorter times, incorporating high levels of automation. The traditional techniques face severe competition due to the development of new techniques, such as pressurized liquid extraction (PLE) [commonly named accelerated solvent extraction (ASE)] and microwave-assisted extraction (MAE). In PLE systems, the liquid state of solvent extractant is maintained at elevated temperatures by application of a moderate pressure. Optimization of extraction conditions is facilitated as organic solvents recommended in traditional techniques can usually be used and pressure has little influence. In addition, these systems offer a high level of automation although only one extraction at a time can be conducted.

A review of the PLE applications for the extraction of moderately and nonvolatile organic pollutants from a variety of solid environmental matrices has been published [29]. In this review we can find studies on the analysis of PCBs, PCDDs and PCDFs in sediments. But PLE has also been applied to the PCA determinations. PLE was tested for the determination of SCCPs in sediment cores from six lakes in Canada [30]. These samples were extracted with CH_2Cl_2 at a temperature of 100 °C and at a pressure of 2000 psi for 30 min. Tomy and Stern [31] proposed a PLE method for the extraction of MCCPs in different environmental matrices. Extractions were carried out with CH_2Cl_2/hexane (1 : 1) at a temperature of 100 °C and at a pressure of 136 atm. The length of the extraction was 30 min and the volume of extract $\sim$ 60 ml. Recoveries of MCCPs were good (79–108%), with coefficients of variation ranging from 14 to 21%. As regards brominated compounds, PLE uses have also been reported [32, 33]. In these reports, CH_2Cl_2 and hexane : CH_2Cl_2 mixtures were applied as solvent extractors. The temperature and pressure used varied from 100 to 150 °C and from 1000 to 1500 psi, respectively. PBDE recoveries obtained using the PLE method are similar to those obtained using the conventional Soxhlet extraction. However, it should be pointed out that lower standard deviations were found with PLE, probably due to the automation of the system.

However, in most of the reported applications of PLE, an exhaustive cleanup of the extracts prior to injection in the chromatographic system is necessary [34, 35]. In an attempt to eliminate this time-consuming step, some authors proposed in-cell cleanup by packing the sample dispersed in an adsorbent, such as modified silica, Florisil or alumina [36–38].

On the other hand, MAE affords the opportunity of performing several simultaneous extractions with a closed-vessel system (pressurized MAE). The cell containing the sample and the liquid solvent is subjected to microwave radiation that enables instantaneous and efficient heating in the presence of

microwave-absorbing compounds. However, this technique requires further filtration to obtain the final extract. MAE has been tested by a number of laboratories for the extraction of PCDDs, PCDFs and PCBs [39, 40]. The considerable saving in extraction time (up to 1 h), solvent consumption (typical volumes are $\sim$ 30 ml) and energy, in addition to the reduction in generated waste, has made MAE a very attractive alternative to the conventional Soxhlet procedure. MAE was also used for the determination of SCCPs in river sediment samples [41]. Maximum extraction efficiencies were obtained using 30 ml of hexane/acetone (1 : 1) as solvent extraction. Extraction time and extraction temperature were also optimized. The highest recovery for SCCPs was found at 15 min, and the recoveries increased with temperature, reaching their maxima at 115 °C. Relative standard deviations of 7 and 9% were obtained for run-to-run and day-to-day, respectively.

However, the major drawback of PLE and MAE is the high investment cost of the commercialized systems.

4.1.2 Purification

The crude extracts contain a substantial amount of interfering substances. As a consequence, subsequent cleanup and fractionation is indispensable. The cleanup step is typically based on solid–liquid adsorption chromatography in open columns using a combination of different adsorbents. Among the stationary phases currently commercially available, the most commonly used are modified silica, Florisil, alumina and different types of carbon (Amoco PX-21, Carbosphere, Carbopack). However, the whole procedure is time and labour intensive, and it represents the bottleneck of the analytical method. The development of sample-handling techniques is directed, on the one hand, toward automation, and from another, toward development of more selective adsorbents such as immunosorbents. Automated cleanup systems were developed based on the use of pressured column chromatographic procedures. This is regarded as an alternative system that offsets most of the disadvantages of the conventional cleanup methods given its capacity for processing automatically different samples simultaneously in approx. 1.5 h [42, 43]. This system was recently tested for PBDE analyses. It is based on the sequential use of multilayer silica and basic alumina adsorbents respectively, pre-packed in Teflon columns. The automated system configuration consists of a valve module, a valve drive module and a pump. The whole system is computer controlled and can be programmed as required (i.e. volume, flow rates, direction of solvent flow ...).

Immunoaffinity chromatography (IAC) is one approach that has been investigated to simplify dioxin cleanup [44–46]. Immunoaffinity columns have been generated from anti-dioxin antibodies and shown to selectively bind dioxins from samples. A monoclonal antibody column showed acceptable re-

coveries and reliable quantification for five of the most toxic dioxins and furans at the sub parts per trillion (ppt) level. When compared to classical cleanup and isolation methods, the IAC procedure was more than 20-times faster and used 100-times less organic solvents, and their selectivity was enormously enhanced. The application of IAC to PCB analysis has shown similar potential in a preliminary study [47]. Limitations of IAC include its incompatibility with high fat matrices and the lack of selectivity for all 17 toxic congeners.

4.1.3 Chromatographic Separation

The method of choice for the determination of many halogenated contaminants is GC because the volatility of these compounds allows a GC determination. The development of capillary columns in GC enables congener-specific determination of a number of these mixtures. The isomer specific elution pattern of PCDDs and PCDFs is well established for such widely used columns as DB-5/DB-5ms (methyl, 5% phenyl polysiloxane), DB-Dioxin (44% methyl, 28% phenyl, 20% cyanopropyl polysiloxane with 8% polyoxyethylene), Sil 88/SP 2331 (100% cyanopropyl polysiloxane) [48–50]. Analogous studies have been reported on the chromatographic separation of PCB congeners using different columns such as DB-5 [51] or SGE HT8 [52]. A few studies were published regarding the PBDE elution pattern [53]. De Boer et al. [54] reported that a good separation can be obtained for most PBDE congeners using 50 m columns. However, more studies are required in order to determine potential coelutions between PBDE congeners. Using the available 46 PBDE standards from mono- to decabromination, a gas chromatography relative retention time model was developed to predict retention times of the 209 individual PBDE congeners [55]. This model concludes that full congener resolution and reliable quantification of all 209 PBDEs on a single column may either require significant advances in column materials and instrumental programming, or simply may not be possible. More and more scientists have become aware of the limitations of single-column capillary GC for the determination of chlorinated and brominated POPs. The chromatographic separation of the toxic isomers from all the non-toxic ones requires the use of at least two columns with different composition and polarity. Injection on two different columns is recommended for an unambiguous determination.

BDE-209 should receive special attention because of its sensitivity for higher temperatures and the higher susceptibility for degradation in the GC system. Analyses were carried out using short GC columns (10–15 m) avoiding long exposures to elevated temperatures [56, 57]. The film thickness of the short column should preferably be 0.1–0.2 μm, again with the aim not to extend the exposure to high temperatures unnecessarily [54]. This means that

the analysis of BDE-209 should occur separately from the analysis of the rest of PBDE congeners.

In recent years, comprehensive two-dimensional chromatography (GCxGC) has been shown to be very useful for the separation of various complex samples. In GCxGC two independent GC separations are applied to the sample. The most obvious advantage of GCxGC is the large peak capacity. Because retentions in the two dimensions are almost independent, the peak capacity that can be achieved is close to the product of the peak capacities of the two individual columns [58]. Another advantage of the GCxGC system is the increase of signal to noise ratios, which leads to an improvement in detection limit. Finally, all peaks in the chromatogram are described by two time co-ordinates, which make the identification more reliable. Some studies examined the potential of GCxGC for the qualitative analysis and characterization of complex mixtures of halogenated contaminants, such as PCBs, with emphasis on the non- and mono-*ortho* PCB congeners [59, 60]. Focant et al. [61] optimized a GCxGC methodology for the determination of PCBs, PBBs and PBDEs. Using this technique, they solve most of the potential coelution problems that can arise during the simultaneous analysis of several classes of halogenated POPs.

Analysis of PCAs is a difficult task because these mixtures contain at least several thousand individual congeners. Complete separation of individual compounds cannot be accomplished by means of single capillary column GC. PCA chromatograms display a characteristic broad envelope indicative of a large number of co-eluting peaks [62]. Coelutions in PCA analyses are cases where the use of GCxGC should be rewarding. Korytár et al. [63] tested the recently introduced rapid-scanning quadrupole mass spectrometer with an electron capture negative ionization (ECNI) option as a detector for GCxGC in the analysis of PCAs. Figure 2 shows the chromatogram obtained for a mixture of polychlorinated decanes with an average chlorine content of 65 wt. %. As can be seen, the separation was much improved, though not yet complete, and an ordered structure with four parallel groups of peaks was observed. More recently, GCxGC with ECNI-time-of-flight (ToF)-MS was used to study the composition and characteristics of SCCP, MCCP and LCCP mixtures [64]. From among the six column combinations tested, DB-1 x 007-65HT was found to be the best choice. The separation of PCA congeners with the same chain length is based on the number of chlorine substituents. When mixtures of PCAs of varying chain length are analyzed, ordered structures are observed which comprise compounds having the same number of carbon plus chlorine atoms (for example, $C_{10}Cl_8$ and $C_{11}Cl_7$, or $C_{12}Cl_6$ and $C_{13}Cl_5$). With the selected column combination, PCAs which differ at least three carbon atoms in their chain length were well resolved. This enables us to distinguish between SCCPs, MCCPs and LCCPs.

HBCD can be determined by GC-MS, using similar methods developed for PBDE determinations. As apparently the response factors of the three diastereomers do not differ very much, HBCD can be quantified as total

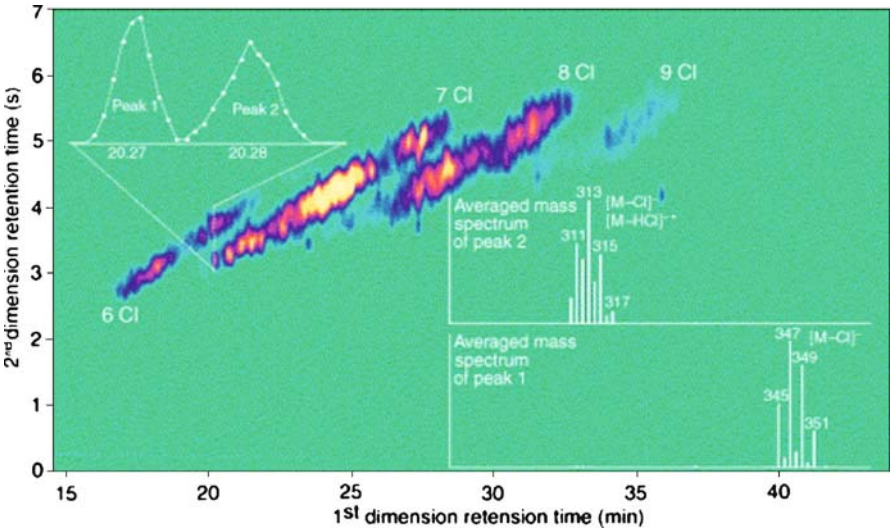


Fig. 2 Full-scan GC×GC-ECNI-MS of a mixture of polychlorinated decanes with average chlorine content of 65 wt. %. *Left-hand-side insert*: separation of hexa- and heptachlorinated decanes in second dimension and number of data points per peak. *Right-hand-side insert*: part of averaged mass spectra of peaks 1 and 2. Reproduced from [63]

HBCD. However, until now the different isomers have not been separated by this technique. Moreover, and because isomers of HBCD are thermally labile (it is known that HBCD decomposition takes place between 240 and 270 °C), elution from a GC column usually results in a broad and diffuse peak. In addition, a number of chromatographic peaks corresponding to different breakdown products were detected. These peaks could interfere with some BFR congeners, such as BDE-99 [65]. To solve these analytical difficulties, recent investigations were made using LC approaches. Actually, LC-MS and LC-tandem MS (MS-MS) are the best methods for measuring HBCD diastereoisomers separately in environmental samples [66, 67]. Chromatographic columns such as C18 were used in the majority of HBCD-isomers studies.

But, the α-, β- and γ-HBCD diastereoisomers are chiral and because of that must be present in the environment as enantiomeric pairs. The enantiomers have identical physicochemical properties and abiotic degradation rates, but may have different biological and toxicological properties and therefore different biotransformation rates. These transformations may result in non-racemic mixtures of the enantiomers that were industrially synthesized as racemates. A chiral chromatographic column must be used in order to obtain an enantiomeric separation. All recent studies showed a good separation using a Nucleodex β-PM (4.0 mm × 200 mm × 5 μm) [68–70]. A representative chromatogram of a standard mixture of three diastereomers of HBCD

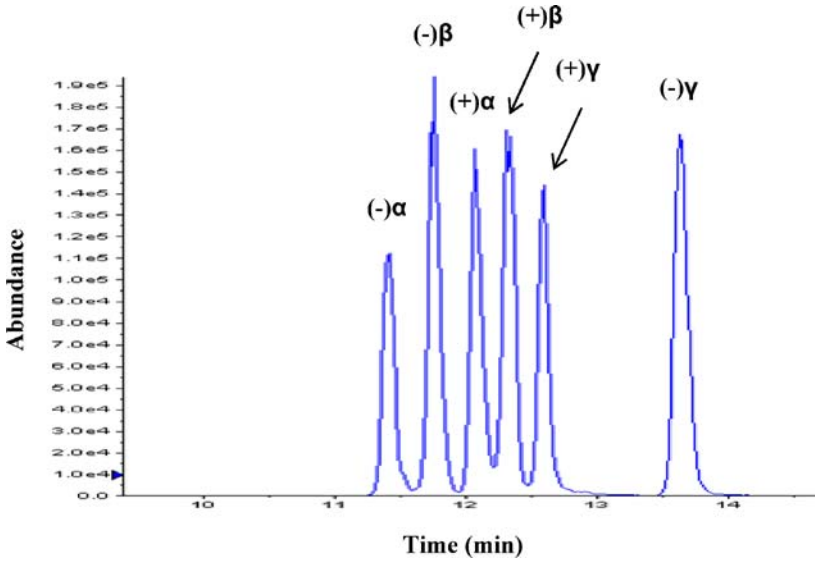


Fig. 3 Enantiomeric separation of a standard mixture of three diastereomers of HBCD (3 pairs of enantiomers) using a chiral column

that results in six peaks is illustrated in Fig. 3. The elution order of each enantiomer was assigned according to Heeb et al. [71]. Good separations were obtained for (–)α-, (–)β- and (–)γ-HBCD. Only in the case of (+)α- and (+)β-, and (+)β- and (+)γ-HBCD separations there are some minor coelutions (at 4.9 and 4.4% of the baseline, respectively).

4.1.4 Mass Spectrometric Detection

The complexity of chlorinated and brominated POP analyses requires a comprehensive approach for their quantitative determination in environmental samples. Moreover, concentrations of some of them, such as PCDDs and PCDFs in environmental samples are decreasing [72, 73] reflecting a general decline in dioxin inputs to the environment owing to tighter controls. The safe values established in different directives and recommendations are more and more restrictive, thus analytical methodologies must be able to achieve the low detection limits now required.

The PCDD, PCDF and PCB analyses involve detection of multiple congeners at the ppt or ppq level for which isotope dilution techniques using GC-HRMS are currently recommended methods (EN Method 1948, US EPA Method 1613, US EPA Method 8290, US EPA Method 1668) [74–76]. HRMS was used operating in the electron ionization (EI) mode at a resolving power of 10 000. Under these conditions, different ions (isotopic labelled included)

were monitored in selected ion monitoring (SIM) mode. Identification of analytes was based on the following restrictive criteria: (1) retention times of chromatographic peaks must be within the appropriate chromatographic windows; (2) simultaneous responses for the two masses monitored must be obtained; (3) signal to noise ratios must be greater than 3; and (4) relative isotopic peak ratios must be within ±15% of the theoretical values. Once these criteria were accomplished, assignment of toxic congeners was performed by comparing the retention times with the corresponding labelled standards added as internal standards. Quantification was carried out by an isotopic dilution technique, based on the addition of labelled standards.

GC-NCI-MS and GC-EI-MS are the approaches more frequently used for PBDE analyses. Mass spectra strongly depend on the type of ionization used. NCI mass spectra of all PBDEs were dominated by the bromine ion $[Br]^-$ and did not show any molecular ion. In contrast, EI provided better structural information, giving the molecular ions and the sequential losses of bromine atoms. For NCI-MS experiments, the two ions corresponding to $m/z = 79$ and 81 ($[Br]^-$) were monitored, whereas for EI-MS experiments, the two most-abundant isotope peaks for each level of bromination, corresponding to the molecular cluster for mono- to tri-BDEs and $[M–Br_2]^+$ for tetra- to hepta-BDEs, were selected.

The main advantage of NCI-MS versus EI-MS is the lower limit of detection (LOD) afforded, but higher specificity and accuracy were obtained using EI-MS. However, both ionization modes are subjected to different types of interferences. Sediment samples are usually polluted with a variety of compounds. The general problem in analysis of complex samples is that the extract obtained by exhaustive extraction techniques typically contains a large number of matrix components, which may co-elute with the analytes and disturb the quantitative analysis. The presence of interfering substances demands either a very selective detection or tedious extract cleanup or even both. The presence of potential interferences in NCI and EI approaches was studied by some authors [77, 78]. In general, EI-MS is affected by chlorinated interferences especially PCBs. Sediments are frequently contaminated with both PBDEs and PCBs. Moreover, analytical procedures for PBDE analyses are mainly based on already available analytical methods for PCBs. Thus, purified extracts contained both PCBs and PBDEs. Few of the ions commonly used for the determination of PBDE homologue groups are isobaric with PCB homologue groups. Moreover, under the chromatographic conditions normally used there are some coelutions, i.e. hepta-CBs eluted in the same chromatographic window as tetra-BDEs.

NCI-MS eliminated chlorinated interferences but presented different brominated interferences well resolved with the EI-MS approach. When PBBs and PBDEs were simultaneously determined, some important coeluting peaks appear. One critical chromatographic pair is BDE-154 and PBB-153 which coelute in many cases [54]. PBB-153 and TBBPA can also coelute with BDE-

154 and BDE-153, respectively, when using non-polar capillary columns and hence interfere with the determination of the corresponding PBDE congeners when monitoring the bromine ions ($m/z = 79$ and 81). Moreover, naturally produced brominated compounds, such as halogenated bipyrrols, brominated phenoxyanisols can be considered as potential interferences.

It was generally believed that the MS-MS technique surpasses others in analytical specificity, but it was not widely used owing to its relatively poor sensitivity and reproducibility. QIT-MS in tandem mode has been confirmed as a low-cost alternative for the analysis of some persistent organic pollutants (POPs) such as dioxins, furans and PCBs [79, 80]. Following this trend, the feasibility of QIT-MS for the analysis of PBDE congeners in sediment samples was tested [81]. The developed method allowed the quantification by isotopic dilution technique. Moreover, the sensitivity obtained using QIT-MS was compared with NCI-MS and EI-MS. LODs ranged from 57 to 128 fg, from 62 to 621 fg, and from 0.7 to 14 pg, for NCI-MS, QIT-MS and EI-MS, respectively. Thus, similar sensitivity was observed for QIT-MS and NCI-MS techniques. Taking into account that the main disadvantage of NCI-MS is the low specificity, and that this problem is well solved by QIT-MS, IT-MS seems to be the alternative offering the best compromise between sensitivity and selectivity.

Since the availability of labelled standards for other chlorinated and brominated POPs, such as PCNs and PBDEs, similar isotope dilution techniques using GC-HRMS were developed for an accurate determination of these contaminants [82, 83].

At the moment, isotope dilution techniques using GC-HRMS are the established and recommended methods. However, other MS techniques, such as time-of-flight (ToF)-MS, are now being developed for this kind of analysis. Two complementary approaches are available in ToF-MS. One employs instruments that provide high resolution but have a moderate scan speed, the other instruments that feature a high storage speed of, typically 100–500 spectra per second but usually provide only unit mass resolution. The first instruments are interesting for high-resolution applications, whereas the second group is used in studies that apply GCxGC. Because of its non-scanning character ToF-MS is a valuable tool for fast GC because this type of instrument can be used to monitor the entire mass range in very short times with high sensitivity. Narrow bore columns are a popular choice when performing complex GC analyses. They produce excellent chromatographic resolution without incurring the penalty of excessive run time. In the field of GC-MS, the disadvantage of short run times where separated components have narrow peak widths is that the desirable data sampling rate exceeds the capability of the MS. Scanning-type mass spectrometers are inherently unsuitable for fast separations, since they are, at best, limited to only 5 or 10 data samples per second. Therefore, when the GC peak widths are below 0.5 s wide, a different type of mass detector must be used. The ToF-MS is ideally suited to this type

of analysis due to the high sampling rates that can be achieved (hundreds of mass spectra per second).

Different recent studies showed the capabilities of ToF-MS for the analysis of PCBs in different type of samples [84–88]. The ToF-MS analyses were generally achieved using an orthogonal acceleration ToF mass spectrometer. Using this state of the art technology it is possible to operate at elevated resolving power (7000). The GC-ToF-MS results were consistent with the GC-HRMS results within the 95% confidence interval limits [86]. These results are very encouraging, and show that GC-ToF-MS allows for analysis times an order of magnitude faster than GC-HRMS methods without a loss in qualitative or quantitative power.

As regards PCA determinations, while a GC-MS method based on SIM of positive ions has been reported [89], the more popular methods have relied on ECNI-LRMS. However, the major problem associated with these methods is lack of selectivity. Since Tomy et al. [90] developed a quantitative method for SCCP analyses in environmental samples based on the use of GC-ECNI-HRMS, this approach was the more frequently used, and it became the standard method for PCA determinations. Using this methodology, molecular composition could be determined by monitoring $[M–Cl]^-$ ions of specific m/z value corresponding to formula groups and by assuming that the integrated ion signals are proportional to molar concentration weighted by the number of chlorine atoms in the formula group. Formula group abundance profile generation is useful because it allows PCA concentrations to be reported according to individual formula and homologue groups.

GC-ECNI-HRMS is a very selective detection method; however, this detection method is not available at many laboratories and is too costly for routine analysis. Therefore, LRMS is also used for the quantification of PCAs. The use of LRMS increases the risk of interferences, which have to be controlled and eliminated. An improved sample cleanup removing other polychlorinated compounds is one possibility. But, disturbances might also occur when mixtures of SCCPs and MCCPs are present. Reth and Oehme [91] studied the limitations of the GC-ECNI-LRMS for the analysis of SCCPs and MCCPs. The analysis can be disturbed by mass overlap caused by congeners with the same nominal mass, but with five carbon atoms more and two chlorine atoms less, for example, $C_{11}H_{17}{}^{37}Cl^{35}Cl_6$ (m/z = 395.9) and $C_{16}H_{29}{}^{35}Cl_5$ (m/z = 396.1). This can lead to an overestimation of congener group quantity and/or total PCA concentration. The authors of the study concluded that the quantification of the most-abundant congeners (C_{11}–C_{14}) is not affected by this interference, if isotopic ratios, retention time changes and shapes of the signals are investigated. As regards sensitivity, LODs were around 1 ng/μl, whereas HRMS provided lower LODs (between 60 and 200 pg) [90]. However, the sensitivity of LRMS is still appropriate for the analysis of PCAs in environmental samples.

Castells et al. [92] also developed a method based on the use of GC-ECNI-LRMS. Since the $[M–Cl]^-$ and $[M–HCl]^-$ cluster ions are subject to interference from other PCA homologues, the $[HCl_2]^-$ and $[Cl_2]^-$ ions have been selected for quantification. Moreover, $[M–Cl]^-$ ions always showed lower responses than the $[HCl_2]^-$ and $[Cl_2]^-$ ions. Nevertheless, other organochlorine compounds could interfere with the determination using these common fragment ions. The authors evaluated the potential interferences between organochlorine pesticides, toxaphenes, PCBs and PCNs with SCCPs, and they concluded that no significant interferences were observed under the proposed GC-MS conditions. This is due to the fact that, under optimized ECNI conditions (methane pressure set at 1.2×10^{-4} Torr, and trap-offset voltage set at 3 V), the formation of $[HCl_2]^-$ and $[Cl_2]^-$ ions from the potential interferences was not favoured. Only some organochlorine pesticides gave a small response, and they did not coelute with SCCPs in the chromatogram. Moreover, the LOD obtained using this method is 0.20 ng, which was lower than those obtained in other studies working with ECNI-LRMS. It should be mentioned that one disadvantage of the proposed method is that obtained information was a total PCA concentration, but not the molecular composition.

The use of ECNI, however, has some disadvantages. Response factors depended strongly on the number of chlorine atoms and their position at the carbon chain. Congeners with a higher degree of chlorination have higher response factors due to a higher electron affinity. Therefore, the use of different technical mixtures as quantification standards can lead to considerable deviations in the results. Additionally, PCAs with low chlorine content are neither sensitively nor completely detected by ECNI-MS. The use of CH_2Cl_2/methane reagent gas mixtures is described as an alternative to conventional GC-ECNI-MS, which used methane as the moderating gas [93]. Using this non-conventional reagent gas, a nearly exclusive formation of $[M+Cl]^-$ ions was observed, enhancing selectivity and sensitivity. Both interferences, PCAs themselves and other chlorinated contaminants, were solved or reduced by selecting $[M+Cl]^-$ ions, enabling the determination by LRMS. Moreover, quite similar response factors were obtained for congeners of different degrees of chlorination, allowing the use of technical mixtures as reference standards for a congener- and homologue-specific analysis. Another interesting advantage is that, in contrast to methane-ECNI, CH_2Cl_2/methane-NICI enabled also the detection of tri- and tetrachloro congeners.

4.2 Bioanalytical Methods

Chemical analysis of contaminants in environmental matrices is essential to assess exposure concentrations of individual compounds, e.g. for (trend) monitoring and for risk assessment purposes. However, with this type of analysis alone, biological effects cannot be predicted, and mixture effects and

contributions of unknown compounds with similar modes of action to the overall effect cannot be taken into account. Therefore, several bioassays have been developed as tools to address the above-mentioned issues. These assays can be used to determine potencies of individual compounds and for measuring the total activity of complex mixtures of compounds. Bioassays can thus be used to determine total activities in sediment samples, without the necessity of knowing all compounds present that contribute to the activity.

Determination of environmental pollutants using biodetectors such as bioassays, biomarkers, enzyme immunoassays or other bioanalytical tools is a continuously growing area. Behnisch et al. [94] reviewed the principles and advantages/limitations of several bioanalytical detection methods for the screening and diagnosis of some chlorinated and brominated POPs, with special emphasis on dioxin-like compounds (DLCs). These methods are based on the ability of key biological molecules (e.g., antibodies, receptors, enzymes) to recognize a unique structural property of the DLC, or on the ability of cells or organisms to have a specific response to DLCs. Bioanalytical methods for measuring DLCs included the EROD bioassay, the AHH bioassay, the enzyme immunoassay (EIA), the reporter gene assay (e.g., CALUX), the gel retardation of AhR DNA binding (GRAB) assay, the reach receptor DELFIA assay kit, the AhR assay with radiolabelled dioxins, and the Ah-immunoassay (AhIA). For several bioanalytical dioxin tests, official methods by governmental authorities have been approved such as EPA Method 4425 (reporter gene assay) or EPA Method 4025 (immunoassay). Moreover, the minimum detection limits reported for 2,3,7,8-TCDD for the bioanalytical methods are similar to those reported for chemical analysis.

The CALUX and EROD methods were two of the most-used bioassays for measuring the dioxin-like activity of environmental samples. The EROD method measured the binding of the dioxin-like compound to the AhR and the subsequent induction of CYP1A related deethylation of 7-ethoxyresorufin to resorufin. In this bioassay, several CYP activities can be measured by using different substrates. Moreover, several cell lines are used for the EROD bioassay, e.g., the rat H4II cell line, the chicken embryo hepatocytes, cultured chicken embryo liver, etc. On the other hand, the CALUX bioassay is based on the induction of reporter genes through AhR binding and the subsequent analysis of light production by luciferase. Similar results were obtained using both bioassays; however, CALUX is faster, has a more stable response with better reproducibility and luciferase is more stable than EROD protein.

4.3 Toxicity Identification Evaluation (TIE) Methods

Assessments of the risk connected to the contamination should rely on a multidisciplinary approach based on both chemical and biological techniques and should be able to address both exposure and effects. Traditional chemical

techniques for the detection and quantification of contaminants provide detailed information regarding the presence and concentration of specific compounds. On the other hand, information often is limited to a small number of compounds that are above the limit of detection and for which analytical standards are available. Furthermore, chemical techniques do not provide any information concerning the biological activity of the contamination. This is particularly relevant when complex mixtures are present at low levels and synergic or antagonistic effects may take place.

In order to identify compounds responsible for specific effects (i.e., endocrine disrupting or AhR ligands) observed in field studies, TIE or bioassay directed analysis approaches have increasingly been applied over the last decade. In such approaches, sensitive bioassays are used to direct the fractionation of a sample extract until its complexity is sufficiently reduced to enable identification of those compounds responsible for the activity measured in the bioassay. This strategy is based on differential extraction and fractionation methods and identification by chemical and biochemical analysis. TIE is a well-established technique having been originally developed by

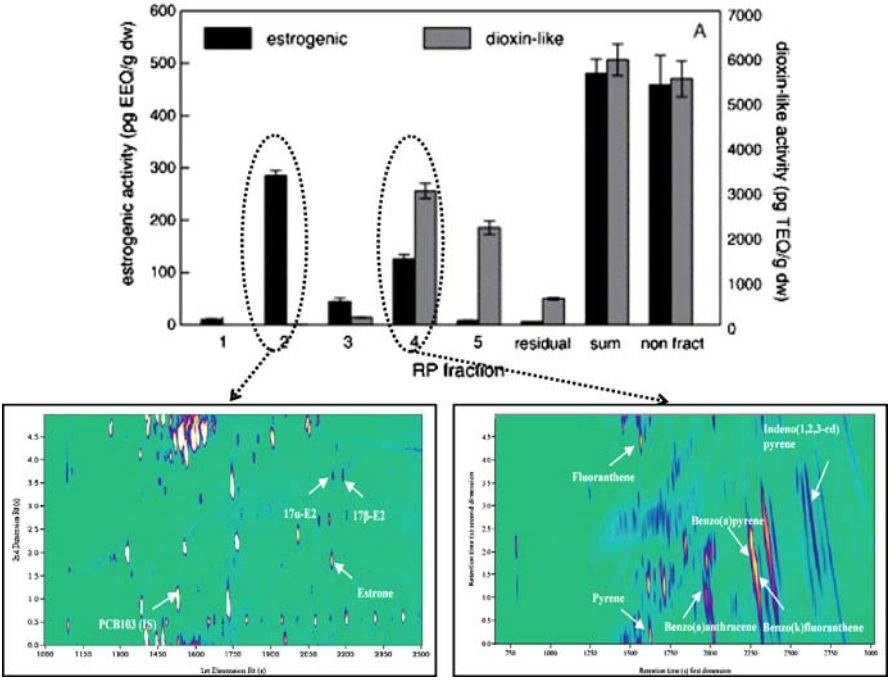


Fig. 4 Estrogenic and dioxin-like activities in fractions of sediment extract from Kierikzee harbour: fraction 2 showed characteristic ions of 17α- and 17β-estradiol and estrone, and fraction 4 showed characteristic ions of various polycyclic aromatic hydrocarbons. Reproduced from [95]

the U.S. Environmental Protection Agency for identifying the cause of toxicity in effluents. More recent applications include some studies related to POP contamination. For instance, Houtman et al. [95] identified estrogenic and DLCs in sediments from Zierikzee harbour with a CALUX assay-directed fractionation combined with GCxGC-ToF-MS. To reduce the complexity of the sediment extracts, total extracts were fractionated (Fig. 4). Five fractions with decreasing polarity were collected and tested for estrogenic and dioxin-like activities. Most estrogenic activity was found in fraction 2 which is known to cover the natural estrogenic hormones. To confirm the presence of natural estrogenic hormones in this fraction, GCxGC-ToF-MS was applied, and the presence of 17α-, 17β-estradiol and estrone was confirmed. Moreover, most dioxin-like activity was found in fraction 4. In this case, the presence of polycyclic aromatic hydrocarbons, which are known to have dioxin-like properties, justified the activity detected in this fraction.

In a more recent work, Puglisi et al. [96] introduced the relevance of using approaches based on non-exhaustive extraction techniques. Usually, approaches are based on total extraction of contaminants, and then, they do not take into account the importance of bioavailability and aging processes. Tenax and cyclodextrin extractions over time were carried out to determine the bioavailable fractions. Results obtained in this study showed that the adoption of a bioavailability-based assessment of contamination led to a large reduction of the toxicity signal (Fig. 5). The total extract had 70 pM; this signal was reduced to 19 pM for the cyclodextrin-assessed bioavailable fraction and to only 3 pM for the Tenax-assessed bioavailable fraction.

From this study, we can conclude that coupling of non-exhaustive extraction and bioanalyses leads to a more realistic and, generally much lower estimated risk for the toxicity of the extracts as compared to commonly

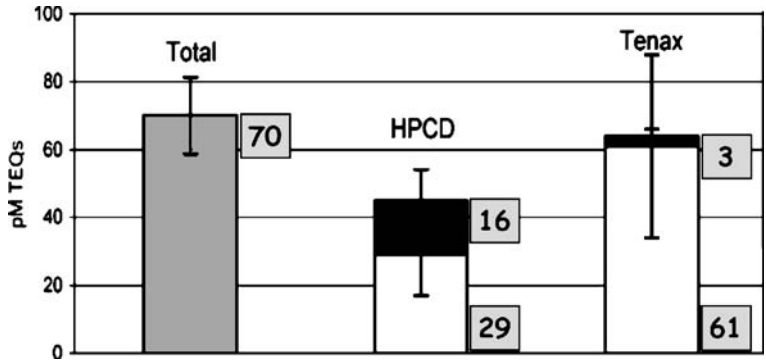


Fig. 5 Comparison between DR-CALUX signals of total, bioavailable, and residual fractions. Bioavailable and residual fractions were collected by hydroxypropyl-β-cyclodextrin (HPCD) or by Tenax extraction. Total extraction was microwave assisted. Reproduced from [96]

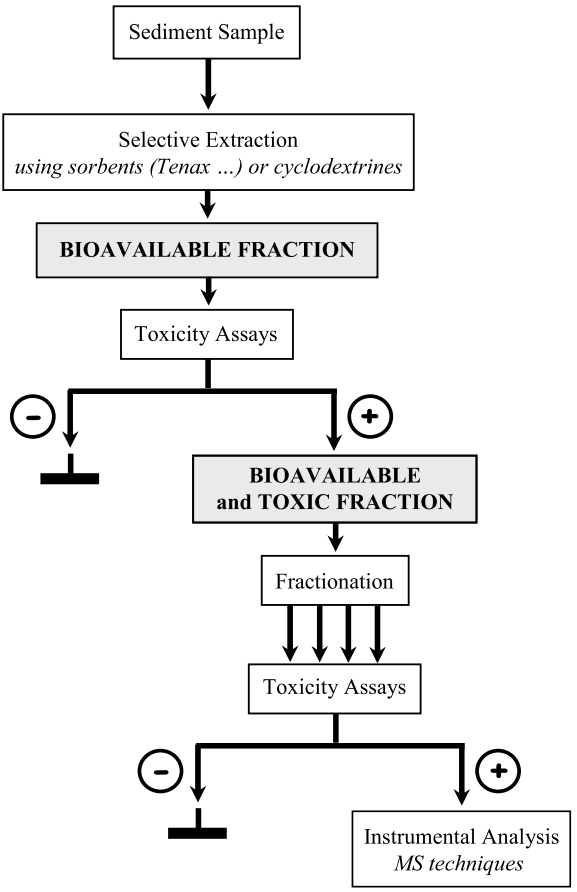


Fig. 6 Different steps to be followed in the new concept of a bioavailable toxicity identification evaluation (BTIE) method

adopted exhaustive techniques. Following these findings, we proposed the introduction of a bioavailable toxicity identification evaluation (BTIE) concept, in which the steps to be followed are similar to those of TIE methods, but following bioavailable extractions (Fig. 6).

