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Macroinvertebrate Ecotoxicity Testing (MET)

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Synonyms

Invertebrate bioassay; Invertebrate toxicity test

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Glossary

Amphipod (or amphipoda) An order of malacostracan crustaceans. General characteristics include no carapace, laterally compressed body, different forms of appendages, size range from 1 to 340 mm in length (most are less than 10 mm), and most are aquatic detritivores (scavengers).

Areas of concern Areas in the (Laurentian) Great Lakes that were identified in the 1970s by the International Joint Commission as having a severely degraded aquatic environment (Grapentine 2009).

Bioavailable The portion of a chemical that is available for uptake by an aquatic organism and reaches the site(s) of toxic action, where it exerts a toxic effect; tissue concentrations of a chemical are generally used as a surrogate measure of bioavailable chemical, as it is not usually feasible to measure the concentration of the chemical at the actual site of toxic action.

Bioaccumulation The process by which chemicals are taken up by aquatic organisms directly from water as well as through exposure through other routes, such as consumption of food and sediment containing the chemicals (Rand 1995).

Critical body concentrations Body concentrations of a contaminant (or contaminants) measured in test organisms that are associated with observed toxicity.

Direct toxicity Toxicity that results from the toxic agent(s) acting directly at the site(s) of toxic action in and/or on the exposed organisms that are exhibiting the adverse biological response in question (Rand 1995).

Dissolved oxygen The amount of oxygen (O_2) dissolved in water, commonly measured as milligrams of O_2 per liter (mg/L), millimoles of O_2 per liter (mmol/L), or percent saturation.

Ecology A branch of biology dealing with the relations between organisms and their environment (Random House College dictionary).

Ecosystem A biological community and its chemical/physical environment.

Ecotoxicology The study of the impact of toxic chemicals on biological organisms (populations, communities, and ecosystems).

Flow-through An exposure system for aquatic toxicity tests in which the test material solutions and control water flow into and out of test chambers on a once-through basis either intermittently or continuously (Rand 1995).

In situ (exposures) Exposure of a defined population of test organisms in confined chambers in the field, under natural or near-natural conditions, followed by measurement of typical toxicity or bioaccumulation test end points. In situ exposures possess more realism than laboratory tests but more control than field studies (Chappie and Burton 2000).

In vivo (tests) Tests using whole, living organisms (as opposed to in vitro tests), which are conducted on organs, tissues, cells, etc.

Indirect toxicity Adverse effects or toxicity that results from the toxic agent(s) acting on and producing changes in the chemical, physical, and/or biological

environment external to the organisms under study (e.g., direct chemical toxicity in prey species may cause indirect toxicity to predator species due to starvation) (Rand 1995).

Invertebrate cultures Continuous maintenance of a population of invertebrates (either collected from a clean location in the environment or purchased from a supplier) in the laboratory under ideal growing conditions for routine harvesting of organisms for use in toxicity tests.

Macroinvertebrate Refers to aquatic invertebrates such as insects, crustaceans, molluscs, and worms that are visible to the naked eye (often greater than 0.5 mm in length).

Mesocosm Large experimental systems designed to simulate some component of an ecosystem. Mesocosms are normally used outdoors, either as physical enclosures of a portion of a natural ecosystem or man-made structures such as ponds or stream channels. They differ from microcosms in that they are larger (volume > 15 m^3 for experimental ponds or length > 15 m for experimental stream channels), are usually located outdoors and are less enclosed, and have a lower degree of control by the researcher. Mesocosms possess more realism than microcosms but are more controlled than field surveys (Kennedy et al. 2003; Newman and Unger 2003).

Microcosm Laboratory systems (usually indoor) designed to simulate some component of an ecosystem (such as multiple species assemblages). Microcosms are generally smaller (volume < 15 m^3 for experimental ponds or length < 15 m for experimental stream channels) and more controlled, but less realistic, than mesocosms (Kennedy et al. 2003, Newman and Unger 2003).

Organotins Organometallic compounds with at least one tin-carbon chemical bond; generally anthropogenic in origin, e.g., tributyltin chloride $(C_4H_9)_3$-Sn-Cl.

Sediment quality triad An effect-based approach to evaluating and assessing pollution-induced degradation due to toxic sediments, consisting of three components: sediment chemistry (measures contamination), sediment bioassay (measures toxicity), and in situ biological assessment (measures effects such as changes in benthic community structure) (Chapman 1990).

Toxicology The science dealing with the effects, antidotes, detection, etc., of poisons (Random House College dictionary).

Abbreviations

AOCs	Areas of concern
ASTM	American Society for Testing and Materials
CCME	Canadian Council of Ministers of the Environment
EC	Environment Canada
EEM	Environmental effects monitoring
ERA	Ecological risk assessment

MET	Macroinvertebrate ecotoxicity testing
TBT	Tributyltin
US EPA	United States Environmental Protection Agency

Definition

Environmental toxicity tests with macroinvertebrates in conjunction with measures of invertebrate community structure and chemical analyses of the ecosystem.

There are a variety of macroinvertebrate species for which standard toxicity test methods have been developed (see Figs. 1–4a,b for examples), including *Daphnia* spp., *Ceriodaphnia dubia*, *Chironomus* spp., Echinoids, freshwater mussels, *Hexagenia* spp., *Tubifex tubifex*, and *Hyalella azteca* (EC 1990, 1992, 1997a, b; US EPA 2000; ASTM 2005, 2006; EC 2007; OECD 2008, 2010). However, environmental toxicity testing has been conducted with a diverse range of organisms; thus, the selection of test species for MET is not limited to those for which standard methods have been published, but will be dictated by the specifics of the assessment involved.

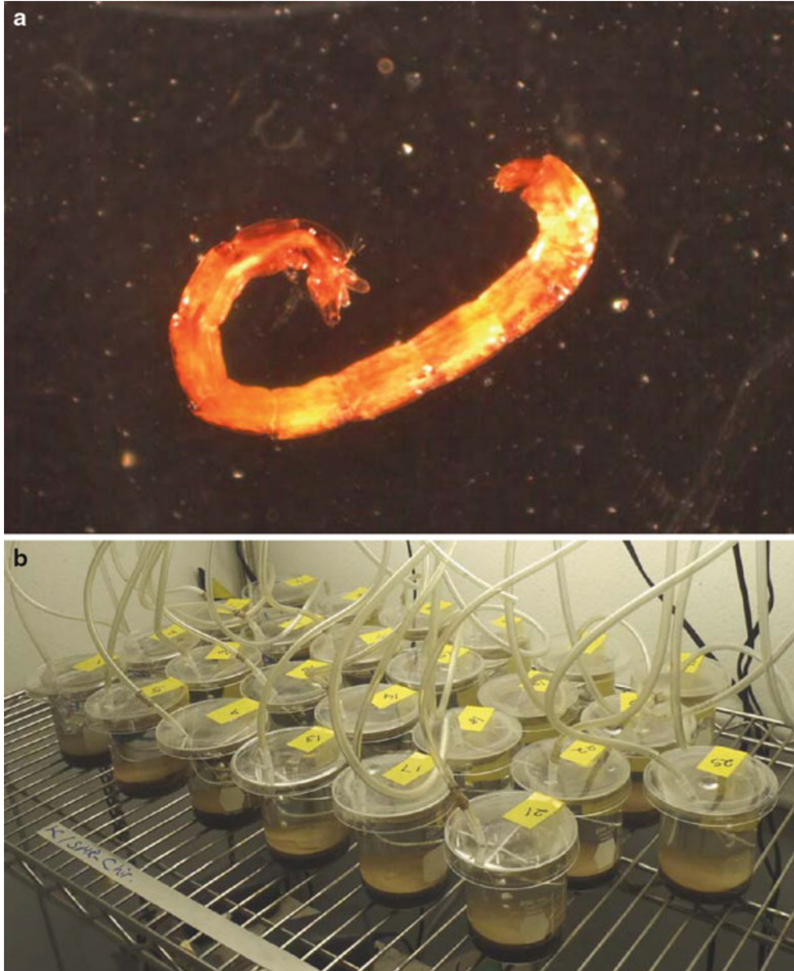
MET is a combination of standard methods (toxicity tests, ecological analyses, and analytical chemistry) tailored for site-specific assessment. MET could involve one or more of the following: use of test species outside the scope of standard tests, simultaneous testing of multiple species, testing mixtures of compounds (field-collected samples and/or laboratory-spiked samples), use of bioaccumulation-toxicity relationships established in the laboratory to link to effects observed in the field, complex chemical analyses, measures of community structure, and conducting long-term exposures (which could include multiple generations). MET incorporates toxicology and ecology and therefore often involves combinations of laboratory testing, field studies, in situ exposures, and analytical chemistry.

Historical Background

According to René Truhaut (1977), in 1969 “during a meeting of an ad hoc Committee of the International Council of Scientific Unions in Stockholm”, he proposed a new branch of toxicology called ecotoxicology. Ecotoxicology was further defined as “the study of toxic effects caused by. . .pollutants, to the biological constituents of ecosystems” (Truhaut 1977). The main focus of ecotoxicology is the assessment of toxic effects on populations. Truhaut also indicated that ecotoxicological investigations require the integration of toxicology, ecology, and analytical chemistry. This led to the development of methods that examine the direct and indirect toxicity on community structure (Chapman 2002), which is different from environmental toxicology (simple tests with individual species and environmental samples, in situ or in vivo). The full integration of analytical chemistry, toxicology,

Macroinvertebrate Ecotoxicity Testing (MET), Fig. 1 (**a**) The freshwater amphipod, *Hyalella azteca* (male) (Photograph courtesy of W. Norwood) (**b**) An example of sediment toxicity tests conducted in polycarbonate Imhoff settling cones with *Hyalella azteca*. Duration of the toxicity test is typically 4 weeks under static conditions with constant aeration of the overlying water. In a standard toxicity test, each Imhoff settling cone contains 1 L overlying water, 15 mL sediment, and 15 juvenile *Hyalella azteca* (0–1 week old at test initiation). Tests are conducted in an environmental chamber at 23–25 °C with a photoperiod of 16 h light:8 h dark (Borgmann and Norwood 1999) (Photograph courtesy of W. Norwood)

and ecology in the evaluation of aquatic ecosystems has been achieved using the sediment quality triad approach (Long and Chapman 1985; Chapman 1990), and Borgmann et al. (2001) have added bioaccumulation measures to the sediment quality triad in order to help identify cause and bioavailability in degraded ecosystems. In all of these cases, benthic macroinvertebrate community structure was evaluated in combination with environmental toxicity tests with relevant



Macroinvertebrate Ecotoxicity Testing (MET), Fig. 2 (**a**) The midge, *Chironomus riparius* (Photograph courtesy of J. Baillargeon) (**b**) An example of sediment toxicity tests conducted in 250-mL glass beakers with *Chironomus riparius*. Duration of the toxicity test is typically 10 days under static conditions with constant aeration of the overlying water. In a standard toxicity test, each beaker contains 125–150 mL overlying water, 50–100 mL sediment, and 15 first instar *Chironomus riparius* (approximately 3 days post-oviposition at test initiation). Tests are conducted in an environmental chamber at 23–25 °C with a photoperiod of 16 h light:8 h dark (Day et al. 1998) (Photograph courtesy of J. Baillargeon)

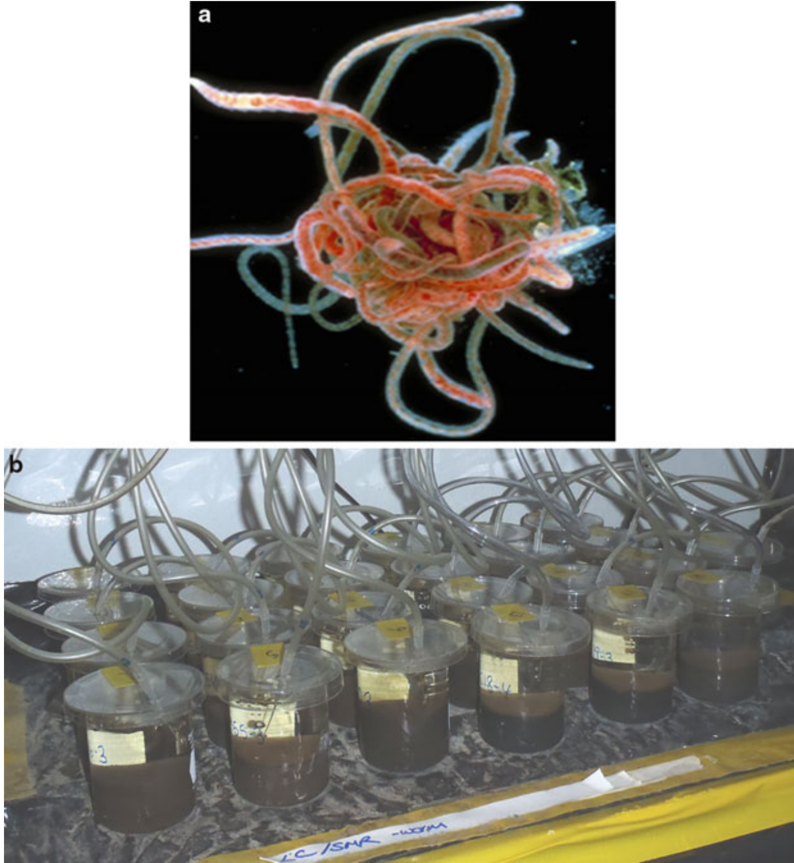
macroinvertebrates and chemical analyses of environmental samples (water, sediment, and biota). Borgmann (2003) outlined four questions that would be applicable to ecotoxicity testing and would require answers in order to fulfill the test:

1. Are contaminants getting into the system?
2. Are the contaminants bioavailable?

Macroinvertebrate Ecotoxicity Testing (MET), Fig. 3 (**a**) The mayfly, *Hexagenia limbata* (Photograph courtesy of D. Milani) (**b**) An example of sediment toxicity tests conducted in 1-L glass jars with *Hexagenia limbata*. Duration of the toxicity test is typically 3 weeks under static conditions with constant aeration of the overlying water. In a standard toxicity test, each jar contains 650 mL overlying water, 125 mL sediment, and 10 *Hexagenia limbata* (1.5–2 months old at test initiation). Tests are conducted in an environmental chamber at 23–25 °C with a photoperiod of 16 h light:8 h dark (Day et al. 1998) (Photograph courtesy of J. Baillargeon)

3. Is there a measurable response?
4. Are the contaminants causing the response?

To answer these questions, the requirements outlined by Truhaut (1977) would be utilized in the following manner. Analytical chemistry would be used to



Macroinvertebrate Ecotoxicity Testing (MET), Fig. 4 (**a**) The oligochaete worm, *Tubifex tubifex* (Photograph courtesy of D. Milani) (**b**) An example of sediment toxicity tests conducted in 250-mL glass beakers with *Tubifex tubifex*. Duration of the toxicity test is typically four weeks under static conditions with constant aeration of the overlying water. In a standard toxicity test, each beaker contains 125-150 mL overlying water, 50-100 mL sediment, and 4 adult *Tubifex tubifex* (8-9 weeks old at test initiation). Tests are conducted in an environmental chamber at 23-25° C in the dark (Day et al. 1998) (Photograph courtesy of J. Baillargeon)

determine if elevated levels of contaminants are in the environment (water and sediment samples) and in biota (bioavailability). A battery of toxicity tests with macroinvertebrates, consisting of both field-collected samples (to determine which sites are toxic, including measurements of bioaccumulation) and laboratory-spiked samples with contaminant(s) of concern at the sites (to determine which contaminants are toxic by establishing relationships between bioaccumulation and toxicity), would be conducted to determine measurable responses and identify cause. Additionally, an ecological assessment (macroinvertebrate community structure analyses) would be used in conjunction with the toxicity tests to identify measurable

responses in the ecosystem. The strengths in this approach lie in the components being tightly linked (chemistry, toxicology, and ecology) and the field and laboratory studies being complementary in nature.

General Characteristics of MET

There are several general characteristics of MET that make this type of testing a widely accepted research tool in ecotoxicology. Typical invertebrate test species have short life cycles, growing from juvenile to adult within a time frame of weeks, so extending the duration of standard test methods to examine reproduction or effects on multiple generations is feasible while remaining time- and cost-effective. Invertebrate cultures are easy to maintain in the laboratory and have minimal space requirements, and most do not need flow-through conditions, which simplifies both culturing and testing procedures. Invertebrates have been shown to be sensitive to various contaminants, including some metals (see links to the CCME (http://ceqg-rcqe.ccme.ca/) and US EPA (http://www.epa.gov/waterscience/criteria/) websites for data used to derive environmental quality guidelines in Canada and the United States, respectively), organotins (Cardwell et al. 1999; Fent 1996, and references therein), and insecticides (Maltby et al. 2005), and thus are an ecologically relevant group of organisms to test in order to adequately protect aquatic ecosystems. Some invertebrates are also tolerant of changing environmental conditions, such as dissolved oxygen and temperature, and therefore, MET is applicable to diverse research requirements. Additionally, aquatic invertebrates have a limited spatial mobility, and therefore, field-collected invertebrates are an accurate representation of site-specific conditions.

Types of MET

All types of MET have the same general design of integrating analytical chemistry, environmental toxicity tests, and invertebrate community structure analyses in the assessment of degraded ecosystems. As well, macroinvertebrates can be used in microcosms and/or mesocosms in combination with the laboratory tests and field studies to assess ecosystem health (Culp et al. 2000; Cash et al. 2003). There are many standardized environmental toxicity tests that utilize a number of pelagic and benthic macroinvertebrate species as outlined in the previous section, and these environmental toxicity tests can be selected and tailored for site-specific applications. For example, in situ test methods have been developed from a number of standard laboratory tests in order to link laboratory to field studies. Species that have been used successfully in situ include *Chironomus* spp., *Lumbriculus variegatus*, *Daphnia magna*, *Ceriodaphnia dubia*, *Hyalella* spp., *Hexagenia* spp., and *Mytilus galloprovincialis* (Salazar and Salazar 1997; Sibley et al. 1999; Maycock et al. 2003; Bervoets et al. 2004; Burton et al. 2005; De Coen et al. 2006).

Applications of MET

MET is a versatile tool that can be used to assess any aquatic ecosystem suspected of degradation due to anthropogenic impacts, to derive environmental quality guidelines for the protection of aquatic ecosystems, and to monitor the success of implemented remediation measures or regulatory efforts. Specific examples of the use of MET include:

1. Areas of Concern (AOCs) evaluation (Grapentine 2009) – MET was used in combination with multiple lines of evidence to evaluate the ecological significance of contaminants in sediment. "Degradation of benthos" is a common beneficial use impairment identified in Great Lakes AOCs. Benthic conditions were assessed using various ecological components, including sediment physicochemistry and grain size, benthic invertebrate community structure, sediment toxicity, contaminant bioaccumulation, and substrate stability. These data can be used to quantify degradation, determine probable cause(s) of degradation, and identify recovery of benthic conditions at AOCs. Based on these data, delisting criteria can then be developed to define targets for restoration of beneficial use. In this way, MET (benthic invertebrate community structure, sediment toxicity, and contaminant bioaccumulation) and multiple ecological components were used to assess AOCs and develop monitoring programs to restore benthic conditions.
2. Environmental Effects Monitoring (EEM) program – Assess impacts on aquatic ecosystems using biological indicators in both the metal mining and pulp and paper sectors (http://www.ec.gc.ca/esee-eem/default.asp?lang=En&n=453D78FC-1).
3. Ecological Risk Assessment (ERA), Level III – Environment Canada (1994) and CCME (1996) guidance documents for ERA outline site-specific data and predictive modeling to derive quantitative information on complex ecosystem responses. Chronic effects, interactions between chemicals, and ecosystem-level studies are encompassed in this assessment.

