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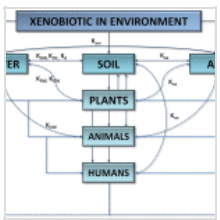
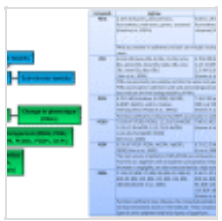
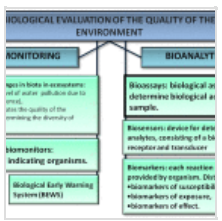
Figures (4)







Tables (5)

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Review article

Bioassays as one of the Green Chemistry tools for assessing environmental quality: A review

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Highlights

- Green chemistry principles should apply to the analysis of environmental samples.
- Toxicity as a summary assessment parameter of the quality of the environment
- Bioassays as a “green” alternative to conventional instrumental methods

Abstract

For centuries, mankind has contributed to irreversible environmental changes, but due to the modern science of recent decades, scientists are able to assess the scale of this impact. The introduction of laws and standards to ensure environmental cleanliness requires comprehensive environmental monitoring, which should also meet the requirements of Green Chemistry. The broad spectrum of Green Chemistry principle applications should also include all of the techniques and methods of pollutant analysis and environmental monitoring. The classical methods of chemical analyses do not always match the twelve principles of Green Chemistry, and they are often expensive and employ toxic and environmentally unfriendly solvents in large quantities. These solvents can generate hazardous and toxic waste while consuming large volumes of resources. Therefore, there is a need to develop reliable techniques that would not only meet the requirements of Green Analytical Chemistry, but they could also complement and sometimes provide an alternative to conventional classical analytical methods. These alternatives may be found in bioassays. Commercially available certified bioassays often come in the form of ready-to-use *toxkits*, and they are easy to use and relatively inexpensive in comparison with certain conventional analytical methods. The aim of this study is to provide evidence that bioassays can be a complementary alternative to classical methods of analysis and can fulfil Green Analytical Chemistry criteria. The test

organisms discussed in this work include single-celled organisms, such as cell lines, fungi (yeast), and bacteria, and multicellular organisms, such as invertebrate and vertebrate animals and plants.

Keywords

Green Analytical Chemistry; Ecotoxicity; Biotests; Battery of biotests

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1. Introduction

1.1. Sources and environmental fates of pollutants

The environment is a highly complex system that is split into biotic and abiotic parts, among which there is a continuous exchange of matter and energy. These processes should remain in balance, and this balance is called homeostasis. This sensitive balance may be disrupted by the release of various chemicals into the environment.

Virtually all human activities can cause environmental pollution, but some of them have important influences on the levels of anthropogenic impacts. Among these activities, the following different industrial branches can be considered: the petrochemical industry, the mining of precious metals and stones, tanneries, the lead battery industry, and industrial and/or municipal discharges. The pollution from other manifestations of human activity such as transportation, housekeeping, agriculture, sewage and municipal waste are not insignificant, either (Nadal et al., 2004; Mehlman, 1992; Kaldor et al., 1984; Cordy et al., 2011; Módenes et al., 2012; Bahadir et al., 2007; Rajaram and Das, 2008).

Environmental pollution does not respect geographic boundaries, and under favourable conditions, it may be transmitted over long distances and “travel” all over the biosphere (Oke, 2002; Walker et al., 1999; Hung et al., 2010). Pollutants may be transferred over long distances by different environmental components such as water and air (as well as particulate matter and aerosols) or by living organisms. Water and air act as a transport medium; however, transport by living organisms strongly depends on the migratory species in question (Lohmann et al., 2007).

Chemicals undergo a number of processes in the environment depending on their physicochemical properties. Hydrophilic substances remain dissolved in water, hydrophobic substances accumulate in soil or/and sediment and volatile compounds pollute the air. Chemicals may be partially bioaccumulated by living organisms (Zenker et al., 2014).

Fig. 1 shows the pathways through which xenobiotics move from the environment into the different levels of the food chain together with an indication of their bioaccumulation and

biomagnification. Human beings make up the last link in the food chain, and we are particularly vulnerable to the adverse effects of accumulated xenobiotics.

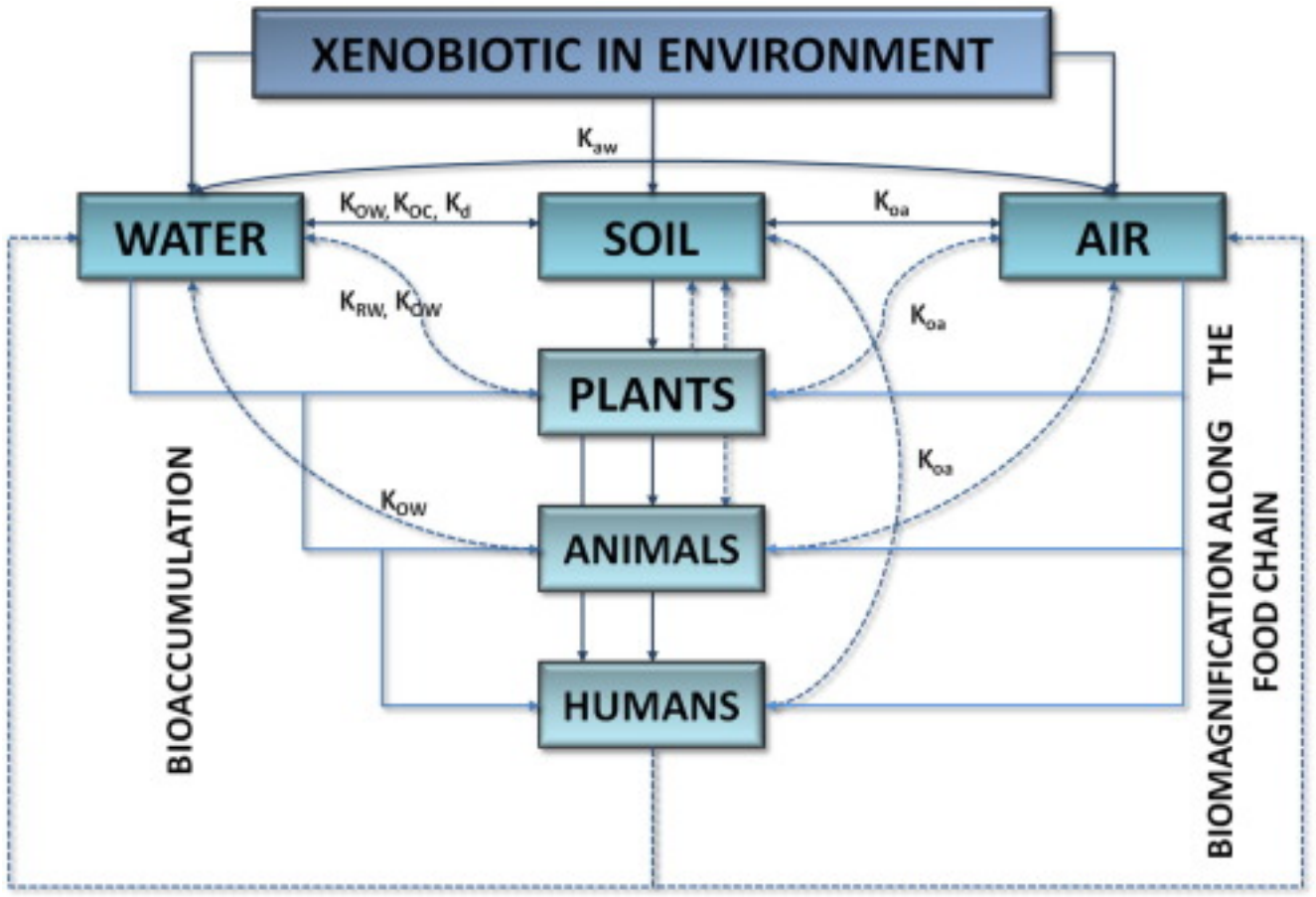

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Fig. 1. Pathways of xenobiotic circulation in the environment (K_{aw} — air-water partition constant, K_{ow} — octanol-water partition coefficient, K_{oc} — organic carbon to water partition coefficient K_{RW} — root to water partition coefficient, K_{oa} — octanol-air partitions coefficient , K_d — solid/liquid partition coefficient , CR — plant/soil concentration ratio).

1.2. Principles of Green Chemistry and Green Analytical Chemistry

To check the applicability of the analytical method, the method must be validated and optimized by determining parameters such as its accuracy, sensitivity, reproducibility, simplicity, cost effectiveness, flexibility and speed. However, none of these parameters helps to reduce the environmental burden of any specific method (Armenta et al., 2008).

At this point, it is not only “dry” validation parameters that are important but also the underlying principles, rules or guidelines are important as well. Compliance with these principles would help to reduce the burden of chemical operations on the environment, and using natural resources in a responsible and sustainable manner should be considered.

The Green Chemistry concept emerged in the 1990s and was aimed at reducing pollution by using so-called green solvents. Planning chemical processes to obtain a final product that would use the same amount of input materials (atom economy and catalysis) is essential to the Green Chemistry approach. In the late 1990s, the Green Chemistry idea

began to expand slowly in Europe and across the ocean in the United States, and its first concerns were chemical synthesis and chemical engineering. In the United States, the Environmental Protection Agency played a significant role in the introduction of new “green” ideas (Anastas and Kirchhoff, 2002).

In 1998, Anastas and Warner proposed a set of twelve Green Chemistry principles that would serve as guidelines, and these guidelines would be focused on reducing the waste that was generated during chemical processes, using non-toxic solvents, applying catalysts (when possible), and designing chemical processes in accordance with the principle of atom economy (Anastas and Warner, 1998).

Over time, the concepts and principles of Green Chemistry came into effect at a smaller scale for laboratory practice. In the Handbook of Green Analytical Chemistry, de la Guardia and Garrigues (2012) state the following five Green Analytical Chemistry strategies:

- remote sensing and direct measurement of untreated samples,
- replacement of toxic reagents,
- miniaturizations of procedures and instrumentation,
- automation, and
- on-line treatment of analytical wastes De la Guardia and Garrigues, 2012).