5 Environmental Levels

The contamination of sediments may pose an unacceptable risk to aquatic organisms, which tend to bioaccumulate chlorinated and brominated POPs, and to wildlife and humans through the ingestion of contaminated fish and shellfish. During the last four decades a large amount of environmental data

has been generated for different chlorinated POPs, such as PCDDs, PCDFs and PCBs. However, for some other chlorinated POPs (e.g. PCAs) literature data are very scarce. As regards brominated POPs, during the last decade a large amount of environmental data has been generated for PBDEs. However, HBCD literature data is scarce.

5.1 Chlorinated Organic Pollutants

In the literature, the PCDD and PCDF results are given as Toxicity Equivalent Quantities (TEQs). Between the 210 PCDD and PCDF congeners, the most toxic molecules are those whose positions 2, 3, 7, 8 are chlorinated. The compounds that meet these conditions number a total of 17, 10 furans and 7 dioxins. Since the individual toxicity of these compounds is different, the real toxicity of a mixture was assessed bearing in mind the relative toxicity of the isomers with respect to the most toxic isomer, the 2,3,7,8-TCDD; a toxicity equivalence factor (TEF) equal to the unit was assigned to the 2,3,7,8-TCDD. For the toxic assessment, the 17 toxic isomers were normalized by multiplying their measured concentrations by the appropriate TEFs. The sum of these products yields the total TEQs, which express these analyte concentrations as a single number, equivalent to that of a toxicity derived exclusively from 2,3,7,8-TCDD.

In order to assess the quality of freshwater sediments, quality objectives for PCDDs and PCDFs have been formulated. Of the eight approaches available, the tissue residue-based (TRB) is the most commonly used. This method involves establishing a safe chemical concentration in sediment which results in an acceptable tissue residue in biota. A NOEC (no observed effect concentration) of 200 pg TEQ/g dw in sediment was derived, but when chronic toxicity data are scarce a safety factor of 10 is applied, which resulted in a safe sediment value of 20 pg TEQ/g dw [97]. Empirically derived sediment quality guidelines (SQGs) were developed on the basis of the associations observed between measures of adverse biological effects and the concentrations of potentially toxic substances in sediments. One set of SQGs developed includes the effects range low (ERL) and effects range median (ERM) values. The ERL value is known to be a concentration that has no harmful effect on biota. An ERL value of 50 ng/g dw was established for total PCBs in sediments [98].

A number of studies have reported PCDD and PCDF levels from sediments in North America, Europe and Asia (Table 2). Generally, the PCDD and PCDF levels in background areas ranged between < 0.1 and the safe sediment value established at 20 pg TEQ/g dw, whereas levels found in polluted areas (i.e., harbours), clearly exceeded the safe value.

Regarding the PCB data, a number of studies have reported levels expressed as total PCBs or as a sum of the seven indicator PCBs; however, the literature on the dioxin-like PCBs is very scant. The concentrations found

Table 2 Levels of PCDDs and PCDFs in sediments from different locations

Location	Levels (pg TEQ/g dw)	Year [Refs.]
Background areas		
11 lakes (USA)	0.1–15.6	1996 [122]
River Danube (upper Austria)	0.4–12	1993 [123]
Oder river (Germany)	0.1–17.5	1997 [124]
Orbetello lagoon (Italy)	0.4–7.3	1998 [125]
Volga riverside (Russia)	0.08–9.4	2000 [126]
Ebro river (Spain)	0.4–3.7	2001 [127]
Llobregat river (Spain)	1.8–7.7	2001 [127]
Umber estuary (UK)	14–24	1996 [128]
12 rivers (Japan)	0.02–24	1998 [129]
Han river (Korea)	0.04–4.4	2000 [130]
Polluted areas		
Black Rock harbour (USA)	223–250	1989 [131]
New Bedford harbour (USA)	10–761	1989 [131]
18 lakes (central Finland)	< 20–230	1990 [102]
Kymijoki river (Finland)	100–59 000	1995 [132]
Chemieharbour (The Netherlands)	434–923	1989 [133]
Laurensharbour (The Netherlands)	352–1849	1989 [133]
Frierfjorden (Norway)	6234–19 444	1989 [134]
(Japan)	1.1–150	1999 [135]

in the background areas ranged between < 0.1 and 28 ng/g dw, always below the ERL value of 50 ng/g dw. However, the levels found in the polluted areas were higher, ranging between 200 ng/g dw and 120 μg/g dw. Khim et al. [99] estimated concentrations of TEQ_{PCB} in sediments from Korea, in the range of 0.05–1.7 pg/g dw. Müller et al. [100] found a mean TEQ_{PCB} value of 20.3 pg g dw in sediments from Germany. Eljarrat et al. [101] reported concentration levels between 0.03 and 25 pg TEQ/g dw in sediments from the Catalonian coast (Spain).

PCNs were quantified in sediment samples from different locations. In sediments from Central Finland [102], the total PCN values ranged from 0.5 to 3.5 ng/g dw. Kjell et al. [103] measured PCN contamination in surface sediments collected in the Gulf of Bothnia (northern Baltic Sea). PCN concentrations were in the range of 0.1–1.9 ng/g dw. PCNs were also studied in two Mediterranean lagoons (Venice and Orbetello, Italy) [104]. The levels of the sum of mono- to octa-CN ranged from 0.03 to 1.5 ng/g dw. In a Swedish sediment study [105], the PCN results ranged between 0.6 and 304 ng/g dw. Kannan et al. [106] determined the concentrations of PCNs in sediments from the upper Detroit and lower Rouge Rivers. Levels ranged from 0.08 to 187 ng/g dw. On average, penta- and hexa-CNs accounted for 60% of the total PCN concentrations in sediments. PCN-71 and 72 (1,2,4,5,6,8- and

Table 3 Levels of PCAs in sediments from different locations

Location	Levels (ng/g dw)	Year [Refs.]
Detroit River (North America)	288	1997 [90]
Canada	4.52–135	1999 [30]
Norway	330–19 400 *	2003 [136]
Besos River (Spain)	0.27–3.26	2004 [41]
North Sea	10–258	2004 [137]
Baltic Sea	209–876	2004 [137]
Lake Thun (Switzerland)	47	2008 [107]

* This results are expressed in wet weight basis

1,2,4,5,7,8-HxCN) were the most abundant in sediment from several locations, followed by PCN-59 (1,2,4,5,8-PeCN) and PCN-69 (1,2,3,5,7,8-HxCN), which collectively accounted for 20 to 30% of the total PCN concentrations.

Literature data on PCA concentration levels in sediments are very scarce (Table 3). Recently, Iozza et al. [107] studied a dated sediment core from lake Thun covering the last 120 years, to get an overview of the historical trend of PCAs. Total PCA concentrations (sum of SCCPs, MCCPs and LCCPs) showed a steep increase in the 1980s and a more-or-less stable level of 50 ng/g dw since then. Comparison of the time profiles of total PCAs, SC-

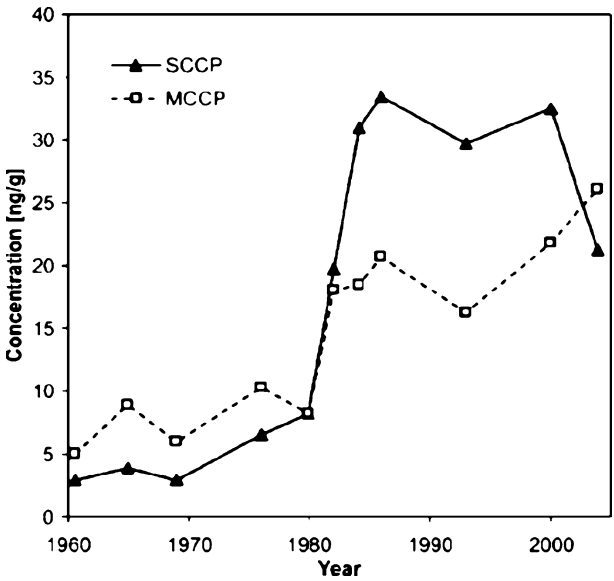


Fig. 7 Historical time trends of SCCP (*solid line*) and MCCP (*dashed line*) concentrations. Reproduced from [107]

CPs and MCCPs revealed that the rapid increase of total PCA concentrations in the 1980s is mainly caused by SCCPs, whereas MCCP levels changed much less (Fig. 7). The maximum SCCP concentration was 33 ng/g dw in 1986, and the level of the surface sediment was 21 ng/g dw of SCCPs. MCCP concentrations increased since 1965 and reached a maximum in the surface sediment (26 ng/g dw). A decrease of SCCPs and a shift to more MCCPs was observed after 2000. Future research must be planned to verify this decrease, which could be attributed to an effect of the regulations of the EU Water Framework Directive and the preceding discussions about a general ban of SCCPs.

5.2 Brominated Organic Pollutants

Several studies have been done on the concentrations and distributions of PBDEs in sediments (Table 4). The major congeners detected were BDE-47, BDE-99, BDE-100, BDE-153 and BDE-209. The concentrations of these compounds were highly variable from location to location, but in general, the concentrations of PBDEs are similar to those of the PCBs. PBDEs were determined in Swedish river sediments at 8–50 ng/g dw [57]. Similar values were found in Japanese river sediments, with concentration levels between 21 and 59 ng/g dw [108]. Sediment samples from a Spanish river collected

Table 4 Reported concentrations (expressed in ng/g dw) of PBDEs in sediment samples

Matrix (location)	Compounds	Concentration	Refs.
River sediments (Japan)	Tetra- + Penta-BDEs	21–59	[108]
Downstream of a plastic industry (Sweden)	BDE-47	490	[138]
	BDE-99	750	
	BDE-100	170	
River with textile industries (Sweden)	BDE-47 + 99 + 100	nd–9.6	[57]
	BDE-209	nd–360	
Sediment (Baltic Sea)	Sum PBDE	nd–1.1	[139]
River mouth sediments (Europe)	BDE-47	< 0.17–6.2	[140]
	BDE-99	< 0.19–7.0	
River sediments (Spain)	Sum PBDE	2–42	[65]
Marine sediments (Spain)	BDE-209	2–132	[38]
River sediments (Danube)	11 PBDEs	0.06–84	[110]
River sediments (Spain)	7 PBDEs	2.5–9.8	[111]
Sediment from estuary (The Netherlands)	13 PBDEs	262–1660	[112]
Industrialized bays (Korea)	Sum PBDE	2.03–2253	[113]
River with chemical industries (Spain)	BDE-209	up to 12 000	[114]
River sediments (Pearl river)	10 PBDEs	1.9–3580	[115]

up- and downstream from a possible point source (chemical industry), presented levels ranging from 2 to 42 ng/g dw [65]. Different marine sediments collected in Spain showed concentration levels of BDE-209 ranging between 2 and 132 ng/g dw [38]. Higher levels up to 1400 ng/g dw were found in a downstream area of a manufacturing plant in the United Kingdom [109] and at 120 ng/g dw downstream of an area with textile industries [57].

More recent studies were also focused on PBDE determinations, and specially, on deca-BDE-209 levels. Sawal et al. [110] determined 11 PBDE congeners (including BDE-209) in sediments from 32 sites along the River Danube. BDE-209 was detected in 93% of the sediment samples and contributed more than 80% to the total PBDE concentration. Total PBDE levels ranged from 0.06 to 84 ng/g dw. Labandeira et al. [111] analyzed seven PBDE congeners (including BDE-209) in sediments from the Anoia and Cardener Rivers in Spain. Total PBDE levels ranged from 2.5 to 9.8 ng/g dw. BDE-209 accounted for 60% of total PBDE. Verslycke et al. [112] determined 13 PBDE congeners (including BDE-209) in sediments from the Scheldt estuary in the Netherlands, with total PBDE concentration levels ranging from 262 to 1660 ng/g dw. Moon et al. [113] analyzed PBDEs in sediments from 111 locations in three industrialized bays in Korea. Total PBDE concentrations ranged between 2.03 and 2253 ng/g dw, and from 2.0 to 2248 ng/g dw for BDE-209. Higher values were found in sediment samples collected downstream of an industrial park in Spain, with BDE-209 values up to 12 μg/g dw [114]. Mai et al. [115] determined ten PBDE congeners (including BDE-209) in 66 surface sediment samples in the Pearl River in 2002. Profiles were again dominated by BDE-209, which ranged in concentration from 1.9 to 3580 ng/g dw.

Regarding HBCD data, some studies have reported levels expressed as total HBCD; however, the literature on the HBCD isomeric distribution is very scant. Klamer et al. [116] determined total HBCD concentrations in the North Sea surface sediments, with values ranging from < 0.2 to 6.9 ng/g dw. Lepom et al. [117] determined HBCD in 12 sediment samples collected in 2002–2005 from the German Bight. Concentrations ranged from 0.03 to 6.5 ng/g dw. Verslycke et al. [112] determined HBCD in sediments from the Scheldt estuary in the Netherlands. At the three sites sampled, concentrations were between 14 and 71 ng/g dw. Evenset et al. [118] determined HBCD in four replicate sediment cores collected in April 2001 from the deepest part of Lake Ellasjoen. HBCD was detected only in the depth interval from 1 to 2 cm (median age 1980). Only the α- and γ-isomers were detected, at 0.43 and 3.9 ng/g dw, respectively. This was an order of magnitude higher than $\sum$ BDE in the same depth slice. Three sediment cores and six surface sediment samples from Tokyo Bay were also analyzed [119]. $\sum$ HBCD were detected for the first time in this region at concentrations ranging from 0.06 to 2.3 ng/g dw, implying widespread contamination. HBCDs first appeared in the mid-1970s and concentrations observed in the cores have increased since then.

PBDDs and PBDFs are often formed in the process of manufacturing BFRs and from the combustion of e-waste products containing BFRs. However, there is only limited information on the environmental concentrations and human exposure of PBDDs and PBDFs. Choi et al. [120] analyzed sediment samples from industrialized areas of Japan collected in 2000. Some 2,3,7,8-tetra- to hexabrominated dioxins and furans were detected in these sediment samples, with different patterns of congeners, indicating that different sources of PBDD/Fs were present. 2,3,7,8-TeBDD was detected above the limit of quantification (LOQ) in only one sediment and 2,3,7,8-TeBDF was identified in three sediments (< 0.2–3.2 pg/g dw). Wang et al. [121] analyzed sediment samples collected in the vicinity of a recycling site for electronic wastes, and they found trace levels of PBFDs (0.025–0.92 ng/g dw) and non-detectable PBDDs.

6 Conclusions and Perspectives

The wide distribution of chlorinated and brominated POP contamination in the environment suggests that monitoring programmes should be extended to include classical POPs, such as PCDDs, PCDFs and PCBs, but also new emerging POPs, such as PCAs and BFRs. Concentrations of deca-BDE and HBCD are rapidly increasing today as the PCB and PCDD/PCDF are declining. In this respect, deca-BDE and HBCD are prime examples for emerging persistent organic contaminants.

Furthermore, brominated dioxin, as well as mixed brominated-chlorinated dioxin, data are needed in order to determine their environmental impact. However, chemical analysis of mixed halogenated dioxins is very difficult due to the large number of possible combinations (there are 4,600 potential mixed congeners). In order to achieve this goal it is necessary to develop analytical procedures that permit determination of different groups of brominated contaminants.

Thus, additional investigation is required in the chemical analysis field, but also for development of new bioassays. The bioanalytical methods offer the chemical analyst the benefits of sensitive and cost/time-effective solutions to diagnose more sufficiently all kinds of environmentally POPs. In the future, these bioanalytical tools will help us to understand more the different classes of AhR agonists/antagonists, their mechanism of toxicity and their potency in the environment. Moreover, research is also needed to develop TIE programmes for the evaluation of toxicity activities in environmental samples. The existence of unknown active compounds has been basically suggested for the explanation of higher bioassay estimates. In order to answer such open questions, as well as contribution analysis of the newly known POPs such as PBDDs and PBDFs, detailed chemical fractionation and identification of the

active compounds will be important using the state-of-the-art fractionation and analytical/bioassay techniques.

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Effects of Static vs. Tidal Hydraulic Conditions on Biogeochemical Processes in Mesocosms: Degradation of Aromatic Hydrocarbons (AHs)

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Abstract This study addressed the feasibility and practical considerations related to the possible use of contaminated sediments in coastal wetland restoration and creation efforts. Dredge and other sediments are frequently contaminated with chemical pollutants, and large volumes of this potentially recoverable resource are disposed of in dumpsites rather than utilized. This work addresses the possible deployment of sediments contaminated with aromatic hydrocarbons (AHs) in salt marsh creation efforts. The experimental system was a hydrodynamic mesocosm developed to study well-drained, permanently saturated-flooded and alternately flooded-drained (tidal) hydraulic effects on biogeochemical and plant processes. The target AHs for this work were naphthalene, phenanthrene, 1-methylnaphthalene, 1,3-dimethylnaphthalene, 3-methylphenanthrene, and 3,6-dimethylphenanthrene. The results of this work confirmed that different hydraulic conditions and the resultant biogeochemical properties of salt marshes influence the rate, transformation-time profile, and completeness of transformation of AHs in sediments. In general, the relative transformation rates for the AHs in the different systems examined here were drained/oxidized Eh $\geq$ tidal/oscillating Eh $\gg$ flood/reduced Eh, as observed previously with other classes of organic chemicals. The generation of hydroxylated AHs coincided with observed compound transformation profiles: decreases in target compounds on sediments were correlated with increases in hydroxylated AHs in

the water. While well-drained aerobic conditions seem to be optimal for the transformation/depuration of AHs in salt marsh sediments, in natural settings this kind of system would not be a "wetland" either geohydrologically or with respect to vegetation. So, when evaluating the use of contaminated sediments in "wetland" construction or expansion, the relevant zones are streamside and marsh interior. This work indicates that AH contaminated sediments can be used, alone or mixed with clean sediments or other industrial materials (e.g., phosphogypsum and bauxite "red mud"), for wetland creation or enhancement. It would seem that either or both marsh locations (interior vs. streamside) are appropriate, at least with respect to rapid degradation and elimination of these AHs from the sediments. The tradeoff involves time and potential offsite risks: at streamside, the removal rates were twice as fast as and somewhat more complete than in the interior, but the action of the tides could mobilize contaminants to the estuary and beyond. The interior sediments will allow for less exchange of the pollutants with the estuarine water, but will take longer to depurate, particularly if the sediments become compacted.

Keywords Aromatic hydrocarbons · Biogeochemistry · Degradation · Dredge · Tidal wetland sediments · Toxic chemicals

1 Introduction

This work addressed questions about the use of dredged and other soils and sediments for coastal wetland expansion, de novo creation, and mitigation. It was of interest to determine the possibility of and extent to which polluted sediments could be deployed in natural tidal wetlands for purposes of system maintenance or expansion, or in systems created completely de novo. To this end, several issues needed to be resolved, including, (1) whether the placement location of the sediments in different wetland zones is an important consideration and, (2) whether in situ physicochemical conditions can be exploited to optimize various design endpoints, such as enhanced degradation of contaminants, while still allowing the system to provide desirable ecological functions [1–7]. From a conceptual standpoint, it was necessary to determine the manner in which these questions could be posed and answered utilizing a tractable experimental system that can be replicated while also allowing for extrapolation to large spatial scales in actual applications.

These questions were explored with respect to tidal salt marsh ecosystems dominated by the cord grass *Spartina alterniflora* [1]. The ecology of these systems is driven largely by sediment-plant biogeochemical processes mediating solid, dissolved, and gas phase partitioning of C, N, O, P, Fe, Mn, and S organic and inorganic chemicals [2, 8, 9]. Tidal saline wetlands are ecologically important in coastal systems, e.g., as breeding and nursery grounds for birds and fish, and are subject to anthropogenic impacts, including contamination from river water, urban runoff, airborne particle deposition, dumping, leaking and spilling of petroleum and chemicals [1–4].

Both natural and engineered salt marshes are dominated by three distinct hydraulic regimes: chronically flooded (marsh interior), well-drained (upland back marsh), and cyclic flooded-drained (streamside). These correspond to different biogeochemical situations with respect to sediment properties and plant biology. Thus, flooded interior areas tend to be permanently anaerobic and electrochemically reducing, upland back marsh aerobic and oxidizing, and streamside alternating aerobic/anaerobic as driven by the tidal water level fluctuations. These biogeochemical differences are correlated with the productivity, health, and physiology of *S. alterniflora* within these zones. Individual plants are tallest at tidal streamside locations and in well-drained back marsh locations (both of relatively high elevation), while plants in the permanently saturated/flooded lower elevation marsh interiors exhibit a stunted, stressed "short form" [1, 2, 4, 5]. Sediment redox potentials (Eh) are significantly more positive in drained and tidal settings than those measured in flooded locations [6]. This measured electrochemical difference corresponds not only to differences in plant size and health but also to the nature and endpoints of microbial processes in situ. Thus, drained oxidized sediments tend to exhibit aerobic microbial and plant root respiration, with end products being primarily CO_2, H_2O and simple organic acids. These areas have larger and more productive stands of *S. alterniflora*, (i.e., the "tall form" of the grass). In flooded, reduced sediments, facultative and obligate anaerobic metabolism dominates the sediments and pore water, with hypoxic/anoxic conditions in the root zone, with endpoints including dissolved and gas phase reduced C, N, S, Fe and Mn species (e.g., methane, ammonia, sulfides, Fe^{2+} and Mn^{2+}). These areas are typified by the short form of the grass.

In much environmental research, tidal salt marshes have been approached as if there are only two basic biogeochemical-hydraulic regimes influencing surface sediments: (1) drained/oxidized/aerobic at stream sides and other elevated zones like the back marsh and, (2) waterlogged/reduced/anaerobic in the nontidal, relatively compacted, marsh interior [6, 10]. Conversely, our work has shown that, while both chronically flooded and drained areas of marshes *do* have stable reduced vs. oxidized Eh values (respectively) when measured in a well-resolved time series, streamside locations only a few meters away can be utterly distinct: these sediments, containing the largest stands of tall form *S. alterniflora* and flourishing microbial systems, showed significant oscillations of Eh as driven by the tidal water level changes (Fig. 1, [6]). Further study confirmed that diurnal tidal pulsing of streamside salt marsh sediment-plant systems stimulated the transformation of several classes of petroleum-and coal derived chemicals in ways similar to well-drained (oxidized) sediments, relative to statically flooded (reduced) conditions [6, 11–13].

Increasingly, dredge and other aquatic sediments and upland soils can be expected to contain moderate or elevated levels of anthropogenic toxic chemicals, including hydrocarbons, pesticides, industrial wastes, metals, and

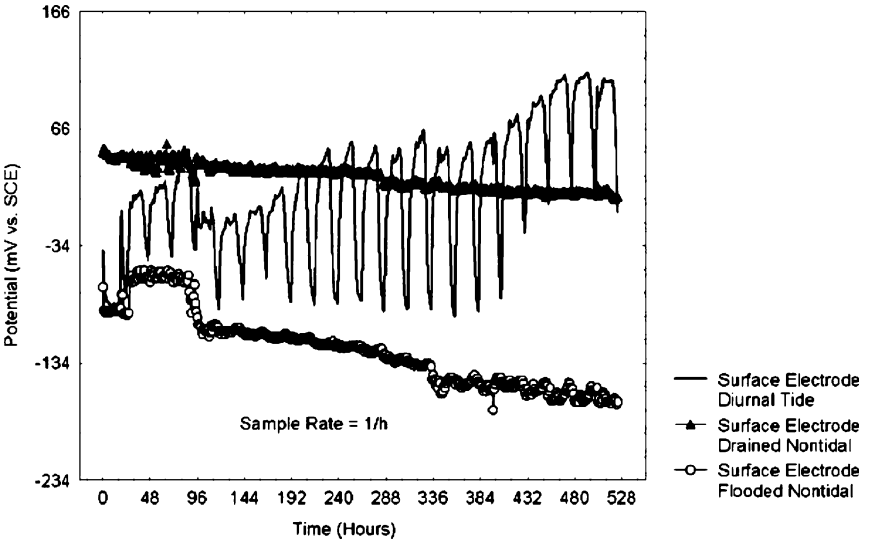

Fig. 1 Time series of redox potential in salt marsh tide simulation microcosms

pyrogenic matter. Frequently, these otherwise serviceable soils/sediments are relegated to dumpsites. It was of interest to determine the potential for recovering these sediments as a resource in a world that is increasingly "sediment-starved". If, like many other classes of pollutant organic chemicals, the degradation of aromatic hydrocarbon pollutants is significantly different under the various hydraulic (and resulting biogeochemical) conditions found in different parts of tidal wetlands, these differences could potentially be exploited to allow for recovery and utilization of marginally or even potentially severely contaminated sediments for use in wetland mitigation and creation.

2 Methods and Rationale

This work required a large amount of subsidiary R&D in (1) hydrodynamic sediment-plant mesocosm design, replication, and monitoring, (2) synthetic and analytical chemistry, including the synthesis of commercially unavailable standards and development analytical approaches to detect minor differences in organic chemicals between time points and treatments and (3) sensor design, time series data acquisition and wavelet analysis of non-stationary series [6], and covariance structure modeling of mesocosm and ecosystem data [1]. Basic questions (e.g., what constitutes a true spatiotemporal replicate in a multivariate, multiply colinear system? What is the minimum number of indicator variables needed to characterize the states of such a system and how often do they need to be sampled in space and time?) arose and had to

be dealt with substantively. This is the case with many large projects whose goals are statistical and analytical (rather than simply descriptive). The main question here was, nevertheless, applied: can sediments that are marginally-contaminated with aromatic chemicals be productively deployed in wetland restoration and creation efforts, and if so, what factors need to be optimized in order to remediate the sediment contamination in situ?

The target AHs for this work were naphthalene, phenanthrene, 1-methylnaphthalene, 1,3-dimethylnaphthalene, 3-methylphenanthrene, and 3,6-dimethylphenanthrene, which are common environmental contaminants found conspicuously in some dredge materials, spilled coal-, petroleum- and pyrogenic mixtures, and sediments near oil production and produced waster discharges. It was expected that AH transformation rates would be fastest and most complete in oxidized and tidal sediments vs. significantly slower and less complete in flooded conditions. In a marsh creation effort using moderately contaminated dredge or other sediments, the flooded/reducing marsh interior would receives the bulk of the contaminants, because the interior constitutes much of the wetland volume.

2.1 Tide Simulation Mesocosm (TSM)

TSMs [6, 15] for this study were established using commercial injection-molded plastic tubs of 300 gallons volume, 1.5 m (i.d.) $\times$ 0.65 m high. Each TSM unit was equipped with one timer-controlled salt water pump that stepped water out of the flooded mesocosms into elevated storage tanks over a 12 h period, simulating a falling tide. A passive siphon was activated upon filling the storage tanks, and this returned the water to the TSM with simple flow restriction controlling the return time (again, 12 h; i.e., a diurnal tide typical of much of the Gulf of Mexico). The water in the storage tanks was bubbled constantly with air, and dissolved sulfate, nitrate, and salinity were monitored daily at flood tide. In addition to the simulated tide, the other managed input was artificial sunlight (via fluorescent grow lamps and rotating high intensity discharge illuminators) which shone for 10 h per day followed by 14 h of relative darkness. The lighting systems (National Biological, Twinsburg OH) were designed to emulate the solar spectrum reaching sea level in the temperate zones of the world, with total radiant energy deliveries around 0.8 kW/m^2. The artificial sunlight cycle was synchronized with external day time because the mesocosms were located in a 2000 ft^2 laboratory annex containing no climate control and a large wall fan open to the outside environment (the laboratory and annex were situated on the top floor of a warehouse). System variables including water and sediment temperature, sediment redox potential and pH, air temperature, relative light intensity, and relative humidity were monitored using custom and commercial loggers and sensors, with sample intervals (scan rates) normally on the order of 1 h or

less. The cost of the each unit with timers, sensors, data loggers and dedicated artificial sunlight units was about $2800 in current US dollars.

Sediments and plants were collected in early spring from a large salt marsh complex in Terrebonne Parish, Louisiana. Bulk sediment samples were collected from shallow water bottoms abutting the natural levees at streamside marsh locations. Chemical analysis of extracts from these sediments confirmed that they were "clean" with respect to the AH target analytes of interest in this work: the few AHs observed in these extracts were present at low parts per billion concentrations (determined by isotopic-dilution gas chromatography – mass spectrometry, below). Also about 250 healthy, mid-sized (3–4 ft) *S. alterniflora* plants were collected, including much of the associated root masses. Harvested plants were replaced immediately by the same number of our greenhouse-reared *S. alterniflora*. Only this dominant plant was collected: no attempt was made to reproduce the vegetative diversity of the marsh by collecting the other typical salt marsh species present in the community (e.g., *Juncus* sp., *Salicornia* sp., *Spartina patens*, etc.).

In the laboratory, the sediments were placed in rectangular plastic agricultural mesh enclosures fitted with interior burlap curtains. These enclosures were positioned on top of layered oyster shells and cobble stones covering the bottoms of the TSMs, with sediments added to a height of 0.6 m above these layers. The plants were transplanted into the sediments enclosures at a stem density similar to that observed in the field (approximately 30 stems/m^2) within 24 h of collection. The resulting six sediment-plant TSMs were randomly assigned to each of the three hydrologic conditions to be examined: *flooded-anaerobic* (oxygen deficient), with water drained and replaced weekly, *drained-aerobic* (well oxygenated), with water flushed into the system and then immediately removed weekly, and (3) *tidal*, pulsed by a simulated diurnal tide (one high and one low per day). The "estuarine water" used was a commercial aquarium mix of virtually identical elemental composition as seawater (Instant Ocean, Greco Veterinary Supply, Baton Rouge LA), and this was inoculated with authentic estuarine water at 20 vol. % to a final salinity of 18 g/kg. The TSMs were acclimated for two weeks under each hydraulic regime prior to the addition of the contaminated sediments (below). These systems ran for two years, and in the tidal and drained TSMs, the annual grass *S. alterniflora* grew to almost 6 ft in height and proceeded through its entire life cycle including producing viable seeds and extensive root masses.

After establishment and equilibration of the TSMs, clean bulk sediments were sieved (3 mm) to remove shells and other debris, slurried into the artificial estuarine water (1 : 8 v/v) and placed in a rotary mixer. While mixing vigorously, the AH target analytes in a concentrated DMSO stock solution were gradually added to the sediments. Next, identical volumes of the slurry were pumped into the TSM enclosures over the 0.6 m of uncontaminated sediments already in the TSM, which provided for a contaminated layer thickness of approximately 0.3 m. After about 12 h of acclimation, the hydraulic regimes

(flood, drain, and tidal) were commenced in duplicate TSMs. Sediment samples from the contaminated layers were taken to a constant depth using solvent-clean 3 cm i.d. corers. The sampling site was marked so as not to inadvertently resample the same area twice. The sediments were immediately processed for solvent extraction and isotopic dilution gas chromatography-mass spectrometry (GC-MS) as given below. Trace gases leaving the sediments in the mesocosm were analyzed using a Buck Scientific Fourier transform infrared (FTIR) spectrometer with a 30 m gas cell. Samples were introduced to the cell under vacuum at ambient temperature. Authentic gas standards (Scott Specialty Gases, Pasadena TX) including methane, hydrogen sulfide, and dimethylsulfide were used for FTIR identification of gaseous compounds in the TSM headspace samples.

2.2 Impulse and Response Signal Acquisition and Analysis

The variations in time of many natural processes are structured, i.e., characterized by observable regularities or geometries [5, 6, 15–17]. The goal of time series data acquisition and analysis is to obtain a discrete record of a continuous function that has high enough resolution to detect all of the information in the output, and then analyze it with techniques that can adequately accommodate transients and spikes. Outputs from processes can be regarded as signals that if appropriately sampled and analyzed, can provide functional metrics of a system. In this sense, a biogeochemical system can be viewed as a signal processor, with exogenous inputs impinging on the many-component system and affecting its observable behavior, that is, the magnitudes and time structuring of some output signals. The inputs typically are impulses or excitation functions acting directly or indirectly on some system process(es) (e.g., thermal and photochemical energy, dissolved nutrients, hydraulic alteration). The outputs are properties or quantities that result from intrinsic system processes. Experiments involving modeling (as in observation of mesocosm function) and temporal structuring of system processes will identify and, ideally, accurately reflect fundamental inputs and outputs. In this work, pH, Eh, temperature, and light levels were monitored using commercial sensors and data loggers. Input variable time series from each mesocosm, including air and water temperature and light level were compared at each sediment sampling time point to determine any major variations or malfunctions. Similarly, output variable time series such as Eh and pH were recorded, and other indicators such as sediment trace gases were used to define the biogeochemical responses to the hydraulic regime.

2.3
Synthetic and Analytical Organic Chemistry

In order to quantify small or subtle differences (i.e., < 10%) in chemical concentrations in sediment extracts, isotopic dilution mass spectrometry is, for many organic compounds, the method of choice. In this work, it was necessary to have affordable access to gram quantities of the perdeuterated AH compounds and their alkyl homologues, and it was found that many compounds were either commercially unavailable or cost prohibitive. As a result, our R&D was targeted in this area. Eventually, these challenges were overcome, and a method for economically perdeuterating a wide selection of AHs and their alkyl homologues in supercritical deuteroxide was validated and patented [12–14]. All AH standards used in this work were purchased (Aldrich, Milwaukee, WI) or synthesized, followed by post-synthetic deuteration using the cited method.

2.4
Sampling and Chemical Analysis

Core samples (glass corers, 3 cm i.d.) from the TSMs were weighed, mixed with anhydrous sodium sulfate, and amended with the isotopic dilution standards in known amounts. The sample then was Soxhlet extracted with ultrapure dichloromethane (DCM, Aldrich, Milwaukee WI) for at least 150 cycles, which took about 36 h. After extraction, the DCM was dried with anhydrous Na_2SO_4 and analyzed using GC-MS. The GC-MS used was a Shimadzu QP500 GC-MS; DB-5 capillary column (30 m; 0.25 mm i.d.; 0.25 μm film); injector 250 °C; temperature program 70 °C (4 min) ramp 4 °C/min to 250 °C (10 min); sampling rate 2 Hz and mass acquisition range 50–300 amu in the full scan mode. Analyte transfer to the mass spectrometer source was at 280 °C and the source energy 70 eV. Target ions spanned the inclusive molecular weight range between C_2-benzenes (106 amu) and coronene (300 amu). Analyte, isotopic dilution standard, and transformation product identifications were performed using (a) comparison of experimental data with authentic standards, (b) interpretation of mass spectra (molecular ions, isotopic structures, and logical fragment losses) and, (c) comparison of spectra with computerized libraries of mass spectra.

2.5
Electrophoresis of Hydroxylated AH Transformation Products [18]

One of the main pathways of microbial and photochemical transformation of AHs is oxidation of an aromatic ring to generate hydroxylated products (e.g., phenols and diols). As bioenergetics and metabolic status of microbial communities varies drastically with change in Eh (which follows prevailing

hydraulic conditions and the availability of dissolved oxygen), an indicator of AH transformation would be the presence of oxygenated AHs in the aqueous phase. It is known that tetrazotized *o*-dianisidine (TODA) forms colored complexes with aromatic alcohols (AHOH) and ketones in water and that the resulting AHOH-TODA complexes can be observed and quantified spectrophotometrically [19]. In mixtures, it is appropriate to separate the components prior to detection, in this case, via capillary electrophoresis. Water samples (10 ml) were taken at the same time as sediment samples, and were filtered through 0.45 μm membranes. The filtrate was mixed with 0.1% TODA (Sigma, St. Louis MO) in 10% ethanol/water, and after 1 h was analyzed using micellar electrokinetic capillary chromatography(MECC). MECC analyses were performed on a Dionex Corporation Capillary Electrophoresis System (68 cm × 25 μm fused silica uncoated; electroinjection at 2500 V for 8 s; voltage control mode; 50 mM sodium dodecyl sulfate electrolyte; UV/Vis detection at 369 nm). Standards included commercial AHOHs including 1-naphthol and *p*-cresol vs. negative controls (no TODA-AH complex formation) naphthalene and *p*-xylene.

