FACULTY OF FISHERIES AND PROTECTION OF WATERS
UNIVERSITY OF SOUTH BOHEMIA IN ČESKE BUDĚJOVICE

Genotoxic potential of foreign substances in ecosystems of surface waters

Genotoxický potenciál cizorodých látek v ekosystémech povrchových vod

Petra Beránková

Czech Republic, Vodňany, 2011

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I thereby declare that I wrote the Ph.D. thesis myself using results of my own work or collaborative work of me and colleagues and with help of other publication resources which are properly cited.

In Brno 28th April, 2011

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Date, hour and place of Ph.D. defence:

14th September 2011 at 10:00 in a small meeting room of Municipality Vodňany

Name: MSc. Petra Beránková
Title of thesis: Genotoxic potential of foreign substances in ecosystems of surface waters

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Ph.D. thesis, USB FFPW, RIFCH, Vodňany, 2011, 103 pages, with the summary in English and Czech.
Graphic design & technical realisation: Lukáš Páral, Czech Republic, Vodňany
ISBN 978-80-87437-17-9

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CHAPTER 1

GENERAL INTRODUCTION

1.1. IMPORTANCE OF AQUATIC ECOSYSTEMS FOR MAN AND POLLUTION THEREOF

Man has been always using aquatic ecosystems for most of his activities. They are the source of drinking water, environment from which food is acquired (mainly fish), it is the source of water for agricultural irrigation and all industry branches, but it is also a means of transport.

In last decades, these systems have also become recipients of a wide range of waste water. Type and extent of pollution has been changing in the course of time according to development of human society, industrial and mining activities and development of new knowledge in the field of waste water treatment. General development of water treatment may seem paradox to laymen – water ecosystems are less and less polluted by „visible" complex of organic substances that are easily decomposable. They were causing mainly oxygen deficiency and other basic phenomena that accompany discharge of well decomposable organic substances into water (e.g. development of algal bloom, muddiness, and bad smell, mortality of water animals due to oxygen deficiency and increased concentration of ammonia). On the other hand, risks related to occurrence of specific pollutants have increased. These also have other than toxic effects. If we analyse sediments, biofilm and water organisms, we discover significant concentration of „traditional" pollutants in them, such as metals, polyaromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs) etc. (Fuksa, 2002) but also of „new" pollutants that were not identifiable or not even produced in a greater extent before (Hajšlová et al., 2002).

Together with industrial development, water consumption designed for its needs also rises. Unfortunately, industrial development goes hand in hand with water pollution. In an extensive review of the literature, Houk documented the genotoxic activities of a wide range of industrial wastes and effluents (Houk, 1992). Several studies have shown contamination patterns (e.g., PAH rations, PAH alkylation) that are clearly associated with nearby industries and/or industrial processed (Baumann and Harshbarger, 1995; Marvin et al., 1999; Marvin et al., 1993; Rapport et al., 1979).

Another source of contamination is municipal wastewater containing sanitary wastes, institutional wastes (e.g., hospitals, restaurants), and storm sewer runoff. This material contains suspended solids, pathogenic organism, decaying organic wastes, nutrients and a myriad of chemicals from small industries, institutions, and households. Several studies have shown that municipal wastewaters are mutagenic, but their mutagenic potency is low in comparison to that of industrial wastewaters (Chen and White, 2004; Meier and Biskup, 1985; Meier et al., 1987).

Not-point sources, although not as well documented as effluents, can also be expected to contribute to the genotoxicity of sediments. For example, dry deposition of particle-bound combustion by products might be expected to enter aquatic system via urban runoff and storm sewer discharge (Chen and White, 2004).

1.2. SUBSTANCES POLLUTING AQUATIC ECOSYSTEMS

In water, we often find a significant amount of residual pollutants characterized by very high chemical stability. This group also includes chlorinated organic compounds (DDT and its derivatives), PCBs, PAHs, groups of chlorine derivatives derived from dibenzodioxin (PCDDs, PCDFs) and heavy metals. Their danger lies in their ability of bio-accumulation in food chains. The extent of danger of contaminant effects escalates in food chains in direction from producers to apex predators. If the organism is affected by a larger amount of contaminants, their mutual interaction may result in increased toxic effect (e.g. influence of PCB and methylmercury on the organism reproduction).

High concentrations of heavy metals are also often identified in water. These include especially mercury (Hg), cadmium (Cd), lead (Pb), chromium (Cr), zinc (Zn) and nickel (Ni). Their significant sources

are industrial waste water, old ecological loads (industry, metallurgy, dump sites) and agricultural production (organomercurial fungicides). Many of these contaminants are stored in bottom sediments. Due to relatively high sediment stability and complexity, their elimination from sediments is very difficult and expensive.

In long-term perspective, heavy metals affect living organisms at low concentrations. We talk about a so-called chronic environmental stress under which the metal does not have immediate lethal effects onto the organism. Presence of heavy metals in water environment significantly affects populations of fish and benthic invertebrate organisms. Toxic effects of heavy metals manifest in fish mainly in terms of disorders in growth, reproduction, immunosuppression, changes in jacket, liver, gills or skeleton deformation. In case of invertebrate organism, entire populations are often eliminated (Berankova et al., 2009).

Organic compounds that are stable (persistent) and are not easily degradable in ecosystems are called persistent organic pollutants (POPs). Gradual deepening of knowledge of effects of such compounds on organisms proved that these are predominantly compounds not solvable in water. On the contrary, most cases show high solubility in lipids (hydrophobicity). Half-life of these substances is in a range of tens or hundreds of years. Their main danger lies within their contamination of food chains and accumulation in adipose tissues of organisms (Ambrozova, 2003).

1.3. NEGATIVE EFFECTS OF SUBSTANCES POLLUTING AQUATIC ECOSYSTEMS

Millions of cubic meters of waste water from all fields of human activity (mainly industry, households and agriculture) are discharged into ponds, rivers, lakes and seas every year. The US EPA´s Toxic Release Inventory for 2001 reported that more than 100000 metric tonnes of chemicals are released into surface waters by industrial use in the United States (Chen and White, 2004). This data show that large quantities of toxic materials are routinely released directly into aquatic systems after industrial usage. Moreover, 800 metric tonnes of chemical released into surface waters are carcinogens ranked as 1, 2A or 2B under the IARC classification system, and most of them are known to have mutagenic and/or clastogenic activity (Waters et al., 1991; Waters et al., 1999).

Group 1	*Carcinogenic to humans*
Group 2A	*Probably carcinogenic to humans*
Group 2B	*Possibly carcinogenic to humans*
Group 3	*Not classifiable as to its carcinogenicity to humans*
Group 4	*Probably not carcinogenic to humans*

These carcinogens are categorized into two types: persistent compounds, which include metals and PAHs and volatile compounds. Mutagenic/genotoxic compounds, including carcinogens, whether known or unknown, become the components of complex environmental mixtures that can have adverse health effect on humans and indigenous biota (Dearfield et al., 2002).

Genotoxic effect of substances is a serious consequence of interaction of organisms and substances in their surroundings. Damage of deoxyribonucleic acid (DNA) may significantly affect quality of life of organisms exposed to the genotoxic effects as well as quality of life of their offspring. Genotoxic effects which occur in somatic cells have the potential to lead to dysfunction and eventually cell death. In contrast, genotoxic effect in germ cells can be passed on to future generations. Phenotypic effects of genotoxin-induced changes in DNA may thereby become temporally decoupled from chemical and radiation exposures so that transgenerational changes ensue. Thus, genotoxicity can result in

rapid alterations in gene frequencies (relative to normal evolutionary rates) in natural populations, the ecological consequences of which are poorly understood, but are likely to be serious (Depledga, 1998).

Usually, genotoxicity is understood as a late effect of the factor studied (chemical, physical or biological). Genome damage subsequently leads to mutagenesis or possibly carcinogenesis, phage induction, cell death, chromosomal aberrations and other less serious consequences. In an adult person, mutations in somatic cells lead to ageing process of the cell and provide the opportunity for tumour genesis.

First information on ability of certain chemical substances to react with DNA structures and therefore induce changes in genetic material originated in the first half of the 20th century. Since then, there has been a major development of detection methods (genotoxicity tests). At present, tens of tests are available to scientists dealing with genotoxicity studies (Chen and White, 2004).

Apart from genotoxic effects that manifest predominantly in long-term periods, contaminants in water environment can also have other serious biological effects.

Toxic effects of these substances are observed in the longest perspective. At present, significance of toxicity tests is sometimes questioned in comparison to more detailed tests mapping specific effects of substances. However, they still prove their unsubstitutable importance in the field of toxicology. Knowing toxic dose of tested substances (e.g. lethal concentration of a test substance for 50% of testing organisms – LC50) allows using such concentrations in tests with water organisms that are not toxic for the testing organisms, but a manifestation of undesirable effects can be expected. Performance of basic and orientation tests is an integral part of each well-planned experiment. This knowledge shows that role of classic genotoxicity tests is unquestionable and that they will also be used in the future.

With the ongoing development of biochemical and detection methods, further effects were clarified in last 20 years. These include for example effects on the endocrine system that may lead to changes in levels of sex hormones in blood of organisms exposed. In extreme cases, these substances may even cause change of sex of organisms of water environments or their sterilisation. These substances have xenoestrogenic or xenoandrogenic effects and their group is called endocrine disruptors. Number of studies have shown that some populations of freshwater fish are being exposed to hormone-like chemicals resulting in disruption of the reproductive physiology of the organism. Anthropogenic chemicals, including synthetic and natural hormones, can disrupt the endocrine systems of wild species (Tyler et al., 1998; Kime et al., 1999; Arukwe, 2001; Zlabek et al., 2009). In fish, hormones play an essential role in gonad development, and display unique seasonal cycles. A study to investigate reproductive parameter responses after exposure to steroid hormones would yield valuable knowledge. Effect of particular chemical substances on aquatic organism often overlap and it is difficult to understand consequential synergistic and antagonistic effects. Biochemical marker and haematological parameters are valuable indicators on such cases (Blahova et al., 2008; Modra et al., 2008). Unfortunately, failed study results are often misleading to interpret. Limited data is available reporting on the effect of environmental endocrine disrupting chemicals (EDCs) using chub (*Leuciscus cephalus*) as a model organism. Chub is a fish routinely used to assess the quality of surface water (Agtas et al., 2007; Christoforidis et al., 2008; Hajslova et al., 2007; Krcca et al., 2007; Stachel et al., 2007; Zlabek et al., 2009).

Occurrence of cancer and development of neoplasm in fish are also associated with contamination of aquatic ecosystems (Baumann, 1998). Neoplasm epizootics in fish from a wide variety of freshwater, marine, and estuarine locations have been associated with genotoxins in sediment or water. The majority of cases have involved benthic or bottom feeding fish living in habitats with sediment contaminated by PAHs. The most common lesions involved in such epizootics include liver neoplasm, both biliary and hepatic, and skin neoplasms. Laboratory research has demonstrated the ability of fish

to metabolize carcinogenic PAHs such as benzo(*a*)pyrene (B(*a*)P) into the ultimate carcinogen with the resulting formation of DNA adducts. Fish dosed with B(*a*)P or sediment extracts containing carcinogenic PAHs have developed skin and liver neoplasm. Neoplasia in freshwater and marine fish had been noted for a long period of time, with reports in the literature becoming common in the late 1800s and early 1900s. The first report that a population of fish from a polluted waterway had an elevated prevalence of neoplasms (epizootic) was be Lucke and Schlumberger (Lucke and Schlumberger, 1941) who described neoplasm on the lip and mouth of brown bullhead (*Ameiurus nebulosus*). These fish had been collected from portions of the Deelawere and Schuylkill rivers, and the authors noted that the lesions were "of common occurrence, – at least in the region about Philadelphia, – and hence easily available". However, Dawe et al., 1964 were the first to suggest that elevated neoplasm prevalence in fish populations (liver lesions in white sucker and brown bullhead) were linked to environmental contamination (Baumann, 1998).

1.4. CLASSIFICATION OF AQUATIC ECOSYSTEMS

Water ecosystems consist of water that fills cavities and channels in the Earth crust, sediments settled on the bottom of water bodies and a biotic constituent. The biotic constituent consists of bacteria, animals and plants. In complex monitoring of the condition of water ecosystems, attention must be paid to all parts of them.

In terms of water, aquatic ecosystems can be classified into several groups according to three different criteria. One of them is classification into salt water and fresh water. Water salinity is one of the determinant factors for the selection of applicable biological tests. For example, the bacterial genotoxicity test Mutatox® assay that uses a sea bacteria Vibrio fisheri (Kwan et al., 1990) requires higher salinity of the tested sample. On the contrary, other organisms (e.g. *Daphnia magna*) can survive only in freshwater habitat.

Another type of classification is between surface water and subterranean water. In terms of choice of testing procedures, there is no difference between these two types of water. Sampling of subterranean waters is usually more difficult than in surface water. In some locations, local wells may be used. However, in cases of mapping of pollution and expansion of pollution, it is necessary to make actual sampling wells.

The third type of classification is division into running water and still water. Still water show lower variability of pollution over time than running water. In conditions of the Czech Republic, still water (mainly ponds and reservoirs) are often used for fish production or as recipients of raw or roughly pre-treated waste water. This manner of utilization includes a great extent of recipient contamination. Both with simple nutrients and with substances with serious negative effects for bacteria, living organisms and plants.

Water as a medium is an unstable indicator of environmental load of the given ecosystem (mainly in running water), as variability of its composition in time is usually higher than in other compounds. Despite that, it is necessary to pay attention to its load. The surface waters, which contain many unknown compounds, are used as a source of drinking water, as well as for agricultural, recreation and religious activities around the world. Consequently, water pollution can be a serious public health and aquatic ecosystem problem (Houk, 1992; Claxton et al., 1998; White et al., 1996a; White and Rasmussen, 1998; White et al., 1996b). Apart from that, water is the carrier constituent of contamination through the entire ecosystem. Substances solved in it or particles diffused in it can be carried many kilometres from the contamination source. Fine particles usually settle in sections of water courses with less intense flow, or in reservoirs, on the bottom and they create so called bottom sediments.

Sediments in freshwaters and marine systems are complex dynamics matrices composed of organic matter in various stages of decomposition, particulate mineral material that varies both in size and chemical composition, and inorganic material of biogenic origin (e.g., diatom frustules and calcium carbonate). Many aquatic pollutants are predominantly associated with fine deposits that are rich in organic matter, and the manner in which these pollutants, particularly semi volatile organic substances (SVOCs), interact with these deposits determines environmental fate, bioavailability, and toxicity (Chen and White, 2004). The degree to which organics pollutants will be associated with aquatic sediment depends on a number of factors including the physicochemical properties of the solute, the proprieties of the sorbent (particulate material), and the environmental conditions. Most natural organic sorbents, such as the fine detrital organic carbon, readily accept non-polar compounds such as organic pollutants. The phenomenon, essentially dissolution of the compound into the organic (carbonaceous) portion of the solid particulate material (Ghosh et al., 2003), can dramatically influence decomposition, exposure and toxicity.

This thesis concerns mainly detection of genotoxic potential and toxic effects of samples of aquatic sediments. Attention has been paid to them due to their aforementioned relatively low variability over time and increased ability to accumulate substances with effects potentially negative organisms, plants and man.

Biota representing the living constituent of aquatic ecosystems allows observation of pollutant effects on organisms in actual environment. At the same time, it is utilized as a natural bio-indicator of pollution. In running waters, benthic macroinvertebrates (macrozoobenthos) are considered as one of the best indicators of habitat quality, very conventionally called as "river health", and this is why they are widely used for water quality assessment (Hellawell, 1986; Rosenberg and Resh 1993; Chessman, 1995; Wright, 1995). The effort to separate the anthropogenic stress effects from differences related to natural conditions (Rossaro and Pietrangelo, 1993) is recently obvious especially in connection with the WFD monitoring programmes (Rollaufs et al., 2004; Helešic, 2006; Adamek et al., 2010). Abundance of species and their number represents not only the extent of pollution in the given locality, but also the type of pollution.

Fish are also very important for pollution monitoring. Their bodies are usually big enough to provide samples of the required amount of individual tissues. Therefore, it is possible to determine the place of effect of pollutants. At the same time, it is possible to observe various types of effects – e.g. genotoxic, carcinogenous, xenoestrogenous, etc. A wide range of permanent or temporary tissue cultures are prepared from fish tissues designed for observation of a specific effect of substances or their mixtures (Kocan et al., 1985; Babich and Borenfreund, 1991).

Plants are an integral part of the water biota. Man is an anthropocentric being and as such has a tendency to deal only with effects affecting him and his possible food. Despite that, we do realise the necessity to utilise plant models in the study of effects of negative substances burdening our environment as well. Planktonic algae and lesser duckweed (*Lemna minor*) are perhaps the most utilized. Algae are an indispensable constituent of zooplankton food which then becomes food of bigger organisms itself.

Macrozoobenthos, fish and certain water algae belong among the most utilized indicators in ecotoxicological studies. Many of these organisms are also used in laboratory tests. Some of them are standardized in forms of the tests OECD and ISO. In order to assess the toxicity and bioavailability of pollutants in the natural environment to aquatic organism, biomarkers have become valuable ecotoxicological tools (Coughlan et al., 2002; Depledge et al., 1995).

Water organisms, mainly benthic, have the ability to release pollutants from sediments and carry them into the food chain. Substances with bio-accumulative characteristics may therefore enter bodies of fish in the form of food and accumulate in them. In this way, substances polluting aquatic

ecosystems may be carried to our plates and influence our health. Plants have the same ability. As far as transport of contaminants into the food chain is concerned, contribution of vascular plants is smaller than of planktonic algae. Planktonic algae are a significant source of food for herbivorous zooplankton and certain fish (e.g. silver carp, *Hypophthalmichthys molitrix*).

1.5. GENERAL CLASSIFICATION OF THE POSSIBILITY OF EVALUATION OF AQUATIC ECOSYSTEMS LOAD

When evaluating contamination of aquatic ecosystems, we can use two approaches differing in principle – a simple chemical analysis and biological tests. Both approaches have their advantages and disadvantages, their supporters and opponents.

Simple chemical analysis provides specific information on concentration of selected substances. Detailed chemical analyses allow us to identify (approximately, at last) the source of pollution (households, agricultural production, paper production facilities, petrochemical industry etc.) and its age (e.g. according to representation of metabolites or decay products – DDT). The problem lies within the selection of substances that will be determined and within the selection of a suitable method of determination. Chemical analyses are costly and that is why only a selected group of substances gets selected (e.g. PAHs, PCBs, heavy metals), total sum of substances of a similar chemical structure (e.g. ∑PAHs, ∑PCDD/F) or substances preferred according to lists recognized internationally (US EPA list of 129 priority pollutants) (White, 2002). Considering the diversity of mixtures contaminating the environment and ongoing degradation processes, it is not possible to perform a complete analysis of all pollutants. Over 1000 industrially-derived chemical contaminants were identified in the biota, water column, and sediment of the Great Lakes (International Joint Commission, 1983) and over 900 organic chemicals in contaminated sediment from an urban bay in Puget Sound, Washington (Malins et al., 1984). The volume of anthropogenic pollutants has necessitated a tier-structured approach to toxicity testing, with preliminary, relatively simple bioassays used to estimate the relative risk of specific chemicals (White, 2002).

Biological (ecotoxicological) tests offer a wide range of tests usable for analysis of pollution effects in constituents of aquatic ecosystems. We can choose from tests using diverse animal, plant or bacterial species. These test use both entire live individuals, permanent or temporary tissue culture or mere enzymes produced by testing organisms. With their aid, we can determine various biological effects of contaminants present – toxicity, hepatotoxicity, neurotoxicity, genotoxicity, carcinogenicity, photosynthesis blocking, endocrine disruption and many more. As it was mentioned above, some of these tests are standardized in international norms ISO or OECD.

In recent years, great attention has been paid specifically to tests that focus on one specific effect. Effort for detailed knowledge of undesirable effects of substances present in the environment or discharged into it leads to an ongoing development of selective biological tests. Therefore, it depends mainly on work conditions and research objective which of the tests will be selected.

Ecotoxicological tests have a major indisputable advantage – their predictive value. The characterize dangers of a xenobiotic in the environment much better than a chemical analysis of the applicable constituent that only gives information on composition of the foreign substance. Furthermore, in an actual ecosystem, there is never only one substance affecting it, but always several substances or their mixtures at the same time. Consequently, this results in their mutual reaction and a type and extent of the effect utterly different than expected.

1.6. TOXICITY TESTS

For the purpose of evaluation of toxic effects of substances discharged into aquatic ecosystems (deliberately or not), a wide range of tests has been developed. These tests use mainly organisms for whom our water bodies are a natural habitat. Because of the necessity to compare the results of various laboratories, these organisms are frequently kept directly in laboratories or in special facilities designed for that particular purpose and their sensitivity towards standard substances used in the given tests is checked regularly.

As well as in genotoxicity tests, the choice of a suitable toxicity test depends mainly on the work facility and research objective. Toxicity tests are very often used in verification of substance effects used for reduction of development of undesirable organisms in aquatic ecosystems. This can involve development of algae, cyanobacteria, raw zooplankton or macrophytes. Mechanical methods (mainly fishing) are usually used to limit presence of fish or other vertebrate organisms. Toxicity is also a parameter very often monitored in samples collected in places of accidental fish mortality. A simple toxicity test with a model organism (e.g. the common carp *Cyprinus carpio* or the rainbow trout *Oncorhynchus mykiss*) clearly determines whether the sample contains substances toxic for fish or not. On the basis of such test, we can decide whether it is suitable to submit the sample for further detailed chemical analysis.

It is also very important to perform a toxicity test before starting long-term testing of substances with water organisms. And mainly in cases when we are interested in some other than toxic effect of the substance in particular (e.g. hepatotoxic, xenoestrogenic, development and increase of oxidative stress and others).

In the Czech Republic, the legislation supports performance of ecotoxicity tests in water environment and aqueous tinctures with:

1) *Daphnia magna* according to ČSN EN ISO 6341. It is an acute toxicity test which monitors inhibition of organisms in water environment.
2) Fish according to ČSN EN ISO 7346-2. It is a regulation specifying acute lethal toxicity for freshwater fish (*Brachydanio rerio*, *Hamilton-Buchanan*).
3) With algae according to ČSN EN 28692. It is a test of inhibition of growth of freshwater algae *Desmodesmus subspicatus* and *Pseudokirchneriella subcapitata*.
4) On seeds of white mustard according to the Methodological instruction of the Ministry of Environment 11/2007 for determination of waste ecotoxicity. It is a test of *Sinapis alba* root growth inhibition.
5) On the growth of a water plant called lesser duckweed according to ČSN EN ISO 20079. It is a test of growth inhibition of duckweed *Lemna minor*.
6) On bacteria *Vibrio fisheri* according to ČSN EN ISO 11348-3. It is a test of inhibition effects of samples of water or tincture on light emission of these bacteria.

1.7. GENOTOXICITY TESTS

A boat range of bioassays has been employed to assess the genotoxic potential of sediments. Assays employed for *ex situ* sediment genotoxicity assessment can be grouped into three major categories: (1) standardized bacterial bioassays such as *Salmonella* mutagenicity test, the SOS Chromotes, and the Mutatox® assay; (2) *in vitro* assays employing eucaryotic cells in culture; (3) *in vivo* assay systems (Chen and White, 2004).

Fast and relatively cheap bacterial genotoxicity tests are used very often for screening studies. *In vitro* tests performed with tissue cultures serve to detect specific damage of cell genetic information (DNA breaks, change of DNA reading purpose, deletion and insertion, replacement of sister chromatids and many more).

Bacterial genotoxicity tests represent a fast and cheap screening tool for detection of genotoxic potential. It is a group of detection systems based on induction of a specific response of a genetically modified procaryotic organism due to interaction with a genotoxic factor. The represent a suitable alternative approach that can often save costs, time and testing animals in studies of genotoxic factors. This group of tests has been going through immense development since the 1970s. During these years, the first bacterial genotoxicity test was published; it was based on induction of reverse mutations in cells of bacteria *Salmonella typhimurium*. Today, references to more than 20 bacterial genotoxicity tests are available in the applicable literature. Samples with positive results signal possible danger also for superior organisms and they are suitable for further study using tests with eucaryotic organisms.