Efforts have been made to create a list of the twelve principles and goals of Green Analytical Chemistry to apply to analytical chemistry practices. The most important aspects were adopted from Green Chemistry, which plays a key role in this approach, and they are related to the elimination or reduction of the use of chemical substances (solvents, reagents, preservatives, additives for pH adjustment and others); the minimization of energy consumption; proper management of analytical waste; and increased safety for the operator (Gałuszka et al., 2013).

Newly emerging chemicals provide a major challenge to the analytical chemist because classical analytical methods involve the use of standards or the uploading of libraries of newly emerging compounds to existing libraries. Continuously decreasing the concentration levels of chemicals does not always make sense because one has to consider environmental samples as a mixture of different chemicals, which do not remain neutral when they interact with one another. The chemicals in a given mixture may act synergistically, antagonistically or additively, resulting in a toxicity shift in the exposed organism. They are active even at ultralow concentration levels (below those set by legal regulations) and may cause adverse effects in ecosystems (Wieczerzak et al., 2015). In these cases, classical analyses (even when conducted according to Green Chemistry principles) is not sufficient and chemical quantitation should be supplemented with

biological tools.

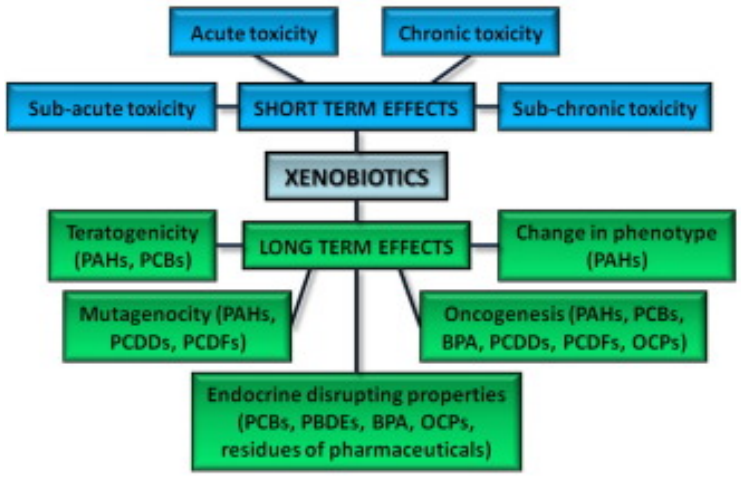
All the issues outlined above reflect the complexity of the problem of determining and removing contaminants from the environment. Green Chemistry is increasingly recognized as an overarching tool that is now included in most chemical operations and chemical analyses. In many countries, there are norms and laws that aim to protect the environment and human health during the design of chemical processes (Anastas and Warner, 1998).

1.3. Evaluating the toxic effects of environmental pollutants

Toxicology is a scientific discipline; it is the study of the toxic properties of chemical substances against living organisms. Through their behaviours, living organisms reflect both the negative and positive effects of stressors. The basis of toxicological studies is dose-response dependence. However, the observed effect is a combination of many factors such as species and individual differences, e.g., age and gender (Traczewska, 2011). The most frequently designated parameters include the ED_x/EC_x (effective dose/concentration), LD_x/LC_x (lethal dose/concentration), and the following parameters related to the threshold dose: the NOEL/NOEC (no observed effect level/concentration), LOEL/LOEC (lowest observable effect level/concentration) and LOAEL (lowest observable adverse effect level).

The pollutants that are present in the environment can affect organisms in many ways. Some compounds are characterized by high toxicity, or they are present in large enough quantities to produce immediate acute toxicity, which can ultimately lead to death.

However, most environmental stressors occur at levels below lethal concentrations or even at trace amounts, causing sub-acute, chronic or sub-chronic toxicity. These changes can be observed after a longer exposure time and within a few generations. The time of exposure to a given compound or to a mixture of compounds is also very relevant, with a prolonged exposure time increasing the chance of the appearance of a distant toxic effect such as teratogenotoxicity and mutagenic effects (see Fig. 2.) (Kuczyńska et al., 2004).



Compounds	logKow	logKoa	logKoc
PAHs	4.18-6.0 (fluorens, phenanthrens, fluoranthens, anthracens, pyrens, chrysene) (Finizio et al., 1997a)	6.68-11.19 (fluorens, phenanthrens, fluoranthens, anthracens, pyrens, chrysene) (Finizio et al., 1997b)	3.02-6.11 (naphthalenes, acenaphthylenes, acenaphthene, fluorenes, phenanthrenes, anthracene, fluoranthene, pyrene, chrysenes, perylenes) (Hawthorne et al., 2006)
	PAHs accumulate in sediments and soils are virtually insoluble in water and undergo biomagnification processes along the food chain.		
PCB	4.63-8.20 (mono-CBs, di-CBs, tri-CBs, tetra-BCs, penta-CBs, hexa-CBs, hepta-CBs, octa-CBs, nano-CBs, deca-CBs) (Han et al., 2006)	6.78-8.99 (4-chlorobiphenyl, 4,4'-diCBP, 2.4.5-triCBP, 2,3.4.5-tetraCBP, 2,2',4,4',6,6'-hexaCBP) (Harner et al., 1995)	5.5-8.5 (mono-CBs, di-CBs, tri-CBs, tetra-BCs, penta-CBs, hexa-CBs, hepta-CBs, octa-CBs, nano-CBs) (Zimmerman et al., 2004)
	PCBs are practically non-volatile and into the water only pass particles with low chlorination. Partition coefficients indicate that PCBs accumulate in sediments and soils and undergo processes of biomagnification along the food chain. Moreover, literature data indicate the low biodegradability of PAHs.		
OCPs	4.70-5.60 (chlordane, 4,4 DDD, 4,4 DDE, 4,4DDT, dieldrin, endrin, lindane, methoxychlor) (Finizio et al., 1997a)	7.26-9.93 (α-HCH, γ-HCH, HCB, p,p'-DDE, p,p'-DDD, p,p'-DDT) (Harner et al., 1995)	data not available
	Partition coefficients indicate that OCPs accumulate in sediments and soils are virtually insoluble in water.		
PCDD	7.02-8.6* (TCDD; PCDDs, 1,2,3,4,7,8-HxCDD, 1,2,3,6,7,8-HxCDD, 1,2,3,7,8,9-HxCDD, 1,2,3,4,6,7,8-HpCDD, OCDD) (Kim et al., 2002)	7.86-11.42 (TCDD, PCDDs) (Harner et al., 2000)	6.38-7.95* (TCDDs; PCDDs, 1,2,3,4,7,8-HxCDD, 1,2,3,6,7,8-HxCDD, 1,2,3,7,8,9-HxCDD, 1,2,3,4,6,7,8-HpCDD, OCDD) (Kim et al., 2002)
PCDF	6.5-8.8* (TCDF; PCDFs; HxCDFs, HpCDFs, OCDF) (Kim et al., 2002)	9.75-12.5 (HxCDF, HpCDF) (Liet al.,2008)	5.86-8.16* (TCDF; PCDFs; HxCDFs, HpCDFs, OCDF) (Kim et al., 2002)
	The main source of pollution PCDFs/PCDD are combustion processes in industry, power industry and in the municipal sector. From the air, together with atmospheric precipitation these compounds get into water soils and sediments. Their presence in the water is negligible, are also characterized by a high toxicity.		
PBDE	5.74-8.27 (BDE-17, BDE-28, BDE-47, BDE-85, BDE-99, BDE-100, BDE-153, BDE-154, BDE-183) (Braekevelt et al., 2003).	9.30-11.97 (BDE-17, BDE-28, BDE-47, BDE-66, BDE-77, BDE-100, BDE-99, BDE-85, BDE-126, BDE-154, BDE-153, BDE-156, BDE-183) (Harner et al., 2002)	6.2-6.5 (BDE-47, BDE-99, BDE-100) (Streets et al. 2006)
	Partition coefficient Kow indicates the strong hydrophobicity of PBDEs. Their amount in the air and water decreases with increase of bromine atoms in the molecule. These compounds are practically insoluble in water and cumulate in the organic layer of soil or sediment and fatty tissues of organisms.		

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Fig. 2. Classification of health effects caused by different types of xenobiotics and the physicochemical parameters of selected representatives of given xenobiotic groups (PAHs, polycyclic aromatic hydrocarbons; PCDDs, polychlorinated dibenzodioxins; PCDFs, polychlorinated dibenzofurans; PCBs, polychlorinated biphenyls; PBDEs, polybrominated diphenyl ethers; PBA, bisphenol A; and OCPs, organochlorinated pesticides (Amarillo et al., 2014; Huang et al., 2014; Salice et al., 2014; Rice et al., 2003; Cohn et al., 2011; Kudłak and Namieśnik, 2008; Wiseman et al., 2011; Vandenberg et al., 2007; Belmeskine et al., 2012; Mrema et al., 2013; Klaassen, 2013; Wink et al., 1991; Park et al., 2011; Jajoo et al., 2014).

Environmental pollution is such a broad issue that a need has arisen for a new field of science that will cover all the problems connected to the environmental fate of pollutants. Ecotoxicology is the branch of science that addresses the study of xenobiotic impact on the environment and covers the entire “life cycle” of toxic substances in the biosphere. At present, ecotoxicological studies are gaining significance, and a new approach from the field of bioanalytics and biomonitoring makes it possible to assess risks and to assess environmental quality quickly; for example, to test the safety of medical products derived from bacterial toxins such as vaccines (Sesardic, 2012).

For these reasons, more and more interest is being devoted by scientists to reducing the harmful chemicals used in environmental monitoring and analyses and to replace at least some of them with biological studies. To support this approach and apply biotests and bioassays to the modern Green Analytical Chemistry field, the most significant information and parameters of both classical and novel tests are presented to facilitate the decision process by less experienced researchers. It is also necessary to account for the transformation and biotransformation of environmental pollutants within the biotic and

abiotic components of the environment, e.g., the compounds that are produced over the course of wastewater treatment, e.g., during ozonation and photocatalysis. Some of these compounds belong to a group of newly emerging contaminants and may be characterized by their greater hazards to and burdens on the environment. The advantage of bioassays in this case lies in their ability to assess the toxicity of a sample as a whole, and it does not matter whether the tested sample contains compounds that are known to humankind.

