

The applications of microbes in environmental monitoring

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Harmful effects of contaminants on the ecosystem and humans can not be assessed by standard chemical analyses of environmental samples, therefore toxicity tests using live organisms or cells represent a vital part of environmental monitoring. Many different biological methods based on native or genetically modified microorganisms as test-species, have already successfully been applied to environmental toxicity/genotoxicity assessment. An important reason is the modern 3R concept (reduction, replacement, refinement) in toxicology and ecotoxicology, which promotes the application of microorganisms in biotests due to simple cultivation in axenic cultures and due to the lack of ethical problems. The article reviews the most important applications of bacteria, protozoa, algae and yeasts in biomonitoring, starting with classical physiology-based toxicity bioassays, proceeding to genotoxicity assays, immunoassays and endocrine disruptor assays and concluding with upcoming approaches of toxicogenomics.

Keywords: environmental monitoring, bioassay, genotoxicity assay, biosensor, immunoassay, endocrine disrupter assay, toxicogenomics

1. Introduction:

In the past few decades, environmental pollution has become one of the world's major concerns. A great number of toxic compounds, originating mostly from industrial and agricultural activities, are being released in to our environment continuously. In some cases harmful chemicals induce strong acute toxic effects to exposed organisms when released to the environment, but frequently the consequences are delayed due to the effects of bioaccumulation and biomagnification. Early detection of toxic chemical compounds in the environment, particularly in water, and their biological effects on organisms has therefore become increasingly important.

The traditional approach to environmental pollution assessment is based on chemical analytical methods which only provide information about the absolute concentrations of known chemicals in the environmental sample without an adequate interpretation of its toxicity to biota in the context of bioavailability, which means it only provides information about their potential, not actual toxicity [1]. Moreover, compounds that are toxic below the detection limit of chemical analytical method or new compounds that are not yet deposited in the databases, can not be detected this way. Another disadvantage of chemical methods is the lack of information about the combined toxicity of different compounds such as additive, synergistic or antagonistic effects. In order to get more relevant information about environmental pollution risk, it is therefore inevitable to supplement the chemical analytical data with the results of methods providing information on biological impacts [2,3,4].

The negative biological effects of pollutants present in all kinds of environmental samples can be assessed using different living organisms or cells as 'analytical devices' [5]. The biological response following the exposure of living organisms or cells to environmental sample usually gives an information on toxicity, genotoxicity, estrogenicity etc. of the whole mixture of chemical compounds present in that particular sample. Besides being sensitive only to the bioavailable fraction of pollutants, biotests also have the power to assess the integrated effect of interacting chemical compounds and to detect the compounds, which are toxic only due to bioactivation [6,7].

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According to the technical principle, methods of biological monitoring can be classified to bioassays, biosensors, immunoassays, estrogenicity tests and ecological methods [8], but there also exist other types of classifications, for example division to biomarkers, whole cell biotests and early warning biological systems [3].

The first biotests for environmental monitoring were based on multi-cellular eucaryotic organisms, in particular fish and mammals [9, 4]. As they were relatively expensive, time-consuming, difficult to standardise and ethically questionable, the need for alternative biological methods for environmental monitoring based on 3R strategy (Reduction, Replacement, Refinement) soon became evident [10]. The development and standardisation of toxicity tests based on procariotic (bacteria) or eucariotic (protozoa, unicellular algae, yeasts) microorganisms instead of higher organisms has enabled fast and inexpensive screening of environmental samples for toxic and genotoxic effects. The first generation of biotests has been based on different naturally sensitive microbes, while the second generation includes genetically modified microorganisms to attain better sensitivity and/or specificity. The next step forward was combining microbial cells or parts of the cells to physicochemical detection elements, forming new integrated devices, called biosensors [11].

2. Microbial toxicity and genotoxicity tests

Bioassay or ecotoxicity assay is an experiment in which living test-species are exposed directly to an environmental sample (soil, sediment, surface water, ground water, waste water..) or extract of an environmental sample to measure a potential biological effect due to the presence of potential contaminants [4].