SOS Chromotest employs a variant of *Escherichia coli* PQ37 that contains a gene fusion between a reporter *lac*Z and *sul*A, a cell division inhibitor gene controlled by the SOS response, to monitor induction of the SOS response to DNA damage. The gene fusion places β-galactosidase under the express control of the SOS regulon and SOS induction is monitored colourimetrically (Quillardet and Hofnung, 1985; Quillard et al., 1982). Test results are usually expressed as SOS induction factor (IF), the ratio of toxicity corrected SOS induction in the samples relative to the solvent control. IF higher than 1.5 is considered significant (in comparison to solvent control).

The WP2 assay procedure with the *E. coli* tryptophan dependent bacteria is the same as those used for the *Salmonella* strains in the Ames test with the exception that limited tryptophan (0.05 mM) instead of histidine is added to the top agar. This chapter provides a historical aspect of the development of the *E. coli* tryptophan reverse mutation assay in addition to guidelines for the use of the WP2 system. The different mutagenicity assay procedures described for the *Salmonella* assay elsewhere in this volume (Mortelmans and Ricco, 2000; Berankova et al., 2009).

Test for chromosomal aberrations in *V. faba* and Test for micronuclei in *V. faba* are short-term genotoxicity tests using plant testing systems. The test uses seeds of the plant *V. faba* (number of chromosomes in the cell nucleus = 12). The tests are performed with meristematic cells of springing primary root. The seeds take 72 hours to spring in the presence of the tested sample. After this time, occurrence of micronuclei and chromosomal aberrations is evaluated microscopically in cells of the root top. The method is very laborious and demanding in terms of accuracy and persistence of the evaluator. However, this exigency is compensated by the statement on effects of the tested samples on genetic material of cells subjected to intense segmentation. Substances with effects that serious may have strong negative impact on development of flora in aquatic ecosystems.

1.8. AQUATIC SEDIMENTS SAMPLE COLLECTION AND PROCESSING

The prerequisite of correct sediment ecotoxicity testing is the correct collection of samples and their processing. In the Czech Republic, there is a valid norm applying to the collection of sediments: ČSN ISO 5667-12 (Water quality – Sample collection – Section 12: Instruction for collection of bottom sediment sampling).

The Eckman-Bridge grab sampler is usually used for sediment collection (Chen and White, 2004). The grab sampler allows collection of a sufficient amount of sediment required for further analysis. In order to preserve as realistic conditions as possible, it is necessary to preserve the sediment in cold and to transport it to the laboratory as fast as possible. If it is not possible to process the sediment

immediately due to technical or time conditions, it can be preserved or frozen. Choice of a preservation method or freezing depends on the analyses required. In the process of preservation, chemical substances are added into the sediment that stop biological processes leading to decomposition and metabolic transformation of substances contained in it. This can also involve lethal and preservative invertebrate organisms living in the sediment (e.g. formaline). In case of utilization of sediments for chemical analyses and biological tests, preservation is not recommended. Many preservative agents may affect chemical analyses or be highly toxic for testing organisms. In such cases, sediment freezing is recommended.

In most biological tests it is not possible to use directly the sample collected, that is why there is the extraction step in the process. The choice of the right procedure is one of the essential moments (Berankova et al., 2011). The choice affect not only the possible toxicity of the actual extract for the testing organism, but also what substances and in what amount will be extracted from the sample. Not all substances combined e.g. in a sediment are available to organisms (especially to bacterial and benthic stock on the bottom) under common conditions. Wrong choice of extraction method can lead us to positively negative or positive results.

Extraction with non-polar solvents such as hexane or dichlormethane (DCM), or solvent mixtures, in the most common method employed to concentrate trace amounts of organic mutagens. In addition to DCM and hexane, frequently used solvents include benzene, methanol, acetone, ether, 2-propanol, and toluene. Commonly used solvent mixtures include DCM/methanol and hexane/acetone. Extractions with more polar solvents such as methanol often employ sonication. Extractions with non-polar solvents such as DCM or hexane usually employ Soxhlet extraction of dry samples extraction solvents are ultimately exchanged for a solvent that is compatible with the selected bioassay (Chen and White, 2004). Many organic solvents used for extraction show serious toxic effects for testing organisms (e.g. DCM, methyl alcohol, acetone). In such case, extracts are transformed into other solvents that combine the ability to solve great amounts of chemical substances and low toxicity for testing organisms (Berankova et al., 2011). One of them is e.g. dimethylsulfoxide (DMSO), which belongs to those utilized the most (Chen and White, 2004).

In case of samples collected from water environment, a simple aqueous tincture is often used for the purposes of testing of toxic effects in water organisms and plants (MŽP, 2007). Aqueous tincture simulates conditions of life and growth of testing organisms in their natural habitat very well. Water is one of polar solvents – therefore substances of lipophilic character do not solve well in it (most carbohydrates, lipids and waxes). Substances that get absorbed in water in the course of extraction reflect the range of substances available to plants, certain bacteria and benthic organisms very well. Therefore, the choice of the most suitable extraction procedure is not easy and clear. It appears that the most suitable approach is to use a combination of solvents or performance of tests with several various extracts. Tests with extracts may also serve as a tool to compare the individual locations (including control locations).

1.9. MONITORING POLLUTION OF AQUATIC ECOSYSTEMS

Long-term monitoring is an inherent part of observation of changes in environment quality. In terms of such monitoring, mainly bottom sediments seem to be a suitable matrix due to their high potential for accumulation of non-polar persistent and toxic substances; they are also highly affected by human activities. Human activity can interfere with natural condition of water (Heiniger et al., 2005). Long-term observation of changes in sediment quality has been a subject of many studies. Most of them concern observation of sediment changes in polluted locations with no comparison to non-polluted locations

(Bertrand-Krajewski et al., 2006; Cachot et al., 2006; den Basten et al., 2003; Heiniger et al., 2005).

Only few studies include sediments from non-polluted so called control/background locations into their range of samples (Heiniger et al., 2005; Berankova et al., 2010). On the other hand, there is a great variability in composition (e.g. content of organic carbon, clay, minerals) and physical-chemical parameters of sediments (e.g. ion exchange capacity, pH, etc.) which need to be considered carefully when comparing samples from different locations. Sediments from a background (non-contaminated) location should therefore be compared always only with such sediments from polluted locations which have similar geochemical characteristics.

Upper parts of rivers whose surroundings do not have industry in it and surrounding areas are not subject to intense agricultural operation are suitable as background locations.

1.10. BIOLOGICAL TESTS AND EXTRACTION METHODS USED IN THIS THESIS

From the wide range of options for extraction methods and ecotoxicological tests selection, I chose the following for my thesis:

extraction methods: Soxhlet extraction into DCM with consequent drying of the sample and solution in DMSO;
genotoxicity tests: SOS chromotest and WP2 test, Test for chromosomal aberrations with *V. faba* and Test for micronuclei with *V. faba*;
toxicity tests: Acute immobilisation test with water crustaceans (*D. magna*), Test of white mustard root growth inhibition (*S. alba*).

THE AIM OF THIS THESIS WAS:

1) To integrate the bacterial genotoxicity tests SOS-chromotest into the regulations of laboratories of the Research Institute of Fishing and Hydrobiology. To verify its functionality with standard substances. To verify its suitability for utilization in testing of genotoxic potential in sediments.
2) To assess the influence of method of sediment extraction on results of selected ecotoxicological tests.
3) To select suitable background location in order to assess contamination of river sediments within the Czech Republic. To verify their suitability using the selected genotoxicity tests.

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CHAPTER 2

THE EFFECTS OF SEDIMENTS BURDENED BY SEWERAGE WATER ORIGINATING IN CAR BATTERIES PRODUCTION IN THE KLENICE RIVER (CZ)

Beránková, P., Schramm, K.W., Bláha, M., Rosmus, J., Čupr, P., 2009. The effects of sediments burdened by sewerage water originating in car batteries production in the Klenice River (CZ). Acta Veterinaria Brno 78, 535–548.

It was allowed by publisher on 26th April, 2011 to include the paper/manuscript in this Ph.D. thesis.

ACTA VET. BRNO 2009, 78: 535-548; doi:10.2754/avb200978030535

The Effects of Sediments Burdened by Sewerage Water Originating in Car Batteries Production in the Klenice River (CZ)

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Received May 26, 2008
Accepted April 6, 2009

Abstract

The aim of this work was to perform tests of genotoxicity and toxicity on samples of riverine sediments from a location subject to motor industry load (car battery production). Together with sediment samples we also collected benthos, biofilm and juvenile fish. Concentration of lead was established in all the samples since the sewage waters discharged from the car battery production plant are heavily polluted with lead. Genotoxicity was tested with two tests of genotoxicity: the SOS chromotest and the *Escherichia coli* WP2 test. The toxicity of sediments was tested with a test of toxicity performed on a water crustacean *Daphnia magna*. A profound toxic influence upon benthic organisms was found; a consequence of the river pollution with waste water and flush water from the car battery production plant. This toxic effect was also proven by an aqueous leach from the test performed with *Daphnia magna*. Both tests of genotoxicity proved a significant genotoxic potential of the sediment samples linked with the growth of the concentration of lead in the sediments (up to 647 $mg \cdot kg^{-1}$). The content of lead also increased in the biofilm (up to 3.37 $mg \cdot kg^{-1}$ of dry mass) as well as in the fish bodies (up to 804.5 $mg \cdot kg^{-1}$ of dry mass). This thesis is the first study of the load imposed on this river as a consequence of the waste water and flush water discharge from the motor industry production plant (car battery production).

Escherichia coli, WP2*, Daphnia magna, SOS chromotest, lead*

Riverine sediments play an important role as pollutants and they reflect the history of river pollution (Jain 2004). Sediments act as both carriers and sinks for contaminants in aquatic environments. Trace elements, especially the so called "heavy metals", are among the most common environmental pollutants and their occurrence in waters and biota indicate the presence of natural or anthropogenic sources (Singh et al. 2005).

Aquatic sediment contaminated by heavy metals and PAHs still presents an unsolved environmental problem.

Metal concentrations in sediments can be linked to high concentrations in living organisms. The bioavailable metal load in sediments may affect the distribution and composition of benthic assemblages (Kress et al. 2004). They may be linked to high concentrations recorded in living organisms (Pempkowiak et al. 1999). The metals can be either absorbed into sediments or accumulated in benthic organism, sometimes at toxic levels (Singh et al. 2005).

The main natural source of metals in the aquatic system is the weathering of soils and rocks and anthropogenic activities, whereby industrial and urban wastes are discharged into water bodies (Pardo et al. 1990; Boughriet et al. 1992; Yu et al. 2001; Kalvins et al. 2000). Car batteries can contain up to 55% of lead. Lead along with sewage water and flush water from production plants may spread into the environment.

Most trace metals and PAHs are potentially genotoxic, which implies that they can damage DNA directly and/or generate reactive species (such as electrophilic metabolites

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or free radicals) which can, in turn, damage DNA. Chronic exposure to such substances at an early stage of life in particular increases mutations, and consequently, dramatically increases the incidence of embryonic mortality and physiological disturbances such as teratogenesis, carcinogenesis, retardation of growth and sexual maturity, infertility, etc. (Cachot et al. 2006).

In the study by Cestari et al. (2004) a substantial negative impact of lead on the genetic material of fish cells was proven. The most common ways of damaging DNA in the tested fish have included various chromosomal abnormalities, comprising chromatid gaps and breaks, chromosomal fragmentation, chromatin decondensation and perientric inversions. Chromatid breaks were the predominant chromosomal aberrations after treatment of lead (Cestari et al. 2004). The mutagenic or clastogenic activities of lead are related to disturbances in enzyme regulation that probably affect the replication, translation and repair of the genetic material. Studies on laboratory animals have shown that exposure to lead at levels of 10 mg $Pb^{2+} \cdot ml^{-1}$ of blood leads to chromosomal aberrations (tetrapolidy, mitotic anomalies, chromatid breaks), and these effects may be related to interference with the mechanisms of replication, transcription and DNA repair (Goyer and Moore 1974).

Lead can be taken up directly from the environment by biota as well as by humans. For the last two decades the concentration of lead in the human peripheral blood has been monitored in the Czech Republic. Especially children are paid great attention to, as the toxic effect of lead endangers mostly them (Černá et al. 1997; Batáriová et al. 2006). One of the possible ways of lead penetrating into human organism is contaminated food (including fish).

The study by Altindag and Yigt (2005) revealed distinctions between the concentrations of selected heavy metals (Cd, Pb, Hg, and Cr) in different elements of aquatic ecosystems (water, plankton, sediment, and fish). With the exception of chromium the concentration of heavy metals decreases in the following order: water > plankton > sediment > fish tissue.

Heavy metals are easily absorbed and tend to accumulate better onto soil particles than PAHs and BCPs, so the soil can be a source of all of these compounds as well as the final sink (Baveye et al. 1999; Stefanutti et al. 2002; Nicholson et al. 2003; Wang et al. 2003; Warman and Termeer 2005).

Lead is among the top 10 US EPA priority pollutants. Ferreira et al. (2004) describe the ability of lead to increase the occurrence of DNA damage. Principally it includes single strand breaks that could possibly initiate double strand breaks. This results in the inactivation or alternation of the repair mechanism. Lead bonds to mitochondrial membranes, penetrates into the mitochondrial matrix space, and is capable of uncoupling oxidative phosphorylation in brain cell mitochondria (Brierley 1977; Holtzman and Hsu 1976). Gebhart (1984) reviewed studies of chromosome damage in cells of human subjects exposed to lead and other heavy metals.

The SOS Chromotest, which measures the induction of the SOS response in *Escherichia coli* PQ37, has been developed by Quillardet et al. (1982) as an alternative to the Ames Test. The SOS Chromotest has been recommended for routine use in environmental applications requiring the assessment of genotoxic activity (Lan et al. 1991; Wong et al. 1994; Helma et al. 1996; Legault et al. 1996; White and Côté 1998). Several studies have also reported its usefulness in monitoring the genotoxicity of complex environmental matrices (Bombardier et al. 2001).

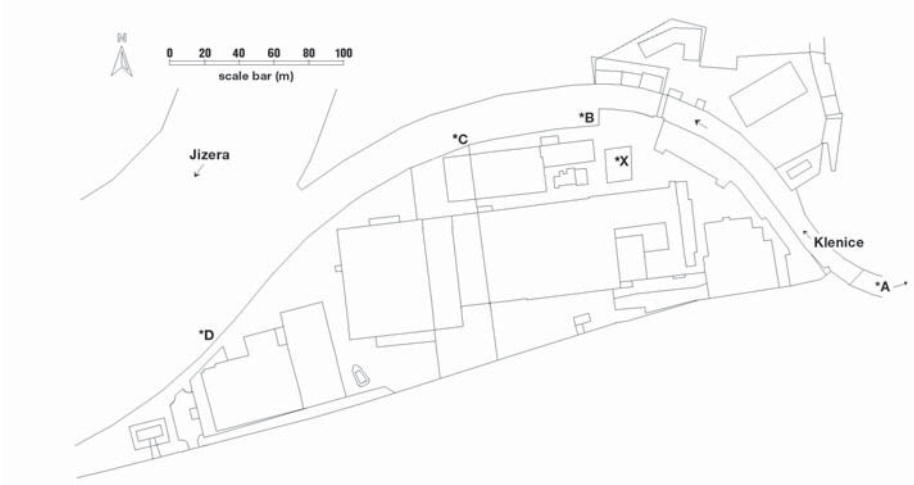
SOS chromotest has been employed by screening environmental studies on a long-term basis. *Escherichia coli* WP2 assay represents a new genotoxicity test that has not been generally employed for testing the environmental matrix yet. As follows from interlaboratory studies (Watanabe et al. 1998ab), it represents a good alternative to the Ames test which is commonly employed for testing genotoxicity of environmental samples.

The study represents the first examination of the burdening of the sediments in the Klenice River (CZ) and is focused on the most detailed covering of the negative effects of sewerage and flush water which contaminate the natural environment. The study combines the results of genotoxicity tests with the analysis of actual biotic samples of the stricken area.

Materials and Methods

Sampling location

The river sediment was sampled in the spring 2007 from the Klenice river (Czech Republic). The Klenice river is the left-bank tributary of the Jizera river in the Labe river basin. Its river basin occupies the area of 169.64 km^2 while its length reaches 29.2 km. The average flow in its mouth amounts to 0.44 $m^3 \cdot s^{-1}$. The Klenice river joins the Jizera river near the town of Mladá Boleslav. The source of pollution is represented in the first place by car industry (namely by production of car batteries).



Situation plan of the sampling location on the Klenice river: *A - sample profile A, *B - sample profile B, *C - sample profile C, *D - sample profile D. Sample profile A is located further upstream of the river (outside the depicted locality).

The samples were collected on four locations of the Klenice river (Scheme):

The sample profile A representing the controlling locations was gathered near the village of Řepov, 4 kilometres above its mouth into the Jizera river. This sample profile is minimally polluted by harmful substances from the town territory and lies out of the reach of pollutants related to car industry.

The sample profile B was gathered under the mouth of a small sewage clarification plant in the area where car batteries are produced. The major source of pollution in this sample profile is represented by repurified sewage water from the area containing PAHs and heavy metals.

The sample profile C was acquired under the mouth of rain water waste pipe from the area. The sample profile is primarily polluted by the water washed off from parking and production areas that contain PAHs and heavy metals.

The sample profile D was obtained under the junction of the Klenice River and the Jizera river. In this case, sewage water and rain water from the car industry area as well as sewage water and sink water from the area of Mladá Boleslav (all including PAHs, heavy metals and common organic pollutants) constitute the main sources of pollution.

Sampling and Treatment

The sampling was carried out in March 2007 using the Eckmann-Bridge grab sampler. Two kilograms of sediment were sampled on each of the locations. These were provided with a code and stored in a plastic bag in a cooling thermobox (4 °C) until laboratory processing. The samples were mechanically homogenized and dried freely at 21 °C.

For the purpose of genotoxicity tests organic extract of the samples was prepared in Soxhlet extractor in contact with dichloromethane (DCM). Ten grams of dry residue were isolated in contact with 150 millilitres of

dichloromethane. The extract was concentrated until its amount reached approximately 5 millilitres; then it was evaporated under a nitrogen flow which was followed by its dissolution in DMSO.

For the purpose of toxicity testing by means of acute toxicity test on the water crustacean *Daphnia magna* (OECD 202) an aqueous leach was prepared. Fifty grams of dry residue were shaken up with 500 millilitres of standard water, conforming to the international norm ISO 9001 (i.e. deionized water) over a period of 24 h. Then the sample was filtered through a filter with a porous diameter of 5 μm. The resultant filtrate was employed in the test.

Chemical analysis

The chemical analysis of samples was performed by the Prague State Veterinary Institute (CZ). Sixteen priority PAHs were determined in the sediment samples in accordance with US EPA using high-performance liquid chromatography (HPLC) with fluorescent detector following the extraction by cyclohexane in compliance with the US EPA regulation.

Lead in all of the samples was determined using mass spectrometry with inductance-coupled plasma (ICP-MS).

Genotoxicity tests

Genotoxicity of the samples was tested by means of two bacterial genotoxicity tests, with and without external metabolism activation (S9 mix), respectively. The S9 blend simulates the mammalian detoxification system. Mammalian liver enzymes can under oxidizing conditions convert some non-genotoxic materials to active genotoxic entities and vice-versa (Fish et al. 1985).

SOS chromotest

The SOS chromotest (Quillardet and Hofnung 1985; 1993) is a colorimetric assay of enzymatic activities following incubating the test strain in the presence of various amounts of sample. The strain used in this study is *Escherichia coli* PQ37 (provided by Prof. Quilardet, Institute Pasteur, Paris, France) that is constitutive for alkaline phosphatase synthesis. This strain exhibits *sfi*A::*lac*Z fusion and includes a deletion of the normal *lac* region, so that β-galactosidase activity is strictly dependent on the *sfi*A expression (Isidori et al. 2004). The assay is quantitative and dose-response curves present a linear region. The slope of the linear region allows the estimation of the SOS-inducting potency (SOSIP), which reflects the inducting activity of the sample (Hofnung and Quillardet 1986; Mersch-Sundermann et al. 1998; Quillardet and Hofnung 1985; 1993).

The SOS chromotest was performed according to a slightly modified method of Xu et al. (1989).

The SOS induction factor (IF) was then calculated for each of the test concentrations. When the induction factor in any of the test concentrations reached 1.5, the test substance was labelled as a significant genotoxin.

Escherichia coli WP2 assay

The mutagenesis assay procedure with the *E. coli* tryptophan-dependent bacteria is the same as those used for the *Salmonella* strains in the Ames test with the exception that limited tryptophan (0.05 mM) instead of histidine is added to the top agar. This chapter provides a historical aspect of the development of the *E. coli* tryptophan reverse mutation assay in addition to guidelines for the use of the WP2 system. The different mutagenicity assay procedures described for the *Salmonella* assay elsewhere in this volume (Mortelmans and Zeiger 2000) are all applicable to the *E. coli* WP2 reverse mutation assay. The only procedural difference is the addition of limited tryptophan (0.05 mM) instead of histidine to the top agar.

The *Escherichia coli* WP2 assay was performed according to a slightly modified method of Mortelmans and Riccio (2000).

The colonies were then counted and the results were expressed as the number of tryptophan revertant colonies per plate (CFU).

Toxicity test: acute immobilization test on water flees (*Daphnia magna*)

Toxicity of samples was tested on the aquatic crustacean *Daphnia magna*. The test was performed in compliance with the methodology OECD 202 "*Daphnia* sp., Acute Immobilisation Test and Reproduction Test" Part I – 24 H EC50 Acute Immobilisation Test.

Biofilm (scabs) sampling

Biofilm (scabs) sampling was performed by means of scraping scabs off the top side of stones taken from the river. The samples were supplied with a code and stored in a plastic bag in a cooling thermobox (4 °C) until their laboratory treatment. The samples were mechanically homogenized and dried freely at 21 °C.

Chemical analysis of the samples was performed by the Prague State Veterinary Institute. Lead was determined with the aid of mass spectrometry including inductance-coupled plasma (ICP-MS).

Sampling and analyzing homogenized bodies of juvenile fish

Collection of juvenile (one-year-old) cyprinids was carried out by an electrical unit. In accordance with the methodology of Randák et al. (2006) a blended sample for chemical analysis was prepared. Whole bodies of juvenile cyprinids were used for the preparation of this sample.

Chemical analysis of the samples was performed by the Prague National Veterinary Institute. Lead was determined using mass spectrometry including inductance-coupled plasma (ICP-MS).

Sampling and treatment of macrozoobenthos (benthos)

The samples of benthos were obtained using the Eckmann-Bridge grab sampler (10 by 10 cm) in the circumlittoral area. The specimens were separated from the substrate by a sieve and fixated in a 4% formaldehyde solution. Qualitative and quantitative sample treatment was performed by the laboratory on the next day. Using a binocular microscope, the specimens were classified as members of families and orders (where possible). Their multitude as well as their abundance (pcs·m^{-2}) and biomass (g·m^{-2}) were established.

Sampling of benthos for chemical analyses was performed accordingly. The samples were supplied with a code and stored in a plastic bag in a cooling thermobox (4 °C) until laboratory processing. The samples were mechanically homogenized and dried freely at 21 °C.

The Saprobic index was introduced by Pantle and Buck (1955), extended by Sládeček (1973) an adjusted by Marvan (1969). The formula for calculation is

$S = \Sigma(Si\text{-}hi\text{-}li)/(\Sigma\ (hi\text{-}li)$

where S is the saprobic index of the whole community,
Si is the individual saprobic species index,
hi is individual species abundance,
li is the species indicative weight.
Saprobity means organic pollution, which is mainly assessed as a level of biochemically degraded substances. Different levels of organic pollution give rise to different community of living organisms.

