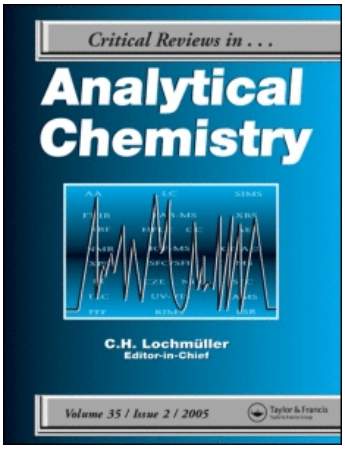

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Application of Biotests in Environmental Research

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The possible applications of tests based on the use of biological materials in the field of environmental research are presented. The known biotests, used for assessing the accumulative pollution level in particular environmental compartments, have been classified. In addition, information collected on the sensitivity of some fauna and flora species to toxic substances present in the environment are presented.

Keywords environmental pollution, pollution assessment, biomonitoring, risk assessment, biotests, bacterial tests, phytotests

INTRODUCTION

Intensive developments of new technologies, the encroaching urbanization and constantly increasing human consumerism lead to detrimental and often irreversible changes in the environment. The continually expanding spectrum of pollutants, mainly originating from anthropogenic sources, gets introduced into the air, surface waters, and soil. At the same time, due to processes such as transport and chemical, photochemical and biochemical pathways, the differentiation of levels of certain pollutants in particular abiotic compartments takes place. From there chemical compounds get into plants, animal organisms and, finally, into human bodies. Such pollutants may cause a variety of adverse effects immediately after the exposure as well as later; the delayed exposure effects are called "long-term toxic effects." Because of the aforementioned reasons analytical chemists are obligated to determine very thoroughly the levels of particular ecotoxins in the biotic and abiotic compartments of the environment.

A comprehensive analytical profiling of the environment, i.e., determining the concentration levels of all (identified and unidentified) pollutants in each compartment would be a sought after solution to many problems. However, a question arises whether such task can be possibly conducted at present, and whether it is purposeful, considering the following:

- number of components that would have to be determined;
- different concentration levels, mainly for the trace and ultra-trace constituents;
- fluctuations in the concentrations of pollutants in time and space;
- complex composition of matrix and the associated with it possibility of interferences;
- complicated, meaning time- and labor-intensive, sample pretreatment procedures;
- additional pollution load to the environment resulting from the reagents used during analysis, and in particular from organic solvents used in the sample pretreatment stage; and
- additional costs in connection to the necessity of buying the high purity reagents, and the utilization or other use of their surplus (leftovers).

To avoid these inconveniences and limitations, it is necessary to introduce into the analytical practice a new approach for estimating the environmental pollution level that employs estimation of the indicators of accumulative pollution level in a given compartment. Such parameters as COD and BOD or total carbon and organic carbon content, that assess the total carbon content in the pollutants present in a studied sample, can be successfully used in environmental analytics (1).

The measure of accumulative load from different types of pollutants, in particular environmental compartments, may be also expressed as the sample toxicity, evaluated with the use of the appropriate biotest.

ASSESSMENT OF TOXIC EFFECTS

Toxicity measurements conducted in environmental samples may form a foundation on which solution to the three basic problems can be based, as follows:

- risk assessment, i.e., estimating the probability of an adverse effect occurrence due to the impact of a given factor on a live organism;

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- assessment of the toxicity level by determining a toxic effect dose; and
- attempt to detect long-term effects caused by the exposure of a live organism to toxic elements such as, mutagens, carcinogens or teratogenic and/or embryotoxic factors.

Ecotoxicological studies can be conducted in two ways by means of (2):

- epidemiological studies consisting of the observations of a given human population being exposed to the particular environmental pollution; that allows for a direct estimation of the exposure risk; and
- application of laboratory methods with the use of different experimental models, and further on, an attempt to apply the obtained results to evaluate the risk of exposure.

Depending on the model used, laboratory methods may refer to the toxocity measurements as

- the whole organism
- a chosen organ

} in vivo methods

- tissue culture
- enzymatic reactions

} in vitro methods

In case of research conducted with the use of simple experimental in vitro models as well as animal studies, a relationship between a dose (concentration) of the toxic factor and biological response, i.e., biological reaction of an organism (or a model used) to the dose. In ecotoxicological studies, the biological response is determined by measuring such parameters as the inhibition of cellular growth, activity of a certain enzyme, accelerated breathing rate of an individual, survival or mortality, etc.

Based on the dose–response relationship, the values of parameters can be calculated that are quantitative estimates of toxicity for the substance in question. The strength of toxic impact on live organisms is expressed by such parameters as EC_{25} and EC_{50} (Effective Concentration), or ED_{25} and ED_{50} (Effective Dose), which describe the concentration or dose of a toxin in the environment that causes 25% or 50% of the maximal biological response. Another used indicator is IC_{50} (Inhibition Concentration), which describes the concentration of a toxic factor in the environment causing 50% inhibition of a given process, e.g., growth.

In case of acute toxicity, the impact of a given substance disrupting biological processes is such that it results in death. The measure of the effect is $\mathbf{LD}_{50}$ (Lethal Dose), i.e., a dose causing death of 50% of individuals in the studied population in a given time. Often a parameter describing lethal concentration of a given substance in water, soil, or air is used, namely $\mathbf{LC}_{50}$ (Lethal Concentration). A determination of acute toxicity is usually a preliminary step in assessing the impact of the substance on the organism; it allows us to set a direction for the further toxicity investigations.

A dose-biological response relationship may also be used when predicting risk, i.e., determining dose and exposure time for which the probability of the toxic effects occurrence is properly low. To this end, threshold concentrations or doses are estimated as follows:

- NOEL or NOEC (No Observed Effect Level/ Concentration), i.e., the highest dose or concentrations of a toxic substance for which no detrimental effect has been observed;
- LOEL or LOEC (Lowest Observed Effect Level/ Concentration), i.e., the lowest dose or concentration for which the first detrimental changes have been observed;
- NOAEL (No Observed Adverse Effect Level), i.e., the highest dose or concentration for which no adverse effect has been detected during the conducted research; and
- LOAEL (Lowest Observed Adverse Effect Level), i.e., the lowest dose or concentration for which the adverse effect has been noted during the conducted research.