MET Case Studies: Use of *Hyalella azteca*

Hyalella azteca (Fig. 1a) is a freshwater amphipod macroinvertebrate widely distributed throughout North America. This species has been extensively used in sediment toxicity tests because of its ubiquitous presence in the North American freshwater environment, ecological importance, contact with sediment, relative sensitivity to contaminants, and ease of culture in the laboratory (Borgmann and Munawar 1989; Ingersoll et al. 1995). Details on the life history and ecology of *H. azteca* have been well documented (e.g., Geisler 1944; Hargrave 1970a, b; De March 1977, 1978), and standardized methods have been published for culturing and conducting toxicity tests (EC 1997b; US EPA 2000; ASTM 2005), which are easily adapted to fit the research needs of specific ecological testing.

MET was used to assess the impacts of sediment contamination in lakes of the Sudbury area of Ontario, Canada (Borgmann et al. 2001). This study incorporated the sediment quality triad approach, which correlates the results of sediment chemical analyses (identification of contamination), in situ benthic macroinvertebrate community composition (identification of impact on populations), and measurement of sediment toxicity, with *H. azteca* environmental toxicity tests (identification of impact on an individual species). In addition to the triad approach, contaminant bioavailability (bioaccumulation in *H. azteca*) was compared to known critical body concentrations in order to identify which contaminants were causing the observed impacts (identification of cause). Analysis of metals in the surface sediments identified Cd, Co, Cu, Ni, Pb, and Zn as contaminants of concern, and both the in situ benthic macroinvertebrate community composition and environmental toxicity tests with *H. azteca*, *Hexagenia limbata*, and *Tubifex tubifex* indicated impacts at sites where metal concentrations were elevated. However, bioaccumulation of metals in *H. azteca* indicated that only Cd, Co, and Ni increased in the impacted areas, and comparison of metal bioaccumulation concentrations to known critical body concentrations indicated that only Ni exceeded its critical body concentration. Therefore, Ni was identified as being the major cause of effects. *H. azteca* was used in each biological component of the assessment (the in situ community composition evaluation, the laboratory environmental toxicity testing, and the bioaccumulation evaluation), linking each component and making this a true ecotoxicity test.

H. azteca was also used as the primary test species in MET to characterize the ecotoxicity of tributyltin (TBT) to freshwater invertebrates. First, chronic, multigenerational sediment toxicity tests were designed to address key issues associated with TBT toxicity, which include long-term reproductive effects at low environmental concentrations (Bartlett et al. 2004). Then, a chronic, multispecies sediment test was designed to compare the toxicity and bioaccumulation of TBT among six invertebrate species, including *H. azteca* (Bartlett et al. 2007). Lastly, bioaccumulation tests were conducted with *H. azteca* using field-collected sediments from TBT-contaminated sites to predict the risk to indigenous invertebrate populations using toxicity-bioaccumulation relationships determined from the previous tests (Bartlett et al. 2005). The results from this study of TBT toxicity can be used as tools for the ecotoxicological evaluation of TBT-contaminated ecosystems and prediction of population-level effects in invertebrates.

In situ studies have been conducted with *H. azteca* and other freshwater amphipods as part of a weight-of-evidence approach to determine the effects of various anthropogenic influences on aquatic ecosystems and to link these effects to those occurring or predicted to occur in indigenous invertebrate populations. This type of MET is an important link between laboratory experiments and field studies and has been used to investigate and predict the impacts of contaminants such as pesticides (Schulz and Liess 1999; Jergentz et al. 2004), metals (Robertson and Liber 2007; Couillard et al. 2008), and stormwater runoff (Grapentine et al. 2004) on aquatic invertebrate populations.

Conclusions and Prospects

The crucial aspect of ecotoxicity testing is the link between toxicology and ecology in order to examine the toxicological impact of contaminants on biological communities, including interactions with the chemical and physical properties of the ecosystem. Therefore, a macroinvertebrate ecotoxicity test must make use of test species that are directly relevant to the ecosystem being studied and must be integrated with a battery of ecosystem analyses (chemical, physical, and biological) in order to fully assess the impact of pollutants on the ecosystem. Standard environmental toxicity tests (i.e., those conducted with one species in the laboratory with environmental samples) cannot, on their own, be considered ecotoxicity tests; however, they are an important component of ecotoxicity testing when considered in combination with ecologically relevant studies. The scope of MET will broaden and develop as the field of ecotoxicology continues to evolve, in order to increase environmental relevance, to address more complex issues, and to become more predictive in nature.

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Cross-References

▶ Active Biomonitoring
▶ Aquatic Mesocosms in Ecotoxicology
▶ Benthic Community Ecotoxicology
▶ Biological Test Methods in Ecotoxicology
▶ Contaminated Sediment Core Profiling
▶ In Situ Bioassays in Ecotoxicology
▶ Polychaetes in Ecotoxicology
▶ Sediment Ecotoxicity
▶ Sediment Quality Guidelines
▶ Sediment Toxicity Identification Evaluation

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Metal Speciation in Aquatic Ecotoxicology

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Synonyms

Chemical and physical forms; Fractionation; Species distribution association

Abbreviations

DOC Dissolved organic carbon
KDa Kilodalton
MINEQL+ Chemical Equilibrium Modeling System (Environmental Research Software, USA)
MINTEQA2 Metal Speciation Equilibrium for Surface and Ground Water (United States Environmental Protection Agency, USA)
WHAM Windermere Humic Aqueous Model (Center for Ecology & Hydrology, Natural Environment Research Council, UK)

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Definition

The distribution of different metal forms is referred to as "metal speciation."

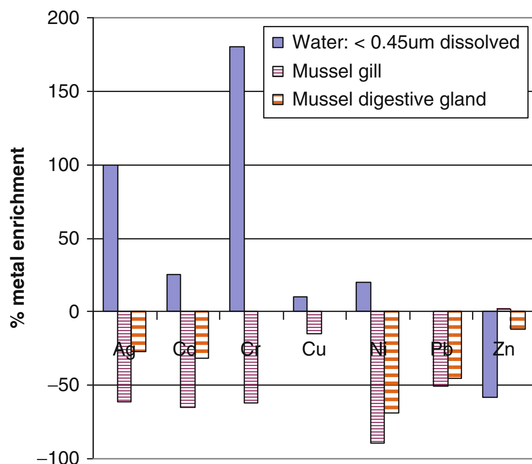
A narrow definition of metal speciation in aqueous media is often given as the quantitative description of the chemical metal forms including free and complexed forms (Stumm and Morgan 1996). Metal species could also be defined as the oxidation state and the complex or molecular forms but could include approaches based on selective extractions, fractionation, and reactivity in the case of solid material such as sediments (Ure and Davidson 2002). Metal speciation can profoundly affect metal bioavailability and toxicity to aquatic organisms.

Historical Background

Due to significant advances in analytical technique development, trace metals can now be quantified in natural waters at concentrations as low as ng L^{-1}. Techniques such as atomic absorption spectrometry (AAS), inductively coupled plasma emission spectrometry (ICP-AES), or mass spectrometry (ICP-MS) are routinely utilized for the determination of total metal concentrations. However, it is well recognized that metal bioavailability and toxicity are poorly correlated with total metal concentration. There is a need, therefore, for determining specific chemical and physical forms of metals in variable environmental matrices to relate to toxicological effects. While there are currently no routine methods to determine metal speciation, several tools are available to provide guidance in the interpretation of metal toxicity data.

Illustration of the Importance of Metal Speciation

The physical and chemical characteristics of waters can affect the speciation of both dissolved and particulate metals (e.g., Lijklema et al. 1993; Gagnon and Saulnier 2003). The bioavailability of metals, in turn, is determined by their speciation in both the dissolved and particulate phases (Luoma 1983; Campbell et al. 1988; Luoma et al. 1992; Gagnon and Fisher 1997). Figure 1 clearly illustrates how total metal concentrations cannot be used to estimate metal exposure to aquatic organisms. In this particular example, metal bioavailability was estimated through the measurement of bioaccumulation of metals in caged mussels. Tissue concentrations of metals, such as Cd, in the exposed mussels were reported to be lower in municipal effluent mixing zones, despite total metal concentrations being higher at those particular sites (Gagnon et al. 2006).



Metal Speciation in Aquatic Ecotoxicology, Fig. 1 Change in water and tissue metal enrichment downstream of a major municipal wastewater effluent (adapted from Gagnon et al. 2006). Percent metal enrichment in water is determined as the difference in metal concentration in upstream site to site located 5 km downstream of the effluent outfall (e.g., $[Ni_{downstream}] - [Ni_{upstream}] / [Ni_{upstream}] \times 100$). Percent metal enrichment in mussel tissue is determined as the difference in metal accumulated in mussel tissues from upstream to the downstream site

Features of Metal Speciation

As metal speciation plays a key role to evaluate the potential fate and toxicity of a given metal, several types of approaches have been developed and refined. The free ion activity of the metal has been recognized in the last 30 years as a key parameter to predict metal toxicity (e.g., Anderson and Morel 1978) which has led to the development of useful predictive tools such as the free ion activity model (Morel 1983) and more recently the Biotic Ligand Model (BLM) (Di Toro et al. 2001; see also the "▶ Biotic Ligand Model" entry in this encyclopedia). Although some pitfalls (e.g., uptake of hydrophobic complexes) have been reported (see Campbell 1995 for a review), the determination of free ion concentrations in natural water is often critical to understand metal bioavailability, and specific methods have been developed to that intent. These methods are either by direct measurements (e.g., electrochemistry or chromatography) or using predictive models (e.g., MINEQL + or WHAM) for ion activity and metal speciation. These models are based on chemical equilibrium constants and predict how water chemistry modifies forms of the metal and in some cases, for example, the BLM predicts, the subsequent changes in toxicity.

Beyond metal forms, a wider definition of metal speciation could include information on operationally defined fractionations of metals such as relative reactivity

for sorbents, sensitivity in voltammetry, and solid-phase association (e.g., binding with sediment particles). The main target is generally the labile metal fractions which usually contain the free ion and easily dissociable complexes and can be more susceptible to potentially interact with an aquatic organism's function (Fairbrother et al. 2007).

Physical Speciation

The most commonly used physical metal speciation term is the operationally defined "dissolved" phase which is obtained by filtration on a membrane of a given pore size such as 0.22 or 0.45 μm. The proportion of metal in the dissolved and particulate fractions can vary greatly from one metal to the other depending on their solubility; it will also depend on the physicochemical characteristics of the surface water. Solubility and changes in water quality could be a major issue for toxicity testing of trace metals, and proper controls and analytical measurements are required. For this reason, development of efficient field techniques for metal speciation is desirable. In any case, the fate and effect of metals are directly related to its physical form. For example, water quality criteria derived to prevent direct toxicity from water borne exposure should be expressed in dissolved rather than total aqueous concentrations. However, it should be noted that the standard micro-filtration (0.22 or 0.45 μm) separates large particles but leaves the colloidal phase in the so-called dissolved fraction. The presence of colloidal matter can significantly influence metal bioavailability (Guo et al. 2001). Metals associated with colloidal material should be considered and distinguished from other forms that are permeable or truly dissolved. This latter fraction has been integrated in ecotoxicological assessments of metals in aquatic environments (Carvalho et al. 1999; Vignati et al. 2005).

Metals can be separated on the basis of their size using micro- and ultrafiltration membranes (Pham and Garnier 1998; Ran et al. 2000; Gagnon and Turcotte 2007). Size distribution can be determined by sequential micro-filtrations and ultrafiltration on membranes with various pore sizes: 0.45 μm through 1 KDa, the latter being considered as truly dissolved metal fraction. Ultrafiltration separations are generally performed with stirred ultrafiltration cells or tangential/cross-flow systems where flow and pressure on the membrane must be closely monitored and controlled (Guo and Santschi 1996). Continuous analytical particle separation techniques which, in combination with suitable detection systems such as ICP-MS, can be utilized to determine metal size distribution (Stolpe et al. 2005). The "sized" metals are quantified following separation by size exclusion or hydraulic chromatography methodologies or field-flow fractionation (FFF), the latter technology being based on the varying diffusion coefficients (Giddings 1993).

Chemical Speciation

Chemical Association of Dissolved Metals

Several analytical methods have been successfully used to assess the chemical speciation of metals in the dissolved phase to provide a characterization of exposure

that goes beyond total dissolved concentrations. The fraction of trace metal that is labile or free will vary greatly from metal to metal and is dependent on the pH and ligand concentrations. For example, zinc could be present almost exclusively as a free metal in natural waters with relatively low pH, while the fraction of copper present as a free ion could be negligible if dissolved organic matter is present in significant concentrations. Publications on measured metal speciation mainly focused on the measurement of labile metals using methods such as diffusive gradients in thin films technique (DGT) (e.g., Davison and Zhang 1994; Unsworth et al. 2006) and competing ligand exchange methods (e.g., Apte et al. 2005). Although labile metal species may be indicators of metal bioavailability (Apte et al. 2005), the free metal concentration is believed to be a better predictor of bioavailability according to the free ion activity model discussed previously (Morel 1983). However, there are a limited number of methods available to reliably measure free metal ion concentrations directly at environmentally relevant concentrations. These methods include the Wageningen Donnan membrane technique (Temminghoff et al. 2000), equilibrium ion-exchange technique (IET; Fortin and Campbell 1998), and some electrochemical techniques such as square wave anodic stripping voltammetry (Ure and Davidson 2002) or direct potentiometric measurements (Rachou et al. 2007). In addition, the metal oxidation state is well recognized as a key factor affecting overall toxicity of a given metal (e.g., chromium III versus chromium VI). Several analytical approaches have been used to measure metals in specific oxidation states including spectrophotometric and chromatographic methods. A very promising approach for such speciation measurements is to hyphenate a chromatographic instrument with an ICP-MS to drastically increase detection limits. In fact, the most common difficulty with most of these metal speciation techniques is that detection limits are often not sufficient for natural waters where concentrations are generally in the subnanomolar range (Sigg et al. 2006). Therefore, there is still no routine method available to directly measure metal speciation at environmentally relevant concentrations, even though the ability to estimate metal speciation and key metal species is critical to predict impacts related to metals.

Chemical Associations of Particle-Bound Metals

The particulate phase plays a key role in the biogeochemical cycle of metals as they can be easily transferred from a solid phase (i.e., particulate and colloidal forms) to an aqueous phase under different environmental conditions. Chemical associations of sediment-bound metals are crucial to assess the mobility and equilibrium of metal forms between the solid and dissolved phases (Jamali et al. 2007). Such exchanges are mainly under the influence of physicochemical factors such as pH, redox potential, salinity, hardness, and organic carbon content (Burgess and Scott 1992).

For the solid phase of sediments, many leaching tests, often single procedures, have been developed and implemented to evaluate the reactivity of metals in sediments (Van der Sloot et al. 1997). However, more sophisticated

mechanism-specific leaching tests such as sequential extraction schemes are required when metal concentrations are controlled by the release rate from several solid phases (Di Toro et al. 1990; Kersten 2002). The intent of sequential extractions is to determine the metals associated with different discrete phases of sediments, and Kersten (2002) pointed out that several methods were adapted from precursor methods by Tessier et al. (1979).

For ecotoxicological purposes, metals in sediments and suspended particles are often determined following a sequential extraction method to operationally separate three key metal fractions: exchangeable and associated with carbonates, associated with iron and manganese oxides, and associated with organics and sulfides (Tessier et al. 1979; Ure and Davidson 2002). Briefly, the first step (a), extractable/carbonates fraction involves shaking sediment with diluted acetic acid at ambient temperature. The second extraction (b) is carried out with a solution of hydroxyl amine. To extract metals associated with organics and sulfides (c), the remaining sediment is agitated with hydrogen peroxide 30%. The residue at end of procedure is considered refractory and not ecologically relevant (Gagnon et al. 2009). For anoxic sediments, which are much more under the influence of reduced sulfur species, labile metals could be released with a solution of diluted chlorhydric acid and are technically named “simultaneously extractable metals” (Di Toro et al. 1990). The latter measurements have been incorporated in some sediment quality guidelines to account for the sequestration of trace metals by sulfides when estimating threshold values for sediment toxicity.

Speciation Models

The development of computer-based programs to estimate metal speciation at equilibrium in complex solutions is tightly linked to the recognition of the importance of chemical speciation regarding metal bioavailability and toxicity. Speciation models are now used for water quality management in several jurisdictions (e.g., US EPA 2007). Programs for metal speciation modeling such as MINEQL+, MINTEQA2, and WHAM are widely used by aquatic chemists and ecotoxicologists. Such computational models, however, require some background knowledge of chemical reactions, good selection of stability constants, and, of course, measurements of key parameters describing water chemistry such as pH, inorganic and organic carbon concentrations, and major ions (Na^+, Ca^{2+}, Mg^{2+}, K^+, SO_4^{2-}, Cl^-). In addition, the presence of other metals can in some cases greatly affect the predicted speciation through competition for complexation sites. Among these parameters, pH and organic carbon can have major impacts on the estimated speciation. A key component of the metal speciation modeling is the estimation of trace metal complexation by both inorganic and organic ligands in solution.

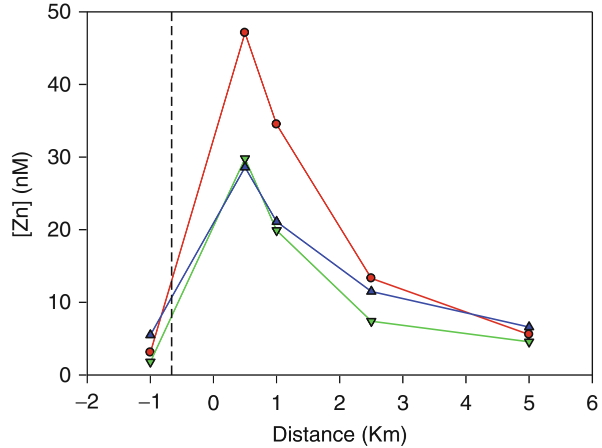
While the use of chemical equilibrium models for complexation by inorganic and synthetic ligands is relatively straightforward, predicting complexation by naturally

occurring dissolved organic matter is more challenging, given its heterogeneous character. In general, humic substances comprise the major component of DOC, ~ 50–80% of DOC (Buffle 1988). Binding of metals by humic substances is affected by factors such as charge, binding site distribution, variable reaction stoichiometry, and competitive nature of the ion binding (Unsworth et al. 2006). The complexity of the modeling approach for the interaction of metals with natural organic matter varies from one model to the other. Tipping (1998) developed the most comprehensive database and approaches to model the interaction of natural organic matter and trace metals. Another limitation to speciation models is that in general, assumptions or calibrations using the composition of organic matter as the fitting parameter are required to run the models. The heterogeneity of natural organic matter is addressed to variable extent by existing models, and it constitutes a potential source of error in the speciation predictions. It should be noted that a fundamental component of the Biotic Ligand Model approach (see the "▶ Biotic Ligand Model" entry in this encyclopedia) to predict metal speciation and metal toxicity is the use of such models.