Next, emphasis should be placed on the problem of sample toxicity changes that are caused during disinfection processes. The list of toxic disinfection by-products covers numerous chemicals, including the following: halogenated organics, phosphines, cyanides, polycyclic aromatic hydrocarbons, metals and organometals, biocides as well as plant and pest control chemicals. According to European regulations, reliable information on the toxicity level of water samples should be based on acute and chronic toxicity determinations that have been performed in algae, macrophytes, *Daphnia*, and fish (those that are characteristic of a given region) to determine the pollution standards for a given ecosystem. For these reasons, bioassays should help with or even constitute the basis for determining the legal safety regulations and procedures of environmental risk assessments for wastes or newly emerging chemicals.

2. Assessment of environmental pollution using bioanalytical tools

2.1. Biomonitoring and bioanalytical methods as tools for environmental quality assessment

The first major contribution in the field of bioindication was made by Carl Linnaeus in the 18th century. Linnaeus discovered that there is a cyclicality and regularity to the processes that occur in nature (Hodacs, 2010; Jardine et al., 1996). Along with increasing knowledge about the processes that occur in the environment and the nature of chemical compounds, it becomes very clear that the study of environmental pollution should be approached comprehensively with consideration of the physical, chemical and biological phenomena that are likely to occur in the environment.

In situ biomonitoring involves the observation of bioindicators and biomonitors, and species of organisms with a narrow range of ecological tolerance for xenobiotics. The changes that are induced in the function, behavior or whole population of bioindicators may point to the degradation of the ecosystem. Further information can be provided by biomonitors (Rainio and Niemelä, 2003; Kevan, 1999; Paoletti, 1999; McCune et al., 1997). Biological environmental monitoring can be performed using a number of techniques and methods and with the appropriate tools (see Fig. 3).

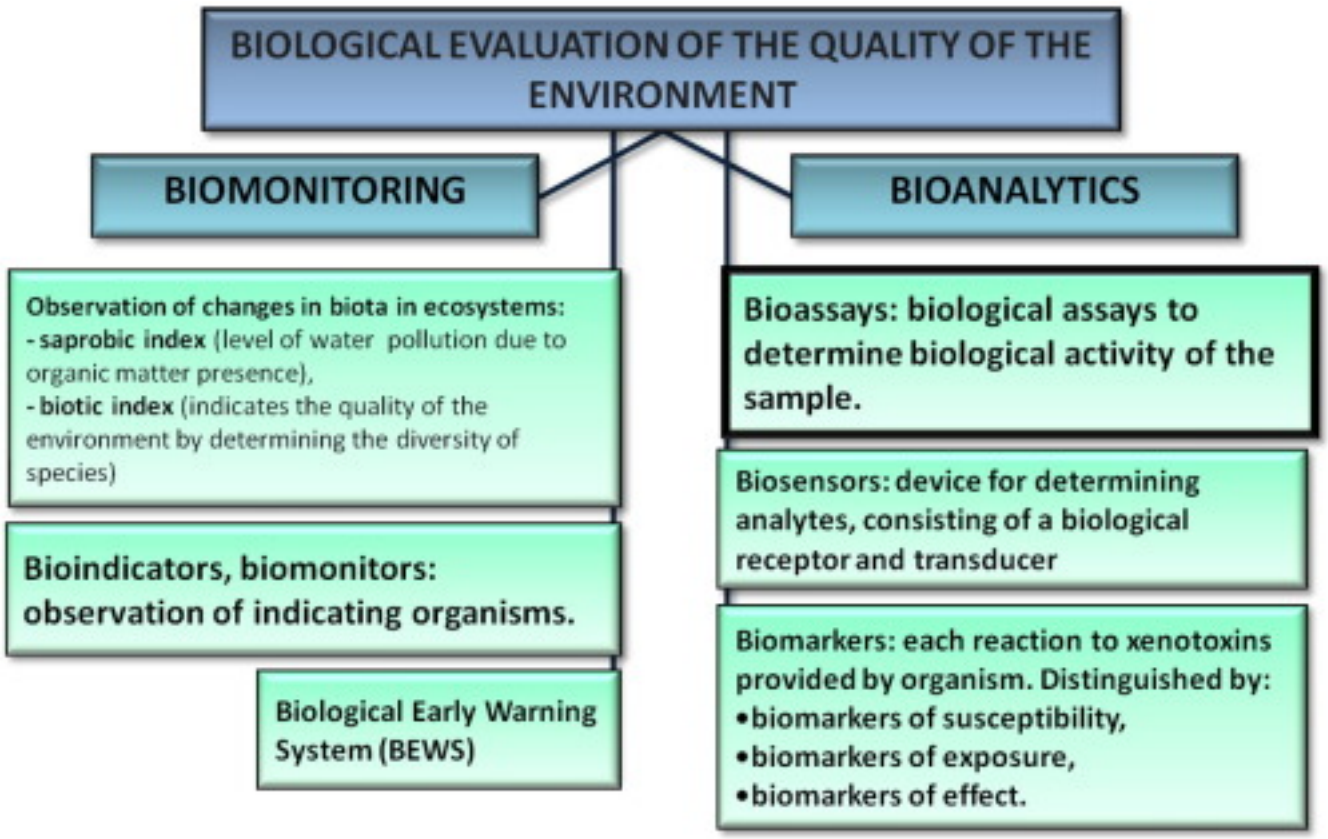

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Fig. 3. Tools of biological methods for estimating the state of the environment.

Environmental samples can also be collected and analysed ex situ using bioanalytical tools such as biosensors or bioassays, and they can be tested for the presence of biomarkers.

Bioanalytics has become a rapidly developing branch of environmental sciences. It began to take shape in the early 20th century, and since that time, it has been used successfully to monitor and evaluate the quality of the environment. At present, a wide range of tools is used for bioanalytical studies, with bioassays being one of them.

Test organisms that dwell in the environment have been “transferred” into the laboratory to allow for more controlled assay conditions. The test organisms must meet a number of requirements. They should be widely and easily available throughout the whole year in large quantities, with little difference in terms of genetics, and they must be free from disease or parasites. Furthermore, they should be sensitive to a wide range of toxins (or sensitive to a group of compounds for identification purposes), and the observed response must be distinctive and reproducible (Kuczyńska et al., 2004; Wardencki, 2004; Kudłak et al., 2012). Classical bioindication often includes observations and measurements of stressors in well-defined bioindicating plants or animals. After several decades of bioindication development, there has been progress in several new areas such as the more frequent inclusion of multi-element total analyses for a thorough investigation of mutual correlations in the sense of the Biological System of Elements, more studies on speciation issues into real effect-oriented environmental sciences, a

focus on integrative bioindication methods from a large number of environmental monitoring problems, and the development of integrative concepts such as the Multi-Markered Bioindication Concept (MMBC) to learn about precautionary environmental protection effects.

By conducting research with bioassays, not only can one find whether a sample contains toxic compounds, but one can also perform a qualitative and/or quantitative assessment by using specially selected organisms or a group of organisms. The biological evaluation of a sample involves the exposure of a selected organism to agents that are contained in the sample, followed by observations of characteristic endpoints. The selection of the appropriate test organism depends on many factors. These important factors are associated with the properties of the tested environmental sample (for example, the physical state, pH, oxidation saturation, ammonia, and sulphate) and the goals of the research. Studies may serve to detect the overall toxicity of the sample, such as non-specific toxicity tests, which are aimed at providing evaluations of the overall toxicity of all the chemical compounds contained in the sample. Specific toxicity tests are focused on a group of chemical pollutants that affect different receptors. Some chemicals may interfere with biomolecules such as DNA, causing genotoxic or mutagenic effects; therefore, reactive toxicity tests should be included (Farré et al., 2013; Pokhrel et al., 2012; Tang et al., 2012). To date, applications have been found for hundreds of different species of test organisms. Various species of plants, animals, fungi, bacteria, and cells are used in bioanalytics. Bioassays can be divided according to the test organism; that is, they are “cellular”, bacterial, animal and plant bioassays.

The difficulties associated with conducting bioassays have often been based on the need to culture the test organisms. Therefore, some bioassays are currently available in the form of ready-to-use *toxkits*, which contain all the necessary reagents and accessories. The production of *toxkits* began in Belgium, and test organisms are supplied by producers in their cryptobiotic forms, i.e., rollers and crustaceans in the form of cysts, plants in the form of seeds, algae immobilized in carrier fluid, bacteria in lyophilized form, and yeast cells (dry) applied to filter paper. To perform this type of test, the organisms in the cryptobiotic form should simply be incubated under appropriate conditions, which can save researchers from needing to conduct various breeding problems. A well-known example is a test based on the bacteria *Vibrio fischeri*, which is used to evaluate water quality. The test was developed in the 1970s and was the first microbioassay to be described. Shortly thereafter, tests based on other species of animals, plants, fungus (in vivo), organs, tissues and even various cells (in vitro) were introduced (Kudłak et al., 2011).

The selection of an appropriate test organism seems to be crucial for the success of this type of study. The results obtained in the laboratory should be easily transferred to the

given environmental conditions. Currently, some countries employ integrated environmental monitoring, which involves a combination of chemical and biological monitoring.

Most protocols that are currently applied to bioassays are based on international standardization guidelines; for example, the ISO (International Standard Organization), OECD (Organization for Economic Co-operation and Development) or U.S. EPA (United States Environmental Protection Agency), among others.

The application of chemical analysis techniques provides results that become a source of information about the state of specific environmental compartments and the processes within these compartments. However, the studies that employ these techniques are usually labor-intensive and time consuming, and they have to be performed by highly qualified employees, all of which significantly influences the cost of conducting the chemical analyses. Moreover, many of those techniques can be used under laboratory conditions only, and this limitation introduces additional delays between the sampling and the sample analysis phases. The application of this “classical” analytical approach does not allow for the inclusion of interaction effects among toxic substances. The selected and basic issues that occur when supplementing instrumental analyses with biotests are presented in Table 1.

