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Bioindicators of wastewater ecotoxicity

Gabriela Jirova [1,2], Zdenka Wittlingerova [1], Magdalena Zimova [1,2], Alena Vlkova [1,2], Martina Wittlerova [2], Marketa Dvorakova [2,3], Dagmar Jirova [2]

1 Faculty of Environmental Sciences, Czech University of Life Sciences Prague, Praha, Czech Republic
2 National Institute of Public Health, Centre of Toxicology and Health Safety, Praha, Czech Republic
3 Charles University in Prague, Third Faculty of Medicine, Prague, Czech Republic

Correspondence to: Gabriela Čárová, M.Sc.
Faculty of Environmental Sciences, Czech University of Life Sciences Prague,
Kamýcká 129, Praha 6 - Suchdol, 165 21, Czech Republic.
TEL: +420 267082564; E-MAIL: carova@fzp.czu.cz

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Abstract

Wastewater, especially containing hospital effluents, exhibits high chemical complexity and specificity since it includes various chemicals, biocides, pharmaceuticals, surfactants, radionuclides, disinfectants and pathogens.
Biological tests provide true evidence of the wastewater quality and unlike chemical analytical tests show comprehensive pollution effects on the environment and human health. Normalized conventional bioassays are not sensitive enough for ecotoxicological evaluation of wastewater and there is a great need for the development of suitable sensitive bioassays in order to characterize properly the residual toxicity of treated effluents.
Provisions of binding EU legislation regarding protection of animals used for scientific purposes and legislation dealing with test methods for identification and classification of health hazard of chemicals, pharmaceuticals, biocides, medical devices and consumer products such as cosmetics for environmental ecosystems and for man require to employ alternative toxicological methods respecting the 3Rs concept with priority given to methods *in vitro*.
The Fish Embryo Test (FET) is identified as a relevant, reliable and efficient alternative test method in vitro for determination of acute toxicity for fish. Using the FET, additional toxicological endpoints may be investigated to assess organ specific bioaccumulation, genotoxicity and mutagenicity, developmental toxicity, teratogenicity, various forms of neurotoxicity or endocrine disruptivity. The addition of multiparametric sensitive endpoints makes the FET a true alternative *in vitro* assay and a powerful tool in toxicology.

REVIEW ARTICLE

INTRODUCTION

Development and use of new pharmaceuticals and the spectrum of new chemicals is increasing due to innovations of industry.The priority of the European Union strategy is to support sustainable use of water and long-term efforts to reach progressive reduction of pollutants released into the aquatic environment. This aim is formulated in the EU Water Framework Directive (2000/60/ES). This directive is considering the water economy from general point of view and its main aim is to

avoid any deterioration of water quality and to protect and improve the state of water ecosystems and adjacent wetlands. Implementation of the directive does not only mean application of new technical standards but also the need to establish completely new and complex management of water and water resources.

Chemical substances and pharmaceuticals which are released into the environment, can have undesirable effects also on organisms, for which they were not originally intended (Oehlmann *et al.* 2006). Wastewater can pose health risk not only for humans, but also for ecosystems of the environment. Series of studies conducted so far with industrial, urban or hospital wastewater were focused only on chemical analysis and detection of selected groups of substances. Simple chemical analysis of wastewater can not reflect all possible reactions of present substances. Toxicity of these substances should be evaluated simultaneously by chemical analysis and by conventional and newly developed biological ecotoxicity tests (Zgórska *et al.* 2011; Wing 2016).

WASTEWATER

Wastewater can be dangerous for human health and for the environment considering a high content of chemicals coming from different types of industries, from urban waste, from household waste, from services or from hospital effluent. A large part of chemical contaminants found in wastewater may pass from wastewater treatment plant directly to the aquatic environment (Fent 2006b).