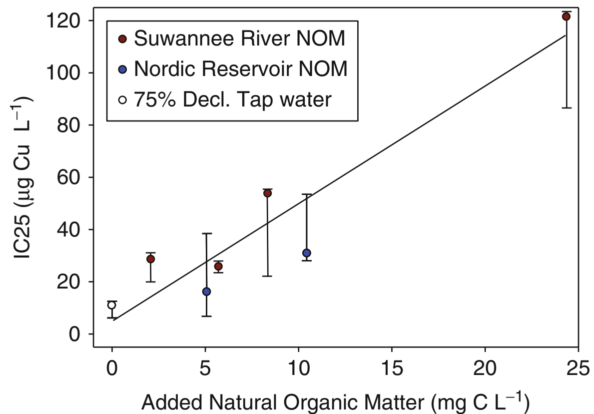
Case Study Examples

The following example illustrates how the size distribution of zinc can change along with concentration when an effluent mixes with receiving water (Fig. 2) to an extent that total concentration provides an incomplete assessment of fate and potential impacts of metals. Total dissolved zinc was measured by filtration, while permeable zinc, less than 10 kDa, was measured by ultrafiltration. Permeable zinc was also estimated based on total dissolved using the chemical equilibrium model WHAM VI and assuming that the zinc predicted to be associated with iron and humic substances was colloidal. In contrast to the upstream and far stream water, total dissolved was much higher than permeable fraction downstream of the discharge point. Permeable zinc measurements and estimations indicate that about 40% of the dissolved zinc is in fact colloidal at 0.5 km downstream of the discharge point, while at 5 km downstream, zinc is back at being predominantly in the truly dissolved fraction.

In another example, the effect of natural organic matter on copper toxicity illustrates the importance of metal speciation in predicting metal toxicity to aquatic organisms, for example, on *Ceriodaphnia dubia* reproduction inhibition (Fig. 3). The laboratory test solution used was 75% dechlorinated tap water with increasing concentrations of commercially available natural organic matter (NOM) from Suwannee River and Nordic Reservoir. The 25% inhibition concentrations (IC25) for copper were determined following a 7d reproduction *C. dubia* bioassay. If metal speciation is ignored, it would be assumed that the IC25 is constant at about 12 μg L^{-1}. In contrast, addition of natural organic matter in an environmentally relevant range resulted in an order of magnitude decrease in the sublethal toxicity of copper at about 25 mg C L^{-1} of NOM. These data have been used to develop a predictive model for copper sublethal toxicity to *C. dubia*, where toxicity is



Metal Speciation in Aquatic Ecotoxicology, Fig. 2 Total dissolved (*circles*), measured permeable (*upward triangles*), and predicted permeable (*downward triangles*) zinc concentrations in the dilution plume of a major municipal effluent (Adapted from Vigneault et al. 2005)



Metal Speciation in Aquatic Ecotoxicology, Fig. 3 Mitigating effect of natural organic matter (NOM) on copper toxicity based on the Ceriodaphnia dubia reproduction bioassay (Adapted from Schwartz and Vigneault 2007)

estimated using the free copper concentration estimated using speciation model WHAM VI, taking into account the effect of food addition in the speciation of copper in the test solutions.

Conclusions and Prospects

In ecotoxicology studies, total metal concentration alone may often be insufficient to quantify exposure and thus to arrive at appropriate conclusions regarding risk. Estimation of metal speciation comprises both experimental evaluations and model calculations that are required for aquatic ecotoxicology studies and range from simple filtration to a complex analytical framework in order to fractionate metal species. The level of effort for metal speciation analysis should be based on an ecological risk driven tiered approach.

Cross-References

▶ Bioavailability of Contaminants
▶ Biology-Based and Population Dynamics Modeling in Ecotoxicology
▶ Biotic Ligand Model
▶ Emerging Issues in Ecotoxicology: Characterization of (Metallic) Nanoparticles in Aqueous Media
▶ Modes of Action of Chemical Pollutants
▶ POCIS Passive Samplers in Combination with Bioassay-Directed Chemical Analyses
▶ Quantitative Structure-Activity Relationship (QSAR) in Ecotoxicology

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Microbial Assay for Risk Assessment (MARA)

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Article Outline

Definition
Historical Background
Bacterial Microbiotests
Features and Validation
Applications
Benefits
Other MARA Platform Tests
Case Study
Conclusions
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Definition

A bioassay performed with an array of microbial strains to assess the toxicity (inhibitory) or enhancement profile of the test substance (sample).

The assay is based on growth of the microbes employing a 96-well microplate format. MARA is a generic test methodology and other assays based on different strains using a particular measurement parameter, for example, bioluminescence (LumiMARA), have also been developed.

The criterion for inclusion of specific phylogenetic strains in the array is the different sensitivity response it exhibits to different toxicants or substances. The collective outcome of the diverse multi-strain array provides the overall profile or fingerprint of the test material (chemical or environmental sample).

Historical Background

The dependency on chemicals for a wide range of services and products has seen significant growth worldwide of the chemical industry in recent decades. Accompanying this development, there has been an increasing concern of the release of

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hazardous substances to the environment. Legislation to control the use of chemicals has been progressively implemented. A prime example of this in the European Union is the REACH regulation concerning the *R*egistration, *E*valuation, *A*uthorisation, and restriction of *CH*emicals (Thompson et al. 2005). For additional details on REACH, see the entry entitled "▶ REACH Legislation in Ecotoxicology."

Fundamental to the process of chemical risk assessment has been the utilization of ecotoxicity testing. This essentially employs bioassays to assess the effect of substances on selective organisms. The organisms that have been predominantly used historically in bioassays are multicellular eukaryotes. These conventional tests are effective tools for ecotoxic assessment but pose some constraints, for example, financial, in the regulatory framework (Wadhia and Persoone 2009). Attempts have been made to develop multispecies assays (Sánchez and Tarazona 2002), but they have not materialized into standardized tests that can be used routinely for monitoring purposes. The need for simple low-cost bioassays fuelled the development of *microbiotests* (Blaise et al. 1998 and entry entitled "▶ Microbiotests in Ecotoxicology").

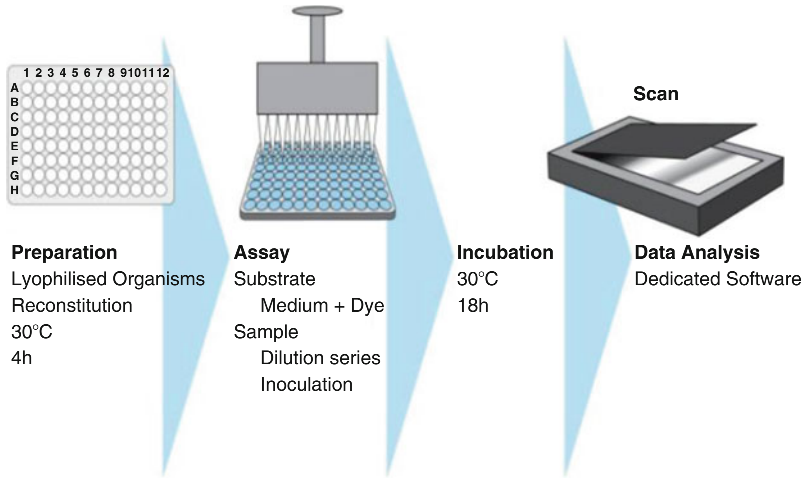
Bacterial Microbiotests

Microbes and in particular bacteria represent the ideal assay organisms for toxicity assessment in microbiotests. Utilization of bacteria in ecotoxicity tests has significant benefits. The relative size of the microorganisms means that concurrent effects measured pertain to large numbers (millions) of test organisms. The duration of the tests is substantially reduced owing to short generation times. The metabolic and physiological activities in bacteria are likely to be impacted by toxicants much more rapidly than those in higher organisms. Ethical issues, particularly associated with vertebrate species, are not a concern, and costs associated with bacterial tests are significantly lower than those of invertebrate and vertebrate ecotoxicity tests.

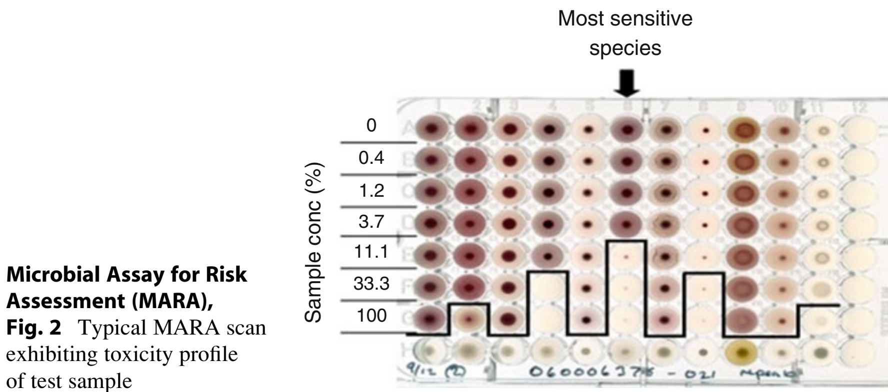
Design of bacterial assays, not unlike conventional invertebrate and vertebrate tests, has essentially been single species based. Toxicity evaluation determined from the response of a single species is unlikely to provide a measure of the toxic impact that would be evident of exposure to a multitude of different species.

Features and Validation

MARA (microbial assay for risk assessment) is an innovative "battery of tests within a test" multispecies assay which allows measurement of toxic effects of chemicals and environmental samples. The test uses a selection of 11 taxonomically diverse microbial strains lyophilized in a microplate (Nałęcz-Jawecki et al. 2010). The strains have been meticulously selected to represent a taxonomically diverse



Microbial Assay for Risk Assessment (MARA), Fig. 1 MARA test protocol



Microbial Assay for Risk Assessment (MARA), Fig. 2 Typical MARA scan exhibiting toxicity profile of test sample

range and, in addition to the 10 prokaryotic species, include a eukaryote (yeast). The diversity of the array is evident with the inclusion of strains from different bacterial groups (gram positive, α-, β-, and γ-gram negative).

A measure of the growth of the organisms over a range of concentrations of the test substance is determined with the reduction of tetrazolium red dye. A flatbed scanner is utilized to capture an image of the test plate (Fig. 1), and the scan is subsequently analyzed using purpose-built software. An array of the 11 different growth determinations gives a consolidated toxic evaluation representing a unique sample "fingerprint" (Fig. 2).

In order to provide a comprehensive and optimal assessment utilizing the significant feature of MARA as a multispecies test, the MARA software computes an

endpoint referred to as the microbial toxic concentration (MTC). The MTC value is determined as follows:

$$MTC = c_{min} \times d^{\left(\frac{p_{tot}}{p_0}\right)-1}$$

Where
c_{min} = lowest concentration in the gradient
p_0 = pellet size in the control well
d = dilution factor
p_{tot} = sum of pellet sizes in all wells

MTC values for MARA are generated for each strain and as a single value for the assay as a whole. The toxic fingerprinting concept in the case of chemicals provides a unique profile that is specific to that particular chemical and is indicative of its mode of toxic action (Gabrielson et al. 2003). This concept using MARA cluster analysis has been utilized to demonstrate that metallic nanopowders can differ in terms of their toxic mode of action (Santos et al. 2009).

Validation of MARA has been implemented with extensive intra-laboratory testing of chemicals and environmental (aquatic and terrestrial) samples. The validation process has also included an international interlaboratory trial involving the participation of laboratories pertaining to academia, regulators, and commercial organizations (Wadhia and Thompson 2009).

Applications

MARA has a broad application scope and can be used to potentially assess the toxicity of:

- Effluents
- Soils
- Waste
- Treat/untreated waters
- Sediments
- Landfill leachates
- Sewage sludges
- Biocides
- Agrochemicals
- Pharmaceuticals

Benefits

- Multispecies
- Simple protocol

- Observations readily made
- Easy image storage
- Software – easy inference
- Concurrent testing of numerous samples
- Minimal space requirement
- Cost-effectiveness

Other MARA Platform Tests

The MARA platform system has been developed further to formulate tests for rapid toxicity assessment (*LumiMARA*) and for utilization in the testing of cosmetics (*DermaMARA*).

The LumiMARA system utilizes 11 naturally bioluminescent bacterial strains. The array of organisms consists of 9 marine and 2 freshwater strains. The LumiMARA assay's principle entails measurement of the decrease in bioluminescence with exposure of the microbial strains (array) to the test sample. Bioluminescence is measured using a luminometer, and the data obtained are expressed as EC_{50} values or % inhibition (Fig. 3).

DermaMARA is a multispecies test employing an array of up to 11 skin microbial species consisting of a range of skin pathogens and commensals. These organisms represent a diverse genetic range exhibiting a spectrum of sensitivities to different skin care products and ingredients.

Other MARA systems offering potential for the testing of antibiotics and disinfectants are also in the process of evaluation.

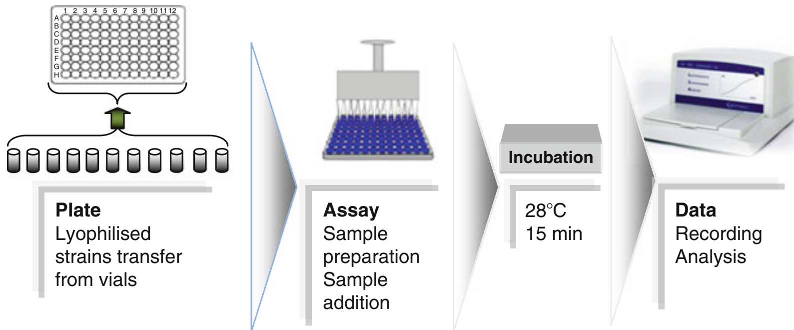
Case Study

The samples tested in this case study pertained to an onshore treatment facility operating to remove traces of residual oil and chemical waste. These constituents were present in the wastewater as a result of offshore oil production activities. The treatment plant's function was to render the wastewater acceptable for onshore discharge.

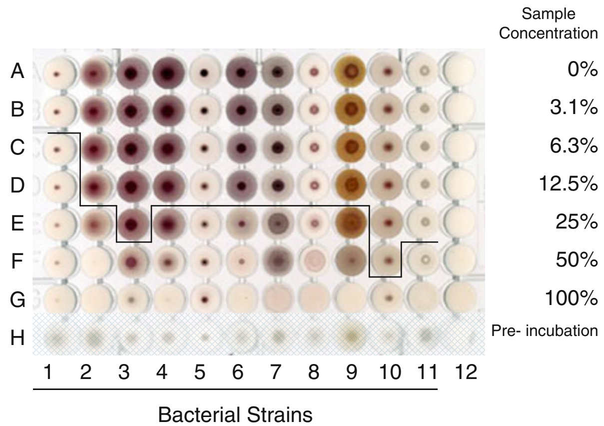
In the context of the treatment facility, representative samples to assess toxicity were taken from:

1. Feed to settling tank
2. Aerated settling tank
3. Produced water

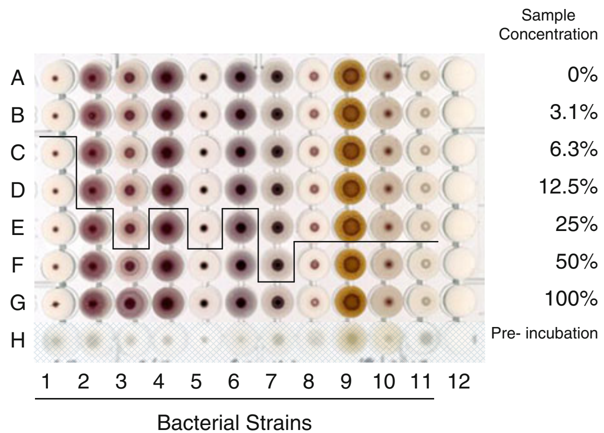
The samples taken from within the oil-water processing plant exhibited significant toxicity as determined with the effect observed on growth of the MARA array (Figs. 4 and 5), and from the inhibitory effect on the bioluminescence activity of the LumiMARA strains (Fig. 6 and Table 1). Toxicity of the samples was attributed to



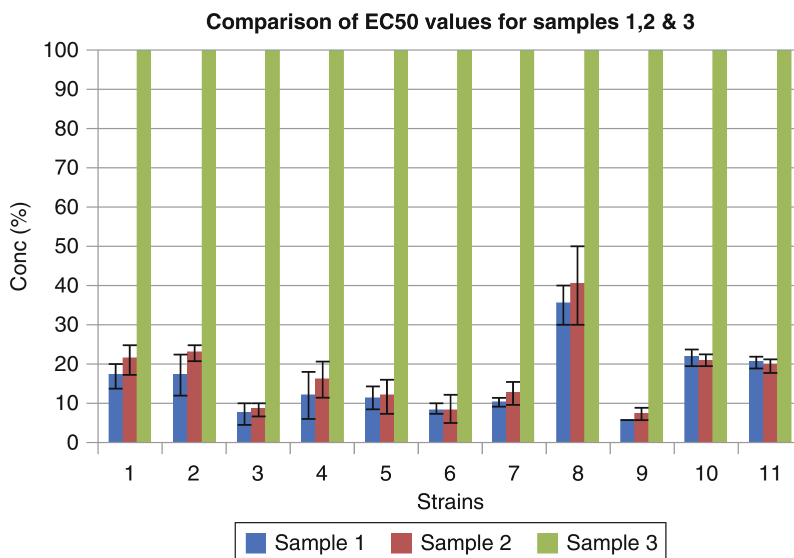
Microbial Assay for Risk Assessment (MARA), Fig. 3 LumiMARA test protocol



Microbial Assay for Risk Assessment (MARA), Fig. 4 MARA scan showing the resultant growth/ inhibition constituting the toxic profile in accordance to the MTC (microbial toxic concentration) values. Scan is of feed to settling tank sample



Microbial Assay for Risk Assessment (MARA), Fig. 5 MARA scan showing the resultant growth/ inhibition constituting the toxic profile in accordance to the MTC (microbial toxic concentration) values. Scan is of aerated settling tank sample



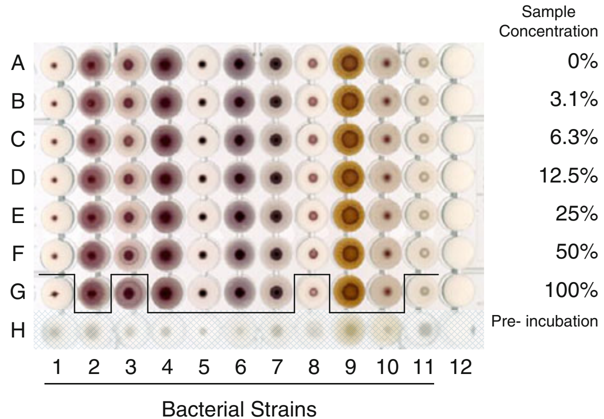
Microbial Assay for Risk Assessment (MARA), Fig. 6 Comparative mean EC_{50} values (with 95% CL) of samples 1, 2, and 3 (analyzed in triplicate) from oil-water treatment plant using LumiMARA array. EC_{50} values for sample 3 were unattainable because of low (<50%) toxicity

Microbial Assay for Risk Assessment (MARA), Table 1 Summary of MARA and LumiMARA data of samples taken from an oil-water processing plant. Figures marked with * indicate samples with high toxicity using these arrays. Figures with # indicate samples with low or no detectable toxicity

		MARA		LumiMARA	
Sample		Min MTC (%)	Mean MTC (%)	Min EC_{50} (%)	Mean EC_{50} (%)
1	Feed to settling tank	5.6*	26*	6*	15*
2	Aerated settling tank	4.0*	25*	6*	19*
3	Produced water	54	ND#	ND#	ND#

levels of sulfide and hydrocarbons evident from data collected (not presented here) at the plant with routine monitoring of these samples. In contrast, the produced water sample was found to show low or no toxicity with MARA (Fig. 7) and LumiMARA (Fig. 6), establishing the effectiveness of the operation of the plant to reduce the toxicant load. Use of MARA and LumiMARA proved to be of value in effective monitoring of the plant's operational activity.