Table 1. Information on supplementing of instrumental methods with bioassays.

	Chemical analyses	Bioassays
Advantages	– Both quantitative and qualitative analysis can be performed. – It is possible to trace the environmental fate of pollutants. – Samples can be stored and archived prior to and after analysis.	– It is possible to conduct tests in situ. – It is not necessary to purchase high-purity reagents and reference materials. – Running most of biotests does not require highly qualified personnel. – Tests supply information on the impact of pollutants on living organisms and ecosystems. – Relatively low cost per analysis. – Possible to perform qualitative and quantitative analysis for individual pollutants that are present in the samples being analysed.

Disadvantages	– Time-consuming and labor-consuming procedures during sample preparation. – High costs associated with the purchase of high-purity non-green reagents, their utilization and the management of their surplus. – Highly qualified personnel is required. – Do not supply information on the impact of pollutants on living organisms and ecosystems.	– Experience required to select battery of bioassays. – More difficult to maintain reproducibility and repeatability. – Necessity to keep clean cultures up.

2.1.1. Ecotoxicological assessment of liquid samples (water quality assessment)

The number of instrumental methods and techniques available for the analysis of aqueous samples is enormous, as are the studied parameters and compounds to be analysed.

The aquatic environment is inhabited by various creatures that have evolved various mechanisms and organs to survive (such as gills in fish). To a large extent, the development of a civilization, society and economy is heavily dependent on having access to a sufficient quantity of fresh clean water. Aquatic ecosystems have many functions, including the dilution, filtration, purification and storage of fresh water, flood prevention, microclimatic balancing and protecting biodiversity (Cardinale et al., 2011; Bernhardt and Palmer, 2011). The aquatic environment often becomes the final reservoir of environmental pollution, in which chemicals may adversely affect organisms that are living in an exposed ecosystem and may interfere with its function (Fleeger et al., 2003). Conventional methods of wastewater treatment are not always sufficient, and very often the process of water disinfection by ozone and chlorination contributes to the formation of pollution.

Depending on its use, water must meet certain standards and criteria that differ for surface water, drinking water, groundwater and bathing water. The directive of the European Union — Water Framework Directive 2000/60/EC and associated documents 98/83/EC (quality of water intended for human consumption), 2006/7/WE (bathing water quality), and 2008/105/WE (environmental water quality standards) clearly establish the objectives, standards and criteria that should be achieved by the Member States in

relation to water policy (EU, 2000, 2008, 2006). The quality of surface water is determined by a number of biological, physicochemical and hydromorphological parameters, and it is also necessary to investigate whether the water samples contain various pollutants (POPs, PAHs, PCBs, EDC, VOC and toxic metals). The aim of biological analysis is to determine the status and diversity of fauna and flora in the study environment and to assess the presence and abundance of sensitive species of fish, phytoplankton, phytobenthos and invertebrates (EU, 2013).

The amount and presence of pollutants in the aquatic environment largely depend on the efficiency of the given sewage treatment plant. Micropollutants pose new challenges to wastewater treatment techniques. The amounts of these compounds are constantly increasing in water; they consist in care products, hormones, pesticides, surfactants and other compounds. In some countries, rules have been established for micropollutants. Water monitoring using classical methods is only a quantitative assessment of water quality. Newly emerged chemical compounds, which are partly the result of transformation or biotransformation, are not detected by chemical water monitoring. It cannot be instrumentally determined whether the substances that are present in the aquatic environment are interacting. Water biomonitoring methods employ many aquatic organisms from multiple trophic levels, and they are used for in vivo and in vitro research. In vivo tests are performed in fish, algae, crustaceans, bacteria, rotifers, and invertebrates (Luo et al., 2014; Repetto, 2013).

The ecotoxicological assessment of water quality involves tests that employ bacteria, plants and animals. Most bacterial tests are based on the *V.fischeri* bacterium. This Gram-negative bacillus occurs in salt water, and its bioluminescence is a natural result of its metabolic processes. These bacteria are obtained in lyophilized form, and to create a suitable osmotic pressure, a solution of NaCl is added to the sample. An incubation period is followed by the reading of the bioluminescence level and the calculation of the EC_{50} parameters by software. MICROTOX® (Modern Water, UK) is currently the most popular kit of its type on the market; there are also kits based on bioluminescent bacteria called LUMITOX and BioTox™ (Marugán et al., 2012). The use of bioluminescent bacteria for research provides the opportunity to determine whether the substances contained in the sample cause acute toxicity against these one-celled organisms.

Other tests based on bacterial or mammalian cells allow for assessments of the genotoxic, mutagenic and carcinogenic properties of the sample. Tables 2–5 provide details on the tests that employ biological materials as their active components.

Table 2. Plant-based bioassays for evaluating environmental quality.

Bioassay -	Toxicity/measure of

production company	Species of the test organism	toxic effects, observed endpoint	Duration of test	Application/sam type
Allium cepa	*Allium cepa* (onion)	Genotoxicity/mutagenicity: clastogenic effects chromosome aberrations, nuclear abnormalities	Depending on sample incubation and purpose of the study	Pesticides and herbicides, exposure effects radiofrequency, electromagnetic fields, coal fly ash contaminated soi sludge from urba sewage treatmen stations, river impacted by industrial effluent metals and dyes, drinking water (Leme and Marin Morales, 2009).
Zea mays	*Zea mays* (maize)	Genotoxicity, mutagenicity: change in phenotype	Depending on sample incubation and purpose of the study	Contaminated so wastewaters, pur chemicals, herbicides (Schm et al., 2001).
Vicia faba	*Vicia faba* (faba bean)	Genotoxicity, mutagenicity: aberrations in meiotic chromosomes		Waters, wastewaters, sediment, contaminated soi river water, mode compounds, heav metals, radiation effect (Iqbal, 201

***Tradescantia* sp.**	*Tradescantia* sp. (spiderworts)	Genotoxicity, mutagenicity: presence of micronuclei		Contaminated so wastewaters, chemicals, pollute air (Traczewska, 2011).
LemnaTest – LemnaTec GmbH, Germany	*Lemna minor*, *Lemna gibba* (duckweed)	Acute and sub-chronic: growth inhibition	7 days	Chemicals, pesticides, aquec samples, substances solub in water (Cayuela al., 2007).
ALGALTOXKIT	*Raphidocelis*	Short-chronic: inhibition of	72 h	Surface waters,

F™ - MicroBioTests Inc., Belgium	*subcapitata*/*Pseudokirchneriella subcapitata* (algae)	growth		pure substances, wastewaters (http://www.microtests.be, 2015a).
ALGALTOXKIT F™ MARINE - MicroBioTests Inc., Belgium	*Phaeodactylum tricornutum* (diatom)	Short-chronic: inhibition of growth	72 h	Pure substances, wastewaters, surface waters ar deep sea waters, contaminated witl salt water and brackish (http://www.micro

tests.be, 2015b).

PHYTOTOXKIT F™ - MicroBioTests Inc., Belgium	Monocot plants *Sorghum saccharatum* (sorghum) and dicot plants *Lepidium sativum* (cress), *Sinapis alba* (mustard)	Short-chronic: inhibition of germination, root or shoot length reduction.	3 days	Soil, sediments, sewage sludge, compost, wastewaters used for irrigation, chemicals and biocides, compos (http://www.micro tests.be, 2015c).

Table 3. Animal-based bioassays for evaluating environmental quality.

Bioassay - production company	Species of the test organism	Toxicity/measure of toxic effects, observed endpoint	Duration of test	Application/sample type	Recomm organiza
Eisenia fetida*, *Eisenia Andrei	*Eisenia fetida*, *Eisenia Andrei* (earthworm)	Acute and chronic toxicity: mortality, inhibitions of reproduction. Bioaccumulation	Depending on sample incubation and purpose of the study	Soils, pure substances (Wang et al., 2012).	OECD, U ISO, AST AFNOR (1984; OE 2004a; U 1989; ISC 2012b,c, ASTM, 2(AFNOR,
Brachydanio rerio	*Brachydanio rerio* (zebra fish)	Acute and chronic toxicity: mortality, behavior, genotoxicity, carcinogenesis, bioaccumulation.	Depending on sample incubation and purpose of the study	Pure substances, groundwaters (Vilar et al., 2011).	OECD; IS (OECD, 1 1992b, 19 2000a; O 2012a; IS 1996a,b,(2007a).
Daphnia magna	*Daphnia magna* (crustaceans)	Acute and chronic toxicity: immobilization or mortality, inhibition of reproduction, inhibition of growth population	Depending on sample incubation and purpose of the study	Wastewaters, surface and deep sea waters (Czech et al., 2014).	DIN, US I EPS, AFI ASTM, IS OECD (D 1989; US 1993; EP 1996; AF 2003, 20(ASTM, 2(ISO, 201 2000b; O 2004b, 2(