A wide range of activities carried out in hospitals, as medical care, diagnostics, hygiene, maintenance or research, requires use of large amounts of chemicals and pharmacologically active substances. Wastewater from hospitals therefore contains a mixture of different drugs, radionuclides, anionic, cationic and amphoteric surfactants, desinfectants and pathogenic microorganisms (Jean *et al.* 2012). The problem with most of drugs is that, even after the cleaning process, they are still present in wastewater (Escher *et al.* 2009). Wastewater from hospitals are loaded with these substances as a result of handling them, or from the patient excreta with remnants of drugs, pathogenic and potentially pathogenic microorganisms. All of these substances and their metabolites end as waste and are released to the environment, where they may further react and interfere. Hospital wastewater is moreover a highly selective environment and it contributes to high resistence of bacteria to antibiotics. It has been demonstrated that higher numbers of , resistance bacteria are found in wastewater from hospitals. Antibiotic resistance was found not only in pathogenic organisms, but also in organisms inhabiting terrestrial and aquatic habitats (Moges, *et al.* 2014).

Wastewater treatment plants eliminate a large number of pollutants, but often show only limited ability to reduce micropollutants (Kümmerer 2004). Even low concentrations of micropollutants may have adverse effects on aquatic organisms (Wick *et al.* 2009).

The activated sludge treatment with an elevated SRT of 18 d was the only process which led to a significant removal of certain beta blockers and psychoactive drugs. The removal efficiency was below 60% for all compounds except for the natural opium alkaloids codeine and morphine being removed by more than 80%.

Sorption batch experiments within this study (Wick *et al.* 2009) and literature data confirmed that sorption onto activated sludge is negligible and thus, any removal of the examined beta blockers and psychoactive drugs can be exclusively referred to as biological transformation.

A serious negative effect with long-term impact is endocrine disruption. The toxicity of various mixtures of micropollutants with additive or synergistic effect raises concern. In the past it was shown that a mixture of estrogenic substances, used in concentrations which separately did not have estrogenic activity, had induced estrogenic activity, as demonstrated for example in trout (Brian 2005). A similar effect was observed in the case of pharmaceuticals in algae and *Daphnia magna* (Fent 2006b). Hormones in the environment can change sex in juveniles animals. For adults they can cause infertility, damage eggs and embryos and may induce tumor growth (Reeder *et al.* 2004). The tests of cattle feedlot effluent demonstrated the effects of feminization in males and defeminization in females for the fish *Pimephales promelas*. Manifestation in males included eg. reduced testosterone production, decrease in testicle size or change in the morphology of head (Knacker *et al.* 2010) . This species is highly susceptible to changes of water pH. Although the decrease of pH did not affect the life span, changes in the behaviour of fish were observed, such as manifestations of stress (hyperactivity, swimming at the surface), or changes in morphology (decreasing of the size of head, changes in colour, increasing the size of the eggs, but also their fragility).

Including an ozonization step after the biological treatment of wastewater can reduce pollutant concentrations and toxic effects in aquatic organisms (Margot *et al.* 2013). However, biological processes and chemical oxidation often lead to degradation of organic matter and the formation of hazardous by-products, such as halo-acid, halo-ketones, bromine, non-halogenated carboxylic acid and other substances. These substances give rise to a reasonable suspicion of inducing a variety of adverse biological effects on aquatic organisms, such as cytotoxicity, genotoxicity and mutagenicity, developmental toxicity, neurotoxicity or toxicity for reproduction (Erbe *et al.* 2011). Through the above effects the original substances (although they are found at very low ineffective concentrations) can impair health and number of population of aquatic organisms and subsequently in the long term ecological balance under conditions of chronic exposure (Fent *et al.* 2006a).

DETERMINATION OF WASTEWATER TOXICITY

Diagnostics of health in populations of aquatic organisms should include sensitive indicators and the use of multi-parameter approach. Conventional classical bioassay is not always sensitive enough to evaluate the ecotoxicity of wastewater in the long term and sublethal doses. Given an excellent correlation of the FET with the acute fish toxicity test and the fact that non-feeding developmental stages of fish are not categorized as protected stages according to the new European Directive 2010/63/EU on the protection of animals purposes, the FET is ready as a true alternative for the acute fish toxicity test, as required for a multitude of national and international regulations (Braunbeck 2014). It is therefore highly desirable to develop more convenient and sensitive bioassays for the efficient characterization of possible residual toxicity after cleaning waste discharges. Sensitive indicators of ecosystem health in a variety of aquatic organisms are particularly cytotoxicity, life span, growth and reproduction, abnormalities in embryos and DNA integrity (Wigh *et al.* 2016). Newly developed, introduced and validated methodologies *in vitro* make it possible to supplement conventional methods with sensitive evaluation of specific indicators of ecotoxicity. Determination of the life span, growth and reproductive ability is complemented by the evaluation of adverse mechanisms, e.g. determination of potential endocrine disrupting activity and genotoxicity.