Both MARA and LumiMARA have also been included in an international project incorporating onshore and offshore studies. The project work was conducted to evaluate whole effluent assessment (WEA) of produced waters in a regulatory framework.



Microbial Assay for Risk Assessment (MARA), Fig. 7 MARA scan showing the resultant growth/inhibition constituting the toxic profile in accordance to the MTC (microbial toxic concentration) values. Scan is of produced water sample

Conclusions

MARA and LumiMARA are valuable tools for the rapid monitoring of effects of complex environmental samples and chemicals or specific toxicants. In addition to conveying a measure of toxicity, the MARA platform assays have innate potential scope with the fingerprint concept to provide information of the mode of toxic action and composition of the test sample.

Cross-References

▶ Bacteria in Ecotoxicology: Microtox Basic
▶ Bacteria in Ecotoxicology: Recombinant Luminescent Bacteria
▶ Biological Test Methods in Ecotoxicology
▶ Microbiotests in Ecotoxicology
▶ Test Batteries in Ecotoxicology

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Microbial Bioremediation of Aquatic Environments

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Article Outline

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Glossary
Definition
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Synonyms

Microbiological treatments

Glossary

Active transport Movement of a substance against its concentration gradient through the cytoplasmic membrane using energy in which specialized transmembrane proteins are involved.

Basal metabolism Minimal cellular organic processes – vital functions – that are necessary for life.

Bioaccumulation Accumulation of substances in cells through an active transport.

Bioaugmentation Addition of indigenous or exogenous microorganisms or addition of a genetically engineered variant to the contaminated sites.

Biodegradation Chemical breakdown of substances by living organisms.

Biosorption Physiochemical process which allows the passive concentration and binding of substances onto a biological matrix.

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Biostimulation Addition of growth rate-limiting factors such as nutrients or electron acceptors to accelerate contaminant degradation rates by indigenous microorganisms.

Biotransformation Chemical modification of chemical compounds by living organisms.

Catabolic diversity Different types of microbial metabolic pathways for the breakdown of organic compounds.

Dystrophic crisis Sudden changes in environmental conditions inducing an abnormal development of living organisms. Eutrophication is an example of dystrophic crisis in which an excess of organic matter induces anoxia due to oxygen consumption and asphyxia.

Genomic plasticity Mechanisms of evolution at the molecular level producing permanent and transmissible mutations of genetic material. Mutations can be caused by copying errors in genetic material during cell division, by exposure to radiation and chemicals, and by acquisition – or deletion – of mobile genetic elements.

In situ/ex situ bioremediation In situ bioremediation: process implemented in place, without transportation of polluted materials. Ex situ bioremediation: process implemented off-site that requires the transportation of polluted materials to specialized areas and devices.

Metabolism The set of chemical reactions in living cells and organisms necessary for maintaining life. These processes allow cells and organisms to grow and reproduce, maintain their structures, and respond to their environments. Metabolism is usually divided into two categories: (1) catabolism breaks down organic matter, for example, to harvest energy in cellular respiration, and (2) anabolism uses energy to construct cell components such as proteins and nucleic acids.

Mineralization Process by which living organisms produce minerals. During the degradation process, mineralization is the transformation of organic matter to its mineral form that usually results in the production of carbon dioxide.

Mobile genetic elements A type of DNA that can move within the genome. It includes transposons, plasmids, bacteriophages, and integrons.

Natural attenuation Reduction of contaminant concentrations in the environment through naturally occurring physical, chemical, and biological processes.

PCBs Polychlorinated biphenyls (PCBs) are a class of organic compounds containing 1–10 chlorine atoms attached to biphenyl (a two-benzene ring molecule). PCBs were used for dielectric fluids in transformers, capacitors, and coolants. PCBs are highly toxic compounds and are classified as persistent organic pollutants.

Physical–chemical remediation Physical remediation includes the following phenomena: advection (transport), dispersion, dilution, diffusion, volatilization, sorption/desorption. Chemical remediation includes the following phenomena: ion exchange, complexation, and abiotic transformation (e.g., photoreaction).

Rhamnolipid A glycolipid, containing the desoxyose rhamnose linked to a β-hydroxydecanoic acid, mainly produced by *Pseudomonas* species. Such molecules present biosurfactant properties due to their amphiphilic character.

Sophorolipid A glycolipid, containing the diholoside sophorose (two glucose units) linked to the oleic acid, produced by yeasts such as *Candida bombicola*. Such molecules present biosurfactant properties due to their amphiphilic character.

Speciation (metal) Specific forms of an element defined as its isotopic composition, electronic or oxidation state, and/or complex or molecular structure. The speciation of an element is its distribution among defined chemical species in a system.

Specific metabolite An organic compound that is a starting material in, an intermediate in, or an end product of metabolism. Intermediary metabolites are by far the most common; they may be synthesized from other metabolites, perhaps used to make more complex substances, or broken down into simpler compounds, often with the release of chemical energy. A specific metabolite is an intermediary metabolite specifically produced during the degradation/transformation of a compound.

Sulfate- reducing bacteria Bacteria that obtain their energy by oxidizing organic compounds or molecular hydrogen (H_2) while reducing sulfates to sulfides, especially to hydrogen sulfide. They are anaerobes that use sulfate as the terminal electron acceptor of their electron transport chain. Other oxidized inorganic sulfur compounds, such as sulfite, thiosulfate, or elemental sulfur, can also be reduced by most sulfate-reducing bacteria. Sulfate-reducing bacteria perform a *dissimilatory sulfate reduction* reducing large amounts of sulfate in order to obtain energy and expel the resulting sulfides as waste. *Assimilatory sulfate reduction* is performed by bacteria that reduce small amounts of sulfates synthesizing cellular components containing sulfur.

Surfactin A bacterial cyclic lipopeptide with powerful surfactant and antibiotic properties produced by *Bacillus subtilis*, for example.

Vadose zone A water unsaturated zone lying between the land surface and the top of an aquifer characterized by pore spaces that are incompletely filled with water.

Definition

Microbial bioremediation is the use of microbial metabolic capacities aiming to reduce toxic effect of pollutants in order to restore polluted environments.

Microorganisms have the capacity to degrade and transform most pollutants. The use of this capacity is an alternative to classical physical and chemical remediation approaches. Microbial bioremediation can occur naturally or can be engineered. Toxic effects are removed or diminished, thereby restoring the environment by this biological process, but not necessarily to its original way of functioning.

Several strategies have been developed including natural attenuation, biostimulation, and bioaugmentation. These strategies have been applied for in situ or ex situ bioremediation. In this entry, we focus exclusively on in situ bioremediation.

Historical Background

As early as the 1960s, microbial bioremediation has been applied (Davis and Raymond 1964), but this approach has become widely used since the 1990s. It constitutes an alternative and presents several advantages compared to conventional remediation techniques since it is a less expensive, noninvasive technique that can also be applied to low-level contaminations (Perelo 2010). Many different pollutants such as crude oil, PCBs, pesticides, and heavy metals have been successfully removed from contaminated marine and freshwater environments using microbial bioremediation (Pandey et al. 2009; Perelo 2010; Vidali 2001).

Remediation Strategies

Three mains strategies have been developed to exploit microbial capacities for in situ remediation of aquatic environments in order to reduce toxic effects of pollutants. These strategies are described herein, and comparisons are provided in Table 1.

Natural attenuation, also known as “intrinsic remediation,” refers to naturally occurring physical, chemical, and biological processes that reduce contamination level and toxicity risk without the need of human intervention. Within these processes, microbial activities play a key role. Natural attenuation is much more than a “wait and see” process, as it is based on appropriate follow-up monitoring to demonstrate the success of natural bioremediation processes in reducing contamination level. It can be applied when evidence of performances of natural processes is demonstrated and remains efficient during the remediation treatment. In comparison to active remediation processes, it can be cost-effective and less disruptive (more respectful) for the environment. There are cases where natural attenuation is the only possible process (e.g., sites that are difficult to access). In contrast, there are cases where it cannot be applied (inefficient bioremediation), and therefore, active bioremediation processes have to be implemented. The use of natural attenuation as a remedial technique has been reported mainly for groundwater ecosystems and vadose zones, contaminated with chlorinated solvents or chemicals, pesticides, and oil compounds such as BTEX (benzene, toluene, ethylbenzene, and xylenes).

Biostimulation involves environmental modification to stimulate indigenous microorganisms capable of bioremediation. As such, it is dependent on the indigenous organisms, their degradation capacities, and their growth requirements. Environmental alteration must have the desired bioremediation effect and avoid a dystrophic crisis (Dibble and Bartha 1979; Xu et al. 2003). Biostimulation can be achieved by adding various forms of rate-limiting nutrients (e.g., carbon, nitrogen, phosphorus) and electron acceptor/donors (e.g., acetate, oxygen, nitrate, sulfate) for enhancing microbial growth and activity. Surfactants can also be added as

Microbial Bioremediation of Aquatic Environments, Table 1 Comparison of in situ bioremediation strategies in aquatic environments

Bioremediation strategy	Advantage	Disadvantage
Natural attenuation	Inexpensive	May be a slow process
	Suitable for sites difficult to access	Requires long-term monitoring
	No human intervention	Effective at a limited number of sites
	Respectful of the environment	Inadequate for heavily polluted sites
Biostimulation	Cost-effective	Dependent on site access
	Use of autochthonous microorganisms	Additives difficult to spread
		Uncertain results depending on indigenous microbial capacities
Bioaugmentation	Introduction of microorganisms with appropriate catabolic pathways	Can be expensive
	Increased rate of remediation	Maintenance of microorganisms not ensured: Dependent on environmental factors (e.g., temperature, pH, presence of electron acceptors) Inhibition by physical and chemical factors (e.g., pH, toxic contaminants, bioavailability) Competition with indigenous bacteria Difficult to follow the fate of added microorganisms

potential agents for enhancing solubility and increasing the bioavailability of contaminants; they include chemical detergents (e.g., Triton X-100, Tween-80) or biosurfactants (e.g., rhamnolipids, sophorolipids, surfactines).

Other biostimulation strategies have been applied, including the addition of substances more amenable to biodegradation than the target contaminant in order to stimulate microbial cometabolic transformation of pollutants, which would otherwise not be degraded (Andreoni and Gianfreda 2007). Alternatively, pollutant removal rates have also been stimulated by generating an optimal balance of physical factors, such as temperature and buffering environmental pH by altering the redox state and electrokinetic state of the contaminated environment (Pandey et al. 2009).

Among the engineered bioremediation processes employed when natural attenuation is inefficient, biostimulation is the most frequently used since it can increase both microbial activities and pollutant bioavailability. For example, in the case of oil spills, biostimulation is the first implemented remediation strategy. Dispersants and surfactants are largely used in order to disperse and solubilize hydrocarbon compounds, together with nutrients for enhancing microbial activity as hydrocarbon degradation is likely to be limited by inorganic nutrient concentration even in high nutrient areas such as estuaries.

Bioaugmentation or bioaddition consists in the addition (augmentation) of microorganisms with specific catabolic abilities that are produced under controlled conditions, to speed up or enable a remediation task in a given environment. Most frequently, the inocula used for bioaugmentation include mixed or pure cultures. The inocula can originate from the polluted environment (indigenous strains) or can be selected from other environments, involving the addition of exogenous microorganisms. Mobile genetic elements or genetically modified microorganisms (GMOs) can also be used. However, their use is still under debate because there is uncertainty on assessing the risk of GMOs for the environment and human health. Consequently, many countries have placed legal barriers on the release of GMOs for site cleanup applications (da Silva and Alvarez 2010).

Bioaugmentation is applied when biostimulation is inefficient or when indigenous strains do not have the metabolic capability to perform the remediation process. However, the efficacy of bioaugmentation is subject to discussion. Bioaugmentation is used to ensure in situ bioremediation of chlorinated solvents (e.g., chlorinated ethenes, such as tetrachloroethylene and trichloroethylene) and is now increasingly being used to enhance the biodegradation of recalcitrant organic pollutants in groundwater ecosystems. In order to ensure the success of bioremediation in aquatic environments, bioaugmentation coupled with biostimulation is often implemented. Indeed, the dynamics of ecosystems induce modifications to both microbial communities and environmental factors; therefore, a successful bioremediation process combines the different approaches in order to place the right microorganisms with the appropriate environmental conditions for an optimal degradation. Thus, the bioremediation treatment must be tailored along the entire process, based on appropriate follow-up monitoring.

Microbial Processes

The strategies described above are based on microbial activity. Microbes act on pollutant transformation directly, resulting in the production of specific metabolites, or indirectly as the consequence of their basal metabolism. The processes involved include biodegradation, biotransformation, and bioaccumulation of pollutants.

Microbial degradation is the breakdown of organic materials and chemicals by microorganisms. Microbes use organic compounds as carbon and energy sources for their development (growth). Complete degradation of an organic compound leads to its mineralization with production of carbon dioxide. However, degradation is at times incomplete resulting in the production of metabolites that are expected to be less toxic than the initial compounds. Microorganisms, due to their genomic plasticity, have an astonishing capacity to adapt their metabolism to diverse environments. As a result, the microbial world presents an extraordinary catabolic diversity leading to the degradation of almost all molecules existing on earth even those

synthesized by humans such as pesticides or pharmaceuticals. The biodegradation process can occur aerobically, with oxygen, or anaerobically, without oxygen. The aerobic process is considered the most efficient, but its efficiency is dependent on the compound itself.

Several catabolic pathways for the degradation of hydrocarbon compounds are well known (Head et al. 2006; Widdel and Rabus 2001). Recently, it has been demonstrated that a group of marine bacteria, namely, the obligate hydrocarbonoclastic bacteria (OHCB), plays a key role in the biological removal of petroleum hydrocarbons from polluted marine waters (Yakimov et al. 2007). The genes involved in aerobic biodegradation processes have been well described – for example, ring-hydroxylating dioxygenases (RHD genes: *phnAc*, *nahAc*; Bordenave et al. 2008) for aromatic compounds and alkane hydroxylase (*alkB*; Van Beilen et al. 1994) for aliphatic compounds – but the anaerobic processes are less understood (e.g., *bss* operon for toluene; Leuthner et al. 1998). Similarly, genes involved in pesticide degradation have been described (e.g., *atz* and *trz* for atrazine, Devers et al. 2007; *opd* for organophosphate compounds, and *lin* operon for lindane; Singh and Walker 2006). Several microbes are also indirectly involved in the degradation of organic compounds; they produce biosurfactants and bioemulsifiers that increase the bioavailability of compounds (Satpute et al. 2010). However, an increased toxicity has been observed to marine life as a result of elevated hydrocarbon dissolution when surfactants are used (Epstein et al. 2000). Thus, increasing bioavailability of chemicals is not necessarily beneficial for remediation of contaminated environments.

Biotransformation usually relates to the transformation of inorganic (mainly metallic) compounds resulting in the modification of their speciation. Microorganisms use their capacity to transform metallic compounds for electron exchange (where metal is used either as an electron donor or acceptor) and/or for detoxification without metabolic advantages. Many metallic compounds are transformed directly by microorganisms, such as mercury, copper, tin, cadmium, arsenic, and others. In some cases, this biotransformation reduces toxicity (e.g., the demethylation of methyl mercury and the oxidation of arsenite into arsenate). Conversely, biotransformation can also produce toxic metallic species (e.g., mercury methylation; arsenate reduction). These biologically mediated transformations are, in some cases, well known and the genes involved characterized (e.g., *mer* operon for mercury biotransformation, *ars* operon for arsenic reduction, *aox* operon for arsenic oxidation; Silver and Phung 2005).

Biotransformation of metals can result from bacterial metabolism, not directly related to specific genes. For example, sulfate-reducing bacteria (SRB) are able to remediate metals or metalloids (such as cadmium, copper, iron, lead, mercury, nickel, zinc, arsenic, antimony, and molybdenum) by production of sulfide that precipitates metals into sulfide mineral complexes. This phenomenon is called "protection by sulfides" (Utgikar et al. 2001). Coprecipitation of metals with phosphate released from hydrolysis of an organic phosphate has also been shown

to be an effective method for metal remediation by forming insoluble metal phosphate complexes on the surface of cells (for a review, see Gadd 2004).

Bioaccumulation is an active process characterized by the intracellular accumulation (absorption) of molecules (organic or inorganic compounds). In contrast, biosorption is a passive adsorption that can be carried out by both dead and living cells. Bioaccumulation occurs in two stages, the first consisting of absorption of molecules to the surface of cells followed by transport of molecules intracellularly. This second stage is slower and frequently involves an active transport system (such as Mer, Ars, Cop transport systems). Bioaccumulation is a metabolism- and energy-dependent process that requires active respiration. Inside the cell, the pollutant binds to intracellular structures, mainly proteins that are generally synthesized in response to its presence. Hence, it is generally accepted that bioaccumulation by adapted microorganisms is more efficient than that from non-adapted microorganisms. Although several bacteria (e.g., *Pseudomonas* or *Escherichia* species) are known for their bioaccumulation capacities, this process is better documented for yeast. Indeed, a large yeast biomass can be obtained at almost no cost from the fermentation industry. For example, strains belonging to *Candida tropicalis, Saccharomyces cerevisiae, and Kluyveromyces marxianus* species are efficient in accumulating both metal ions (Cd(II), Cu(II), Cr(III), Cr (VI)) and organic compounds such as various textile dyes (Remazol Blue, Reactive Black, and Reactive Red) (Aksu and Dönmez 2000; Aksu 2005; Chojnacka 2010).

Bioremediation Monitoring

Monitoring bioremediation processes is essential to demonstrate efficiency. Different indicators for chemical degradation and microbial activities, as well as toxicological and ecotoxicological risks, have to be implemented. Microbial activity indicators include metabolic activity (e.g., respiration, photosynthesis), identification of intermediate (e.g., benzylsuccinate for anaerobic toluene biodegradation) and final metabolites, and catabolic gene expression (e.g., ring-hydroxylating dioxygenase for aerobic aromatic hydrocarbons biodegradation).

Examples of Microbial Bioremediation Strategy Applications

Hydrocarbon-polluted sites and oil spill catastrophes provide several examples of microbial bioremediation in aquatic environments.

A first example is provided by the construction of retention basins to improve natural attenuation in coastal areas. This system has been used in order to limit pollution in the Etang de Berre (Mediterranean coast, France), which receives effluents from a petrochemical factory. Monitoring of the remediation process

indicated that this basin had an efficient retention effect and that sediments showed effective biodegradation. Concomitantly bacterial community structures were correlated with the level of oil contamination (Païssé et al. 2008).

During the Exxon Valdez oil spill in March 1989, bioaugmentation by seeding with cultured microorganisms and biostimulation by modifying environmental conditions were applied as bioremediation strategies. Although the efficiency of bioaugmentation could not be demonstrated, addition of fertilizers providing nitrogen, phosphorus, and surfactant proved to be a useful bioremediation approach (Atlas 1995). Indeed, the number of oil-degrading microorganisms increased and biodegradation rates were enhanced as a result. Page et al. (2002) demonstrated with the standard amphipod bioassay using *Rhepoxynius abronius* that toxicity of weathered oil was reduced 1 year after the accident. The effectiveness of various organic fertilization treatments was also evaluated during the 10th month of a bioremediation experiment performed in situ in a subantarctic environment. Using the Microtox solid-phase test, Pelletier et al. (2004) showed that toxicity of oiled residues was significantly reduced during the first 6 months of the process; however, it increased again in the last months of the experiment.