The observed toxicity effects may regard changes in morphology, life activities, growth, development, or a life stage of the investigated organisms. The aforementioned parameters are expressed in mg or μg of a given substance per 1 kg of body weight per 24 hours; LOAEL is used when the value of NOEL remains undetermined (under assumption of appropriately larger uncertainty coefficient).

The determination of a minimal exposure level by measuring the above parameters is not reciprocal to the fact that the toxic effect cannot occur; that is due to the fact that the threshold values are estimated with a certain probability. Moreover, in case of some chemical compounds, no dose (concentration) is permissible, therefore the threshold values cannot be established. In order to assess risk, effective threshold concentrations, EC_{10} or EC_{15}, are used that cause a given biological effect at 10% and 15%, respectively, of its maximal value (3).

A toxic effect of the chemical substance can also be influenced, in a decisive way, by its low propensity to be metabolized that, in connection to lipophilic properties, significantly obstructs its excretion form the organism that results in accumulation. A propensity of the toxic to accumulate in the organism can be evaluated by comparing the values of, for example, CLD_{50} (Cumulative Lethal Dose) and LD_{50}.

A longer observation period and descriptive character of the results are typical for the methodology of sublethal and chronic toxicity research. Such studies concern the organism as a whole, and consist of the observations of cellular and biochemical processes, physiological functions, and individual behavior. In turn, after the death of the animals, macro- and microscopic observations of changes that had occurred in organs due to toxic exposure are conducted. In addition, chronic toxicity research encompasses so-called specialized studies as the investigated

substance may have carcenogenic properties causing abnormalities in tissue growth; mutagenic properties that result in the alteration of inherited traits due to the DNA damage; or embryotoxic and/or teratogenic properties resulting in the embyonic or fetal death.

HEALTH AND ECOLOGICAL RISK ASSESSMENT

The process of conducting risk assessment has been initiated by the U.S. Environmental Protection Agency. EPA published an initial set of five risk assessment guidelines (relating to cancer, mutagenic effects, developmental effects, exposure assessment, and chemical mixtures) in 1986 as recommended by the National Academy of Sciences. EPA continues to revise its risk assessment guidelines and to develop new guidelines as experience and scientific understanding evolve (see Table 1).

The Way to Conduct Risk Assessment of Exposure to Toxic Substances in Humans

According to the ordinance of Minister of Health, dated 18 February 2003 (Dz. U. No. 52, position 467, appendix 1), in the process of assessing the risk to humans due to the exposure to toxic substances, the following types of harmful impact should be considered (4):

1. acute toxic effect; LD_{50} or LC_{50} should be determined, or when the determined dose methodology has been used, a differentiating dose should be estimated (differentiating dose is a dose that causes visible toxic effect, but not death, at one of four dose levels which were determined using the proper toxicity methodology, i.e., 5, 50, 500, or 2,000 mg/kg body weight (5)),
2. irritating action
3. caustic action
4. allergenic action

   } It should be established whether the substance has such properties.
5. chronic toxic effect; a dose-response and NOAEL should be determined; in case when NOEAL value cannot be established then LOAEL should be determined.
6. mutagenic action
7. carcinogenic action

   } It should be established whether the substance has such properties; in case of the substance identified as carcenogenic but not genotoxic, it is purposeful to determine NOAEL or LOAEL.
8. harmful effect on reproduction; a dose-response relationship and NOAEL values should be established; in case NOAEL cannot be determined, the value of LOAEL should be measured.

Next, values of the above mentioned parameters, i.e., NOAEL or LOAEL, should be compared to the estimated doses or concentrations to which the populations are going to be exposed (the ratio of exposure level to NOAEL or LOAEL values is calculated).

TABLE 1
U.S. EPA guidelines for risk assessment

No.	Risk assessment guideline	Source
1.	Guidelines for Carcinogen Risk Assessment	Federal Register 51 (185) 33992-34003, 24 September 1986
1A.	Proposed Guidelines for Carcinogen Risk Assessment	Federal Register 61 (79) 17960-18011, 23 April 1996
2.	Guidelines for Chemical Mixtures Risk Assessment	Federal Register 51 (185) 34014-34025, 24 September 1986
2A.	Supplementary Guidance for Conducting Health Risk Assessment of Chemical Mixtures	Risk Assessment Forum, Washington, DC, EPA/630/R-00/002, 2000.
3.	Guidelines for Ecological Risk Assessment	Federal Register 63 (93) 26846-26924, 14 May 1998
4.	Guidelines for Neurotoxicity Risk Assessment	Federal Register 63 (93) 26926-26954, 14 May 1998
5.	Guidelines for Reproductive Toxicity Risk Assessment	Federal Register 61 (212) 56274-56322, 31 October 1996
6.	Guidelines for Exposure Assessment	Federal Register 57 (104) 22888-22938, 29 May 1992
7.	Guidelines for Developmental Toxicity Risk Assessment	Federal Register 56 (234) 63798-63826, 5 December 1991
8.	Guidelines for Mutagenicity Risk Assessment	Federal Register 51 (185) 34006-34012, 24 September 1986

The Way to Conduct Environmental Risk Assessment

According to the ordinance of Minister of Health, dated 18 February 2003 (Dz. U. No 52, position 467, appendix 3), regarding conducting environmental risk assessment, the purpose of determining the relationship between the dose (concentration) and biological response is to estimate the concentration of a substance present in particular components or compartments of the environment below which the harmful effects should not be expected (4). Such concentration is defined as Predicted No Effect Concentration (PNEC). The value of PNEC is calculated by using the appropriate Uncertainty Factor (UF), and the results from the studies on live organisms such as parameters LD_{50}, LC_{50}, EC_{50}, IC_{50}, NOEL, or NOEC, LOEL or LOEC, or other compatible parameters. Next, the PNEC, i.e., Predicted Effect Concentration, value is compared to the value of PEC. Based on the PEC/PNEC ratio, conclusions can be drawn whether, among others, the undertaken remedial actions to diminish risk have been sufficient.