3 Results and Discussion

Within two weeks of the initial establishment of the hydraulic conditions of the TSMs, three distinct biogeochemical conditions were observed, and these were reproducible between the duplicate mesocosms (Fig. 2). Over the course of this study, the input variables of air temperature, water temperature and light regime were constant between the six TSMs, i.e., there were no anomalies or input biases affecting the TSMs. Water and sediment temperatures followed air temperature very closely in time and magnitude, and these time series were highly reproducible between duplicates in all treatment groups. Wavelet transform analyses of the time series from these variables confirmed a tidal signal of 24.05 h, with Eh, pH, and sediment temperatures following the tide and showing similar periodicities.

Under static flooded (reducing) and drained (oxidizing) conditions, output variables, such as Eh and pH were more or less constant, with an insignificant downward trend in both over a period of months (i.e., Eh slowly became more negative/reducing, and pH also slowly decreased). Nevertheless, in both static hydraulic treatments, Eh and pH were fully described by elementary statistics: there were no significant fluctuations or structured dynamics (i.e., the error terms after a linear fit were random). Thus, the Eh; pH values for the static systems were: drained (+ 167 ± 35 mV; 7.8 ± 0.8) and flooded (– 310 ± 50 mV; 6.6 ± 0.7), with the Eh values given vs. Ag/AgCl reference electrodes and sample sizes for both indicators of $n = 5376$ (hourly samples for a 32 week period). On the other hand, the tidal systems had

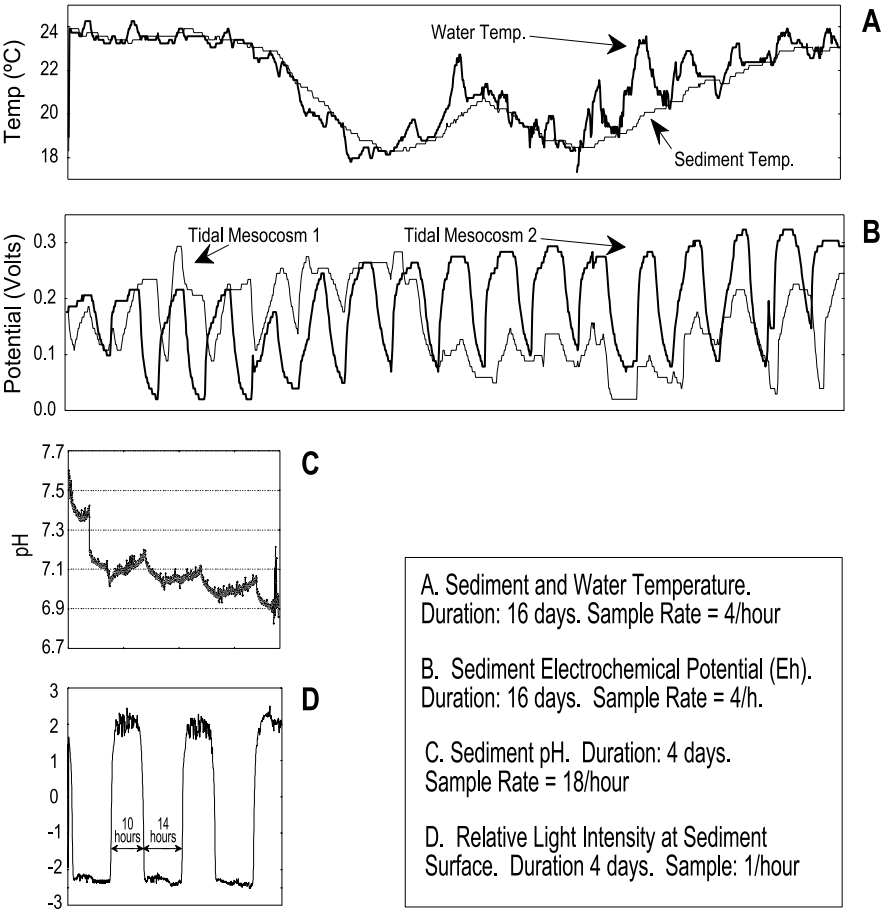

Fig. 2 High resolution time series of input and output variables from the TSMs. Air and water temperature (**A**) and redox potential Eh (**B**) from duplicate TSMs over a sixteen day period. Time series of sediment pH in tidally-forced sediments (**C**) showed clear diurnal variation in response to the tides (no structured variation was observed in static systems). The light (10 h)–dark (14 h) illumination cycle as detected at the sediment surface of a TSM (**D**)

reproducible daily fluctuations of central variables (such as Eh), and this was solely due to the tides (Fig. 2). In the tidal systems, Eh fluctuated daily between + 300 and – 50 mV daily. The duplicate systems required a pre-equilibration period of weeks for both phase and amplitudes of the Eh sinusoids to be satisfactorily synchronized. After about six months, however, the duplicate systems tended to meander, particularly with respect to irregular changes in Eh amplitude over the tide cycle. As the sensors were permanently placed for data logging, we interpreted these changes to be due to (1) the development of patches (heterogeneity) in previously well-homogenized sys-

tems and/or (2) progressive passivation of the electrode, although electron micrographic examination of new and used Pt electrode surfaces showed no differences.

These data have ecological relevance, in that plant productivity and survival were much higher in both drained and tidal conditions, and are consistent with field observations. In these mesocosms, above-ground plant heights averaged approximately two meters, while those in the static flooded systems averaged only about half that height. Seed production and root mat development also were obviously far greater in the drained and tidal systems than in the flooded ones. These observations reflect *S. alterniflora* growth patterns observed in natural systems, where the tall form *S.alterniflora* is found at higher elevations such as back marsh and streamside levees, while the short form of the grass occurs just a few meters away in the low-lying, flooded, marsh interior. Biogeochemical trace gases (Fig. 3) leaving the sediments evidenced the dominant metabolic processes occurring in each hydraulic treatment as expected. Aerobic metabolic processes were predominant in the drained and tidal TSMs, as the major gaseous product was CO_2. Anaerobic metabolism was predominant in the flooded systems, as indicated by an abundance of CH_4 and mixed sulfides, with smaller amounts of CO_2. These indicators were constant over the period of this work.

The time course of recovery of AH analytes from the TSMs under drained, flooded, and tidal hydraulic conditions are shown in Figs. 4–6. The range for these recoveries from duplicate TSM samples in all cases was < 16%, and in most cases < 10%. It seems from Figs. 4–6 that the AH transformation time course profiles from the drained and flooded treatments reflect limiting cases, with the tidal system somewhere in between, albeit most similar to the drained system, as the other biogeochemical indicators (e.g., Eh, trace gases) suggested. Under drained/aerobic conditions, most of the AH transformation occurred in the first month of the experiment, with much slower rates of transformation through week 10, and virtually none after that point, as shown in Fig. 4. Interestingly, the rate of transformation and the overall AH removal efficiency in this system both were inversely related to the molecular weight of the AH: as the molecular weight increased from naphthalene (128 amu) to C_2-phenanthrene (206 amu), rates of transformation and overall efficiency decreased. Naphthalene was 99% degraded to levels below 1 ppm in 10 weeks, while 3,6-dimethyl phenanthrene was degraded to about half its initial concentration in ten weeks, but remained constant after that point.

On the other hand, flooded anaerobic conditions (Fig. 5) did not support the rapid initial transformation of the AHs as seen in the drained TSM, with concentrations of all analytes remaining within 80% of their initial concentration for 20 weeks. Interestingly, between 20 and 30 weeks, an apparent burst of metabolic activity caused all AHs to be degraded to levels below 60 ppm. Relative to drained conditions, the observed AH transformation was

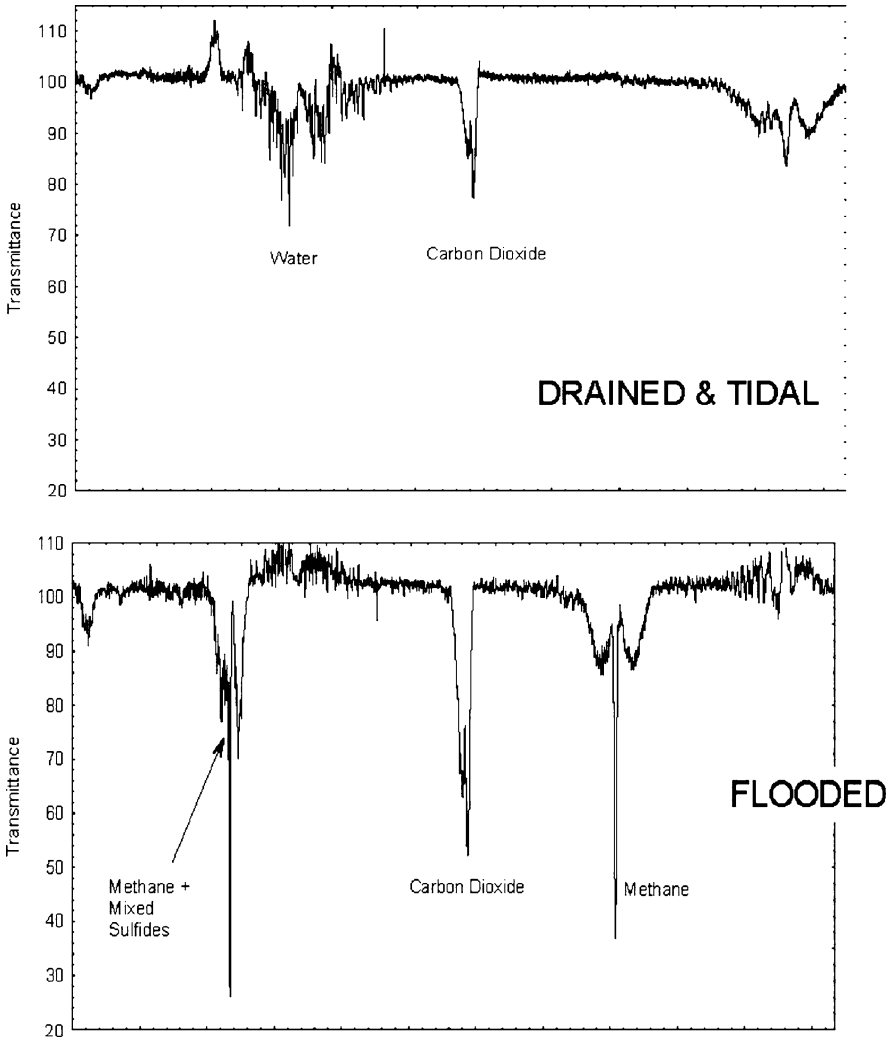

Fig. 3 Fourier transform infrared absorbance spectra of trace gases leaving the sediments of the TSMs. *Top*: tidal, *bottom*: chronically flooded. The spectra of gases from the drained TSM was essentially identical to the spectrum shown for the tidal TSM (*top*)

slower and less complete in the flooded TSMs. Under drained conditions, the AHs all were degraded to below 40 ppm by week 10, with naphthalene, 1-methylnaphthalene, 1,2-dimethylnaphthalene, and phenanthrene all below 20 ppm by 30 weeks. It took 28 weeks for the flooded reduced system to perform this function. Conversely, the clear dependence of degradation rate and efficiency on molecular weight of the AH observed under drained conditions was not observed in flooded treatments.

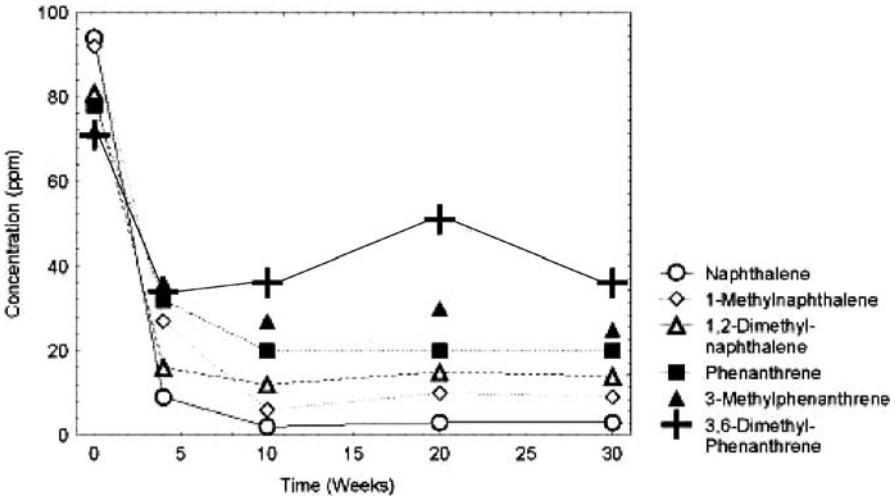

Fig. 4 Time course transformation profiles of AHs in the drained/aerobic TSMs. *Data points* are means of duplicates, with the maximum range of the trace given by the diameters of the *symbols*

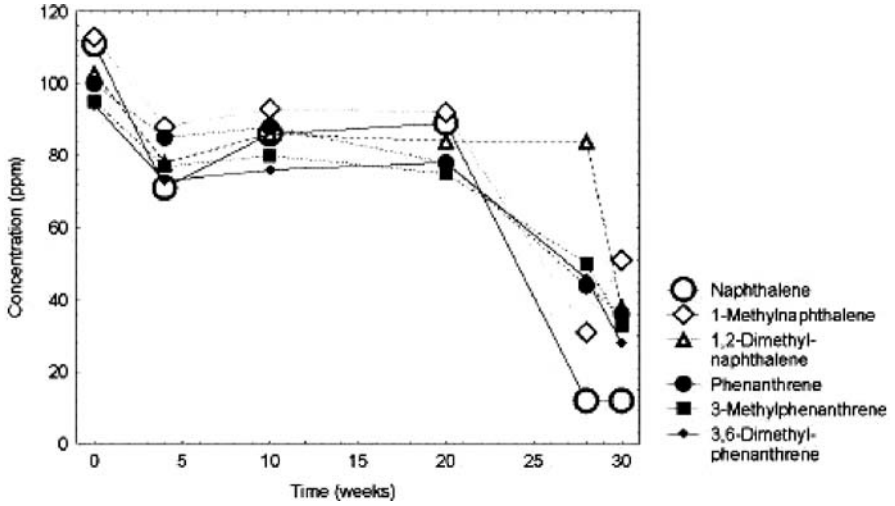

Fig. 5 Time course transformation profiles of AHs in the flooded/anaerobic TSMs. *Data points* are means of duplicates, with the maximum range of the trace given by the diameters of the *symbols*

The AH transformation profiles from the duplicate tidal TSMs (Fig. 6) resembled those seen in the drained TSMs: The apparent transformation rates in the first ten weeks were somewhat slower in the tidal systems, but the inverse relationship between molecular weight and transform-

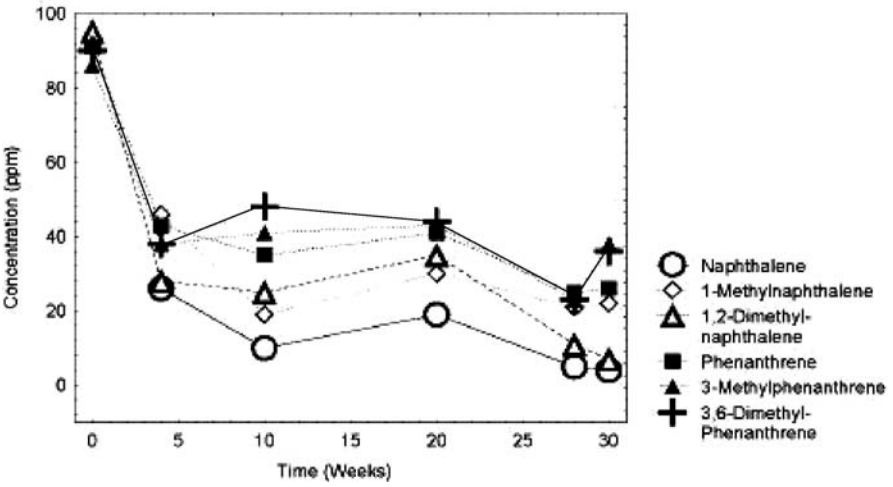

Fig. 6 Time course transformation profiles of AHs in the tidal TSMs. *Data points* are means of duplicates, with the maximum range of the trace given by the diameters of the *symbols*

ation was again observed. Also, the AH concentrations remained static between weeks 10 and 20 as in drained systems, but after 20 weeks there was evidence of further transformation, more like what was observed in the flooded systems. The tidal system then, in some ways resembled the drained TSMs (biogeochemical gases, AH transformation profile, grass productivity) and in other ways resembled the flooded/reduced TSMs (increased relative transformation rates after 20 weeks). In one respect at least, the tidal systems were unique: Their Eh and pH profiles were sinusoidal, rather than static and linear as seen in the drained and flooded TSMs.

Oxygenated (oxidized) AHs entering the aqueous phase were visualized using TODA and separated using MECC as shown in Fig. 7. Figure 7 shows an aqueous sample from a sterilized slurry containing the AHs, but no viable microbes, after treatment with TODA (no peaks detected), a standard phenol-TODA complex (one peak), and an aqueous phase sample from the drained TSM at week 5 (the last showing perhaps eight different TODA complexes). It was found that the number of MECC-resolved and detected TODA-AHOH complexes increased in the aqueous phases of the drained and tidal TSMs around the times that rapid AH transformation was observed (Table 1), but were constant or decreasing when transformation was ebbing. These changes were also seen in the flooded systems, but to a somewhat smaller degree.

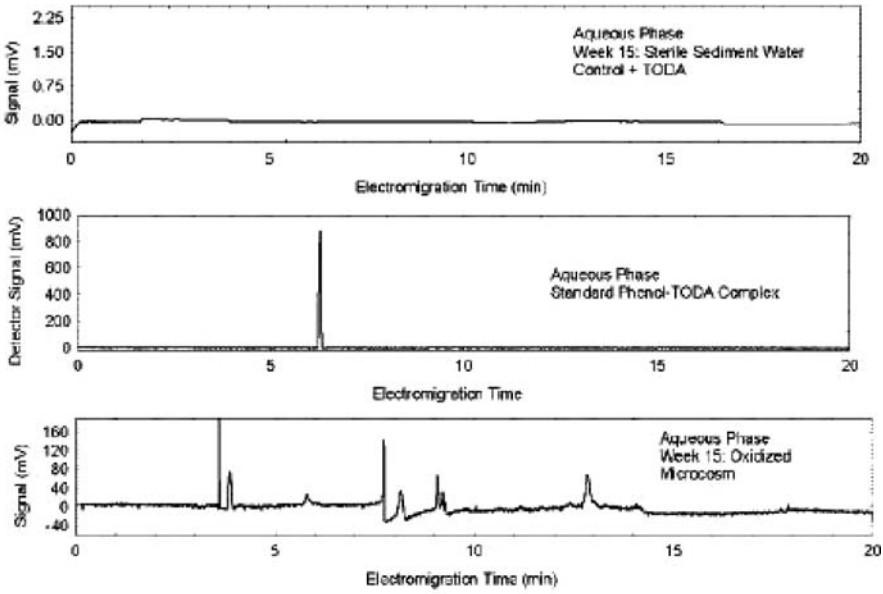

Fig. 7 MECC of TODA treated water samples from a sterile sediment water slurry containing the AHs (*top*), standard *o*-cresol-TODA complex (*middle*) and TODA-AHOH from the drained TSM at 5 weeks, where some eight resolved peaks are evident (*bottom*)

Table 1 Numbers of AHOH-TODA complexes resolved using MECC at different times during the experiments. Data are from duplicate TSMs for each hydraulic regime

Time (weeks)	Sterile control*	Drained/ oxidized	Flooded/ reduced	Tidal/ alternating
0	0	0, 0	0, 0	0, 0
4	0	9, 7	1, 2	6, 4
10	0	3, 2	2, 3	3, 3
20	0	1, 2	1, 1	1, 0
28	0	1, 1	5, 8	5, 4
30	1	1, 0	7, 6	2, 1

* $N = 1$; drained.

4 Concluding Remarks

The data generated in this work indicate the following:

- Hydraulic conditions and the resultant biogeochemical properties of tidal saline wetlands influence the rate, transformation-time profile, and completeness of transformation of AHs. In well-matched replicate systems,

these data are reproducible with acceptable standard deviations (< 20% of means in duplicate runs) for at least 30 weeks.

- Drained, flooded and tidal conditions can be established and maintained in the laboratory with true replication. The vegetative and biogeochemical behavior of the TSM sediment-plant assemblage is similar to natural field settings [6], so extrapolation of results from these systems to real-world ecosystem volumes is supported.
- In general, the relative transformation rates for the AHs in the different systems examined here was drained/oxidized Eh $\geq$ tidal/oscillating Eh $\gg$ flood/reduced Eh. This trend has been observed before in compounds including mixtures of N-, O-, and S-heterocycles [11–13].
- The generation of hydroxylated and probably other oxygenated AHs coincides with increases in observed compound transformation rates.
- While well-drained aerobic conditions seem to be optimal for the transformation/depuration of AHs and many other organic compounds, in natural settings this kind of system would not really be a "wetland", either geohydrologically or with respect to vegetation. In dry parts of impounded systems, and in back marsh areas cut off from regular salt water inputs (salt water is toxic to unadapted plants), upland and invasive plant species such as *Phragmites* sp. can take over and completely alter the ecosystem in a few years. In a real world wetland restoration or creation project, the available options are limited to flooded marsh interior locations, where AH transformation is slow, and streamside tidal locations, where transformation is about twice as fast. However, in both settings, the AHs are degraded to acceptable levels after about 7 months. Thus, water and wetland based fish and wildlife monitoring could be deployed during the first year or two, or until chemical analysis and toxicity testing validated an acceptable level of remediation of the sediments. So, when discussing "wetland" construction or expansion in this regard, the zones of relevance are streamside and marsh interior.
- AH contaminated dredge and other sediments are abundant and frequently not utilized or recycled for beneficial purposes. This work indicates that these sediments can be used, alone or mixed with clean sediments or other industrial materials (e.g., phosphogypsum and bauxite "red mud"), for wetland creation or enhancement. It would seem that either or both marsh locations (interior vs. streamside) are appropriate, at least with respect to these AHs. The tradeoff involves time and potential offsite risks: at streamside, the removal rates are twice as fast as and somewhat more complete than in the interior, but the action of the tides could mobilize contaminants to the estuary and beyond. The interior sediments will allow for less exchange of the pollutants with the estuarine water, but will take longer to depurate, particularly if the sediments become compacted.

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Marine Sediment Toxicity Identification Evaluations (TIEs): History, Principles, Methods, and Future Research

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Abstract A common method for determining whether contaminants in sediments represent an environmental risk is to perform toxicity tests. Toxicity tests indicate whether contaminants in sediments are bioavailable and capable of causing adverse biological effects (e.g., mortality, reduced growth or reproduction) to aquatic organisms. Several environmental management and regulatory programs concerned with contaminated sediments use this approach for assessing risk. However, a limitation of the toxicity testing approach is that the results indicate only if toxicity is present in a given sediment sample. Toxicity test results do not provide information on the cause of toxicity; that is, what specific toxic chemicals are responsible for the effects observed. Information on the cause of the effect is important in performing risk assessments and determining remedial actions at contaminated sediment sites. Methods called Toxicity Identification Evaluation

(TIE) procedures were originally developed for industrial and municipal effluents to determine the causes of toxicity in waters affected by these discharges. The TIE approach combines toxicity testing with simple chemical manipulations to selectively alter the toxicity of specific classes of toxicants in a sample. These aqueous TIE methods were later adapted for use with contaminated sediment interstitial waters. Now, whole sediment TIE methods have been developed for both freshwater and marine sediments. This chapter will focus on the development of TIE methods for marine contaminated sediments. Like the aqueous-based TIE methods, the whole sediment TIE methods combine toxicity testing with chemical manipulations to selectively alter the toxicity of potential classes of sediment contaminants. By selectively altering the toxicity of potential classes of toxicants followed by comparison to the toxicity of an unmanipulated sample, it is possible to characterize and identify the causes of sediment toxicity. Currently, interstitial water and whole sediment TIE methods are designed to detect toxicity caused by cationic and anionic metals, nonionic organic compounds, and ammonia, although methods for other toxicants including hydrogen sulfide and specific pesticides are under development. This chapter will provide an overview of the TIE approach and methods for both marine interstitial waters and whole sediments. The chapter will conclude with a brief discussion of new applications for sediment TIEs as well as research needs for the continued development of sediment TIE methods.

Keywords Identification · Interstitial waters · Sediments · TIE · Toxicity · Toxicity identification evaluation · Whole sediments

Abbreviations

COC	Contaminants of concern
EDTA	Ethylendiaminetetraacetic acid
K_{ow}	Octanol-water partition coefficient
NPDES	National Pollutant Discharge Elimination System
NSI	National Sediment Inventory
PAHs	Polycyclic aromatic hydrocarbons
PCBs	Polychlorinated biphenyls
PCC	Powdered coconut charcoal
PCDDs	Polychlorinated dibenzodioxins
PCDFs	Polychlorinated dibenzofurans
PCPs	Personal care products
SI	Stressor identification
TMDL	Total maximum daily loading
TIE	Toxicity Identification Evaluation
U.S. EPA	United States Environmental Protection Agency

1 Introduction

Sediments are a major sink for many anthropogenic contaminants entering coastal marine systems. The presence of these chemicals in sufficient quantities and under certain conditions may result in sediment toxicity to benthic

and epibenthic organisms. Sediment toxicity is widely recognized as an environmental problem in many regions of the world including North America, Europe, Australasia and Asia [1–6]. For example, in an assessment of estuarine sediments in the United States, 11% of whole sediments and 43% of interstitial waters were determined to be toxic [7]. Further, the National Sediment Inventory (NSI) reported 10% (or nearly 1 billion cubic meters) of sediments in the US were contaminated and represented a risk of causing toxicity [8].

Sediment toxicity assumes many forms but is most often measured by acute endpoints such as mortality [9–13], and sublethal endpoints like reduced growth and reproduction [7, 14]. Sediment toxicity is also manifested as population declines [15, 16] and community impairments [17, 18]. While knowledge of the presence of sediment toxicity is important, the ability to identify the cause(s) of toxicity in sediments is also important and has many applications. Identification of toxicants in toxic sediments allows environmental managers to make informed decisions on appropriate remediation and disposal options for dredged material, identify important stressors in impaired benthic communities, develop stressor-response relationships for ecological risk assessments, and determine sources of continuing contamination in order to develop appropriate environmental management and clean-up strategies.

For all of these reasons, the U.S. Environmental Protection Agency (U.S. EPA) along with its research partners have developed an approach called sediment Toxicity Identification Evaluation (TIE) to identify specific toxicants or classes of toxicants responsible for causing toxicity in sediments. The approach consists of several phases encompassing both toxicity testing and chemical manipulations used in parallel and iteratively until the active toxicants or classes of toxicants are identified. While freshwater methods have been developed in parallel to the marine methods, this chapter discusses the history, basic principles, methods, and future research for marine sediment TIEs.

2 History

The TIE approach was originally developed in the 1980s and 1990s to identify toxicants in industrial and municipal effluents and their receiving waters. In the United States, TIEs were first developed for freshwater applications to complement regulations of the Clean Water Act to reduce the release of toxic chemicals into the nation's waters (i.e., National Pollutant Discharge Elimination System (NPDES) permitting) [19–21]. Later, similar methods were developed for marine applications [22]. The TIE approach has three phases (Fig. 1). Phase I (Characterization) characterizes the classes of toxi-

cants causing observed adverse biological effects. For effluent and receiving water TIEs, the classes of toxicants include cationic metals (e.g., cadmium, copper, zinc), organic chemicals (e.g., pesticides, polychlorinated biphenyls (PCBs), polycyclic aromatic hydrocarbons (PAHs), volatile chemicals, filterable toxicants, pH-dependent toxicants like ammonia, and oxidizing chemicals (e.g., chlorine) [20, 22]. Phase II (Identification) seeks to identify the toxicants characterized in Phase I and strives to answer questions such as "If a cationic metal is causing toxicity, which metal is it?", and "If it is an organic chemical, is it polar or non-polar" [19]? Phase III (Confirmation) [21] validates the identify of the toxicant(s) characterized and identified in Phases I and II using a variety of approaches including correlation, toxic units, symptoms, spiking, and deletion. Finally, because effluent and receiving water TIE methods were developed primarily to support the Clean Water Act (at least in the United States), the toxicity testing species used were frequently the same species used in NPDES permitting. For example, in marine effluents testing, these species included acute methods with the epibenthic mysid *Americamysis bahia* and sub-lethal fertilization tests with echinoderms (sea urchins) [23]. Sediment TIEs use this same basic approach developed for effluent and receiving water TIEs [19–21]; however, due to the difference in matrices and the types of chemicals accumulating in sediments relative to those occurring in water, different specific chemical manipulations and toxicity tests have been developed and applied. In 2007, the U.S. EPA released

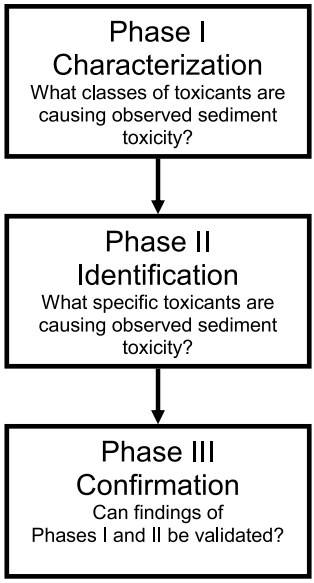

Fig. 1 Basic structure and questions asked at each phase of a toxicity identification evaluation (TIE)

a sediment TIE guidance document [24] that includes information on the performance of Phases I, II, and III for marine and freshwater interstitial waters and whole sediments.

Sediment TIE methods fall generally into two classes: interstitial water (IW) and whole sediment [24]. Interstitial, or pore water, methods have been largely adapted from the effluent and receiving water methods discussed above and used over the last 10 to 15 years [25–31]. However, a number of concerns associated with interstitial water toxicity testing have arisen [32]. These issues include changes in metal concentrations and consequently toxicity that may occur during interstitial water isolation procedures such as centrifugation. Specifically, changes in the ratio of sediment particles to water that occur during centrifugation may result in metal concentrations greater than the concentrations in the actual sediment interstitial waters [32]. Further, the routine practice of aerating interstitial waters during toxicity testing may result in oxidation of metals and other toxicants causing changes in interstitial water pH due to CO_2 volatilization. It is also likely that interstitial water toxicity testing results in under-exposure of moderate to high octanol-water partition coefficient (K_{ow}) organic chemicals because of sorption to the test chamber walls and volatilization into the atmosphere. Finally, there is potential for over-exposure of organisms not normally experiencing 100% interstitial water but rather a mixture of overlying and interstitial waters. Further, for some organisms, elimination of important exposure routes, such as sediment particle ingestion, may occur. For all of these reasons, as well as the fact that testing the whole sediment matrix more closely approximates the type of toxicity testing performed to initiate the use of sediment TIEs (e.g., dredging protocols [33], whole sediment TIE methods have been developed [24, 34–40].

3 Principles

3.1 Whole Sediment TIE

In the whole sediment TIE, the objective is to change the toxicity of the manipulated sediment relative to the un-manipulated treatment (called the "baseline"). This change should be performed in a reproducible and consistent manner. Sediment TIE chemical manipulations can be categorized into two basic types: additions and alterations.

Addition manipulations are introduced into the exposure by mixing a manipulation chemical or phase into the sediment or placing the phase above the sediment in the exposure system. The added phase has a high affinity and selectivity for the contaminant of interest and most importantly

sequesters the contaminant in a non-bioavailable form. If the addition manipulation is effective, the presence of this added phase results in a reduction in the bioavailability of the class of toxicant of interest as compared to the baseline treatment. The manipulations discussed below for characterizing organic chemicals and cationic metals are addition manipulations. Similarly, one of the manipulations for ammonia, the macroalgae *Ulva lactuca*, which removes ammonia from the exposure by uptake, is also an addition manipulation (see section on *Ulva lactuca* addition). For these manipulations, the addition of a new phase (a resin or algae) results in reduced bioavailability expressed as reduced toxicity that can be compared to the baseline toxicity.

In the alteration manipulations, the toxicant is changed from a toxic form to a less toxic form through the TIE chemical manipulation. In general, like the addition manipulation, the alteration manipulation is introduced into the sediment by mixing. The TIE manipulation results in the removal of the toxicant from the exposure and ultimately a decrease in toxicity. The manipulation described below for characterizing ammonia toxicity in which the mineral zeolite is added exemplifies this type of manipulation. The addition of zeolite to the sediment results in ammonium adsorbing to the zeolite. Because ammonia and ammonium exist in equilibrium in water (see Eq. 1 in Ammonia section), the removal of ammonium from the water also causes the ammonia concentrations to decline in order to re-establish the equilibrium. The reduction in ammonia concentrations results in reduced toxicity.

As discussed, the whole sediment TIE manipulations are all designed to reduce the bioavailability of specific sediment contaminants and their associated toxicity. By using these manipulations together in a whole sediment TIE, it is possible to begin to characterize the classes of contaminants causing toxicity in environmentally contaminated sediments.

3.2 Interstitial Water TIE

The interstitial water TIE methods, like the whole sediment methods, can be categorized into two basic types: removals and alterations. Because interstitial water is a liquid, the interstitial water TIE often involves combining the sample with a manipulatory chemical or phase for a relatively brief period of time. This is in contrast to the whole sediment TIE in which the manipulatory chemical is actually amended into a sample, in most cases, for the duration of exposure. In the interstitial water TIE, column chromatography is the most common technique for accomplishing this process. In this technique, the toxicants of interest are transferred from the interstitial water sample to the column stationary phase and removed from the aqueous phase. The post-column effluent is then tested for toxicity. In some cases, the toxicants can be

eluted from the stationary phase as part of Phase II. The *U. lactuca* addition manipulation discussed above is performed nearly identically in the interstitial water TIE and can also be considered a removal method.

In the interstitial water TIE, the alteration manipulations are similar to the alteration manipulations in the whole sediment TIE. For example, changing the interstitial water pH is used to alter the toxicity of ammonia in one of the interstitial water TIE manipulations. The change in interstitial water pH causes an alteration of the ammonium and ammonia equilibrium (equation 1 in Ammonia section) which affects the concentration of toxic ammonia in the interstitial water. By altering the ammonia concentrations as a function of pH, it is possible to determine if ammonia is associated with observed toxicity.

4 Marine TIE Methods

This section summarizes commonly used marine sediment TIE methods. While equivalent freshwater methods have been developed [37–39, 41] this chapter focuses on Phase I (Characterization) marine methods developed for both interstitial water and whole sediments [24]. Phase II sediment TIE methods (Identification) are under development (e.g., [37–39, 41] but will not be discussed here). Phase III confirmation methods are not unique to sediments and the techniques developed for effluents and receiving waters can be used [21].