Chironomus riparius*, *Chironomus tentans	*Chironomus riparius*, *Chironomus tentans* (midge)	Acute and chronic toxicity: mortality (larval stage - water test), inhibition of reproduction Endocrine disrupting properties, genotoxic properties	Depending on sample incubation and purpose of the study	Pure substances, insecticides, sediments, polluted water (Stefani et al., 2014).	EPS; US OECD; A AFNOR (1997a; U 2000; OE 2004c; A 2010a,b; AFNOR,
Oryzias latipes	*Oryzias latipes* (ricefish)	Acute toxicity: mortality. Endocrine disrupting properties. inhibition of reproduction	Depending on sample incubation and purpose of the study	Pure substances, sediments, contaminated waters (Hsu et al., 2014).	OECD (C 1992a,b, 2000a; O 2012a)
Ceriodaphnia dubia	*Ceriodaphnia dubia* (crustaceans)	Acute and chronic toxicity: mortality inhibition of reproduction inhibition of growth	Depending on sample incubation and purpose of the study	Pure compounds, effluents, sediments, surface and ground waters, wastewaters (Kokkali and Van Delft, 2014).	AFNOR, EPS (AFI 2009; AS 2010a,b; 1997b)
Daphnia pulex	*Daphnia pulex* (crustaceans)	Acute toxicity: immobilization or mortality of the test organisms	Depending on sample incubation and	Wastewaters, surface and deep sea waters (Kokkali and Van Delft,	US EPA; OECD (U 1993; EP 1997b; O

			purpose of the study	2014).	2004a)
Hyalella azteca	*Hyalella azteca* (amphipoda)	Acute and chronic toxicity: mortality, inhibition of growth, inhibition of reproduction	10 days	Freshwater, sediments (Gómez-Oliván et al., 2012)	ASTM; U (ASTM, 2010a,b; EPS, 200
Apis mellifera	*Apis mellifera* (honey bee)	Acute toxicity: mortality.	Up to 96 h	Plant protection products. (Traczewska, 2011).	OECD; E (OECD, 1998b,c; 2013)
Tubifex tubifex	*Tubificidae* sp. (sludge worm)	Acute and chronic toxicity: mortality, inhibition of growth, inhibition of reproduction	Depending on sample incubation and purpose of the study	Sediments, soils, pure chemicals (Pasteris et al., 2003).	ASTM (A 2010a,b).
Folsomia candida	*Folsomia candida* (collembola)	Chronic toxicity: inhibition of reproduction	Depending on sample incubation and purpose of the study	Soils, pure substances, pesticides (Santos et al., 2012)	ISO (ISO 2014b)
Drosophila melanogaster	*Drosophila melanogaster* (diptera)	Acute and chronic toxicity: cytotoxicity,	Depending on sample incubation	Polluted air, pure substances (Traczewska, 2011)	Lack of recomme organizat

		genotoxicity, chromosomal aberrations, recombination and gene mutations. Sex-linked recessive lethal mutations. Endocrine disrupting properties.			and purpose of the study	
DAPHTOXKIT F™	*Daphnia magna*	Acute and chronic	24 h	Wastewaters,		ISO; OEC

***magna* - MicroBioTests Inc., Belgium**	(crustaceans)	toxicity: immobilization or mortality, inhibition of reproduction, inhibition of growth population		surface and deep sea waters (http://www.microbiotests.be, 2015d).	(ISO, 201 OECD, 2
ROTOXKIT F™, ROTOXKIT F™ short-chronic/MicroBioTests Inc., Belgium	*Brachinious calyciflorus* (cotifers)	Acute and chronic toxicity: mortality, reduction of reproduction by the action of roller skates toxins	24 h/48 h	Pure substances, wastewaters, surface water and groundwaters (http://www.microbiotests.be, 2015e,f).	ASTM; A ISO (AST 2012b; A 2000b; IS 2008)
DAPHTOXKIT F™ pulex - MicroBioTests Inc., Belgium,	*Daphnia pulex* (crustaceans)	Acute toxicity: immobilization or mortality of the test organisms	48 h	Wastewaters, surface and deep sea waters (www.ebpi.ca, 2015a).	OECD (2
OSTRACODTOXKIT F™ - MicroBioTests Inc., Belgium	*Heterocypris incongruens* (crustaceans)	Acute and sub-chronic toxicity: mortality, growth inhibition	6 days	Freshwater sediments, also applied to the soil and solid waste (Kudłak et al., 2011; http://www.microbiotests.be, 2015g).	ISO (ISO 2012e)

RAPIDTOXKIT™ - MicroBioTests Inc., Belgium	*Thamnocephalus platyurus* (crustaceans)	Acute toxicity: reduction or complete cessation of food intake by organisms test	30–60 min	Assessment of water pollution (http://www.microbiotests.be, 2015h).	ISO (ISO
CERIODAPHTOXKIT F™ - MicroBioTests Inc., Belgium	*Ceriodaphnia dubia* (crustaceans)	Acute and chronic toxicity: mortality: inhibition of reproduction inhibition of growth	24 h	Pure compounds, effluents, sediments, surface and ground waters, wastewaters (http://www.microbiotests.be, 2015i).	Lack of recomme organizat
PROTOXKIT F™ - MicroBioTests Inc., Belgium	*Tetrahymena thermophila* (crotozoa)	Chronic toxicity: inhibition of growth		Pure substances, wastewaters, surface and deep sea waters	

				(http://www.microbiotests.be, 2015j).
ARTOXKIT M™-MicroBioTests Inc., Belgium	*Artemia franciscana* (crustaceans)	Acute toxicity: mortality	24–48 h	Pure substances, marine waters, estuaries and coastal waters (http://www.microbiotests.be, 2015k).

Table 4. Single-cell organisms-based bioassays for evaluating environmental quality.

Bioassay - production company	Species of the test organism	Toxicity/measure of toxic effects, observed endpoint	Duration of test	Appl type
***Umu*-Chromotest**	The mutant strains of *Salmonella* Typhimurium TA1535/pSK10002	Mutagenicity/genotoxicity: *umu*C gene induction overall SOS response	up to 48 h	Surfa sedir sludg (afte chem comp (Trac
Ames test	Modified strains of *Salmonella*	Mutagenicity/genotoxicity:	48 h	Surfa

	Typhimurium TA 98, TA 100, TA 102, TA 104, TA 1535, TA 1538, YG 1012, YG 1021, YG 1024 and NM 2009, *Escherichia coli* WP2	growth of bacterial colonies on the substrate poor in histidine, point mutations comprising a substitution, addition or deletion of one or more base pairs		sedir sewa soils extra subs (Trac
SOS-Chromotest	The mutant strains of *Escherichia coli*	Mutagenicity/genotoxicity: *lexA* and *recA* genes induce overall SOS response	24 h	Pure indus grou wast (Trac
GFP-receptor yeast assay	*Saccharomyces cerevisiae* (genetically mutated yeast cells)	Endocrine disrupting properties	Depending on sample incubation and purpose of	Envi samp subs (Bec

				the study
MICROTOX®/MICROTOX®-solid-phase test/DeltaTox® II, Modern Water, UK	*Vibrio fischeri* formal name *Photobarterium phosphoreum* (bacteria)	Acute toxicity: decrease in bioluminescence	5–30 min.	Petro conta drink indus salty wate phar minir and v from deter swim wate solid (www m, 2(
LUMISmini®/LUMIStox®- Hach Lange GmbH, Germany				Pure sewa landf (Kok Delft
Mutatox®- Modern Water,	*Vibrio fischeri* formal name	Acute toxicity: emission	16–24 h	Surfa

UK	*Photobarterium phosphoreum* (bacteria) (“dark mutant strain”)	of bioluminescence		grou wast leach sedir (www
Ames MPF™, Ames MPF™ 98/100, Ames MPF™ PENTA I, XENOMETRIX AG, Switzerland,	The mutant strains of *Salmonella* Typhimurium TA98, TA100, TA1535, TA1537, *Escherichia coli* WP2	Mutagenicity/genotoxicity: growth of bacterial colonies on the substrate poor in histidine, point mutations comprising a substitution, addition or deletion of one or more base pairs	48 h	Surfa sedir sludg extra chem (www , 201
Mini Ames Test - Cyprotex, UK,	The mutant strains of *Salmonella* Typhimurium TA98, TA100			Surfa sedir sludg (after chem comp (www

UmuC Easy AQ/UmuC Easy CS - XENOMETRIX AG, Switzerland	The mutant strains of *Salmonella* TyphimuriumTA1535/pSK10002	Mutagenicity/genotoxicity: *umu*C gene induction overall SOS response	30 h	Aque conc samp comp wast drink (www 2015
***Umu*-ChromoTest - EBPI Inc., Canada**	The mutant strains of *Salmonella* Typhimurium TA1535/pSK10002		30 h	Phar indus surfa grou potal subs their (www 2015
SOS-ChromoTest™ - EBPI Inc., Canada	The mutant strains of PQ37 *Escherichia coli*	Mutagenicity/genotoxicity: *lexA* and *recA* genes induce overall SOS response	24 h	Sedi chem comp cosm wast potal chem comp cosm wast potal (www 2015
LumiMARA- MARA NCIMB, UK	11 different microbial species (10 bacteria and 1 yeast)	Acute toxicity: Bioluminescence		Com efflu

		inhibition/growth inhibition		wate and/ (www 2015
XenoScreen YES/YAS® - XENOMETRIX AG, Switzerland	*Saccharomyces cerevisiae* (genetically mutated yeast cells)	Endocrine disrupting properties, cytotoxicity: growth arrest, lysis of yeast cells tested	48 h	Surfa wast aque and chem (www , 201

Table 5. Cell, enzyme and antibody-based bioassays for evaluating environmental quality.