CLASSIFICATION OF BIOLOGICAL METHODS APPLIED IN ENVIRONMENTAL RESEARCH

Analytical methods employing biological material are becoming increasingly competitive in relation to the classical ones, mainly because of their specificity, quickness, and the possibility of using them in situ and in an on-line mode. Their application allows the measurement of the cumulative pollution load in the samples containing different pollutants and complex matrices without labor- and time-consuming pretreatment of the collected environmental material (6). In general, there are two groups of biological methods (7):

- biomonitoring that can be realized as follows;
  - by performing analytical studies of biological samples (biota) that are treated as passive samplers accumulating the pollutants; and
  - by observing bioindicators that are properly chosen faunal and floral organisms;
- bioanalytics that employ biologically active substances acting as receptors of certain pollutants. Considering the particular use of a biological substance, one can distinguish
  - biosensors; biologically active element such as, bacteria, viruses, enzymes, antibodies, etc., is the active part of an appropriate sensor; and
  - biotests; biological material constitutes the "original" measuring device.

The abrupt development of biological methods was observed in both methodological and practical aspects. The classification of these methods has already been attempted; however, thus far, it only introduced confusion that has led to many misunderstandings in the field of terminology.

Biotest (Greek *bios* − life + Latin *testari* − testify) can be defined as an experimental biological sample used to prove the presence of toxic substances in the environment or to check for its toxicity by quantitative evaluation of the sample's impact on a live organism (based on a comparison with the control sample). Toxicity measurement is yet another example of a relative measure that is so commonly used in classical chemical analytics.

METHODS OF CONDUCTING STUDIES WITH THE APPLICATION OF BIOTESTS

In literature, information is found on three main ways of conducting research using biotests to obtain data on pollution in a given environmental compartment, as follows:

1. Toxicity tests realized in the laboratory during which the toxic substance is introduced into pure water or sediment. Such tests are a source of information on the toxicity of a given substance under controlled conditions; they are conducted in order to obtain the standard biotest that will be later used to evaluate toxicity in real samples (8–10).
2. Toxicity tests conducted in the laboratory on the basis of real samples (water, soil, and sediment). Toxicity of real samples is compared to that of the standard samples (11–14).
3. in situ tests with the use of animal populations living under natural conditions (15–18).

Basic information about the degree of contamination in a given environmental element is obtained from the biotests performed on one plant or animal species (single species tests) that is representative of a particular trophic level. Tests are conducted according to standard procedures under specific laboratory conditions that are optimal for a given test organism (11, 19–24). To more thoroughly investigate the complex interactions between potentially toxic chemical compounds and the organisms inhabiting certain ecosystems, experimental work is conducted in microcosms. In such case, the communities of organisms are introduced into large containers of a volume of a few hundred liters (25–27). During the experiments, the natural conditions—predominant for a given environmental compartment—are being simulated. Microcosms can be a source of information about the impact of toxic substances on different species and trophic levels as well as the entire communities of organisms. Much less frequently the influence of toxins on plant and/or animal populations, and their structure and functioning is researched in costly mesocosms to which chemical compounds have been introduced under controlled conditions. Mesocosms are subjected to the natural variability of the environment, i.e., winds, temperature, and insolation (27–31).

According to other classification (32), biotests can be conducted under the following conditions:

- static state; the same water or sediment are used throughout the duration of the test (8, 10, 33–35).
- semistatic; the exchange of medium takes place at particular time intervals, e.g., every 24 hrs or once a week. Tests on a crustacean *Daphnia magna* are conducted this way (11–13, 14, 36).
- dynamic state; tests are conducted with the constant exchange of water during mesocosm experiments and in situ, mainly with the use of fish as a biological element (29, 30).

Biotests used in analytics can be, obviously, classified in reference to the type of organism that constitutes the active part of the test. The most frequently used organisms are

- plants
- bacteria
- animal organisms.

BACTERIAL TESTS

Biotests are an important element of bioanalytics and biomonitoring, i.e., these fields of chemical analysis that presently undergo the abrupt development. Bioluminescence in

marine bacteria *Vibrio fischeri* (formally *Photobacterium phosphoreum*) has found broad application in toxicity testing. Tests based on this phenomenon are a useful tool applied to evaluate the pollution level in water (22, 37–42), benthic sediments (21, 43), and soil (44). Toxicity in each of the compartments is evaluated based on the bioluminescence measurements in bacteria that emit light during their life activities (45). Luminescence measurements are performed before and after the bacterial suspension's exposure to the sample in question.

Mechanisms behind the toxic action of particular chemical compounds are variable and extremely complex. According to the available information, toxicity may result from (46)

- interaction between the toxin and cellular receptors;
- disruption of the plasmalemma functioning;
- chemical reactions within the cellular elements; and
- inhibition/competition of the enzymatic cycles.

In addition, the interactions (antagonistic or synergistic) occurring among chemical substances may significantly influence the test results (47, 48). Also, the use of reagents that increase the solubility of some compounds can change the real toxicity of the sample (49). Some substances may indirectly cause damage to the bacterial cells due to their dissociation and decomposition in aqueous environment into more toxic products. Through ionization, highly acidic or alkaline solutions may form whose pH becomes the main toxicity factor. On the other hand, after adding buffer to the solution, the same substance may turn nontoxic (50).

At present, the most frequently used commercially available devices that make use of the bioluminescence phenomenon in *Vibrio fischeri* are:

- ToxAlert 10 (Merck);
- ToxAlert 100 (Merck);
- Microtox (Azur Environmental); and
- LUMIStox (Dr. Bruno Lange).