Conclusion

Management of microbial resources is becoming a promising strategy for the in situ bioremediation of aquatic environments. Different human intervention levels – from basic monitoring to microorganism additions and more sophisticated treatments – have been successfully applied. However, further research is still needed to gain knowledge in microbial metabolic capacities and in the ecology of microorganisms for elucidating structure–function relationships and to increase the culture collection of microorganisms with remedial abilities. Such knowledge will provide the basis for successful interventions into environmental processes, leading to improved strategies for bioremediation with optimal removal rates and efficient reduction of toxicity.

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Cross-References

▶ Bioavailability of Contaminants
▶ Biodegradability in Ecotoxicology
▶ Environmental River Biofilms as Biological Indicators of the Impact of Chemical Contaminants
▶ Monitoring of Oil-Degrading Bacteria by Stable Isotope Probing

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Microbiotests in Ecotoxicology

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Article Outline

Synonyms
Glossary
Definition
Historical Background
Characteristics of MBTs
Types of MBTs
Illustration of a MBT
Applications
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Synonyms

Microbioassay; Microscale test; Microtest; Small-scale toxicity test

Glossary

Acute bioassay An aquatic toxicity test yielding a measurable effect response in a relatively short period of time. For example, an acute effect for exposed fish usually occurs within a 96 h exposure. For microalgae, such a response can occur within 4 h after exposure. The time of exposure leading to an acute effect is thus taxon specific.

Biomonitoring Employing living organisms (flora and/or fauna) as sentinel species in the surveillance of water quality to evaluate (temporal or spatial) changes in an effluent or receiving water body in order to verify whether biota may be at risk.

Chronic bioassay An aquatic toxicity test yielding a measurable effect response after a relatively long period of time which can span for a few days to years depending on the life cycle of the aquatic species considered. For example,

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a chronic effect for exposed fish might be measured only after several months or years of exposure. For microalgae, a chronic toxicity response is usually measured after two or more days of exposure and is linked to its cell division cycle. The time of exposure leading to a chronic effect is thus taxon specific.

Cryptobiotic preservation Relating to the dormant stage of a particular microorganism or organism. Examples include cyst formation in micro-invertebrates such as water fleas (e.g., *Daphnia magna*) or the embedding of physiologically active algal cells (e.g., *Selenastrum capricornutum*) in an alginate matrix to produce algal beads. Water fleas can later be hatched "on demand" to conduct biological testing, as can be algal cells once they are removed from their beaded matrix. (Blaise and Férard 2005a).

Effects-based approach A strategy in ecotoxicology whereby the toxic potential of a liquid (effluent, receiving water) or solid (sediment) sample is determined by measuring effects resulting from the exposure of living organisms to such samples.

Decomposers An organism (e.g., a bacterium or protozoan) that feeds on dead or decaying plants and animals, transforming them chemically, thereby contributing to recycling (in)organic materials to the environment. (Blaise and Férard 2005).

Intercalibration exercise (round-robin, ring test) A multi-laboratory testing exercise designed to assess the reproducibility of a toxicity test method. This is a necessary step required in the validation process of a bioassay procedure to confirm its reliability. Successful intercalibration exercises can eventually lead to the international recognition and standardization of the test method being evaluated.

Lyophilization Process which extracts water from biological products or field samples, so that they remain stable over time. It is carried out using a principle called sublimation, which is the transition of a substance from the solid to the vapor state. Synonymous term is freeze-drying. (Blaise and Férard 2005).

Phyla (plural of phylum) A taxonomic grouping of animals based on general body features (e.g., form, development, or internal organization). For example, crabs belong to the phylum *Arthropoda*, whereas earthworms are part of the phylum *Annelida*. Other major animal phyla are the following: *Mollusca* (e.g., bivalves), Porifera (e.g., sponges), *Cnidaria* (e.g., hydra), *Platyhelminthes* (e.g., flat worms), *Nematoda* (e.g., round worms), *Echinodermata* (e.g., star fish), and *Chordata* (e.g., human beings).

Portability Said of a MBT whose compactness and robustness allow it to be transported and used in a field situation.

Primary consumers Animals that eat, for example, green plants or algae in a food chain. (Blaise and Férard 2005).

Primary producers Autotrophic organisms (plants and algae) which synthesize organic matter from inorganic materials (e.g., algae photosynthesize sugars from CO_2). (Blaise and Férard 2005).

QA/QC QA (quality assurance): a laboratory program designed to ensure accurate and precise generation of toxicity data which includes, for example, the proper selection and use of technical procedures, laboratory equipment, and collection and

preservation of samplesQC (quality control): specific requirements designed to provide information linked to the QA program, such as standardization, calibration, replicate testing, built-in controls, and statistical validation of produced data.

Secondary consumers Animals that eat other animals (e.g., primary consumers) in a food chain.

Species A specific type of organism found in an aquatic ecosystem (e.g., bacterium, alga, invertebrate).

Taxa (plural of taxon) A taxon refers to a group of organisms that share common characteristics (e.g., bacterial species as opposed to protozoans or invertebrates). Defining what belongs to a taxonomic group, and what criteria should be considered to distinguish a taxon from another, is based on classification systems proposed by taxonomists.

Taxonomic groups Groups of organisms that are classified into specific units (taxa) based on features that set them apart from other groups. Taxonomy is the science that distinguishes animals or plants and places them into logical arrangements or classes.

Water quality criterion The maximum concentration of chemical or other water constituent deemed safe to protect an organism, an aquatic community, or a prescribed water use. If exceeded, an aquatic community, or a part thereof, may be at risk.

Definition

Microbiotesting: The Exposure of a Unicellular or Small Multicellular Organism to a Liquid or Solid Sample to Measure a Specific Toxic Effect (in the Context of Aquatic Toxicology).

The wide array of (micro)organisms available to conduct toxicity testing can comprise representatives of different species of taxonomic groups commonly found in either freshwater or marine aquatic ecosystems. They can include decomposers (bacteria, protozoans), primary producers (microalgae, small macrophytes), primary consumers (micro-invertebrates), secondary consumers (small fish or life stages thereof, cnidarians), as well as various types of cell lines.

The application of aquatic microbiotests (MBTs), quite often employed as initial screens to assess and rank the toxic potential of chemicals and environmental samples (e.g., effluents and sediments), is frequent owing to their attractive features which include cost-effectiveness and ease of testing.

Historical Background

As early as the 1960s, fish bioassays were initially employed to assess the hazard/risk of pollutants as an important complement to chemical analysis (Blaise et al. 1988).

In ensuing decades, particularly from the 1980s onward, a large number of assays representative of different levels of biological organization were developed so as to detect the full toxic potential of chemicals and complex environmental media (liquids and solids) owing to the fact that toxicity can be trophic level-specific. Hence, numerous microbiotests (MBTs) have become available to users desirous of applying an effects-based approach to ensure aquatic environmental protection.

Characteristics of MBTs

Attractive features of most MBTs that have made their use popular internationally are numerous and include, for example, simplicity of testing, cost-effectiveness, high sample throughput, low sample volume requirements, availability of maintenance-free cultures owing to lyophilization or cryptobiotic preservation of (micro) organisms, sensitivity and rapid turnaround time to results, robotic initiation of procedure and of postexposure endpoint measurement (particularly for microplate-based MBTs), as well as portability (Blaise 1991, 1998; Blaise et al. 1998a, b).

Types of MBTs

Table 1 describes several MBTs currently employed in the field of aquatic toxicology. Thanks to sustained efforts on the part of individuals and standards organizations, reliable MBTs such as these have resulted, after having been fully validated via intercalibration exercises and/or built-in QA/QC in their experimental protocols. While this list of MBTs is far from exhaustive, the reader will appreciate that MBTs span across different taxa/phyla and that toxicity responses are evaluated from a broad spectrum of species. This is crucial to ensure sound hazard assessment of contaminants that may impact aquatic ecosystems.

Illustration of a MBT

A typical protocol of how a MBT can be initiated is illustrated in Fig. 1. Miniaturization, modest bench space, and ease of testing by employing a 96-well microplate format and multichannel pipetting highlight this particular MBT. Figure 2 shows the recommended experimental configuration for dispensing micro-volumes into the microplate.

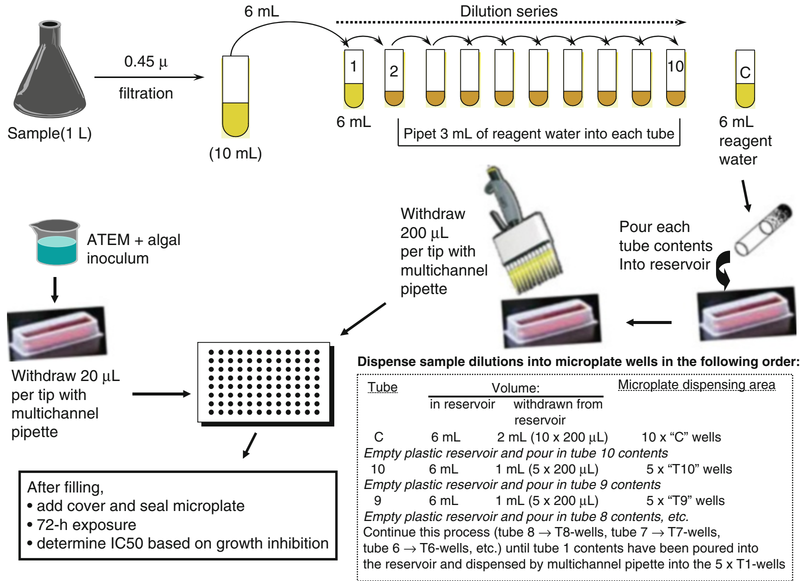
Applications

Applications are versatile, and along with other tools and approaches in ecotoxicology, microbiotesting contributes to the protection and conservation of the aquatic

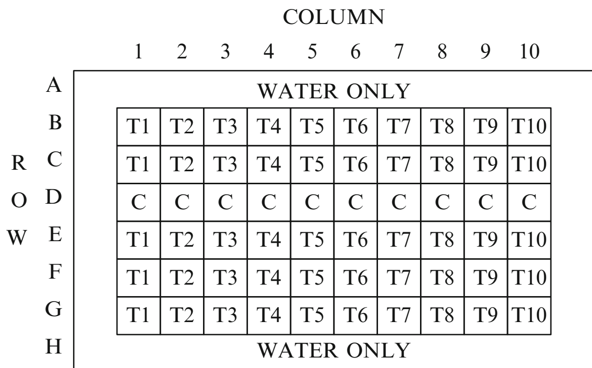
Microbiotests in Ecotoxicology, Table 1 Characteristics of some current MBTs used in aquatic toxicological studies

Trophic level	Toxicity test	Assessment endpoint	Measurement endpoint	References
Liquid phase assays				
Decomposer	Bacterial test *Vibrio fischeri* (Microtox® toxicity test)	Acute sublethal light inhibition	15 min-IC25	Environment Canada (1992)
Decomposer	MARA (Microbial array for risk assessment) assay	Growth inhibition of 11 microbial species	18 h-MTC[a]	Gabrielson et al. (2003)
Primary producer	Algal test (*Pseudokirchneriella subcapitata* microplate assay)	Chronic sublethal growth inhibition	72 h-IC25/ IC50	Blaise and Vasseur (2005)
Phototrophic assay	Luminotox assay with PECs[b]	Inhibition of photosynthetic efficiency	15 min-IC20	Boucher et al. (2005)
Primary consumer	Microcrustacean *Thamnocephalus platyurus* test (ThamnoToxkit assay)	Acute lethality	24 h-LC50	Microbiotests Inc., http://www.microbiotests.be/
Secondary consumer	Cnidarian test (*Hydra attenuata* assay)	Acute sublethality indicated by morphological changes	96 h-EC50	Blaise and Kusui (1997)
	Fish cell test (rainbow trout primary hepatocyte test)	Acute cytotoxicity	48 h-TEC[c]	Gagné (2005)
Solid-phase assays				
Decomposer	Bacterial test *Vibrio fischeri* (Microtox® toxicity test)	Acute sublethal light inhibition	20 min-IC25	Environment Canada (2002)
Phototrophic assay	Luminotox assay with PECs[b]	Inhibition of photosynthetic efficiency	15 min-IC20	Dellamatrice et al. (2006)
Primary producer	ASPA (algal solid-phase assay) with *Pseudokirchneriella subcapitata*	Chronic sublethal inhibition of esterase activity	24 h-IC50	Blaise and Ménard (1998)

[a]MTC or microbial toxic concentration (essentially corresponding to a 50% effect: see Gabrielson et al. 2003)
[b]PECs: photosynthetic enzyme complexes isolated from spinach leaves
[c]TEC (threshold effect concentration) for cytotoxicity as manifested by a significant reduction in cell viability = $(\text{NOEC} \times \text{LOEC})^{1/2}$, where NOEC = no observed effect concentration and LOEC = lowest observed effect concentration



Microbiotests in Ecotoxicology, Fig. 1 Typical sample dilution procedure for undertaking the algal microplate toxicity test listed in Table 1 (Blaise and Vasseur 2005). *ATEM* algal test enrichment medium (Reproduced with permission from Springer)



Microbiotests in Ecotoxicology, Fig. 2 Recommended configuration for dispensing micro-volumes into a 96-well microplate for phytotoxicity testing (Blaise and Vasseur 2005). C = control wells; T = test concentration wells. (Reproduced with permission from Springer)

environment. For example, small-scale acute and chronic bioassays have served to rank and screen chemicals in terms of their hazardous potential, to undertake biomonitoring studies, to derive water quality criteria for safe release of specific chemicals into receiving ecosystems, and to assess industrial effluent quality in support of compliance and regulatory statutes. Several key publications have emphasized and detailed the comprehensive ways in which MBTs have served, and continue to serve, the scientific community at large in the field of ecotoxicology (Wells et al. 1998; Blaise et al. 2000; Persoone et al. 2000; Blaise and Férard 2005).

MBTs in the World of Science

Initiated in 1983, the ISTA symposium (International Symposium on Toxicity Assessment) is a leading forum dedicated to research and development activities conducted in the area of microbiotesting. While the present themes it promotes are diverse and linked to current issues in ecotoxicology, MBT investigations continue to be an important component of its scientific program. ISTA symposia are also associated with international peer-reviewed Journals, such as Environmental Toxicology (Wiley Publishers) and Environmental Science and Pollution Research (Springer Publishers), from which dedicated post-ISTA special issues are published and in which MBT studies are often highlighted.

Conclusions and Prospects

MBTs comprise practical effects-measurement diagnostic tools for aquatic ecotoxicology. They are an essential component of environmental management programs to assess toxicity of both liquid and solid media. The field of microbiotesting should markedly expand in the future in part owing to breakthroughs in instrumental technology and robotization that will continue to enhance their sample throughput and reliability.

Acknowledgments The author is indebted to ***Springer publishers*** for reproduction of Fig. 1 taken from Blaise and Vasseur (2005).

Cross-References

- ▶ Bacteria in Ecotoxicology: Microtox Basic
- ▶ Biological Test Methods in Ecotoxicology
- ▶ Cell Lines in Aquatic Toxicology
- ▶ Hydra in Ecotoxicology
- ▶ Landfill Leachate Ecotoxicity
- ▶ Microbial Assay for Risk Assessment (MARA)
- ▶ Sediment Ecotoxicity
- ▶ Test Batteries in Ecotoxicology

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Suggested Resources

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Mixture Effects in Ecotoxicology

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Article Outline

Synonyms
Glossary
Definition
Historical Background
Mixture Theory Evaluated by Testing
Ecological Risk Assessment of Mixtures
Conclusions and Prospects
Cross-References
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Synonyms

Mixture ecotoxicity; Multiple stressor ecotoxicity

Glossary

Antagonism A class of interactive joint action between compounds where the potency effects of the mixture are lower than expected, i.e., where the joint action is interactive.

Assessment endpoint Parameter measured after a test exposure on a given organism, e.g., mortality and reproduction. A measurement endpoint designates calculated values such as NOEC or EC50.

Baseline toxicity Baseline toxicity is the minimal toxicity of any given chemical. It is due to a nonspecific mode of action that exerts a narcotic toxicity. For example, the toxicity of triazines on insects or fish can be seen as baseline toxicity. Indeed triazines act as photosynthesis inhibitors, but this process has no effect on these organisms.

Concentration addition (CA) The concentration addition (or dose addition) model is commonly used to predict mixture toxicity for similarly acting compounds (with a similar mode of toxic action). This concept was originally described by Loewe and Muischnek (1926).

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Independent action (IA) or response addition (RA) Independent action (or response addition) is commonly proposed to predict mixture toxicity for substances with dissimilar actions (with different modes of toxic action). This concept was originally described by Bliss (1939).

Interactive joint action When two or more compounds in a mixture interact, they will affect each other's toxicity. For example, one compound can make a complex with another substance and prevent it from exerting its toxic action.

Mode of action (MoA) This is a general term to describe a chemical action. The primary mode of action, often exerted at the receptor level, is often used to predict the toxicity of a mixture of substances. An example of primary mode of action is photosystem II inhibition exerted by triazine herbicides. It should be recalled that the mode of toxic action of many substances is largely unknown, especially for environmental species.

NOEC No observed effect concentration. It refers to the highest tested concentration, for which the average response of the organism is not statistically different from the control.

Sites of action Generally, the site where a substance will act, i.e., it will affect the system and exert its toxicity.

SSD Species sensitivity distribution curves representing the distribution of sensitivity to a given substance among different environmental species.

Synergism A class of interactive joint action between compounds where the potency effects of the mixture are greater than expected, i.e., where the joint action is interactive. Synergism can be observed in certain formulations of substances involved in plant protection or biocidal or pharmaceutical products.

Taxonomic group Taxonomic groups classify organisms in an ordered system that identifies their natural relationships. This can be at the level of the class, family, genus, etc.

Definition

Combined effects of different stressors that may impact living organisms.

Organisms in the environment are typically exposed to a large variety of stressors. They are inherent to their changing environment (temperature, nutrients, light, etc.) or linked with chemical pollution (organic and inorganic compounds, nanoparticles, etc.).

Growing production and use of chemicals is expected to generate increasing environmental concerns in the future. As a result, organisms might permanently be exposed to a multitude of substances that could affect their life cycle. Even if each single compound of a mixture is present at, or below, its NOEC level, they may in combination exhibit a significant adverse effect, as has been shown by several researchers (Altenburger et al. 2000; Silva et al. 2002; Arrhenius et al. 2004). Mixture effects assessment has therefore been of growing interest for several years. Indeed, traditional ecotoxicological testing was usually designed to assess

a single chemical and did not take into account joint actions with other substances. Mixture toxicity depends on several factors such as the number, kind, and concentration of each compound present in the mixture as well as on the organisms exposed according to the sensitivity of their receptors. Prediction of mixture toxicity is therefore not a trivial endeavor.

Effects owing to joint action between substances are generally called mixture effects, whereas effects linked to interactions between substances and environmental changes or between different environmental changes are called multiple stressor effects.

Historical Background

The first authors who described joint actions of compounds were Loewe and Muischneck (1926) and Bliss (1939). Plackett and Hewlett (1952) later resumed these concepts and proposed a classification of joint effects of substances and toxicological models to predict these effects (Fig. 1).