Data evaluation

Using ANOVA factorial the correspondence between the results of the particular tests was evaluated. Normality of the data was assessed by distribution fitting test. Differences were considered significant if $p \leq 0.05$.

Results

Chemical analysis of the sediment samples

Sixteen priority PAHs and lead were determined in the sediment samples in accordance with US EPA (Table 1).

Table 1. Results of chemical analysis of sediment samples of the Klenice River (CZ)

		LOD µg·kg^{-1}	RSD %	Sample A	Sample B	Sample C	Sample D
Lead	mg·kg^{-1}	mg·kg^{-1} 0.05	8	7.42	307.00	647	126
Benzo(a)anthracene	µg·kg^{-1}	0.05	10	< 0.05	< 0.05	< 0.05	< 0.05
Benzo(a)pyrene	µg·kg^{-1}	0.05	6	< 0.05	< 0.05	< 0.05	< 0.05
Benzo(b)fluoranthene	µg·kg^{-1}	0.10	7	< 0.10	< 0.10	< 0.10	< 0.10
Benzo(k)fluoranthene	µg·kg^{-1}	0.02	6	< 0.02	< 0.02	< 0.02	< 0.02
Indenol(1,2,3-cd)pyrene	µg·kg^{-1}	0.30	11	< 0.30	< 0.30	< 0.30	< 0.30
Dibenzo(a,h)anthracene	µg·kg^{-1}	0.10	5	< 0.10	< 0.10	< 0.10	< 0.10
Benzo(g,h,i)perylene	µg·kg^{-1}	0.10	7	< 0.10	< 0.10	< 0.10	< 0.10
Chrysene	µg·kg^{-1}	0.05	5	< 0.05	< 0.05	< 0.05	< 0.05
Naphthalene	µg·kg^{-1}	0.25	14	0.83	11.25	10.61	12.94
Acenaphtene	µg·kg^{-1}	0.20	12	0.11	0.57	0.29	0.32
Fluorene	µg·kg^{-1}	0.10	12	0.20	1.12	0.45	0.39
Phenanthrene	µg·kg^{-1}	0.05	9	0.72	1.95	0.64	0.69
Anthracene	µg·kg^{-1}	0.01	11	0.03	0.31	0.11	0.11
Fluoranthene	µg·kg^{-1}	0.40	8	0.08	0.31	0.13	0.17
Pyrene	µg·kg^{-1}	0.20	5	0.05	0.19	0.08	0.09
Σ PAHs	µg·kg^{-1}			2.02	15.7	12.31	14.71
Dry mass	g·100g^{-1}			99.19	96.33	95.08	98.00

LOD – detection limit [mg·kg^{-1}] for lead and [µg·kg^{-1}] for PAHs
RSD – repeatability of the metod [%]

The highest concentration of PAHs was detected in the second sample profile, located under the mouth of a small sewage clarification plant in the area where car batteries are produced. In the third sample profile located under the mouth of rain water waste pipe from the area the highest concentration of lead (647 ± 8 mg·kg^{-1}) was measured. A marked decline in the concentration of the monitored substances was noted in the third sample profile located under the junction of the Klenice and the Jizera rivers. At this point the Klenice water gets diluted by the Jizera water.

Genotoxocity Tests

SOS Chromotest

In the SOS chromotest the concentrations of 0.15, 0.075 a 0.0375 g·ml^{-1} were tested.

The results of the SOS chromotest for the version excluding S9 fraction (Fig. 1a) show significant differences between the tested samples ($F = 17.098$; $DF = 3$; $p < 0.05$) as well as the tested concentrations ($F = 8.315$; $DF = 2$; $p < 0.05$). In all concentrations of various samples the value of the inductive factor (IF) reflects the same tendency ($F = 2.088$; $DF = 6$; $p = 0.925$). All of the tested samples demonstrated a genotoxic effect (IF > 1.5) in all of the tested concentrations (Fig. 2a). Only sample D at the lowest concentration did not reach the limit.

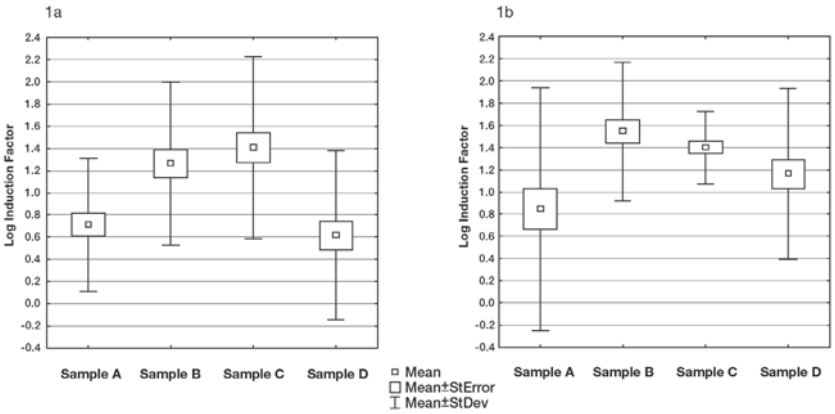


Fig. 1. Results of SOS chromotest of organic extract of sediment samples acquired from the Klenice River (CZ). Variability is expressed by Standard Deviation (SD). Fig. 1a represents the version without the addition of S9 fraction, Fig. 1b represents the version with the addition of S9 fraction.

The results of the SOS chromotest for the version including S9 fraction (Fig. 1b) show significant differences between the tested samples ($F = 38.482$; $DF = 3$; $p < 0.05$) as well as the tested concentrations ($F = 70.625$; $DF = 2$; $p < 0.05$). A consonant tendency for all concentrations of various samples was not determined ($F = 7.382$; $DF = 2$; $p < 0.05$). All of the tested samples demonstrated a genotoxic effect (IF > 1.5) in all of the tested concentrations (Fig. 2b). Only sample A at the lowest concentration did not reach the limit.

Escherichia coli WP2 sssay

In *Escherichia coli* WP2 assay the concentrations of 0.2; 0.1; 0.05 and 0.025 g·ml^{-1} were tested.

The results of *Escherichia coli* WP2 assay for the version excluding S9 fraction (Fig. 3a) show significant differences between the tested samples ($F = 35.16$; $DF = 3$;

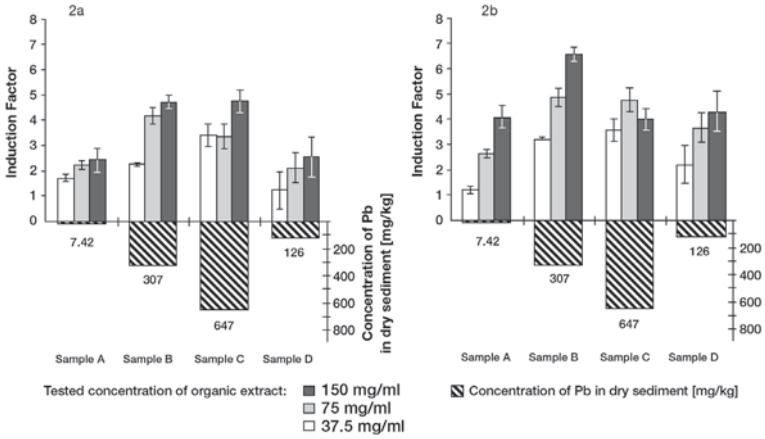


Fig. 2. Results of SOS chromotest of tested concentration of organic extract of sediment samples acquired from the Klenice River (CZ) and concentration of lead in dry sediment. Fig. 2a represents the version without the addition of S9 fraction, Fig. 2b represents the version with the addition of S9 fraction.

$p < 0.05$) as well as the tested concentrations ($F = 4.86$; $DF = 3$; $p < 0.05$). In all of the concentrations of all samples the number of CFU reflected the same tendency ($F = 0.59$; $DF = 9$; p 0.79). All of the tested samples demonstrated a genotoxic effect (CFU > number of CFU in the negative control, CFU of the negative control = 24) at all of the tested concentrations (Fig. 3b). Only sample A at the lowest concentration did not reach the limit.

The results of *Escherichia coli* WP2 assay for the version including S9 fraction (Fig. 3b) show significant differences between the tested samples ($F = 82.58$; $DF = 3$; $p < 0.05$) as well as the tested concentrations ($F = 10.01$; $DF = 3$; $p < 0.05$). In all of the concentrations of all samples the number of CFU reflected the same tendency ($F = 0.95$; $DF = 9$; $p = 0.498$). All of the tested samples demonstrated a genotoxic effect (CFU > number of CFU in the negative control, CFU of the negative control = 24) at all of the tested concentrations (Fig. 4b). Only sample A at the lowest concentration did not reach the limit.

Toxicity test: acute immobilization test on water flees (*Daphnia magna*)

In acute immobilization test on water flees the sample concentration of 1 $g \cdot ml^{-1}$ was tested. Sample A did not produce any toxic effects (0% mortality). Sample B demonstrated strong toxic effects (100% mortality). Sample C showed toxic effects (30% mortality). Sample D produced moderate toxic effects (10% mortality).

Biofilm

In biofilm samples the content of lead was determined (Table 2). The highest lead concentration was detected in the sample acquired from profile C (804.5 $mg \cdot kg^{-1}$ dry weight) and the lowest concentration was detected from profile A (9.50 $mg \cdot kg^{-1}$ dry weight). A marked decline in lead concentration followed the junction with the Jizera river (131.0 $mg \cdot kg^{-1}$ dry weight).

Table 2. Results of assessment of lead concentration ($mg \cdot kg^{-1}$ of dry mass) in biofilm and juvenile fish acquired from the Klenice River (CZ)

Matrix	Sample A	Sample B	Sample C	Sample D
Biofilm	9.50	383.1	804.5	131.0
Juvenile fish	0.89	3.38	3.37	1.74

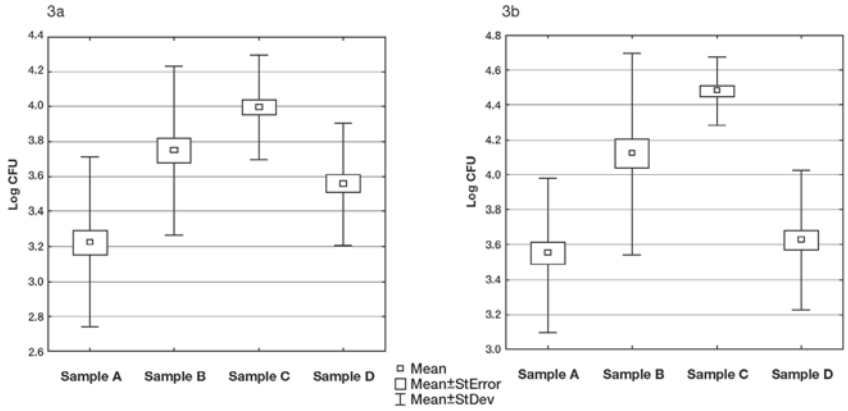


Fig. 3. Results of *Escherichia coli* WP2 assay of organic extract of sediment samples acquired from the Klenice River (CZ). Variability is expressed by Standard Deviation (SD). Fig. 3a represents the version without the addition of S9 fraction, Fig. 3b represents the version with the addition of S9 fraction.

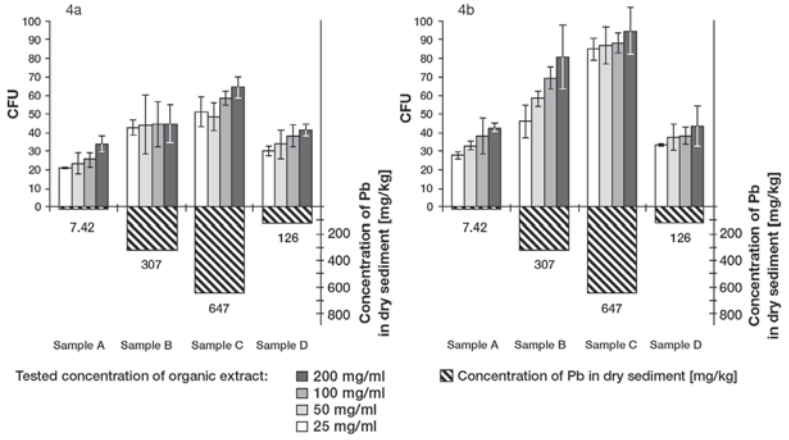


Fig. 4. Results of *Escherichia coli* WP2 assay of tested concentration of organic extract of sediment samples acquired from the Klenice River (CZ) and concentration of lead in dry sediment. Fig. 4a represents the version without the addition of S9 fraction, Fig. 4b represents the version with the addition of S9 fraction.

Juvenile fish

In the samples of juvenile fish the content of lead was determined. As with the biofilm, the highest concentration was detected in the sample from profile C (3.37 mg·kg^{-1} dry weight) and the lowest concentration was detected from profile A (0.89 mg·kg^{-1} dry weight). A marked decline in lead concentration again followed the junction with the Jizera river (1.74 mg·kg^{-1} dry weight).

Benthos

Eight kinds of benthic organisms ranked into 4 groups (Table 3) were found present in the sample from the inspection area (sample profile A). The total saprobic index (S = 3) corresponds to a biotop with an increased amount of organic substances coming from anthropogenic activity (alphamesosaprobity). The indicator character for

Table 3. Results of quantitative and qualitative analysis of benthos samples of the Klenice River (CZ)

	Phylum	Group	Family	Species	Abundance pcs·m^{-2}	Biomass g·m^{-2}	Indicative weight of species	Saprobic index of species
Sample A	Annelida	Oligochaeta	Tubifexidae		200	5.3	4	3.8
		Hirudinea		*Piscicola geometra*	100	3.3	2	2.1
				Erpobdella sp.	400	27.3	2	3
				Glossiphonia sp.	200	1.7	2.5	2.6
	Crustacea	Isopoda		*Asselus aquaticus*	400	21	3	2.8
	Insecta	Odonata	Zygoptera		100	2.3	3	2
		Diptera	Chironomidae		600	3.1	4	3.5
	Mollusca	Gastropoda	Physidae	*Physa fontinalis*	100	4	4	1.6
				Σ	2100	68		
Sample B	Annelida	Hirudinea		*Erpobdella* sp.	100	1.8	2	3
	Crustacea	Isopoda		*Asselus aquaticus*	200	6.9	3	2.8
	Mollusca	Bivalvia	Sphaeridae	*Sphaerium corneum*	100	empty shell	3	2.4
		Gastropoda	Lymneidae	*Lymnea* sp.	100	empty shell	3	2
				Σ	500	8.7		
Sample D	Annelida	Oligochaeta	Tubifexidae		200	5.3	4	3
	Insecta	Diptera	Chironomidae		100	0.6	4	3.5
				Σ	300	5.9		

No sample of benthos was acquired from the profile C, therefore qualitative and quantitative analysis of benthic population was not carried out.

alphamesosaprobity was detected in the water louse (*Asellus aquaticus*) only, the remaining species can be distinguished by greater quantivalence.

No benthic organisms were located in sample profile B. In order to assess the effect of the drains emptied into the profile in question more accurately, the occurrence of benthos was investigated also directly above the drain. Three groups of organisms and four species were discovered (Table 3). Saprobic index (S = 2.6) corresponds to bottom bound of alphamesosaprobity on the verge with betamesosaprobity.

No benthic organisms were located in sample profile C.

Two groups of organisms were located in sample profile D (Table 3). Saprobic index (S = 3.2) corresponds to alphamesosaprobity. The presence of heddle (*Tubifex tubifex*) indicates rather polysaprobity of which strong pollution by organic substances is typical.

Discussion

The results of chemical analyses and genotoxicity tests confirmed the same tendency of the pollution development progressing in the direction from the spring towards the river mouth. This direction was established by the general rules of the increase of concentration of pollutants due to the flow of waste waters. The least genotoxic effect was displayed by a sample from the control location 4 km up the river from the first production plant discharge outlet (MIF for the SOS chromotest without fraction S9 2.4 with fraction S9 4.1). The sample collected under the confluence of the Klenice river and Jizera river (MIF for the SOS chromotest without fraction S9 2.5 with fraction S9 4.3) where the waters mixed and diluted, also displayed low genotoxicity.

The increase of MIF after the addition of the S9 fraction is determined by the increase of genotoxic effects of PAHs contained in the sample after their metabolic activation. In sampling profile B (under the discharge outlet of a small wastewater treatment plant belonging to the production area) there was, in accord with the chemical analysis, an increase of the genotoxic effect (MIF for the SOS chromotest without fraction S9 4.7 with fraction S9 6.6). Under the inflow of the wastewater from the car battery production area (profile C) there was an increase of the genotoxic potential on MIF for the SOS chromotest without fraction S9 4.8 with fraction S9 4.8. In this sample there was no increase of the genotoxic effect due to the metabolic processes simulated by the addition of the rat liver homogenizer.

There was a lower concentration of detected PAHs in sample C compared with sample B. PAHs could be activated by mammalian enzymes therefore no difference in the reactions between the versions with and without the addition of the S9 fraction.

The increase of genotoxic reaction after the addition of the S9 fraction is in accordance with other studies in which sediments were tested with the SOS chromotest (White et al. 1998; Langevin et al. 1992; Lan et al. 1991; Buchman 1990).

Both the SOS chromotest and the *Escherichia coli* WPS test displayed the lowest genotoxic potential in sample A (the controlling locations) and sample D (the junction of the Klenice and Jizera rivers); the tests gave these results both in the version with and in the version without the S9 fraction.

After adding the S9 fraction to samples B, there was a significant increase of the genotoxic potential (under the inflow from the wastewater treatment plant of the car battery production area) and to samples C (the influx of waste waters from the car battery production area). In the literature available at present, there are no studies published which performed tests of natural sediments in the same testing system.

The published studies imply that the reaction of the *Escherichia coli* WPS test is in a good accord with the classic Ames test TA102 a TA2638 (Wilcox 1990; Watanabe et al. 1998a; 1998b). Sediments of the Po river were tested negative on TA98, TA100 and TA102

with or without S9 (Vigano et al. 2002). However, for sediment testing the Ames test uses more frequently TA98, TA100 and TA97 strains (White et al. 2004).

We assume on the basis of our observations and monitoring that an essential part of the genotoxic effect in the sediment samples from the Klenice river is played by the increased content of lead. We have two reasons for this assumption:

1. Concentration of 16 detected PAHs (Σ 16 US EPA PAHs 2.2–14.71 $\mu g \cdot kg^{-1}$, Table 1) in the sediment samples is lower than in the industry-loaded locations (Svobodová et al. 2004). The concentration of PAHs placed according to the IARC into groups 2A and 2B [benzo(a)anthracene, benzo(a)pyrene, benzo(b)fluoranthene, benzo(k) fluoranthene, indenol (1,2,3,-c,d) pyrene a dibenzo(a,h)antracene] is found even under the detection limit (Table 1). Concentration of PAHs in sediments that is so low is clearly due to the absence of industrial plants and large human residences in the basin area of the Klenice river. In their work, Frouin et al. (2007) report a concentration of 129 $ng \cdot g^{-1}$ in the control sediment and a concentration of 22,550 $ng \cdot g^{-1}$ in smelter soot sediment. In sediments from different parts of the USA, concentrations of PAHs were determined in the range of 644 to 55,612 $ng \cdot g^{-1}$ (Jarvis et al. 1996). In the mouth of the Yangtze River (China), Liu et al. (2000) measured a concentration of PAHs from 0.08 to 11.4 $\mu g \cdot g^{-1}$.

2. In the work of Cestari et al. (2004) a significant negative influence of lead upon the genetic material of fish cells was proven. The most frequent ways of damage to the DNA of the test fish were various chromosomal abnormalities, including chromatid gaps and breaks, chromosomal fragmentation, chromatin decondensation and perientric inversions. Chromatid breaks were the predominant chromosomal aberrations after treatment of lead (Cestari et al. 2004). The mutagenic or clastogenic activities of lead are related to disturbances in enzyme regulation that probably affect the replication, translation and repair of genetic material (Goyer and Moore 1974). Studies in laboratory animals have shown that exposure to lead at levels of 10 mg $Pb^{2+} \cdot ml^{-1}$ of blood leads to chromosomal aberrations (tetrapolidy, mitotic anomalies, chromatid breaks), and these effects may be related to interference with the mechanisms of replication, transcription and DNA repair (Goyer and Moore 1974).

The content of lead detected in the samples of sediments collected from the Klenice river (7.42–647 $mg \cdot kg^{-1}$) (Table 1) is in comparison with the available data higher than in other rivers in the Czech Republic, e.g. the Tichá Orlice river reaches a range of lead content of 31.3 to 36.6 $mg \cdot kg^{-1}$ (Svobodová et al. 2004). The increase of the content of lead under the discharge outlets of the car battery production plant in comparison to the control location located 4 km up the river from the first production plant discharge outlet (7.42 $mg \cdot kg^{-1}$) is due to the nature of waste water pollution in this location. These waters are being highly polluted with lead during the production process, because car batteries contain up to 55% of lead. A similar situation is also in other parts of the world. For example, Singh et al. (2005) measured in the sediments of the river Ganges (India) a content of lead in the range of 6.27 to 68.73 $\mu g \cdot kg^{-1}$, in the Po river (Italy) a range of 32 to 98.5 $mg \cdot kg^{-1}$ (Farkas et al. 2007) and Cachot et al. (2006) measured in the Seine river (France) 2–76.6 $mg \cdot kg^{-1}$. Johnson (1998) found lead in suspended sediment to range around 40 $\mu g \cdot kg^{-1}$ in rural streams and rivers but reached 150 to 350 $\mu g \cdot kg^{-1}$ in urban industrial areas.

Altindag and Yigt (2005) describe differences in concentrations of selected heavy metals (including Pb) in various elements of water ecosystems. According to the authors the concentration of these heavy metals decreases as follows: water > plankton > sediment > fish tissue. This sequence is maintained even with the samples from the Klenice river: biofilm (5.5–804.5 $mg \cdot kg^{-1}$ of dry mass) > sediment (0.05–647 $mg \cdot kg^{-1}$) > alevin (0.89–3.38 $mg \cdot kg^{-1}$ of dry mass).

In the tissues of *Abramis brama* L. of the west bank of the Lake Balaton (Hungary) the lead concentration ranging from 44 to 3.24 $mg \cdot kg^{-1}$ of dry mass was determined by Franks et al. (2007). In the study by Begum et al. (2005) the average lead concentration of 2.08 $mg \cdot kg^{-1}$ of dry mass was found in the tissues of the fish *Tilapia nilotica*, *Cirrhina mrigala* and *Clarirus batrachus of* Dhanmondi Lake in Bangladesh. The maximal limit of lead accepted in fish meat by European Union (EU) is 0.96 $mg \cdot kg^{-1}$ of dry mass.

The screening benchmark has been selected utilizing numerous criteria. Priority has been given to values based on direct toxicity over food chain modelling, as site-specific food chain exposure modelling (MacDonald et al. 2000). The values of environmental screening risk (ESR) calculated for observed sampling localities have exceeded critical value 1 (location B ESR = 8.58, location C ESR = 18.07, and location D ESR = 3,52). In case of exceeding ESR = 1 the usage of biological tests for the confirmation of negative biological effects is recommended. The ESR value for sample A (rear location) has also reached 0.21.

There is no presence of macrozoobenthos under sewerage water outfall which could be related to high concentration of lead or other undetected substances and their negative influence on condition of benthic organisms (Robson 2006; Fargasova 1994). Mebane et al. (2008) performed toxicity test of lead on mortality and fertility level in chironomid larvae and reported negative effects even at lower lead concentrations than in the Klenice river. Namely, chironomid larvae are able to build up some tolerance on heavy metal concentration in water, as are copper or zinc, but in the case of cadmium and lead this ability is minimal.

The results of the performed monitoring indicate that the ecosystem of the Klenice river is heavily damaged in consequence of pollution with waste waters containing high concentrations of lead. Lead is being stored in the bottom sediments. From there, it can be released through the activity of benthic organisms or from the sedimental swirling caused by an increased flow. In both cases, the probability of lead entering the food chain increases.

The production of lead car batteries in the observed location has been terminated. Further contamination of the Klenice river can therefore only take place by flush water from the former production area.