Microbial bioassays can roughly be divided to (general) toxicity assays and genotoxicity assays. The purpose of ecotoxicity bioassays is to assess the integral effect of an environmental sample on general physiological state of the test-species, while genotoxicity tests specifically show the effects resulting in changes of genetic material [12].

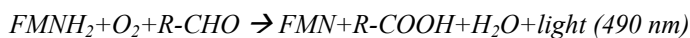
Regarding the exposure time of the test-species to the investigated sample, bioassays can be subdivided into acute and chronic. The first group is formed of bioassays, where exposure time does not exceed 96 hours, while the chronic assay subgroup includes tests with longer exposure times. Common parameters calculated from acute bioassay data are EC_{50} (estimated toxicant concentration in a sample at which 50 percent of the test organisms show an effect following a given exposure time) and LC_{50} (estimated toxicant concentration in a sample, at which 50 percent of the test organisms die following a given exposure time), while the chronic test reference parameter is NOEC value (represents the highest toxicant concentration at which no significant effect can be detected when compared to the control sample).

2.1 Toxicity bioassays

Several toxicity bioassays applying bacteria, microalgae, protozoa and yeast have already been developed. Many of them are also standardized and commercially available.

Most common parameters measured by microbial toxicity assays are population growth, substrate consumption, respiration, ATP luminescence and bioluminescence inhibition.

Vibrio fischeri bioluminescence inhibition assay has been most frequently used and is claimed to be the most sensitive across a wide range of chemicals compared to other bacterial assays (nitrification inhibition, respirometry, enzyme inhibition and ATP luminescence) [13]. *Vibrio fischeri* is gram negative marine bacterium possessing natural bioluminescence properties. Light production in a culture of test-species is directly proportional to the metabolic activity of the bacterial population, therefore any inhibition of enzymatic activity causes a corresponding decrease in bioluminescence. The biochemical principle behind the bioluminescence reaction is the oxidation of reduced flavin mononucleotide (FMNH₂) in to FMN and H₂O upon reaction with molecular oxygen in the presence of aldehyde and luciferase enzyme. The surplus energy formed in this reaction is emitted as blue-green light of wavelength 490 nm [14], that can be measured by a luminometer.



Vibrio fischeri bioluminescence inhibition assay is applicable for almost all kinds of environmental samples such as surface and groundwater, sediments, municipal and industrial waste effluents, etc. [13].

Other commonly used bacterial bioassays are based on assessing the inhibitory effect of a sample on β -galactosidase activity in *E. coli*. Commercially available variations of this assay use different chromogenic β -galactosidase substrates for colorimetric determination of enzymatic activity products. A similar principle is applied with *Bacillus spp.* dehydrogenase activity assay, where redox-potential induced changes in tetrazolium salt colour indicate inhibition of microbial respiration [4].

Bioassays with unicellular algae have found wide application in environmental biomonitoring, too. A generally recognized method for testing the effects of pollutant chemicals, especially pesticides, is based on measuring the growth inhibition of green microalga *Scenedesmus supspicatus* following 72 hours exposure. Beside various modifications of this standardized test, several new algal bioassays based on different approaches have also been developed. An improvement on the standard microalgal growth-inhibition test has been made by using flow cytometry. This is a rapid and sensitive method for quantitative measurement of light scattering and fluorescence of flowing cells, which enables lower cell density algal cultures to be used for the assay. Algae contain *chlorophyll a*, a pigment molecule which autofluorescences when excited by blue light, therefore additional fluorophores for cell count experiments are not needed. By using biochemically specific fluorescent dyes it is possible to get additional information about the physiological status of the cells and mechanisms of toxicant action. Staining of algal cells with fluorescein diacetate (FDA) enables measurement of algal esterase activity as an indicator of their physiological status. Healthy cells take up and hydrolyse FDA producing fluorescent fluorescein, therefore decreased fluorescence at 530-560 nm indicates damage of algal cells, such as impaired enzyme activity or loss of cell membrane integrity [15].