Bioassay - production company	Species of the test organism	Toxicity/measure of toxic effects, observed endpoint	Duration of test	Application/sample type	Rec orga
Comet assay	Various types of cells comprise a cell nucleus	Genotoxicity: The amount of DNA that had moved and formed the so-called tail of a comet	Depending on sample incubation and purpose of the study	Surface water and sediments, sewage, sludge phase, soil (after extraction), substances and chemical compounds (Fairbairn et al., 1995).	AST (AS OEC
CALUX/(DR)CALUX	Specially crafted cells (analytes induce expression of luciferase)	Endocrine disrupting properties and dioxin-like coumpaunds: the level of bioluminescence	24 h	Soil, sediment, water, exhaust gases; biological fluids, food, consumer products (Murk et al., 1996).	US I EPA
Chromosome aberration test	Mammalian cells, microbial cultures	Cytotoxicity/genotoxicity: changes in chromosome structure	up to 48 h	Suspected cancerogens, pharmaceuticals, cosmetics and environmental samples, nutrients (Miller et al., 1998).	OEC 201 201
MTT/XTT/MTS/WST – dyes		Metabolic cytotoxicity: activity of the respiratory chain	Depending on sample incubation and purpose of the study	Pure compounds, pharmaceuticals, anti-cancer drugs, cosmetics and environmental samples (Tominaga et al., 1999).	OEC 201

SBR, CVDE – dyes	Cytotoxicity: total protein synthesis, cell proliferation	up to 3 h	Pharmaceuticals, environmental samples, nutrients (Traczewska, 2011).	Lacl recc orga
GLU test	Cytotoxicity: glucose consumption	1 h	Pharmaceuticals, cosmetics and environmental samples, nutrients (Traczewska, 2011).	
LDHe test	Cytotoxicity: membrane integrity, cell viability			
Sister chromatid exchange test (SCE)	Genotoxicity: detection of reciprocal exchanges of DNA between two sister chromatoids	Depending on sample incubation and purpose of the study	Suspected cancerogens, pharmaceuticals, cosmetics and environmental samples, nutrients (Traczewska, 2011).	
pNPP (PAC test), NR – dyes	Mammalian cells, Cytotoxicity: lysosomal activity, cell membrane	up to 4 h	Pharmaceuticals, cosmetics and	

	microbial cultures (adhesive cells)	permeability		environmental samples, nutrients (Traczewska, 2011).	
Micronucleus test	Mammals bone marrow cells	Genotoxicity/mutagenocity: chromosomal damage the appearance of micronuclei	Depending on sample incubation and purpose of the study	Suspected cancerogens, pharmaceuticals, cosmetics and environmental samples, nutrients (Traczewska, 2011).	OEC 201
Direct/indirect/sandwich ELISA	Different antigens or antibodies	Presence of specific proteins		Biological material, plasma, serum (Akamizu et al., 2005).	Lacl recc orga
Platinum™ ELISA -				Cell culture	

eBioscience, Inc., USA				supernatant, serum, plasma (citrate, heparin) (www.ebioscience.com, 2015).
Single Endpoint Kits GLU Glucose, LDHe Extracellular Lactate dehydrogenase NR Neutral Red SRB Sulforhodamine B XTT Tetrazolium Hydroxide XENOMETRIX AG, Switzerland	Mammalian cells, microbial cultures	Cytotoxicity: membrane integrity metabolic activity respiratory chain activity total protein synthesis, DNA content, lysosomal activity	up to 7 h	Pharmaceutical, chemicals, environmental compounds, nutrients (http://amestest.cz, 2015).

Multiple Endpoint Kits **NR-SRB** **NR-CVDE** **LDHe-GLU-XTT-SRB** **LDHe-GLU-XTT-PAC** **XTT-SRB-CVDE** **LDHe-XTT-NR** **XENOMETRIX AG, Switzerland**	Mammalian cells, microbial cultures (adherent cells)

The most common quantitative determination of cytotoxic activity involves the use of various types of cell lines in vitro. This measurement can relate to a number of cells, their ability to divide, the cell membrane functionality, mitochondrial activity, incorporation of dyes into lysosomes, the total protein or DNA contents in the cell, and the inhibition of DNA synthesis.

To determine the cytotoxic activity of the given compounds, several methods that employ various dyes can be applied; for example, MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide), SRB (sulphorhodamine B), DAPI (4′, 6-diamidino-2-phenylindole), propidium iodide, and trypan blue. Each test consists of the same stages; there is cell incubation with the aqueous sample, followed by the indication of a parameter associated with cellular processes, depending on the dye colour change or discoloration, which indicates the cytotoxic activity (Žegura et al., 2009; Vichai and Kirtikara, 2006). The *Umu*- and Ames tests are used to detect mutagens and possible carcinogens. The MTT assay is quite popular among colometric methods of determining cytotoxicity, it is based on the ability of mitochondrial dehydrogenase enzyme to convert the orange-yellow water-soluble tetrazolium salt (MTT) to formazan which is violet colored product of the above reaction. Formazan is insoluble in water and has to be dissolved in organic solvents, e.g. DMSO. Improvement of the MTT test is MTS assay, where the reaction product (tetrazolium salt) is converted by dehydrogenase in the presence of PMS (phenazine methosulfate) and fully soluble in water. Only living cells are capable of producing formazan, which enables quick and accurate determination of the percentage of the functional cells and the effect of a test agent on the viability of any cell line. Tests are performed by adding a given amount of the reagent directly to the cell

cultures in tests plats (incubated for 1–4 h) and measuring the absorbance (Tubaro et al., 1996).

In studies on mutagenicity and cancerogenocity of liquid samples *Umu*- and Ames tests are utilized. Ames test was established in the early 1970s by Bruce Ames. This test uses cell stains from *Salmonella* Typhimurium auxotrophic mutants, which are characterized by a gene mutation that prevents the synthesis of L-histidine, an amino acid that is necessary for bacterial growth. Exposing these *S.* Typhimurium mutants to a sample that contains mutagens can result in the reversal of the mutation, and then the bacteria begin to synthesize L-histidine and are able to grow on a medium that is poor in this amino acid. In addition, these bacteria feature mutations that increase the permeability of the cell wall (*rfa* mutation) to allow for the better penetration of mutagens and the deletion of *uvr*B, which results in the loss of the gene-encoding enzymes that are involved in cutting out pyrimidine dimers, which in turn reduces the cell's DNA repair ability through cutting. By using different strains of bacteria, it is possible to detect different mutation mechanisms. For example, the *S.* Typhimurium strains TA 98 and TA 1537 are used to detect the translational frameshifting mechanism, and the TA100, TA 102, and TA 1535 strains can be used to detect single base-pair substitutions (Resende et al., 2012).

An alternative to the Ames test is the use of an *Escherichia coli* WP2 strain with a lacZ^{-} allele encoding the inactive form of β-galactosidase, which was developed in the 1990s. Mutagens cause reversion to *trp*$^{+}$, and as a result, the bacteria are able to grow on a glucose minimal medium with trace tryptophan content. The Ames test procedure usually involves making a mixture of the test sample from the test bacterial strain, trace amounts of L-histidine, and optionally, the S9 microsomal fraction of rat liver (to activate promutagens). Following incubation, the number of bacterial colonies is counted, and a large number of colonies can indicate the strong mutagenicity of the compounds in the sample (Kwasniewska et al., 2012).

In 1976, Ames et al. examined approximately 300 compounds, among which there were known human carcinogens as well as non-carcinogenic substances. These studies have shown the usefulness of a test based on *S.* Typhimurium bacteria as a tool for the preliminary assessment of the carcinogenic potential of compounds in water samples, given that most carcinogens caused mutations in the bacteria (Ames et al., 1975; McCann and Ames, 1976).

Among the new methods, the Ames II and Ames MPF™ assays are worth mentioning (Xenometrix, Switzerland). Modifications of the usual Ames test (which are based on the fluctuations method referenced in OECD guideline 471) are now sold as commercially available kits. The standardized testing procedures and the use of 384-well microplates makes it easier to assess the mutagenicity of a sample visually after 48 h of incubation

(OECD, 1997).

The *Umu*-test is another method that can be used to analyse water samples for potential genotoxicity. Guidelines on genotoxicity studies in water and wastewater are found in ISO 13829 (Dizer et al., 2002; ISO, 2000a).

The *Umu*-test was developed and published in 1985 to evaluate the genotoxic potential of chemicals. This test is based on the ability of DNA-damaging agents to induce the expression of the *Umu* operon. A plasmid (pSK 1002) containing the *Umu*C gene in association with the *lacZ* receptor gene was introduced into the *S.* Typhimurium strains. The induction of the *Umu* gene, which is associated with the *lacZ* gene, is a measurement of the sample's genotoxicity. This genotoxicity can be evaluated through a colorimetric determination of the β-galactosidase activity, as measured by the conversion of a colourless substrate called ONPG (o-nitrophenyl-β-*D*-galactopyranoside) into a yellow solid called *o*-nitrophenyl. Currently, there are many varieties of the *Umu*-test, e.g., *umu*C Easy AQ with S9 and Positive Controls and *umu*C Easy CS with S9 and Positive Controls (Xenometrics, Switzerland) on the market (Oda et al., 1985).

The application of an SOS Chromotest is recommended as an alternative or a complement to the Ames test. It is considered to be a rapid, short-term, cost-effective test for genotoxic potential determination. The simplicity in its performance and its rapidity make the SOS Chromotest a good tool for sample screening. The complementation of the Ames test involves the detection of genotoxic compounds that were false negatives, and false positive results from the Ames test could be detected. The principle of this test is the occurrence of the SOS reaction, which plays a key role in the response of *E. coli* to compounds with genotoxic properties. A gene that plays a vital role in the SOS response is *lexA*, which encodes a repressor for all the genes in the system in addition to *recA*, which encodes a protein that is able to cleave the *lexA* repressor upon activation by an SOS-inducing signal. One of the simplest assays consists of monitoring the expression of an SOS gene by using a fusion with *lacZ*, the structural gene for *E. coli* β-galactosidase (Quillardet and Hofnung, 1985 and 1993).

The comet assay, which is also known as the single cell gel electrophoresis assay, is one of many tests used for identifying genotoxic activity. In comparison with the other previously mentioned tests, this assay employs eukaryotic cells. The test name comes from the appearance of damaged cells (an intact cell is round, and after exposure to a genotoxic agent, it takes on an elongated form and resembles a comet with a head and a tail). This test is a standard technique for assessing DNA damage/repair and biological monitoring. It involves the encapsulation of cells in a low-melting point agarose cell suspension, the lysis of the cell membrane in a neutral or alkaline (pH > 13) buffer and the electrophoresis of lysed cell samples (Tice et al., 2000).

A number of reports in the literature indicate that the comet assay is being used more and more because of its sensitivity in comparison with other biomarkers that are commonly used in genetic ecotoxicology. Unfortunately, because of the lack of standardization, there is still a wide range of individual procedures being used in laboratories, as shown in the literature, and therefore, the evaluation and comparison of results is very difficult (Frenzilli et al., 2009).

With increased pollutant emissions into the environment, it is important to explore all the possible effects of endocrine-active compounds. In the scientific literature, one can find mentions of the growing amount of research on endocrine activity. Pesticides, personal care products, plasticizers, and drugs could all potentially turn out to be endocrine-active substances. The idea of creating a bioassay to measure endocrine activity based on the yeast cell *Saccharomyces cerevisiae* is not a novelty. However, the lack of procedures and reagent standardization has caused difficulties in assessing and comparing the results, as in the case of the Comet Assay.