Fish are an indispensable part of routine examinations of toxicity to aquatic environment. Globally they are the most widely used species for ecotoxicity testing. Fish are consumers of higher order in the food pyramid of aquatic organisms and play an important role in the regulation of aquatic ecosystems. Increased production of chemicals and waste, however, has increased the number of ecotoxicological tests and thus the use of fish as vertebrates for ecotoxicity tests. The current, obligatory legislation for testing the hazardous properties of chemicals and consumer products strictly requires the use of alternative toxicological methods without tests on vertebrate animals, if available. The current EU strategy on the protection of animals includes the implementation of the concept called 3Rs, to reduce the number of animals in experiments (Reduction), to reduce the deprivation in the course of the experiments (Refinement) and the target is to completely replace testing on vertebrate animals (Replacement) by alternative *in vitro* methods in practice of testing and research facilities. If an alternative method is available, it must always be used instead of conventional toxicology test on live vertebrates. 3Rs strategy is part of the REACH Regulation (EU 2006) and CLP Regulation (EU 2008) for chemicals, the Cosmetics Regulation (EU 2009), and in particular the directive on the protection of animals used for scientific purposes (EU 2010). In the case of fish, the optimal method that meets the ethical requirement to replace vertebrate testing scheme by an alternative method is the Fish Embryo Test (FET) (OECD 2013).

TRADITIONAL/CLASSICAL ECOTOXICITY TESTS

The authors of recent scientific publications (Wigh 2016, Braunbeck 2014) suggest rational assembly methods for ecotoxicity tests which allow individual methods to evaluate long-term adverse effects of substances on aquatic ecosystem. The tests with different types of organisms are included in test strategies. They represent several trophic levels in the hierarchy of the ecosystem such as decomposers bacteria, algae and higher plants as primary producers, primary consumers such as zooplankton and fish as secondary consumers. The principle of conventional tests, which are generally performed in the Czech Republic in accordance with harmonized standards, is the determination of toxicity based on the inhibitory or lethal effects of the test substance on the whole organism. Zgorska (2011) used a battery of tests with bacteria, algae and crustaceans to evaluate the ecotoxicity of wastewater. Results of the study showed that for each organism the wastewater is differently toxic. The most sensitive to wastewater toxicity was the alga *Pseudokirchneriella subcapitata*.

Test on bacteria *Vibrio fischeri* is described in the standard EN ISO 11348-1, 2, 3 and ISO 21338. The principle of the test is the determination of the inhibition of light emission of luminescent marine bacteria after exposure to the test material for 5 min, 15 min or 30 min. The test result is the concentration of test substance which causes 20% and 50% inhibition of luminescence.

Toxicity testing using freshwater algae is carried out according to the standard EN ISO 8692. *Desmodesmus subspicatus* or *Pseudokirchneriella subcapitata* can be used as the test organisms. The method is based on determination of the test substance concentration causing 50% inhibition of specific growth rate relative to control samples growing under the same standard conditions for 72h.

Seeds of *Sinapis alba* are used to determine the acute toxicity in seeds of higher plants. The principle of the method is the assessment of root growth in the initial development of the plants in comparison with control samples. By measuring the root length after exposure of 72 hours, the inhibitory concentration for 50% of organisms can be calculated.

Test for acute toxicity of substances on freshwater crustacean *Daphnia magna* Straus is carried out according to the standard EN ISO 6341. The merit of the method is to determine the concentration of the test substance that causes 50% of newborns immobilization of *Daphnia magna* Straus, gained by acyclic parthenogenesis.