4.1 Toxicity Tests

As the TIE approach is an iterative and interactive process between toxicity testing and chemical manipulations, the choice of toxicity testing organisms and protocols can profoundly affect the outcome of the TIE. Factors influencing the choice of the testing organism for a TIE are the same factors that influence the choice of toxicity testing organisms used in many applications including conducting field surveys and assessing dredged sediments. These factors include species sensitivity, selectivity, regional or nation-wide availability (i.e., cultured, field-collected, or commercially available), geographic distribution, ecological niche, the organism's ability to act as a surrogate and, an additional consideration for TIEs, tolerances to the TIE chemical manipulations. Tolerances to TIE chemical manipulations have been documented for some lifestages of the following toxicity testing organisms: amphipods *Ampelisca abdita*, *Leptocheirus plumulosus* and *Eohaustorius estuarius*, mysid *Americamysis bahia*, bivalves *Mercenaria mercinaria*, *Crassostrea gigas*, *Mytilus californianus*, *Mytilus galloprovincialis* and *Mulinia lateralis*,

Table 1 Marine TIE toxicity testing species. Each of these species has been tolerance tested for the common TIE chemical manipulations and conditions

Species	TIE lifestage or size	Intersitial water or whole sediment	Geographic location	Refs.
Amphipod, *Ampelisca abdita*	0.7 mm	Both	Atlantic, Gulf and Pacific coasts	[22, 24]
Amphipod *Eohaustorius estuarius*	Subadult	Both	Pacific coast	[40]
Amphipod *Leptocheirus plumulosus*	Subadult	Both	Atlantic, Gulf and Pacific coasts	D. Fischer Univ. of Maryland, personal communication
Mysid *Americamysis bahia*	48 h old	Both	Atlantic coast	[22, 24]
Bivalve *Mercenaria mercenaria*	> 0.7 and < 1 mm	Both	Atlantic coast	Ho et al. in prep
Bivalve *Crassostrea gigas*	Embyo – larval development	Interstitial water	Pacific coast	[20]
Bivalve *Mytilus californianus*	Embyo – larval development	Interstitial water	Pacific coast	[20]
Bivalve *Mytilus galloprovincialis*	Embyo – larval development	Interstitial water	Pacific coast	[20]
Bivalve *Mulinia lateralis,*	Embyo – larval development	Interstitial water	Atlantic coast	[20]
Echinoderm *Arbacia punctulata*	Fertilization	Interstitial water	Atlantic coast	[20]
Echinoderm *Strongylocentrotus purpuratus*	Fertilization	Interstitial water	Pacific coast	[20]
Echinoderm *Dendraster excentricus*	Fertilization	Interstitial water	Pacific coast	[20]

echinoderms *Arbacia punctulata*, *Strongylocentrotus purpuratus* and *Dendraster excentricus* (Table 1) [22, 40]. Consideration of an organism's origins should be answered with respect to the specific TIE's objective. For example, "Is a particular organism appropriately representative of the benthic environment being evaluated in a given study, or is a surrogate organism acceptable?"

4.2 Whole Sediment TIE Methods

Whole sediment TIE methods have been developed to address three major classes of toxicants: organic chemicals, cationic metals, and ammonia (Fig. 2). The following section discusses the specific TIE methods for these classes of toxicants. These methods are discussed in detail elsewhere [24, 34–37, 40, 42].

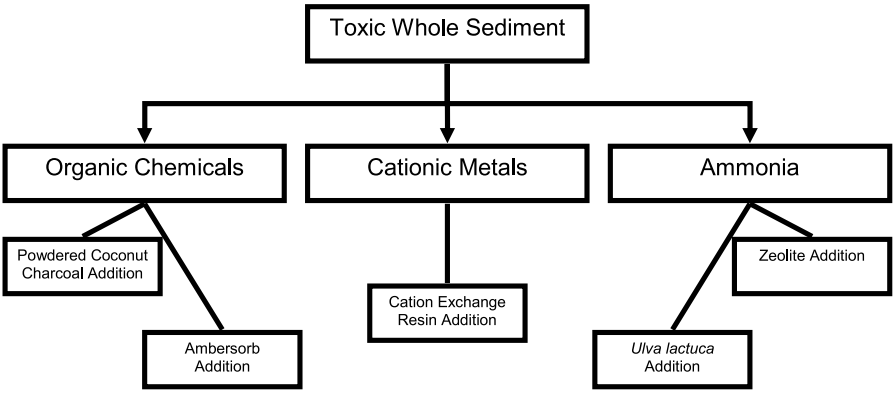

Fig. 2 Whole sediment toxicity identification evaluation (TIE) toxicant classes and manipulations

4.2.1 Organic Chemicals: Powdered Coconut Charcoal Addition and Ambersorb™ Addition

Sediments are major sinks for many contaminants, particularly medium to high $\log K_{ow}$ (e.g., >4) non-polar, hydrophobic organic chemicals entering coastal marine systems [2–4, 43]. Both powdered coconut charcoal (PCC) and Ambersorb 1500™ additions were developed to be used as Phase 1 manipulations for characterizing such non-polar organic chemicals. Because sediments themselves contain natural organic matter (generally <1–10% by weight) capable of absorbing organic chemicals, developing a TIE manipulation to distinguish and reduce the bioavailability of anthropogenic organic contaminants without altering the natural organic matter or creating artifactual sediment toxicity represented a significant challenge. Both Ambersorb 1500™ (Rohm and Hass, Philadelphia, PA, USA) and PCC (Calgon Carbon Corp, Pittsburg, PA, USA) proved to be effective manipulations because of their ability to adsorb organic toxicants and not impart any artifactual toxicity of their own [35].

Calgon PPC consists of pyrolyzed, activated coconut husks ground to a specific size (i.e., 90% to 96% <45 μm). Ambersorb 1500™ (estimated particle size

2–30 μm) [44] is a carbonaceous resin designed to adsorb organic chemicals. Both materials can be added directly to sediments at concentrations of 10–20% wet weight. The sediment-resin or sediment-PCC mixture is allowed to equilibrate for 24 h before organisms are added. The PCC or resin is left in place during the toxicity test and discarded with the sediment after test termination. Both materials have proven to be relatively specific in reducing organic chemical bioavailability as compared to cationic metals and ammonia bioavailability. Further, both manipulations are non-toxic to the marine organisms tested and effective at removing a range of different organic toxicants [35]. The majority of whole sediment TIE research has focused on PCC because Ambersorb 1500™ is no longer manufactured by Rohm and Hass.

4.2.2
Cationic Metals: Cation Exchange Resin Addition

Like organic contaminants, several toxic cationic metals including cadmium, copper, nickel, lead and zinc produced by industrial activities, including mining, accumulate in marine sediments [2, 45]. At sufficiently high levels these metals can be toxic to sediment organisms [46]. The cation exchange resin addition manipulation was developed to characterize the toxicity caused by these metals [47]. In the sedimentary marine environment, there are several cations like calcium, sodium and magnesium which occur naturally and are biologically required [48, 49]. This is especially true in seawater where sodium occurs at very high concentrations [49]. When these cations are removed or altered relative to the "natural" composition of seawater, toxicity can occur due to the phenomena called ionic imbalance [50]. Consequently, the Phase 1 TIE manipulation for toxic cationic metals needed to demonstrate a high degree of selectivity to avoid removing biologically important cations. Similarly, if the manipulation was not selective, it could be completely saturated by naturally occurring and non-toxic ions like sodium and magnesium and thus be ineffectual. ResinTech SIR-300 cation exchange resin (West Berlin, NJ, USA) was specifically designed to have the greatest affinity for chemically bonding (i.e., chelating) with divalent transition cationic metals (i.e., cadmium and the others). This affinity results from the use of the functional group aminodiacetate as the chelator. When a cationic metal like copper is chelated by this functional group it is strongly sequestered to the resin and only removed by strong acidification. The resin, when prepared in a high purity form, is not toxic to marine toxicity testing species.

Like PCC or Ambersorb 1500™, the cation exchange resin is added to the sediment at concentrations of 15 to 20% of the sediment wet weight and mixed thoroughly. After a 24 hour equilibration period, the toxicity testing organisms are added to the exposure. The resin is left in the sediment throughout the exposure. When the toxicity test is terminated, the resin can be sieved-out, rinsed with deionized water, and the metals extracted with acid in order to determine

which metals were removed from the exposure [47]. This last step of rinsing the resin with acid is considered a Phase II manipulation.

4.2.3 Ammonia

Ammonia in sediments occurs naturally as a result of the decomposition of nitrogenous organic matter introduced into the benthos [34]. Like many chemicals, ammonia is an important nutrient for aquatic organisms but at high concentrations can be toxic [51]. Excessive anthropogenic loadings of nutrients and organic carbon can disrupt the natural nitrogen cycle and cause the build-up of elevated and toxic concentrations of ammonia. In marine waters, ammonia occurs in two forms: "ammonia" (NH_3) and "ammonium" (NH_4^+). Ammonia and ammonium are distributed in seawater as a function of pH, temperature and salinity:

$$NH_3 + H_2O \longleftrightarrow NH_4^+ + OH^- . \quad (1)$$

Because of ammonia's log base dissociation constant (pK_b) of approximately 9.00, at low pHs, ammonium is dominant and at pHs greater than 9.00, ammonia dominates. Ammonia is generally the form toxic to aquatic organisms while ammonium is relatively non-toxic [34, 51]. A number of methods exist to characterize ammonia caused toxicity in interstitial waters (see sections on pH manipulation and zeolite column chromatography). The two methods used most commonly in marine sediment TIEs are the addition of the macroalgae *Ulva lactuca* and the mineral zeolite.

4.2.3.1 *Ulva Lactuca* Addition

Ulva lactuca is a cosmopolitan attached macrophyte with the ability to absorb, store, and use large amounts of ammonia as a nutrient [36, 52]. This ability to absorb and use large amounts of nutrients over a short period of time is called "glut" or "luxury uptake" and this trait has made *U. lactuca* useful as a biofilter for fish pond waste resulting from aquaculture [53, 54]. Within a TIE context, clean *U. lactuca* is introduced into the overlying water above whole sediments or directly into isolated interstitial water (see discussion in interstitial water section). The *U. lactuca* is allowed to interact with the water or water-sediment system for 24 h under fluorescent light (75–100 $\mu E/m^2/s$) at <15 °C. While in the water, or water–sediment system, *U. lactuca* absorbs large amounts of ammonia. The *U. lactuca* (and absorbed ammonia) is then removed. The system is allowed to come to normal test temperature (~20 °C) and toxicity testing organisms added. The presence of *U. lactuca* in the exposure container has not demonstrated any artifactual toxic effects.

4.2.3.2 Zeolite Addition

Zeolite is a hydrated aluminosilicate mineral composed of symmetrically stacked alumina and silica tetrahedra forming an open and stable three-dimensional structure with a negative charge [55, 56]. The negative charges allow for the adsorption of certain positively charged ions [56]. In aqueous solution, the negative charge is generally neutralized by Na^+; however, NH_4^+ is preferentially adsorbed by zeolite.

Like many of the other TIE manipulations, zeolite (SIR-600, ResinTech, West Berlin, NJ, USA) is added directly to the sediment 24 h before the addition of the test organisms [34]. The amount added is generally 15 to 20% of the sediment wet weight. Like the PPC, Ambersorb 1500™ and cation exchange resin, the zeolite is left in the sediment during the organism's entire exposure. At toxicity test termination, the zeolite is disposed of along with the sediment.

4.3 Interstitial Water TIE Methods

Interstitial water methods have the advantage of being more directly transferable from effluent and receiving water TIE methods, about which there is a wealth of literature and technical guidance [19–22, 24–31, 40]. While some of these methods are used as they would be for effluents and receiving waters (e.g., ethylendiaminetetraacetic acid (EDTA) addition for toxic metals) others such as the C_{18} solid phase extraction used for characterizing organic chemicals are altered slightly to accommodate the type of toxicants found in interstitial waters. Concerns over the use of interstitial water testing were addressed early in this chapter. Conversely, advantages of using interstitial water for performing sediment TIEs include: (i) the previously mentioned wealth of information and manipulations that exist for effluents and receiving waters, (ii) the ease of moving from Phase I (Characterization) to Phase II (Identification), where the majority of existing TIE manipulations are designed for an aqueous or liquid matrix (i.e., not sediment particles), and finally, (iii) some organisms are not amenable to a sediment matrix and can only be tested in water (i.e., echinoderm fertilization tests). The latest U.S. EPA sediment TIE guidance document also provides methods for conducting interstitial water TIEs [24].

As with whole sediment TIE methods, the interstitial water TIE methods are designed to characterize three main classes of toxicants known to accumulate in sediments: non-polar organic chemicals, cationic metals and ammonia (Fig. 3). The methods discussed below are described in more detail in [22, 24].

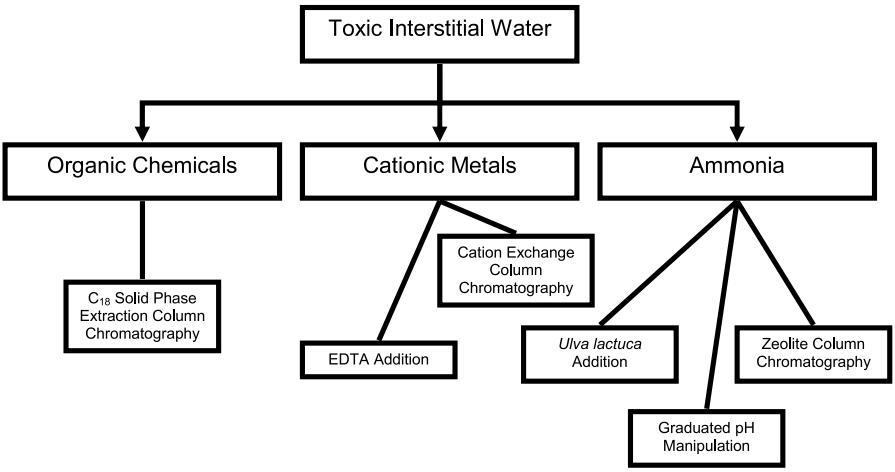

Fig. 3 Interstitial water toxicity identification evaluation (TIE) toxicant classes and manipulations

4.3.1 Organic Chemicals: C_{18} Solid Phase Extraction (SPE) Column Chromatography

C_{18} SPE is used to determine if non-polar organic contaminants are responsible for toxicity. C_{18} SPE is a type of reverse-phase chromatography using an octadecyl stationary phase through which particle-free interstitial water is passed. The column is relatively inexpensive and disposable. If there is a difference in toxicity between the post-column effluent and the baseline treatment, non-polar organic compounds, as a class, are considered likely toxicants. A difference between this manipulation performed on effluents and receiving waters, relative to interstitial waters, is that in effluents the sample is filtered prior to passing it through the column. When used with interstitial water, the sample is not filtered, but needs to be relatively particle-free (usually accomplished through double high-speed centrifugation of the sample). Elution of contaminants from the C_{18} SPE column using organic solvents is a Phase II manipulation applying analytical chemistry and toxicity testing to identify specific organic toxicants.

4.3.2 Cationic Metals

4.3.2.1 Ethylendiaminetetraacetic Acid (EDTA) Addition

Metal toxicants in marine interstitial waters are addressed using the same methods used for marine effluent and receiving waters [22]; that is, by add-

ition of ethylendiaminetetraacetic acid (EDTA). EDTA is an organic chelating agent that acts to complex with any initially dissolved cationic metal ions (Me^{2+}). The metal-EDTA complex is non-toxic. Several studies with marine samples have demonstrated the efficacy of this procedure to substantially reduce the adverse effects of metals to marine organisms [57]. EDTA preferentially binds with divalent cationic metals such as copper, nickel, lead, zinc, cadmium, mercury, and other transition metals [58].

To perform this manipulation, a non-toxic concentration of EDTA is added to the interstitial water sample and the EDTA and water sample are allowed to interact for 3h before adding organisms [22]. If metal toxicity is present in the samples, decreased toxicity should be apparent after comparison to the baseline.

4.3.2.2
Cation Exchange SPE Column Chromatography

Like the C_{18} SPE, cation exchange SPE column chromatography uses an interstitial water TIE procedure [59]. This procedure uses cation exchange functionalities as the stationary phase upon which divalent cationic metals are chelated and removed from the sample. The procedure is very similar in performance to the C_{18} SPE in which the post-column effluent is tested for toxicity as compared to a baseline. Following the column chromatography, the metals on the column can be eluted with strong acid to begin a Phase II identification procedure.

4.3.3
pH Dependent Toxicants

4.3.3.1
Graduated pH Manipulation

The graduated pH test is designed to characterize toxicants that have different toxicities at different pHs. The most notable toxicants in sediments would be ammonia and hydrogen sulfide. Both of these naturally occurring and human-enhanced toxicants demonstrate pH-dependent toxicity. In addition, a number of metals also display different toxicity depending upon pH [60, 61]. The graduated pH manipulation involves conducting toxicity tests on three sub-samples at pHs of approximately 7.0, 8.0, and 9.0 for marine TIEs [22]. This range is physiologically acceptable for most marine organisms. The chemistry of ammonia toxicity was discussed earlier in the *Whole Sediment TIE* section. Generally, as the pH of a sample increases, NH_3 is the dominant form of ammonia in aqueous solution. As NH_3 is also the more toxic form of ammonia, the sample toxicity will also increase [62]; therefore, we would expect to observe increasing toxicity at higher pHs in the graduated pH manipulation.

Like ammonia, hydrogen sulfide (H_2S) exists in two forms at environmental pHs, H_2S and the short-lived form HS^-:

$$H_2S \leftrightarrow HS^- + H^+ . \quad (2)$$

Based on a log acid dissociation constant (pK_a) of 7.0 for hydrogen sulfide, as the sample pH decreases, H_2S concentrations increase and toxicity increases [63, 64]. Because of these unique speciation trends, the graduated pH manipulation can be used to characterize ammonia and hydrogen sulfide toxicity as well as other toxicants showing this type of behavior (e.g., some metals).

Seawater has a relatively strong pH buffering capacity that is largely controlled by the concentration of CO_2 in water according to the reaction:

$$CO_2 + H_2O \leftrightarrow H_2CO_3 \leftrightarrow H^+ + HCO_3^- \leftrightarrow H^+ + CO_3^{-2} . \quad (3)$$

We take advantage of this relationship in order to implement the graduated pH manipulation by altering the amount of CO_2 in the atmosphere over the test chambers. As the CO_2 concentration increases, the equation is driven to the right, causing a greater concentration of H^+ ions and decreasing the pH of the water. Conversely, if the CO_2 concentration decreases, the H^+ ions associate with the carbonic ions (CO_3^x) and the pH increases. During the graduated pH manipulation, interstitial water samples are placed into atmospheric chambers (i.e., generally, small plexiglass chambers). These atmospheric chambers are not completely sealed but maintain a positive pressure ensuring external gases (i.e., regular air) does not enter. By increasing the CO_2 concentration in the chambers, low pH conditions (~7.0) can be maintained in the interstitial water samples. To increase the interstitial water pH to approximately 9.0, "CO_2 free" air is allowed to flow into the atmospheric chamber. The pH 8 treatment is considered ambient pH conditions. Because of seawater's strong buffering capacity, pHs need to be checked daily and readjusted if necessary throughout the toxicity test.

4.3.3.2 Zeolite Column Chromatography

Recently, a new method was developed for characterizing ammonia toxicity in interstitial water [42]. The method involves the use of the natural mineral zeolite, as described in the *Whole Sediment TIE* section. In the method, rather than adding the zeolite directly to the sample, the interstitial water is charged through a chromatography column composed of zeolite. As in the whole sediment application, ammonium adsorbs to the zeolite resulting in an overall reduction in ammonia concentrations. Following the manipulation, the zeolite is discarded and the post-column effluent tested for toxicity.

4.3.3.3 Ulva Lactuca Addition

Like the whole sediment TIE manipulation discussed above, *U. lactuca* can also be used in the interstitial water TIE. In this version of the manipulation, the algae is added directly to the interstitial water for 5 h and then removed. The toxicity test organisms are then added to the interstitial water sample and the toxicity test is performed.

5 Future Research

5.1 Applications of Sediment TIEs

As discussed above, sediment TIEs have several current uses including assessing dredged materials and performing risk assessments. However, there are other applications for the TIE approach including the performance of diagnostic investigations as part of the Total Maximum Daily Loading (TMDL) process. TMDLs are discussed in the Clean Water Act as a process for remediating environmental impairments to water bodies around the United States. The TMDL process involves a diagnostic component needed for determining which stressors are causing observed impairments. Whereas, the sediment TIE focuses exclusively on identifying toxic chemicals causing toxicity, the TMDL diagnostic investigation includes nutrients, toxic chemicals and other classes of environmental stressors (e.g., siltation). The U.S. EPA has developed guidance for performing causal analysis in aquatic systems [65, 66]. These approaches provide broad guidance on performing diagnostic investigations and can include the use of sediment TIEs as well as other diagnostic tools. The future evolution of such causal analysis approaches offers exciting applications for sediment TIE methods.

5.2 Needed Development

The sediment TIE approach discussed above has two primary components: toxicity testing and chemical manipulations. Currently, the marine whole sediment TIE toxicity tests use acute endpoints (i.e., survival) except for an experimental growth endpoint with the bivalve *Merceneria mercenaria* which is under development. In principle, acute endpoints are the least sensitive, and increasing the number of toxicity tests with sublethal endpoints like reproduction and growth that can be successfully paired with TIE manipulations would enhance TIE sensitivity. Consequently, the development of

toxicity tests for whole sediment TIEs with sublethal endpoints would be very desirable. Several of the marine interstitial water TIE toxicity testing methods apply sublethal endpoints; however, for the reasons discussed above regarding concerns about interstitial water TIEs, the use of whole sediment TIE methods are encouraged.

Further, the toxicity tests generally employed with TIE chemical manipulations are not sensitive to dioxins or toxicants which have a dioxin-like mode of action affecting the Ah receptor [67]. This class of toxicants includes polychlorinated dibenzodioxins (PCDDs), polychlorinated dibenzofurans (PCDFs) and planar PCBs. Similarly, current whole sediment TIE toxicity tests are not sensitive to mercury toxicity. Given that these contaminants tend to accumulate in sediments and many contaminated sediment sites contain significant quantities of dioxin, dioxin-like chemicals, and mercury, the inability of sediment TIE methods to determine if these toxicants are causing adverse effects is highly unsatisfying and appropriate toxicity testing methods for application in the TIE approach need to be developed. However, it should be noted that toxicity tests do exist which are sensitive to dioxin and dioxin-like chemicals [68, 69] but these tests are not generally part of the TIE approach. Incorporation of such toxicity tests into a sediment TIE approach would be very useful (Fig. 4).

The other area of method development is new chemical manipulations. Currently, as discussed above, marine sediment TIE methods are available for organic chemicals, cationic metals, and ammonia. The cation exchange method will reduce the bioavailability of divalent mercury (Hg^{2+}) but not the more toxic methyl- and dimethyl-mercury forms: $CH_3 - Hg^+$ and $(CH_3)_2Hg$, respectively. There has been no research on the efficacy of PCC or Ambersorb addition for the removal of methylated mercury. In addition, there is another class of metals known as anionic metals. These metals form anionic ions in water because of their association with hydroxide molecules in solution [70]. Among these metals, including arsenic and selenium, the toxicity of chromium is the best understood. Recently, Burgess et al. [71] described whole sediment TIE addition methods for the anionic metals arsenic and chromium. This manipulation needs to be fully vetted with freshwater sediments and test organisms. Further, a common sediment toxicant associated with anthropogenically enriched nutrient conditions is hydrogen sulfide (see discussion in the *Interstitial Water TIE Methods* section). Current whole sediment TIE manipulations are not designed to affect hydrogen sulfide bioavailability. Because hydrogen sulfide is rapidly volatilized and oxidized under oxic conditions, frequently toxicity associated with this toxicant will decrease under regular toxicity testing conditions (aeration) with sufficient time. However, this approach is not easily controlled and can require several days. Finally, the suite of emerging contaminants of concern (COC) such as personal care products (PCPs) and pharmaceuticals has not been addressed in TIE methodology. These emerging contam-

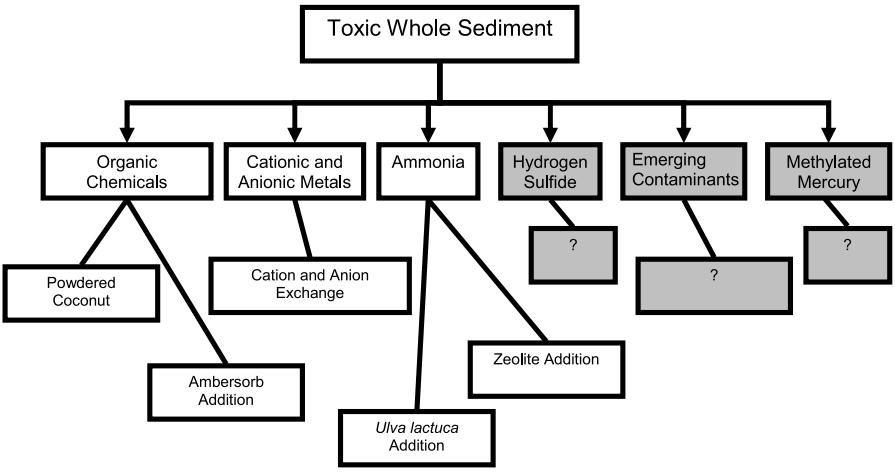

Fig. 4 Whole sediment toxicity identification evaluation (TIE) toxicant classes and manipulations including methods under-development or needed (in *grey tones*)

inants are a group of chemically disparate compounds often designed to have biological effects. The consistent nature of their discharge in effluents warrants further research as to their effects in the marine environment.

6 Summary

TIEs are an effective method for determining which stressors cause sediment toxicity. Methods have been developed for many common sediment contaminants [23], however, research remains to be performed on methods to identify several notable sediment contaminants such as methyl mercury, anionic metals and other chemicals of concern (COCs). The TIE methodology can be an important tool paired with other methods to look at a wide range of stressors affecting estuarine and marine systems in a diagnostic approach.

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Nucleic Acid-Based Techniques for Studying Diversity and Activity of Bacterial Communities in Oil-Contaminated Sediments

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Abstract Recent developments in molecular techniques have provided an outstanding opportunity to investigate in situ diversity and activity of bacterial communities in various ecosystems. These techniques are based on the detection of nucleic acids within living cells or after extraction directly from field material. They include several DNA

fingerprinting, hybridization and analytical PCR-based techniques. Molecular techniques were designed to circumvent the limitations associated with traditional microbiological methods, which often involved characterization of readily cultivated species. The use of molecular methods has contributed significantly to a comprehensive understanding of the microbiology of ecological systems.

The application of molecular techniques to oil-contaminated sediments has provided valuable information on the diversity of oil-tolerant bacteria and the identification of field dominant species that were significantly involved in the degradation of pollutants. This has led to the discovery of numerous novel bacterial species that were not previously described among isolates. Furthermore, using molecular techniques, the activity of oil-degraders could be assessed by following the expression of relevant functional genes. The obtained knowledge has facilitated the selection of appropriate microorganisms and the design of suitable treatments for bioremediation purposes. In bioremediation studies, molecular techniques have served as monitoring tools to follow changes and responses of bacterial communities to a given treatment. Thus, correlations between microbial community compositions and the progress of biodegradation could be determined.

Herein we focus on the description of various molecular techniques and give examples of their application in oil biodegradation and bioremediation studies. The advantages as well as the disadvantages of each technique will be discussed so that the reader is well aware of the potential and the problems encountered during the application of these techniques. The main aim is to provide scientists in this field with extra tools to learn more about the role of bacteria in the cleanup of contaminated sediments. Moreover, this work will enable scientists to choose from these techniques the best suited for their applications.

Keywords Biodegradation · Contaminated sediments · Microorganisms · Molecular techniques · Nucleic acids · 16S rRNA

Abbreviations

A	Adenine
ARDRA	Amplified ribosomal DNA restriction analysis
BAC	Bacterial artificial chromosome
bp	Base pair
C	Cytosine
CARD-FISH	Catalyzed reporter deposition-fluorescence in situ hybridization
cDNA	Complementary DNA
CLSM	Confocal laser scanning microscope
DGGE	Denaturing gradient gel electrophoresis
DNA	Deoxyribonucleic acid
FISH	Fluorescence in situ hybridization
G	Guanine
HCl	Hydrochloric acid
HRP	Horseradish peroxidase
Kbp	Kilo base pair
MAR-FISH	Microautoradiography-fluorescence in situ hybridization
MPN-PCR	Most probable number-polymerase chain reaction
mRNA	Messenger ribonucleic acid
ORF	Open reading frame
PAH	Polyaromatic hydrocarbons

PCR	Polymerase chain reaction
PFLA	Phospholipids fatty acids
qPCR	Quantification polymerase chain reaction
RAPD	Random amplification of polymorphic DNA
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
RT	Reverse transcription
SSCP	Single-strand conformation polymorphism
T	Thymine
TGGE	Temperature gradient gel electrophoresis
T-RFLP	Terminal restriction fragment length polymorphism
tRNA	Transfer ribonucleic acid

1 Introduction

Oil spills have drastic effects on the environment especially when they occur over large areas. They result in a significant contamination of marine water and coastal sediments [1–3]. Petroleum compounds are destructive to various forms of marine life. Dissolved aromatic components of oil, even at low concentrations, can lead to elimination of many marine species as a result of disruption of chemoreception, which is essential for feeding and mating responses [4]. Some polynuclear components of petroleum that are recalcitrant and carcinogenic can move up marine food chains and taint fish or shellfish. Compounds like gasoline and low-viscosity distillation products can seep into soils leading to pollution of groundwater. Vegetation is also adversely affected by oil spills due to contact toxicity, and because hydrocarbon biodegradation in the soil renders plant root zones anoxic. The lack of oxygen and accompanying H_2S evolution kills the roots of most plants including large trees [5].

In the water column, microbial degradation of oil is often limited due to the hydrophobic nature of oil, which leads to decreased surface area for bacterial colonization and also because of the limited availability of nutrients such as nitrogen and phosphate [6, 7]. The volatile hydrocarbon fractions evaporate quickly, leaving longer-chain aliphatic and aromatic components [8]. As a result, the viscosity of oil increases and ultimately oil sinks to form layers on sediment surfaces. These contaminated sediments are expected to harbor complex microbial communities that are capable of degrading oil compounds [3, 9, 10]. Both aerobic and anaerobic microorganisms are typically present in oil-contaminated sediments increasing the possible interaction between the two processes in the elimination of toxic oil compounds.

The dramatic effects of oil spills reflect the emerging need for development of techniques that deal with oil pollution both at sea and on shorelines [3]. There are well-established different physical and chemical methods for dealing with oil spills. Observations in the field have shown that many indigenous microorganisms have the potential to effectively degrade hydrocarbons [2, 11, 12]. This has opened a new field of utilizing such microorganisms in the cleanup of polluted sites and elimination of environmental pollutants, a process known as bioremediation [10, 13–15]. The use of bacteria to clean up polluted sediments is a rapidly expanding field of environmental biotechnology. Bioremediation approaches are classified into two categories namely biostimulation and bioaugmentation [16]. Biostimulation involves the addition of nutrients or other growth enhancing cosubstrates to stimulate the growth of indigenous oil degraders [7, 17, 18]. Biostimulation could also be achieved by optimizing the environmental conditions, e.g. salinity and temperature, at which the degrading bacteria exhibit their maximum activity. Bioaugmentation, also known as seeding, involves the addition of oil-degrading bacteria to supplement the existing microbial communities [19, 20]. Both bioremediation methods were successfully applied to different contaminated sediments.

Since bioremediation approaches depend mainly on microbial populations inhabiting or inoculated to polluted sites, comprehensive knowledge of their diversity and physiological capabilities in the field becomes indispensable. It has been a challenge for microbiologists to analyze bacterial communities in situ. Conventional methods were restricted to readily isolated strains, which in many cases did not represent important microorganisms in the field. Recent developments in molecular tools have opened a new horizon to microbial ecologists to understand the dynamics of bacterial populations that are dominant in situ and to explore, at the genetic level, their physiological potential. With the help of these techniques we are now able to explore the diversity of bacterial communities and to monitor shifts occurring in them over a period of time or in response to certain treatments, for example, addition of nutrients in case of biostimulation. Populations of interest can now be specifically targeted under in situ conditions. Their abundance, distribution and spatial arrangement can be further illustrated. Furthermore, the existence and the diversity of certain functional genes, that are relevant to the degradation of specific compounds, can be revealed. Furthermore, the expression of these genes can be quantified.

Herein, various molecular tools are described and their applications in biodegradation studies are discussed. These methods are classified into four categories namely fingerprinting, hybridization, analytical PCR and activity techniques. The use of these techniques will enable researchers to gain more insights into the diversity of bacterial communities and their role in contaminated sediments. An overview of the molecular techniques described herein is presented in Fig. 1.

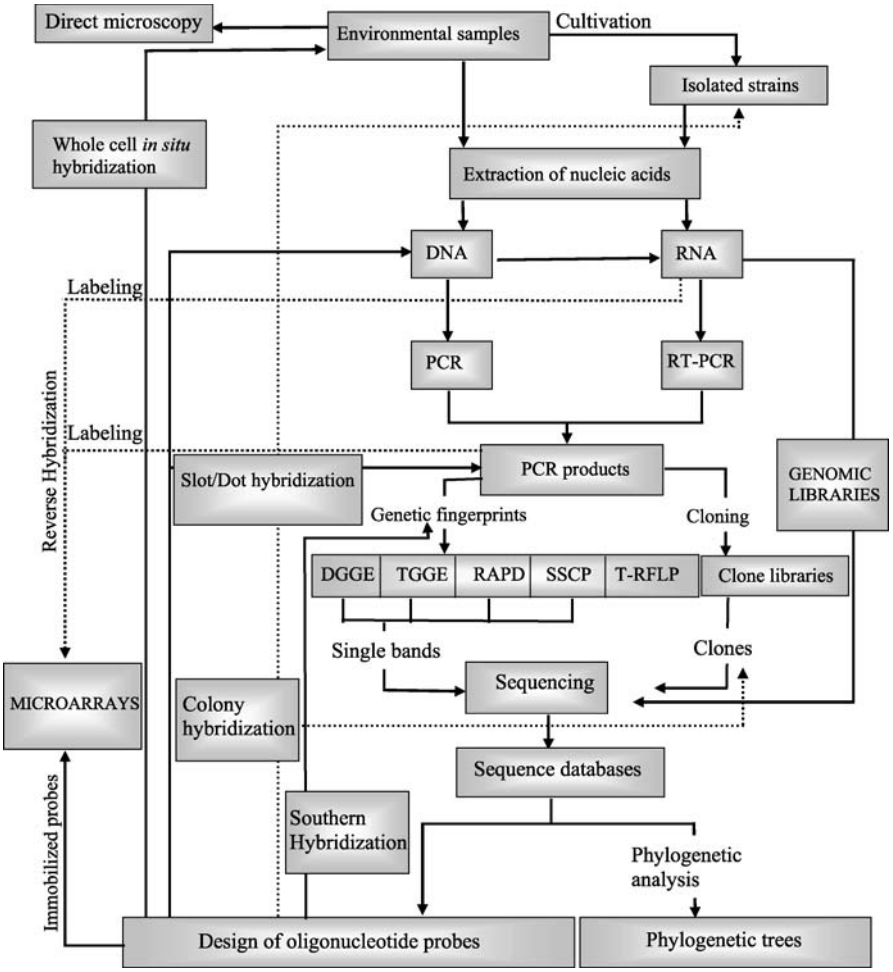


Fig. 1 Overview of various molecular techniques used to study bacterial communities in environmental samples including contaminated sediments (adapted and modified from [21])

2 Molecular Versus Conventional Techniques

For many years, traditional microbiological techniques such as direct microscopy and enrichment cultivation have been used to study microbial diversity in contaminated ecosystems. Assessing the bacterial diversity by direct microscopy is very difficult due to the simple morphology of bacteria and the lack of conspicuous traits that distinguish them from each other. Isolation of oil-degrading bacteria was, and still is, an essential discipline in biodegra-

dation and bioremediation studies. A major advantage of obtaining axenic oil-degrading strains is the ability to perform physiological studies and to explore the isolates degradation capabilities. Furthermore, to a limited extent, the interaction among microorganisms in biodegradation of a certain compound could be studied by performing co-culture experiments [22–24]. In spite of these advantages, enrichment isolation has been proven to underestimate the real diversity of oil-degrading bacteria thriving under natural conditions. The isolated strains may even represent an insignificant proportion of all microorganisms. The isolates are often selected by the supplied culture conditions. Thus, we may succeed in isolating microorganisms which fit the culturing conditions, but fail to recover important community members. Additionally, the behavior of microorganisms in cultures, where all growth requirements are supplied, may not be comparable to their behavior under selective field conditions. In the isolation of oil-degrading bacteria, additional problems are encountered such as the water insolubility of certain petroleum compounds, such as aromatics, in the culture medium and the toxicity of these compounds. Water-insolubility renders these compounds inaccessible to many bacteria.