Conclusions from the research indicate that toxicity data obtained from the same compounds with the use of different commercially available tests show some discrepancies (51). This can result from the differences in analytical procedures used in particular tests, composition of the reagents, and the preparation mode of the bacteria, employed by a manufacturer, i.e., liquid-dried or freeze-dried. Therefore, it is critical to perform a control test on a blank sample, consisting of zinc vitriol [Zn^{++}]. As recommended by the manufacturer, a ca. 50% decrease in the bacterial bioluminescence after a 30-minute incubation should occur at the [Zn^{++}] concentrations ranging from 2.11 to 25.0 mgL^{-1}, depending on the test type.

TESTS WITH THE USE OF PLANTS

In the case of toxicity tests based on the use of plants (phytotests) as the reactive elements, algae (green algae, cyanobacteria, and diatoms), duckweed, and rooted terrestrial and aquatic macrophytes (the plant and its seeds) are used. These plant organisms are representative for their natural habitats; they produce oxygen, promote the cycling of organic matter, control water quality, and the equilibrium of soil and benthic sediments. They also become food, shelter, and habitat for other organisms such as, insects, invertebrates, fishes, amphibians, birds, and mammals (52, 53). Changes undergoing in the plant may directly influence the structure and functioning of the entire ecosystem.

The adverse effect of pesticides on plants, herbicides in particular, raises a pronounced interest because of their common and increasing use that results in a widespread pollution of surfacewaters and groundwaters.

Throughout the years, in situ biomonitoring has been employed as popular means to use plants for testing the quality of aqueous environment. Aquatic plants were also used to eliminate suspended matter, heavy metals, nutrients (nitrogen and phosphorus), and toxic organic compounds as well as bacteria present in the runoff waters from mines, landfills, agricultural land, and storm water systems (52). Only recently the aquatic plants have been applied, in the form of phytotests, to evaluating the risk resulting from the aquatic environment pollution; this issue has been discussed further on in the ensuing chapter. In literature, there is a wealth of information on bioaccumulation of chemical pollutants in algae and macrophytes. These plant organisms have been used as in situ bioindicators in water quality control due to their propensity for accumulating chemical compounds as well as because their biomass constitutes a significant immobile part of the aquatic environment. A comprehensive study, dealing with the use of algae in biotests for determining the content of copper in the environment, has been published (3). Also, studies on algal uptake of metals such as, Mn^{6+}, Mo^{6+}, Ni^{2+}, and V^{5+}, were conducted (54).

The Use of Algae as an Active Element of Phytotests

The choice of an alga for a given test depends on the algal availability, culturing requirements, and the ease of its application. Based on these criteria, it has been recommended to use microalgae (in the form of a microscale biotest) from among which the two most frequently tested species belong to the family Chlorophyta, namely, *Selenastrum capricornutum* (55), *Scenedesmus quadricauda*, and *S. subspicatus* (56). Blue-green algae and diatoms are used less frequently because of their slow growth and demanding conditions for culturing.

The application of flow cytometry in testing based on microalgae use has been described in literature (57). Flow cytometry is a rapid method for the quantitative measurement of individual cells in a moving fluid. Thousands of individual cells are passed through a light source (lamp or laser) and measurements of light scatter and fluorescence properties are collected simultaneously. Although it is widely used in medical and oceanographic applications, flow cytometry has only recently been applied to ecotoxicological studies. This technique can be used to help overcome the limitations of the standard algal-growth inhibition tests. These limitations include:

- high density of cells, unattainable under natural conditions, which leads to aging of the species;
- lack of the techniques that allow performing cell counts with concomitant identification of live and dead cells, and suspended matter;
- impossibility to measure, at the same time, more than one parameter for a given species; and
- impossibility to obtain information about the toxicity mechanism resulting from pollution.

Microalgae are used successfully in flow cytometry because of their single-cell structure and the presence of a photosynthetic pigment, chlorophyll *a*, that is excitable by blue light. In the natural aquatic environment, an algal population rarely consists of one species (with an exception of algal blooms); therefore, the application of flow cytometry allows us to distinguish specific species, based on the received fluorescent signal, and in this way, the realization of biotests with the use of couple algal species is possible. In literature, many studies have been conducted, attempting to compare the results obtained from tests on single and multiple species, that were exposed to the same toxic substance (54, 55, 58, 59).

Another technique of conducting tests using microalgae involves immobilizing the cells on a special support. Such cultures retain stable respiration and photosynthetic processes, and after 12 months of storage at 4°C in the dark, they are capable of normal growth. Until now, immobilized algal cells were used mainly for removing heavy metals, and phosphorus and nitrogen compounds from wastewater. Recently, research has been undertaken to apply such cultures to water quality control in fish farming (56). In this respect, immobilization prevents washing out of algal cells and their subsequent consumption by herbivorous animals.

The application of chosen algal species in tests used for evaluating the toxicity of environmental samples is presented in Table 2.

The Use of Higher Plants as an Active Element of Phytotests

Toxicity studies using tests employing higher plants are conducted mainly in regard to pesticides, polycyclic aromatic hydrocarbons (PAHs), and heavy metals. Publications regarding the application of chosen plant species as an active element of appropriate biotets have been presented in Table 3.

Duckweed. Only extremely rarely do the macrophytes find an application in toxicity testing. However, if this plant group is mentioned in the published literature, then the presented studies most frequently deal with duckweed (*Lemna minor* and *L. gibba*), which is a species representative of all higher plants. Duckweed is free-floating, not rooted in the substratum. Its small dimensions, simple culturing requirements, and short generation time (doubling time of 1–4 days) had become decisive factors for the species' application as biological material. Relative sensitivity of different duckweed species (as well as duckweed sensitivity) in comparison to that of algae and other aquatic plants has not been established explicitly. In the literature, none of the described species, i.e., duckweed *Lemna minor*, and an alga *Selenastrum capricornutum*, showed significant sensitivity (33, 70). The investigations conducted on a numerous group of algae and microphytes with regard to herbicides indicate that the sensitivity of these plants is comparable (34). Upon the exposure to PAHs during the comparative tests, duckweed (*Lemna gibba)* displayed a sensitivity level similar to that of *Myriophyllum spicatum* (71).