For these authors, the mode of action (MoA) determines the type of joint actions. Basically, if the substances have a similar MoA, but do not interact with each other (have no interactive joint action), they will have a simple similar action, also called "concentration addition (CA)":

$$ECx_{mixCA} = \left(\sum_{i=1}^{n} \frac{p_i}{EC_{xi}}\right)^{-1} \text{ with } p_i = \frac{c_i}{c_{mix}} \quad (1)$$

ECx_{mixCA}: mixture concentration having x% of effects following CA
c_i: concentration of compound *i*
c_{mix}: concentration of the mixture
EC_{xi}: effect concentration x% for compound *i*

Even for a mixture of substances with different specific MoAs, CA can be applied if the organisms exposed do not have the specific receptors. Substances in this case may exhibit the same nonspecific MoA, referred to as a baseline toxicity. It may hold also for substances at extremely low concentrations, below a threshold at which they may not exert their specific MoA (de Zwart 2005a).

If substances act by a dissimilar MoA on a given species, and do not interact, they will have an independent joint action, also called "independent action (IA)":

$$E(c_{mixIA}) = E(c_1 + \cdots + c_n) = 1 - \prod_{i=1}^{n} \left[1 - E_{(c_i)}\right] \quad (2)$$

$E(c_{mixIA})$: predicted effect of the mixture following IA
$E_{(ci)}$: effect of compound i at concentration c

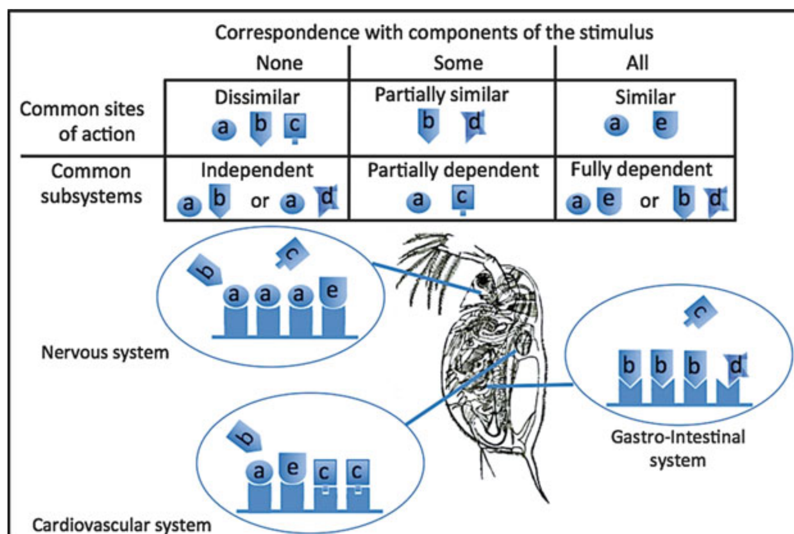
Mixture Effects in Ecotoxicology, Fig. 1 Possible joint actions between substances following Plackett and Hewlett (1952) and Hewlett and Plackett (1959). The mode of action of substances determines the type of joint actions

	Similar joint action	Dissimilar joint action
Non-interactive	Simple similar action	Independent joint action
interactive	Complex similar action	Dependent joint action

When applying this model, we assume that no correlation exists in the tolerance of individual organisms within a single species population to different pollutants (Bliss 1939). Indeed, a correlation coefficient of organism tolerance (r) exists between two substances and can cover a range between -1 (where individuals most susceptible to one pollutant are the least susceptible to the other) and 1 (where individuals most susceptible to one pollutant are also the most susceptible to the other) (Könemann 1981). When we assume no correlation for the IA model, we assume an r coefficient equal to 0. For mixtures with more than two chemicals, Könemann (1981) considers that coefficient r varies between 0 and 1 and defines two extreme cases: (1) the coefficient is equal to 0 and we can apply Eq. 2, and (2) the coefficient is equal to 1 and mixture effects are driven by the most toxic chemicals within the mixture. The author calls this case "no addition." Note that the IA model is applied without evaluating the r coefficient in most cases.

If the substances interact, we can observe synergism and/or antagonism, which is difficult to model and to predict. However, it is possible to detect such effects by comparing results of mixture toxicity experiments and prediction by models CA and IA. Furthermore, Könemann (1981) proposed a "mixture toxicity index" that provides information on the type of mixture toxicity according to a scale of five different classes: antagonism, no addition, partial addition, concentration addition, and supra-addition.

A classification scheme in strictly four types of joint action (Fig. 1) was criticized by de Zwart and Posthuma (2005), in particular, because it was defined to predict effects of binary mixtures. A more complex classification strategy was proposed by Ashford (1981). It integrates the fact that an organism is composed of several more or less complex subsystems (e.g., nervous, endocrine, cardiovascular), each containing different sites of action, at which the compounds can act fully, partially, or not (Fig. 2). The inhibition of each subsystem according to the mixture contributes differently in intensity to an overall effect on a critical endpoint. If this classification outlook is interesting and better describes the complexity of the system, it is hard to apply in ecotoxicology owing to lack of data. For example, sites of action of specific compounds for the wide variety of species present in ecosystems are largely unknown.



Mixture Effects in Ecotoxicology, Fig. 2 Joint actions in complex mixtures (as proposed by Ashford 1981) depending on the type of substances, the type of subsystems, and the type of sites of action. An illustration is given for five different substances (a, b, c, d, e) in different subsystems of a daphnid. The substances can have a common site of action (a & e), commonly called primary MoA, or can have effects on common subsystems without having a similar primary MoA (a & c). In contrast, they can be completely dissimilar at the site of action level (a & c) or at the subsystem level (a & b). Several situations are possible between these two extremes. For example, a subsystem could also be partially inhibited (for instance, the cardiovascular system if substance c is not in the mixture). The inhibition of each site of action/subsystem will contribute differently to an overall effect that could impact growth, reproduction, mortality, etc.

Mixture Theory Evaluated by Testing

In aquatic ecotoxicology, CA and IA models were successfully used to predict the effects of similarly and dissimilarly acting compounds, respectively (for reviews, see Backhaus et al. 2003 and Belden et al. 2007), but on a limited number of species. In general, CA showed a better prediction than IA for mixtures composed of similarly acting substances and conversely for mixtures with dissimilarly acting chemicals (Altenburger et al. 2000; Backhaus et al. 2000). However, the use of both models to predict the toxicity of mixtures of heterogeneous compounds (similarly and dissimilarly acting substances) is still rare. In the case of complex mixtures, application of the CA concept alone is often advised as a “worst case” prediction. Indeed, different studies showed a slight overestimation of observed toxicity with this model (Backhaus et al. 2003). However, overestimation seems not to be overprotective for pesticide mixtures under realistic exposure scenarios (Junghans et al. 2006).

Nonetheless, if studies conducted until now seem to support the use of CA and IA in case of similarly/dissimilarly acting compounds, a systematic evaluation of these theoretical models is still missing, especially regarding the different species and different endpoints that may be involved in the process. Cedergreen et al. (2007) showed that mixture effects can vary between the assessment endpoints measured, notably between the response measured at the biomarker level and at the population level, thereby displaying the complexity involved in mixture effect predictions.

Ecological Risk Assessment of Mixtures

Some authors recently proposed new approaches to include mixtures in risk assessment by combining SSD approaches with the CA/IA models (de Zwart 2005b; Chèvre et al. 2006). One of the main assumptions underlying these approaches is that mixture models proposed for single species effect assessment can also be used for risk predictions at the ecosystem level. In other words, all species exposed to the mixture will react similarly to the compounds, that is, following the CA and/or the IA model. If this assumption seems to hold ground for organisms from the same taxonomic group (e.g., algae regarding the toxicity of photosystem II inhibitors), it seems to be less defendable when assessing effects on both fish and algae, for example. Therefore, CA/IA should be applied to group of species that react similarly to a specific kind of substances.

A second important assumption is linked with correlation of species sensitivities to the different compounds of the mixture. For CA, the classification of species sensitivity should be the same for all substances included in the mixture. For IA, in contrast, the most likely probability is that they are independent (de Zwart 2005a). Furthermore, one important limit regarding the application of these concepts is the lack of knowledge on different MoAs, especially considering the diversity of organisms that may be affected. A more systematic testing of the hypothesis underlying the proposed approaches needs to be undertaken.

However, comparison of mixture toxicity predictions with field investigations has shown encouraging similarities (Posthuma and de Zwart 2006). Microcosm or mesocosm studies have also allowed interesting comparisons between mixture toxicity predictions and community observations (Knauert et al. 2009). Such studies, however, are still rare, and there is a genuine need to validate risk prediction with field observation.

Conclusions and Prospects

It is now recognized that mixture effects have to be considered in risk assessment, in view of the wide variety of substances present in the environment. However, implementation strategies are still being debated, and the development of robust

approaches is challenging. In particular, some attention should be drawn to the following points in future:

- The current mixture models, CA/IA, should be tested more systematically on different species and also in combination.
- The observation of different mixture effects at different organism levels should be conducted to consider the actual mixture models critically. This could be done in collaboration with toxicologists who investigate mixture effects on humans.
- Further joint effects, calling into play synergism and antagonism, should also be considered in mixture effects evaluation.
- The assumptions underlying the use of mixture models for risk assessment should be posed rigorously and, if possible, tested. More comparisons between predicted and observed effects should also be conducted.

Cross-References

► Biology-Based and Population Dynamics Modeling in Ecotoxicology
► Evaluating Impacts of Multiple Stressors on Aquatic Ecosystems Using Isobolic Models
► Modes of Action of Chemical Pollutants

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Modes of Action of Chemical Pollutants

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Article Outline

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Glossary

Adverse outcome pathways (AOP) Conceptual framework that leads from the initiating event of interaction between a toxicant and a receptor in an organism over cellular and organ response to an adverse outcome at organism or population level (Ankley et al. 2010).

Baseline or nonspecific toxicity Minimal toxicity that any compound exhibits by partitioning into biological cell membranes, causing nonspecific disturbance of the integrity and functioning of cell membranes.

Biologically effective dose The biologically effective dose (BED) or the amount that actually reaches cells, sites, or membranes where adverse effects occur may represent only a fraction of the delivered dose, but it is obviously the best one for predicting adverse effects (cited from Paustenbach DJ 2000, The practice of exposure assessment: A state-of-the-art review. J Toxicol Environ Health B Crit Rev 3:179–291).

Dose (external) The dose of a toxicant that is external to the organism that can be used to quantify adverse effects (e.g., LD_{50}).

Dose (internal) That amount of a toxicant accumulated by an organism expressed as a tissue concentration in mass or molar units. The internal dose can be used to quantify adverse effects and is based on whole-body, organ-specific, or receptor-specific concentrations.

Excess toxicity (Te) Synonym to toxic ratio TR.

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Intrinsic potency A measure of the degree of specific effect, for example, how much more potent a compound is as compared to its baseline toxicity. A quantitative measure of the intrinsic potency is the toxic ratio.

Mechanism of toxic action Crucial biochemical processes and/or xenobiotic-biological interactions underlying a given mode of action (Rand G, Wells P, McCarty LS 1995, Introduction to aquatic toxicology. In: Rand G (ed.) Fundamentals of Aquatic Toxicology, 2nd edn. Taylor & Francis, Washington, DC, pp. 3–67).

Mode of toxic action or Mode of action (MOA) A common set of physiological and behavioral signs that characterize a type of adverse biological response (Rand G, Wells P, McCarty LS 1995, Introduction to aquatic toxicology. In: Rand G (ed) Fundamentals of Aquatic Toxicology, 2nd edn. Taylor & Francis, Washington, DC, pp. 3–67).

Narcosis mode of action Physiological and behavioral responses elicited by baseline toxicants and subcategories include nonpolar and polar narcosis and ester narcosis. Narcosis in this context refers to minimum toxicity that any compound exhibits and is not related to narcosis/anesthesia in clinical medicine.

Nonspecific mode of action Physiological and behavioral responses elicited by baseline toxicants, often used synonymously to "narcosis mode of action."

Physiological mode of action A set of observable effects on the life-history traits, such as feeding, growth, development, reproduction, and survival, a more specific definition of mode of toxic action.

Primary mechanism, primary effects The type and degree of interaction of a toxicant with biomolecules at the target site triggers the toxic effect and determines the primary mechanism of toxic action.

QSAR Quantitative structure-activity relationship.

Reactive toxicity Mode of toxic action that is associated with chemical reactions where covalent bonds are formed. Can be either direct reactivity of electrophilic chemicals with biological nucleophiles like DNA bases or proteins or indirect reactivity via reactive oxygen species that are formed indirectly from chemical pollutants.

Specific mode of toxic action A mode of toxic action that causes higher toxicity than baseline toxicity, either caused by specific interaction with receptors or enzymes or by reactive toxicity.

Toxic ratio (TR) Ratio between the effect concentration (e.g., EC50 or LC50) predicted by a baseline toxicity QSAR and the experimental effect concentration for the same endpoint. The TR is a measure of specificity of effect. A $TR < 10$ indicates nonspecific toxicity, while a $TR > 10$ indicates a specific mode of action. The value of TR is associated to the intrinsic potency of a chemical.

Toxicodynamics Processes linking the concentration at the target site to the observed effect.

Toxicokinetics Processes of absorption, distribution, metabolism, and excretion of a toxicant.

Definition

A mode of action (MOA) or mode of toxic action is a common set of physiological and behavioral signs that characterize a type of adverse biological response, which can be caused by a variety of toxic mechanisms that are defined as the crucial biochemical processes and/or xenobiotic-biological interactions underlying a given mode of action (Rand et al. 1995).

Historical Background

It was early recognized that information on the mode of toxic action is a vital piece of information for the prediction of aquatic toxicology of chemical pollutants (Könemann 1981; Hermens 1989). Consequently, a series of classification schemes have been developed over the years. In the following, five general approaches are distinguished that allow a systematic evaluation and classification of modes of toxic action:

1. MOA classification according to chemical structure and QSARs (quantitative structure-activity relationships) (Verhaar classification)
2. MOA classification according to physiological observations (fish acute toxicity syndromes (FATS) and beyond)
3. MOA classification according to "ecotoxicity profiles" and QSARs
4. MOA classification according to interaction with the target site
5. MOA classification based on genomics and proteomics information

A detailed overview of all these classification schemes and their historical development is given below. Approaches 1–3 have evolved simultaneously, have been refined for decades, and are still being refined. The Verhaar classification, the FATS classification, and the ecotoxicity profiles were recently compared (Escher et al. 2011), and a compilation is given in Table 1. Approach 4 builds up on all of these approaches in that it combines information on the target site with mechanistic information. As such it is not a really separate approach but integrates information on all previous. While "omics" technologies listed as approach 5 have the potential to be used for mode of action classification, a comprehensive and systematic classification scheme has not yet been developed up to date, presumably due to the difficulty to clearly assign upregulation of genes with a specific response. Therefore, they are discussed below, and promising examples are summarized, but they are not incorporated in the overview table.

It must be noted that the MOA is not a universal property of a chemical but related to the target organism. A given chemical may exhibit multiple mechanisms and different MOA for acute and chronic exposure as well as different MOAs in different organisms.

Information on the MOA of a chemical pollutant is not sufficient for risk assessment, but it provides valuable information in several steps during the risk

Modes of Action of Chemical Pollutants, Table 1 Comparison of the primary mechanism and mode of action classification schemes of [c](Escher et al. 2002), [d](Verhaar et al. 1992, 1996), [e](McKim et al. 1987a; Bradbury 1994) (*FATS* fish acute toxicity syndromes), and [f](Nendza et al. 1995, 2006). Note that it is not in all cases possible to come up with an unambiguous assignment. [a]m = membrane, *c* cytosol and other aqueous compartments in the cell, [b]*ns* nonselective, *s* selective (This table is reprinted from Escher et al. (2011) and reprinted with permission from John Wiley & Sons Ltd)

Target site (general)	Specific target site	Domain[a]	Interaction[b]	Molecular mechanism(s)[c]	Mode of action [c]	Verhaar classification[d]	FATS classification[e]	Ecotoxicity profiles[f]
Biological membrane	All membranes	m	ns	Nonspecific disturbance of membrane structure and functioning	Baseline toxicity	Nonpolar narcosis	Nonpolar narcosis	Nonpolar nonspecific toxicity
						Polar narcosis	Polar narcosis Ester narcosis	Polar nonspecific toxicity
	All membranes	m	s	Formation of reactive intermediates (e.g., reactive oxygen species) causing peroxidation of membrane lipids and proteins	Degradation of membrane lipids and membrane proteins	Reactive MOA		
	Energy-transducing membranes	m	ns, s	Ionophoric shuttle mechanisms	Uncoupling	Specific MOA	Uncoupling	Uncoupling
		m	s	Blocking of quinone and other binding sites on the mitochondrial respiratory chain	Inhibition of the electron transport chain	Specific MOA	Respiratory inhibitors	Respiratory inhibition
		m	s	Blocking of proton channels and other transport channels	Inhibition of ATP synthesis/ depletion of ATP	Specific MOA		
	Photosynthetic membranes	m	s	Blocking of photosynthetic electron transport	Photosynthesis inhibition	Specific MOA		Photosynthesis inhibition

Proteins, peptides	All proteins and peptides	m, c	ns, s	Electrophilic reactivity, alkylation and oxidation of proteins and glutathione (GSH)	Damage and depletion of biomolecules	Reactive MOA	Electrophilic/ proelectrophilic reactivity	SH-alkylation of non-protein SH reactive toxicity
	Specific enzymes and receptors	m, c	s	Non-covalent or covalent binding to enzymes and receptors	Inhibition or competition, e.g., acetylcholine esterase, estrogen receptor, and AH receptor	Specific MOA	Acetylcholine Esterase inhibition, central nervous system seizure	Acetylcholine Esterase inhibition, estrogenic activity
	Specific enzymes and receptors	c	s	Non-covalent or covalent binding to enzymes of the nucleic acid metabolism, effect on replication or repair	Indirect mutagenicity (DNA repair, recombination, regulation)	Reactive MOA		
DNA, RNA	All	c	ns, s	Base modification and damage: electrophilic (alkylation) and oxidative damage, bulky adducts	Direct mutagenicity (frameshift, cross-links, strand breaks, deletion, etc.)	Reactive MOA		

assessment process (Williams et al. 2009). Ankley et al. (2010) advocated the framework of "adverse outcome pathways" (AOP), which can help to better understand the differences between mechanism and mode of action and looks at mechanistic information of the molecular events triggering an adverse effect on organism or population level in a risk assessment framework. Recent developments to include mechanistic modeling in the risk assessment process rely heavily on information on modes of toxic action (Grimm et al. 2009; Preuss et al. 2009).

MOA Classifications

MOA Classification According to Chemical Structure and QSARs (Quantitative Structure-Activity Relationships)

QSAR analysis allows the discrimination between nonspecifically and specifically acting and reactive compounds. Nonspecific mechanisms (baseline toxicity) encompass nonpolar and polar narcosis. Nonpolar compounds conform to a QSAR that follows minimal toxicity, with all other mechanisms yielding higher toxicity compared to this QSAR (Lipnick 1991). Polar narcotic compounds are 5–10 times more toxic than estimated by the narcosis QSAR (Verhaar et al. 1992). Specifically acting and reactive compounds are 10–10,000 times more toxic (Verhaar et al. 1996). Specifically acting and reactive chemicals cannot be differentiated by QSAR analysis. Verhaar et al. (1992) developed structural rules to differentiate these two groups.