Determination of the acute lethal toxicity of substances for freshwater fish is done according to the standard EN ISO 7346-1, 2, 3. Suggested species are *Poecilia reticulata* or *Danio rerio* Hamilton-Buchanan. The essence of the method is the determination of the concentration of a test substance, which after 96 h of exposure causes 50% mortality of the test organism under the method conditions.

ALTERNATIVE METHODS IN THE EVALUATION OF ECOTOXICITY

Fish Embryo Test (FET)

One of the most renowned alternative approaches to testing on fish is the fish embryo test (FET) (Lammer *et al.* 2009). FET gained importance and application in relation to the estimated up to 20 –fold increase in the ecotoxicity tests within the testing strategies of chemicals according to REACH (Hartung a Rovida 2009) and the current requirement of obligatory use of alternative *in vitro* tests instead of experiments on vertebrates.

Fish embryo test on freshly fertilized ova of fish *Danio rerio* (zebrafish) was introduced in the EU initially in Germany as so called "fish egg test" in 2001. It became a part of DIN standard (DIN 2001). Originally, the test was performed for 48 hours and (as indicators of toxicity) it included the evaluation of acute lethality, coagulation of embryos and formation of intraembryonal opaque clots, inseparation of tail bud and disappearance of heartbeat. According to the current test methodologies the test is completed within 96 hours, which is reliably 2/3 of the time of embryo development,when the development of the nervous system reaches the stage when vertebrates may begin to feel pain. FET therefore fully meets the definition of alternative methods in terms of compensation (Replacement) experiments on living vertebrate animals (fish). *Danio rerio* are fish, which are easy to treat and produce transparent, non-adherent eggs (diameter about 1 mm) throughout the year.

According to the methodology of OECD TG 236 (OECD 2013) the freshly fertilized embryos are exposed to the test material for 96 hours. Every 24 hours (four observation periods) four main indicators of toxicity (Braunbeck *et al.* 2005b; Knobel *et al.* 2012; Braunbeck 2014) are evaluated and LC50 is determined, i.e. the concentration which causes toxic changes in 50% of individuals. The indicators that are assessed as positive at any time of observation are: coagulation of fertilised eggs, lack of somite formation, lack of detachment of the tail-bud from the yolk sac and lack of heartbeat. In order to capture the very early negative effects of the test material on the development of embryos it is necessary to perform exposure as soon as possible, i.e. 1.5 hours after fertilization.

Validation studies demonstrated an excellent correlation of FET with conventional acute toxicity test on fish (Belanger *et al.* 2013; Knobel *et al.* 2012). Certain restrictions of FET were reported in the case of high-molecular substances such as polymers, or substances subject to biotransformation, such as allyl alcohol (Braunbeck 2014).

Braunbeck *et al.* (2005) in their earlier work reported that the FET principle can also be applied using other types of eggs of fish, e.g. *Oryzias latipes* or *Pimephales promelas*. Further they demonstrated that the variability between the results of FET on zebrafish embryos and other species of fish is not higher than the variability of the conventional test of acute toxicity for fish using various species of fish. Unlike the Fish Embryo Test which shows excellent correlation of results with tests carried out on adult fish for chemicals and wastewater (Castano 2003), cell cultures derived from fish, which have been used by some laboratories, showed generally lower sensitivity compared to the complete body of fish (Scholz *et al.* 2013).