Many biodegradation studies have been performed in the laboratory under simulated field conditions. However, the inability to duplicate natural conditions in the laboratory often leads to the development of bacterial communities different from those present under in situ conditions. Therefore, there is an emerging need for techniques that reflect the real picture in the field. Recent developments in molecular techniques have contributed to a large extent to filling this gap. The general advantages of molecular (i.e. culture-independent) over culture-dependent techniques are summarized in the following:

- Studying bacterial communities without the need to cultivate them, thus saving time.
- Provide information on bacterial community composition and their metabolic status under field (in situ) conditions.
- Extraction of nucleic acids from environmental samples accounts for a large fraction of the microorganisms that is not culturable but may be responsible for major biodegradation activities.
- The possibility to target a specific bacterium or bacterial subgroups using specific probes.
- Provide information on the abundance, distribution of bacteria and their spatial arrangements.
- The possibility to quantify bacterial genes.
- Studying the diversity of functional genes and monitoring their expressions by quantification assays.

Molecular methods complement basic microbiological methods and do not substitute them. These techniques, when combined with microbiological

ones, will provide a comprehensive overview of the microbiology of contaminated systems. It should also be kept in mind that, molecular tools have their own limitations, which will be discussed with each technique.

3 16S rRNA as a Phylogenetic Biomarker

In bacterial cells, nucleic acids are found in the form of deoxyribonucleic acid (DNA) and ribonucleic acid (RNA). DNA carries the genetic blueprint for the cell and RNA acts as an intermediary molecule to convert the blueprint into proteins [8]. RNA has three forms namely, ribosomal, messenger and transfer RNAs. All the three types of RNA are essential for protein synthesis. Ribosomal RNA is the most abundant macromolecule, next to proteins, in an actively growing prokaryotic cell. It is a major component of the ribosome, the cellular machinery used to synthesize new proteins. There are three ribosomal RNA molecules in prokaryotes namely 5S (ca. 120 nucleotides), 16S (1500 nucleotides), and 23S (2900 nucleotides).

The first attempts in studying microbial communities from environmental samples were performed using 5S RNA molecules [25–27]. The 5S RNAs from different community members were separated using electrophoresis and subsequently sequenced and phylogenetically analyzed. A major problem associated with this approach was the limited sequence information obtained from the short 5S RNA molecule. Therefore, attention was directed towards longer molecules such as 16S and 23S rRNA [28]. The 16S rRNA molecule is considered as an excellent genetic tool for community analysis and phylogenetic comparison for the following reasons:

- It is present in all organisms in high numbers.
- It is long enough (ca. 1500 bp) to contain sufficient information for reliable phylogenetic analysis by sequence comparison.
- It possesses both conserved and variable sequences, which enables proper alignment of sequences and easy design of specific primers and probes for covering different phylogenetic ranges.
- Production is growth-rate regulated.
- There is rather little horizontal transfer of genes between species and it is highly conserved among closely related species.

16S rRNA has been widely used as a phylogenetic marker and the method is now well developed. There are large (and growing) public databases available which allow comparison of newly obtained sequences to already existing ones.

4
Nucleic Acid Extraction

Extraction of nucleic acids is the first and probably the most crucial step in examining the bacterial diversity and catabolic genes by molecular techniques. Subsequent steps are very much dependent on this step. Thus, a careful evaluation of different extraction protocols is needed. Several methods were described for the extraction of both DNA and RNA (e.g. [29–33]). Several treatments have been described for the lysis of bacterial cells including mechanical disruption using glass homogenizers, bead-mill homogenization, freeze (at – 120 °C in liquid nitrogen) and thaw (at 65 °C) and enzymatic treatments (lysozymes and proteinase K). Following cell lysis, nucleic acids are obtained by successive extractions using acidic phenol (in case of RNA) or phenol-chloroform-isoamyl alcohol (in case of DNA) followed by precipitation using ethanol or isopropanol. A recent protocol has been described for the extraction of nucleic acids without using phenol products [34]. Nowadays, many commercial kits are available for nucleic acid extraction.

The ideal nucleic acid extraction should:

- Represents the total nucleic acids of all bacterial community members.
- Produces long fragments of DNA and/or RNA. This is particularly necessary for working with functional genes and for construction of clone libraries where long sequences are required.
- Not involve harsh treatments that lead to shearing of the DNA.
- Be fast and simple to avoid degradation of nucleic acids, particularly RNA. Prokaryotic RNA are known to have a short half life.
- Produces nucleic acids that are sufficiently pure for reliable PCR amplification, reverse transcription and enzyme digestion. In case of high concentrations of salts or humic acids, the DNA can be purified using purification columns that are commercially available.

5
Polymerase Chain Reaction (PCR) and Reverse Transcription (RT-PCR)

Many molecular techniques involve polymerase chain reaction (PCR). The purpose of PCR is to amplify genes in order to have enough starting template for subsequent analyses. PCR consists of three major steps; denaturation, annealing and extension (Fig. 2). The denaturation step is performed at 94 °C, during which the paired strands of the double helix DNA are separated. In the annealing step, the primers (short DNA oligonucleotides of a specific sequence) bind to their complementary sites on the template DNA and then a DNA polymerase enzyme binds to the template and starts copying. Once there are a few bases built in, the ionic bond is so strong between the tem-

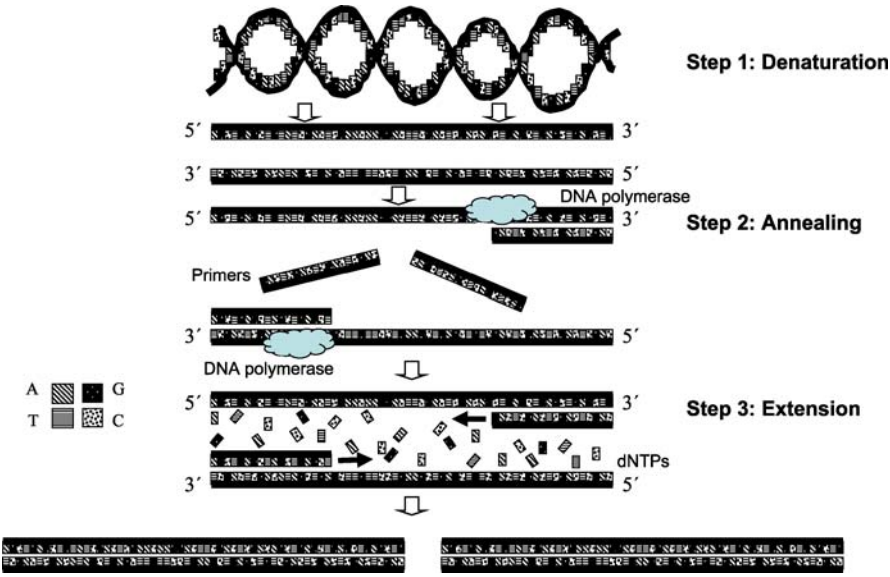


Fig. 2 Working principle of polymerase chain reaction (PCR). PCR has three main steps namely denaturation, annealing and extension

plate and the primer, that it no longer breaks. The extension step is performed at 72 °C, which is the optimum working temperature for most commercial polymerases. The copying process is completed during this step. The previous three steps are repeated several times until the maximum amplification of the target DNA is reached.

A PCR reaction can be designed to amplify a specific gene or multiple genes from a particular microorganism or a group of microorganisms. This selectivity depends on the specificity of the primers used in the reaction. Primers that are domain-specific and primers that target a single microorganism or a group of bacteria are now available. A list of selected primers to amplify the 16S rRNA from bacteria that are present in oil-contaminated systems is presented in Table 1.

Reverse-transcriptase-PCR (RT-PCR) is a method used to amplify complementary DNA (cDNA) copies of RNA. Single-stranded RNA is copied to DNA using the enzyme reverse transcriptase. This is achieved by hybridizing an oligodeoxynucleotide primer to the RNA followed by its extension with a reverse transcriptase. The created cDNA can be amplified by PCR as described above.

Table 1 List of selected primers used to amplify small subunit ribosomal RNA used for DGGE. The target sites, sequences and specificity of the primers are shown. R (reverse) and F (forward) indicate the orientation of the primers. Y indicates C/T, W indicates A/T, M indicates A/C, R indicates A/G, S indicates C/G. A GC clamp (GC-rich sequence) is attached to the 5′-end of the forward primers to prevent complete dissociation

Primers	Nucleotide positions	Sequence (5′-3′)	Target microorganism/s	Refs.
GM5F	341–357	CCTACGGGAGGCAGCAG	Bacteria	[35]
907RC	907–926	CCGTCAATTCCTTTGAGTTT	Bacteria	[36]
907RM	907–926	CCGTCAATTCMTTTGAGTTT	Bacteria	[36]
CYA359F	359–378	GGGGAATYTTCCGCAATGGG	Cyanobacteria	[37]
CYA781R	781–805	GACTACWGGGGTATCGAATCCCWTT	Cyanobacteria	[37]
ARC344F	344–363	ACGGGGYGCAGCAGGCGCGA	Archaea	[38]
915R	915–934	GTGCTCCCCCGCCAATTCCT	Archaea	[38]
Paer16SH	449–473	AGGGCAGTAAGTTAATACCTTGCTG	*Psuedomonas aeruginosa*	[39]
Paer16SIR	667–643	CCACCTCTACCGTACTCTAGCTCAG	*Psuedomonas aeruginosa*	[39]
Xmal16SL	452–473	GCTGGTTAATACCTGGTTGGGA	*Stentrophomonas (Xanthomonas)*	[39]
Xmal16SKR	671–647	CTACCCTCTACCACACTCTAGTCGT	*maltophilia*	[39]
Smar16SM	462–485	TGGTGAACTTAATACGCTCATCAA	*Serratia marcescens*	[39]
Smar16SNR	674–654	CCCCTCTACGAGACTCTAGCT	*Serratia marcescens*	[39]
Sphing 43F	43–63	TCACTTCGGTGGTAGTGGCG	*Sphingomonas chlorophenolica*	[40]
Sphing750R	750–772	CTGAAATGCCATGCACCCCAGC	*(strain RA2 and ATCC39723)*	[40]
IC427F	442–459	ACCATGACGAAGCATTAT	*Corynebacterium* sp. IC10	[41]
IC 974R	998–1015	ACGGCCTAGCCGCGTCTC	*Corynebacterium* sp. IC10	[41]
Ps-for	n.a.	GGTCTGAGAGGATGATCAGT	*Pseudomonas* species	[42]
Ps.rev.	n.a.	TTAGCTCCACCTCGCGGC	*Pseudomonas* species	[42]
Sphingo 108F	108–128	GCGTAACGCGTGGGAATCTG	*Sphingomonas* strains	[43]
Sphingo 420R	420–401	TTACAACCCGAAGGCCTTC	*Sphingomonas* strains	[43]
Myc16-1F	n.a.	TGGAGAGTTTGATCCTGGCTC	*Mycobacterium* strains	[44]

Table 1 (continued)

Primers	Nucleotide positions	Sequence (5′-3′)	Target microorganism/s	Refs.
Myc16-4R	n.a.	GACGACAGCCATGCACCACC	*Mycobacterium* strains	[44]
DFM140	140–158	TAGMCYGGGATAACRSYKG	*Desulfotomaculum* species	[45]
DFM842	823–842	ATACCCSCWWCWCCTAGCAC	*Desulfotomaculum* species	[45]
DBB121	121–142	CGCGTAGATAACCTGTCYTCATG	*Desulfobulbus* species	[45]
DBB1237	1215–1237	GTAGKACGTGTGTAGCCCTGGTC	*Desulfobulbus* species	[45]
DBM169	169–183	CTAAGRCCGGATRAAGTCAG	*Desulfobacterium* species	[45]
DBM1006	986–1006	ATTCTCARGATGTCAAGTCTG	*Desulfobacterium* species	[45]
DSB127	127–148	GATAATCTGCCTTCAAGCCTGG	*Desulfobacter* species	[45]
DSB1273	1252–1273	CYYYYYGCRRAGTCGSTGCCCT	*Desulfobacter* species	[45]
DCC305	305–327	GATCAGCCACACTGGRACTGACA	*Desulfococcus-Desulfonema-*	[45]
DCC1165	1144–1165	GGGGCAGTATCTTYAGAGTYC	*Desulfosarcina* species	[45]
DSV230	230–248	GRGYCYGCGTYYCATTAGC	*Desulfovibrio-Desulfomicrobium*	[45]
DSV838	818–838	SYCCGRCAYCTAGYRTYCATC	species	[45]

6
Clone Libraries

The purpose of constructing a clone library is to reveal the diversity of a single gene among different bacteria constituting a microbial community. The target gene can be amplified from different community members using specific PCR primers. This results in a pool of different sequences of the same gene, which originate from different bacteria. The separation of these gene types is a prerequisite for further sequencing work. This is achieved by inserting one gene at a time into a plasmid (circular DNA present in some bacteria), which is then transferred into a living bacterial cell. Extensive sequencing of the clones generates a library of sequences, which reflects the diversity of bacteria in the studied microbial community. Clone libraries are useful to obtain a comprehensive overview of the structure of complex microbial communities or to reveal the composition of bacterial consortia.

6.1
Method

The construction of a clone library involves the extraction of nucleic acids from environmental samples followed by PCR-amplification of the target gene

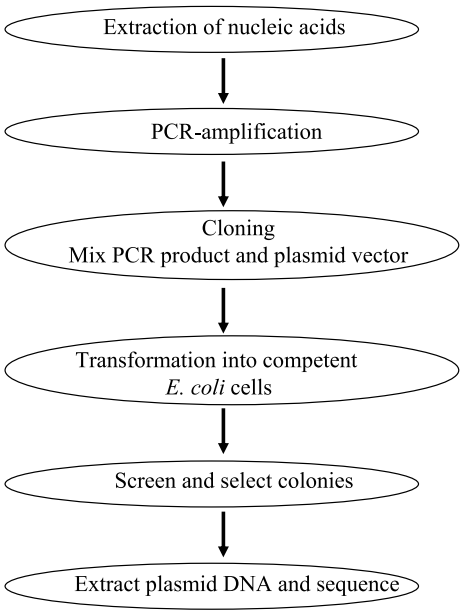


Fig. 3 Experimental outline of molecular cloning using bacterial plasmid vectors

(e.g. 16S rRNA gene or functional gene such as dioxygenase) using specific primers (Fig. 3). Cloning is then performed by mixing the PCR product and the plasmid vector under defined salt concentration. Subsequently, the target gene-plasmid construct is then inserted into competent *E. coli* cells either by heat shock or electroporation, a process known as transformation. The transformed versus non-recombinant *E. coli* cells are distinguished by plating on a selective medium. Antibiotic resistance, coded on the plasmid, could be used as a trait for the selection of recombinant cells. Plasmids are extracted from transformed cells and then sequenced.

6.2 Applications

In oil biodegradation and bioremediation studies, clone libraries have several applications. This includes revealing the genetic diversity of microbial communities in polluted environments as well as identification of yet-uncultured microorganisms. In addition, changes in microbial communities following the application of a bioremediation process could be monitored. For example Ogino et al. [46] used this technique to follow succession in microbial communities during a biostimulation process. The community composition of the fertilized sites showed remarkable differences in comparison to control sites. In a study by Jeon et al. [47] a clone library of 16S rRNA genes was constructed from ^{13}C labeled DNA obtained from sediment pre-exposed to ^{13}C labeled naphthalene. This approach provided information on the bacteria that were involved in the degradation of naphthalene. Eriksson et al. [48] also used cloning to study bacterial diversity of a biofilm growing on pyrene and phenanthrene.

Furthermore, cloning is a powerful tool to determine the structure of bacterial consortia, which often contain several bacteria living together in close association. The degradation of certain petroleum compounds is often performed by a consortium rather than by individual microorganisms. Ulrich and Edwards [49] used cloning to characterize the bacterial communities of methanogenic and nitrate-reducing cultures capable of anaerobic benzene-degradation. The sequences revealed that the methanogenic consortium was composed of one sulfate-reducing bacterium and four different Archaea while the nitrate-reducing consortium was composed only of bacteria and no Archaea were found. In a similar fashion, a hydrocarbon-degrading consortium enriched from fuel-contaminated Arctic soil was characterized by cloning [50]. The enrichment was grown on Jet A-1 fuel at 7 °C. Most of the obtained sequences from cloning were closely related to *Rhodococcus erythropolis*, *Sphingomonas* and *Pseudomonas synxantha*. Schlotelburg et al. [51] used cloning to determine the bacterial diversity of an anaerobic, 1,2-dichloropropane-dechlorinating consortium. Phylogenetic analysis of the recovered sequences revealed significant sequence similarities to several

uncultured bacterial species found in other reductively dechlorinating freshwater consortia but not to any of those consortia from marine habitats. One clone showed close similarity to *Dehalobacter* species, known from similar habitats.

6.3 Advantages and Disadvantages

The use of clone libraries is recommended when an in-depth knowledge of the structure of bacterial communities is desired. It is also useful in revealing the composition of bacterial consortia. The decision to use this method depends on the number of samples since the construction of a clone library requires a lot of time and effort. A major advantage of clone libraries is the generation of long sequences, which enables phylogenetic analysis and the design of probes and primers needed for further specific detection.

Among the disadvantages of clone libraries are the high costs of sequencing, and the ability to analyze only a few samples. The results of clone libraries are very much dependent on PCR, which could be preferential and has many biases. Because of the PCR step, clone libraries might fail to detect all bacteria in the studied sample.

7 Fingerprinting Techniques

7.1 Denaturing Gradient Gel Electrophoresis (DGGE)

DGGE was first introduced by Muyzer et al. in 1993 as a method for profiling complex environmental microbial communities [35]. Since then, this technique has been used extensively in microbial ecology, including biodegradation studies. A detailed overview of DGGE applications in microbial ecology is given in Muyzer and Smalla [52]. Here, we will only emphasize its applications in biodegradation studies.

7.1.1 Method

DGGE is an electrophoresis method that separates DNA fragments along a denaturing gradient. The working principle of DGGE is illustrated in Fig. 4. DNA is a double-stranded polymer in which the two strands are held together by hydrogen bonds between complementary bases on the opposite strands. The interaction between hydrogen bonds weakens upon increasing the concentration of certain denaturing chemicals (formamide and urea are

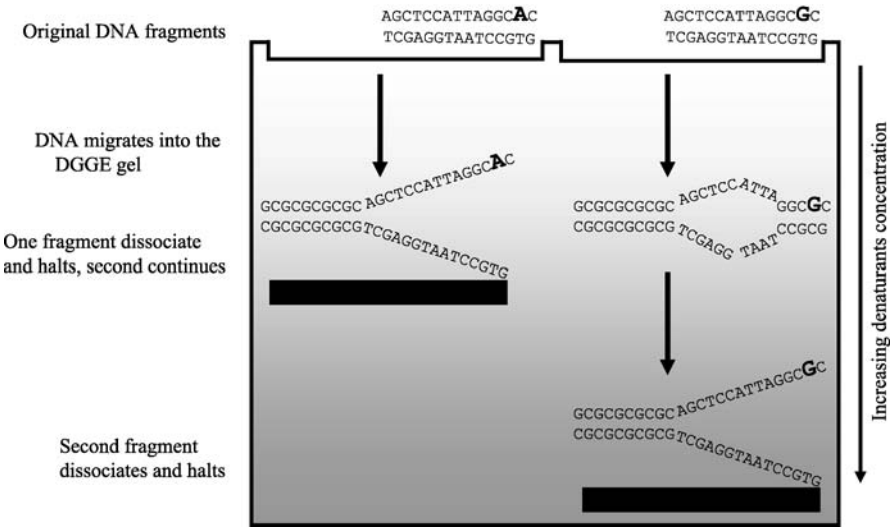


Fig. 4 Illustrates the working principle of DGGE. The example shows the separation of two DNA fragments which differ in one base pair. The two bands halt migration at different positions along an increasing concentration of gradients

usually used in the case of DGGE) or temperature (in the case of TGGE, Temperature Gradient Gel Electrophoresis). Consequently, the two strands of DNA separate, a process known as DNA denaturation. The denaturants concentration (or temperature) at which this occurs is determined by the nucleotide sequence. For example, DNA fragments that are rich in the bases G and C (which pair with three hydrogen bonds) are more stable than fragments rich in the bases A and T (which pair with only two hydrogen bonds). Identical DNA molecules, which differ in a single base within a "weak" domain will have different overall melting temperatures. DGGE is performed on a polyacrylamide gel with increasing gradient of chemical denaturants. The migration of DNA molecules through the gel will be halted when they reach a concentration of denaturants that cause the strands to dissociate. This is because single-stranded molecules become entangled in the gel matrix. In order to improve the sensitivity of electrophoretic separation, a complete strand separation is prevented by the addition of a GC-rich stretch with a very high melting domain (GC clamp) at one end of the molecule during PCR.

7.1.2 Analysis of Microbial Communities by DGGE

A scheme of the steps involved in community analysis by DGGE is presented in Fig. 5. The first step is the extraction of total nucleic acids, DNA and RNA, from environmental samples. The extracts are then subjected to

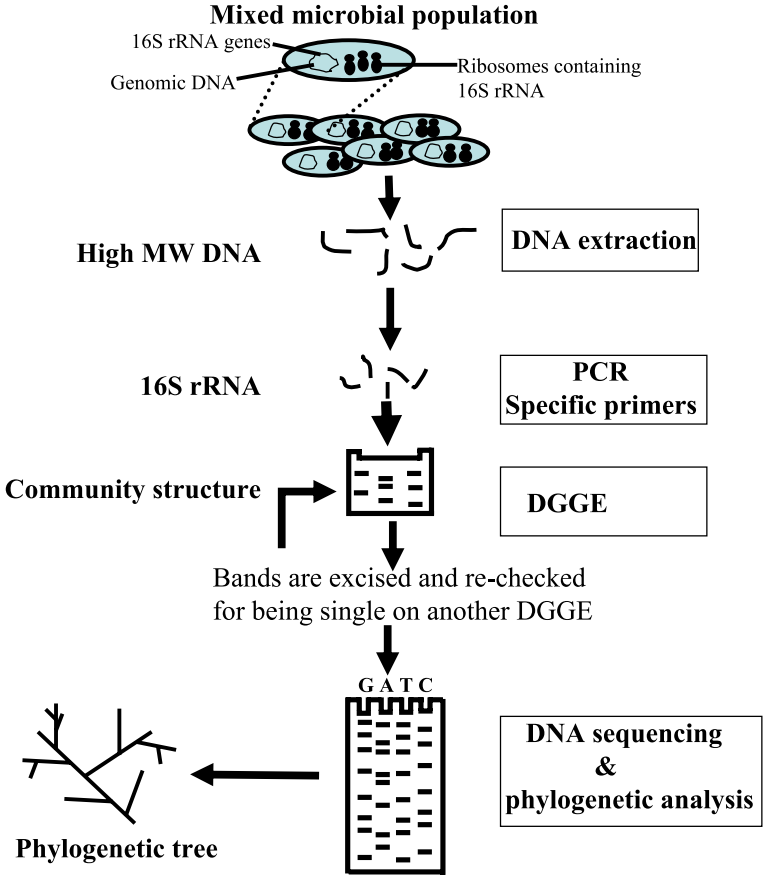


Fig. 5 An overview of different steps involved in microbial community analysis by DGGE (modified after [53]). Nucleic acids from an environmental sample are extracted, amplified and analyzed by DGGE. The obtained bands are cut, re-amplified and then sequenced

amplification by polymerase chain reaction (PCR) using specific primers. Several primers, which amplify the 16S rRNA genes of all microorganisms of the domain *Eubacteria* or specific subgroups of bacteria are available. The PCR products are loaded on the DGGE gel and the electrophoresis is then performed at 60 °C and a constant voltage of 200 V for 3.5 h. Different denaturant gradients, temperatures and running periods may be used for different primers. After electrophoresis, the various 16S rRNA fragments stop at different locations on the polyacrylamide gel, yielding a pattern of DNA bands, which typifies the sample. The bands can then be excised from the gel, re-amplified with the same primers. The amplification product is then run on a second DGGE gel to confirm that it forms a single band. Clean single bands are sequenced and the obtained 16S rRNA sequences

are phylogenetically analyzed in order to identify the respective microorganisms. Detailed protocols of the above-mentioned steps can be found in [36].

7.1.3 Applications

7.1.3.1 Bacterial Diversity in Oil-Polluted Sites

DGGE has been used to determine the structure of microbial communities and particular populations in various polluted ecosystems, or following oil spills. Kasai et al. [54] used DGGE to study the long-term effect on marine bacterial populations of the huge *Nakhodka* oil spill, which resulted in the contamination of more than 500 km of the Japan Sea coastline with more than 5000 tons of heavy oil. Samples were collected from contaminated sediments at different time intervals over a period of two and a half years. Most of the DGGE bands identified derived from the *Cytophaga/Flavobacterium/Bacteriodes* phylum, *α-Proteobacteria* or cyanobacteria. Community composition in the seawater samples was different from that in the oil paste samples. These communities included strains phylogenetically related to oil degraders such as *Sphingomonas subarctica* and *Alcanivorax borkumensis*. DGGE was also used to study the cold-adapted bacterial communities in petroleum-contaminated sediments from two Canadian environments [55]. The analysis showed that, compared to pristine controls, the bacterial diversity was lower at one contaminated site but the same or higher at the other. Phylogenetic analysis of the excised bands suggested that the community was dominated by high G+C Gram positive *Actinomycetales* (63.6%) and *Proteobacteria* (36.4%). Based on DGGE analysis, the authors concluded that species diversity was determined by geographical origin of the samples rather than by the level of oil pollution.

DGGE has also been used to study the role of anaerobic microorganisms in oil biodegradation. For example, Kleikemper et al. [56] investigated the response of sulfate-reducing bacteria in a petroleum-contaminated aquifer to the addition of different carbon sources. In all treatments, sulfate reduction rates were stimulated following carbon source addition, whereas DGGE profiles indicated no changes in the community structure. Watanabe et al. [57] used DGGE to investigate the bacterial populations in petroleum- contaminated groundwater. Their results suggested that novel members of the epsilon subclass of the *Proteobacteria* were major populations in the studied groundwater.

7.1.3.2 Population Dynamics

Addition of oil induces dramatic changes in the composition of microbial communities. DGGE is a suitable technique to monitor such changes. For example, Macnaughton et al. [58] followed changes in bacterial communities following a simulated coastal oil spill. The DGGE patterns showed no significant changes in uncontaminated controls, while the bacterial community in the oiled plots apparently underwent substantial shifts. The community composition also changed when nutrients were added in addition to oil. Oil treatment was found to stimulate the growth of Gram-negative microorganisms within the *α-Proteobacteria* and *Cytophaga/Flavobacterium/Bacteriodes* phyla. *α-Proteobacteria* were not detected in uncontaminated controls, suggesting a possible involvement of this group in the degradation of oil. In a study by Duarte et al. [59], sediment samples were exposed to varying concentrations of sulfurous oil. The number of DGGE bands decreased with increasing oil concentration, indicating the toxic effect of oil. Sequence analysis showed that the organisms present at high oil concentration were related to *Actinomycetes* sp. and *Arthrobacter* sp. as well as an unknown bacterium.

Monitoring DGGE patterns in biodegradation studies may provide hints of who is (are) mainly responsible for the observed breakdown of oil compounds. For example, Abed et al. [60] demonstrated the ability of

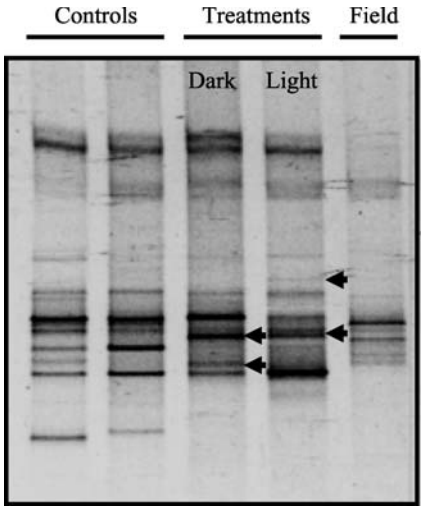


Fig. 6 DGGE profiles of PCR-amplified 16S rRNA fragments, using universal bacterial primers, obtained from the heavily polluted microbial mats of Wadi Gaza (WG) and mats collected at the end of a petroleum compounds-biodegradation experiment. The DGGE shows the enrichment of certain populations (marked by arrows) which are not detected in the controls. Sequencing of these bands showed that they belonged to the *Holophaga/Geothrix/Acidobacterium* phylum and *chloroflexus* group [60]

cyanobacterial mats originating from Wadi Gaza to degrade phenanthrene and dibenzothiophene completely and pristane and *n*-octadecane partially within three days of incubation. It was concluded that members of the *Holophaga/Geothrix/Acidobacterium* phylum might have been responsible for the degradation of the four model compounds. The DGGE patterns showed that members of this group were apparently enriched in the presence of the model compounds, during both light and dark incubations, but did not appear in any of the controls (Fig. 6). The analysis also indicated a possible involvement of green non-sulfur bacteria in the biodegradation process. Cyanobacteria seem to play an indirect role in the breakdown of petroleum compounds by supplying oxygen for the aerobic heterotrophic degraders. However, it should be kept in mind that such conclusions based on DGGE analysis remain speculative unless proven by isolation of the respective organisms. In some cases, the community may not undergo changes even when oil components are added. An example is the study by Grötzschel et al. [61], in which cyanobacterial mats were incubated for 18 weeks in the presence of oil compounds. In spite of the observed oil biodegradation and the long incubation period, no changes in community composition were detected by DGGE analysis. The authors concluded that these mats were inhabited by robust microbial communities.

7.1.3.3 Bioremediation Studies

Bioremediation can be achieved either by bioaugmentation or biostimulation. It is essential to follow the fate of added microorganisms in the case of bioaugmentation and the impact of the added stimulators on the present community in the case of biostimulation. Ogino et al. [46] used DGGE to follow microbial succession during a biostimulation process. They found that the community structure was disturbed by the biostimulation treatment but recovered immediately after the end of fertilization. The impact of different levels of inorganic nutrients on biodegradation rates and on bacterial community composition was studied by Roling et al. [62]. Oil biodegradation was stimulated upon addition of nutrients, irrespective of their concentration. Different microbial communities were apparently selected in all treatments, but this could not be correlated with nutrient levels. It was concluded that bioremediation treatments dramatically reduced bacterial diversity by selecting for oil-degrading bacteria.

7.1.3.4 Monitoring of Enrichment Cultures and Characterization of Oil-Degrading Consortia

DGGE can be used for screening of isolates and for comparing them to dominant phylogenetic signatures obtained from their original ecosystems.

Several studies have demonstrated the use of DGGE to monitor the development of oil-degrading enrichment cultures [63–66]. Bonin et al. [67] followed denitrifying bacteria in a squalene enrichment. The composition of the enrichment culture changed significantly through 8 months of incubation. The isolated strains were compared to the dominant phylotypes on DGGE gels. Eight isolates were obtained from the 12 phylotypes dominating the enrichment cultures. In another example, Burns et al. [68] isolated *tert*-butyl ether-degrading bacteria and used DGGE to show that it was apparently dominant in compost biofilter enrichments.

In some cases, biodegradation of petroleum compounds is performed by a consortium rather than by an individual microorganism. In this case, DGGE can be used to characterize the composition of this consortium without the need to separately cultivate its members, which can be difficult. For example, Koizumi et al. [69] used DGGE to study the composition of a mesophilic toluene-degrading consortium and an ethylbenzene-degrading consortium. Their findings revealed that, one member of both consortia was affiliated with the family *Desulfobacteriaceae*, whereas the other was related to an uncultured non-sulfate reducing soil bacterium. Similarly, the structure of an anaerobic pentachlorophenol-degrading consortium was revealed by DGGE in a study by Tartakovsky et al. [70]. The consortium was found to consist of *Clostridium* and *Syntrophobacter*/*Syntrophomonas* spp.

7.1.3.5
Detection of Active Members of a Community by RT-DGGE

PCR-DGGE analyses can be performed not only with DNA but also with RNA. DNA-based DGGE provides information on the presence of different bacterial populations while RNA-based DGGE may give an indication of which populations are active. The first step in RNA-based DGGE is the reverse transcription (RT) of RNA to form double-stranded cDNA. PCR is then used to amplify the DNA. This technique has been implemented in several studies to follow changes in active populations upon addition of various organics [71, 72]. Instead of targeting the 16S rRNA, RT-DGGE can be performed, in a similar fashion, using functional genes. For example, in a study by Hoostal et al. [73], DGGE was performed on RT-PCR amplified fragments of the *bph*A1 gene that encodes the large subunit of biphenyl dioxygenase.

7.1.4
Advantages and Disadvantages

DGGE has received many applications in biodegradation studies. The advantage of DGGE is the possibility to process many samples at the same time and to compare them on one gel. This makes DGGE an attractive technique to monitor changes in a single community composition or to compare differ-

ences among various communities. The possibility to sequence DGGE bands enables phylogenetic analysis of the obtained sequences and subsequently the identification of corresponding bacteria.

DGGE, like other methods, is not free of limitations. Biases can be introduced by sample handling, uneven cell lysis during DNA extraction as well as preferential amplification of certain sequences in the PCR step. The DGGE pattern fails to recover all bacteria in a community. DGGE is not quantitative and the intensity of bands does not necessarily reflect the abundance of the respective bacteria. The obtained sequences from DGGE are short and sometimes difficult to get. Other limitations include the detection of heteroduplex molecules and the co-migration of fragments with different sequences [52].

7.2 Terminal Restriction Fragment Length Polymorphism (T-RFLP)

7.2.1 Method

Terminal restriction fragment length polymorphism (T-RFLP) is a technique that has been described for revealing the composition of complex bacterial communities [74–76]. The method involves the extraction of DNA from environmental samples followed by subjecting the extracted DNA to PCR-amplification using specific primers (Fig. 7). One of the primers should be fluorescently labeled, which subsequently leads to the generation of PCR products carrying the fluorescence label at one terminal. The PCR products are digested with selected restriction endonucleases producing fluorescently labeled terminal fragments with different sizes. These fragments are separated on high-resolution sequencing gels. The separation of fragments is based on the different sized PCR products. The sizes of fragments are represented by peaks; each corresponds to a bacterial species. Each microbial community is characterized by a unique peak profile.

7.2.2 Applications

7.2.2.1 Bacterial Diversity of Contaminated Sites

T-RFLP has been used to study microbial diversity in contaminated sediments and to monitor changes in community composition following exposure to pollutants. In a study by Covert and Moran [77], the structure of bacterial communities growing at high-molecular weight (HMW) and at low-molecular weight (LMW) dissolved organic carbon was compared by T-RFLP.