Short period incubation algal tests for toxicity estimation are based on 'in vivo' chlorophyll prompt (PF) and delayed fluorescence (DF) measurements, which indicate photosynthesis inhibition due to toxic chemicals. Many herbicides and some other chemical compounds like mercury and 3,5-dichlorophenol have already been detected in environmental water samples this way [16, 17, 18]. An important difference between the 72-h algal growth inhibition test and shorter period tests is, that the first group of tests involve multiple cell generations, whereas the second group of tests only determine the effects of tested chemicals on one cell generation, which might result in lower sensitivity of short period tests to chemicals affecting multiple cell generations [18].

Protozoa are eucaryotic microorganisms, known to be very sensitive to environmental changes, therefore being an ideal early-warning indicators of aquatic ecosystem deterioration, as well as an important test-species for toxicological assays. Non-pathogenic, free-living ciliates *Tetrahymena pyriformis* and *Tetrahymena thermophila*, being the first protozoa cultured axenically, are the organisms of choice for most toxicological studies. Common end points assessed in ecotoxicity protozoan assays are growth, viability/mortality, grazing ability, ATP (adenosine-5'-triphosphate) content, ACP (acid phosphatase) activity and MMT (3-[4,5-dimethyl-thiazol-2-yl]-2,5-diphenyl tetrazolium bromide) reduction capacity [19,20,21].

Growth impairment bioassays are traditionally based on microscopic observations of morphological changes (cell shape and motility), what makes them simple and inexpensive techniques. Nevertheless, problems such as underestimating the true number of viable cells because of the assumption that all non-motile or shape-altered cells are dead, forced the development of an alternative method to standard direct counting. A novel method is based on using two fluorescent dyes: calcein/AM - nonfluorescent substance, which diffuses passively into cells where it is converted to green fluorescent calcein by intracellular esterases of viable cells and EthD-1, that enters only damaged (dead) cells and represent a source of red fluorescence when bound to DNA. Biotests based on detection of changes in metabolic state of protozoa often include colorimetric determination of acid phosphatase or dehydrogenase activity (MMT/tetrazolium reduction capacity) and determination of ATP content [21].

2.2 Genotoxicity assays

Genotoxicity assays are used to measure the potential of environmental sample to induce changes in genetic material of test organisms. Types of DNA damage that may be assessed using these tests include mutagenicity, clastogenicity and aneuploidy [12]. Mutagenicity results in changes of one or a small number of DNA base-pairs (point mutations), comprising substitutions, additions and deletions of base pairs. Clastogenicity involves structural changes in larger areas of chromosome, while changes in number of whole chromosomes lead to aneuploidy [22].

Some widely used microbial genotoxicity assays are based on bacterium *Salmonella typhimurium*. The most widespread is the Ames test, which has also been established as a routine method of environmental water monitoring. It is based on a histidine dependent strain of *S. typhimurium* (TA98). Mutagenicity of the sample is determined by frequency of back mutations, which enable the growth of revertants on the medium without histidine [23]. The umu-test is also a standardized and validated method, based on *S. typhimurium*. Genotoxicity is detected by measuring the transcription of SOS-response genes, which code for enzymes involved in DNA repair. Fusion of SOS-response genes with β -galactosidase encoding reporter gene enables colorimetric detection of genotoxic compounds [24]. The same principle is applied in SOS-chromotest, which applies *Escherichia coli* as test-species [25] and is frequently used, too, because of its high sensitivity to certain groups of pollutants, such as chloride pesticides and chlorophenols [8].