Rooted Plants. Submerged and immersed rooted plants are used very rarely in toxicity tests. Large size, slow growth, and the lack of established rules for testing procedures have been influencing the sporadic use of these plants as biological material. In the literature, information on the use of *Myriophyllum spicatum* and *M. hydrophyllum* can be found. The plants are cultured in the laboratory or obtained from their natural environment. A description of culturing requirements for the chosen submersed microphyte species on natural substratum is presented in (72).

TESTS BASED ON THE USE OF ANIMAL ORGANISMS

Based on the literature data, it can be stated that acute toxicity tests using animal organisms are conducted much more frequently than phytotests. Only 10% of all the information that has been obtained on the subject of toxicity, until now, comes from phytotests. According to *Aquatic Toxicity Information Retrieval* (AQUIRE), from among the 20 most frequently described tests organisms, a green alga has placed only 13th. Thus far, plant species were considered less sensitive to chemical substances compared to animal organisms. Such a point of view has not yet been supported by the explicit research results. On the contrary, it has been established that the relative sensitivity of plants and animals greatly depends on environmental conditions such as organic matter, pH, temperature, water hardness, the presence of ligands, and the interactions among toxic substances. Studies conducted to compare the sensitivity of plants and animals are numerous (78–81).

The application of chosen animal species in toxicity testing of environmental samples has been presented in Table 4. The organisms were assigned according to the classification proposed in [82].

NEW TRENDS IN THE USE OF BIOTESTS IN ENVIRONMENTAL ANALYTICS

In literature, more information has appeared that deals with

- the development of new types of biotests;
- the implementation of commercially available biotests to the practice of analytics; and
- new uses of the known types of biotests.

Also, from information on new directions in biotest development has been published, the most important issues are discussed further here.

TABLE 2
Application of chosen algal species in tests used for evaluating environmental sample toxicity

Family	Species	Measured parameter and determined value	Test duration	Number of replicates	Recommending organization/ original source	Application	Literature cited
Chlorophyta	*Selenastrum capricornutum* (also known as: *Pseudokirchneriella subcapitata*, *Raphidocelis subcapitata*)	Growth inhibition, EC50	72 h	6	ISO 8692 (1989)	Comparison of selectivity and sensitivity of different algal species to herbicides and inorganic metal-containing compounds	(60)
				1	ISO/DIS 8692 (1987)	Impact of agricultural activities on the water quality in rivers (Japan)	(20)
		Growth inhibition, EC_{10} EC_{50}	7 d	6 (control), 1 (test)	ISO 8692 (1989)	Determination of toxicity of the antibiotics used in animal farms	(55)
		Growth inhibition, EC_{50}	24 h	3 (batch test) 2 (continuous test)	US EPA (1985)	Comparison of algal sensitivity to toxic metals based on static and dynamic cultures	(61)
		Fluorescence intensity, EC_{10}	2 d	3	(83)	Toxicity assessment of soil contaminated with PAHs	(62)
		Cell count, fluorescence intensity, EC_{10}, EC_{50}, LOEC, TU_{10}	48 h	6 (control), 3 (test)	ISO 8692 (1989)	Impact of agricultural activities on the quality of surface waters (Thailand)	(63)
		Chlorophyll content, EC_{20}, TDS (total dissolved solids)	72 h	3	Not specified	Impact of zinc and gold mining on the quality of freshwater (Alaska)	(37)
	Scenedesmus quadricauda	Growth inhibition, EC_{50}	72 h	6	OECD (1984)	Comparison of selectivity and sensitivity of different algal species to herbicides and inorganic metal-containing compounds	(60)
		Growth inhibition, respiratory rate, total chlorophyll, chlorophyll *a* and *b* contents, EC_{50}	12 d	3	Not specified	Uptake of metals (Cu^{+}, Cu^{2+}, Mn^{6+}, Mo^{6+}, Ni^{2+}, V^{5+})	(54)

(Continued on next page)

TABLE 2
Application of chosen algal species in tests used for evaluating environmental sample toxicity *(Continued)*

Family	Species	Measured parameter and determined value	Test duration	Number of replicates	Recommending organization/ original source	Application	Literature cited
	Scenedesmus subspicatus (new name:*Desmodesmus subspicatus)*	Growth inhibition, EC_{50}	72 h	6	ISO 8692 (1989)	Comparison of selectivity and sensitivity of different algal species to herbicides and inorganic metal-containing compounds	(60)
		chlorophyll *a* contents	14 d	3	ISO 8692 (1989)	Assessment of the riverine water quality with the use of *in situ* biotest (Scotland)	(64)
		Cell count, growth inhibition, EC_{50}	3 d	6 (control), 3 (test)	ISO 8692 (1989)	Impact of the light-absorbing substances on growth inhibition in algae	(65, 66)
		Biomass, fluorescence	72 h	6 (control), 3 (test)	DIN 38412 - part 33 (1989)	Influence of nutrient concentrations (nitrogen and phosphorus) on growth in algae	(19)
		Growth inhibition, EC_{50}	7 d	4	ISO (1989)	Obtaining data on the toxicity of fluoranthene and its biodegradation metabolites	(59)
	Stichococcus bacillaris	Growth inhibition, EC_{50}	72 h	6	OECD (1984)	Comparison of selectivity and sensitivity of different algal species to herbicides and inorganic metal-containing compounds	(60)
	Chlamydomonas reinhardtii	Growth inhibition, EC_{50}	72 h	6	OECD (1984)	Comparison of selectivity and sensitivity of different algal species to herbicides and inorganic metal-containing compounds	(60)
		Photosynthesis, chlorophyll content	4 h	3	Not specified	Toxicity assessment of the run-off from hazardous waste stored in salt mines	(67)
	Chlorella kessleri	Growth inhibition, EC_{50}	72 h	6	OECD (1984)	Comparison of selectivity and sensitivity of different algal species to herbicides and inorganic metal-containing compounds	(60)