With regard to the possibility of fish migration, it could be appropriate to fish out the Jizera river above and under the mouth of the Klenice river and determine concentration of lead in fish samples.

Účinky sedimentů zatížených odpadními vodami z výroby autobaterií, řeka Klenice (ČR)

Cílem této práce bylo provedení testů genotoxicity a toxicity vzorků říčních sedimentů z lokality zatížené automobilovým průmyslem (výroba autobaterií). Zároveň s odběrem sedimentů byl proveden i odběr bentosu, biofilmu a juvenilních ryb. Ve všech vzorcích byla stanovena koncentrace olova neboť odpadní vody z výroby baterií jsou silně znečištěny olovem. Genotoxicita byla testována dvěma testy genotoxicity: SOS chromotestem a *Escherichia coli* WP2 testem. Toxicita sedimentů byla testována testem toxicity na vodním korýši *Daphnia magna*. Byl zjištěn silný toxický účinek na bentické v důsledku znečištění toku odpadními a splachovými vodami z areálu výroby autobaterií. Silný toxický účinek vykázal i vodný výluh sedimentů v testu s *Daphnia magna*. Oba testy genotoxicity prokázaly významný genotoxický potenciál vzorků sedimentů v návaznosti na nárůst koncentrace olova v sedimentech (až 647 $mg \cdot kg^{-1}$). Zvýšený byl i obsah olova v biofilmu (až 3.37 $mg \cdot kg^{-1}$ sušiny) a těle ryb (až 804.5 $mg \cdot kg^{-1}$ sušiny). Tato práce je první studií

zatížení tohoto toku v důsledku vypouštění odpadních a splachových vod z areálu automobilového průmyslu (výroba autobaterií).

Acknowledgements

This study was supported by the project MSM 6007665809 (Ministry of Education, Youth and Sports of the Czech Republic) and the project SP/2e7/229/07 (Ministry of Environment of the Czech Republic) and cooperation of Research Centre for Environmental Chemistry and Ecotoxicology, Faculty of Science, Masaryk University, Brno, Czech Republic and German Research Centre for Environmental Health, Institute of Ecological Chemistry, München, Germany

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CHAPTER 3

INFLUENCE OF SELECTED METHODS OF EXTRACTION OF RIVER SEDIMENTS ON RESULTS OF SELECTED ECOTOXICOLOGICAL TESTS

Beránková, P., Máchová, J., Kolářová, J., Randák, T., Poláková, S., 2011. Influence of selected methods of extraction of river sediments on results of selected ecotoxicological tests. Chemical Letters 105 (6), 476–481. (in Czech)

It was allowed by publisher on 22th April, 2011 to include the paper/manuscript in this Ph.D. thesis.

There is a translation of the article. The full text in original version in its original form see Appendix No. 1.

INFLUENCE OF THE METHOD OF EXTRACTION OF RIVER SEDIMENTS ON RESULTS OF THE SELECTED ECOTOXICOLOGICAL TESTS

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ABSTRACT

Sediments can serve as a reservoir of toxic materials which continue to endanger the aquatic organisms' health and life. The sediment is the place where translation of these materials between inorganic and biological part of the environment. With respect to their character (solid particles settled at the bottom of the river) and lower variability in time, sediments are in terms of longterm monitoring suitable for monitoring aquatic ecosystems. The present study is aimed to initiate a long-term monitoring of the sediment quality in selected rivers of the Czech Republic. The river sediments has been sampled in autumn 2007 from three rivers in the Czech Republic – Ploučnice River (above and below the town Cvikov), Mže River (above and below the town Tachov) and Blanice River (above and below the town Prachatice). Organic extract and water eluates of the sediments were tested. There were 5 tests employed for testing of samples of river sediments: 3 tests for evaluation of genotoxic effect (SOS-chromotest, test for chromosome aberration in *Vicia faba* and test for micronuclei in *Vicia faba*) and 2 tests for evaluation of toxic effect (OECD 202 acute immobilization test with *Daphnia magna* and toxicity test with *Sinapis alba*). Genotoxic potential of the tested samples did not significantly differ from the negative control values. Similar results have come out also of toxicity tests.

INTRODUCTION

Sediments are a heterogenous polyphase system containing both organic and inorganic matter, water and various gases. In their essence, sediments are a mixture of hard particles settling on the bottom of water bodies. Substances contained in water can bind to their surface in various ways. Mobility, bio-availability and biological effects of these substances depend on the way of this binding.

Sediments represent a significant indicator of water ecosystem pollution. The contents of toxic elements in sediments of water bodies and reservoirs reflect the overall contamination of the given locality better than immediate concentration of pollutants in the water[1]. Therefore, sediments are a significant indicator of pollution of water ecosystems.

Two approaches can be adopted in the study of sediment pollution. Chemical approach, which determines chemical composition and substance concentration of the analysed sample. Ecotoxicological approach which determines the effect on biological systems[2]. Both approaches have their advantages and disadvantages. In chemical analysis, it is impossible to grasp presence of all substances in the mixture and their reactions, on the other hand, the ecotoxicological approach does not acquire information on which substance was the cause of the toxic effect observed. Another disadvantage of the ecotoxicological approach is the choice of the manner of preparation of the tested sample, as not all tests can be performed directly with the sediment, i.e. without prior extraction.

The manner of extraction and the extraction agent determine which substances contained in the

sediment will be transferred into the extract. In the extract acquired using a polar solvent, higher content of polar substances can be expected. On the other hand, with a non-polar solvent, higher content of non-polar substances can be expected. That is why the choice of the extraction agent plays a very important role influencing the final results of ecotoxicological tests. Mainly in samples of unknown character, parallel extraction with different extraction agents is recommended.

A simple aqueous tincture and extraction using an organic solvent are the most frequent manners of sediment extraction. Water is a typical polar solvent. It is suitable for solving polar substances whose molecules feature charge placed irregularly. These include most of inorganic salts, carboxylic acid, alcohols and a great part of carbohydrates.

As far as organic solvents are concerned, dichlormethane (DCM) is one of the most used along with its mixtures with other solvents (hexane, methanol, acetone). DCM is a non-polar solvent. It solves non-polar substances very well whose molecules feature charge placed regularly. These include most carbohydrates, lipids and waxes and some inorganic substances (e.g. iodine).

However, many organic solvents used in extraction manifest significant toxic effects for test organisms (e.g. DCM, methanol and acetone). In such cases, the extracts are transferred into different solvents which combine the ability to solve large amounts of chemical substances and low toxicity for test organisms. One of them is dimethyl sulphoxide (DMSO) which is used the most. DMSO appears to be a suitable solvent on the basis of results of tests using various organisms[4,5,6].

When choosing the manner of extraction, it is necessary to consider not only the character of the testing sample. With regards to the fact that the actual manner of extraction can significantly influence results of ecotoxicological test and reality of their response against the original sample, our observations focused primarily on the phase of sample preparation.

The interpretation of test results with extracts for entire ecosystem may be difficult because it is impossible to grasp interactions between the substances, sediments and organisms living in and on the sediments precisely (bio-availability versus substance extractability), but they represent a valuable piece of information on the condition of our environment.

The submitted study compares: aqueous tincture which used for testing of hard waste and two extracts into DCM. Extraction into DCM was performed using Soxhlet extraction and sonication. Both methods are very frequently used extraction procedures.

EXPERIMENTAL PART

1. SEDIMENT SAMPLING, PREPARATION AND PROCESSING

The sediments were taken from the Klenice river (Czech Rep.). Klenice is a left confluence of the Jizera river; it joins it in Mladá Boleslav. The main sources of pollution in the Klenice river basin are human settlements and runoffs from agricultural areas and communications.

Samples were collected near the town of Řepov, approx. 4 km above the estuary into Jizera. It was a fine-grain sediment with low rate of hard sand grains (approx. 20 weight %).

A Bridge-Eckman dredge was used for the collection of sediments. Approx. 2 kg of water sample were collected. The sample was packed, labelled individually and stored in an ice box (4 °C) until its processing in the laboratory. The sample was dried using the method of freeze-drying (lyophilisation). Consequently, the sample was homogenized mechanically and sucked through a sieve with 2 mm eye diameter.

2. SEDIMENT EXTRACTION

Three most frequently used methods[3] of extraction were used for sediment extraction. Every method of extraction was carried out in five autonomous repetitions. Every extract acquired was treated as an autonomous sample.

2.1. Preparation of an aqueous tincture

Aqueous tincture was prepared by means of shaking 100 g of freeze-dried sediment with 1000 ml of distilled water for a period of 24 hours. The aqueous tincture acquired was filtered through a paper filter with 5 μm pore diameter.

2.2. Extraction into DCM

The advantage of the organic extract is an easy preparation of concentrated samples (according to character of substances contained in a unit up to tens of grams in a millilitre). This allows acquisition of information on presence of toxic and genotoxic substances whose concentration is under the detection limit of analytical devices.

2.2.a. Extraction using a Soxhlet extractor

50 g of freeze-dried sediment was being extracted for 8 hours in 250 ml of DCM in the Soxhlet extractor. The acquired extract was densified to approx. 5 ml, consequently desiccated and transferred into DMSO.

2.3.b. Extraction using sonication

50 g of dry sediment was being extracted for 30 minutes in 50 ml of DCM in an ultrasound bath. The acquired extract was decantated and stored. The extraction was performed four times. The acquired extract was densified to approx. 5 ml, consequently desiccated and transferred into DMSO.

3. ECOTOXICOLOGICAL TESTS

The influence of the method of extraction on the biotest results was evaluated using three selected tests (Acute immobilisation test with water crustaceans – *Daphnia Magna*), Test of white mustard (*Sinapis alba*) root growth inhibition and SOS-chromotest). These tests are usually used for evaluation of toxic and genotoxic parameters of environmental samples and they are very frequently used worldwide.

All test were performed with non-diluted aqueous tincture and DCM extracts. Tested concentration of DCM extracts was identical with concentration of the non-diluted aqueous tincture so that effects of extracts acquired through various means of extractions could be compared.

Every test was prepared in three autonomous repetitions for every extract prepared. The acquired results were evaluated statistically. In tests featuring preliminary modification of the testing sample using DMSO, 2 controls were applied concurrently: i. one using diluting water with no addition of the tested substance, ii. one using diluting water solution and DMSO in maximum concentration which was applied in preparation of the testing solution. Response of testing organisms towards the testing sample was connected to the corresponding negative control and furthermore the condition of the organisms was compared in both controls in order to evaluate to what extent were testing organisms influence by the solvent itself.

3.1. Acute immobilisation test with water crustaceans (*Daphnia Magna*)

Acute immobilisation test with *D. magna* was carried out according to the standard OECD 202[7]. Mortality and immobilisation of *D. magna* individuals was the subject of the test. There were 10 individuals of *D. magna* installed in every repetition.

The test used crustaceans of laboratory breeding, their sensitivity was verified using a test with the standard substance $K_2Cr_2O_7$ p.a. The discovered value of 24h-EC50 for $K_2Cr_2O_7$ = 1.13 mg.l^{-1} falls within the limit (0.6–1.7 mg.l^{-1})[8] allowed for this substance.

3.2. Test of white mustard (*Sinapis alba*) root growth inhibition

Test of *S. alba* root growth inhibition was performed according to the Methodological Regulation of the Ministry of Environment (ME) from 2007[9]. Seed germination and primary root growth were the main parameters of this test. There were 30 *S. alba* seeds planted in every repetition. Missed seeds were counted into the calculation of average length of the primary root as a zero value according the Methodological Instruction of the ME from 2007[9].

3.3. SOS-chromotest

It concerns the bacterial genotoxicity test and it is based on detection of production of beta-galactoside in consequence of the launch of SOS reparation system of the bacterial cell. The SOS reparation system is activated in case of DNA damage.

The principle of the SOS-chromotest[10,11] is a colorimetric detection of changes in enzyme activity in consequence of influence of the tested sample onto the testing organism. The test was performed according to the methodology described in the work of Xu et al., 1989[12]. There was an induction factor (IF) calculated for each of the tested samples. If the IF value exceeds 1.5, the tested sample is considered significantly genotoxic[10,11].

The testing organism is a genetically modified bacteria (*Escherichia coli* K12 PQ37) which is subject to the applicable laws and regulations of the CR[13–14].

4. STATISTIC EVALUATIONS

Differences in results which were acquired in ecotoxicological tests (Acute immobilisation test with *D. magna*, Test of the *S. alba* root growth inhibition and SOS-chromotest), while using the individual methods of extraction, were tested with the non-parametric Kruskal-Wallis test in terms of statistic significance. The statistic analyses used software STATISTICA 8.0[15].

RESULTS AND DISCUSSION

The results acquired in ecotoxicological test using the individual methods of extraction are summarized in Chart No. I in the form of averages of three repetitions and standard deviation (all methods of extraction were carried out in five autonomous repetitions).

In the acute immobilisation test with *D. magna*, there was a 100% mortality or immobilisation of tested individuals in the aqueous tincture and both DCM extracts, therefore no statistical difference was discovered between them. Mortality and immobilisation of testing organisms in negative controls

(average 0 and 3.3%) met the conditions of validation of the applied test. The response of testing organisms towards the test samples differed probatively from responses towards negative controls ($N = 51$, $df = 4$, $H = 50$, $p < 0.001$). The influence of the actual method of extraction on the results of the selected tests was insignificant.

In the test of *S. alba* root growth inhibition, no difference significant statistically between the effects of the tested sample acquired in the individual methods of extraction was proven ($N = 51$, $df = 4$, $H = 36$, $p < 0.001$) (Figure 1). Differences in average lengths of roots in the individual test in comparison with the applicable negative control have not exceeded 30%; therefore the results acquired in this test were evaluated as negative. (The method of evaluation stems from the Methodological Instruction of the ME from 2007[9] for evaluations of waste ecotoxicity, where differences in root growth in the tested tincture up to 30% in comparison to the control are considered negative).

In the SOS-chromotest with an aqueous tincture, no difference in response in testing and control organisms was detected, i.e. the result of this test was negative. On the other hand, there were significant statistic differences between the effects of DCM extracts acquired using Soxhlet extraction and extracts acquired using sonication ($N = 51$, $df = 4$, $H = 43.5$, $p < 0.001$). Effects of the DCM extracts acquired in both was were mutually different, but they also differed from the results acquired using aqueous tincture (Figure 2).

It is therefore obvious that different ecotoxicological tests and different methods of extraction of tested matrix bring diametrically opposed results. While using the test with white mustard, negative results were acquired with all methods of extraction, the results of all extracts were positive in the test with *Daphnia magna*. When evaluating genotoxic effects using the SOS-chromotest, positive results with non-polar extracts were acquired; however the results were negative with aqueous tincture. This fact proves that when evaluating ecotoxicological characteristics of a certain matrix, various methods of extraction must be used as well as various types of ecotoxicological tests. Otherwise there is a risk that possible undesirable effects of substances contained in the sediment will not be „detected" and the evaluation will be performed on the basis of falsely negative results.

In published studies concerning influence of method of extraction on results of the Ames test (bacterial genotoxicity test using the *Salmonella typhimurium* bacteria), the authors used various solvents and their mixtures (e.g. mixture water/ether, methyl alcohol, toluene, DCM and distilled water)[16–20]. Authors of one of the stated studies[16] have performed tests with sediments from the Tama river (Japan) and declare that polar extracts from these sediments manifested stronger genotoxic effects in the Ames test than non-polar extracts (mixture water/ether > ethyl acetate > methyl alcohol ~ n-hexane). On the other hand, a study carried out with sediments from the German part of the river Elbe proves stronger effect in the Ames test in non-polar extracts (toluene) than in extracts into methyl alcohol and much stronger than in aqueous tinctures.

Inconsistency of these results shows that using only one type of solvent (polar x non-polar) is not suitable for objective grasp of effects of sediments tested. If we do not know the nature of pollution of the location concerned, using extraction with both polar and non-polar solvents or their mixtures seems to be more suitable. However, we always have to respect the requirements of the selected ecotoxicological test that is to be used for evaluation of ecotoxicological effects of sediments tested.

CONCLUSIONS

1) Results acquired that when evaluating toxic and genotoxic characteristics of sediments it is necessary to select various methods of extraction in order to allow discharge of as complete range of substances present in the sediment as possible. Furthermore, it is necessary to subject the prepared

extracts to ecotoxicological tests which use various testing organisms.

2) Methods of sediment extraction described in the submitted thesis seem suitable for toxicity tests with *D. magna*, *S. alba* and also in SOS-chromotest, as chemical substances used in preliminary treatment of the samples are not toxic for these testing organisms (proved by behaviour of these organisms in controls with solvents without the testing substance).

3) In sediment evaluation, it is necessary to use several methods of their extraction (both aqueous tincture and DCM extracts, or some other combination of polar and non-polar solvents), as substances solvable in water (polar) primarily discharge in aqueous tinctures; they can have other effects than macerated non-polar substances. Although aqueous tincture does simulate real exposition of water organisms towards substances contained in the sediment, it proved not to be suitable for SOS-chromotest. This is so due to the known fact that most substances damaging DNA are of lipophilic character; they are therefore extractable with non-polar solvents more easily.

The authors would like to thank for the financial grant from the Ministry of Education and Sports of the Czech Republic No. MSM 6007665809, CENAKVA No. CZ.1.05/2.1.00/01.0024 and GAJU No. 047/2010/Z, grant of the National Agricultural Research Agency NAZV QH82117 and the grant from the Ministry of Environment of CR No. SP/2e/229/07.

CHAPTER 4

TOXICITY AND GENOTOXICITY ASSESSMENT OF SEDIMENT FROM SELECTED SMALL STREAMS IN THE CZECH REPUBLIC – PILOT STUDY OF LESS AFFECTED SITES

Beránková, P., Kolářová, J., Máchová, J., Randák, T., 2010. Toxicity and genotoxicity assessment of sediment from selected small streams in the Czech Republic – pilot study of less affected sites. Bulletin RIFCH Vodňany 46 (1), 5–11. (in Czech)

There is a translation of the article. The full text in original version in its original form see Appendix No. 2.

TOXICITY AND GENOTOXICITY ASSESSMENT OF SEDIMENT FROM SELECTED SMALL STREAMS IN THE CZECH REPUBLIC – PILOT STUDY OF LESS AFFECTED SITES

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INTRODUCTION

Long-term monitoring is an inherent part of observation of changes in quality of the environment. From the point of view of long-term monitoring of changes in quality in water ecosystems, mainly bottom sediments appear to be a suitable template; they have a high potential for accumulation of non-polar, persistent and toxic substances and are highly influenced by human activities that can disrupt the natural condition of water (Heiniger et al., 2005).

A wide range of studies is dedicated to the long-term monitoring of sediment quality. Many of them are devoted to monitoring of changes in sediments in polluted locations with no comparison to unpolluted locations (Bertrand-Krajewski et al., 2006; Cachot et al., 2006; den Basten et al., 2003; Heiniger et al., 2005). Only few studies includes also sediments from unpolluted , i.e. reference / background locations (Aouadene et al., 2008; Frouin et al., 2007). On the other hand, there is a great variability in composition (e.g. amount of organic carbon, clay, minerals) and physical-chemical parameters of sediments (e.g. ion exchange capacity, pH, etc.) which have to be considered carefully while comparing samples from various locations. Sediments of reference (non-contaminated) location should therefore be compared always only with such sediments from polluted locations which have similar geochemical characteristics.

Upper parts of river courses around which there is no industrial operation and surrounding areas are not subject to intense agricultural activities are suitable as background localities. Such sections often feature fish species very sensitive with respect to water quality, such as brown trout (*Salmo trutta*) and grayling (*Thymallus thymallus*) (Armstrong et al., 2003).

This paper is the first step towards monitoring selected rivers in the Czech Republic in terms of their condition and quality changes. The goal of the paper was to select potential suitable background locations, sediment sampling and sediment sample processing from these locations and performing tests for toxicity and genotoxicity. In the following years, sediments will be sampled from the central and lower parts of courses of selected rivers in the Czech Republic in parallel with repetitive samplings from background locations. Extension of this study will lead to mapping of the condition of sediments in river courses in the Czech Republic and their variability over time. With regard to different physical-chemical characteristics of sediments, locations with similar sediment characteristics will be compared.

MATERIAL AND METHODOLOGY

Sampling locations – river sediments were sampled in autumn of 207 from three rivers in the Czech Republic – from the Ploučnice river (above and under the city of Cvikov), the Mže river (above and

under the city of Tachov) and the Blanice river (above and under the city of Prachatice). A waste water treatment plants are located in all of the three cities and they all treat municipal waste water.

Sampling and sample processing – sediments were collected using the Eckmann-Bridge dredge. At each location, approx. 2 kg of water sample were collected. The samples were packed, individually labelled and stored in an ice box (4 °C) until their processing in a laboratory. The samples were dried up at the ambient temperature of 21 °C. Consequently, they were mechanically homogenized and sucked through a sieve with 2 mm mesh diameter.

An organic extract was prepared for the genotoxicity tests using the Soxhlet extractor. Dichloromethane (DCM) was the extraction agent. Fifty grams of dry sediments was extracted 250 ml of DCM. The extract was thickened to approx. 5 ml and consequently remade into dimethyl sulfoxide (DMSO).

An aqueous tincture was prepared for the toxicity test by shaking 100 g of dry sediment with 1000 ml of standard water prepared according to the norm ISO 9001 for the period of 24 hours. The resulting aqueous tincture was filtered through a paper filter with 5 μm pores. The resulting filtrate was used in the toxicity tests.

Chemical analysis – the chemical analysis was carried out by the Department of Food Chemistry and Analysis of the Institute of Chemical Technology in Prague. Indicator congener polychlorinated biphenyls (PCB 28, 52, 101, 118, 138, 153 a 180) and selected organochlorinated pesticides (HCB, OCS and DDT derivatives) (Hajšlová et al., 1995).

TOXICITY TEST

Acute immobilization test on water crustaceans (water fleas such as *Daphnia magna*) – toxicity of samples was assessed using the toxicity test on the water crustacean *Daphnia magna*. The test was carried out according to the standard OECD 202 (1996).

Test of inhibition of growth of roots of white mustard (*Sinapis alba*) – toxicity was tested on seeds of *Sinapis alba* in early stages of germination according to a methodical instruction of the Ministry of Environment from 2007. Inhibition of seed germination and growth of primary root was observed.

GENOTOXICITY TEST

SOS-chromotest – SOS-chromotest (Quillardet a Hofnung, 1985; Quillardet a Hofnung, 1993) is a fast colorimetric test based on monitoring changes in enzymatic activity caused by incubation of the testing bacterial strain in a medium with added testing sample. This test uses the bacterial strain Escherichia coli K12 PQ37. The test was carried out according to methodology described in the work of Xu et al. (1989). For each of tested concentrations, an induction factor (IF) was calculated. If the IF value exceeds 1.5, the tested sample will be considered significantly genotoxic.

Test for chromosomal aberration in *Vicia faba* and test for micronuclei in *Vicia faba* – these are short-term genotoxicity tests in a plant system (Kihlman, 1975; Kanaya, 1994). Seeds of the plant *Vicia faba* are used for the test (number of chromosomes in the cell nucleus = 12). The experiments are performed on meristematic cells of germinated primary root. After the application of the testing sample, the seeds are cultivated in the dark in temperature 21 °C for 72 hours. After cultivation, a microscopic specimen is made of the root tops in which the chromosomal aberrations and occurrence of micronuclei are assessed.

RESULTS

CHEMICAL ANALYSIS

Results of the chemical analysis of the samples are shown in Table No. 1. Concentration of assessed substances is in $\mu g.kg^{-1}$.

TOXICITY TEST

Acute immobilization test on a water crustacean (*Daphnia magna*) – results of the acute immobilization test are shown in Table No. 2. Aqueous tincture with concentration of 100 $g.l^{-1}$ was tested.

Test of the Sinapis alba root growth inhibition – results of the test of the *Sinapis alba* root growth inhibition are shown in Table No. 2 together with the results of the acute immobilization test on *Daphnia magna*. Aqueous tincture with concentration of 100 $g.l^{-1}$ was tested.