The genotoxicity bioassay developed by Zimmermann et al. [26] is based on recombinant *Saccharomyces cerevisiae* strains (D7, D61,...) and enables not only detection of mutations, but also recombinations and loss of chromosomes [27]. A novel commercially available yeast genotoxicity reporter assay has been developed recently. GreenScreen assay (GSA) is sensitive to broad spectrum of mutagens and clastogenes. In this assay, the reporter system in yeast cells employs the DNA damage inducible promoter of the *RAD54* gene, fused to green fluorescent protein [28]. Genotoxicity indicator assay, which has recently attracted much attention, is Comet assay (also called the Single-Cell Gel Electrophoresis Assay), which primarily measures single and double-DNA strand-breaks in single cells [29]. Adapted protocols enable also the detection of oxidized bases and abasic sites [30, 31]. The protocol has originally been developed for detection of DNA damage in blood cells, but it has later been adapted for eukaryotic microorganisms, too. *T. thermophila* have already been used for the purposes of environmental and drinking water genotoxicity monitoring by comet assay [32,33]. Yeast cells have been applied equally successfully in monitoring of wastewater genotoxicity reduction by biological wastewater treatment plants [34].

3. Microbial biosensors

A biosensor is defined as a self contained, integrated device, consisting of a biological recognition element interfaced to a physical signal transducer, that together reversibly respond to a chemical species in a concentration-dependant manner. A wider definition also includes some other forms of biological sensors, including genetically engineered microorganisms, which respond in observable ways to target analyte or group of related analytes [11, 35].

A wide range of biological recognition elements have already been used in biosensors constructed for potential environmental applications. Whole microbial cells, cellular organelles and molecules such as enzymes, antibodies, different kinds of receptors or DNA are the most common biorecognition elements of microbial origin. Regarding the type of transducer, biosensors could roughly be classified to electrochemical, optical, thermometric and piezoelectric [35].

Microbial biosensors for environmental applications range in their development stages from proof-of-concept to full commercial availability. Regarding the target detection specificity they may fall in one of two groups [36]:

- Biosensors, which measure general biological effects/parameters or
- Biosensors for specific detection of target compounds

The first group of biosensors is aimed to measure an integral toxicity, genotoxicity, estrogenicity or other general parameters of the sample, which affect living organisms. They essentially include whole microorganisms as biorecognition elements. The most often reported cell-based biosensors include genetically modified bacteria with artificially constructed fusions of particular regulatory system (native promoter) with reporter genes. The presence of an effector (non-specific stressor such as DNA damaging agents, heat shock, oxidative stress, toxic metals, organic environmental pollutants) results in transcription and translation of fused target genes, generating recombinant proteins which produce some measurable response. Frequently used reporter genes are *lux* (coding for luciferase) and *gfp* (coding for green fluorescence protein), expression of which correlates with luminescence or fluorescence based light emission [37]. Colorimetric determination of target gene expression is possible by fusing it to reporter genes coding for β -galactosidase (*lacZ*) or alkaline phosphatase (*phoA*).

Recently, *E. coli* biosensor capable of detecting both genotoxic and oxidative damage has been developed. This was achieved by introducing two plasmids: first one with fusion of *katG* (gene encoding for an important antioxidative enzyme) promoter to the *lux* reporter genes, and another with *recA* (gene encoding crucial enzyme for DNA repair) promoter with the *gfp* reporter gene [38].

Besides genetically modified microorganisms (also named bioreporters), some other types of cellular biosensors have also been constructed. An example is an algal biosensor, based on amperometric monitoring of photosynthetic O₂ evolution - the process affected by toxic compounds, which has been developed by coupling Clark electrode to cyanobacterium *Spirulina subsalsa* [39].

Biosensors for specific determination of chemical compounds frequently contain molecules like enzymes, receptors and metal-binding proteins as recognition elements. A number of enzymes have been shown to be inhibited by toxic metals, pesticides and some other important contaminants, like endocrine disrupting compounds. Limitations for the potential applications of many enzyme biosensors include limited sensitivity and selectivity, as well as interferences by environmental matrices [11]. One recently introduced strategy to overcome the first two of these limitations uses inhibition ratio of two enzymes for the detection of specific compounds. Acetylcholinesterase and urease, co-entrapped in the sol-gel matrix with the sensing probe, FITC-dextran, have successfully been used for Cu, Cd and Hg detection, for example [40]. Beside molecular biosensors, bioreporter cells may also be used for detection of specific target compounds. Recently, for example, a biosensor for nitrate monitoring has been constructed by transformation of *E. coli* with plasmide containing nitrate reductase operon fused to *gfp* reporter gene [41].

