

INTEGRATED USE OF DIFFERENT FISH RELATED PARAMETERS TO ASSESS THE STATUS OF WATER BODIES

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Summary: Freshwater bodies receive high levels of different toxicants. Since fish are typically situated at the top of aquatic food chains, they have a good potential to be used as indicators of water pollution. Assessment of the presence of pollutants and their toxicity can be efficiently performed by the combined use of analytical chemistry, bioassays and applied mathematics. In this study, we present the general approach of the integrated use of different fish related parameters to assess the status and pollution levels of waterbodies. We discuss our previous experiences within the field of analytical toxicology, toxicological pathology and toxicity testing, as well as biomathematical and statistical methods that are able to provide for integration of results acquired by each of the specific methods. We discuss advantages and shortcomings of each of the methods, and present necessary future steps in the method development. Since the industrial and domestic wastewaters in Serbia are still not processed before being released into watercourses, they pose a serious risk for aquatic ecosystems and public health. Consequently, described pollution indicators and genotoxicity parameters represent an essential tool for efficient monitoring of aquatic ecosystems. Methodological approach presented here might be of interest for scientists and managers dealing with the ecotoxicological research and monitoring of freshwater ecosystems.

Key words: freshwater fish; heavy metal; histopathology; genotoxicity; PCA; CDA

Introduction

Freshwater ecosystems are nowadays exposed to high anthropogenic impacts from untreated or poorly treated industrial and communal wastewaters, runoff from agricultural lands and mining sites, as well as from numerous other sources connected with human activities (1, 2). As a result, freshwater bodies receive high levels

of different toxicants. Assessment of the presence of pollutants and their toxicity can be efficiently performed by the combined use of analytical chemistry, bioassays and applied mathematics. In such an approach, analytical toxicology would provide information regarding the identification and assay of toxicants in environmental and biological materials, toxicological pathology would assess the effects of toxicants and their metabolites on cell and tissue morphology, while genotoxicity testing would use living systems to estimate genotoxic effects. Since these

approaches commonly deal with a substantial amount of acquired data, they also require the use of advanced biomathematical and statistical methods for data analysis.

Fish have a potential to be utilized as indicators of water bodies' pollution status, as different fish species occupy different habitats and belong to different trophic levels (3, 4). According to the Water Framework Directive (EU Directive 200/60/C), fish are one of the most important biological quality elements for the assessment of the ecological status of water bodies. However, only a few fish related parameters, such as fish species composition, abundance and age structure, have been commonly included in such assessments so far.

In this study, we present the general approach of the integrated use of different fish related parameters to assess the status and pollution levels of waterbodies. We present our previous experiences within the field of analytical toxicology, toxicological pathology and toxicity testing, as well as biomathematical and statistical methods that are able to provide integration of the results acquired by each of the specific methods. We discuss advantages and shortcomings of each of the methods, and present necessary future steps in the method development.

Analytical toxicology

Heavy metals are considered to be among major pollutants in the Danube River basin in Serbia (5). Since fish are typically situated at the top of aquatic food chains, they are able to accumulate large amounts of some metals (6). Heavy metals can be either ingested through food or absorbed from the water through gills and skin (7). Following their absorption, heavy metals and trace elements demonstrate tissue specific accumulation patterns in fish (8, 9).

The two most widely used techniques for heavy metal and trace element analyses are Atomic Absorption Spectrometry (AAS) and Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES). Each of the two methods is characterized by certain advantages and drawbacks when compared with the other one. Although ICP-OES has better detection limits than the flame-AAS, it has significantly lower sensitivity than the graphite furnace-AAS. Both methods are generally considered as inexpensive, although ICP-OES has

somewhat higher running costs of analysis. On the other hand, ICP-OES provides a much greater speed of the analysis, when compared with the standard AAS methods. This feature represents an important advantage in the field of analytical toxicology and environmental monitoring, which typically deal with studies that are based on analyzing a large number of samples. As a result, ICP-OES is gaining ground in ecotoxicological research as a more dominant method, and will be further described here.

Sample preparation procedure for the ICP-OES analysis is as follows: caught specimens are dissected and samples of different tissues are quickly removed, washed with distilled water and stored on -20°C prior to analysis. Samples are freeze-dried, and 0.2-0.5 g dry weight sample portions are subsequently processed in a microwave digester, using 6 ml of 65% HNO_3 and 4 ml of 30% H_2O_2 at a 100–170 $^{\circ}\text{C}$ temperature program. The problem with the potential presence of trace elements in chemicals used for digestion is commonly resolved by using a number of blank samples, while the analytical process quality can be controlled by the use of reference material. Following the cooling to room temperature, digested samples are diluted with distilled water to a total volume of 25 ml.