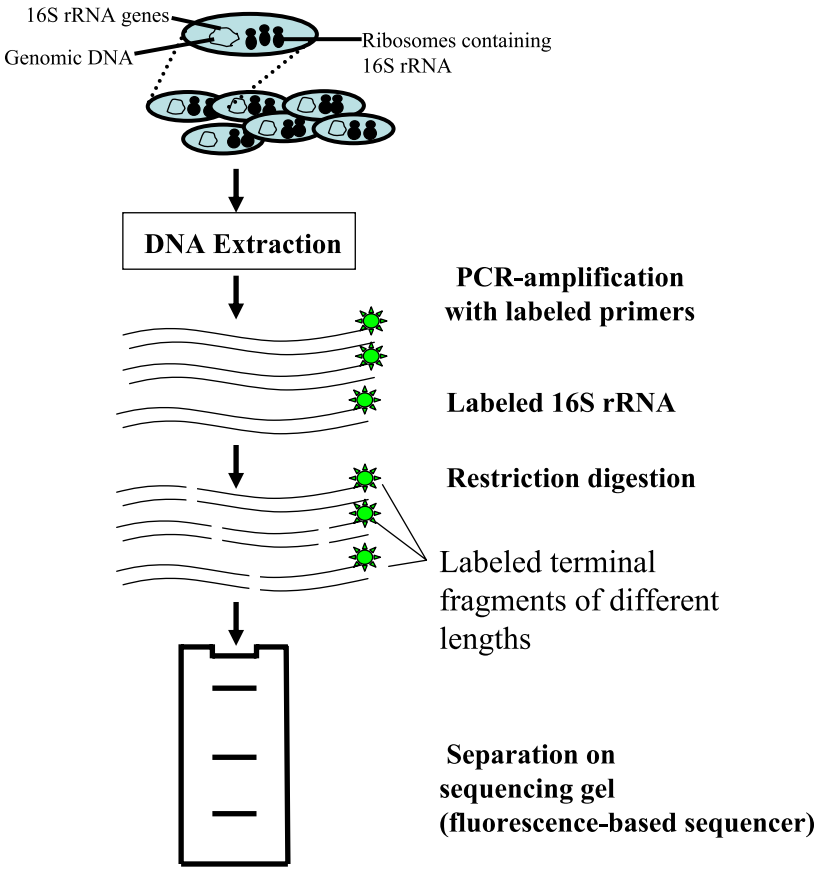


Fig. 7 Principle of the T-RFLP technique. Following extraction of nucleic acids, PCR amplification is performed using one fluorescently labeled primer. The amplified products are then subjected to restriction digestion, which leads to the generation of fluorescently labeled fragments of different lengths. The fragments are separated on a sequencing gel

The results showed a dominance of gamma and epsilon *Proteobacteria* in the case of LMW but a dominance of alpha, beta, and gamma *Proteobacteria* in addition to members of the C*ytophaga/Flavobacterium/Bacteriodes* group in the case of HMW. Konstantinidis et al. [78] used T-RFLP in order to estimate the bacterial diversity in sediments contaminated with heavy metals. Their analysis revealed a conserved community structure over a depth profile of 15 cm.

T-RFLP could also be used to follow shifts in bacterial community structure. Turpeinen et al. [79], used this technique to study the impact of contamination by arsenic, chromium and copper on microbial communities. The T-RFLP analysis showed that contamination with metals induces permanent changes in the microbial community structure. Rousseaux et al. [80] also re-

ported significant changes using T-RFLP in soil bacterial communities upon exposure to the herbicide, 4,6-dinitroorthocresol .

7.2.2.2
Community Changes During Bioremediation Studies

The impact of addition of fertilizers on the structure of indigenous bacteria (in the biostimulation process) as well as the fate of added microorganisms (in the bioaugmentation process) could be well followed using T-RFLP. An example is a study by Jernberg and Jansson [81] in which this technique was used to assess the impact of added *Arthrobacter chlorophenolicus* A6L into 4-chlorophenol-contaminated soil. Simultaneously, the impact of 4-chlorophenol alone or the inoculation of *A. chlorophenolicus* into non-contaminated sediments was compared. The study could differentiate the various bacteria, which were stimulated or repressed by the different treatments. The relative abundance of certain populations could be inferred by the peak areas of terminal restriction fragments. For example, the abundance of *A. chlorophenolicus* was found to be significantly higher in soils contaminated with 4-chlorophenol.

7.2.2.3
Characterization of Consortia

T-RFLP has been used to identify bacteria living in consortia. An example of this is the identification of an aerobic microbial consortium that reductively dechlorinates trichloroethene completely to ethane [82]. T-RFLP patterns suggested that the consortium was dominated by populations belonging to three phylogenetic groups; *Dehalococcoides*, *Desulfovibrio* and members of the *Clostridiaceae*. This composition was further confirmed by cloning.

7.2.3
Advantages and Disadvantages

T-RFLP provides a sensitive and rapid technique for assessing amplification product diversity within a single community as well as comparative distribution across communities. Comparison of data obtained by this technique to those obtained by DGGE showed comparable results with T-RFLP showing slightly higher resolution than DGGE [83]. However, the advantage of DGGE over T-RFLP is the possibility to obtain sequences and perform phylogenetic analysis.

T-RFLP has several limitations due to the preferential extraction of DNA and PCR amplification biases. These problems have been discussed in detail in [84, 85]. Another problem is the incomplete and non-specific restriction digestion. However, this can be solved by including an amplified product from

a known isolate (using a primer with a different fluorescence). T-RFLP fails to provide reliable quantitative data, even though some researchers consider the peak area as a good measure of abundance. Using T-RFLP it is not possible to obtain sequence information.

7.3 Random Amplification of Polymorphic DNA (RAPD-PCR)

Random Amplified Polymorphic DNA (RAPD) is a commonly used molecular technique in genetic diversity studies. Unlike standard PCR, RAPD reactions are PCR reactions, which amplify unknown DNA fragments.

7.3.1 Method

The principle involved in generating RAPDs is that a single or a pair of short oligonucleotide primers (ca. 10 bp), which bind to many different loci, are used to amplify random sequences from a complex DNA template (Fig. 8). The PCR is then performed at low stringency for primers annealing. The amplified fragments generated by PCR depend on the size of both the primer and

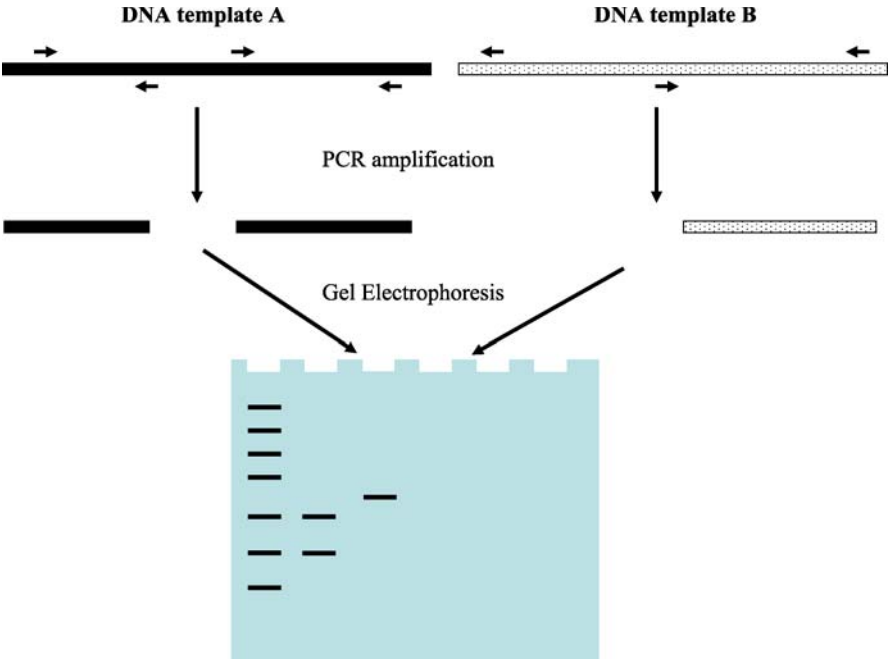


Fig. 8 Principle of random amplification of polymorphic DNA (RAPD-PCR)

the target DNA. Amplification will occur as a result of the presence of sites on both strands of the DNA that are complementary to the primers. The amplified products (of up to 3.0 kb) are usually separated on agarose gels and visualized by ethidium bromide staining. The use of a single 10 bp oligonucleotide primer promotes the generation of several discrete DNA products that are considered to originate from different genetic loci. Polymorphisms result from mutations or rearrangements either at or between the primer binding sites and are detected as the presence or absence of a particular RAPD band.

7.3.2 Applications

7.3.2.1 Comparing Microbial Communities and Monitoring Their Changes

RAPD can be used to compare the community composition of different contaminated sites or to follow changes in a community upon addition of a certain contaminant or due to a remediation process. This technique has been used to follow the bacterial succession associated with anaerobic degradation of phenol in a fed-batch culture [86]. The microbial community was shown to change following each phenol amendment. The changes suggested a microbial acclimation towards faster degradation rates upon repeated amendments. In this study, a comparison of RAPD and T-RFLP was performed. Their conclusion was that RAPD was faster, simpler, had a higher resolution and is more suitable for studies of microbial community shifts. Wilkstrom et al. [87] showed with this technique that amendments with hydrocarbons previously unknown to a microbial community fostered the growth of unique microbial communities while the community remained unchanged after amendments with hydrocarbons that were known to the community. RAPD can also be used to study the genetic similarity of communities from different sites. For example, Franklin et al. [88] used RAPD to determine the genetic similarity of microbial communities from several groundwater wells.

7.3.2.2 Screening of Oil-Degrading Bacteria

Different bacteria are expected to display different genetic fingerprints thus making RAPD suitable for screening of bacterial strains. Cavalca et al. [89] used RAPD to screen 15 isolated strains from a BTEX (benzene, toluene, ethylbenzene, xylene)-contaminated subsoil. The isolates were found to belong to different species of the genera *Pseudomonas* and *Alcaligenes*. Similarly, Lloyd-Jones and Hunter [90] clustered their bacterial isolates obtained from two sites contaminated with polycyclic aromatic hydrocarbons into two

groups based on RAPD analysis. These results were further supported by phenotypic properties of the obtained isolates.

7.3.3 Advantages and Disadvantages

RAPD is an easy and quick assay that requires low quantities of DNA (5–50 ng per reaction). The design of primers does not require sequence data since the primers sites are randomly distributed throughout the genome. Each reaction generates multiple bands that are often sufficient to reveal changes. Reading of RAPD patterns is amenable to automation.

The disadvantages of this technique are that a purified high molecular weight DNA is required. Extensive precautions need to be taken to avoid contamination because short random primers are used, which are able to amplify DNA fragments from a variety of organisms. Highly standardized experimental procedures are needed because of sensitivity to reaction conditions. Because of the length of the primers, their random sequence and the PCR conditions, RAPD is not expected to be reproducible. All these serious limitations of the technique makes DGGE and T-RFLP better techniques for studying complex microbial communities.

7.4 Amplified Ribosomal DNA Restriction Analysis (ARDRA)

7.4.1 Method

ARDRA can be used for the characterization of bacterial isolates and has the potential for analyzing mixed bacterial communities. The working principle of the technique is based on the fact that the 16S rRNA operon has restriction sites that are conserved according to phylogenetic patterns. The restriction patterns are obtained by subjecting the amplified rDNA to restriction endonuclease digestion leading to the production of smaller DNA fragments (Fig. 9). The obtained banding patterns serve as fingerprints for the identification of respective bacteria.

7.4.2 Applications

7.4.2.1 Screening of Bacterial Strains and Clones

Han et al. (2003) [91] used ARDRA to screen 36 strains of phenanthrene-degrading bacteria that were isolated from polycyclic aromatic hydrocarbon-

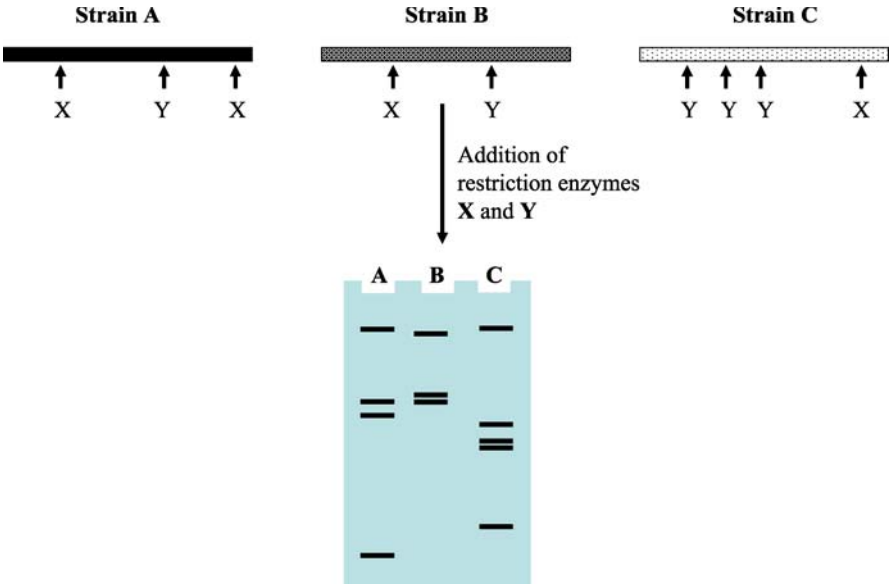


Fig. 9 Principle of amplified ribosomal DNA restriction analysis (ARDRA). The example demonstrates the use of ARDRA in the screening of three bacterial strains (A–C). Two restriction enzymes are used (designated as X and Y)

contaminated sites. The strains were found to fall into four different ARDRA types. Most of these strains belonged to the genus *Sphingomonas*. Bakermans and Madsen [92] also used ARDRA in combination with cloning to study the bacterial diversity of contaminated aquifers. After cloning, clones were randomly selected and screened by ARDRA for differences. Their analysis showed that 60% of 400 picked clones contained unique ARDRA patterns.

7.4.2.2 Studies of Bacterial Communities and Consortia

ARDRA patterns of mixed microbial communities are expected to be very complex. The degree of pattern complexity depends substantially on the type and number of restriction enzymes used. Fries et al. [93] used the technique to follow the succession of phenol and toluene-degrading strains in an aquifer. Thomassin-Lacroix et al. [50] used the technique to study the structure of a hydrocarbon-degrading consortium. ARDRA patterns suggested that the consortium is constituted of three phylotypes. Further cloning showed that these three phylotypes were *Rhodococcus erythropolis*, *Sphingomonas* and *Pseudomonas*.

7.4.3
Advantages and Disadvantages

ARDRA is a very useful technique for screening clones or isolates before sequencing all of them. This helps in revealing the similar ones and thus minimizes sequencing costs. For analyzing complex microbials, ARDRA is not recommended. This is due to the very complex banding pattern since one species may generate many bands.

The application of ARDRA requires previous sequence information in order to select the appropriate restriction enzymes. Incomplete restriction digestion leads to changes in banding pattern.

7.5
Single-Strand Conformation Polymorphism (SSCP)

7.5.1
Method

Single-strand conformation polymorphism was originally described by Orita et al. [94] for the detection of point mutations. The idea of SSCP is to perform electrophoresis on a non-denaturing polyacrylamide gel using small PCR products after denaturation of the DNA. As the PCR product moves through the gel, it will regain a secondary structure that is sequence dependent (similar to RNA secondary structure). The mobility of the single-stranded PCR products depends on their secondary structure. Therefore, PCR products that possess sequence differences (mutations, insertions or deletions) will have different mobilities.

7.5.2
Applications

SSCP has been used in bioremediation studies in order to follow population dynamics. Wenderoth et al. [95] used this technique to monitor changes in microbial communities of chlorobenzene-contaminated groundwater subjected to biostimulation and bioaugmentation processes. The fate of the three introduced *Pseudomonas* strains was followed in the case of bioaugmentation. SSCP patterns were statistically analyzed and showed that the indigenous community was specific to the introduced strain, inoculum size and physicochemical conditions. Beaulieu et al. [96] used SSCP to study the impact of pentachlorophenol on soil microbial communities upon enrichment with these compounds. The SSCP patterns suggested a decrease in community complexity. They also used SSCP to screen several clones of 16S rDNA libraries. A great part of the clones had the same SSCP profile, suggesting a selection of a little phylogenetic diversity during the enrichment process.

These clones were related to the genus *Sphingomonas*. In one particular study by Cho et al. [97] SSCP patterns were followed by computer-assisted cluster analysis of the community fingerprints. Such modification facilitates comparative analysis of microbial communities using SSCP.

7.5.3 Advantages and Disadvantages

The main advantage of SSCP is the ability to screen many PCR products for variation simultaneously. This helps in reducing the amount of sequencing needed to detect new alleles. However, the difference in position of two different PCR products doesn't necessarily reflect the degree of sequence difference between them. Additional limitation of SSCP is the optimal size for the detection of most point mutations, which is rather small (ca. 200 bp). Using SSCP for studying complex communities is problematic because of the bad resolution of the obtained banding pattern. This makes DGGE a better technique for profiling complex bacterial communities.

8 Hybridization Techniques

8.1 Whole-Cell in situ Hybridization

8.1.1 Method

In situ hybridization was first described more than a decade ago [98–102] but has since then undergone significant developments. The technique is based on individual population detection using labeled oligonucleotide probes either with a fluorescent dye (termed as FISH, fluorescence in situ hybridization) (Fig. 10), a radioactive label, an enzyme or a marker molecule like digoxigenin. The probes are about 18 base pair (bp) in length and can be designed to target a specific organism or a group of organisms. A list of selected probes relevant to detect bacteria in contaminated sites is presented in Table 2. The probe binds to its complementary binding site within the fixed bacterial cell. Hybridization probes are added to a defined, stringency-determined buffer to maximize specific probe binding.

The hybridization procedure involves the following main steps (detailed protocols can be found in [103]:

1. Sample fixation: This is a crucial step needed to permeabilize the cells for penetration of the fluorescent probes into the target cells and to preserve

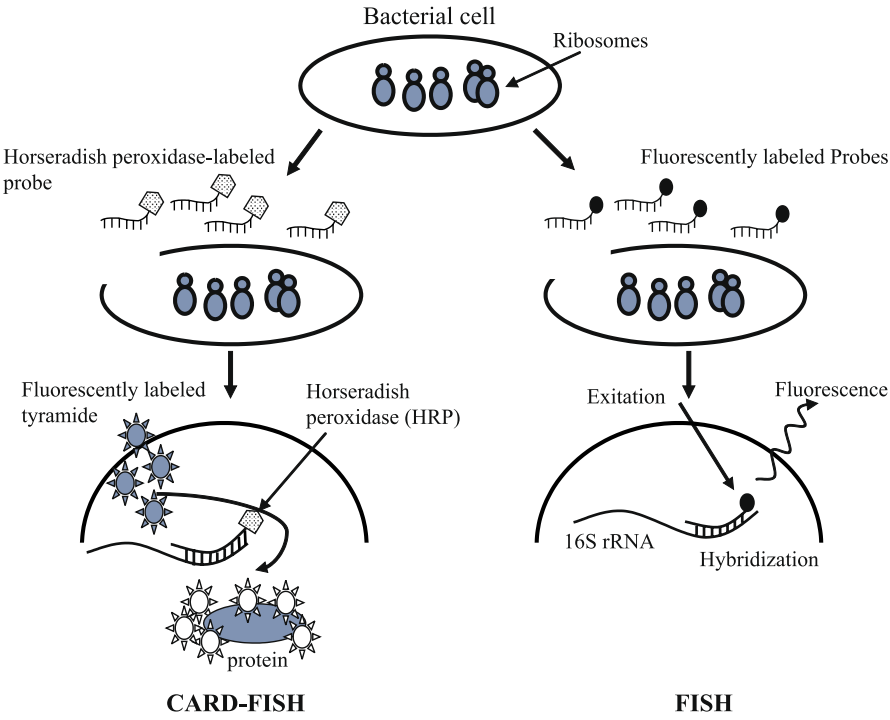


Fig. 10 The difference between FISH and CARD-FISH

the RNA from degradation by endogenous RNAses. Fixation is performed using ethanol or methanol or aldehydes such as paraformaldehyde or gultaraldehyde (3–4% v/v). Fixation conditions depend very much on the sample as well as on the target cells.

2. Pretreatment: In this step, samples are placed on a gelatin-coated glass slide in order to ensure strong attachment. Bacterial cells may also be collected on filters (0.2 μm diameter). The bacterial cells are then dehydrated in an ethanol series. Lysozyme treatment is often applied particularly in the case of Gram-positive bacteria in order to perforate the peptidoglycon layer. Some permeabilization steps may involve treatments with 1 M hydrochloric acid (HCl).
3. Hybridization with probes: Hybridization must be carried out under stringent conditions to ensure specific annealing of the probe to the target sequence. Stringency is adjusted by varying either the formamide concentration in the hybridization buffer or the hybridization temperature. The hybridization takes place by applying the fluorescently labeled probes to the samples in the presence of a hybridization buffer. Hybridization should be performed in a dark humid chamber usually at temperatures ranging from 27 to 50 °C.

Table 2 List of selected oligonucleotide probes specific for microorganisms or groups of bacteria present in oil-contaminated sites or relevant to certain biodegradation processes. The target sites, sequences and specificity of the probes are shown

Probes	Nucleotide Sequence (5′-3′)	Target microorganism/s	Target site	Refs.
ARCH915	GTGCTCCCCCGCCAATTCCT	Archaea	16S; 915–934	[104]
EUB338	GCTGCCTCCCGTAGGAGT	Bacteria	16S; 338–355	[104]
CF319a	TGGTCCGTGTCTCAGTAC	*Cytophaga-Flavobacterium* cluster of CFB phylum	16S; 319–336	[105]
HGC69a	TATAGTTACCACCGCCGT	High G+C Gram-positive bacteria	23S; 1901–1918	[106]
ALF1b	CGTTCGYTCTGAGCCAG	α-Subclass of *Proteobacteria*/most *Spirochaetes* several members of δ-subclass of *Proteobacteria*	16S; 19–35	[107]
BET42a	GCCTTCCCACTTCGTTT	β-Subclass of *Proteobacteria*	23S; 1027–1043	[107]
GAM42a	GCCTTCCCACATCGTTT	γ-Subclass of *Proteobacteria*	23S; 1027–1043	[107]
DFM228	GGGACGCGGAYCCAT	*Desulfotomaculum* species	16S; 228–242	[45]
DBB660	GAATTCCACTTTCCCCTCTG	*Desulfobulbus* species	16S; 660–679	[108]
DBM221	TGCGCGGACTCATCTTCAAA	*Desulfobacterium* species	16S; 221–240	[108]
DSB623	TGTTTCAAGTGCWCTTCCGGGG	*Desulfobacter* species	16S; 623–644	[45]
DCC868	CAGGCGGATCACTTAATG	*Desulfococcus-Desulfonema-Desulfosarcina* spp.	16S; 868–885	[45]
DSV687	TACGGATTTCACTCCT	*Desulfovibrio-Desulfomicrobium* species	16S; 687–702	[108]
Azo644	GCCGTACTCTAGCCGTGC	strains of *Azoarcus toulyticus* [1]	16S; 644–661	[109]
Azo1251	CGCGCTTTGGCAGCCCT	strains of *Azoarcus evansii* [1]	16S; 1251–1267	[109]
AT1458	GAATCTCACCGTGGTAAGCGC	*Azoarcus*/*Thauera* cluster within β-Subclass of *Proteobacteria* [2]	16S; 1458–1478	[110]
Ade441	GCGCCGTTTCTTCCCTGC	*Alcaligenes defragans* [3]	16S; 441–458	[110]
ALV461	GTACTCATCCGTATTAAGG	strains of *Alcanivorax borkumensis* [4]	16S; 461–480	[111]
ALV735	CGTCAATGTCAGTCCAGGAGG	*Alcanivorax* strains and *Fundibacter jadensis* [4]	16S; 735–750	[111]

Table 2 (continued)

Probes	Nucleotide Sequence (5′-3′)	Target microorganism/s	Target site	Refs.
ACA	ATCCTCTCCCATACTCTA	*Acinetobacter venetianus* strain T4 [4]	16S; 652–669	[112, 113]
Pp16S-R	TTGCCAGTTTTGGATGCAGT	species *Pseudomonas putida* (strain F1) [5]	16S; n.a.	[114]
JS16S-R	GATGCAGTCACCAATGCAGT	species *Burkholderia* strain JS150 [5]	16S; n.a.	[114]
Bs16S-R	GTTCCCCAGTTTCCAATGACCC	species *Bacillus* (strain ATCC7003) [5]	16S; n.a.	[114]
Pa16S-R	AGCTCAGTAGTTTTGGATGCA	species *Pseudomonas aeruginosa* [6]	16S; n.a.	[95]

[1] denitrifying bacteria degrading toluene and *m*-xylene; [2] denitrifying bacteria degrading alkybenzene and alkanes; [3] denitrifying bacterium degrading α-pinene and unsaturated monoterpenes; [4] aerobic bacteria degrading alkanes; [5] aerobic bacteria involved in aromatics biodegradation; [6] aerobic bacteria degrading chlorobenzene

4. Washing step: In this step, the slides are washed with a washing buffer to remove unbound probe. Stringency of the washing buffer can be regulated by varying salt concentrations.
5. Visualization and documentation of the results: The slides are then dried and mounted. Observation of the hybridization signals can be performed by a conventional epifluorescent microscope or by a confocal laser scanning microscope (CLSM). CLSM is particularly recommended in the case of thick sections, or samples with high fluorescence background or if further image analysis (quantification, 3D-structure) is required.

Recently, a series of modifications have been introduced to the in situ hybridization technique. This includes the development of a new in situ hybridization technique based on horseradish peroxidase (HRP)-labeled probes for the identification of individual cells of cyanobacteria, which could not be detected due to the strong autofluorescence of their cells [115]. This technique (known as CARD-FISH, Catalyzed reporter deposition-fluorescence in situ hybridization) has been further optimized for the detection of other groups of bacteria [116] (Fig. 10). The introduction of this method has led to increased detection of hybridized cells, brighter hybridization signal and higher sensitivity. An automated system for the quantification of hybridized cells in suspensions has recently been developed [117]. In situ hybridization on rRNA has been successfully combined with in situ hybridization on mRNA for the detection of environmental bacteria [118].

8.1.2 Applications

8.1.2.1 Microbial Diversity of Contaminated Systems

This technique has been successfully used to characterize multispecies population in a laboratory-scale trickle bed bioreactor used for the biodegradation of a mixture of polyalkylated benzenes. Interestingly, the in situ hybridization results revealed that the aromatic-degrading cells constitute less than 10% while 60% of the cells were saprophytes and about 30% were inactive cells [119, 120]. These saprophytes were believed to utilize intermediate compounds and cell lysis products.

In situ hybridization could also be used in bioremediation studies. For example, the fate of added microorganisms in the case of bioaugmentation could be followed and the development of these bacteria could be quantified. Wenderoth et al. [95], performed a microcosm experiment to assess biostimulation and bioaugmentation for groundwater contaminated with chlorobenzenes. Biostimulation was performed by the addition of air and nitrate while bioaugmentation was through the addition of aerobic

chlorobenzene-degrading bacteria (*Pseudomonas putida* GJ31, *Pseudomonas aeruginosa* RHO1, and *Pseudomonas putida* F1DeltaCC). The microbial community was followed using several molecular techniques including in situ hybridization. In the case of biostimulation, a specific change in the natural bacterial community composition was observed, which corresponded to the aerobic or anaerobic denitrifying conditions. *P. putida* GJ31 as well as *P. putida* F1DeltaCC were capable of growing in groundwater and in the presence of natural bacterial communities, and stimulated biodegradation of chlorobenzene. *P. putida* GJ31 disappeared when the xenobiotics were depleted but *P. putida* F1DeltaCC persisted even in the presence of chlorobenzenes.

In situ hybridization was also used to reveal the structure of microbial consortia. Wang and Hegemann [121], used this technique to study the structure of a consortium capable of reductively dechlorinating trichlorophenol. Their analysis showed that the consortium contain major subpopulations that are phylogenetically affiliated to the gamma and delta subclass of *Proteobacteria*.

8.1.2.2
Targeting Specific Bacteria

Oligonucleotide probes could be designed to target specific populations that are relevant in certain biodegradation processes. For example, Yanog and Zeyer [122] designed specific probes to detect *Dehalococcoides* species that were responsible for the complete dehalogenation of chloroethenes. They used these probes to monitor the presence, distribution, fate of these organisms, as well as to quantify members of this group.

FISH is also an excellent technique to detect yet-uncultured microorganisms that have been detected only by DNA fingerprints. For example, Haruta et al. [123] used this technique to detect a population which was always encountered and formed a major band on their DGGE gels. They designed a specific probe for this population using the sequence information obtained from the corresponding DGGE band. Quantification of this population showed that they account for 30% of the total eubacterial cell count.

8.1.2.3
Spatial Distribution of Bacterial Populations

FISH is also suitable for revealing the spatial distribution of certain populations. For example, the *Desulfitobacterium frappieri* PCP-1 used for the anaerobic degradation of pentachlorophenol in an anaerobic upflow sludge bed (UASB) system was found to densely colonize the outer biofilm layer as revealed by FISH [124].

8.1.3 Advantages and Disadvantages

FISH has facilitated the identification of specific bacteria under in situ conditions and has been successfully used to study their abundance, distribution and spatial arrangement. FISH is a PCR-independent technique and simple to handle.

Using FISH, sequence information and an updated database with the function of probe design are required. The use of specific probes, which are designed based on known sequences, limits the analysis only to previously known bacteria. The specificity of probes can sometime be problematic, therefore a careful optimization and evaluation of the selected probes is required. The application of FISH in complex communities is tedious and may encounter several problems such as high fluorescence background and unclear signals. Autofluorescence of some bacteria may disturb the hybridization signal.

8.2 Blotting Hybridization

8.2.1 Method

Dot-blot and slot-blot hybridizations are PCR independent approaches that are applied directly to nucleic acid extracts (DNA and/or RNA) from environmental samples. The extracted nucleic acids are fixed on a membrane, usually a charged nylon membrane, and hybridized with radioactively labeled oligonucleotide probes selected to identify and quantify the relative abundance of specific rRNA sequences in the original samples. The amount of target samples is estimated by comparing the intensity of signals emitted by dots containing the test samples with standards containing known concentrations of the target sequence.

Northern hybridization is used to locate the RNA species of interest. The RNA is immobilized and fixed on a membrane. Hybridization with specific probes is then performed under optimized conditions and the obtained image of the distribution of the tightly bound probe on the membrane is read. At the end of the procedure, the probe may be stripped from the membrane and the membrane could be used for another hybridization.

Southern blot hybridization is used to study the organization of genes within genomes. This is achieved by mapping restriction sites in and around segments of the genomic DNA for which specific probes are available. The different steps of southern blot hybridization include, extraction of genomic DNA followed by restriction digestion with one or more restriction enzymes. The obtained fragments are separated on agarose gel. The DNA fragments are then transferred from the gel to a nylon membrane. Hybridization of the

immobilized DNA fragments to a radiolabeled DNA, RNA or oligonucleotide probe is then performed and the obtained signals are read by autoradiography. By estimating the size and the number of the bands generated after digestion of the genomic DNA with single or several restriction enzymes, it is possible to place the target DNA within a context of restriction sites.

8.2.2 Applications

8.2.2.1 Slot and Dot Blot Hybridization

Slot and dot blot hybridizations are used mainly for quantification purposes. Slot blot hybridization has been successfully used to estimate the relative abundance of four nitrate-reducing bacteria that constitute a consortium capable of anaerobic degradation of benzene [49]. Interestingly, the results showed that the bacteria that formed around 52% of the clone library, accounted only for 4% of the culture while one of the cloned 16S rRNA gene sequences comprised 70% of the culture. It is also possible to use this technique to follow the expression of functional genes. Sakai et al. [125] used slot blot hybridization to follow the expression of four newly described 2,3-dihydroxybiphenyl dioxygenases genes in *Rhodococcus* sp. RHA1 that were involved in the degradation of polychlorinated biphenyls. RNA slot blot hybridization showed that only one type of the four was transcribed in *Rhodococcus* cells grown on biphenyl and ethylbenzene.

Dot blot hybridization has been used to monitor the alkB gene in n-alkane-degrading bacterial isolates [126]. The gene was shown to be widely spread among short-chain alkane degraders. Dot blot hybridization is also suitable for monitoring microbial communities after a bioremediation process [127].

8.2.2.2 Northern and Southern Blot Hybridization

In biodegradation studies, northern blotting hybridization has been used to follow the expression of functional genes. Taguchi et al. [128] used this technique to monitor the expression of eight genes encoding extradiol dioxygenase (bphC1-8). Kahng [129] used a naphthalene dioxygenase gene fragment cloned from *Pseudomonas* strain KK1 as a probe for northern blot hybridization. The results showed that the naphthalene dioxygenase gene was expressed in cells grown on phenanthrene to a larger degree than in those grown on naphthalene.

Southern blot hybridization has been used to examine the presence of certain genes as well as to study the organization of genes. Fedi et al. [130] used this tool to study the homology of bph operon obtained from *Pseu-*

domonas pseudoalcaligenes strain KF707 and fifteen biphenyl-degrading bacteria. The results showed homology only within four strains. Similarly, Danne et al. [131] used this technique to screen for genes responsible for PAH degradation among isolates from salt marsh rhizosphere. In a study by Murakami et al. [132], the localization of two catA genes coding for catechol 1,2-dioxygenases were studied by southern blot hybridization. On the basis of the southern blot hybridization, the authors concluded that one gene was located on the chromosomal DNA whereas the other was located on large plasmid DNA.

8.2.3 Advantages and Disadvantages

Blotting techniques are useful to quantify the genes involved in biodegradation and to study their organization. Sequence databases are needed in order to design specific probes and these probes should be carefully evaluated prior to use. The use of radioactivity requires extra precautions. The transfer of RNA or DNA to a membrane may be incomplete leading to inaccurate quantification results. Reading radioactivity signals require special instrumentation. Blotting techniques are tedious and time-consuming.

8.3 Colony Hybridization

8.3.1 Principle

The colony hybridization technique was first described in 1973 [133] for screening *E. coli* transformed clones for possessing a specific gene. The technique has undergone subsequent modifications appropriate for different applications in microbial ecology. This method relies on the growth of bacterial colonies on nutrient agar. Nutrient agar plates are first inoculated with an environmental sample and then incubated at optimum conditions (Fig. 11). The grown colonies are transferred to a nylon or nitrocellulose membrane and then subjected to cell lysis. The cellular debris is removed by washing the membranes, which are subsequently hybridized with the target probe. This probe is radioactively labeled, enabling detection by exposure to X-ray film. Recently, non-radioactive probes have been successfully used as well.

8.3.2 Applications

Most of the applications of colony hybridization in biodegradation studies are associated with screening the presence of catabolic genes. The genes

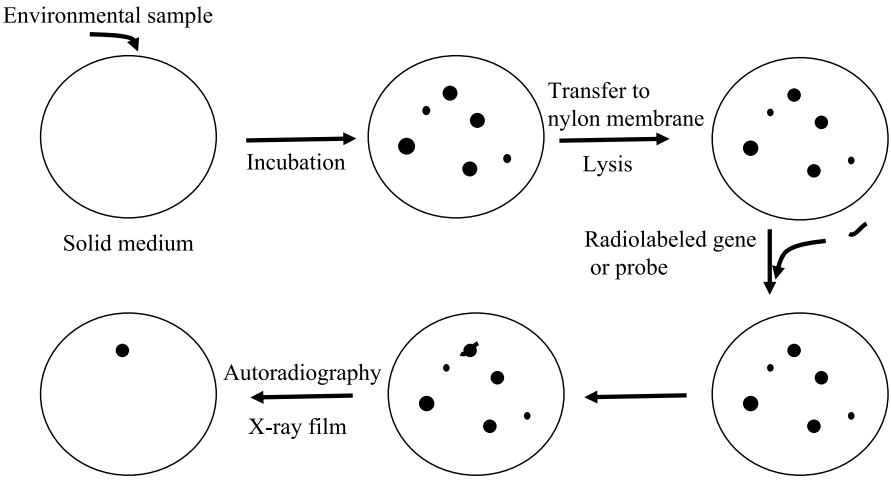


Fig. 11 Steps in colony hybridization. Hybridization is performed using radiolabeled probes and checked by autoradiography

involved in biodegradation of alkanes have been the focus of several studies [126, 134, 135]. Whyte et al. [134, 135] studied the distribution and determined the relative abundance of alkane-degrading genes in cold environments. In one study they determined the presence of four alkane monoxygenase genotypes (*Pseudomonas putida* GPo1, PpalkB; *Rhodococcus* sp. strain Q15, Rh alkB1 and Rh alkB2; and *Acinetobacter* sp. Strain ADP-1, Ac alkM) in hydrocarbon-contaminated cold-adapted and mesophilic populations from Arctic and Antarctic soils [135]. The relative abundance of the studied genes revealed that *Rhodobacter* spp. are the most dominant alkane-degraders in those sediments followed by *Pseudomonas* and then *Acinetobacter*. Similarly, probes targeting aromatic-degrading genes were also used for colony hybridization. Milic-Terzic et al. [136] used this technique to study the presence and distribution of (xylE and ndoB) genes involved in degradation of aromatics in diesel, toluene and naphthalene-degrading microbial consortia.