GENOTOXICITY TEST

SOS chromotest – induction factors found in individual samples were not statistically significantly different from the results of the negative control (ANOVA, $F = 1.297$; $DF = 6$; $p = 0.269$). Organic extracts with concentrations 0.15; 0.075; 0.037 a 0.018 $g.l^{-1}$ was tested.

Test for chromosomal aberrations in *Vicia faba* and test for micronuclei in *Vicia faba* – the same as in the SOS-chromotest, no significant statistic difference was observed in the induction of chromosomal aberrations in *Vicia faba* in comparison to negative control (for chromosomal aberrations: ANOVA, $F = 1.325$; $DF = 6$; $p = 0.199$). Occurrence of micronuclei was not detected in monitored cells. Aqueous tincture with concentration of 100 $g.l^{-1}$ was tested.

ARGUMENTATION

Chemical analysis confirmed a slight increase in river pollution in water courses of the studied cities. This increase was recorded in units, maximum tens of $\mu g.kg^{-1}$ (max. increase sum PCB under Cvikov 61.7 $\mu g.kg^{-1}$). Influence of these residential areas is therefore minimal due to their size (Prachatice 12000 inhabitants, Tachov 12500 inhabitants, Cvikov 4500 inhabitants) and due to use of waste water treatment plants. There are no industrial plants in these areas that would significantly influence the quality of water ecosystems.

Low concentration of pollutants detected in the sediments is apparently given by the absence of industrial plants and major human residential areas in the river basins of upper parts of the selected rivers. In their work (2007), Frouin et al. state concentration of PAHs of 129 $ng.g^{-1}$ in the reference sediment and concentration of 22550 $ng.g^{-1}$ in the sediment polluted by smut from high furnaces. In sediments collected from the system of St. Lawrence River in Canada (Côté et al., 1998), the concentration of PCB was under the detection limit in most cases (0.10 $\mu g.g^{-1}$) the highest concentration measured was 1.90 $\mu g.g^{-1}$. Also the low IF of the SOS-chromotest performed by the authors corresponded to the low pollution. In the acute immobilization test with water crustaceans (*Daphnia magna*), a strong toxic effect was observed in the sample from the location above Tachov (100% inhibition after only 24 hours

of exposition). The strong toxic effect was also observed in the sample from the location above Cvikov; there was a 100% inhibition after 48 hours of exposition). In the sample from the location under Cvikov, a 50% inhibition was observed after 48 hours of exposition. In other samples, very low or zero amount of inhibition of *daphnia* mobility (*Daphnia magna*). Higher toxicity of samples collected above the selected cities may be caused by presence of non-detected substances, e.g. substances from runoffs from fields. Sediments taken in Brazil in the lower part of the river course showed increased toxicity in the test with *Daphnia magna* in comparison with the samples from the upper part of the river course (Mitteregger Junior et al., 2006).

No negative influence of aqueous tinctures of the samples was observed in the test of the *Sinapis alba* root growth inhibition. On the contrary, a slight stimulation of the primary root growth was observed, possibly caused by increased concentration of nutrients in the tinctures in comparison with negative reference (ISO water).

The genotoxicity tests did not prove any significant genotoxic effect of the tested samples, in any of the tested concentrations in any of the tested samples. This response complies with the results of the chemical analysis and with the presumption of low pollution of upper river courses in foothill areas.

Low pollution of the selected locations is proved by their placement into the trout belt. Salmonlike fish are very sensitive towards water pollution by organic and toxic substances and also towards a sufficient oxygen concentration.

However, available literature offers little data on monitoring of unpolluted locations which could be considered reference locations in further assessments of changes in environment quality.

Selected river courses and profiles are suitable background locations for further monitoring of condition of rivers in the Czech Republic due to results of selected ecotoxicological tests. Nevertheless, it is necessary to consider the concrete situation (see the note in the introduction) and to compare the locations in a suitable way in order to retrieve information on actual differences – mainly quantitative differences between variously polluted sections in lower river courses. Concordance of results of the test used proves the hypothesis on applicability of their placement into the battery of ecotoxicological tests designed for this monitoring.

SUMMARY

In water environment, sediments serve as a reservoir of toxic substances that can endanger health and lives of water organisms. With regards to their character (small firm particles on the river bottom) and relative stability over time (in comparison with the flowing water), the sediments appear to be a suitable template for monitoring of pollution of water ecosystems. This paper focused on the initial stage of monitoring of condition of sediments in the river system of the Czech Republic. The sediments were taken from three rivers – the Ploučnice river (above and under the city of Cvikov), the Mže river (above and under the city of Tachov) and the Blatnice river (above and under the city of Prachatice) in autumn 2007. Organic extract and aqueous tincture were subject to tests. Five tests were used in order to assess the sediment pollution: 3 genotoxicity tests (SOS-chromotest, test for chromosomal aberrations in *Vicia faba* and test for occurrence of micronuclei in *Vicia faba*) and 2 toxicity tests (OECD 202 acute immobilization test of water crustaceans *Daphnia magna* and test of *Sinapis alba* root growth inhibition. Genotoxic potential of the tested samples was not statistically significantly different from the negative control. The toxicity tests shown a similar result (except for one of the locations above Tachov and above Cvikov, where significant mobility inhibitions of *D. magna* were observed). Selected locations proved to be applicable in the tests we selected as chosen background locations for further assessment of sediment pollution of small water courses in the Czech Republic.

ACKNOWLEDGEMENTS

This paper was elaborated with support of research goal No. MSM6007665809, CENAKVA No. CZ.1.05/2.1.00/01.0024 and GAJU No. 047/2010/Z.and of a grant from the Ministry of Environment of the Czech Republic No. SP/2e7/229/07.

CHAPTER 5

INDUCTION OF VITELLOGENIN AND GONADAL IMPAIRMENT IN CHUB (*Leuciscus Cephalus* L.) AFTER EXPOSURE TO 17β-ESTRADIOL AND TESTOSTERONE

Žlábek, V., Beránková, P., Randák, T., Kolářová, J., Svobodová, Z., Kroupová, H., 2009. Induction of vitellogenin and gonadal impairment in chub (*Leuciscus cephalus* L.) after exposure to 17β-estradiol and testosterone. Neuroendocrinology Letters 30 (Suppl. 1), 225–229.

Neuroendocrinology Letters *Volume 30 Suppl. 1 2009*

ORIGINAL ARTICLE

Induction of vitellogenin and gonadal impairment in chub (*Leuciscus cephalus L.*) after exposure to 17β-estradiol and testosterone

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Submitted: 2009-07-19 Acteptted: 2009-09-25 Published online: 2009-10-15

Key words: **chub; fish; EDCs; gonads; ELISA**

Neuroendocrinol Lett 2009; **30**(Suppl 1): 225–229 **PMID:** 20027175 NEL300709A38

Abstract

OBJECTIVES: A controlled laboratory study was carried out to quantify vitellogenin (VTG) concentrations in a common cyprinid freshwater fish, the chub (*Leuciscus cephalus* L.), exposed to steroid hormones.
DESIGN: The effect of 17β-estradiol, testosterone and testosterone-estradiol mixture was investigated on vitellogenin induction. Gonad status was also determined.
RESULTS: Oral exposure to estradiol and a testosterone-estradiol mixture increased ($p < 0.01$) blood plasma concentrations of VTG in blood plasma of both sexes. The testosterone-estradiol mixture had a negative effect on the investigated chub gonads. The effects were signified by histological changes when compared to control fish.
CONCLUSION: Our results showed a significant VTG increase in blood plasma of both sexes, indicating that vitellogenic response in the chub is sensitive to steroid hormones.

INTRODUCTION

A number of studies have shown that some populations of freshwater fish are being exposed to hormone-like chemicals resulting in disruption of the reproductive physiology of the organism. Anthropogenic chemicals, including synthetic and natural hormones, can disrupt the endocrine systems of wildlife species (Tyler *et al.* 1998; Kime *et al.* 1999; Arukwe, 2001). In fish, hormones play an essential role in gonad development, and display unique seasonal cycles. A study to investigate reproductive parameter responses after exposure to steroid hormones would yield valuable knowledge. Effects of particular chemical substances on aquatic organisms often overlap and it's difficult to understand consequential synergistic and antagonistic effects. Biochemical markers and haematological parameters are valuable indicators in such cases (Blahova *et al.* 2008; Modra *et al.* 2008).

Unfortunately, field study results are often misleading to interpret. Limited data is available reporting on the effect of environmental endocrine disrupting chemicals (EDCs) using chub (*Leuciscus cephalus*) as a model organism. Chub is a fish routinely used to assess the quality of surface water (Agtas *et al.* 2007; Christoforidis *et al.* 2008; Hajslova *et al.* 2007; Krcca *et al.* 2007; Stachel *et*

Abbreviations & units

eDCs	– endocrine Disrupting Chemicals
E_2	– 17β-estradiol
T	– testosterone
VTG	– vitellogenin
GSI	– gonadosomatic index
HSI	– hepatosomatic index
ELISA	– enzyme-linked immunosorbent assay
MJ	– mega joule

al. 2007). However, there is a lack of fundamental data from the controlled laboratory studies for evaluation of field biochemical monitoring of surface water pollution. Presently the effect of steroid hormones exposure on vitellogenin induction in chub is limited to only two studies (Flammarion *et al.* 2000; Zlabek *et al.* 2009).

To date, there is missing data regarding the effects of endocrine disruptors (EDCs) on vitellogenin synthesis and gonad development in chub, which is essential for following successful reproduction processes. First, the effect of chemicals must be studied under controlled conditions. Laboratory studies of individual chemical effects on fish are essential to understand its impact on aquatic animals living in the natural environment. Fish exposure to chemicals, which are considered to be the standards for estrogenic and androgenic modulation in laboratory conditions, deliver valuable data about the effect of a single chemical and their mixtures on the physiological status of the fish.

In the present study, estrogenic (17β-estradiol; E_2) and androgenic chemical (testosterone; T) were chosen as test compounds. In female teleosts, 17β-estradiol and testosterone are the dominant sex steroids in plasma during oogenesis, while T and 11-ketotestosterone are elevated during spermatogenesis in males (Skjæraasen *et al.* 2004). The sex steroid 17β-estradiol activates the production of vitellogenin (VTG) by the liver. This protein is then incorporated into maturing oocytes (Kime *et al.* 1999). Vitellogenin synthesis normally takes place only in mature females, but it is possible to induce it in males by exposing them to estrogens or estrogenically active chemicals. Since estrogen receptors are present in the liver of male fish, non-physiological induction of vitellogenesis caused by exogenous steroids may occur. Therefore, vitellogenin induction in males has been shown to be a specific biomarker for exposure to endocrine disrupting chemicals (Örn *et al.* 2003; Holbech *et al.* 2006). Both endogenous and synthetic estrogens are suspected to play a key role in the field observations of elevated vitellogenin concentrations and intersex in fish (Jobling & Tyler 2003). The major part of the estrogens detected in fresh water systems originates from women who excrete conjugated estrogens that are deconjugated in sewage treatment plants and from livestock operation (Holbech *et al.* 2006). Finally, the structural integrity of gonads can be altered by xenobiotics. Abnormal gonadal development, such as delayed maturation, high levels of atresia or intersexuality may also be detected by histological analysis. Such parameters are frequently investigated in fish exposed to anthropogenic chemicals or living in contaminated environments (Jobling & Tyler, 2003; Bateman *et al.* 2004; Mikula *et al.* 2006).

Initially we investigated whether chub (*Leuciscus cephalus*) is suitable to indicate the presence of endocrine-disrupting chemicals. The aim of the present study was to evaluate the impact of estrogenic and androgenic model substances on vitellogenin induction and gonad status in chub. Additionally, a mixture of steroid hormones with different modes of action was used to simulate effects of pollutant "cocktails" present in the aquatic environment.

Table 1. The main characteristics of sampled fish (n = number of fish, mean ± standard deviation)

Group	Sex	n	Total Length (mm)	Weight (g)
Control	♀	14	226 ± 68.6	140 ± 168.5
	♂	8	213 ± 41.0	101 ± 72.8
T	♀	11	220 ± 36.4	106 ± 57.4
	♂	11	244 ± 41.2	146 ± 74.7
E_2	♀	10	202 ± 24.8	88 ± 30.1
	♂	11	211 ± 23.9	95 ± 31.3
T+E_2	♀	14	223 ± 50.3	115 ± 97.1
	♂	8	205 ± 36.1	83 ± 44.5

MATERIAL AND METHODS

Compound and treatment preparation. The test chemicals, 17β-estradiol (E_2) and testosterone (T) were purchased from Sigma Aldrich Chemical Company, and were dissolved in 99.5% ethanol. The ethanol solutions were mixed with the feed resulting in an E_2 concentration 20 mg kg^{-1} feed, T concentration (0.1 g kg^{-1} feed) and E_2+T mixture (20 mg E_2 + 0.1 g T kg^{-1} feed) diets. The control diet was treated with ethanol only. After thorough mixing, the ethanol was evaporated from the feed. An Ecolife 15 (Biomar, 3 mm, 45 % protein, 16% fat, energy 20.8 MJ kg^{-1}) fish feed was used. The dose was calculated at the end of exposure, based on total weight of fish from each group and amount of feed consumed. The estimated dose of 17β-estradiol was 9.1 mg kg^{-1} fish, the testosterone dose was 39.3 mg kg^{-1} fish and the mixture dose was 8.9 mg kg^{-1} and 44.6 mg kg^{-1} of 17β-estradiol and testosterone, respectively.

Animals and study design. The fish from pond aquaculture were transferred to experimental 200-liters aquariums for a 7 day adaptation period. The main characteristics of the 3 years old fish are shown in ***Table 1***. The fish were exposed to the chemicals through *per-oral* route for 30 days; under semi-static water conditions. Aerated tap water was used daily for the renewal of the test volume. The water temperature was 18-21°C with

a pH range of 6.9-7.8 throughout the test procedure. Experimental diets were fed *ad libitum* three times a day. All exposures, including controls, were performed in duplicates with 11 fish per aquarium.

After 30 days of exposure, the fish were collected for VTG analyses from each duplicate aquarium. Blood samples were collected from the caudal vein into heparinized tubes. Samples were centrifuged and blood plasma samples were frozen in liquid nitrogen and stored at -80°C until analysis. The length and body weight were recorded. The gonads and livers were dissected from the fish, and the gonadosomatic index (GSI) as well as the hepatosomatic index (HSI) were calculated as described by Hecker *et al.* (2002):

GSI (%) = [gonad weight / body weight (without viscera) × 100]
HSI (%) = [liver weight / body weight (without viscera) × 100]

The measurements of VTG in the blood plasma samples were performed using a pre-coated ELISA kit (Biosense laboratories® Norway). The procedure was implemented in compliance with the manufacturer's instructions. The use of carp vitellogenin ELISA for determination of vitellogenin in chub was validated by Flammarion *et al.* (2000). The absorbance was measured using a SLT Spectra (A5082) instrument set at wavelength 492 nm. Twenty individuals from each group were sampled for histological analysis. The gonads were fixed in phosphate-buffered formalin and embedded in paraffin. The sex was confirmed by light microscopic evaluation of hematoxylin–eosin stained sections. Gonads with the presence of both previtellogenic oocytes and testicular tissue were classified as intersex.

Statistical analysis. Measured parameters were analysed using Kruskal-Wallis test. All analyses were performed using Statistica for Windows 7.1 (Statsoft Inc., 2005), with a significance of $p < 0.01$.

RESULTS

VTG in Females. The VTG concentrations given in ng ml^{-1} blood plasma of females are shown in ***Fig.1***. Significant vitellogenin induction ($p<0.01$) was registered in the dietary group exposed to E_2 (555 ± 35.7 μg ml^{-1}) and the E_2+T (511 ± 64.0 μg ml^{-1}) mixture group compared to the control group (468 ± 354.1 ng ml^{-1}). No significant differences were found between the control and the experimental group exposed to testosterone; however, T group showed a higher vitellogenin concentration (1135 ± 553.8 ng ml^{-1}).

VTG in Males. Exposure of males to only testosterone resulted in increased induction of VTG (72.1 ± 34.8 ng ml^{-1}) compared to the control group (13.2 ± 4.4 ng ml^{-1}), but was not significant. A highly significant vitellogenin induction ($p<0.01$) was registered in the group exposed to E_2 (487 ± 80.2 μg ml^{-1}) and the E_2+T (446 ± 131.2 μg ml^{-1}) mixture group. Blood plasma concentrations of VTG after exposure to estradiol and a testosterone-estradiol mixture surprisingly reached the levels of VTG in exposed females**.**

GSI and HIS. The gonadosomatic index and the hepatosomatic index are presented in ***Fig. 3***. The highest GSI of males was found in the control group. The males from all exposed groups showed lower GSI although not significantly different from the control group. Females did not show any differences in GSI. The lowest HSI was found in both sexes from the control group. The fish from all exposure groups expressed higher HIS although not significantly different from the control.

Histology. Early vitellogenic oocytes were characteristic for ovaries of females from the control group (***Fig. 4 A***). Oocyte degeneration was observed in testosterone treated females (***Fig. 4B***). Perinucleolar oocytes predominated in ovaries of females after exposure to 17β-

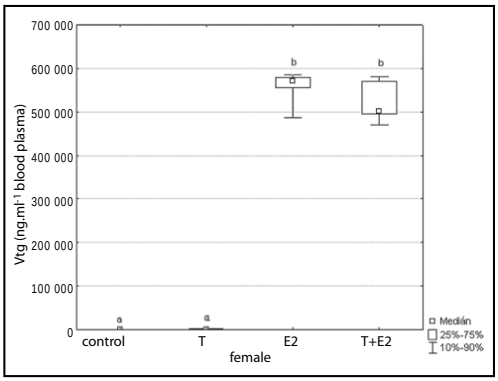

Figure 1. The blood plasma concentrations of vitellogenin (VTG) in female chub after the exposure to testosterone (T), 17β-estradiol (E_2) and their mixture T+E_2. Different superscript letters indicate significant differences (Kruskal-Wallis, $p < 0.01$) between groups.

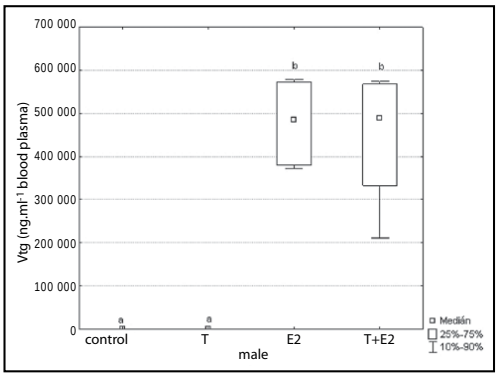

Figure 2. The blood plasma concentrations of vitellogenin (VTG) in male chub after the exposure to testosterone (T), 17β-estradiol (E_2) and their mixture T+E_2. Different superscript letters indicate significant differences (Kruskal-Wallis, $p < 0.01$) between groups.

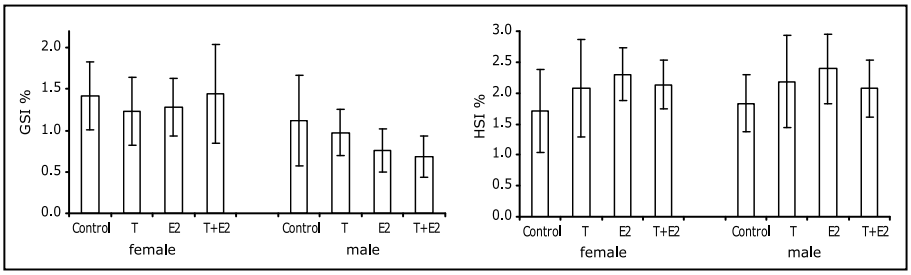

Figure 3. The gonadosomatic index (GSI) and the hepatosomatic index (HSI) of chub after the exposure to steroid hormones.

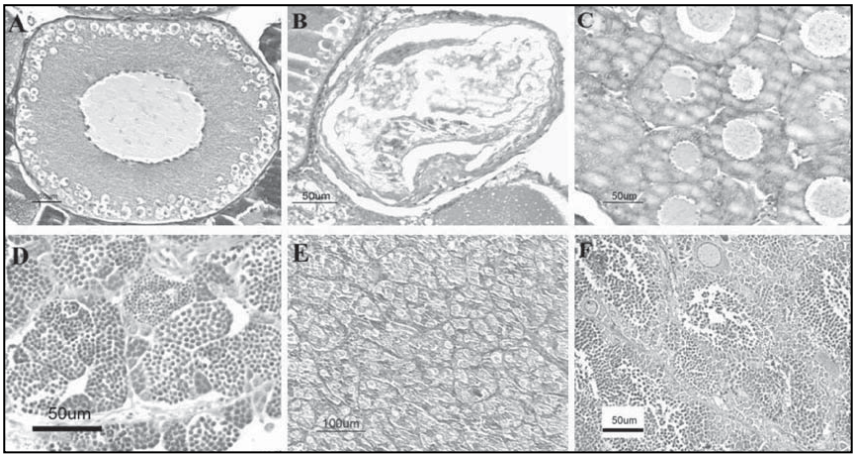

Figure 4. Light micrograph showing: (A) an early vitellogenic oocyte in ovary of female chub from the control group, (B) a degenerating oocyte in female chub after exposure to testosterone, (C) perinucleolar oocytes in female chub after exposure to 17β-estradiol (D) testes of male chub from the control group, containing spermatocytes and spermatids. (E) Regressed testes containing only spermatogonia in male chub after the exposure to 17β-estradiol, (F) intersex in chub after the exposure to T+ E_2 mixture. Perinucleolar oocytes are dispersed in testicular tissue. H&E stain.

estradiol (***Fig. 4C***). Testes of male chub from the control group contained both spermatocytes and spermatids. Testicular tissue of males exposed to E_2 was dominated by the presence of spermatogonia, typical for the pre-spermatogenic stage of testes development (***Fig. 4 E***). One intersex individual (***Fig. 4 F***) was found in T+E_2 exposure group. No evidence of ovotestes was present in either the E_2, T or the control group.

DISCUSSION

Published data on the effects of steroid hormones on reproductive functions are sometimes contradictory. One of the most sensitive responses for estrogens in fish is the induction of vitellogenesis (Arukwe & Goksoyr, 2003). An immunological assay was used to identify vitellogenin induction in chub to provide evidence for the endocrine disruption in our study.

Our results showed a significant VTG increase in blood plasma of both sexes, indicating that vitellogenic response in the chub is sensitive to estrogens. Significant induction of vitellogenin was also found after exposure to combination of estradiol and testosterone. Females and males showed 1000 and 10000 fold increase, respectively. Registered VTG concentrations in blood plasma of males from the control group are comparable with the values found by Flammarion *et al.* (2000) in control male chubs. On the other hand, concentrations of VTG in females are more than 10 times higher than expected in mature female fish. Reported plasma VTG concentrations in male and female chub reached more than 1 mg ml^{-1} in exposed groups (Flammarion *et al.* 2000), while exposed fish in our study reached slightly lower concentrations close to 0.5 mg ml^{-1}. This might be due to different exposure methods. Even higher peroral dose in our experiment resulted in lower VTG induction when compared to intraperitoneal application of 2mg E_2 kg^{-1} (Flammarion *et al.* 2000). A similar result was also found in our previous study with juvenile chub (Zlabek *et al.* 2009). Exposure of fish only

to testosterone resulted in the increased induction of VTG compared to the control group, but was not significant. Considering the above fact, the effect observed in the group, which was exposed to testosterone, may be explained by a possible conversion of testosterone into estrogens. Iwamatsu *et al.* (2006) reported that aromatizable testosterone in high concentrations may induce a significant increase in E_2 content in embryos of medaka (*Oryzias latipes*) and also paradoxical sex reversal. In general, it is believed that the paradoxical effect of androgens on sex differentiation results from the conversion of androgens into estrogens (Örn *et al.* 2003). Contrary to our previous study (Zlabek *et al.* 2009) no significant effect of testosterone was found in the T + E2 exposed group.