4. Immunoassays

Immunochemical methods are based on specific and reversible binding of immunoglobulin molecules (antibodies) to their target antigens. The most popular immunochemical technique in environmental analyses today is immunoassay, which has been shown to detect and quantify many compounds of environmental interest such as pesticides, industrial chemicals, and products of xenobiotic metabolism.

Basic immunoassays are performed by detection of a specific marker molecule immobilized either to antibody (Ab) or the antigen (Ag). Marker molecules may be in the form of fluorescent or chemiluminescent compounds, radioisotopes or enzymes. Enzyme-based immunoassay offer many advantages over other immunotechniques, because of the great amount of product molecules, which results in signal amplification. The main enzymes used are horseradish peroxidase, alkaline phosphatase and β -galactosidase. A widely used immunoassay for environmental purposes is enzyme-linked immunosorbent assay (ELISA), which can be carried out according to different formats - direct competitive, indirect competitive or sandwich-type. Competitive assays are most common and can be performed in different ways. Analyte and the tracer (direct competitive ELISA) or analyte and the

immobilised ligand (indirect ELISA) may compete for a limited number of binding sites. Sandwich-type ELISA is non-competitive assay, in which the analyte is recognised by two different antibodies-immobilized Ab and marker Ab [42, 8].

Flow-injection immunoassay (FIIA) is a technique, based on the introduction of the sample into carrier stream, which enters the reaction chamber where the immunoreaction takes place. FIIA has been successfully used for detection of different pollutants, e.g. triazines. At present, this method is integrated into different immunosensors [8].

The role of microorganisms, related to immunoassays is mostly indirect, but still significant. They are used as artificial factories (expression systems) for recombinant antibody production. Since *Escherichia coli* provides the most popular expression system, much research has been done to maximise the expression levels of recombinant antibodies (rAbs) in this system. Main problems associated with procaryotic expression systems are reducing environment inside microbial cell, that does not favour disulfide bond formation and leads to production of insoluble recombinant proteins in the form of inclusion bodies. The current approach to overcome this problem is to export the rAbs to the periplasm of *E.coli*. However, this strategy is still limited by the amount of proteins that can be exported. Eucaryotic expression systems are also in use. They enable higher levels of Ab expression, whereas the functionality of Ab produced is highly dependent on individual single-chain antibody fragments. Different microscopic fungi have been used for recombinant antibody production, including yeast species *Saccharomyces cerevisiae* and *Pichia pastoris* [43].

Besides being used as antibody-production systems, microorganisms may also represent a source of marker enzymes (alkaline phosphatase, β -galactosidase) used in certain type of immunoassays.

5. Endocrine disruptor (EDC) assays

Endocrine disrupting compounds are a newly defined category of environmental contaminants, which interfere with the endocrine system function, which results in altering the reproductive systems in wildlife and humans. Compounds, acting as agonists or antagonists of hormone(estrogene, androgene) receptors include a wide range of molecules, such as organochlorine pesticides, pthalates, alkylphenols, phyto- and mycoestrogens, pharmaceutical estrogens and many other [44].

Estrogens are hormones, that play crucial functions in growth, differentiation and homeostasis of male and female reproductive organs. Besides, they also influence non-reproductive tissues, such as bone, liver and the cardiovascular system [44].

When estrogens enter the cells, they bind to specific receptors, forming homodimeric complexes. Ligand-receptor complexes induce the transcription of target genes by binding to specific regions on DNA, called ERES [45,46]. Other mechanisms of action, which do not include hormone receptors also exist. Faster responses to compounds with estrogenic activity, which take place in the cytoplasm or on membranes and involve different effector molecules are also of importance [47].