8.3.3 Advantages and Disadvantages

The main advantage of this technique is that the isolate that contains the target gene can be recovered for further phylogenetic and physiological studies. This is achieved by duplicating the colonies before transferring them to the membrane.

Colony hybridization is a culture-dependent approach that determines only the microorganisms that are readily cultivated. Therefore, a significant part of the microbial community will be overlooked.

8.4 DNA Microarrays

8.4.1 Method

DNA microarrays or DNA chips are new devices (< 10 years) that allow researchers to rapidly screen for the presence or absence and for expression levels of a huge number of genes [137, 138]. Using 16S rRNAs as target genes enables the detection of bacterial diversity [139]. The application of DNA microarrays requires firstly the preparation of the chip. This is done by spotting and immobilization of high-density nucleic acid samples, usually cDNAs or oligonucleotides, on a glass slide. Subsequently, hybridization is performed on the resulting DNA chip using fluorescently labeled nucleic acids derived from a desired source. A hybridization pattern representing gene expression is obtained by analyzing the signal emitted from each spot with digital imaging software. The obtained pattern is then compared to that of a control for differential analyses.

8.4.2 Applications

DNA microarrays have many applications in microbial ecology (for details refer to Ye et al. [140]), however their applications in biodegradation studies have been so far limited. Koizumi et al. [69], used this technique to identify the bacterial composition of toluene and ethylbenzene-degrading communities. They designed oligonucleotide probes specific for the 16S rRNAs of the target microorganisms. The DNA microarray data were consistent with DGGE analysis revealing that two populations belonged to the family *Desulfobacteriaceae* and the other to uncultured non-sulfate-reducing soil bacterium. Koizumi et al. [69] also applied the DNA microarray technology to oil-contaminated sediments. These attempts were partially successful as they could not get any hybridization signals with probes specific for sulfate-reducing bacteria even though other methods showed that they were present. The authors realized the limitations of using this technology for environmental samples.

In another study by Dennis et al. [141], the DNA microarray technology was used to monitor the expression of catabolic genes related to 2,4-dichlorophenoxyacetic acid (2,4-D) degradation within pure and mixed cultures of the 2,4-D-degrading bacterium *Ralstonia eutropha* JMP134. With this technique, it was shown that two of five 2,4-D catabolic genes were induced upon addition of the pesticide. The study demonstrated the potential of DNA microarrays for the detection of functional genes in complex environmental systems.

8.4.3
Advantages and Disadvantages

DNA microarrays are powerful tools because of the large number of hybridizations that can be performed simultaneously on a glass slide (around 100 000 spots per cm^2) [21]. Compared to traditional hybridization techniques, glass slide-based microarrays have the additional advantages of low cost, rapid detection, high sensitivity and low background and automation.

DNA microarray technology has been widely applied to study numerous bacterial species [140]. However, the application to environmental samples has encountered several problems [142]. Among those, is the high bacterial diversity of environmental samples, which might lead to unspecific hybridizations. The quality of the environmental nucleic acids, which are often contaminated with humics and organic contaminants as well as the low bacterial biomass, compared to pure cultures, pose additional problems.

8.5
Magnetic Capture Hybridization

8.5.1
Method

Magnetic beads have previously been used for purification and sequencing of PCR products, construction of cDNA libraries, affinity purification of DNA-binding proteins, hybridization of covalently attached oligonucleotides and many other applications (for details see [31]). Recently they have been optimized to specifically isolate different classes of rRNAs from mixtures of total RNA for stable isotopic characterization [143]. Magnetic capture hybridization involves the use of streptavidin-coated magnetic beads and biotin-labeled oligonucleotides. Magnetic beads are non-porous, superparamagnetic, polystyrene beads with a magnetic core and a diameter of $\sim 2.8\,\mu m$. The RNA sample is mixed with a specific biotin-labeled oligonucleotide probe under defined conditions in order to allow hybridization between the probe and the target RNA (Fig. 12). The hybridized RNA is separated from the unhybridized RNA by mixing with streptavidin-coated magnetic beads, which have a very high biotin binding capacity. The target RNA is then obtained after heat-dissociation from the magnetic beads.

8.5.2
Advantages and Disadvantages

Magnetic capture hybridization offers several advantages among them are speed and strong binding between biotin and streptavidin. However, the high cost of magnetic beads and low recovery of the RNA are often the major draw-

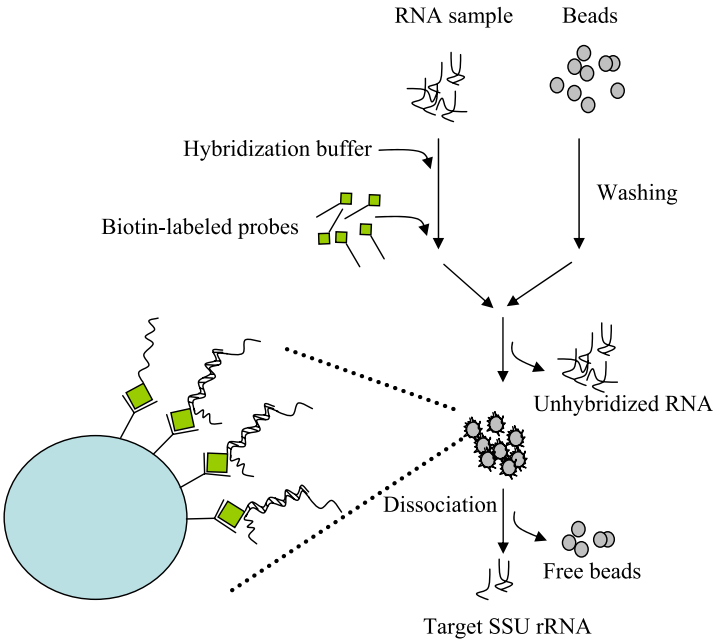


Fig. 12 Description of the magnetic capture hybridization method. Hybridization is first performed between RNA and biotin-labeled probes. The hybridized RNA is separated from the unhybridized RNA using streptavidin-coated magnetic beads

backs. Magnetic beads capture bears a powerful potential in biodegradation studies, nevertheless, it has not been so far used in such studies. Among possible applications are the specific isolation of RNA from a single or a group of bacteria relevant to the degradation of a certain compound(s). In combination with radioactive or stable isotope labeling, this technique could be used to follow the incorporation of ^{13}C- or ^{14}C-labeled hydrocarbon into the rRNA of microbial communities.

9 Analytical PCR Techniques

9.1 Real-Time PCR

9.1.1 Method

Real-time PCR or quantitative polymerase chain reaction (qPCR), is a highly sensitive technique that can be used to quantify genes of interest (functional

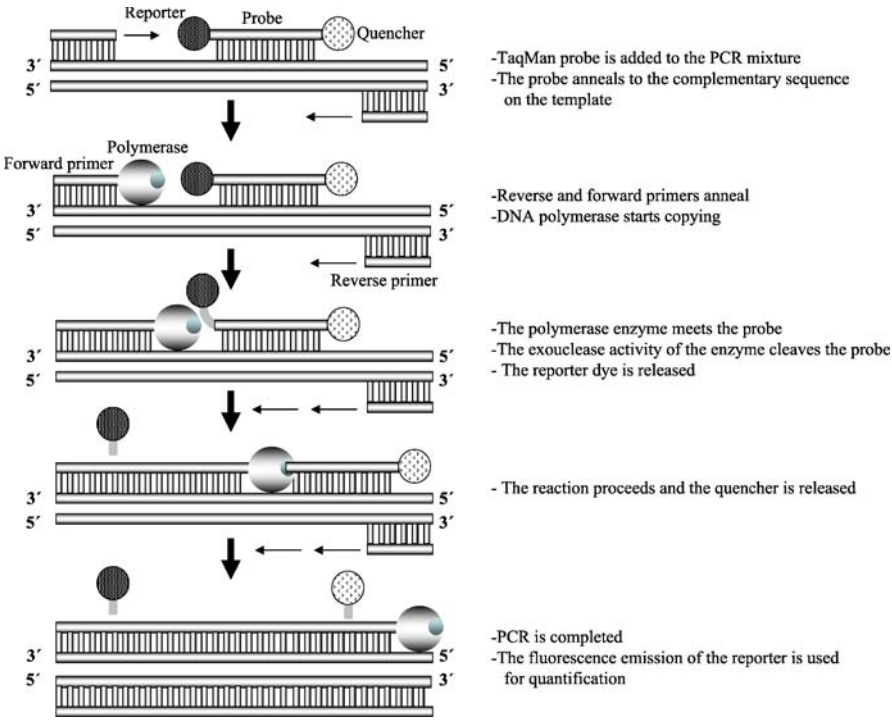


Fig. 13 Steps in quantitative real-time PCR

or 16S rRNA genes). The technique is based on the detection of the fluorescence produced by a reporter molecule, which increases as the reaction proceeds (Fig. 13). This occurs due to the accumulation of the PCR product with each cycle of amplification. These fluorescent reporter molecules include non-specific dyes such as SybrGreen dye [144] or sequence-specific TaqMan probes [145]. TaqMan probes contain a high-energy dye termed as a reporter and a low-energy molecule termed as a quencher. The reporter dye emission, when exited with a light source, is suppressed by the quencher dye due to the proximity between them. When the polymerase enzyme collides with the probe, the reporter dye is released due to the nuclease activity of the enzyme. Thus, the distance between the reporter and quencher increases and the transfer of energy stops. This leads to an increase in the fluorescence emission of the reporter.

An additional advantage of qPCR is the ability of quantification of several sequences in one sample by using different fluorophores. The detection limit of the method depends on the target of interest, sample purity, PCR conditions and other factors but theoretically allows the detection of a single DNA molecule [146]. RNA can, similar to DNA, be quantified after reverse transcription.

9.1.2 Applications

Real-time PCR has been used in oil biodegradation studies mainly in the quantification of functional genes that are involved in the breakdown of certain hydrocarbons. An assay based on real-time PCR has been developed by Wilson et al. [147] to quantify naphthalene dioxygenase genes that are involved in catabolizing naphthalene. The method was applied to ground water microorganisms capable of degrading naphthalene at a coal tar waste-contaminated site. Several primer sets were designed to cover the entire diversity of *nahAc* genes and were used in a combination with a digoxigenin-labeled probe mixture. The obtained PCR products were cloned and sequenced in order to obtain knowledge of the diversity of these genes. The results showed that the diversity of these genes as revealed by real-time PCR is much higher than that detected by culture-based approaches. Sequences showed two major groups related to dioxygenase genes *ndoB* and *dntAc* known previously from *Pseudomonas* and *Burkholderia* strains. In a more recent study, Baldwin et al. [148] developed a real-time assay to quantify aromatic oxygenase genes including naphthalene dioxygenase, biphenyl dioxygenase, toluene dioxygenase, xylene monoxygenase, phenol monoxygenase and ring-hydroxylating toluene monoxygenase genes. Such assays enable researchers to monitor in situ biodegradation of petroleum compounds by indirectly monitoring the activity of degrading bacteria through their genes, which are involved in the degradation process. Impact of remediation technologies on the indigenous microbial population could also be monitored using these assays.

Real-time PCR was also used to quantify catabolic genes involved in anaerobic degradation of petroleum compounds [149]. The assay was based on the *bssA* gene that is associated with the first step of anaerobic toluene and xylene degradation and codes for the α-subunit of benzylsuccinate synthase. The method had a detection limit of about five gene copies and could detect genes within a range of copy numbers of up to seven orders of magnitude. The method was used to monitor the indigenous toluene-degrading bacteria in microcosms inoculated with aquifer sediments and incubated with BTEX (benzene, toluene, ethylbenzene and xylenes) and nitrate. The results showed a good correlation between the extent of toluene degradation and number of *bssA* copies. The number of the *bssA* copies increased from 100- to 1000-fold over the time period during which most of the toluene was degraded.

9.1.3 Advantages and Disadvantages

Real-time PCR is a powerful technique to accurately quantify genes involved in biodegradation processes. Among the advantages of this technique is the

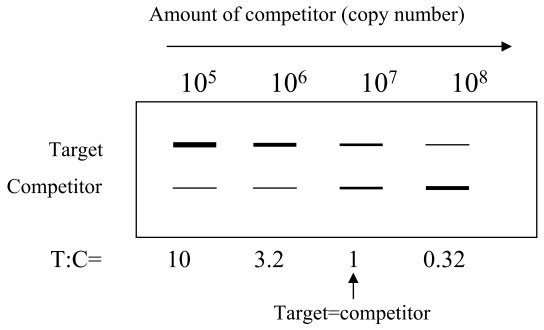
ability to monitor the reaction as it progresses. Minimal amounts of nucleic acid can be amplified and the end product can be accurately quantified. Moreover, there is no need for post-PCR processing (electrophoretical separation of amplified DNA), which saves resources and time. Real-time assays are easy to perform and have high sensitivity (detection is capable down to a two-fold change).

The application of real-time PCR requires sequence information to enable the design of primers and probes. Before designing a real-time quantification assay, extensive optimization is required.

9.2 Competitive PCR

9.2.1 Method

Competitive PCR is also used for the quantification of different genes. The technique is based on the addition of a known amount of a competitor (i.e. a DNA fragment) (Fig. 14). The competitor should be amplified by the same primers used in the amplification of the target and should have different size or restriction fragment pattern so that it can be easily distinguished from the target DNA. Amplification of the target DNA and the competitor in the reaction mixture will lead to a competition of both templates for the same set of primers. Because of this competition, the ratio of the amounts of the two amplified products will reflect the ratio of the amounts of the target DNA and competitor. Since the amount of the competitor is known, the amount of the target DNA could be estimated from the T : C ratio (the amount of amplified



T: amount of amplified product from DNA or RNA
C: amount of amplified product from competitor

Fig. 14 Principle of quantification using competitive PCR. Quantification is performed using a pre-quantified competitor in the PCR mixture

product of the target DNA or RNA [T] vs. competitor [C]). When the T : C ratio equals 1, then the amount of the target DNA or RNA will be equal to the competitor concentration.

9.2.2 Applications

Competitive PCR has been used to quantify genes coding for catechol 2,3-dioxygenase enzymes that are involved in at least one pathway of the degradation of benzene, toluene, xylenes, phenol, naphthalene and biphenyl [150]. The technique has been successfully applied to monitor the increase in catechol 2,3-dioxygenase genes in petroleum-amended soils. Similarly Laurie et al. [151], used this technique to quantify *phnAc* and *nahAc* genes in contaminated soils in New Zealand. Competitive PCR showed that the *phn* genotype had a greater ecological significance than the *nah*-like genotype, which was claimed to be dominant in the field as revealed by culture-based techniques.

In bioremediation studies, competitive PCR was applied to monitor the development and metabolic activity of bacterial communities following specific treatment. For example, Ka et al. [152] used this technique to follow the number and metabolic response of microbial communities of Arctic soil after biostimulation with fertilizers. Quantification was performed using universal bacterial primers and was applied to DNA and RNA extractions. The copy number of 16S rDNA was relatively stable during the treatment whereas the copy number of 16S rRNA increased substantially. This suggested that the treatment did not influence the bacterial number but instead led to an increase in the overall metabolic activity of existing bacteria. Tartakovsky et al. [153] used competitive PCR to follow the development of *Desulfitobacterium frappieri* PCP-1 bacteria used in the augmentation of an anaerobic bed reactor. This strain was capable of degrading pentachlorophenol. The abundance of this strain increased from 10^6 to 10^{10} cells/g of volatile suspended solids within 70 days. The increase in bacterial abundance was accompanied with increased pentachlorophenol degradation efficiency.

9.2.3 Advantages and Disadvantages

The competitive PCR technique, like real-time PCR has been mainly used for quantification of functional genes. However, real-time PCR is more sensitive and accurate. In competitive PCR, quantification is relative to the competitor, which has to be carefully designed and accurately prequantified. The results of competitive PCR can be affected by the PCR protocol itself. Inhibition can occur because of the high concentrations of non-amplifying DNA relative to target DNA or due to the contamination with humic acids and organics.

9.3 In Situ PCR

In situ PCR, as the name implies, is the amplification and the detection of target genes inside individual bacterial cells (Fig. 15). This starts with subjecting the bacterial cells to a permeabilization step using enzymes such as lysozyme and proteinase K. This step is crucial in order to allow penetration of the PCR reagents into the cells. Fluorescently labeled dNTPs are used, which facilitate the detection of the amplified target genes. The advantage of the in situ PCR technique is that it enables the detection of functional genes that are present in low copy number. These genes are often not detectable by in situ hybridization (FISH). The application of this technique in biodegradation studies has been limited in spite of its potential to detect various catabolic genes, which are involved in biodegradation steps. The reason for this might be the inconsistency of the results, which could be attributed to biological as well as instrumental variables. This tech-

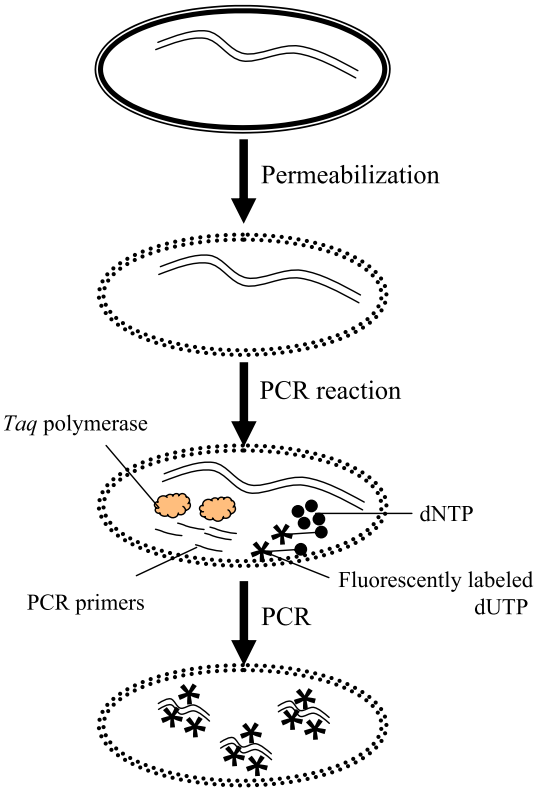


Fig. 15 Principle of in situ PCR

nique, when combined with in situ reverse transcription, can be used to monitor the expression of genes in response to changes in environmental conditions. For example, Chen et al. [154] used this technique to detect the expression of *todC1* gene in *Pseudomonas putida* upon exposure to toluene vapor.

9.4 MPN-PCR

9.4.1 Method

This technique is used to quantify genes based on the most probable number concept. This concept depends on the assumption that elements to be quantified must be in solution, randomly distributed and do not form clusters and one single copy of these elements is detectable. The quantification using MPN-PCR starts with the DNA extraction directly from the sample. The obtained DNA is purified, serially diluted and PCR is carried out with three replicates for each dilution. The PCR products are checked on an electrophoresis gel. The most probable number of the target gene is estimated from the number of positive and negative amplifications in various triplicates using the published MPN tables. The total number of bacterial cells per gram of sample could subsequently be calculated.

9.4.2 Applications

MPN-PCR has been used to estimate the abundance of oil-degrading bacteria. Thomassin-Lacroix et al. [50] used this technique to study the abundance of three phylotypes belonging to the genera *Rhodococcus*, *Sphingomonas* and *Pseudomonas* and forming a hydrocarbon-degrading consortium enriched from fuel-polluted soils. The results showed a change in the relative abundance of the three phylotypes during long-term incubation of the consortium. In another study, Leung et al. [155] used MPN-PCR to quantify the pentachlorophenol-degrading *Sphingomonas* sp. UG30 in soils. They used the tetrachlorohydroquinone reductive degalogenase gene (pcpC) as the target gene. With this technique, they had a detection limit of 3 CFU/g dry soil. In bioremediation studies, MPN-PCR has also been used. Chandler et al. [156] used the technique to assess the degradative potential at a jet fuel contaminated site undergoing bioremediation. The target genes were *nahAc*, *alkB* and *xylE*. All previous studies showed the potential of MPN-PCR in quantifying genes relevant to biodegradation.

9.4.3
Advantages and Disadvantages

MPN-PCR is an easy quantification method, which does not require a standard like in competitive PCR or special instrumentation like in real-time PCR. However, the quantification based on MPN-PCR encounter many problems, particularly when applied to environmental samples. This is due to the uneven distribution of bacteria and their tendency to aggregate, which makes it difficult to obtain homogenous suspensions. In addition, known biases associated with conventional PCR may render quantification based on MPN-PCR unreliable.

10
Activity Techniques

Relating identity of microorganisms to their specific roles in the field has been one of the challenging topics in environmental microbiology. The previously described techniques dealt mainly with the identity of bacterial communities without estimating how important they are in the field and what role they are playing there. Therefore, there is a need to design techniques, which contribute to filling this gap. Recently, a few techniques have been described for that purpose but their application to environmental samples still encounters enormous problems. The application of such techniques to contaminated sediments has been very restricted as a result of the complex nature of contaminants and the high diversity of microbial communities. Herein, examples of these techniques will be described and their potential for biodegradation and bioremediation studies will be illustrated.

10.1
Microautoradiography Combined with in situ Hybridization (MAR-FISH)

10.1.1
Method

This technique is based on the simultaneous detection of microbial populations that assimilate a certain radioactively labeled substrate [157, 158]. The detection of bacteria is performed by applying FISH (fluorescent in situ hybridization) whereas the substrate incorporation is followed by microautoradiography. Both techniques are combined on a single slide. The MAR-FISH technique can be used to quantify subgroups in the community and to find out which bacteria are actively involved in substrate uptake.

10.1.2 Applications

MAR-FISH was used to follow the degradation of ^{14}C-labeled *o*-nitrophenol in a coculture composed of *Pseudomonas putida* B2 and *Sphingomonas stygia* [159]. The technique allowed the identification of the bacterium involved in degradation of *o*-nitrophenol. Microautoradiography was also used by Francisco et al. [160] to monitor the microbial activity of *Candida albicans* and *Pseudomonas aeruginosa* in an oil-polluted tropical bay on the Atlantic Ocean.

10.1.3 Advantages and Disadvantages

MAR-FISH has been successfully applied to well-defined systems and bacterial isolates and helped in the detection of bacteria that are involved in the uptake of certain substrates under in situ conditions. However, the application of MAR-FISH to environmental samples in general and to oil-polluted ones in particular has encountered several problems including high background and unspecificity. A limiting factor in applying this technique in biodegradation studies is the high cost of radioactively labeled petroleum compounds and the difficulty to label all carbon atoms of the compound of interest. Thus, more studies have been performed with simple organic compounds compared to oil components. MAR-FISH fails to provide quantitative data on the uptake rates of the studied substrate.

10.1.4 Other Techniques

Continuous attempts are being made to develop new techniques for linking bacterial identity to their in situ activities. This task is not simple since environmental samples contain an enormous number of physiologically diverse bacteria. Degradation of petroleum compounds is not necessarily performed by a single bacterium but rather by consortia. Furthermore, the intermediate compounds produced from degradation of a single compound by certain bacteria may be utilized by another. Recently, new techniques have been developed to facilitate linking identity to activity but they still await application in oil biodegradation studies. Among these techniques is the identification of bacteria that respond to specified stimuli by targeting the incorporation of bromodeoxyuridine (BrdU), a thymidine nucleotide analog, into newly synthesized DNA [161, 162]. This is achieved by incubating the environmental sample with bromodeoxyuridine and the target compound, followed by immunocapture of the BrdU-labeled DNA. Subsequently, this DNA is subjected to molecular analysis and phylogenetic identification of the active bacteria

during the treatment. Recently, this technique has been coupled with in situ hybridization (CARD-FISH) in order to allow simultaneous microscopic identification of individual DNA-synthesizing populations [163].

Radioactive and stable isotopes have contributed significantly to relating activity to identity of specific microbial populations. This was accomplished by following the incorporation of ^{13}C or ^{14}C into specific biomarkers such as phospholipid fatty acids (PFLA) or nucleic acids (RNA and DNA). Stable isotopes are preferred over radioactive ones due to their easy handling and safety, thus receiving enormous applications in the field of microbial ecology [164]. Several studies were performed using a ^{13}C isotope label with the purpose of identifying toluene-degrading bacterial populations. For example, Hanson et al. [165] found out that 27% of the PFLAs extracted from (^{13}C) toluene pre-incubated soil were labeled compared to 91% upon incubation with (^{13}C) glucose. The majority of labeled PFLAs obtained from the sediment on toluene were identical to those contained in a toluene-metabolizing bacterium isolated from the same sediment. Using the same approach, the anaerobic toluene metabolizing population in an oil-contaminated aquifer was identified [166]. The PFLA profiles suggested that this population contains the *β-Proteobacteria Azoarcus* spp. and related species. A new variation of this technique based on the incorporation of ^{13}C into the DNA of microrganisms when grown on ^{13}C-enriched carbon substrate was developed [167]. The ^{13}C-DNA is separated from ^{12}C by density-gradient centrifugation. The resolved ^{13}C-DNA can then be subjected to gene probing and sequence analysis in order to identify microorganisms involved in the utilization of the labeled compound. This technique has been used to identify methanol-utilizing bacteria in soil [167]. It potentially allows the identification of bacteria responsible for biodegradation processes. Nevertheless, such application has not been so far demonstrated. In a similar fashion, the incorporation of ^{14}C into the RNA of microorganisms could be followed (Abed et al., unpublished). Initial experiments demonstrated that the incorporated ^{14}C is detectable. This approach had several problems, in addition to the difficulty of working with radioactive substances. The concentrations of isolated RNA are small and the work with these low concentrations without PCR amplification results in the loss of the radioactive label. The separation of labeled from unlabeled RNAs is a serious challenge in using this technique. These problems render the ^{13}C isotope more suitable for identification of bacterial communities that play a vital role in metabolic processes.

11 Conclusions and Future Prospects

The use of molecular techniques in the context of biodegradation and bioremediation studies has enriched significantly our knowledge of bacterial di-

versity in polluted sites. Furthermore, additional insights into the diversity of functional genes that are actively involved in biodegradation processes and their expression have been gained. One of the major achievements attributed to molecular techniques is the ability to demonstrate that most bacteria degrading pollutants under in situ conditions are different to those obtained by enrichment cultivation. This finding has directed us to a new diversity of oil degraders that was not known before. However, it remains a challenge to isolate these bacteria in order to allow detailed physiological characterization. Molecular techniques also provided monitoring tools for bioremediation treatments and changes associated with them. This includes the response of indigenous bacterial communities to addition of inorganic and organic fertilizers and the fate of added consortia to polluted soils. The empirical knowledge obtained from these monitoring programs has helped in estimating the optimal environmental conditions, which ensure high bacterial biodegradation activities, thus facilitating the design of more efficient bioremediation strategies.

The selection of the appropriate molecular technique depends on the question being addressed. For example, quantification techniques such as blot hybridization and analytical PCR techniques enable studies on the abundance and distribution of certain functional genes. For studies of bacterial diversity, techniques like 16S rRNA clone libraries, DGGE, T-RFLP, FISH, SSCP and ARDRA are suitable. Clone libraries and DGGE have the advantage of providing sequence information of community members. However, it should be kept in mind that there is no single molecular technique available today that can target and describe the total diversity of microbial communities. Each technique is associated with certain limitations and biases that could be introduced at each step of the procedure. Molecular techniques do not provide complete information on the physiology of microorganisms, which is better studied on isolated strains. Thereby, it is clear that basic microbiological techniques provide information that modern molecular ones do not. Therefore, scientists should adopt a polyphasic approach that combines molecular and basic microbiological techniques to study microbial communities.

In the last century, biodegradation studies focused mainly on enrichment, isolation and physiological characterization of oil-degrading bacteria. This research was further extended to phylogenetic sequence analysis of 16S rRNA genes and short fragments of other functional genes. Molecular techniques have enabled scientists in this field to realize the great extent of microbial diversity in contaminated systems and to generate an unlimited number of 16S rRNA sequences. This has resulted in the identification of a large diversity of yet-uncultured oil-degrading bacteria. We know almost nothing about the physiological capabilities and roles of these bacteria, which are only identified by their 16S rRNA sequences, in the field and the interaction among different bacteria. One way around this problem is to extrapolate the information available from related species. However, it is well known that closely related

species based on 16S rRNA phylogeny may have tremendous differences in genome composition and physiological criteria. An alternative approach to solve this problem is the emerging field of environmental genomics, otherwise known as metagenomics. Genomic libraries are constructed by inserting large DNA fragments (size is up to 120 kb) obtained directly from the environment into vectors such as BAC (bacterial artificial chromosome) and fosmids. This approach has contributed significantly to the understanding of how bacterial communities control various metabolic processes. Furthermore, the use of BACs has allowed for the identification of novel metabolic processes and recognition of genetic potential and probably lifestyle of yet uncultured target microorganisms. The implementation of this method in the future biodegradation-related research may open a new horizon in revealing many interesting degradation enzymes, pathways and proteins involved in the degradation processes. However, a major challenge in working with environmental samples is the successful assembly of all sequence data so that a large contiguous piece of DNA with operons or links to phylogenetic markers can be generated.

In recent years, sequencing technologies have improved significantly and sequencing of whole microbial genomes has become common. Handling and assembly of generated sequences have become realistic and easier with the help of computers. Many databases are currently available, facilitating the process of identification of ORFs (open reading frames) and annotation of genes. With detailed bioinformatics analysis, many biochemical pathways and the metabolic capacity of the studied microorganism could be identified. This approach provides a huge amount of information on the potential physiology of the studied bacteria and their likely role in the environment. In the last few years, several international projects have focused on obtaining the complete genome sequence of diverse bacteria that occupy a range of environmental niches, and that are responsible for an array of environmental processes. Future research on oil-degrading bacteria should consider this option and be directed towards obtaining the full genome sequence of important degraders. Genome sequencing should particularly focus on extremophiles due to their ecological as well as biotechnological significance. Extremophiles with the ability to degrade petroleum compounds at extreme environmental conditions have an intriguing physiology and contain interesting enzymes, which could be exploited for industrial applications. Computer analysis of the genome sequence could guide us to new operons or novel degradation pathways, the enzymes involved, and even how the different processes are regulated. In this context, Wackett and his colleagues from the University of Minnesota have established an interactive web page (http://umbbd.ahc.umn.edu), which deals with every aspect of biodegradation pathways and operons [168]. This web page also provides a prediction system, mainly used in the annotation of genome sequences and the prediction of the fate of chemicals in the environment [169].

Regardless of the advancements made in the field of genomics and bioinformatics, it has not been possible to know the function of all genes obtained after sequencing the whole genome of a single bacterium. It has been estimated that almost 40% of sequence information in entire genomes remain hypothetical. This requires further proteomics analysis in order to study the expression of genes and protein–protein interactions. Two-dimensional protein electrophoresis and microarrays are becoming standard tools for investigation of gene expression under different conditions and the presence or absence of genes in different species. Our success in assigning functions to unknown genes would make the annotation process in the future much easier. This emerging field of genomics and proteomics carries a strong potential for the future research in the field of environmental microbiology and its application to contaminated ecosystems is expected to result in many breakthroughs.

The molecular techniques described herein provide researchers with many tools suitable to study several aspects related to mixed complex bacterial communities in contaminated environments. There are continuous efforts to improve these techniques and to develop new ones. The applications of these techniques in biodegradation studies are tremendous; however some techniques have not been sufficiently implemented. Studies on the diversity of bacteria in contaminated sites are numerous whereas little research has been performed to illustrate the in situ activity of degrading bacteria. New developments of activity techniques are required even though this task poses a real challenge. More information on the interaction between bacteria in the degradation processes is needed. Our vision is that genome sequencing will contribute significantly to our knowledge about the metabolic capabilities of oil-degrading bacteria.

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Steady-State Model of Chemical Migration in a Sediment Cap

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Abstract Capping with clean media represents one of the few available means of reducing exposure and risk to contaminated sediments in situ. Effective design of a chemical isolation cap layer requires modeling tools capable of describing the operative processes in a cap and the resulting chemical fate and transport processes. An analytical model developed by the lead author is commonly used for this purpose but is limited in the types of capping scenarios that can be evaluated. In particular, the standard model cannot deal with multiple layers in the cap with different transport rates and sorption coefficients. This is especially important in estimating surficial sediment concentrations in the presence of bioturbation. Additional analytical and numerical models are described that can relax the assumptions of the existing model. The models are compared and recommendations are provided to guide model usage for various cap simulation needs. A simple model employing conservative steady-state assumptions is proposed specifically for the case of predicting long-term containment by a cap and surficial sediment concentrations. The simple model is compared to more sophisticated numerical models that can deal with a wide range of conditions.

Keywords Bioturbation · Containment · Flux · In situ sediment capping · Steady-state model

1 Introduction

Capping with clean materials is one of the few methods of managing contaminated sediments in situ. Caps can be used to improve substrate diversity and quality to enhance the habitat value of the surficial sediments, or used to change the fundamental character of a water body by creating emergent wetlands or additional land area (e.g., a port expansion). The primary purposes of a cap over contaminated sediments, however, are to:

1. Armor contaminated sediments to ensure they are not resuspended in high flow conditions
2. Physically isolate contaminated sediments from benthic organisms that typically populate only the upper few centimeters of sediments
3. Provide a mass transfer resistance to advective and diffusive processes that encourage chemical release from the sediments

Because most sediment contaminants are highly sorptive, their migration through a cap is retarded due to transient accumulation on the clean cap material. Thus, the time for typical sediment contaminants to migrate through a cap can be hundreds or thousands of years if the cap is designed and maintained to retain its integrity throughout that period. A model of chemical fate and transport is typically used to evaluate the long-term effectiveness of a cap as defined by its ability to provide chemical isolation in a subaqueous environment [1]. These models assume that the cap is armored such that erosion of the cap does not provide the primary means of contaminant migration. In addition, the biologically active zone in which contaminants are transported by organism reworking is assumed to be confined to a small layer above the chemical isolation layer. The primary means of contaminant transport within the chemical isolation layer are the physicochemical processes of advection and diffusion in the porewater. The driving force for chemical transport is the contaminant concentration within the porewater of the underlying sediment. As a conservative estimate of the chemical flux through a capping layer, it is normally assumed that the contaminant concentration in the overlying water is effectively zero, which maximizes the driving force for chemical diffusion. In addition, the concentration in the underlying sediment is assumed constant, without degradation or reduction due to chemical migration out of the sediments.