	Dunaliella tertiolecta	AGP (algal growth potential)	4 d	3	US EPA (1974)	Assessment of the quality of coastal marine waters with the use of microbiotests (Italy)	(68)
Diatom	*Skeletonema costatum*	Cell count, fluorescence, growth inhibition, EC_{10}, EC_{50}, EC_{90}	3 d	6 (control), 3 (test)	ISO 102 53 (1995)	Assessment of the diatom sensitivity, with regard to recommendations of OSPAR (Oslo and Paris Commissions)	(58)
Rhodophyta	*Ceramium strictum, Ceramium tenuicorne* (also known as: *C. gobii)*	SMA: the final length (from the first branch to the longest tip); CIA: number of cells, the algal area, the length (includig branches); for both methods: EC_{10}, EC_{20}, EC_{25}, EC_{50}	7 d	SMA: 6 (control), 4 (test); CIA: 6 (control), 5 (test)	Not specified	Development of the methodology for 2 tests based on the use of macroalgae, i.e., SMA (*stereo microscope analysis*) and CIA (*computer image analysis*)	(69)
Cyanophyta	*Synechococcus leopoliensis (Anacystis nidulans)*	Growth inhibition, EC_{50}	96 h	6	OECD (1984)	Comparison of selectivity and sensitivity of different algal species to herbicides and inorganic metal-containing compounds	(60)
	Microcystic aeruginosa	Growth inhibition, EC_{50}	7 d	6 (control), 1 (test)	ISO (1989)	Determination of toxicity of the antibiotics used in animal farms	(55)

DIN-Deutsches Institut für Normung.
ISO-International Standard Organization, Geneva, Switzerland.
OECD-Organization for Economic Co operation and Development.
US EPA-United States, Environmental Protection Agency.

TABLE 3
Application of higher plant in tests used for evaluating environmental sample toxicity

Species	Measured parameter and determined value	Test duration	Number of replicates	Conditions	Recommending organization/ original source	Application	Literature cited
Duckweed *Lemna minor*	Frond count, biomass	96 h	3	Static		Comparison of the relative sensitivity of duckweed and a green alga to 16 herbicides	(34, 70)
						Impact of agricultural activities on the riverine water quality (USA)	(33)
Duckweed *Lemna gibba*	Frond count, chlorophyll *a* and *b* contents, growth inhibition, EC_{50}	8 d	3	Static	ASTM (1998)	Comparison of the sensitivity of duckweed to PAHs	(71)
		Not specified				Assessment of risk to aquatic organisms resulting from the presence of herbicides in water courses (USA)	(73)
Ceratophyllum demersum, *Elodea canadensis*, *Myriophyllum heterophyllum*, *Najas*	Wet weight increase	14 d	3	Static	Not specified	Comparison of the relative sensitivity of 5 species of macrophytes and 6 species of algae to 4 herbicides	(34)
Myriophyllum spicatum	Shoot length, number of leaf nodes of the main shoot and side shoots, number and length of all roots, chlorophyll *a* and *b* contents, growth inhibition, EC_{50}	12 d	3	Static	ASTM (1998)	Comparison of the sensitivity of duckweed to PAHs	(71)
Lactuca sativa	Inhibition of root elongation, IC_{50}	120 h	3	Static	NWRI	Comparison of the test results obtained during inter-laboratory research by WaterTox Network; and further on, quality assessment of drinking water	(74, 75)
Ryegrass *Lolium perene* Radish *Raphanus sativum*	Growth inhibition, number of emerged plants, dry weight	14 d	4	Static	OECD	Assessment of toxicity and mobility of heavy metals in sandy soil (Belgium)	(76)
Willow trees *Salix viminalis x schwerinii*	Growth inhibition, transpiration, efficiency of water use	336 h/ 381.5 h/ 361.5 h		Not specified		Toxicity assessment of soil contaminated with PAHs (Denmark)	(77)

APHA-American Public Health Association.
ASTM-American Society for Testing and Materials.
NWRI-National Water Research Insitute, Burlington, Ontario, Canada.
OECD-Organization for Economic Cooperation and Development.

TABLE 4
Application of chosen animal species in tests used for evaluating environmental sample toxicity

Phylum	Class	Species	Measured parameter and determined value	Test duration	Conditions	Recommending organization/ original source	Application	Literature cited
Ciliata	*Holotricha*	*Tetrahymena pyriformis*	Cell count, growth inhibition, EC_{50}	46 h	Static	Not specified	Development of the methodology for a growth inhibition test conducted on multiple, consecutive generations of protozoans	(35)
Annelida	*Oligochaeta*	Tubificid sludge-worms (*Tubifex tubifex, Limnodrilus hoffmeisteri*)	Concentration of substance in animals and sediment, BAF, BSAF	12 d	Static	Not specified	Bioaccumulation of lindane and hexachlorobenzene under laboratory conditions	(8)
	Hirudinea	Medicinal leeches (*Hirudo medicinalis L.*)	Mobility, avoidance reaction, changes in body shape, feeding activity	7/21 d	Static	Not specified	Toxicity assessment of water, benthic sediments in the lake and model mix of heavy metals based on the observations of behavioral changes in leeches (Lithuania)	(9)
Mollusca	*Bivalvia*	Glochidial larvae of a freshwater clam (*Anodonta cygnea zellensis*)	Viability	24/48/72 h	Static	Not specified	Influence of pH and water hardness on Cd, Cu and Zn toxicity to the larvae of freshwater clam (glochidium)	(10)
		Zebra mussel (*Dreissena polymorpha*)	Mortality, reattachment success, condition index, gonadic index	2 months	Dynamic	Not specified	Determination of genotoxicity of the riverine water based on micronuclei (MN)inhibition; in situ test (France, Belgium, Luxembourg, Germany)	(15)

(Continued on next page)

TABLE 4
Application of chosen animal species in tests used for evaluating environmental sample toxicity *(Continued)*