The general idea of the Verhaar approach was used to develop a series of classification models based on "toxicophores" (Rosenkranz et al. 1999), also called "reactive substructures" (Jackel and Nendza 1994; Nendza and Muller 2007), "biophores" (Rosenkranz et al. 1999), or "structural alerts" (von der Ohe et al. 2005). Toxicophores are structural subunits of a molecule that are responsible to trigger a given MOA. For example, the thiophosphate group with at least one good leaving group is a structural alert for acetylcholinesterase inhibition or an activated bond in alpha position to a double bond is a structural alert for reactive toxicity. A given structural alert is related to a defined MOA for a given species. Phenylurea functions, for instance, are structural alerts for photosynthetic organisms only because they usually are responsible for direct inhibition of photosynthesis, a MOA that is lacking in other organisms. Classification schemes based on the toxicophore concept are particularly relevant for genotoxicity and mutagenicity (Kazius et al. 2005).

Later, expert systems were developed that integrate chemical-specific mode of action classification and associated QSAR selection for estimating potential toxicological effects of organic chemicals (Bradbury et al. 2003), and computational approaches were improved to discriminate better between different MOAs. A prominent example is (M)Case (http://www.multicase.com), which uses similarities between structural subunits associated with a specific QSAR model as

a measure of mechanistic similarity (Klopman et al. 1999; Rosenkranz et al. 1999). However, even refined statistical methods cannot overcome the problem of multiple MOAs, that is, a chemical acting not only on one target site but on several different ones causing a variety of different modes of toxic action (Spycher et al. 2004). More recently, the Verhaar methods have been revisited, and improved rules were proposed (Enoch et al. 2008). Based on the same concept is ToxClust that allows clustering of chemicals using concentration-response data of single or multiple endpoints and derives a pattern dissimilarity of concentration-response curves between chemicals and their relative toxic potency (Zhang et al. 2009). The Verhaar classification has been implemented in the public domain software ToxTree (http://toxtree.sourceforge.net/).

MOA Classification According to Physiological Observations

The "fish acute toxicity syndromes" (FATS) were developed in the 1980s by the US EPA group in Duluth (McKim et al. 1987a; Bradbury 1994). Eight different MOAs were defined after discriminant function analysis of physiological (McKim et al. 1987a) and behavioral (Drummond and Russom 1990) responses of fish like heart rate or locomotive activity (McKim et al. 1987b).

The principle of discrimination of physiological response of chemicals was taken up by Adler et al. (2007), who made use of flow cytometry to differentiate phytotoxic modes of action in algae. Neuwoehner et al. (2009, 2010) used a combination of QSARs and endpoint pattern and time dependence to classify a number of specific and nonspecific endpoints in algae and applied this scheme successfully to fluoxetine and diuron and their transformation products.

MOA Classification According to "Ecotoxicity Profiles" and QSARs

Nendza et al. proposed to classify contaminants' mode of action based on in vitro assays (Nendza et al. 1995; Wenzel et al. 1997) and later developed so-called ecotoxicity profiles, which are fingerprints for chemicals with known MOAs in a series of in vivo and in vitro test systems (Nendza and Wenzel 2006). These ecotoxicity profiles can be used in combination with information on chemical structure to predict the MOAs of unclassified chemicals (Nendza and Müller 2000). This concept was applied and expanded by several groups, for example, for the in vitro assessment of modes of toxic action of pharmaceuticals toward aquatic organisms (Escher et al. 2005), and has more recently found applications in test batteries for water quality assessment (Cao et al. 2009; Escher and Leush 2011).

MOA Classification According to Interaction with Target Site

It is possible to classify toxic mechanisms based on the type and degree of interaction of a chemical pollutant with the target molecule or target site (Escher and Hermens 2002). The main targets for environmental pollutants are (membrane) lipids, proteins and peptides, and DNA. Depending on the type of interaction with the target, one can differentiate between nonspecific effects, when only partitioning

to the target site is involved, and specific effects, where interactions are three-dimensional and include specific H-donor/acceptor interactions as well as ionic interactions between the chemical and a target molecule. If covalent bonds are formed between the chemical and its target, the MOAs are classified as reactive mechanisms (Escher and Hermens 2002). This generic classification scheme can be further refined by differentiation between more specialized target sites, for example, specific enzymes and receptors.

The affinity to and the degree of interaction with the target site determine the toxic potency. This classification allows for multiple modes of action of a given molecule, and while also QSARs are applied in this approach, they differ from the previously mentioned ones in that they aim to account for the underlying mechanism and use descriptors that are directly related to the MOA (Spycher et al. 2008a, b). This principle is also useful when it comes to multiple modes of toxic action: for example, hydrophobic reactive chemicals are accumulated in membranes and elicit baseline toxicity, while their hydrophilic reactive counterparts will remain in the cytosol and can attack peptides and DNA (Freidig et al. 1999; Freidig and Hermens 2001).

MOA Classification Based on Genomics and Proteomics

More recently, with the maturation of gene profiling technology, the concept of linking physiological observation with mode of toxic action has been advanced to using responses on the gene expression level (DNA arrays and RT-PCR) for MOA classification (Keiter et al. 2010). A successful example of using microarray technology was the identification of the MOA of a hydroxylated metabolite of a brominated flame retardant as uncoupler of oxidative phosphorylation in zebrafish embryonic fibroblast cells (Van Boxtel et al. 2008).

Further applications of transcriptomics and metabolomics for mode of action classification are reviewed in Schirmer et al. (2010). While the field is probably most advanced for fish (Ankley et al. 2009; Iwaiashi et al. 2009; Van Aggelen et al. 2010), the availability of gene profiles has made MOA classification based on transcriptomics possible for other aquatic organisms, for example, *Daphnia magna*(Watanabe et al. 2007; Garcia-Reyero et al. 2009), *Caenorhabditis elegans* (Swain et al. 2010), and green algae (Kluender et al. 2009).

Adverse Outcome Pathways (AOP)

The terms “mechanism” and “mode of action” are often used in an ambiguous way. While the definition given above is the one used most often, the actual assignment of a mechanism or a mode of action is not clear-cut, as is also evidenced in Table 1. The US EPA group in Duluth proposed a comprehensive conceptual framework that can rationalize all steps leading from an initiating event on the molecular level to an adverse outcome at the organism or population level (Ankley et al. 2010).

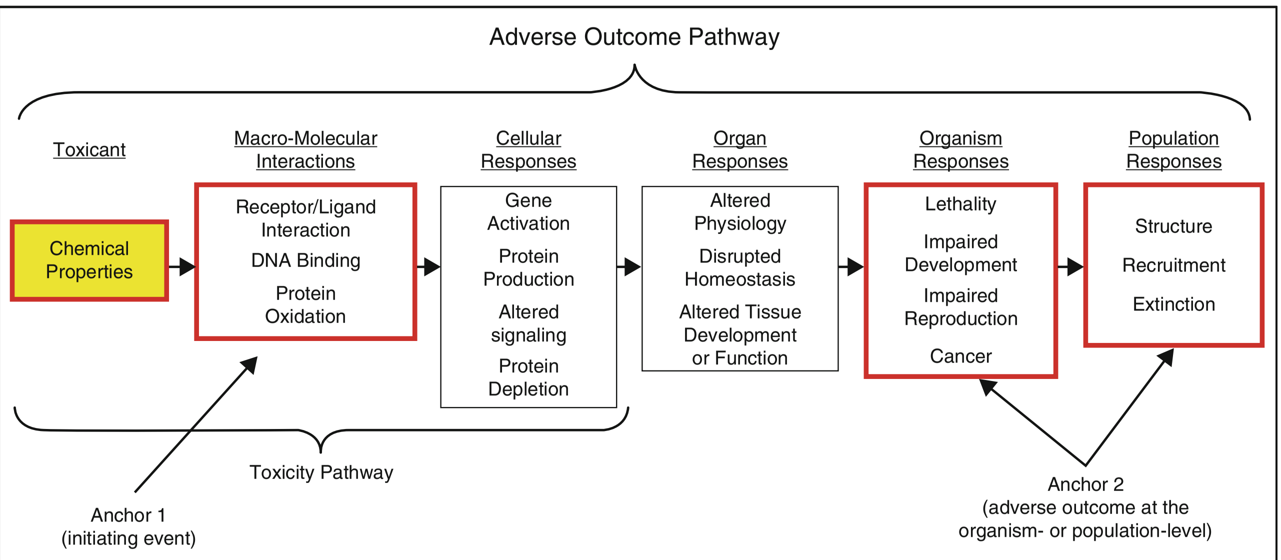
The first steps are aligned with the "toxicity pathways" established in the National Toxicology Program for human health (National Toxicology Program 2004), which are defined as the cellular response pathways after chemical exposure expected to result in adverse health effects (Collins et al. 2008). The initiating event is the macromolecular interaction between the toxicant and receptors and other biomolecules (Fig. 1). This is consistent with the classification via target interaction discussed above (Escher and Hermens 2002). This interaction triggers a cellular response, for example, activation of certain genes, production or depletion of proteins, or altered signaling (Fig. 1). The AOP then goes beyond the toxicity pathways in that it relates the cellular response to an adverse effect considered to be relevant in risk assessment, that is, the response on the organism level (lethality, reproduction failure) or on the population level (Fig. 1).

Modes of Action in Relation to Toxicokinetics and Toxicodynamics

While the AOP is a conceptual model that can help rationalize chains of events and modes of toxic action, for a translation into risk assessment, it is necessary to have a quantitative link between exposure and effect. Toxicokinetic (TK) and toxicodynamic (TD) models have the potential to close this gap (Grimm et al. 2009; Preuss et al. 2009; Escher et al. 2010; Ashauer and Escher 2010). The toxicokinetics describe all processes that lead from external to internal and target site concentration, that is, uptake, excretion, internal distribution, and metabolism. The toxicodynamics are the link to observed effect, which in principle encompasses the entire AOP. In TK-TD models, a series of differential equations can be set up to mathematically quantify and describe the relationship between exposure and effect (Lee et al. 2002; Ashauer and Brown 2008). At present, most applications of these models lack mechanistic implications on the toxicodynamics but have the potential to include systematic information on the mode of action and the reversibility of effect as a variant of a TK-TD model demonstrated, which explicitly included the receptor kinetics that are associated with the inhibition of acetylcholinesterase by organophosphates (Jager and Kooijman 2005). Kretschmann et al. (2011a, b) developed a mechanistically based TK-TD model describing the inhibition of acetylcholinesterase by diazinon in *Daphnia magna*, where the TD part was parameterized by a combination of in vitro and in vivo experiments (Fig. 2).

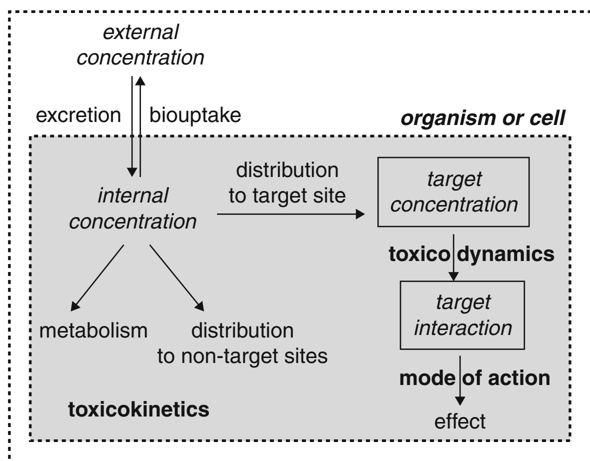
Modes of Action and Mixture Toxicity

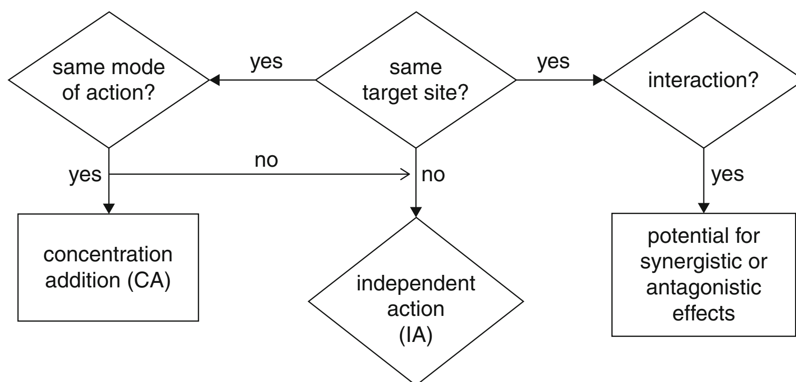
The mode of toxic action plays a crucial role, when it comes to mixture toxicity (Altenburger et al. 2003; Borgert et al. 2004; McCarty and Borgert 2006). Chemicals that share a target site and act according to the same mode of action are generally expected to act together in a concentration additive manner (Fig. 3).



Modes of Action of Chemical Pollutants, Fig. 1 Conceptual diagram of an adverse outcome pathway (AOP). Each of the first three boxes is the parameter that defines a toxicity pathway, which begins with a molecular-initiating event in which a chemical interacts with a biological target (anchor 1), leading to a sequential series of higher-order effects to produce an adverse outcome with direct relevance to a given risk assessment context (e.g., survival, development, and reproduction; anchor 2) (This figure is reprinted with permission from Ankley et al. (2010))

Modes of Action of Chemical Pollutants, Fig. 2 Definition of the processes in a cell or organism that link external to internal and target site concentrations (toxicokinetics) and link the target concentrations with effect (toxicodynamics) (This figure is adapted from Escher et al. (2011) and reprinted with permission from John Wiley & Sons Ltd)





Modes of Action of Chemical Pollutants, Fig. 3 The relationship between mode of action and mixture toxicity concepts

Those chemicals that do not share a target site will act through independent action, also called response addition. Only if chemicals interact somehow, either during the toxicokinetic phase or during toxicodynamics, there is potential for synergism and antagonism.

Cross-References

▶ Biology-Based and Population Dynamics Modeling in Ecotoxicology
▶ Biotic Ligand Model
▶ Evaluating Impacts of Multiple Stressors on Aquatic Ecosystems Using Isobolic Models

▶ Mixture Effects in Ecotoxicology
▶ Quantitative Structure-Activity Relationship (QSAR) in Ecotoxicology
▶ Toxic Units (TU) Indicators

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Monitoring of Oil-Degrading Bacteria by Stable Isotope Probing

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Glossary

16S rRNA gene A gene that encodes for the ribosomal RNA of the small subunit of the ribosome involved in the translation of messenger RNA sequences into amino acid chains in prokaryotes. This gene is universally present but sufficiently variant to allow comparison among all bacterial taxa. Many molecular tools are based on its phylogenic resolution capacity.

Bacterial taxon (plural: bacterial taxa) A population, whether named or not, of organisms which are usually inferred to be phylogenetically related and having characters in common which differentiate the unit (e.g., a geographic population, a genus, a family, an order) from other such units. A taxon encompasses all included taxa of lower rank and individual organisms. Today, bacterial taxa are largely defined by their 16S rRNA gene sequence variations.

Biostimulation Modification of an environment carried out to stimulate indigenous bacteria capable of degrading pollutants. This can be done by addition of

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various limiting nutrients or electron acceptors, such as phosphorus, nitrogen, oxygen, or even carbon sources.

Cross-feeding Synthrophic interaction in which an organism depends on or benefits from one or more growth factors or nutrients provided by another organism.

Cultivation Refers to various methods for multiplying microbial organisms by letting them reproduce in predetermined culture media under controlled laboratory conditions. It allows isolating organisms from a complex environmental sample and maintaining them in pure culture.

Density gradient A solution in which the concentration of solute is lowest at the top and gradually becomes denser toward the bottom.

Hydrocarbon biodegradation Total or partial decomposition of hydrocarbons by biological processes, which results in a minor loss of functional groups, in a fragmentation into components, or in a complete degradation to CO_2 and minerals. Hydrocarbon biodegradation is mainly performed by bacteria.

Hydrocarbonoclastic Refers to the ability of certain microorganisms to metabolize one or several hydrocarbons.

Metabolic capacities All chemical reactions carried out in aid of specific enzymes within a cell.

Metagenomics Studies that aim to characterize the partial or entire genomes of whole communities of organisms rather than individual species.

Microbial consortium Physical association of two or more different microorganisms interacting through the exchange of signals and molecules.

Molecular approaches Methods based on the exploration of genetic material pools (gene structure and function), by opposition to culture-based methods.

Molecular fingerprinting methods Methods that give a snapshot of the entire microbial community at once, by differential electrophoretic migration on agarose or polyacrylamide gels, which depend on their size fragments (T-RFLP, ARISA, LH-PCR) or sequence variations (DGGE, CE-SSCP). The result is a profile (fingerprint) of the community structure that can be compared to other samples treated in the same way.

Most probable number (MPN) A method for quantifying a functional group out of a total bacterial community. This method is based on the dilution/extinction cultivation technique with a particular substrate, and results are given by using a correspondence table giving the most probable number of bacteria able to grow on this substrate.

Phylogenetic affiliation Positioning an organism on the basis of its evolutionary distance to the closest related microorganism using their gene sequence homologies (16S rRNA genes in general).

Polymerase chain reaction (PCR) A molecular technique using a polymerase enzyme to exponentially amplify a DNA fragment until thousands or even millions of copies of the sequence are produced. PCR is the basis of a wide range of genetic analyses avoiding limitations in DNA quantities.

Pyrosequencing A massively parallel DNA sequencing method based on the sequencing-by-synthesis principle, which relies on efficient detection of the sequential incorporation of natural nucleotides during DNA synthesis. Due to the short read length generated by the 454 platform and in order to increase sequencing capacity, new strategies for exploring microbial diversity by 16S pyrosequencing are currently being developed. While the first generation 454 Life Sciences apparatus (GS20) provided up to 25 megabases of data in a single run with an average read length of 100 base pairs (bp), the new GS FLX Titanium provides up to 400 megabases of data in a single run with an average read length of 400 bp. The widespread availability of 454 pyrosequencing, a technology roughly an order of magnitude less expensive than classical Sanger sequencing in terms of cost per base, has changed the landscape of genomics.

Real-time PCR (quantitative PCR) A technique used to amplify and simultaneously quantify a targeted DNA molecule as absolute number of copies or relative amount when normalized to DNA input or additional normalizing genes. The procedure follows the general principle of a polymerase chain reaction. Its key feature is that the amplified DNA is detected as the reaction progresses in *real time*, a new approach compared to standard PCR, where the product of the reaction is detected at its end.

Stable isotope-labeled molecule A nonradioactive natural or synthesized stable molecule containing one or several atoms enriched in one or several neutrons. Different isotopes of the same element have nearly the same chemical characteristics and therefore behave almost identically in biology. The mass difference, due to a difference in the number of neutrons, leads to a partial separation of the light isotopes (unlabeled molecules) from the heavy isotopes (labeled molecules) during physical processes such as ultracentrifugation.

Abbreviations

16S rRNA	16S ribosomal ribonucleic acid
ARISA	Automated ribosomal intergenic space analysis
BTEX	Benzene, toluene, ethylbenzene, and xylene
CE-SSCP	Capillary electrophoresis-single-strand conformation polymorphism
CsCl	Cesium chloride
CsTFA	Cesium trifluoroacetate
DGGE	Denaturing gradient gel electrophoresis
DNA	Deoxyribonucleic acid
FISH	Fluorescent in situ hybridization
GC/MS	Gas chromatography/mass spectrometer
LSA	Liquid scintillation analyzer
MDA	Multiple displacement amplification
MPN	Most probable number

mRNA	Messenger ribonucleic acid
NA-SIP	Nucleic acid-stable isotope probing
PAH	Polycyclic aromatic hydrocarbon
PCB	Polychlorinated biphenyl
PCR	Polymerase chain reaction
PLFA	Phospholipid fatty acid
RNA	Ribonucleic acid
SIP	Stable isotope probing
T-RFLP	Terminal-restriction fragment length polymorphism

Definition

Utilization of new molecular techniques for the identification of oil-degrading bacteria.