Human oestrogen receptor (YES) and androgenic screens (YAS) have been integrated into the chromosomes of brewer's yeast. A substance that has endocrine-active properties binds to a receptor and induces the synthesis of β-galactosidase in the plasmid *lacZ* cells. Yeasts that are exposed to EDCs (Endocrine Disrupting Compounds) secrete β-galactosidase into the medium, which contains a CPRG (chlorophenol red-β-D-galactopyranoside) dye. The β-galactosidase then catalyses the disconnection of a galactose molecule from a CPRG molecule, causing a change in the substrate's colour from yellow to purple (Sanfilippo et al., 2010).

To meet market demands, Xenometrix has issued two bioassays to measure the endocrine-disrupting properties of liquid samples by using modified yeast cells as follows: XenoScreen YES/YAS® and XenoScreen YES/YAS XL®. The tests provided by Xenometrix are thought to identify both the agonist and the antagonist as well as oestrogen and androgen-related properties. The additional advantages of these tests are their minimal consumption of samples and the lack of a need for cell culturing (Fic et al., 2014).

The CALUX® test (*C*hemical *A*ctivated *LU*ciferase gene e*X*pression) is considered to be more sensitive than the YES/YAS yeast test. This test uses special recombinant human cells. There are four variations of this assay as follows: ER-CALUX® is used to detect oestrogenic agonists, antiER-CALUX® is used to detect oestrogenic antagonists, AR-CALUX® is used for detecting androgenic agonists and antiAR-CALUX® is used for detecting androgenic antagonists. There is a variety of CALUX assays (DR) CALUX® (Dioxin Responsive) oriented towards the detection of dioxin and dioxin-like compounds, which has applications in the food industry. The endocrine-active compounds bind to the Ah-receptor on the cell surface, resulting in the transport of the PHAH-Ah receptor

complex to the cell nucleus, where the complex bonds to specific sequences in the DNA (responsive elements, or REs). This chemical bonding to the receptor initiates the expression of RE-associated genes, e.g., luciferase gene expression is increased (Houtman et al., 2004).

Tests that utilize single-cell bacterial/fungal organisms (or other cells) can provide a lot of information about the sample tested. Researchers are not only able to find out whether the sample contains mutagenic, genotoxic, oncogenic and endocrine substances but also, on the basis of used test, can identify mechanism resulting in observable effects. Classical instrumental methods have certain limitations with respect to biological methods, one of them is that they are not able to detect new and emerging substances and products of biotransformation and/or metabolism.

Tests that employ algae and diatoms as cell-based assays are also worth mentioning. Chronic toxicity microbiotests that are used to determine growth inhibition are most often based on the green algae *Selenastrum capricornutum* and the diatom *Phaeodactylum tricornutum*. These microbiotests comply with OECD and ISO guidelines (Ren and Frymier, 2003).

The evaluation of aquatic ecosystem quality should be comprehensively approached. Therefore, studies should be performed on fish, aquatic invertebrates, macrophytes, phytoplankton, and sediment-dwelling organisms. The selection of an appropriate test organism does not always reflect the effects caused by environmental pollution with respect to the organism's role in the ecosystem and sensitivity to test compounds as well as bacterial cell models. Within the framework of biomonitoring, it is appropriate to apply higher organisms such as plants and animals.

Phytotoxkit F™ is a 3-day acute toxicity soil and sediment microbiotest that evaluates the inhibition of germination and root growth in the monocotyledonous *Sorghum saccharatum* and dicotyledonous *Lepidium sativum* and *Sinapis alba* plants. This test does not require specialized equipment or trained personnel (as opposed to the tests listed above, for which basic knowledge of cell culture is required). This test can be used to determine the phytotoxicity of water samples, sediments and soils (without the need to extract solid samples) (Czerniawska-Kusza and Kusza, 2011).

Allium cepa (common onion) is the most commonly used organism among the higher plants for environmental monitoring applications. *Allium cepa* is frequently used as a genetic model to detect environmental mutagens and is often used in control tests to assess DNA damage, such as chromosomal aberrations and abnormal mitotic cycles. Because of its low price, common onion is used to evaluate the toxicity levels of numerous chemicals. The detection of mutagens dates back to the 1940s by Levan and contributes to their increasingly controlled release into the environment use in increasing

the environmental control. The mitotic index and certain nuclear abnormalities are used to assess cytotoxic agents, and micronucleus analysis is used to verify the mutagenicity of different chemicals. In addition, tests based on *A. cepa* provide information for evaluating the mechanisms of action of xenobiotics and their effects on genetic material (clastogenic effects and/or aneugenic effects) (Leme and Marin-Morales, 2009; Fiskesjö, 1985; Levan, 1938; Bolle et al., 2004).

Given their prevalence, invertebrates and crustaceans are frequently used as indicator organisms. The most commonly used organisms are *Daphnia magna* (DAPHTOXKIT F™ *magna*) and *Daphnia pulex* (DAPHTOXKIT F™ *pulex*), which play very important roles in the trophic chain, bridging the gap between the producers and consumers of higher orders (Illés et al., 2014). Both tests serve to assess the acute toxicity of pure substances, wastewater, surface water and groundwater. *D. magna* and *D.pulex* are very sensitive to toxic substances, have a short generation time, multiply quickly, and are easily acclimatized to the laboratory. They are grown in a small space and can be measured within a relatively short period of time, and they comply with OECD Guideline 202 and ISO 6341 (Koivisto, 1995).

Assays based on plants, algae and crustaceans are inexpensive and do not require skilled personnel as well as specific incubation conditions. Selected test organisms are an important link in the trophic chain and are sensitive to a broad spectrum of pollutants, particularly to pesticides, PAHs, metals, drugs (e.g., antibiotics used in animal farming). These tests can be applied to all types of liquid samples, without any sample preparation.

Ecotoxicological testing with fish can be performed on approximately 150 species, the most common of which are zebrafish (*Brachydanio rerio*), fathead minnow (*Pimephales promelas*), carp (*Cyprinus carpio*), Japanese rice fish (*Oryzias latipes*), Guppy (*Poecilia reticulata*), Bluegill (*Lepomis macrochirus*), and rainbow trout (*Oncorhynchus mykiss*). The zebrafish is an important vertebrate model in genetics, neurophysiology and biomedicine, and because of this great interest, the zebrafish is one of the first vertebrates whose genome was sequenced. This fish species is easy to farm because of its low cost and small size. Females can lay eggs every 2–3 days, at several hundred at a time. Zebrafish eggs are quite large and transparent. Their development from egg to adult usually takes 3 to 4 months. A biotest based on *D. rerio* can provide important information on the presence of potential serious human xenobiotics that could be found in drinking, ground and surface waters (Spence et al., 2008; Martins et al., 2007).

The biological evaluation of water quality is the most developed branch of environmental monitoring because of the number of aquatic organisms and the same properties of the medium. However, the selection of an appropriate test requires a great deal of ecotoxicological knowledge. The application of bioassays as standard methods for monitoring the aquatic environment has become common practice, and these tests can

be used for screening or indicating dangers, and, if necessary, they can be supplemented by instrumental analysis.

2.1.2. Ecotoxicological assessment of soil and sediment quality

Most of the bioassays described in this section on assessing water quality can also be applied successfully to assess the quality of soils and sediments. For example, the United States Environmental Protection Agency recommends screening with a CALUX® assay to detect dioxins and dioxin-like compounds in soils and sediments (US EPA, 2014). Unfortunately, in most cases, the use of assays designed to study aqueous samples is associated with the need to extract the solid samples that are most common in water. Some environmental pollution shows strong hydrophobic properties accumulating in the soil or sediments. Research on extracts (aqueous) may not always reflect the toxicity of a given soil or sediment sample. Therefore, it seems obvious that soil and sediment research should be conducted with bioassays, in which the test organisms would be indigenous.

To determine the toxicity of sediments, the bottom-dwelling organism *Heterocypris incongruens* (OSTRACODTOXKIT F™) can be used. The literature also describes the application of *Tubificidae*, with the use of the earthworms *Eisenia fetida* and *Folsomia candida* (springtail) to evaluate sediment toxicity. Biological tests using *H. incongruens*, *E. fetida* and *F. candida* are simple and can be performed with accessible and cheap equipment. The endpoints of the given tests have included studies on genotoxicity, immunotoxicity, mortality and reproductive toxicity (Bierkens et al., 1998; Reynoldson et al., 1991).

To assess the quality and toxicity of soil samples, it seems natural to use higher plants such as *Vicia faba*, *Zea mays*, *Tradescantia* L., *Nicotiana tabacum*, *Crepis capillaris*, *Hordeum vulgare*, and plants included in the Phytotoxkit F™ (Płaza et al., 2005).

Because of the complexity of the matrix and other concerns, each sample or batch of samples, particularly those of environmental origin, require an individual approach. For this reason, there are a number of tests, a multitude of test organisms and sometimes different protocols for the same test in different types of samples. Tables 2, 3, 4 and 5 summarize the most frequently described toxicity and ecotoxicity tests with respect to bioassays based on plants, animals, single-cell organisms and cells/enzymes/antibodies, respectively. Some of these tests are certified, commercially available and recommended by various organizations such as the ISO, OECD and others.