Phylum	Class	Species	Measured parameter and determined value	Test duration	Conditions	Recommending organization/ original source	Application	Literature cited
		Fingernail clam *(Sphaerium fabale)*	Length (initial, final), growth rate, natality, survival	70–135 d	Dynamic	Not specified	Use of 2 types of cages for an in situ test (USA)	(16)
		Asiatic clam *(Corbicula fluminea)*	Wet weight, metallothionein concentration	21/49/85/120/ 150 d	Dynamic	Not specified	Investigations of Cd and Zn bioaccumulation based on metallothionein (MT) levels in clams relocated to the area contaminated with heavy metals (France)	(23)
			Length of amphipods, sex, presence of eggs, survival	28 d	Semi-static	Environment Canada (1993)	Toxicity assessment of benthic sediments contaminated with copper; test conducted in a mesocosm (New Zealand)	(28)
	Insecta	Mayfly nymphs (*Hexagenia bilineata*)	Growth, survival	21 d	Static	Not specified	Determination of bioaccumulation of mercury originating from the pollutants in riverine sediments (USA)	(87)
		Mayfly nymphs (*Hexagenia limbata*)	Wet weight, mortality, metal concentrations	21 d	Static	Not specified	Investigations of spatial and temporal variation in concentrations of metals polluting benthic sediments (Canada)	(88–90)
		Chironomid larvae (*Chironomus tentans*)		10 d				

Chordata	*Pisces*	Bluegill *(Lepomis macrochirus)*	Total length, wet weight, mortality, mean liver weight (per fish), mean concentration of cadmium (per gram of liver), mean total cadmium–binding capacity of hepatic MBP per fish, LOEC	28 d	Semi-static	Not specified	Toxicity assessment of riverine sediments contaminated with cadmium (USA)	(24)
			Mean length, mean weight, survival	6 weeks	Dynamic	Not specified	Investigations of end-points and effects of a triazinone herbicide metribuzin in the aquatic environment; a mesocosm test (USA)	(30)
		Flounder *(Platichthys flesus)*	Mean length and weight, concentration of metals in liver and fillet of flounder, TEQ (toxic equivalency factor—final concentration of PCDF/PCDD)	3 months	Dynamic	Not specified	Determination of the pollution level with chloroorganics and metals in benthic sediments; in situ and mesocosm testing (Norway)	(29)
		Channel catfish *(Ictalurus punctatus)*	Hematocrit levels, hemoglobin, δ-aminolevulinic acid dehydratase activity, plasma glucose, plasma chloride, DNA strand breakage, condition factor (CF = total body length/ weight), liver somatic indices (LSI = liver weight/ total body weight), spleen somatic indices, (SSI = spleen weight/ total body weight)	1/84 d	Dynamic	Not specified	Impact of coal mining on the pollution level of waters and benthic sediments; in situ test (USA)	(18)

(Continued on next page)

TABLE 4
Application of chosen animal species in tests used for evaluating environmental sample toxicity *(Continued)*

Phylum	Class	Species	Measured parameter and determined value	Test duration	Conditions	Recommending organization/ original source	Application	Literature cited
	Mammalia	Female rats	[1]Activities of cytochrome P450 1A1 (as EROD) and P450 2B (as PROD), liver weight, [2]microsomal protein content, serum total thyroxine (T4), thyroid follicle colloid area and follicular cell height	22 d	Static	[1][91] [2] [92]	Toxicity assessment of the soil extracts containing high concentrations of PCB, originating from a decommissioned landfill (USA)	(93)
Arthropoda	*Crustacea*	*Daphnia magna* (cladocera)	Average of total number of offspring produced per parent animal alive at the end of test, survival rate	21 d	semi-static	OECD (1998)	Assessment of the riverine water quality (Japan)	(11)
							Toxicity assessment of storm water (Japan)	(12)
			Immobility, EC_{50}	24 h	Semi-static	ISO 6341 (1996)	Determination of a relationship between the age of test organisms and the results of acute toxicity test	(36)
			Immobility, 24-h $LC_{50,}$ 48-h LC_{50}	24/48 h	semi-static	NMX-AA-087 (SCFI 1995)	Efficiency assessment of wastewater treatment from textile industry (Mexico)	(13)
						NOM-074-ECOL-1994	Toxicity assessment of treated industrial wastewater and untreated hospital wastewater (Mexico)	(14)
			Algal density, feeding rates, survival rate	Exposure period: 24 h, post-exposure period: 4 h	Dynamic	[84]	Toxicity assessment of the riverine water based on the observations of decreased appetite due to the earlier exposure; in situ test (Scotland)	(17)

Hyalella azteca (copepod)	Mortality	10 d	Static	ASTM (1990)	Impact of the activities within the golf course facilities on the pollution level of benthic sediments in the nearby bay (USA)	(85)
Mysidopsis bahia (mysid)		4/7 d	Dynamic	US EPA (1994, 1996)		
Leptocheirus plumulosus (amphipod)		28 d	Static	US EPA (1998)		
Paracorophium excavatum (amphipod)	Length of amphipods, sex, presence of eggs, survival	10 d	Static	Not specified	Toxicity assessment of benthic sediments contaminated with copper; a 28-day test conducted in a microcosm (New Zealand)	(26)
		28 d	Semi-static			
	Survival, reburial ability	10 d	Static	Environment Canada (1992)	Determination of pollution level of benthic sediments contaminated with copper (New Zealand)	(86)

ASTM-American Society for Testing and Materials.
ISO-International Standard Organization, Geneva, Switzerland.
OECD-Organization for Economic Cooperation and Development.
SCFI-Secretaria de Comercio y Fomento Industrial.
US EPA-United States, Environmental Protection Agency.

Battery of Biotests

The choice of a proper biotest for toxicity testing depends on the type of information required, condition and physicochemical properties of the analyzed sample, type of toxic substance, as well as sensitivity of the test organism. In the case of single-species biotests, the estimated toxicity reflects the sensitivity of individuals belonging to one species only. Such testing procedure bears a risk of underestimating the toxicity of a given substance in regard to the entire environment. The risk can be diminished by employing a battery of biotests (sometimes called also "test battery") that use organisms of varying sensitivity and representative of different trophic levels. This approach is often applied when complex mixes of substances of unknown properties are being investigated. The comparisons of relative sensitivity among the species used in a test battery can be performed based on the values of the parameter "w", calculated from the following equation [78]:

$$w = \frac{NOEC_{\mathrm{x}}}{NOEC_{\mathrm{mean}}} \qquad [1]$$

where

$NOEC_{\mathrm{x}}$—the concentration of toxic substance for which there is no observed adverse effect (No Observed Effect Concentration), obtained from the test used,

$NOEC_{\mathrm{mean}}$—arithmetic mean value of NOEC, calculated from all tests used in the test battery.