The GSI in fish from the control group were somewhat lower than expected of mature fish (Flammarion *et al.* 2000), but comparable with GSI reported for wild roach (Hecker *et al.* 2002) in the autumn period. Lower GSI in males from all exposed groups reflects a negative effect of the tested hormones on normal development of gonads.

The developmental stages of gonads in both males and females from the control group were typical for maturing fish. Histological analyses of the testes from the males of the control group confirmed active spermatogenesis. On the contrary, exposure of males to E_2 resulted in regressed testes containing only spermatogonia. Similar effects were also registered in males exposed to testosterone and T+ E_2 mixture. Additionally, one intersex individual was found in the T+E_2 exposure group. Presence of intersex after exposure to steroid hormones is surprising for an already differentiated gonad; however, this was a single finding**.** Lower maturation stages of ovaries were found in females exposed to E_2 compared to the control fish. Absence of fully matured gonads in fish from hormone exposed groups is a sign of disruption of pituitary-gonadal axis in both sexes. Moreover, high incidence of degenerating oocytes reflects developmental disorder in females after exposure to testosterone.

CONCLUSIONS

In the present study, steroid hormones were used as standards to simulate the effect of endocrine disruptors on fish organism under controlled conditions. Positive vitellogenin induction in the E_2 and the T+E_2 exposed groups is indicating that vitellogenic response in the chub is sensitive to steroid hormone exposure. Negative histological changes of fish gonads in exposed groups proved the sensitivity of chub to chemicals with endocrine disrupting properties. Endocrine-disrupting chemicals may adversely affect the hormone systems and thereby the homeostasis, development and reproduction of exposed organisms. Such observations provide important insight into potential impacts from endocrine disruptors, and can provide useful monitoring tools.

Acknowledgements

This study was supported by the USB RIFCH no. MSM6007665809 and the Czech Science Foundation 525/06/P234.

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CHAPTER 6

GENERAL DISCUSSION • ENGLISH SUMMARY • CZECH SUMMARY • ACKNOWLEDGEMENTS • LIST OF PUBLICATIONS • TRAINING AND SUPERVISION PLAN DURING STUDY • CURRICULUM VITAE

GENERAL DISCUSSION

Aquatic ecosystems, a part of the Earth that is essential to man, are still used for waste disposal for all liquid waste produced by man, despite all development and level of our civilization. Pollution is originated in various sources and it brings about a wide range of effects both for man and for the environment as a whole.

The largest surface water discharges of toxic material can be attributed to the chemical, food, and metal smelting industries. These three categories account for almost 73% of the total toxic discharge for 2002 in United States. The top seven industrial categories (i.e., including petroleum refining, pulp and paper, electric, gas, sanitary services, and electronic equipment manufacture) account for almost 90% of the total 2002 discharges (Chen and White, 2004).

Thousand of synthetic compounds are currently registered for use in industry, commerce, agriculture and the home, and thousand of tones of these are produced annually in the world. Portions of these chemicals are released either deliberately or unintentionally into the atmosphere, land, rivers, lakes and seas, and numerous xenobiotics are ultimately found in the surface waters and sediments. Carcinogens are also released into the environment and ultimately migrate into surface waters and accumulate in sediments. Xenobiotics dissolved or suspended in water or sediments enter through the gills, the skin, or the gastrointestinal tract in fish or epidermal cells or root hairs in plants inhabiting chemically polluted aquatic environments. Pollack et al. (2003) indicated that environmentally persistent chemicals pose not only an ecological threat but a health hazard inducing cancer in humans (Ohe et al., 2004).

Houk (1992) documented the genotoxic activities of a wide range of industrial wastes and effluents. Industries that were shown to discharge effluents that elicit a positive response on a genotoxicity bioassay were divided into several major categories: chemical and allied products, pulp and paper manufacturing, defence and munitions, petroleum refining, and primary metal refining and founding.

Moreover, several studies on specific mutagenic chemicals have shown contamination patterns (e.g., PAH rations, PAH alkylation) that are clearly associated with nearby industries and/or industrial processes (Baumann and Harshbarger, 1995; Marvin et al., 1993; Marvin et al., 1999; Prahl and Carpenter, 1983).

In their work, Kennet et al. (1999) assessed toxic effects of sediments collected rom the Bay des Anglais on the St. Lawrence estuary. Waste water from grain storage facility, aluminium smelter, pulp and paper mill and municipal sewage is all discharged in this estuary. Sediments were collected from three locations – ca 1000 (Site 1), 3000 (Site 2) and 6000 meters (Site 3) from these sources of pollution. The highest degree of pollution was measured in sediment collected closest to the source of pollution. With increasing distance from the source, the degree of pollution was decreasing. Although gradients were observed, differences were attributed to natural variability in sediment particle size and organics carbon disruption at these sites. The range of heavy metal concentrations measured at all three sites was not higher than values found at other locations on the St. Lawrence estuary far removed from any point source of contamination (Gobeil and Cossa, 1993; Gobeil et al., 1987; Gobeil et al., 1995). A similar tendency of pollution decrease in the direction from its source was also observed in case of detected PCBs, PAHs and PCDFs. The toxicity of the contaminants in these sediments was directly reflected in the micro scale bioassay (Kenneth et al., 1999).

Genotoxins in paper mill effluents are believed to originate from chlorination of residual lignin (Kringstad et al., 1981; Rannug et al., 1981) kraft pulping results in a higher mutagenic activity than sulphite pulping and the mutagenicity increased with increasing Kappa number (Møller et al., 1986). Relatively little is known about the genotoxicity of effluents from straw pulping in contrast to wood pulping. However, this study (Møller et al., 1986) revealed that genotoxins are produced in the pulping process as well as in the chlorine dioxine bleaching process, indicating that the genotoxic activity may

to some extent by non chlorinated compounds. The presence of genotoxic substances in the chlorine dioxide bleached paper pulp effluent, as found in this study, is noticeable, since substitution of chlorine with chlorine dioxide have been shown to reduce the mutagenicity (Rannug et al., 1981; Møller et al., 1986; Ander et al., 1977), and Ames mutgenic activity has not be found in an unconcentrated chlorine dioxide hardwood kraft pulp bleaching effluent (Eriksson et al., 1979; Wrisberg and van der Gaag, 1992).

Although the study by White et al. (1996) demonstrated that the genotoxicity loading from a municipal wastewater treatment facility is far greater than of the majority of industrial facilities, one must acknowledge that municipal wastewaters are complex mixtures of wastewaters from a variety of source. For example, municipal wastewater from the Montreal Urban Community contains domestic wastes, commercial and institutional wastes, industrial wastes, groundwater infiltration, and source runoff. Moreover, some researches have suggested that the genotoxicity of municipal wastewaters is proportional to the industrial contribution (Rappaport et al., 1979; Meier and Bishop, 1985; Meier et al., 1987). However, attempts to demonstrate a statistical association between municipal wastewater genotoxicity and industrial contribution have been unsuccessful (Babish et al., 1983). In addition volumetric contribution of industrial facilities to municipal wastewater streams in large urban centers' with more than 200000 inhabitants is usually les than 25% (White and Rasmussen, 1998). Genotoxicity and pollution of some industrial wastewaters, domestic wastewaters constitute a greater genotoxic hazard to aquatic systems and their associated biota.

The PAH results also shoved an increasing concentration gradient from rural through residential to industrial land uses supporting the link with vehicles found by Maltby et al. (1995) and van Metre et al. (2000). However, results from this study show clearly that this is not always true and depends on the individual element (Beasley and Kneale, 2002). Naphthalene does appear to adhere to this general pattern; the highest concentration were recorded in stream sediments below industrial and motorway inflows. However, certain residential sub catchments posed a greater contamination risk than either the industrial or motorway source areas. These residential areas with residential areas with roadside parking accumulate higher concentration of total PAHs largely because of high concentrations of fluoranthene, phenanthrene and pyrene, which originate from crankcase oil drippings leaked onto the road surface (Latimer et al., 1990). Differences in stream characteristic have undoubtedly influenced the levels of contamination in study of Bleasley and Kneale (Beasley and Kneale, 2004) and one would presume the ecological quality. It is hypothesised that a silt laden, shallow, slow flowing stream would support a relatively depauperate macroinvertebrate community structure (Beasley and Kneale, 2004).

Waste and runoff water from the are of car battery production plant polluting the Klenice river (CR) bring mainly compounds of lead that has not been used until recently in car battery production. Concentration of lead measured in sediments under the waste water discharge amounted to 307 mg. kg^{-1}, under the runoff water inlet in the area even up to 647 $mg.kg^{-1}$. However, the content of lead in sediment collected from a control location ca 4 km further against the Klenice river flow from the waste water discharge from the premises of the car battery production plant slightly exceeded 7 $mg.kg^{-1}$ (Berankova et al., 2009). These waters are being highly polluted with lead during the production process, because car batteries contain up to 55% of lead. The content of lead detected in the samples of sediments collected from Klenice river (7.42–647 $mg.kg^{-1}$) is in comparison with the available data higher than in other rivers in the Czech Republic, e.g. the Tichá Orlice river reaches a range of lead content of 31.3 to 36.6 $mg.kg^{-1}$ (Svobodova et.al., 2004). A similar situation is also in other parts of the world. For example, Singh et al. (2005) measured in the sediments of river Ganges (India) a content of lead in the range of 6.27 to 68.7 $mg.kg^{-1}$, in the Po river (Italy) a range of 32 to 98.5 $mg.kg^{-1}$ (Farkas et al., 2007) and Cachot et al. (2006) measured in the Siene river (France) of 2 to 76.6 $mg.kg^{-1}$. Johnson (1998) found lead in suspended sediment to range around 40 $\mu g.kg^{-1}$ in rural streams and rivers but reached

150 to 350 $\mu g.kg^{-1}$ in urban industrial areas.

Lead is among the top 10 US EPA priority pollutants. Ferreira et al. (2004) describe the ability of lead to increase the occurrence of DNA damage. Principally it includes single strand breaks that could possibly initiate double strand breaks. This results in the inactivation or alternation of the repair mechanism. Lead bonds to mitochondrial membranes, penetrates into the mitochondrial matrix space, and is capable of uncoupling oxidative phosphorylation in brain cell mitochondria (Holtzman et al., 1987). The genotoxic potential of sediment samples collected from the Klenice river (CR) complies with this information on negative effects of lead on DNA (Berankova et al., 2009).

PAHs are substances with proven negative effect on DNA: Due to their impact on DNA, the DNA becomes damaged and some cases result even in occurrence of cancer proliferation. Liver tumors have also been induced in fish using a variety of known carcinogens, including two PAHs, dimethylbenzanthracene and benzo(a)pyrene (BaP) (Black, 1984; Couch and Harsberger, 1985; Metcalfe, 1989). Waterborne exposure to BaP produced liver tumors in guppy (*Poecilia reticulata*) (Hawkins et al., 1988). Similarly, dietary exposure and intraperitoneal injections of BaP produced liver tumors in rainbow trout (*Oncorhynchus mykiss*) (Hendricks et al., 1985). Both skin and liver tumors have also been inducted by exposure to extract from PAH-contaminated sediment. Extract from Buffalo river, NY, sediment painted on brown bullhead produced skin tumors at an incidence of 38% after two years. This same extract, as an additive in commercial trout food, produced both biliary and hepatic tumors when fed to brown bullhead. These lesions could not be distinguished from those examined in wild fish from PAH-contaminated locations (Baumann, 1998).

Although PAHs are known pro-mutagens, few investigations and contradictory results concerning plant sensitivity to PAH mutagenic effects have been obtained until now. This may be due to the activation of chemicals to nonmutagenic quinones by different enzyme systems (Higashi, 1988; Sandermann, 1988; Minissi et al., 1998).

It is not surprising that many researchers have attempted to relate measured mutagenicity with PAH contamination. All published studies with PAH concentration and genotoxicity data from 4 or more sites were scrutinized in an attempt to identify empirical relationship between sediment genotoxicity and PAH contamination (Chen and White, 2004). White et al. (1996) noted a highly significant relationship between SOS genotoxicity (+S9) and PAH concentration across nine sites in the St. Lawrence system.

Genotoxicity tests are commonly used for assessment of genotoxic effects of sediments and their extracts. The literature suggests a wide range of genotoxicity tests. Most common methods of classification of these tests is according to the organisms used in them (e.g. bacteria, mammalian cells, plants, etc.) and endpoints (mutation, DNA damage, cytogenetic abnormalities). Although many studies agreed that the *Salmonella* mutagenicity assay is the most versatile and well-validated tool for assessing the mutagenic hazards of complex environmental samples, several studies compared *Salmonella* results with the results of similar bacterial mutation and DNA damage tests such as the SOS Chromotest, the Mutatox® test, the *umu* test etc. Extensive comparisons between the SOS Chromotest and the *Salmonella* mutagenicity test based on pure compounds have shown a high degree of correspondence (Quillardet and Hofnung, 1993; White and Rasmussen, 1996); however, comparisons between the two tests for sediment analyses have yielded inconsistent and contradictory results. In their analyses of five sediments, Bombardier et al. (2001) noted good agreement between the SOS Chromotest (+S9) and the *Salmonella* fluctuation assay with TA98 and TA100 (+S9). However, the authors also noted that the SOS Chromotest appeared to be more sensitive and generated results that were more consistent with the reported contamination gradient. In a study of seven sites along the Welland river, Lan et al. (1991) used the *Salmonella* test (TA98 +S9) to verify a series of positive SOS Chromotest results. The results of the *Salmonella* test were entirely negative suggesting, according to the authors, that the SOS Chromotest is a more sensitivity assay. However, it should be noted that White et al. (White

et al., 1999) in their study of water and suspended sediments from the Providence river (RI), reached the opposite conclusion. They concluded that sensitivity to bacteriostatic effects resulted in reduced sensitivity of the SOS Chromotest and an inability to detect potent mutagenicity in selected samples (Chen and White, 2004).

Results of the work of Wristberg and Gaag (Wrisberg and van der Gaag, 1992), who used micronucelus assay with mussels and sister-chromatid exchange assay with fish to test genotoxicity of waste water from a wheat and rye paper pulp factory, show that aquatic *in vivo* tests are capable of detecting genotoxic substances from water with a sensitivity comparable of with or even higher than the commonly used Ames test (Wrisberg and van der Gaag, 1992). These test system utilize various aquatic species which have been exposed to a range of different waters: drinking water (Jaylet et al., 1987), waste waters (Das and Nanda, 1986), river water (Prein et al., 1978; Alik et al., 1980; Hooftman, 1981) and marine waters (Al-Sabti and Kurelec, 1985; Brunetti et al., 1988; Scarpato et al., 1990).

Therefore, aquatic *in vivo* genotoxicity tests are efficient system, which could prove to be important, complementary tools for future ecotoxicological evaluations of effluents.

Due to the different chemical and physical properties of mutagenic substances, their linkage and bioavailability in sediment differs greatly. As a consequence, the results of the detection of mutagenic effects in complex environmental mixtures like sediment are strongly influenced by the methods of sample preparation and extraction. Different ecotoxicological test and different extraction methods of tested matrix may show diametrally different results. The literature suggests several studies concerning impact of extraction methods on Ames test results (a *Salmonella* test) in which the authors used various solvents and their combinations (e.g. water/ether, methyl alcohol, toluene, dichlormethane and distilled water) (Suzuki et al., 1982; Vahl et al., 1997; Picer et al., 2001; Berankova et al., 2011). Authors of one of the studies published (Suzuki et al, 1982) performed tests with sediments from the Tama river (Japan) and concluded that polar extracts of these sediments showed higher genotoxic effect in the Ames test than the non-polar extracts (combination water/ether > ethyl acetate > methyl alcohol ~ n. hexan). On the other hand the study carried out with sediments from the German part of the River Elbe proves higher genotoxic effect in the Ames test in non-polar extracts (toluene) than in extracts into methyl alcohol and much higher than in aqueous tincture (Vahl et al., 1997).

Monitoring the changes of environment quality should also inherently include long-term monitoring. For its purpose, monitoring of changes in sediment contamination is very recommended in terms of aquatic ecosystems. In comparison with running water where sediments are more stable and less variable over time. At the same time, they have the ability to accumulate non-polar, persistent and toxic substances. These can be released anywhere in the future (e.g. during floods) and they can decrease the environment quality temporarily or even in a long-term perspective. Unfortunately, the available literature suggests relatively few studies concerning integrated monitoring of changes of quality in aquatic sediments (Bertrand-Krajewski et al., 2006; Cachot et al., 2006; den Basten et al., 2003; Heiniger et al., 2005). Even fewer studies include ALSO sediments from non-polluted (so called control) locations into their range of tested samples (Aouadene et al., 2008; Frouin et al., 2007; Berankova et al., 2010).

Developing an understanding of where and why contaminants accumulate is essential for making informed management decisions about uses of these coastal areas and for developing sound strategies for monitoring environmental change.

All rivers flowing through the area of the Czech Republic also spring there. Therefore, we are at the presumable beginning of pollution of aquatic ecosystems of Europe. With respect to that we should pay greater attention to their pollution and protection against it. We should also know in what condition we hand our water over to our neighbours and children and we should be careless about its protection.

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ENGLISH SUMMARY

Genotoxic potential of foreign substances in ecosystems of surface waters

Petra Beránková

Human activities have negative impact on our environment. Surface waters are not left out of that impact either. Every year, thousands of hectolitres of waste water is discharged into them in various degree of preliminary or ordinary treatment and contaminated by various combinations of substances. Many of those contaminants are harmless for the environment and living organisms within it, including man. However, due to their chemical structure and physical characteristics, many substances have the ability to damage or influence the quality of the environment and life of organisms within it.

One of very serious negative impacts of these substances is the genotoxic impact. Genotoxic impact is usually defined as the ability of substances to react with cell DNA and damage its structure or change its meaning. Due to impact of such substances (genotoxic factors), cell functions may become disrupted, the cell can die or cancer proliferation may start. If there is a DNA damage in somatic cells, "only" life of the given individual is influenced. If there is a DNA damage in sex cells, the damage occurred may get transferred onto the progeny. Genotoxic factors can therefore be responsible for fetus damage and development of congenital development defects in progeny.

With regards to these facts, monitoring of presence of substances with genotoxic effects is very important in our environment. As substances present in the environment may travel as far as onto our tables through food chains.

Surface waters, mainly running waters, are a system very variable over time. In terms of long-term monitoring of surface water quality, sediments appear to be a suitable matrix. They are a more stable matrix in comparison to water. In fact, they are tiny particles settled on bottoms of river courses. On the surface of these particles, hydrophobic substances get absorbed and therefore they are preserved and accumulated in the sediment. PAH, PCB, PCDD or PCDF also belong into this group of substances. Many of them have also genotoxic effects, among other things. Substances contained in sediments can be released back into the environment. This can happen through simple mechanical re-suspendation of sediment, through change of chemical characteristics of sediments (e.g. PH) or through bacteria, plants or benthic organisms.

Bio-availability of substances combined in sediments is one of the key factors determining the extent of negative effects of these substances in real environment. And precisely this is the problem we have encountered in case of testing sediment effects through their extraction. The literature describes a wide range of possibilities of extract preparation. Results of the study of influence of sediment extraction methods on selected ecotoxicological tests shows that this particular step is a key factor in determination of relevance of test results. The objective of this paper is to compare results of two toxicity tests (Acute immobilisation test with *Daphnia magna* and Test of Sinapis alba root growth inhibition) and one genotoxicity test (SOS-chromotest) depending on the extraction method used (aqueous tincture, extraction into dichlormethane (DCM) using sonication and extraction into DCM using Soxhlet extraction). The SOS-chromotest showed a significant difference between effects acquired using extraction into DCM in comparison with aqueous tincture. The test of *Sinapis alba* root growth inhibition has not shown any statistically relevant difference in effects of extracts acquired in various methods. The test of acute immobilisation of *D. magna* showed 100% inhibition effect in all extracts prepared. A similar tendency can also be found in expert literature. The study results show the need for testing of sediment effects using several types of extracts (including aqueous tincture).

Some substances do not act directly as mutagens, but they can impact DNA repair or signal transmission for triggering the repair indirectly. Lead is one of these substances. Sediments with high content of lead and its combinations are located mainly near discharges from facilities processing lead or using lead combination in their production process (e.g. car battery production). These locations include also the lower section of the Klenice river (CR) right before it flows into the Jizera river (CR). Extract of these sediments showed high genotoxic effect both in SOS chromotest and in WP2 test. In comparison to benthic systems from the background location of the Klenice river (CR) (ca 4 km above the place of inflow of waste water from the car battery production plant) a very high toxic impact on benthic invertebrate organisms was observed in the section polluted by lead. In the place of collection directly under the dicharge of runoff water from the premises, no benthic organisms were found.

Selection of a background location for the purpose of assessment of negative effects of sediment is a very difficult task. It is necessary to choose locations that are only polluted by human activities to a minimum extent (including air pollution). At the same time, it is desirable to find a location with similar characteristics. Sediments are very heterogenous and the differ mainly in grain composition, content of organic carbon and pH. Selection of a background location for long-term monitoring of sediment condition in the rivers of the Czech Republic was the third objective of the study. Locations in upper parts of river courses of Ploučnice, Mže and Blanice were selected. Samples were always collected above and below the town situated on the river. All sediments collected showed suitability for utilisation as a background location for the future monitoring. Their disadvantage may be that (in comparison to sediments of lower river courses) they have lower content of organic carbon and therefore a lower ability to combine pollutants in their volume.

Monitoring of pollution of sediments in terms of genotoxic effect should become an inherent part of monitoring of water ecosystem pollution.

CZECH SUMMARY

Genotoxický potenciál cizorodých látek v ekosystémech povrchových vod

Petra Beránková

Lidská činnost negativně ovlivňuje naše životní protředí. Výjimkou nejsou ani povrchové vody. Každoročně jsou do nich vypouštěny tisíce hektolitrů odpadních vod v různé míře před či vyčištěním kontaminované různou směsí látek. Mnohé z těchto kontaminantů jsou pro životní prostředí a v něm žijící organismy i pro člověka neškodné. Mnohé však díky své chemické struktuře a fyzikálním vlastnostem nesou schopnost poškozovat či ovlivňovat kvalitu životního prostředí a život v něm žijících organismů.

Jedním z velmi závažných negativních účinků těchto látek je genotoxický účinek. Genotoxický účinek bývá definován jako schopnost látek reagovat s buněčnou DNA a poškozovat její stavbu či měnit její smysl. V důsledku působení těchto látek (genotoxických faktorů) může docházet k poruše funkce buňky, a následně i k její smrti či propuknutí rakovinového bujení. Dojde-li k poškození DNA v buňkách somatických, je negativně ovlivněn „pouze" život daného jedince. Dojde-li k poškození DNA v buňkách pohlavních, může být vzniklé poškození přeneseno na potomstvo. Genotoxické faktory tedy mohou být zodpovědné za poškození plodu a rozvoj vrozených vývojových vad u potomstva.

Ve světle těchto skutečností se jeví sledování výskytu látek s genotoxickým účinkem v našem životním protředí jako velmi významné. Neboť látky, které se vyskytují v životním protředí, mohou prostřednictvím potravních řetězců pronikat až na náš stůl.

Povrchové vody, zejména tekoucí, jsou v čase velmi proměnlivým systémem. Z hlediska dlouhodobého sledování kvality povrchových vod se jako vhodnou matricí jeví sedimenty. V porovnání s vodou se jedná o stabilnější matrici. Jedná se de facto o drobné částečky usazené na dně vodních těles. Na povrchu těchto částeček dochází k sorbci hydrofobních látek, které jsou takto v sedimentu uchovávány a kumulovány. Do této skupiny patří například PAH, PCB, PCDD, PCDF. Mnohé z nich vykazují mimo jiné i genotoxické účinky. Látky obsažené v sedimentech mohou být uvolněny zpět do vodního prostředí. K tomu může docházet prostou mechanickou resuspendací sedimentu, změnou fyzikálně chemických vlastností sedimentu (např. pH) či prostřednictvím bakterií, rostlin a bentických organismů.