Matching bacterial taxa with specific metabolic capacities in natural environments remains one of the biggest challenges for microbial ecologists. Stable isotope probing (SIP) coupled with uncultured-based molecular biology techniques is a new powerful approach allowing the identification of a microbial consortium actively involved in specific biogeochemical processes, such as hydrocarbon biodegradation. This method relies on the uptake of stable isotope-enriched substrates (^{13}C-phenanthrene or a mix of ^{13}C-petroleum hydrocarbons, for example) by microorganisms able to metabolize and incorporate these substrates into their cellular components (DNA, RNA, polar lipid-derived fatty acids, amino acids, and protein) with minimum disturbance for microorganisms. Separation of isotope-enriched DNA or RNA (heavy) from others (light) is performed by density gradient ultracentrifugation after nucleic acid extraction, and the phylogenetic affiliation of heavy nucleic acid sequences reveals the composition of the hydrocarbonoclastic microbial community.

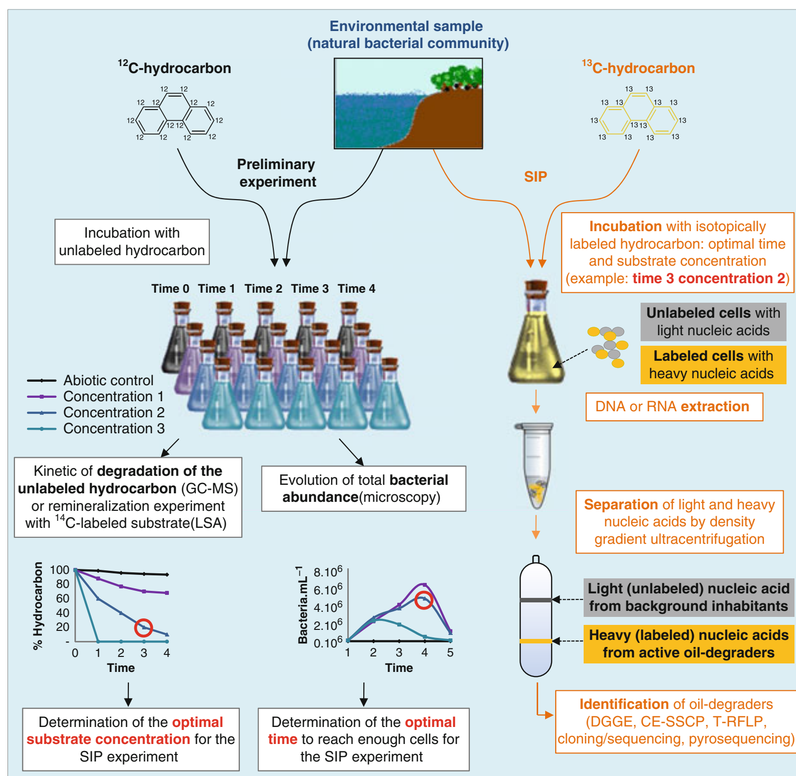
Historical Background

The ultimate degradation of complex petroleum hydrocarbons in the environment mainly depends on the complementary metabolic capabilities of different hydrocarbonoclastic bacteria (Head et al. 2006). For a long time, the difficulty for microbial ecologists to discriminate microbes responsible for in situ oil-biodegradation processes has been hampered by the high complexity of microbial assemblages and the limitations of culture-based identification methods. The cultivation of microorganisms is still a very useful technique for the discovery of new hydrocarbonoclastic bacterial strains (Prince 2005; Rodríguez-Blanco et al. 2010b) but remains clearly incomplete since it allows the identification of only 0.1–1% of the total bacterial community (Giovannoni et al. 1990). The appearance of polymerase chain reaction

(PCR)-based molecular approaches (16S rRNA gene or functional gene-based methods) from in situ DNA extracts has allowed to shunt culture-dependent biases to explore the spatial and temporal variation of microbial assemblages in soil (Ranjard et al. 2003) as well as in seawater (Ghiglione et al. 2005, 2007, 2008; Lami et al. 2009). With these approaches, links between changes in bacterial community structure and oil biodegradation have been demonstrated (Rodríguez-Blanco et al. 2010a). However, direct identification of hydrocarbonoclastic bacteria was not feasible by using classical molecular methods. Hanson et al. (1999) first revealed toluene degrader's identity from in situ soil samples by using stable isotope probing (SIP) with ^{13}C-toluene-labeled substrates. Originally developed by Meselson and Stahl (1958) to demonstrate the semiconservative mechanism of DNA replication, SIP technique has received a growing interest in the last 10 years because of its potential to be coupled with new molecular methods to identify organisms involved in the metabolism of a given substrate. Until now, the technique has been principally used in soil and sediment, whereas studies in seawater are scarce (Neufeld et al. 2008). Several authors suggested that SIP coupled with new metagenomic tools is leading to major progress in microbial ecology for its potential to reveal new diversity-function relationships of uncultivated microorganisms (see Chen and Murrell 2010 for a review).

Protocol

Nucleic acid-stable isotope probing (NA-SIP) first consists in setting up a microcosm or an in situ incubation of an environmental sample with a stable isotope-labeled molecule (see Fig. 1 for a global view of SIP protocol). The stable isotope can be ^{13}C, ^{15}N, ^{2}H, or ^{18}O, but the carbon atom is the most commonly used in oil-degradation NA-SIP studies since it is the most important element in hydrocarbons and nucleic acids. An important step concerns the incubation period with the labeled substrate that should be optimized for sufficient intracellular stable isotope incorporation but not too long to avoid unspecific incorporation by cross-feeding. Incubation time is comprised between a couple of hours and 2 months according to the hydrocarbonoclastic capacity of the environmental sample and the bioavailability of the substrate. The success of the experiment is improved by the utilization of totally labeled substrate (i.e., stable isotope enrichment of all carbon atoms). Separation of heavy and light nucleic acids is performed in cesium chloride (for DNA recovery) or cesium trifluoroacetate (for RNA recovery) density gradient by isopycnic ultracentrifugation step (from 10 to 90 h) using vertical, near-vertical, and more occasionally fixed-angle rotors. Before ultracentrifugation, extracted DNA or RNA can be systematically spiked with a marker (such as *Escherichia coli* DNA or RNA if this species is not present in the environmental sample) as a control for the ultracentrifugation separation efficiency of labeled and unlabeled nucleic acids. Recovery of light and heavy bands can be performed by different



Monitoring of Oil-Degrading Bacteria by Stable Isotope Probing, Fig. 1 Typical procedure of stable isotope probing for monitoring oil-degrading bacteria (See references Neufeld et al. (2007) and Whiteley et al. (2007) for complete DNA and RNA-SIP protocols, respectively)

methods. Some operators localize the two bands by ethidium bromide incorporation and observation with a transilluminator for direct sampling of the bands. Others retrieve fractions of the gradient by pricking the bottom of the centrifuge tube with a needle or by successive pipetting on the top of the tube. Then classical DNA or RNA quantification allows the recovery of the light and heavy nucleic acid fractions. In both cases, a purification step eliminates cesium chloride (CsCl) from DNA or cesium trifluoroacetate (CsTFA) from RNA for subsequent analysis. A large panel of molecular techniques can be used to identify the hydrocarbonoclastic bacteria from the purified stable isotope-labeled DNA or RNA. All of them are based on PCR or reverse transcription-PCR amplification of the 16S rRNA gene of the heavy fraction of DNA or RNA, respectively. Difference in the nucleic acid composition of labeled DNA or RNA can be observed by classical molecular fingerprinting methods such as DGGE (Röling et al. 2002), CE-SSCP (Rodríguez-Blanco et al. 2009), ARISA (Maron et al. 2005), or T-RFLP (Bordenave et al. 2007) coupled with the taxonomic identification of bands or peaks. A better picture of the

diversity with a better coverage can be obtained by the clone library (Giovannoni et al. 1990) or by the new massively parallel pyrosequencing technology (Rogers and Venter 2005).

A comprehensive view of the experiment requires that several parameters be measured before and/or during the course of incubation (Fig. 1). First, the assimilation of the substrate is followed by chemical analysis such as gas chromatography coupled with a mass spectrometer (GC/MS) or by a remineralization experiment following the decrease of ^{14}C-labeled substrate by radiorespirometry with a liquid scintillation analyzer (LSA) (Singleton et al. 2006). Such analysis gives indication about the amount of labeled substrate degraded during the course of the experiment and about the optimal substrate concentration and optimal time for NA-SIP incubation. Second, the evolution of total bacterial abundance is often followed by microscopy counting in order to determine a minimal bacterial abundance for DNA extraction. Eventually, the quantification of hydrocarbonoclastic microorganisms can be performed in parallel by using the most probable number (MPN) method based on the incubation of replicated cultures across several serial dilution steps with the substrate (Delille et al. 2009). A more precise estimation can be done by real-time PCR (if specific PCR primers are available), a quantitative method for the determination of copy number of genes involved in the transformation of the substrate (Singleton et al. 2006).

Applications

Stable isotope probing requires no foreknowledge about the studied microorganisms and no cultivation step and minimizes the disturbance of the microbial population. It is not a complicated technique to implement, and it offers many advantages. Even if we focus herein on its evident application to monitor oil degraders in situ, it can be applied to many other topics, as long as labeled substrates are available and can be incorporated by bacteria. Until now, NA-SIP has been successfully employed to explore soil or sediment bacterial communities (Table 1), but its use in marine environment has only been performed to explore active marine methylotrophs (Neufeld et al. 2008). The identification by NA-SIP of marine bacteria able to degrade recalcitrant hydrocarbons such as polycyclic aromatic hydrocarbons (PAH) with more than four rings is in process (Ghiglione, personal communication).

DNA-SIP analysis is undertaken with a range of 250 ng to 10 μg of DNA per milliliter of CsCl. High concentrations of loaded DNA allow easier visualization/recovery of heavy and light bands and allow further investigation by a wide range of molecular analysis methods. It enables the monitoring of oil degraders within the entire bacterial community based on their 16S rRNA gene affiliation. RNA-SIP can offer the same sequence-based phylogenetic resolution as DNA-SIP. Its main advantage is reduction of incubation time due to its faster synthesis, which is of particular interest for natural samples containing active but nonreplicating cells or

Monitoring of Oil-Degrading Bacteria by Stable Isotope Probing, Table 1 Overview of studies using DNA/RNA-SIP for identification of active hydrocarbon degraders

Substrate	Environment	Biomarker	Phylogenic groups identified	Biomarker analysis	Additional study features	References
^{13}C-naphthalene	PAH-contaminated sediments	DNA	*Polaromonas vacuolata*	T-RFLP	Discovery of a bacterium hosting a naphthalene dioxygenase gene	Jeon et al. (2003)
^{13}C-naphthalene ^{13}C-caffeine ^{13}C-phenol ^{13}C-glucose	Agricultural experimental soil	DNA	*Pseudomonas*, *Acinetobacter* and *Variovorax*	T-RFLP	Use of a mix of ^{13}C-labeled compounds minimizing microbiological artifacts caused by nutritional disturbance	Padmanabhan et al. (2003)
^{13}C-benzoate	Sediment	DNA	No identification (Study of the activity of benzoate-utilizing denitrifying population)	T-RFLP	Use of Archaeal carrier DNA in the density gradient reducing labeling incubation time	Gallagher et al. (2005)
^{13}C-naphthalene ^{13}C-phenanthrene	Bioreactor treating PAH-contaminated soil	DNA	*Acidovorax*, *Pseudomonas* and *Ralstonia*	DGGE	Use of DNA from *E. coli* K-12 as an indicator of separation efficiency of ^{12}C and ^{13}C-DNA during ultracentrifugation	Singleton et al. (2005)
^{13}C-naphthalene ^{13}C-glucose	Soil	DNA	*Acidovorax*, *Pseudomonas* and *Intrasporangium*	Real-time-T-RFLP	Adaptation of a quantitative assay for linking microbial community function (Q-FAST) and structure T-RFLP	Yu and Chu (2005)
^{13}C-methane	Forest soil	DNA	*Methylocystis*	Cloning in bacterial artificial chromosome plasmid	Methane mono-oxygenase operon revealed by metagenomic tools	Dumont et al. (2006)
^{13}C-benzene	Gasoline-contaminated groundwater	RNA	*Azoarcus*	DGGE	Supplementation by ^{13}C-benzene and an electron acceptor (benzene degradation under denitrifying conditions)	Kasai et al. (2006)

^{13}C-pyrene	Bioreactor treating soil	DNA	*Sphingomonas*, *β* and *ϒ*-*Proteobacteria*	DGGE and real-time PCR	Use of DNA from *E. coli* K-12 as an indicator of separation efficiency of ^{12}C and ^{13}C-DNA during ultracentrifugation	Singleton et al. (2006)
^{13}C-benzene	Coal gasification soil	DNA	*Deltaproteobacteria*, *Clostridia* and *Actinobacteria*	T-RFLP and real-time PCR	Evidence of a novel clade of Gram-positive iron-reducers in anaerobic benzene degradation	Kunapuli et al. (2007)
^{13}C-phenanthrene ^{13}C-pyrene	Bioreactor treating soil	DNA	*Acidovorax*	DGGE and real-time PCR	Mixture of ^{13}C-substrates	Singleton et al. (2007)
^{13}C-methane	Acidic peatlands	DNA	*Methylocystis* and *Methylocella*/*Methylocapsa*	DGGE and MDA for a fosmid metagenomic library	Discovery of a methanol dehydrogenase	Chen et al. (2008)
^{13}C-pyrene	PAH-contaminated Soil	DNA	*β* and *ϒ-Proteobacteria*	DGGE	SIP coupled with biostimulation assays	Jones et al. (2008)
^{13}C-benzoate	Agricultural soil	RNA	*Pseudomonas*, *Bacillus*, *Acinetobacter*, *Variovorax* and *Burkholderia*	DGGE	Resource availability influence on bacterial diversity	Langenheder and Prosser (2008)
^{13}C-benzoic acid	Agriculture soil	DNA	*Burkholderia*	T-RFLP, real-time PCR, isolation/ cultivation	Use of MPN-PCR technique to quantify a close relative of the species *Burkholderia*	Pumphrey and Madsen (2008)
^{13}C-benzene	BTEX-contaminated Groundwater	RNA	*Acidovorax* and *Malikia*	DGGE	Comparison between community fingerprinting technique and SIP	Aburto and Ball (2009)
^{13}C-toluene	BTEX-contaminated aquifer	RNA	*Desulfocapsa*	T-RFLP	Combination of *in situ* microcosm and SIP technique	Bombach et al. (2009)
^{13}C-benzene	Coarse sand	DNA	*Cryptanaerobacter*/ *Pelotomaculum* *ξ-proteobacteria*	T-RFLP	Relationship between methane production and benzene degradation	Herrmann et al. (2009)

(*continued*)

Monitoring of Oil-Degrading Bacteria by Stable Isotope Probing, Table 1 (continued)

Substrate	Environment	Biomarker	Phylogenic groups identified	Biomarker analysis	Additional study features	References
^{13}C-naphthalene	Groundwater	RNA	*Pseudomonas Acidovorax*	DGGE and FISH-Raman microspectroscopy	mRNA analysis	Huang et al. (2009)
^{13}C-toluene	Agricultural soil	DNA	Organism belongs to the candidate phylum TM7	T-RFLP	Identification of a novel toluene-degrading bacterium	Luo et al. (2009)
^{13}C-benzene	Lotus field soil	DNA	Hasda-A	DGGE and real-time PCR	Putative identification of a novel benzene-degrading bacterium	Sakai et al. (2009)
^{13}C-toluene	BTEX-contaminated soil	DNA	*Polaromonas*	T-RFLP and real-time PCR	First *Polaromonas* strain growing on toluene	Sun et al. (2010)
^{13}C-xylene	Groundwater	DNA	*Ramlibacter*, *Paenibacillus* and *Bacillus*	T-RFLP	Identification of novel *m*-xylene degrading bacteria	Xie et al. (2010)

with low bacterial growth rates. It increases the sensitivity of the technique by labeling more efficiently and rapidly the RNA biomarkers (up to 6.5 times faster, Manefield et al. 2007). However, RNA-SIP is limited to a maximum loading of 500 ng of RNA per mL of CsTFA, a sufficient concentration for the 16S rRNA analysis that constitutes the major fraction of the RNA, albeit more laborious for mRNA-based analysis. Such a constraint complicates its recovery and its analysis, making the labeled-transcriptome exploration relatively complicated at this time, even if it represents an exciting challenge for the future (Dumont and Murrell 2005). Only one study thus far has managed to partially reveal the transcriptome of hydrocarbonoclastic bacteria in a polyaromatic hydrocarbon-contaminated environment by using an mRNA-SIP approach (Huang et al. 2009). Advance information on the succession of populations using the labeled substrate can be obtained by coupling the advantages of the use of DNA- and RNA-based SIP, as proposed by Lueders et al. (2004) and Manefield et al. (2007).

Conclusions and Prospects

Stable isotope probing is a powerful technique to open the “microbial black box” by matching diversity of bacteria and their hydrocarbonoclastic function in natural environments. SIP technique offers a large potential in terms of prospects. For example, a modification of SIP technique enabled the identification of active predators of stable isotope-labeled *Prochlorococcus* and *Synechococcus* (the two most abundant marine cyanobacteria) in surface waters of the Pacific Ocean (Frias-Lopez et al. 2008). This assay opens up the field of exploring the diversity of bacterial predators responsible for “top-down” control of hydrocarbonoclastic bacteria during oil spill pollution events (Kota et al. 1999).

Limitation of resources known as “bottom-up control” has also received very little attention thus far in SIP studies. Nutrient resources have a direct effect on oil-biodegradation processes by limiting hydrocarbonoclastic bacterial activities (Atlas and Bartha 1972), and addition of nutrients has been successfully used to improve oil degradation in natural environments. However, very few studies have addressed the question of which bacteria were responsible for such biostimulation. Kasai et al. (2006) showed that supplementation of groundwater with ^{13}C-benzene together with or without nitrate as electron acceptor resulted in a selection of a phylotype affiliated with the genus *Azoarcus*, a denitrifying bacterium able to degrade benzene only when nitrate was added. In contrast, Jones et al. (2008) found that pyrene-degrading bacterial diversity remained unchanged under nitrogen-amended conditions in an aged PAH-contaminated soil, even if biostimulation increased the rate of pyrene degradation. Further comparison between species labeled by ^{13}C-hydrocarbon or nutrients + ^{13}C-hydrocarbon should be conducted for a better identification of nutrient-limited bacteria in oil-biodegradation processes.

Another promising prospect can involve detection of rare species and that of novel enzymes and bioactive compounds by coupling DNA-SIP with new metagenomic approaches. To our knowledge, prescreening of the metagenomic library based on hydrocarbon substrate incorporation has never been tried, even if this approach has already been used for polychlorinated biphenyl (PCB)-degrading bacteria identification (Sul et al. 2009). The application of SIP and metagenomic tools is largely conceivable to investigate hydrocarbonoclastic bacterial genes such as PAH dioxygenase (Cébron et al. 2008) especially because of the recent improvement of the sequencing technique with massively unparallel pyrosequencing technologies (Rogers and Venter 2005). Coupling SIP and metagenomics holds promise for extending the SIP application toolbox to expand discoveries in the exploration of functional microbial communities.

Cross-References

► Bioavailability of Contaminants
► Biodegradability in Ecotoxicology
► Environmental River Biofilms as Biological Indicators of the Impact of Chemical Contaminants
► Microbial Bioremediation of Aquatic Environments

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