METHODS *IN VITRO*

Determination of genotoxicity and teratogenicity

FET test on *Danio rerio* embryos can be preferably enriched by determination of genotoxic potential of waste material for cells of an embryo using Comet assay. Comet assay is a known method which is used for measuring DNA breakages in cells. This test is now widely accepted as a standard method of assessing DNA damage and is used in a wide range of applications from biomonitoring in humans, genotoxicology testing, to monitoring of genototoxicity in ecology (Kosmehl *et al.* 2008). The method is based on gel electrophoresis of individual cells. The presence of DNA breaks causes local relaxation of supercoiled structure. When a small electrical charge passes through the gel, relaxed structure of DNA loops migrates toward the anode, creating the so-called Comet's tail. Head of the comet represents undamaged DNA, which for its size cannot proceed in gel (Liao *et al.* 2009). The advantage of the test is the possibility to obtain information on the level of individual cells, which is crucial for robust statistical analysis. Further advantages are the need to have only a small quantity of cells for testing an individual sample, high sensitivity of detection of DNA damage and suitability of the method for any eukaryotic cells. Limitation of the method is the requirement for cell viability. If the cell suspension contains mostly necrotic or apoptotic cells it is not possible to obtain accurate information on the presence of lesions and breaks. After completion of the 96-hour exposure, i.e. at the end of the FET test, it is possible to isolate the cells from whole embryos and evaluate primary DNA damage, e.g. using a modified Comet assay with FPG (formamidopyrimidine DNA glycosylase). The Comet assay showed, according to some authors, an increased sensitivity for the detection of DNA damage due to oxidation and alkylation.The combination of FET and Comet assay using cells isolated from the whole

embryo is advantageous for multiple assessment to detect embryotoxicity and genotoxicity (Kienzler *et al.* 2012; Wigh 2016).

Zebrafish embryo is a useful model for studying vertebrate evolution because of its transparency, low cost of breeding and known transcription of the genome. It is proposed as a model for screening of teratogenicity not only for fish (Hruscha *et al.* 2013; Hwang *et al.* 2013), but also for mammals (Brannen *et al.* 2013). The relationship between developmental toxicity and neurotoxicity during exposure to industrial chemicals was documented in studies that described the use of FET for identifying e.g. subclinical brain dysfunctions, spinal malformations, defects of the eye and other organ failures during the development of vertebrates (Selderslaghs *et al.* 2013; Sipes *et al.* 2011).

A separate, recently widely proposed test of acute toxicity and genotoxicity for the soil and aquatic ecosystems, is a test using the bulbs of *Allium cepa L*. The principle of the test is to determine ratio of chromosomal aberrations in cells of the bulb root after exposure to wastewater. The advantage of the test is no need of wastewater sample modification before application on bulbs and low cost of the assay. The indicator of general toxicity of the test material is inhibition of length of roots grown from bulbs after exposure to the wastewater sample (Firbas 2011; INVITTOX – PROTOCOL 8, IP-8, 1989).

At present the most widely used tests of genotoxicity are based on bacteria *Salmonella typhimurium* and *Escherichia coli* known as Ames test or UmuC test or SOS chromotest. Ames test and its liquide version Ames fluctuation test are the longest used bacterial reverse mutation assays for evaluation of the mutagenic potential of chemicals or mixtures by detecting their effect on histidine deficient strains *Salmonella typhimurium* in the absence and presence of liver metabolic activation. Addition of liver metabolic activation enables to detect promutagenic properties of the test material in cases when the genotoxic effect occures only after metabolic activation. The Ames test has been recognized as advantageous for identification of genotoxicity of different types of wastewater, including urban or hospital effluents (Sharma *et al.* 2015).

Endocrine disruption and neurotoxicity

Zebrafish embryos can serve as a suitable model for identifying endocrine disruption, namely for estrogenic activity (Schiller *et al.* 2014). Typical biomarkers of estrogenic activity in fish such as vitelogenin, receptors for estradiol α and β and zona pelucida protein can be easily identified and measured already in early stages of zebrafish development (Braunbeck 2014). Besides estrogenic activity it is also possible to monitor anti-estrogenic, androgenic and anti-androgenic effects. Some reports suggest the possibility to use zebrafish embryos for screening of negative effects on the thyroid (Wang 2013).

For the determination of endocrine disruptive activity other methods can be also used, e.g. Melno cell line or MDA-KB2, which are capable of identifying androgen and glucocorticoid agonists and antagonists (Wilson *et al.* 2002). The stabilized cell line of human origin MDA-KB2 is expressing androgen receptors and glucocorticoid receptors and contains a luciferase reporter gene linked to the MMTV promoter derived from MMTV (Mouse Mammary Tumour Virus) which is activated by these two receptors. A positive response in the test means that there was a response by binding to androgen or glucocorticoid receptors. In order to distinguish whether it is the activation of the androgen receptor or a glucocorticoid, complementary exposure with specific androgen antagonist (flutamide) or glucocorticoid antagonist (RU486) is carried out. Information about stimulation of receptors for glucocorticoids is significant, as municipal and hospital wastewater are known sources of substances from the group of glucocorticoids, which are then identified in water systems (Creusot *et al.* 2014).