Biodostupnost látek vázaných v sedimentech je jedním z klíčových faktorů určujících míru negativních účinků těchto látek v reálném prostředí. Právě s tímto problémem se setkáváme v případě testování účinků sediemntů prostřednictvím jejich extrakce. V literatuře je popsáno široké spektrum možností přípravy extraktů. Z výsledků studie vlivu způsobu extrakce sediementů na vybrané ekotoxikologické testy vyplývá, že právě tento krok je klíčovým faktorem určujícím relevantnost výsledků testování. Cílem této práce bylo porovnat výsledky dvou testů toxicity (Akutní imobilizační test na *Daphnia magna* a Test inhibice růstu kořene *Sinapis alba*) a jednoho testu genotoxicity (SOS chromotest) v závislosti na použitém způsobu extrakce (vodný výluh, extrakce do dichlormethanu (DCM) pomocí sonifikace a extrakce do DCM Soxhletovou extrakcí). V SOS chromotestu byl prokázán významný rozdíl mezi účinky extraktu získaného extrakcí do DCM v porovnání s vodným výluhem. V testu Inhibice růstu kořene *S. alba* nebyl pozorován statisticky významný rozdíl v účincích extraktů získaných různým způsobem. V akutním imobilizačním testu na *D. magna* byl u všech připravených extraktů pozorován 100% inhibiční účinek. Obdobný trend je možné nalézt i v odborné literatuře. Z výsledků provedené studie lze odvodit potřebu testování účinků sedimentů prostřednictvím několika druhů extraktů (včetně vodného výluhu).

Některé látky nepůsobí přímo jako mutageny, ale mohou nepřímo ovlivňovat opravu DNA či přenos

signálů pro její spuštění. Mezi tyto látky patří například olovo. Sedimetny silně zatížené olovem a jeho sloučeninami se nacházejí zejména v blízkosti výpustí z podniků zpracovávajících olovo či používajících olověné sloučeniny při výrobě (např. výroba autobaterií). K těmto lokalitám patří i dolní úsek řeky Klenice (ČR) těsně před ústím do řeky Jizery (ČR). Extrakt těchto sediemntů vykázal vysoký genotoxický účinek v SOS chromotestu i ve WP2 testu. V porovnání s bentickými společenstvy z pozaďové lokality řeky Klenice (ČR) (cca 4 km nad místem vtoku odpadních vod z výrobny autobaterií) byl v olovu zatíženém úseku pozorován velmi závažný toxický účinek na bentické bezobratlé. V místě odběru přímo pod výpustí splachových vod z areálu nebyly nalezeny žádné bentické organismy.

Výběr pozaďové lokality pro účely posuzování negativních účinků sediemntů je obtížným úkolem. Je nutno vybrat lokality, které jsou jen minimálně zatíženy lidskou činností (včetně exhalací). Zároveň je žádoucí nalézt lokalitu s podobnými vlastnostmi. Sedimenty jsou totiž velmi heterogenní a liší se zejména zrnitostí, obsahem organického ulíku a pH. Výběr pozaďové lokality pro dlouhodobý monitoring stavu sedimentů v řekách České republiky byl cílem třetí studie. Byly vybrány lokality v horních částech toků řek Ploučnice, Mže a Blanice. Vzorky byly odebírány vždy nad a pod městem ležícím na řece. Všechny odebrané sedimenty prokázaly vhodnost pro použití jako pozaďová lokalita pro budoucí monitoring. Jejich nevýhodou však může být (v porovnání se sedimenty dolního toku řek) nižší obsah organického uhlíku, a tedy i nižší schopnost vázat ve svém objemu polutanty.

Sledování zatížení sedimentů z hlediska genotoxických účinků by se mělo stát nedílnou součástí monitoringu znečištění vodních ekosystémů.

ACKNOWLEDGEMENTS

Many people helped me and contributed to my work in different ways. One list devoted to acknowledgements is maybe not sufficient to express my thanks to all of them namely. I am grateful to all the people who helped, supported my study and work.

First of all, I would like to express my thanks to my family.

I have to and I want to express my thanks to my colleagues from Research Institute of Fish Culture and Hydrobiology (Faculty of Fisheries and Protection of Waters). All of them, from professors to technicians, helped me a lot. Thank you.

I am very grateful to all collaborators. Thank you very much.

This study was supported by the project MSM 6007665809, the project SP/2e7/229/07, the project CZ.1.05/2.1.00/01.0024 and the project GA JU 047/2010/Z.

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Beránková, P., 2008. Detetion genotoxic and toxic effects of the sediments in the Klenice River (Czech Republic). In: Book of abstract. Environmental changes and biological assessment IV, Ostrava, Czech republic, p. 54 (oral presentation).		2008
Beránková, P., Kolářová J., 2008. Detection of genotoxic and toxic potential of river sediments. In: Book of abstract. SETAC Europe, Warsau, Poland, p. 106 (poster).		2008
Beránková, P., Kolářová, J., Poláková, S., 2009. Impact of sediment samples extraction methods on results of ecotoxicological tests. In Book of abstracts. 11th Interdisciplinary Toxicology Conference, TOXCON 2009, Brno, Interdisciplinary Toxicology 2, p. 92 (poster).		2009

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Detection of the genotoxic potential of water ecosystem elements, toxicity tests on water organisms

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APPENDIX No. 1.

Beránková, P., Máchová, J., Kolářová, J., Randák, T., Poláková, S., 2011. Influence of selected methods of extraction of river sediments on results of selected ecotoxicological tests. Chemical Letters 105 (6), 476–481. (in Czech)

Chem. Listy *105*, 476–481 (2011) Laboratorní přístroje a postupy

LABORATORNÍ PŘÍSTROJE A POSTUPY

VLIV ZPŮSOBU EXTRAKCE ŘÍČNÍCH SEDIMENTŮ NA VÝSLEDKY VYBRANÝCH EKOTOXIKOLOGICKÝCH TESTŮ

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Klíčová slova: dichlormethan, vodný výluh, Soxhletova extrakce, sonifikace, *Daphnia magna*, *Sinapis alba*, SOS-chromotest, *Escherichia coli*

Úvod

Sedimenty jsou heterogenní polyfázový systém obsahující anorganickou i organickou hmotu, vodu a různé plyny. Ve své podstatě jsou směsí tuhých částic usazujících se na dně vodních těles. Na jejich povrch se mohou různým způsobem vázat látky obsažené ve vodě. Mobilita, biodostupnost a biologické účinky těchto látek závisí právě na způsobu této vazby.

Sedimenty představují významný indikátor znečištění vodních ekosystémů. Obsah toxických prvků v sedimentech vodních toků a nádrží odráží celkovou kontaminaci dané lokality lépe než okamžitá koncentrace znečišťujících látek ve vodě[1]. Proto představují sedimenty významný indikátor znečištění vodních ekosystémů.

Při studiu znečištění sedimentů je možno použít dva přístupy. Přístup chemický, kdy se stanovuje chemické složení a koncentrace látek vyskytujících se v analyzovaném vzorku. Přístup ekotoxikologický, kdy se stanovuje účinek na biologické systémy[2]. Oba dva přístupy mají svá pro a proti. Při chemické analýze nelze postihnout přítomnost všech látek přítomných ve směsi a jejich vzájemné interakce, ekotoxikologický přístup naopak nepodává informaci, která látka byla příčinou pozorovaného toxického účinku. Dalším úskalím ekotoxikologického přístupu je volba způsobu přípravy testovaného vzorku, neboť ne všechny testy lze provádět přímo se sedimentem, tedy bez předchozí extrakce.

Způsob extrakce a použité extrakční činidlo určují, které látky obsažené v sedimentu přejdou do extraktu. V extraktu získaném pomocí polárního rozpouštědla lze očekávat vyšší obsah látek polárních a naopak u rozpouštědla nepolárního vyšší obsah látek nepolárních. Proto je volba extrakčního činidla velmi významným faktorem ovlivňujícím konečné výsledky ekotoxikologických testů. Zejména u vzorků neznámé povahy je vhodné volit paralelní extrakci různými extrakčními činidly.

K nejčastěji používaným způsobům extrakce sedimentů patří prostý vodný výluh a extrakce organickým rozpouštědlem[3]. Voda je typickým polárním rozpouštědlem. Dobře se v ní rozpouštějí látky polární, v jejichž molekulách je náboj rozložen nepravidelně. Patří mezi ně většina anorganických solí, karboxylové kyseliny, alkoholy a velká část cukrů.

Z organických rozpouštědel patří k nejpoužívanějším dichlormethan (DCM) a jeho směsi s dalšími rozpouštědly (hexan, methanol a aceton). DCM patří mezi nepolární rozpouštědla. Dobře se v něm rozpouštějí látky nepolární, v jejichž molekulách je náboj rozložen rovnoměrně. Patří mezi ně většina uhlovodíků, tuky a vosky a některé anorganické látky (např. jód).

Mnoho z organických rozpouštědel používaných pro extrakce však vykazuje závažné toxické účinky pro testovací organismy (např. DCM, methanol a aceton). V takovém případě jsou extrakty převáděny do jiných rozpouštědel, která kombinují schopnost rozpouštět velké množství chemických látek a nízkou toxicitu pro testovací organismy. Jedním z nich je i dimethylsulfoxid (DMSO), který zároveň patří k nejčastěji používaným. DMSO se jeví jako vhodné rozpouštědlo na základě výsledků testů využívajících různé organismy[4–6].

Při výběru způsobu extrakce je třeba zohlednit povahu testovaného vzorku. Vzhledem k tomu, že vlastní způsob extrakce může významně ovlivnit výsledky ekotoxikologických testů a reálnost jejich odpovědi vzhledem k původnímu vzorku, byla naše pozorování zaměřena právě na tuto fázi přípravy vzorku.

Interpretace výsledků testů s extrakty na celé ekosystémy je sice problematická, neboť nelze přesně postihnout interakce probíhající mezi látkami, sedimentem a organismy žijícími v sedimentu a na sedimentu (biodostupnost *versus* extrahovatelnost látek), přesto je pro nás cennou informací o stavu našeho životního prostředí.

V předložené studii jsou srovnávány: vodný výluh, který je dlouhodobě používán pro testování tuhých odpadů a dva extrakty do DCM. Extrakce do DCM byla provedena Soxhletovou extrakcí a sonifikací. Obě metody patří mezi nejčastěji používané extrakční postupy.

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Experimentální část

Odběr, úprava a zpracování sedimentu

Sediment byl odebírán z řeky Klenice (ČR). Klenice je levostranným přítokem řeky Jizery, do které ústí v Mladé Boleslavi. Hlavními zdroji znečištění v povodí řeky Klenice jsou lidská sídla a splachy ze zemědělských ploch a pozemních komunikací.

Vzorek byl odebrán v blízkosti obce Řepov, přibližně 4 km nad ústím do Jizery. Jednalo se o jemnozrnný sediment s malým podílem hrubých pískových zrn (přibližně 20 hm.%).

K odběru sedimentu byl použit Bridge-Eckmanův drapák. Odebrány byly přibližně 2 kg zvodnělého vzorku. Vzorek byl zabalen, individuálně označen a uložen v chladícím boxu (4 °C) až do doby jeho zpracování v laboratoři. Vzorek byl vysušen lyofilizací. Následně byl mechanicky homogenizován a přesát přes síto s průměrem ok 2 mm.

Extrakce sedimentu

Pro extrakci sedimentů byly vybrány tři nejčastěji používané způsoby[3].

Každý způsob extrakce byl proveden v pěti nezávislých opakováních. S každým získaným extraktem bylo nakládáno jako se samostatným vzorkem.

Příprava vodného výluhu

Vodný výluh byl připraven vytřepáním 100 g lyofilizovaného sedimentu s 1000 ml destilované vody po dobu 24 hodin. Získaný vodný výluh byl přefiltrován přes papírový filtr o průměru pórů 5 μm.

Extrakce do DCM

Výhodou organického extraktu je snadná příprava koncentrovaných vzorků (dle povahy obsažených látek jednotky až desítky gramů v mililitru). To umožňuje získání informace i o přítomnosti toxických a genotoxických látek, jejichž koncentrace je pod mezí detekce analytických přístrojů.

Extrakce na Soxhletově extraktoru

50 g lyofilizovaného sedimentu bylo po dobu 8 h extrahováno 250 ml DCM v Soxhletově extraktoru. Získaný extrakt byl zahuštěn na přibližně 5 ml, následně odpařen a převeden do DMSO.

Extrakce sonifikací

50 g suchého sedimentu bylo po dobu 30 min extrahováno 50 ml DCM v ultrazvukové lázni. Získaný extrakt byl dekantován a uchován. Extrakce byla opakována čtyřikrát. Získaný extrakt byl zahuštěn na přibližně 5 ml, následně odpařen a převeden do DMSO.

Ekotoxikologické testy

Vliv způsobu extrakce na výsledky biotestů byl hodnocen pomocí tří vybraných testů (Akutní imobilizační test na vodních korýších (hrotnatka velká – *Daphnia magna*), Test inhibice růstu kořene hořčice bílé (*Sinapis alba*) a SOS-chromotest. Tyto testy jsou běžně používány pro hodnocení toxických a genotoxických vlastností vzorků životního prostředí a patří k celosvětově nejčastěji používaným.

Všechny testy byly provedeny s neředěným vodným výluhem i DCM extrakty. Testovaná koncentrace DCM extraktů byla shodná s koncentrací neředěného vodného výluhu, aby bylo možno srovnávat účinky extraktů získaných různým způsobem extrakce.

Každý test byl proveden ve třech nezávislých opakováních pro každý připravený extrakt. Získané výsledky byly statisticky vyhodnoceny. V testech, kde byla provedena předúprava testovaného vzorku za použití DMSO, byly souběžně nasazovány 2 kontroly: *i*) za použití ředicí vody bez přídavku testované látky, *ii*) za použití roztoku ředicí vody a DMSO v maximální koncentraci, která byla použita při přípravě testového roztoku. Odezva testovacích organismů na testovaný vzorek byla vztahována k odpovídající negativní kontrole a dále byl v závěru testu srovnáván stav organismů v obou kontrolách, aby bylo možné posoudit, do jaké míry byly pokusné organismy ovlivněny samotným rozpouštědlem.

Akutní imobilizační test na vodních korýších (hrotnatka velká – Daphnia magna)

Akutní imobilizační test na *D. magna* byl proveden dle normy OECD 202 (cit.[7]). Zjišťována byla mortalita a imobilizace jedinců *D. magna*. V každém opakování bylo nasazeno 10 jedinců *D. magna*.

K testům byli použiti korýši laboratorního chovu, jejichž citlivost byla prověřena testem se standardní látkou $K_2Cr_2O_7$ p.a. Zjištěná hodnota 24h-EC50 pro $K_2Cr_2O_7$ = 1,13 mg l^{-1} spadá do povoleného limitu (0,6–1,7 mg l^{-1})[8] pro tento test a standardní látku.

Test inhibice růstu kořene hořčice bílé (Sinapis alba)

Test inhibice růstu kořene *S. alba* byl proveden dle Metodického pokynu Ministerstva životního prostředí (MŽP) z roku 2007 (cit.[9]). Byla zjišťována inhibice klíčení semen a růstu primárního kořene. V každém opakování bylo nasazeno 30 semen *S. alba*. Nevyklíčené semeno bylo v souladu s pokyny Metodického pokynu MŽP z roku 2007 (cit.[9]) započítáno do výpočtu průměrné délky primárního kořene jako nulová hodnota.

SOS-chromotest

Jedná se o bakteriální test genotoxicity, založený na detekci produkce β-galaktosidasy v důsledku spuštění SOS reparačního systému bakteriální buňky. K aktivaci SOS reparačního sytému dochází v případě poškození DNA.

Principem SOS-chromotestu[10,11] je kolorimetrická detekce změny enzymatické aktivity v důsledku vlivu testovaného vzorku na testovací organismus. Test byl proveden dle metodiky popsané v práci Xu a spol. 1989 (cit.[12]). Pro každý z testovaných vzorků byl vypočítán indukční

faktor (IF). Překročí-li hodnota IF hodnotu 1,5, je testovaný vzorek považován za významně genotoxický[10,11].

Testovacím organismem je geneticky modifikovaná bakterie (*Escherichia coli* K12 PQ37), na kterou se vztahuje příslušná legislativa ČR (cit.[13,14]).

Statistické vyhodnocení

Rozdíly ve výsledcích, které byly získány v ekotoxikologických testech (Akutní imobilizační test na *D. magna*, Test inhibice růstu kořene *S. alba* a SOS-chromotest), při použití jednotlivých způsobů extrakce, byly z hlediska statistické významnosti testovány pomocí neparametrického Kruskal-Wallisova testu. Ke statistickým analýzám byl použit program STATISTICA 8.0 (cit.[15]).

Výsledky a diskuse

Výsledky získané v ekotoxikologických testech při použití jednotlivých způsobů extrakce jsou shrnuty v tab. I ve formě průměrů ze tří opakování a směrodatné odchylky (všechny způsoby extrakce byly provedeny v pěti nezávislých opakováních).

V akutním imobilizačním testu na *D. magna* došlo k 100% mortalitě případně imobilizaci testovacích jedinců ve vodném výluhu i v obou DCM extraktech, a tudíž mezi nimi nebyl zjištěn statistický rozdíl. Mortalita a imobilizace testovacích organismů v negativních kontrolách (v průměru 0 a 3,3 %) splnila podmínky validace použitého testu. Odpověď testovacích organismů na testované vzorky se průkazně lišila od odpovědi na negativní kontroly ($N = 51$, $df = 4$, $H = 50$, $P<0{,}001$). Vliv vlastního extrakčního postupu na výsledky vybraných testů byl zanedbatelný.

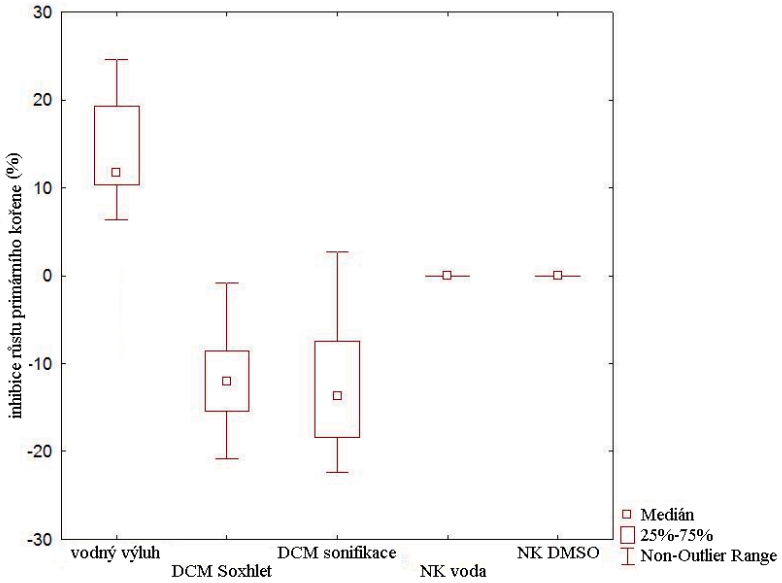
V testu inhibice růstu kořene *S. alba* nebyl prokázán statisticky významný rozdíl mezi účinky testovaného vzorku získaného jednotlivými způsoby extrakce ($N = 51$, $df = 4$, $H = 36$, $P<0{,}001$) (obr. 1). Rozdíly průměrné délky kořenů v jednotlivých testech v porovnání s příslušnou negativní kontrolou nepřesáhly 30 %, a proto byly výsledky získané v tomto testu posouzeny jako negativní. (Způsob hodnocení vychází z Metodického pokynu MŽP z roku 2007 (cit.[9]) pro hodnocení ekotoxicity odpadů, kde rozdíly v růstu kořene v testovaném výluhu ve srovnání s kontrolou do 30 % se považují za negativní).

V SOS-chromotestu při použití vodného výluhu nebyl zjištěn rozdíl v odezvě u pokusných a kontrolních organismů, tzn., že výsledek tohoto testu byl negativní. Naproti tomu byly prokázány významné statistické rozdíly účinku

Tabulka I
Přehled hodnot získaných ve vybraných ekotoxikologických testech[a]

Ekotoxikologický test	Způsob extrakce			Kontrola	
	vodný výluh	DCM soxhlet. extrakce	DCM sonifikace	H_2O	DMSO
Akutní imobilizační test na *D. magna* – uhynulí a imobilizovaní jedinci [%]	100 (0)	100 (0)	100 (0)	0 (0)	3,33 (5,77)
	100 (0)	100 (0)	100 (0)		
	100 (0)	100 (0)	100 (0)		
	100 (0)	100 (0)	100 (0)		
	100 (0)	100 (0)	100 (0)		
Test inhibice růstu kořene *S. alba* – inhibice klíčení a růstu primárního kořene [%]	14,89 (5,93)	–5,38 (8,18)	–10,24 (11,31)	0 (0)	0 (0)
	15,21 (3,52)	–10,17 (6,43)	–10,06 (5,03)		
	13,23 (6,25)	–9,07 (7,39)	–13,82 (7,67)		
	9,19 (2,22)	–13,77 (1,52)	–8,76 (11,05)		
	14,42 (9,35)	–26,17 (4,60)	–16,05 (2,99)		
SOS-chromotest – hodnota IF	1,02 (0,01)	1,62 (0,11)	1,94 (0,09)	1,01 (0,02)	1,03 (0,04)
	1,01 (0,01)	1,62 (0,05)	2,06 (0,18)		
	1,01 (0,01)	1,55 (0,13)	1,86 (0,32)		
	1,03 (0,01)	1,63 (0,08)	1,95 (0,20)		
	1,02 (0,01)	1,63 (0,05)	2,00 (0,16)		

[a] V tabulce jsou uvedeny průměrné hodnoty získané ze tří opakování testu pro každý testovaný extrakt. V závorkách je uvedena směrodatná odchylka vypočteného aritmetického průměru. Inhibice/stimulace klíčení a růstu primárního kořene *S. alba* byla vypočítána porovnáním průměrné délky primárního kořene v testovaném vzorku s délkou primárního kořene příslušné kontroly. Kladné hodnoty znamenají inhibiční účinek testovaného výluhu v porovnání s kontrolou, záporné hodnoty znamenají stimulační účinek v porovnání s kontrolou.



Obr. 1. **Výsledky statistického vyhodnocení vlivu způsobu extrakce sedimentu na výsledky Testu inhibice růstu primárního kořene *S. alba*** (Kruskal-Wallis test: N = 51, df = 4, H = 36, *P*<0,001)

DCM extraktů získaných Soxhletovou extrakcí a extraktu získaného sonifikací (N = 51, df = 4, H = 43,5, *P*<0,001). Účinky DCM extraktů získaných oběma způsoby se lišily vzájemně a lišily se i od výsledků s vodným výluhem (obr. 2).

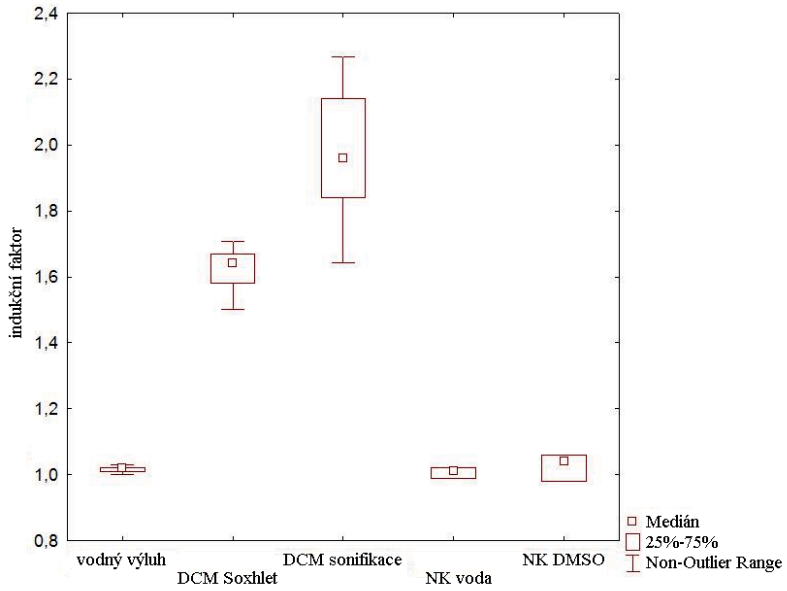
Jak se tedy ukazuje, rozdílné ekotoxikologické testy a rozdílné způsoby extrakce testované matrice přinášejí diametrálně rozdílné výsledky. Zatímco při použití testu na hořčici bílé byly získány negativní výsledky u všech způsobů extrakce, v testu na *Daphnia magna* byly výsledky všech extraktů pozitivní. Při posuzování genotoxických účinků SOS-chromotestem byly získány pozitivní výsledky s nepolárním extraktem a negativní výsledek s vodným výluhem. Tento fakt svědčí o tom, že k posuzování ekotoxikologických vlastností určité matrice musí být použity různé způsoby extrakce, ale i různé typy ekotoxikologických testů. V opačném případě existuje riziko, že případné nežádoucí účinky látek obsažených v sedimentu nebudou „odhaleny“ a hodnocení bude provedeno na základě falešně negativních výsledků.