2.2. Other applications of bioassays and future development trends

Currently, the application of biotests extends beyond the boundaries of environmental monitoring. A simple, quick search in publication databases leads to a number of examples. New bioassays are being developed to detect the endocrine-disrupting

properties of food additives and contaminants (Connolly et al., 2011; Bejrowska et al., 2015). Bioassays were also found to be applicable in medicine, e.g., for the detection of viral pathogens (Pan-Viral Microarray Assay (Virochip) Screen) (Chen et al., 2011) or milk production analytics for antibiotic detection. New development trends in bioassays employ molecular biology tools to give deep insights into the abnormalities that are induced in living organism by environmental pollutants. In the cases studied to date, our observations have improved our knowledge about the disrupted balance of various biological systems induced by environmental stressors. It is certain that untapped potential lies in analytical methods that are based on changes in the activity of nuclear receptors and other transcription factors. As indicated above, the strongest potential seems to be found in methods that employ markers of gene expression modulation (e.g., the gene-reporter assay). A genetically constructed recombinant organism can be designed to express the appropriate receptor, and a fast screening of receptor ligands in probes can be made into a standard analytical test. Another trend in development is the miniaturization and transfer of bioassays to households as “green” and user-friendly products to study the toxicity level or endocrine potential of food products, water, and, e.g., baby products. These advanced and rapid immune-based tests are being prepared for introduction in a commercially available form. They will be used to detect the stressors that are responsible for endocrine threats in groups of societal interest (such as pregnant women, newborns, and adolescents) and to help reduce the risk of adverse impacts from everyday products on human beings.

In view of all the advantages of bioassays, one must bear in mind that the greatest challenge lies in selecting the proper battery of organisms to perform proper evaluations. For this reason, legal regulations should be issued to select organisms reliably for toxicity evaluations at the geographical and developmental levels.

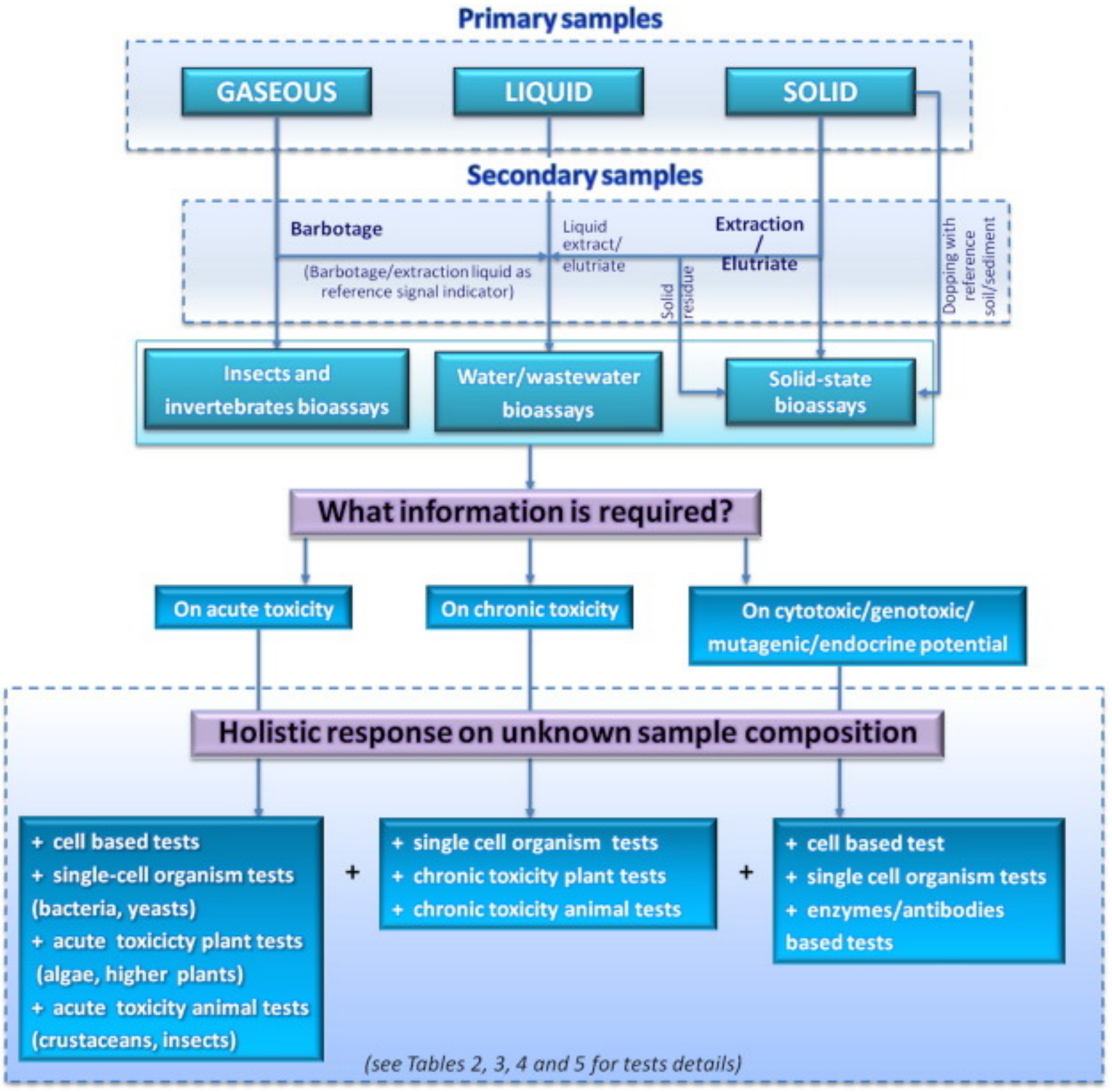
2.3. Battery of bioassays

Assays based on living organisms can provide a counterbalance to classical chemical analyses. However, the evaluation of results may pose a problem even for the most experienced researcher. One cannot ignore the fact that there is a difference in sensitivity between species and even between organisms of the same species. Research on one species will reflect the sensitivity of that organism. Therefore, there is a significant risk of the underestimation or overestimation of the overall toxicity of a sample in relation to the entire ecosystem. That risk can be reduced by using a group (battery) of organisms. A bioassay battery consisting of organisms from different trophic levels reduces the risk of errors. The use of bioassay batteries has another advantage; it allows users to determine whether the analysed sample contains compounds that have been characterized by more than one type of toxicity.

A battery of bioassays can be an effective tool for analyzing complex samples or

examining the risk of environmental exposure to substances that have not yet entered into circulation. The set of organisms that is selected for bioassay batteries largely depends on two different purposes for which these batteries are used; one goal is protection and the other is the early detection of chemical risk (see Fig. 4. for schematic representation of methodology how to select bioassaying organisms). The methodology proposed relies on responding to several basic questions every scientist must ask:

- in what state is the primary sample present — is it gaseous, liquid or solid;
- should any sample treatment be conducted – barbotage, elution, extraction, preservation, doping etc. – it will produce secondary samples that may undergo bioassaying;
- what information is required — on acute, chronic toxicity? cytotoxic, genotoxic, endocrine potential? or is it holistic response of bioassays to sample of unknown origin and composition?



Download full-size image

Fig. 4. Schematic diagram presenting process of decision making with guidelines on how to select battery of bioassays.

There is very limited number of bioassays capable of dealing with gaseous samples, they are most often based on insects and invertebrates and quite tricky to handle with. If one is able to perform barbotage of know volume of gas state sample in liquid of known (low) toxicity then such secondary sample can be toxicologically assessed with numerous biotests, depending on parameter one is willing to measure. Vast number of bioassays is validated and commonly used for liquid samples — as presented in Tables 2–5. Very often (e.g. in case of shale gas exploration and extraction and impact assessment of this process) the sample may take 2 forms: original suspension solidifies with small amount of

liquid present above the sediment. Again, such sample can undergo studies with tests validated for solid samples and liquid ones; furthermore, such complex matrices can be eluted/extracted with liquid (of known toxicological parameters playing role of background signal in such situation) and comprehensive information about extractable/bioavailable component. In case of necessity of determining ecotoxicological impact on environmental samples of unknown composition it is strongly advised to select both acute and chronic bioassays from the entire trophic level, namely bacteria, algae, yeasts, lower and higher plants and invertebrates. In this way, it is possible to both give information on particular pollutants presence and their summary impact on living organisms if present in complex mixture. Such holistic response/knowledge is impossible to be performed and gained barely with instrumental techniques as they do not respond to interactions occurring between pollutants and metabolic processes they may undergo.

An example of a bioassay battery that is available on the market is the MARA test (Microbial Assay for Risk Assessment). Toxicity measurements are performed with eleven genetically diverse microorganisms (ten strains of bacteria and one yeast) in lyophilized form inside a microtiter plate. The growth of microorganisms in the matrix is measured as a loss/reduction of the dye (Gabrielson et al., 2003).

3. Conclusions

It is possible to obtain quantitative and qualitative information and to determine the toxicity of a given sample using modern bioanalytics. This possibility does not mean that one should abandon instrumental techniques. At this stage, the best solution is to combine data obtained from these two sources because they will form a complete picture of the environmental conditions. Furthermore, bioassays can be used for a separate study, screening, and a preliminary examination prior to standard instrumental analysis. Actions were undertaken to combine biotests for screening purposes followed by instrumental analyses in the case of problem detection as well as the incorporation of this approach into the legal system (in Poland), although this project is still at a very early stage.

Biotests meet most of the principles of Green Chemistry. These methods are characterized by their speed, and in most cases they lack a sample preparation step (which reduces waste and solvent usage), and they are cheap and user-friendly. In the case of potential extraction, only green extraction media are used.

Bioassays are continuously being made easier to use, and the certified and validated *toxkits* that are entering the market now are equipped with instructions, appropriate treatment and QA/QC protocols, necessary reagents and accessories. Thanks to biotests, it is possible to evaluate test samples in a comprehensive manner without conducting numerous chemical studies. Bioassays also have some restrictions resulting

from differences in the sensitivities of different trophic-level organisms or the fact that in some of the tests, GMOs (Genetically Modified Organisms) are used. Still, even those drawbacks cannot justify a failure to regard biotests as the green analytical tools of the future.

In summary, to fulfil the Green Analytical Chemistry guidelines and QA/QC protocols, toxicity evaluations should have the following characteristics:

- be environmentally benign,
- be generally accepted by scientific centres as indicators of regional ecosystems stability,
- be utilizable in modelling and risk assessment studies,
- enable pollutant group selection for eventual instrumental studies,
- constitute a basis for risk assessment of chemicals and their transformation products (and also in relation to other co-existing organisms),
- be comprehensively studied, representative, reliable, and repeatable, and
- be economical, easy-to-perform and sensitive (depending on the sample).

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