Several bioassays have been developed to assess substances with estrogenic activity. Most of the 'in vivo' assays are based on a variety of endpoints and are therefore time-consuming, expensive and require sacrifice of numerous animals. Consequently, 'in vitro' assays applying microorganisms have been developed for the purpose of large-scale screening. Most of them are based on simple cell models, that express hormone receptor coding genes coupled to reporter genes, such as β -galactosidase or luciferase, when induced by estrogenic-like compounds [44, 8].

The estrogenic induced signaling pathways are highly conserved in yeast and mammalian cells, which makes yeast cells a suitable system for modeling cellular response of mammalian cells when exposed to endocrine disruptors. Besides being less expensive and easier to culture, one important advantage of using yeasts instead of mammalian cells is their resistance to different contaminants, usually present in environmental samples. Numerous tests, using genetically modified yeasts for the detection of estrogenic and androgenic compounds have been developed. They monitor either the transcriptional activation of the steroid receptor itself or its ligand-induced interaction with a transcriptional coactivator [48]. Commercially available assay for estrogenic screening (YES/Yeast Estrogen Screen by Glaxo) is based

on genetically engineered *Saccharomyces cerevisiae* with human estrogen receptor (hER) fused to *lacZ* reporter gene [49].

6. Ecotoxicogenomic approaches in environmental monitoring

Rapid progress in the fields of genomics is lately beginning to provide tools that may assist our understanding of how chemicals can impact human and ecosystem health. A new scientific discipline, which integrates genomics (transcriptomics, proteomics and metabolomics) into ecotoxicology is named ecotoxicogenomics. It is defined as the study of the response of the genome to environmental toxicant exposure [50]. Although the application of gene and protein expression analysis to ecotoxicology is still at an early stage, this holistic approach seems to have several potentials in different fields of ecological risk assessment.

The most important advantage in using a gene expression profiling approach compared to most standardized methods used to assess the potential impact of chemicals on organisms, is the power of an insight into the precise mode of action (MOA) of toxicants. This may potentially be useful for prioritization of substances for extensive testing regarding their MOA and may therefore allow optimization of resources and limit the use of animals for testing purposes. Moreover, the knowledge of precise toxicity pathways may reveal novel molecular biomarkers for early detection of environmental stress [51].

Comparison of gene expression profiles of different test microorganisms and higher organisms may provide useful information about the possibility of extrapolation of the effects of toxic chemicals across species. Determining similarities and dissimilarities in toxicity mechanisms across species would give the answer where the extrapolation of chemical hazards from one species to another is technically valid [52]. The knowledge about conservation of toxicity mechanisms in organisms will therefore enable to choose appropriate model organisms at lower levels of biological organization (e. g. microorganisms) for relevant monitoring of specific environmental toxicants.

The use of microarray, proteomic and metabolomic techniques may also provide the possibility to predict toxic potential of unknown chemicals by comparing specific patterns of gene expression (fingerprints), reflecting mode of action of unknown chemical, with expression profiles of known toxicants [51, 52].

To conclude, by providing both, mechanism of action and predictive tools, ecotoxicogenomic approach seems especially promising for studying the effect of pollutants at low, environmentally relevant concentrations, improvement of toxic mixture analysis and long term exposure assessment of organisms [51].

7. Conclusion

The use of biological methods in environmental monitoring is essential in order to complement chemical analysis with information about actual toxicity or genotoxicity of environmental samples. Microorganisms are widely applied test-species in different bioassays because of the ease and low costs of their culturing as well as the lack of ethical issues often accompanying the use of higher organisms. Combining biology to engineering skills has enabled the development of biosensors - new generation of analytical devices coupling biological recognition elements to physical signal transducers. Besides the direct application of whole microorganisms or their isolated parts for general toxicity assessment or detection of specific compounds, genetically modified microbes also represent an important source of recombinant antibody production, which makes them important also when talking about immunoassays. With the development of toxicogenomic approaches, the use of microorganisms for environmental monitoring purposes is expected to become even more extensive because of better knowledge about potential analogies in toxicity mechanisms between higher organisms and microbes.

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