The cell line MELN (Balaguer *et al.* 1999) stably expresses the luciferase reporter gene under the transcriptional control of an endogenous estrogen receptor. Methodology for determining the hormonal activity of wastewater can be advantageously used according to various recent publications (Kinane *et al.* 2010, Creusot *et al.* 2010). In principle, wastewater is first extracted using an extraction column, eluated in methanol / dichloromethane and dissolved in DMSO. The hormonal activity is determined in a microplate against standards / controls, namely estradiol, dexamethasone, and dihydrotestosterone. The result is expressed as an equivalent of hormonal activity of standard substances.

A method based on genetically modified strains of baker's yeast *Saccharomyces cerevisiae*, commercially available from company Xenometrics as a standard kit Xenoscreen YES/YAS, can be used for screening of both activating (agonistic) and inhibitory (antagonistic) effects of chemical substances and their mixtures. Suitable samples are aqueous solutions, extracts and tinctures from different materials, and also the wastewater. Agonistic or antagonistic activity is always set relatively to the values for the physiological steroids (17-β estradiol for method Xenoscreen YES and 5α-dihydrotestosterone method for Xenoscreen YAS). Commercial kit Xenoscreen YES / YAS contains genetically modified strains of baker's yeast *Saccharomyces cerevisiae*, in which the DNA sequences of genes for the human estrogen receptor (gene hERα) and androgen receptor (Har gene) are stably integrated into the chromosomes of the cells. The cells also contain a plasmid carrying a reporter gene, lacZ encoding the enzyme β-galactosidase and estrogen (YES) or androgen (YAS) responsive elements (Routledge & Sumpter 1996). The method detects also cytotoxic effect, which leads to growth arrest or cell lysis. Cytotoxicity is measured as a reduction in light scattering measured at 690 nm. Cur-

rently, validation studies of methods based on similar systems – yeast cells – are ongoing. In the future this method can be useful as a marker for endocrine disruptive potential of different sample types, including wastewater.

CONCLUSIONS

Wastewater, particularly from health care facilities, can be dangerous for human health and components of the environment due to the complex of chemicals, pharmacologically active substances, biocides, radionuclides, detergents, disinfectants and pathogenic microorganisms. Unlike chemical analyses, biological tests provide true information on the quality of wastewater and its effect on environmental ecosystems and human health. Currently it is desirable to develop and use new sensitive methods capable of correct characterization of residual toxicity. By performing simple chemical analysis it is not possible to assess cross-reaction between substances and their actual effects on biological systems.

According to binding regulations on determination of hazardous properties of chemicals, biocides, medical devices and consumer products, and respecting the EU directive on the protection of animals used for experimental purposes, it is the responsibility of testing laboratories to use alternative toxicological methods based on 3Rs principle. The priority is given to methods *in vitro* instead of conventional tests on vertebrates.

Conventional classic bioassays are not always sensitive enough to evaluate the ecotoxicity of wastewater, especially under conditions of prolonged exposure and in sublethal doses. Diagnostics of health in populations of aquatic organisms should include sensitive indicators and the use of multi-parameter approach. It is therefore desirable to supplement the conventional method with a sensitive evaluation of specific indicators of ecotoxicity, such as cytotoxicity, life span, growth, reproduction, and the ability to evaluate the mechanisms of action of tested noxious agents for example by determining potential endocrine disrupting activity and potential genotoxicity.

For ecotoxicity bioassays the Fish Embryo Test (FET) can be used as an alternative method to tests on fish because it meets the ethical requirement to replace vertebrate testing scheme by an alternative method, and it is a good tool for efficient detection of possible residual toxicity. FET allows to determine not only the acute toxicity for fish, but also developmental toxicity, endocrine disruption, neurotoxicity and integrity of DNA in a genotoxicity test.

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