The standard method of estimating chemical migration in a cap is via a transient advection–diffusion model as described by Palermo et al. [1]. This model is applied to the chemical isolation layer of a cap, which is the cap thickness after removing components for porewater expression via consolidation of underlying sediment, consolidation of the cap, and bioturbation of the upper cap layers. Normally, an analytical solution to the mass conservation equation, assuming that the cap is semi-infinite, is employed in such an

analysis. Such a model is, in general, not applicable after the conditions at the top of the cap (such as benthic activity or changing organic content) influence contaminant behavior. This is a serious limitation in that the protectiveness of a cap is largely defined by the contaminant behavior in the biologically active zone. This zone is subject to significantly different transport processes and rates than in the underlying cap layer and may exhibit significantly different physical and chemical characteristics, such as increased organic carbon content and sharp gradients in reduction–oxidation conditions. When the upper boundary of the cap begins to influence the contaminant migration, a numerical model is normally required to describe transient behavior. An alternative approach is to consider only steady-state conditions, in which it is possible to consider the complexities of the upper boundary and still employ relatively simple analytical solutions to the chemical transport equations. The estimation of flux through a cap at steady state is conservative as the contaminant flux is a maximum at steady state. To further ensure conservatism, the concentration beneath the cap layer (that is, in the underlying contaminated sediment) is assumed to maintain a constant concentration with no depletion due to chemical reactivity or migration into the cap. This maximum flux can then be used to estimate concentrations in the biologically active layer. Chemical reactivity in the cap or biologically active layer can be incorporated for appropriate compounds either by employing measured rates of degradation or by considering conservative estimates from the literature. Through use of the steady-state design approach, it is possible to estimate the maximum contaminant concentration that may ever be achieved within the biologically active zone. The presumption is that if predicted contaminant concentrations under steady-state conditions do not exceed levels of concern or excessive risk, then a cap would be a protective management approach for the contaminated sediments. The goal of this paper is to develop a simple and easy to use steady-state model based upon this framework that can be used to make conservative estimates of a cap's protectiveness at long times.

If a feasible cap is not deemed sufficiently protective by such an analysis, the assumptions can be relaxed and a more sophisticated analysis (e.g., numerical solution to the transport equations) employed to better estimate the effectiveness of the cap. As indicated above, a significant source of conservatism in the modeling framework outlined is the assumption that the concentration beneath a cap remains constant for all time, reduced neither by chemical reactions nor by the flux into and through the cap. A simple indication of the appropriateness of assuming constant underlying conditions can be made by evaluating the time required to achieve steady state or the time required before the contaminant migrates through the entire capping layer. Due to sorption onto the capping materials for many sediment contaminants, this time may be very long, and the mass accumulated in a cap under steady-state conditions may be large compared to the total mass of contaminants in the underlying sediment. Thus, the calculated steady state may never

be achieved due to fate or dilution processes influencing the contaminant in the underlying sediment. Contaminants that reach steady-state conditions relatively rapidly are reasonably well described by the model, but the model would likely over predict concentrations and fluxes for contaminants requiring very long times to achieve steady state.

2 Model

2.1 Driving Force for Chemical Migration

The driving force for chemical migration through a cap, the concentration of contaminant in the porewater of the underlying sediments, can be measured via porewater sample collection or estimated from sediment data through the development of a site- and contaminant-specific partition coefficient. This partition coefficient can then be used to estimate porewater concentrations in locations where porewater data are not available. The site-specific partition coefficient between solid and water matrices, K_d^{obs}, can be estimated through use of the relationships

$$K_d^{obs} = \left(\frac{W_s}{C_{pw}}\right)_{known} \qquad C_0 = \frac{W_s}{K_d^{obs}}, \tag{1}$$

where C_0 is the contaminant concentration in the porewater in the sediment underlying a cap and W_s and C_{pw} are known values of total sediment concentration (e.g., in mg/kg) and porewater concentration (e.g., in mg/l), respectively. Typically, the observed partition coefficient is estimated from a few simultaneous measurements sediment and porewater concentration and then used to estimate contaminant concentration elsewhere from measured sediment concentration.

For organic contaminants, the contaminant partition coefficient in the cap can be estimated by

$$K_d^{cap} = K_{oc} f_{oc}, \tag{2}$$

where K_{oc} is the organic carbon based partition coefficient, a tabulated compound-specific measure of hydrophobicity, and f_{oc} is the fraction of organic carbon in the cap materials which tends to be the dominant location for contaminant sorption. For sand, the organic carbon fraction tends to be small, on the order of 0.1%. At these low organic carbon contents, mineral sorption of even organic contaminants tends to become important, so the assumption of 0.1% organic carbon is likely a lower bound to the effective sorption of organic contaminants on cap materials.

2.2 Effective Cap Thickness

The various layers in a cap are shown in Fig. 1. The effective cap thickness, h_{cap}, is given by

$$h_{cap} = h_0 - \frac{\Delta h_{sed}}{\varepsilon R_f} - \Delta h_{cap} - h_{bio}\,, \tag{3}$$

where h_0 is the initially placed cap thickness, Δh_{sed} is the consolidation distance in the underlying sediment, Δh_{cap} is the consolidation thickness of the capping material itself, and h_{bio} is the layer influenced by bioturbation by benthic organisms. The product εR_f, or porosity times the retardation factor, is the ratio of the total contaminant in an elementary volume of the cap material to the contaminant in the mobile phase (the porewater volume). Its role in Eq. 3 is to emphasize that the mass of contaminant expressed by sediment consolidation is both sorbed onto the cap material as well as mobile in the porewater. The net effect is to reduce the contaminant migration into the cap relative to the porewater volume expressed. This same effect slows the rate of chemical migration through the cap after consolidation. The retardation factor is estimated by the relationship

$$R_f = 1 + \frac{\rho_b K_d^{cap}}{\varepsilon}\,. \tag{4}$$

Here ε is the porosity of the cap layer (typically about 40% for unconsolidated sand), ρ_b is the bulk (dry) density of the cap layer (typically about 1.3–1.5 g/cm^3 for sand), and K_d^{cap} is the contaminant partition coefficient in the capping materials.

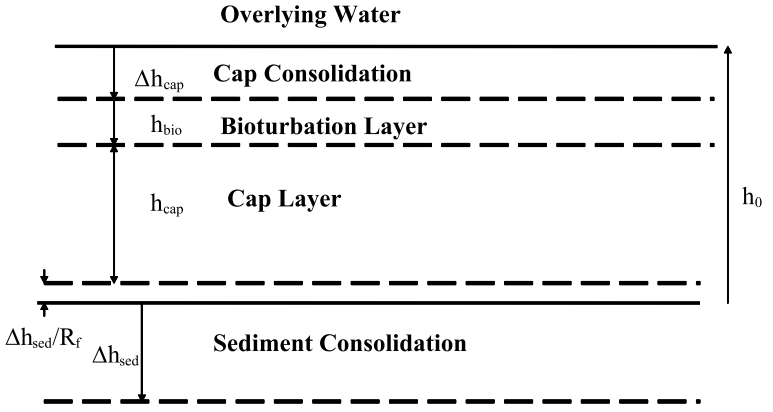

Fig. 1 Conceptual model of a sediment cap

Consolidation occurs relatively quickly (over a period of months to years), essentially instantaneously compared to the design lifetime of a cap. The modeling conducted here assumes that any porewater expression of contaminants leads to an instantaneous reduction of effective cap thickness, as indicated by Eq. 3. While appropriate for the evaluation of the dynamics of contaminant migration, this phenomenon results only in transient effects on cap performance. Any short-term chemical migration due to consolidation of the underlying sediment will be overcome by the long-term chemical migration characteristics of a cap. Thus, for steady-state migration through a cap, the effective cap thickness in Eq. 3 is replaced with

$$h_{\text{cap}} = h_0 - \Delta h_{\text{cap}} - h_{\text{bio}} \,. \tag{5}$$

Although consolidation of the cap is accounted for in Eq. 5, it is minimal with most capping materials such as sand and is neglected hereafter.

2.3 Advection–Diffusion Model of Transport in a Cap

Within the effective chemical isolation thickness of a cap, as defined by Eq. 4 under transient conditions or Eq. 5 under steady-state conditions, the chemical migration processes are limited to advection and diffusion (that is, no significant bioturbation or erosion). The dynamics of the chemical migration behavior within this layer can be estimated by the advection–diffusion equation. Traditionally, the cap is often approximated as semi-infinite and the transient behavior estimated using an analytical solution of the advection–diffusion equation [1]. The approach can be extended to reactive contaminants using the solution of van Genuchten [2]

$$\begin{aligned} C = C_{\text{i}} \exp\left(-\frac{\lambda t}{R_{\text{f}}}\right) \left\{1 - \frac{1}{2}\text{erfc}\left[\frac{R_{\text{f}} z - vt}{2\sqrt{D' R_{\text{f}} t}}\right] - \frac{1}{2}\exp\left(\frac{vz}{D'}\right)\text{erfc}\left[\frac{R_{\text{f}} z + vt}{2\sqrt{D' R_{\text{f}} t}}\right]\right\} \\ + C_0 \left\{\frac{1}{2}\exp\left[\frac{(v-u)z}{2D'}\right]\text{erfc}\left[\frac{R_{\text{f}} z - ut}{2\sqrt{D' R_{\text{f}} t}}\right] \right. \\ \left. + \frac{1}{2}\exp\left[\frac{(v+u)z}{2D'}\right]\text{erfc}\left[\frac{R_{\text{f}} z + ut}{2\sqrt{D' R_{\text{f}} t}}\right]\right\} \end{aligned} \tag{6}$$

where:

C_{i} = initial contaminant concentration in the cap (assumed to be zero)

C_0 = contaminant concentration in the underlying porewater (e.g., mg/l)

λ = reaction term or biodegradation rate (e.g., /year)
z = vertical distance in the cap (chemical isolation layer thickness, h_{cap}) (e.g., cm)
v = porewater velocity (e.g., cm/year)
t = time (e.g., year)
D' = effective diffusion/dispersion through porous media (e.g., cm^2/year)
$u = \sqrt{v^2 + 4\lambda D'}$

and:

$$D' = av + \frac{D_w}{\varepsilon^{-1/3}},$$

where:

a = dispersivity (e.g., cm)
D_w = molecular diffusion of chemical species in water (e.g., cm^2/year)

and:

$\varepsilon^{-1/3}$ = hindrance parameter

The solutions given by Palermo et al. [1] or van Genuchten [2], however, assume a semi-infinite cap layer and, as indicated earlier, cannot account for changes in conditions in the near surface cap layer, for example, due to changes in organic carbon content in the surface layer or changes in rate of migration due to bioturbation or other processes. They can be used to estimate concentrations within the cap underlying the biologically active zone during the transient period. They are also limited to constant concentration in the underlying sediment, which, as indicated previously, can be a significant source of conservatism. The analytical models of Choy and Reible [3] allow variable conditions in space or time for both initial concentrations and transport coefficients, but these models are generally limited to diffusion and cannot address the important transport mechanism of advection.

The semi-infinite models of Palermo [1] or van Genuchten [2] are accurate predictions of contaminant migration and resulting concentrations only until near steady-state conditions are reached and the influence of the conditions at the upper boundary can no longer be ignored. The time required to achieve steady state can be estimated from the relationships below. A separate relationship is provided for advectively dominated transport and diffusion

dominated transport:

$$\tau_{ss,adv} = \frac{R_f h_{cap}}{U}, \qquad (7)$$
$$\tau_{ss,diff} = 3.69 \frac{h_{cap}^2 R_f}{\pi^2 D'}.$$

For times approaching or exceeding these times under either advectively dominated or diffusion dominated conditions, a more complete model that includes the transport processes at the upper boundary is necessary to accurately predict fluxes and contaminant concentrations. Typically, a numerical solution is necessary but it is also possible to take a conservative approach and develop an analytical solution for the case of steady-state behavior. This model is discussed in more detail below.

2.4 Analytical Steady-State Model of Transport in a Cap

Advection and diffusion in the cap materials are subject to retardation by transient sorption as is contaminant migration in groundwater. Under steady conditions, sorption does not influence the flux of contaminants through the cap materials. Thus, the steady-state fluxes (rates per unit area) of diffusion and advection in the cap are given simply by

$$\text{Flux}_{adv} = UC_0, \qquad \text{Flux}_{diff} = \frac{D}{h_{cap}}\left(C_0 - C_{bio}\right). \qquad (8)$$

Here, U represents the superficial or Darcy velocity of the groundwater flow through the cap ($U = \varepsilon v$), D represents the effective diffusion (i.e., D' includes dispersion but not D per earlier convention) coefficient in the cap, and C_{bio} represents the porewater concentration within the bioturbation layer (C_{bio}, as well as other location-specific parameters discussed in the following sections are depicted in Fig. 2).

For diffusion dominated processes, D is the molecular diffusivity in water of the contaminant of interest multiplied by the porosity and divided by the tortuosity (or hindrance factor, H_p) of the sediment ($D = D_w\varepsilon/H_p$). The Millington and Quirk model (as referenced in Palermo [1]) suggests $H_p \sim \varepsilon^{-1/3}$, which for a cap with 40% porosity is about 1.4. The molecular diffusion coefficient in water is a function of temperature and can be estimated by the methods defined by Lyman [4]. In the presence of advection D is increased due to mechanical dispersion in the medium (typically modeled as some dispersivity, α, multiplied by the interstitial velocity, v). The dispersivity is an empirical parameter but is related to the mean particle size in nearly

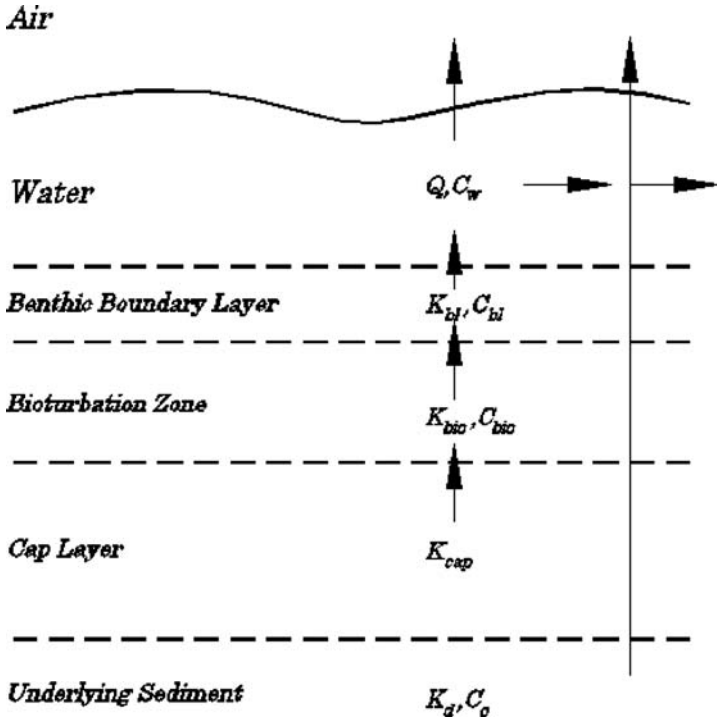

Fig. 2 Contaminant migration layers and associated notation

uniform sand beds:

$$D = \frac{D_w \varepsilon}{H_p}, \qquad D' = \frac{D}{\varepsilon} + \alpha v . \tag{9}$$

Bioturbation in the upper layers of a cap can lead to rapid chemical migration. The normal feeding and burrowing activities of benthic organisms result in the rapid movement of particles and the contaminants with which they are associated, as well as the movement of porewater. The layer that is effectively mixed by organisms, h_{bio}, is relatively small. More than 90% of the 240 observations of bioturbation mixing depths in fresh and salt water reported by Thoms [5] were 15 cm or less, and more than 80% were 10 cm or less. In freshwater systems, most measurements of mixing depth are of the order of 3–5 cm. Most of these measurements were based upon particle-associated radionuclides and the observed effective particle diffusion coefficients, D^{p}_{bio}. The observed effective particle diffusion coefficients fell within the range of 0.3–30 cm^2 per year more than two-thirds of the time. Thibodeaux [6] also reported a range of particle diffusion coefficients that ranged from 1.4 cm^2/year to more than 470 cm^2/year in all but the deep ocean where effective bioturbation diffusion coefficients were less. As these particles are mixed and trans-

ported by the benthic organisms, sediment contaminants are also transported at an equivalent rate. The flux of contaminants associated with particles in the bioturbation layer can be estimated by

$$\text{Flux}_{\text{bio}}^{\text{p}} = \frac{D_{\text{bio}}^{\text{p}}}{h_{\text{bio}}} \varepsilon R_{\text{f}} \left(C_{\text{bio}} - C_{\text{bl}} \right) , \tag{10}$$

where C_{bl} is the concentration at the cap–water interface at the bottom of the benthic boundary layer of the lake. The factor εR_{f} is the ratio of the total concentration in an elementary sediment volume to that in the porewater. The product of this quantity and the porewater concentration represents the total quantity of contaminants per unit volume of sediments.

The ratio of $D_{\text{bio}}^{\text{p}}/h_{\text{bio}}$ represents an effective mass transfer coefficient, $k_{\text{bio}}^{\text{p}}$. Thoms [5] report 38 measurements in freshwater systems for which this ratio can be calculated. The average value is 130 cm/year and about 60% of the measurements exceed 1 cm/year and 90% exceed 0.1 cm/year. In shallow waters such as would be observed nearshore, Boudreau [7] provided correlations between bioturbation and depth that suggest a 2.54 and 29.5 cm/year effective particle mass transfer coefficient. A value of 1 cm/year appears to be a reasonably conservative estimate of the effective particle bioturbation mass transfer coefficient in a clean shallow system, such as the top of a nearshore sediment cap. A coefficient of the order of 1 cm/year or more has also been observed by Thibodeaux [8].

In addition to particle mixing, organisms also irrigate the surficial sediments through direct porewater exchange with the overlying water. Filter feeders process water for food, while other organisms cause water exchange through simple movement or respiration processes. Thibodeaux [6] and Boudreau [7] reported porewater mixing coefficients associated with the exchange of porewater by benthic organisms. The porewater mixing rate tends to be much higher numerically than particle effective diffusion coefficients, 1000–6000 cm^2/year, but is generally of lesser importance than particle reworking due to the strongly sorbing nature of most sediment contaminants. However, in some locations there are relatively soluble contaminants such as chlorobenzene, where porewater irrigation may be more important:

$$\text{Flux}_{\text{bio}}^{\text{pw}} = \frac{D_{\text{bio}}^{\text{pw}}}{h_{\text{bio}}} \left(C_{\text{bio}} - C_{\text{bl}} \right) . \tag{11}$$

The mass transfer coefficient associated with this process is termed the bioirrigation coefficient and can be defined as $D_{\text{bio}}^{\text{pw}}/h_{\text{bio}} = k_{\text{bio}}^{\text{pw}}$. There are fewer direct measurements of bioirrigation mass transfer coefficients and its estimation is thus subject to greater uncertainty. A reasonably conservative value of this coefficient is 100 cm/year.

C_{bl} in Eqs. 10 and 11 is the concentration at the cap–water interface and this can be related to the flux via a similar equation:

$$\text{Flux}_{bl} = k_{bl}\left(C_{bl} - C_w\right) . \tag{12}$$

Here, k_{bl} is the benthic boundary layer mass transfer coefficient and C_w is the concentration of contaminant in the overlying water. As indicated previously, the maximum flux will be estimated when $C_w = 0$.

Under steady conditions, the fluxes through the chemical isolation layer of the cap, the bioturbation layer, and the benthic boundary layer are all equal. This provides a basis for estimating the steady-state concentrations in each layer as described below.

The flux through the chemical isolation layer of the cap tends to be dominated by either advection or diffusion as indicated by the Peclet number

$$N_{Pe} = \frac{vh_{cap}}{D'} . \tag{13}$$

Here $N_{Pe} > 1$ means that transport in the chemical isolation layer is dominated by advection while $N_{Pe} < 1$ implies that transport is dominated by diffusion. Advection and diffusion in either the cap isolation layer or bioturbation layer are not independent because advection tends to reduce diffusion gradients and diffusion tends to reduce the advective flux. In the cap isolation layer, a reasonable approximation is to assume that the flux is well-estimated by the dominant flux (either advection or diffusion). Solutions to the steady-state transport equations considering both diffusion and advection with and without reaction are feasible, but are algebraically more complicated and deviate significantly from solutions assuming only the dominant process in the relatively narrow range of approximately $0.3 < N_{Pe} < 3$. Even within this range, the dominant process correctly estimates the flux within a factor of 2.

The total flux through the bioturbation layer is the sum of the flux via porewater and particle processes because they act independently. The particle processes are solely the result of bioturbation (characterized by k_{bio}^{p}) in the absence of erosion. Of the potential porewater processes in the bioturbation layer, diffusion tends to be very small compared to bioturbation and will be neglected. In an advection dominated system, however, it is possible that advection remains important relative to porewater irrigation. The porewater processes include those driven by bioturbation (characterized by k_{bio}^{pw}) and advection (driven by hydraulic gradients and characterized by the seepage velocity U), which also act independently of each other, i.e., organisms do not significantly influence advection nor does advection at the rates of a few hundred cm/year (< 1 cm/day) significantly influence the organisms. Thus, the total flux through the bioturbation layer is the sum of all three processes (particle and porewater irrigation by bioturbation and advection).

Similarly, the advective flux at the cap–water interface (vertical seepage into the overlying water) is largely independent of the benthic boundary layer mass transfer coefficient (driven by horizontal velocity shear and turbulence above the surficial sediments). The total flux through this surficial sediment layer is thus the sum of the two processes. The benthic boundary layer mass transfer coefficient is a function of the turbulence and speed of water flow over the surface. A reasonable order of magnitude estimate is 1 cm/h. This rarely controls the overall contaminant release from the sediments and thus the benthic boundary layer concentration tends to be small (if the overlying water concentration is small or assumed zero as here in order to estimate the maximum flux to the overlying water).

Setting the fluxes in the chemical isolation layer, the bioturbation layer, and the overlying benthic boundary layer equal at steady state, the fluxes through the various layers are related by

$$\begin{aligned}\text{Flux} &= \text{Max}\left\langle \text{Flux}_{\text{adv}}, \text{Flux}_{\text{diff}} \right\rangle = \text{Flux}_{\text{bio}}^{\text{p}} + \text{Flux}_{\text{bio}}^{\text{pw}} + \text{Flux}_{\text{adv}} \\ &= \text{Flux}_{\text{bl}} + \text{Flux}_{\text{adv}} \,. \end{aligned} \tag{14}$$

From these relationships and by analogy to Eqs. 19–22 in Appendix B of Palermo et al. [1], the concentration in the bioturbation layer can be estimated. In Appendix B, however, only diffusion and bioturbation by particles were considered. Here the more complicated case is necessary because of the additional operative processes. Rewriting Eq. 14, defining the steady-state flux as the maximum of the diffusive or advective flux in the chemical isolation layer

$$\begin{aligned}\text{Flux} &= \left(k_{\text{bio}}^{\text{p}} \varepsilon R_{\text{f}} + k_{\text{bio}}^{\text{pw}} + U \left[\frac{C_{\text{bio}}}{C_{\text{bio}} - C_{\text{bl}}} \right] \right) (C_{\text{bio}} - C_{\text{bl}}) \\ &= \left(k_{\text{bl}} + U \left[\frac{C_{\text{bl}}}{C_{\text{bl}} - C_{\text{w}}} \right] \right) (C_{\text{bl}} - C_{\text{w}}) \,. \end{aligned} \tag{14a}$$

The concentration in the overlying water, C_{w}, is assumed to be 0 to estimate a maximum flux. Similarly, we will estimate a maximum concentration in the bioturbation layer (subject to zero overlying water concentration) by assuming that $C_{\text{bio}} \gg C_{\text{bl}} \gg C_{\text{w}}$. Then we can write

$$C_{\text{bio}} - C_{\text{bl}} = \frac{\text{Flux}}{k_{\text{bio}}^{\text{p}} \varepsilon R_{\text{f}} + k_{\text{bio}}^{\text{pw}} + U} \,, \qquad C_{\text{bl}} - C_{\text{w}} = \frac{\text{Flux}}{k_{\text{bl}} + U} \,, \tag{14b}$$

$$C_{\text{bio}} - C_{\text{w}} = \frac{\text{Flux}}{k_{\text{bio}}^{\text{p}} \varepsilon R_{\text{f}} + k_{\text{bio}}^{\text{pw}} + U} + \frac{\text{Flux}}{k_{\text{bl}} + U} \tag{14c}$$

and since C_w is approximately 0, the predicted concentration in the bioturbation layer is given by

$$C_{bio} = \text{Flux}\left[\frac{1}{k_{bio}^{p}\varepsilon R_f + k_{bio}^{pw} + U} + \frac{1}{k_{bl} + U}\right] . \quad (15)$$

The solid concentration in the bioturbation layer, which can then be compared to sediment quality standards, is given by

$$W_{bio} = K_d C_{bio} , \quad (16)$$

where K_d is an effective partition coefficient appropriate for the bioturbation layer. If the top layer of the cap constitutes the bioturbation layer, then this may be the effective partition coefficient in the cap. It may be appropriate to have a surficial sediment layer that contains additional organic carbon or has other characteristics different from those of the cap. For the purposes of this evaluation, it was assumed that the bioturbation layer contains additional organic carbon. Initially the surface layer would have the same organic carbon content as the bulk of the cap, i.e., 0.1%. Over time, the deposition of fresh organic material may increase the surficial cap layer to the order of that of existing sediments, e.g., of the order of 1% organic carbon. A major advantage of the proposed steady-state model is that the differing conditions that may occur in the surface layer of the cap can be still be explicitly included in a simple, analytical framework.

2.5
Steady-State Model for Reactive Contaminants

For degrading contaminants, the flux through the cap and to the bioturbation layer is reduced. The degradation rate of a contaminant is related to its half-life in the environment

$$\lambda_{=}\frac{\ln(2)}{\tau_{1/2}} . \quad (17)$$

Half-lives that are reported for most compounds represent reactivity in the media of concern. Half-lives reported in soils or sediments represent the time required for the soil or sediment concentration to be halved. For the purposes of this model it is assumed that the half-lives apply to the porewater concentrations of the contaminant and local equilibrium between solid and porewater is assumed. This assumption neglects any additional reaction that occurs in the solid phase or slow desorption into the porewater that may slow the overall degradation rate.

2.6 Advectively Dominated Conditions

Under advectively dominated conditions, the contaminant degrades according to its residence time in the cap, h_{cap}/v, where v is the interstitial velocity in the cap (U/ε). The concentration at the top of the isolation layer (but not necessarily the bottom of the bioturbation layer) is given by

$$C = C_0 e^{-\frac{\lambda h_{cap}}{v}} . \tag{18}$$

The flux through the cap is then

$$\text{Flux}_{adv} = UC = UC_0 \, e^{-\frac{\lambda h_{cap}}{v}} , \tag{19}$$

and Eqs. 15 and 16 may still be used to estimate the porewater and solid concentration, respectively, at the bottom of the bioturbation layer.

2.7 Diffusively Dominated Conditions

Under diffusively dominated conditions, the problem is more complicated. Degradation of the contaminant decreases both concentrations and flux in the cap but the decrease in flux is partially offset by increased concentration gradients (i.e., increasing the driving force for diffusion). A simple model of this process can be derived by assuming that the concentration at the top of the cap is small compared to the concentration in the underlying sediment, C_0. The model equations and boundary conditions can be written as

$$D\frac{\partial^2 C}{\partial z^2} = \lambda \varepsilon C \quad \begin{cases} C(z = h_{cap}) \ll C_0 \\ C(z = 0) = C_0 \end{cases} . \tag{20}$$

The solution to Eq. 20 written in terms of flux at the top of the cap is

$$\text{Flux}_{diff} = DC_0 \left(\frac{\lambda_\varepsilon}{D}\right)^{1/2} \left(\sinh\left(\left(\frac{\lambda_\varepsilon}{D}\right)^{1/2} h_{cap}\right) - \frac{\cosh\left(\left(\frac{\lambda_\varepsilon}{D}\right)^{1/2} h_{cap}\right)}{\tanh\left(\left(\frac{\lambda_\varepsilon}{D}\right)^{1/2} h_{cap}\right)} \right) . \tag{21}$$

This flux with Eqs. 15 and 16 may be used to estimate the porewater and solid concentration at the bottom of the bioturbation layer for a reacting, diffusion dominated system.

3 Comparison to Numerical Model of Chemical Transport Through a Cap

All of the models presented presume steady-state conditions, when the flux and bioturbation layer concentration values are at their highest levels. Note that the models assume that the underlying sediment concentration remains constant throughout the transient period leading up to steady state. Thus, the calculated steady state may never be achieved if there are fate or dilution processes influencing the contaminant in the underlying sediment. Contaminants that reach steady-state conditions relatively rapidly are reasonably well described by the model, but the model would likely over predict concentrations and fluxes for contaminants requiring very long times to achieve steady state.

The applicability and limitations of the model can be seen by comparison of the results to a full numerical model of chemical migration through a cap. Such a model was developed using the finite element modeling tool FEMLAB®. An implementation of this model has been made available on the internet at www.capping.ce.utexas.edu. The advantages of a numerical model include the ability to predict transient chemical conditions for all conditions to which the analytical steady-state model is applicable as well as address a number of situations for which analytical models are unavailable or have not been presented. Such conditions include chemical or physical parameters that are distributed in time or space. Most important of these is the ability to address chemical concentrations in the underlying sediment that are variable in space or time.

The comparison between the full numerical simulation of the transient flux and the predicted flux by the steady-state model is shown in Fig. 3a–c, which shows the predicted flux under diffusion controlled conditions (Fig. 3a), advective controlled conditions (Fig. 3b), and near equal diffusion and advection (Fig. 3c). The chemical parameters are for a PCB, which is a highly hydrophobic, low reactivity organic compound, and a typical sediment contaminant. The cap simulated is 2 ft of sand. Note that the time required to achieve steady state is of the order of 10^{11}–10^{12} s or more than 3000–30 000 years in each simulation. The steady-state analytical model is shown with and without reaction.

The model does an excellent job of predicting fluxes through caps and concentrations in the bioturbation layer in either diffusion and advection dominated conditions and also provides reasonable agreement when both diffusion and advection are important. Note that the predicted concentration via both the transient numerical model and the steady-state model is directly proportional to the predicted flux, and the predicted concentrations are in agreement as long as the model predicted fluxes are in agreement. As a result of the assumption of the constant concentration with the steady-state

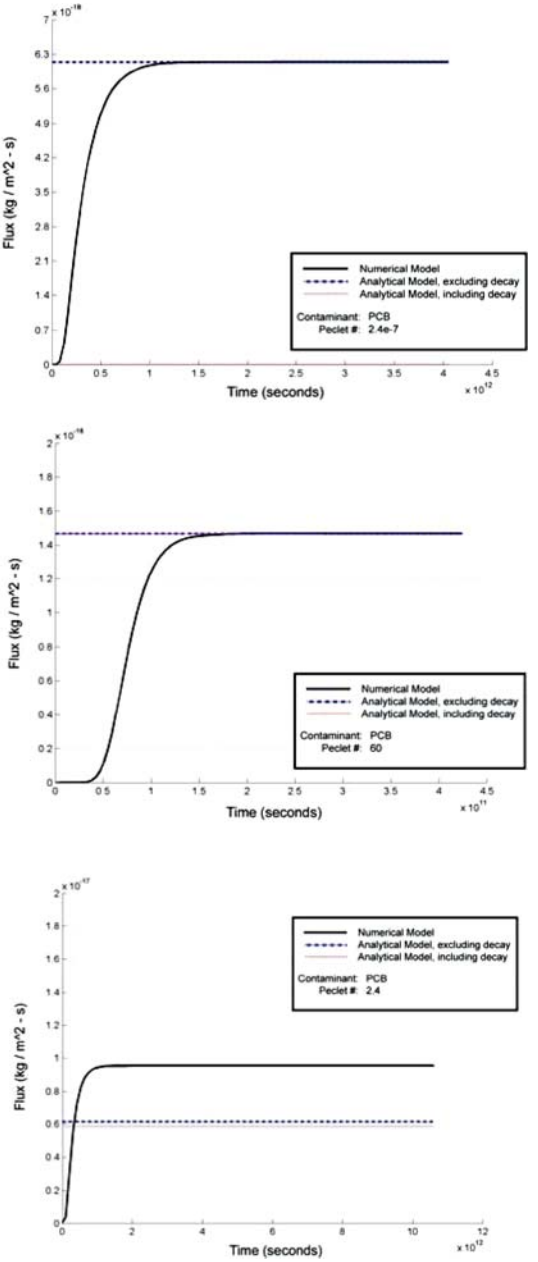

Fig. 3 Flux under **a** diffusion control, **b** advective control, and **c** advection–diffusion

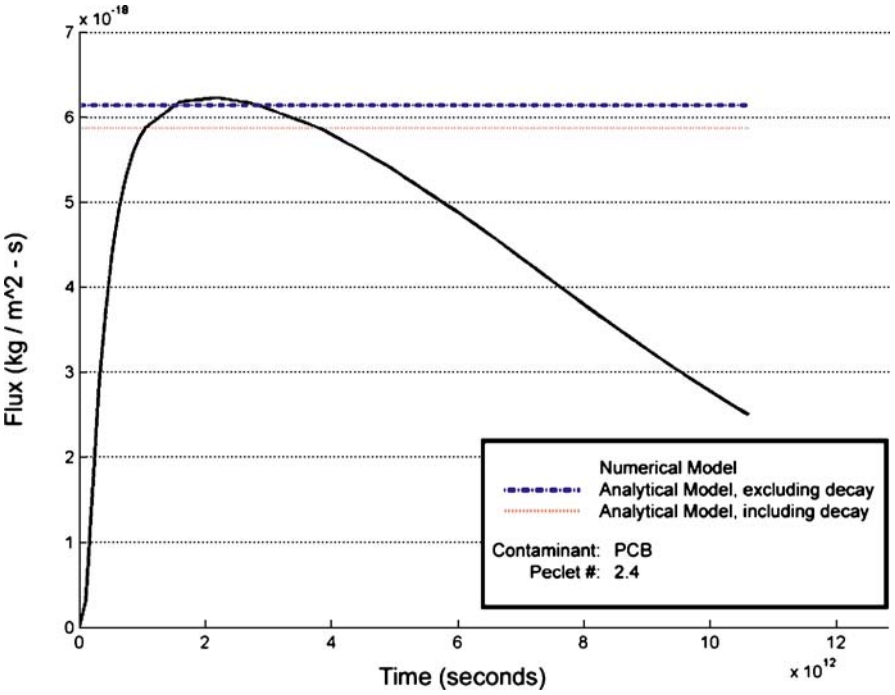

Fig. 4 Predicted PCB flux through the cap assuming advection–diffusion and allowing mass conservation to deplete mass in the underlying sediment layer as it migrates into the cap

model, the actual predicted flux would be less than simulated in Fig. 3a–c. Figure 4 compares the steady-state model prediction with the transient flux prediction assuming that an infinite layer of contaminated sediment underlies the cap layer. A concentration profile is assumed to develop in this infinitely thick sediment layer as mass moves into the cap in the transient model. The peak flux predicted by the transient model at Peclet number of order 1 is in excellent agreement with the estimated steady-state model flux assuming a constant underlying concentration. This is not a general result although simulations using a variety of compounds and conditions (Peclet numbers) showed that the steady-state model approximately equaled or overestimated the maximum fluxes out of the cap when compared to the transient flux that included material depletion beneath the cap. Overestimation of the actual flux means that the steady-state model is conservative in that it would overestimate the risk associated with chemical migration through the cap. The steady-state model is, of course, also conservative, in that it does not recognize that the flux through the cap is essentially zero until shortly before steady conditions are achieved.

In summary, the proposed steady-state model provides a useful and conservative predictor of long-term cap flux through the cap. As such the model, although simple in form, can be used to evaluate the long-term effectiveness of a cap and provides a conservative indicator of long-term cap performance. The model can quickly determine whether a given cap is capable of achieving flux and sediment concentration targets at all times. If a cap is found to be a feasible means of achieving these risk based targets, a more complete design can be undertaken to evaluate any geotechnical issues associated with placement and stability to ensure the long-term physical integrity of the cap.

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