Conclusions about the biotest sensitivity are drawn based on the value of the parameter "w." For values $w < 1$, the test organisms are characterized as having high relative sensitivity to the toxic substance in question.

Microbiotests

The need to analyze a large number of environmental samples in a relatively short period of time resulted in the increased significance of miniaturized toxicity tests, known as microbiotests, alternative tests, or the second-generation tests (94). Microbiotests are based on the use of single-cell or small metazoan organisms that react in a specific way to the contact with a liquid sample. Because of numerous advantages, alternative tests are most frequently used in a form of aforementioned battery of biotests that have been based on the application of the organisms from different trophic levels. Due to the fact that microorganisms constitute a basic trophic level in the food chain, any adverse changes taking place within them may, directly or indirectly, influence the organisms at higher trophic levels, and further on, at the ecosystem level. Because of high specific surface and a direct contact of plasmalemma with the analyzed medium, microorganisms display higher sensitivity to toxic substances than invertebrates or fishes. In general, toxicity is the function of exposure time, therefore, the long-term testing plays a particularly significant role in ecotoxicology. However, conducting the long-term testing on long-lived species is troublesome. The microorganisms characterized by a short generation time are a convenient solution that helps to elucidate the impact of long-term exposure to a toxic substance. Moreover, the parameters such as elimination of culturing; low cost of analysis per sample; possibility to analyze multiple samples in parallel; fast generation of results; small individual sample volume; low laboratory space demand for the proper equipment; and possibility of field-testing have been definitely causing the increased interest with this method of evaluating the environmental pollution.

In Table 5, the information on commercially available microbiotests, the so-called "toxkits," has been presented (95). The scientific team from the University of Ghent, Belgium is the pioneering group that has originated and developed the methodology for the tests employing microorganisms without requiring their culturing. The organisms are delivered to the laboratory in the cryptobiotic form, i.e., rotifers as cysts, crustaceans as resting eggs, and algae as cells immobilized on a proper medium and covered with special liquid that prevents their growth; such preparations can be stored in a refrigerator for up to a couple months. Before starting the test, cysts are placed in water where, under the strong light conditions, a development of resting stages occurs. After 18–96 hours (this time is species-dependent), the hatching of young individuals that are ready for testing takes place. The elimination of culturing permits lowering the costs of analysis. The use of standard organisms allows researchers to standardize a given test and to obtain repeatable results by different laboratories.

CONCLUSION

The results of chemical analysis are the source of qualitative and quantitative data about different forms of pollution that occur in the analyzed environmental samples. Also, a comparative analysis of the determined values and the appropriate standard values allows research to obtain a general information about the toxicity level of the material in question. However, these results cannot be treated as a direct data source about the toxic effect, both acute and chronic, on a live organism. Such direct data can be obtained from appropriate biotests.

At present, the majority of tests is based on the use of animal organisms as an active element. However, it seems that the importance of phytotests will be on the increase in connection to:

- the sensitivity of plant organisms to the changing conditions; and
- an increasing number of legal regulations that recommend the use of phytotests.

Numerous publications dealing with biotests contain information about the experiments conducted in micro- and mesocosms. In turn, the quick generation of data on the pollution level in a given environmental compartment can be facilitated with the use of microbiotests. To avoid the underestimation of toxicity in the analyzed sample, a battery of biotests is widely used. Very detailed information on bioindicators and biomonitors, used in

TABLE 5
List of commercially available microbiotests (*toxkits*)

Test	Taxon	Species	Test duration	Type of test	Recommending organization
Tests for terrestrial and freshwater environments					
ALGALTOXKIT F™	*green alga*	Selenastrum capricornutum (renamed: Raphidocelis subcapitata lub Pseudokirchner-iella subcapitata)	72 h	*growth inhibition*	OECD, ISO
DAPHTOXKIT F™ magna	*cladoceran crustacean*	*Daphnia magna*	24–48 h	*acute toxicity*	OECD, ISO
DAPHTOXKIT F™ pulex	*cladoceran crustacean*	*Daphnia pulex*	24–48 h	*acute toxicity*	OECD
CERIODAPHTOXKIT F™	*cladoceran crustacean*	*Ceriodaphnia dubia*	12 h	*acute toxicity*	US EPA
THAMNOTOXKIT F™	*anostracan crustacean*	*Thamnocephalus platyurus*	24 h	*acute toxicity*	
ROTOXKIT F™	*Rotifer*	*Brachionus calyciflorus*	24 h	*Acute toxicity*	ASTM
ROTOXKIT F™ short-chronic			48 h	*short-chronic (reproduction)*	AFNOR
PROTOXKIT F™	*ciliate protozoan*	*tetrahymena thermophila*	24 h	*Chronic (growth inhibition) toxicity*	OECD
OSTRACODTOXKIT F™	*ostracod crustacean*	*Heterocypris incongruens*	6 d	*chronic (mortality/growth inhibition) toxicity*	—
Tests for estuarine/marine environments					
ROTOXKIT M™	*rotifer*	*Brachionus plicatilis*	24–48 h	*acute toxicity*	ASTM
ARTOXKIT M™	*anostracan crustacean*	*Artemia franciscana (formerly Artemia salina)*	24–48 h	*acute toxicity*	—

ASTM-American Society for Testing and Materials.
AFNOR-Association Française de Normalisation.
ISO-International Standard Organization, Geneva, Switzerland.
OECD-Organization for Economic Cooperation and Development.
US EPA-United States, Environmental Protection Agency.

environmental studies, is contained in recently published fundamental monography (96).

Without a doubt, the area of application of biotests will be expanding, and the resulting data might become the basis for undertaking the investigations with the use of "classical" analytical methods.

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