V publikovaných studiích zabývajících se vlivem způsobu extrakce na výsledky Amesova testu (bakteriální test genotoxicity používající bakterii *Salmonella typhimurium*) použili autoři různá rozpouštědla a jejich směsi (např. směs voda/ether, methanol, toluen, DCM a destilovanou vodu)[16–20]. Autoři jedné z uvedených studií[16] prováděli testy na sedimentech z řeky Tama (Japonsko) a uvádějí, že polární extrakty z těchto sedimentů vykazovaly v Amesově testu vyšší genotoxický účinek než extrakty nepolární (směs voda/ether > ethyl acetát > methanol ~ *n*-hexan). Naproti tomu studie provedená na sedimentech z německé části řeky Labe dokazuje vyšší genotoxický účinek v Amesově testu u nepolárních extraktů (toluen) než u extraktů do methanolu a mnohem vyšší než u vodného výluhu[18].

Nekonzistentnost těchto výsledků naznačuje, že použití pouze jednoho typu rozpouštědla (polární × nepolární) není vhodné pro objektivní postihnutí účinků testovaných sedimentů. Neznáme-li povahu znečištění zkoumané lokality, jeví se jako vhodnější použití extrakce polárními i nepolárními rozpouštědly, případně jejich směsí. Vždy však musíme respektovat požadavky zvoleného ekotoxikologického testu, jenž má být použit pro hodnocení ekotoxikologických účinků testovaného sedimentu.

Závěry

1) Získané výsledky potvrzují, že při posuzování toxických a genotoxických vlastností sedimentů je nutné volit různé způsoby extrakce tak, aby došlo k uvolnění co možná nejúplnější škály látek přítomných v sedimentu.



Obr. 2. **Výsledky statistického vyhodnocení vlivu způsobu extrakce sedimentu na výsledky SOS-chromotestu** (Kruskal- Wallis test: N = 51, df = 4, H = 43,5, *P*<0,001)

Dále je třeba připravené extrakty podrobit ekotoxikologickým testům, při kterých jsou použity rozdílné testovací organismy.

2) Způsoby extrakce sedimentu popsané v předložené práci se jeví jako vhodné pro testy toxicity na *D. magna*, *S. alba* i v SOS-chromotestu, neboť chemické látky používané při předúpravě vzorku nejsou toxické pro pokusné organismy (dokladováno chováním organismů v kontrolách s rozpouštědly bez testované látky).

3) Při hodnocení sedimentů je zapotřebí používat více způsobů jejich extrakce (vodný výluh i DCM extrakty, případně jinou kombinaci polárních a nepolárních rozpouštědel), neboť do vodného výluhu se uvolňují převážně látky ve vodě rozpustné (polární), které mohou mít jiné účinky než vyluhované nepolární látky. Vodný výluh sice vhodně simuluje reálnou expozici vodních organismů látkám obsaženým v sedimentu, na druhé straně se ukázal jako ne příliš vhodným pro SOS-chromotest. Je tomu tak v důsledku známé skutečnosti, že většina látek poškozujících DNA je lipofilní povahy, a jsou tedy snáze extrahovatelné nepolárními rozpouštědly.

Seznam použitých zkratek

DCM	dichlormethan
DMSO	dimethylsulfoxid
IF	indukční faktor

Autoři děkují za finanční podporu grantu Ministerstva školství a tělovýchovy ČR č. CZ.1.05/2.1.00/01.0024, grantu Grantové agentury Jihočeské univerzity v Českých Budějovicích č. 047/2010/Z, grantu Národní agentury pro zemědělský výzkum NAZV QH82117 a grantu Ministerstva životního prostředí ČR č. SP/2e/229/07.

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P. Beránková, T. Randák, J. Kolářová, J. Máchová, and S. Poláková *(University of South Bohemia, České Budějovice, Faculty of Fishery and Protection of Waters, South Bohemian Research Center of Aquaculture and Biodiversity of Hydrocenoses, Vodňany):* **Influence of Methods of River Sediment Extraction on the Results of Selected Ecotoxicological Tests**

River sediments are contaminated with a wide range of substances from the water environment. Such contaminants may be buried for a long time and then gradually released into the environment. For this reason, attention must be paid to their load. Ecotoxicological tests are usually used to assess toxic and genotoxic effects of sediments; the sediment extracts are adjusted for these purposes. The aim of this article is to assess the effect of three common methods of sediment extraction on results of three selected standardized ecotoxicological tests. The samples were prepared by shaking with water, Soxhlet extraction with dichloromethane (DCM) or extraction with DCM under sonication. Toxicity was tested using the acute immobilisation test on water flees (*Daphnia magna*) and the test based on inhibition of growth of the *Sinapsis alba* root. The SOS-chromotest was used as a genotoxicity test. The experiments performed show that the selected method of extraction can significantly influence the results of ecotoxicological tests.

APPENDIX No. 2.

Beránková, P., Kolářová, J., Máchová, J., Randák, T., 2010. Toxicity and genotoxicity assessment of sediment from selected small streams in the Czech Republic – pilot study of less affected sites. Bulletin RIFCH Vodňany 46 (1), 5–11. (in Czech)

Beránková a kol. / ***Bulletin VÚRH Vodňany 46,*** *2010/1, 5–11*

POSOUZENÍ TOXICITY A GENOTOXICITY SEDIMENTŮ Z MALÝCH VODNÍCH TOKŮ ČESKÉ REPUBLIKY – PILOTNÍ STUDIE MÁLO ZATÍŽENÝCH LOKALIT

TOXICITY AND GENOTOXICITY ASSESSMENT OF SEDIMENTS FROM SELECTED SMALL STREAMS IN THE CZECH REPUBLIC – PILOT STUDY OF LESS AFFECTED SITES

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ABSTRACT

Sediments can serve as a reservoir of toxic materials which continue to endanger the aquatic organisms' health and life. The sediment is the place where translation of these materials between inorganic and biological part of the environment. With respect to their character (solid particles settled at the bottom of the river) and lower variability in time, sediments are in terms of longterm monitoring suitable for monitoring aquatic ecosystems. The present study is aimed to initiate a long-term monitoring of the sediment quality in selected rivers of the Czech Republic. The river sediments has been sampled in autumn 2007 from three rivers in the Czech Republic – Ploučnice River (above and below the town Cvikov), Mže River (above and below the town Tachov) and Blanice River (above and below the town Prachatice). Organic extract and water eluates of the sediments were tested. There were 5 tests employed for testing of samples of river sediments: 3 tests for evaluation of genotoxic effect (SOS-chromotest, test for chromosome aberration in ***Vicia faba*** *and test for micronuclei in* ***Vicia faba****) and 2 tests for evaluation of toxic effect (OECD 202 acute immobilization test with* ***Daphnia magna*** *and toxicity test with* ***Sinapis alba****). Genotoxic potential of the tested samples did not significantly differ from the negative control values. Similar results have come out also of toxicity tests.*

Klíčová slova: *SOS-chromotest, Vicia faba, Daphnia magna, Sinapis alba*
Keywords: *SOS-chromotest, Vicia faba, Daphnia magna, Sinapis alba*

ÚVOD

Dlouhodobý monitoring je nedílnou součástí sledování změn kvality životního prostředí. Z hlediska dlouhodobého sledování změn kvality vodních ekosystémů se jako vhodná matrice jeví zejména dnové sedimenty, které mají vysoký potenciál pro akumulaci nepolárních, persistentních a toxických látek a jsou silně ovlivňovány lidskou činností, která může narušovat přirozený stav vod (Heiniger a kol., 2005). Dlouhodobému sledování kvality sedimentů je věnována řada studií. Řada z nich se věnuje sledování změn sedimentů v zatížených lokalitách bez porovnání s lokalitami nezatíženými (Bertrand-Krajewski a kol., 2006; Cachot a kol., 2006; den Basten a kol., 2003; Heiniger a kol., 2005).

Jen méně studií zahrnuje do palety vzorků i sedimenty z nezatížených, tzv. kontrolních/pozaďových lokalit (Aouadene a kol., 2008; Frouin a kol., 2007). Na druhou stranu existuje velká variabilita ve složení (např. obsah organického uhlíku, jílu, minerálů) a fyzikálně-chemických parametrech sedimentů (např. iontově výměnné kapacity, pH atd.), které musí být pečlivě uvažovány při srovnávání vzorků z různých lokalit. Sediment pozaďové (nekontaminované) lokality by tedy měl být srovnáván vždy jen s takovými sedimenty ze zatížených lokalit, které mají obdobné geochemické vlastnosti.

Jako pozaďové lokality jsou vhodné horní části toku řek, v jejichž okolí není rozšířen průmysl a okolní plochy nejsou intenzivně zemědělsky obdělávány. V těchto úsecích se často vyskytují ryby silně citlivé na kvalitu vody, jako jsou pstruh obecný (*Salmo trutta*) a lipan podhorní (*Thymalus thymalus*) (Armstrong a kol., 2003).

Tato práce je prvním krokem k monitoringu stavu a změn kvality vybraných řek v České republice. Jejím cílem bylo vytipování potenciálních vhodných pozaďových lokalit, odebrání a zpracování vzorků sedimentů z těchto lokalit a provedení testů toxicity a genotoxicity. V následujících letech budou odebrány sedimenty ze středních a dolních částí toku vybraných řek na území České republiky souběžně s opakovanými odběry z pozaďových lokalit. Rozšíření této studie povede k zmapování stavu sedimentů v říčních tocích na území České republiky a jejich proměnlivosti v čase. Vzhledem k rozdílným fyzikálně-chemickým vlastnostem sedimentů bude provedeno srovnání lokalit s podobnými vlastnostmi sedimentů.

MATERIÁL A METODIKA

Odběrové lokality – říční sedimenty byly odebrány na podzim roku 2007 ze tří řek na území České republiky – z řeky Ploučnice (nad a pod městem Cvikov), řeky Mže (nad a pod městem Tachov) a řeky Blanice (nad a pod městem Prachatice). Ve všech třech městech se nachází čistírna odpadních vod, v níž jsou čištěny komunální odpadní vody.

Odběr a zpracování vzorků – sedimenty byly odebírány Eckmann-Bridge drapákem. Na každé lokalitě byly odebrány přibližně 2 kg zvodnělého vzorku. Vzorky byly zabaleny, individuálně označeny a uloženy v chladicím boxu (4 °C) až do doby jejich zpracování v laboratoři. Vzorky byly vysušeny při pokojové teplotě 21 °C. Následně byly mechanicky homogenizovány a přesáty přes síto s průměrem ok 2 mm.

Pro testy genotoxicity byl připraven organický extrakt pomocí Soxhletova extraktoru. Extrakčním činidlem byl dichlormethan (DCM). Padesát gramů suchého sedimentu bylo extrahováno 250 ml DCM. Extrakt byl zahuštěn na přibližně 5 ml a následně převeden do dimethylsulfooxidu (DMSO).

Pro testy toxicity byl připraven vodný výluh vytřepáním 100 g suchého sedimentu s 1000 ml standardní vody, připravené dle normy ISO 9001 po dobu 24 hodin. Získaný vodný výluh byl přefiltrován přes papírový filtr o průměru pórů 5 µm. Získaný filtrát byl použit v testech toxicity.

Chemická analýza – chemickou analýzu vzorků provedl Ústav chemie a analýzy potravin při VŠCHT v Praha. Stanoveny byly indikátorové kongenery polychlorovaných bifenylů (PCB 28, 52, 101, 118, 138, 153 a 180) a vybrané organochlorové pesticidy (HCB, OCS a deriváty DDT) (Hajšlová a kol., 1995).

TESTY TOXICITY

Akutní imobilizační test na vodních korýších (hrotnatka velká – *Daphnia magna*) – toxicita vzorků byla hodnocena pomocí testu toxicity na vodním korýši *Daphnia magna*. Test byl proveden dle normy OECD 202 (1996).

Test inhibice růstu kořene hořčice bílé (*Sinapis alba*) – toxicita byla testována na semenech rostliny *Sinapis alba* v počátečních stádiích klíčení dle metodického pokynu Ministerstva životního prostředí z roku 2007. Byla pozorována inhibice klíčení semen a růstu primárního kořene.

TESTY GENOTOXICITY

SOS-chromotest – SOS-chromotest (Quillardet a Hofnung, 1985; Quillardet a Hofnung, 1993) je rychlý kolorimetrický test založený na sledování změny enzymatické aktivity v důsledku inkubace testovacího bakteriálního kmenu v médiu s přídavkem testovaného vzorku. V tomto testu je využíván bakteriální kmen Escherichia coli K12 PQ37. Test byl proveden dle metodiky popsané v práci Xu a kol. (1989). Pro každou z testovaných koncentrací byl vypočítán indukční faktor (IF). Překročí-li hodnota IF hodnotu 1,5, je testovaný vzorek považován za významně genotoxický.

Test na chromozómové aberace u *Vicia faba* a test na mikrojádra u *Vicia faba* – jedná se o krátkodobé testy genotoxicity na rostlinném systému (Kihlman, 1975; Kanaya, 1994). Pro testy jsou používána semena rostliny *Vicia faba* (počet chromozomů v jádře buňky = 12). Experimenty jsou prováděny na meristematických buňkách naklíčeného primárního kořene. Po aplikaci testovaného vzorku jsou semena kultivována ve tmě při teplotě 21 °C po dobu 72 hodin. Po kultivaci je z kořenových špiček připraven mikroskopický roztlakový preparát, v němž jsou hodnoceny chromozómové aberace a vznik mikrojader.

VÝSLEDKY

CHEMICKÁ ANALÝZA

Výsledky chemické analýzy vzorků jsou uvedeny v tabulce č. 1. Koncentrace stanovovaných látek je uvedena v $\mu g.kg^{-1}$.

Tab. 1. *Výsledky chemických analýz vzorků sedimentů.*
Tab. 1. *The results of chemical analyses of sediment samples.*

Lokalita	sušina %	Σ PCB $\mu g.kg^{-1}$	HCB $\mu g.kg^{-1}$	OCS $\mu g.kg^{-1}$	DDE $\mu g.kg^{-1}$	DDD $\mu g.kg^{-1}$	DDT $\mu g.kg^{-1}$
nad Cvikovem	*72,5*	*10,2*	*0,22*	*0,07*	*1,20*	*1,40*	*4,37*
pod Cvikovem	*54,7*	*71,9*	*1,78*	*0,26*	*5,23*	*8,85*	*10,01*
nad Tachovem	*72,2*	*1,7*	*0,11*	*< 0,02*	*0,46*	*3,23*	*0,42*
pod Tachovem	*31,2*	*11,5*	*1,45*	*0,05*	*3,35*	*2,89*	*1,97*
nad Prachaticemi	*54,1*	*8,6*	*0,28*	*0,08*	*1,97*	*2,05*	*3,74*
pod Prachaticemi	*62,2*	*29,1*	*1,53*	*0,08*	*2,13*	*2,59*	*5,43*

TESTY TOXICITY

Akutní imobilizační test na vodním korýši (*Daphnia magna*) – výsledky akutního imobilizačního testu jsou uvedeny v tabulce č. 2. Testován byl vodný výluh o koncentraci 100 g.l^{-1}.

Test inhibice růstu kořene *Sinapis alba* – výsledky testu inhibice růstu kořene *Sinapis alba* jsou uvedeny v tabulce č. 2 spolu s výsledky akutního imobilizačního testu na *Daphnia magna*. Testován byl vodný výluh o koncentraci 100 g.l^{-1}.

Tab. 2. *Výsledky akutního imobilizačního testu na vodní m korýši (*D. magna*) a testu inhibice růstu kořene* S. alba.
Tab. 2. *The results of Acute immobilisation test with water flea (*D. magna*) and test of the growth inhibition of* S. alba *root.*

Lokalita	% inhibice *Daphnia magna* po 24 hodinách	% inhibice *Daphnia magna* po 48 hodinách	% inhibice růstu kořene *Sinapis alba*
nad Cvikovem	*60*	*100*	*-14*
pod Cvikovem	*20*	*50*	*-38*
nad Tachovem	*100*	*100*	*-39*
pod Tachovem	*0*	*10*	*-26*
nad Prachaticemi	*0*	*0*	*-27*
pod Prachaticemi	*0*	*30*	*-26*

TESTY GENOTOXICITY

SOS chromotest – indukční faktory zjištěné v jednotlivých vzorcích nebyly statisticky významně odlišné od výsledků negativní kontroly (ANOVA, F = 1,297; DF = 6; p = 0,269). Testován byl organický extrakt o koncentracích 0,15; 0,075, 0,037 a 0,018 g.l^{-1}.

Test na chromozómové aberace u *Vicia faba* a test na mikrojádra u *Vicia faba* – stejně jako u SOS-chromotestu nebyl u indukce chromozómových aberací u *Vicia faba* sledován významný statistický rozdíl v porovnání s negativní kontrolou (pro chromozómové aberace: ANOVA, F = 1,325; DF = 6; p = 0,199). Vznik mikrojader nebyl v pozorovaných buňkách detekován. Testován byl vodný výluh o koncentraci 100 g.l^{-1}.

DISKUSE

Chemický rozbor potvrdil mírné zvýšení znečištění řek po průtoku u studovaných měst. Tento nárůst se pohyboval v jednotkách, maximálně desítkách µg.kg^{-1} (max. nárůst suma PCB pod Cvikovem 61,7 µg.kg^{-1}). Vliv těchto sídel je tedy díky jejich velikosti (Prachatice 12 000 obyvatel, Tachov 12 500 obyvatel, Cvikov 4500 obyvatel) a využití čistíren odpadních vod minimální. V sídlech se také nenachází průmyslové podniky, které by svou činností mohly výrazně ovlivnit kvalitu vodních ekosystémů.

Nízká koncentrace škodlivin detekovaných v sedimentech je zřejmě dána absencí průmyslových podniků a větších lidských sídel v povodí horní části vybraných řek. Frouin a kolektiv ve své práci (2007) uvádí koncentraci PAHs v kontrolním sedimentu 129 ng.g^{-1} a koncentraci 22 550 ng.g^{-1} sedimentu zatíženém sazemi z vysokých pecí. V sedimentech odebraných ze systému St. Lawrence River v Kanadě (Côté a kol., 1998) byla celková koncentrace PCB ve většině případů pod detekčním limitem (0,10 µg.g^{-1}), nejvyšší naměřená koncentrace byla 1,90 µg.g^{-1}.

Takto nízkému znečištění odpovídal i nízký IF SOS-chromotestu, který autoři pozorovali. V akutním imobilizačním testu s vodními korýši (*Daphnia magna*) byl pozorován silný toxický účinek u vzorku z lokality nad Tachovem (100% inhibice již po 24 hodinách expozice). Silný toxický účinek byl také pozorován u vzorku z lokality nad Cvikovem, u něhož došlo ke 100% inhibici po 48 hodinách expozice. U vzorku z lokality pod Cvikovem byla po 48hodinové expozici pozorována 50% inhibice. U ostatních vzorků byla pozorována nízká nebo nulová míra inhibice mobility hrotnatek (*Daphnia magna*). Vyšší toxicita vzorků odebraných nad vybranými městy může být dána přítomností nedekovaných látek, například látek ze splachů z polí. Sedimenty odebrané v Brazílii v dolní části toku v porovnání se vzorky z horního toku vykazovaly v testu s *Daphnia magna* zvýšenou toxicitu (Mitteregger Junior a kol., 2006).

V testu inhibice růstu kořene rostliny *Sinapis alba* nebyl pozorován negativní vliv vodných výluhů vzorků. Naopak byla pozorována mírná stimulace růstu primárního kořene, zřejmě v důsledku vyšší koncentrace živin ve výluzích v porovnání s negativní kontrolou (ISO voda).

Testy genotoxicity neprokázaly významný genotoxický účinek testovaných vzorků, v žádné z testovaných koncentrací u žádného z testovaných vzorků. Tato odpověď je ve shodě s výsledky chemického rozboru i s předpokladem nízkého zatížení horních toků řek v podhorských oblastech. O nízkém zatížení vybraných lokalit svědčí i jejich zařazení do pstruhového pásma. Lososovité ryby jsou silně citlivé k znečištění vod organickými a toxickými látkami a na dostatečnou koncentraci kyslíku. V dostupné literatuře je bohužel málo údajů o monitoringu čistých lokalit, které by mohly být při dalším posuzování změn kvality životního prostředí považovány za lokality kontrolní.

Vybrané toky a profily jsou vzhledem k výsledkům vybraných ekotoxikologických testů vhodnými pozaďovými lokalitami pro další monitoring stavu řek na území České republiky. Je však třeba zohlednit konkrétní situaci (viz zmínka v úvodu) a vhodně srovnávat lokality, aby byly získány informace o skutečných rozdílech – zejména kvantitativních rozdílech mezi různě zatíženými úseky na dolních tocích. Shoda výsledků použitých testů potvrzuje hypotézu o vhodnosti jejich zařazení do baterie ekotoxikologických testů určených pro tento monitoring.

SOUHRN

Sedimenty slouží ve vodním prostředí jako rezervoár toxických látek, které mohou ohrožovat zdraví a život vodních organismů. S ohledem na jejich charakter (drobné pevné částečky uložené na dně řeky) a relativní stabilitu v čase (ve srovnání s tekoucí vodou) se sedimenty jeví jako vhodná matrice pro monitoring zátěže vodních ekosystémů. Tato práce byla zaměřena na počáteční fázi monitoringu stavu sedimentů v říčním systému České republiky. Sedimenty byly odebrány na podzim roku 2007 ze tří řek – řeky Ploučnice (nad a pod městem Cvikov), řeky Mže (nad a pod městem Tachov) a řeky Blanice (nad a pod městem Prachatice). Byl testován organický extrakt a vodný výluh. Pro hodnocení zatížení sedimentů bylo použito 5 testů: 3 testy genotoxicity (SOS-chromotest, test na chromozómové aberace u *Vicia faba* a test na vznik mikrojader u *Vicia faba*) a 2 testy toxicity (OECD 202 akutní imobilizační test na vodních korýších *Daphnia magna* a test inhibice růstu kořene *Sinapis alba*). Genotoxický potenciál testovaných vzorků se statisticky významně nelišil od negativní kontroly. Podobný výsledek daly i testy toxicity (s výjimkou jedné lokalit nad Tachovem a nad Cvikovem, kde byly pozorovány významné inhibice mobility *D. magna*). Vybrané lokality prokázaly v námi vybraných testech svou vhodnost jako zvolené pozaďové lokality pro další hodnocení zatížení sedimentů malých toků na území České republiky.

PODĚKOVÁNÍ

Tato práce byla provedena za podpory výzkumného záměru č. MSM6007665809, projektů CENAKVA reg. č. CZ.1.05/2.1.00/01.0024 a GA JU 047/2010/Z a grantu Ministerstva životního prostředí České republiky č. SP/2e7/229/07.

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UNIVERSITY OF SOUTH BOHEMIA IN ČESKE BUDĚJOVICE

ISBN 978-80-87437-17-9