Our previous research within this field has been mainly focused on the assessment of heavy metal and trace element accumulation levels in different localities (3), in different fish species (4, 8, 9), along the aquatic food chains (i.e., in fish and piscivorous birds; 7), as well on general accumulation patterns in different fish tissues. Research included assessment of the following heavy metals and trace elements: Ag, Al, As, B, Ba, Cd, Co, Cr, Cu, Fe, Hg, Li, Mg, Mn, Mo, Ni, Pb, Si, Se, Sr and Zn. The major tissues that have been monitored were fish muscles, gills and liver. Gills represent an important tissue for water pollution monitoring due to their direct contact with the water, while the liver has a high accumulation potential and as such represents an important pollution indicator (10, 11). Although muscles typically accumulate low or even minimal elemental concentrations, they often represent the major focus of toxicological research since they are the main part of the fish that is consumed by humans (12, 13). Other fish tissues that have a potential to be used as pollution indicators are gonads, intestines and kidneys.

Figure 1 presents general accumulation patterns of heavy metals and trace elements in muscles, gills and liver of fish from the Danube River basin, based on our previous research. It comprises the data for the Pontic shad (*Alosa immaculata*; 8), sterlet (*Acipenser ruthenus*; 9), barbel (*Barbus barbus*; 4), Prussian carp (*Carassius gibelio*; 7), silver carp (*Hypophthalmichthys molitrix*), bream (*Abramis brama*), white bream (*Blicca bjoerkna*), carp (*Cyprinus carpio*) and wels catfish (*Silurus glanis*; 14). As can be seen in the Figure, gills represent the center of the accumulation of Al, B, Ba, Mn and Sr, liver is the accumulation center of As, Cu and Mo, while both tissues have approximately equal frequency as accumulation centers of Fe and Zn. Muscle was most frequently the tissue with lowest elemental concentrations, with the exception of Sr and As, and partly Al, B, Ba and Cu. Future research within this field should include the assessment of additional fish tissues, of a wider spectra of fish species from various habitats and with differing life histories, as well as experimental approach that would establish relationship between elemental concentrations in the water and those in fish tissues.

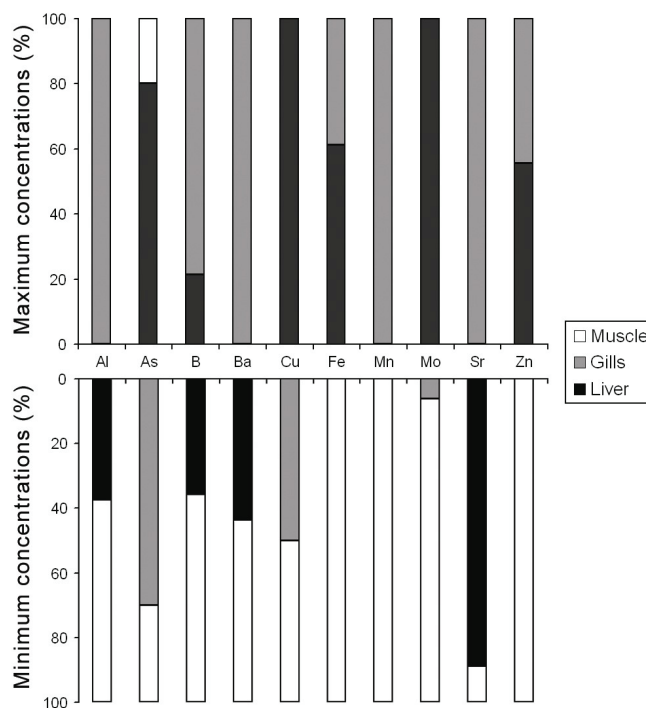


Figure 1: Ratio of the assessed fish species from the Danube River basin that had the maximum and minimum concentrations detected in a particular tissue, presented for each of the analyzed heavy metals and trace elements; data represent results from previous research (4, 7, 8, 9, 14)

Toxicological pathology

Toxicological pathology represents the study of the effects of pollutants on fish organs, tissues and cells. For the sample analysis, both light (LM) and electron microscopy (EM) can be used, although the use of light microscope is the most common approach for routine examinations and will be further discussed here.

Histological method procedure comprises sampling of fish organs, followed by immediate sample fixation (aldehyds or alcohols are commonly used LM fixatives) and dehydration in progressively higher ethanol concentrations. Following sample dehydration and clearing with xylene, samples are embedded in paraffin, sectioned using a microtome and stained with various histological stains (15). At the end of the protocol, the cells and tissues remain the same as they were at the moment of sampling, enabling examination and detection of tissues' alterations.

Advantages of the histopathological method lie in the fact that fish are located at the top of the trophic pyramid in the aquatic environment, which makes them suitable as markers

of bioconcentration and biomagnification. Histopathology allows the assessment of target organs, namely those in immediate contact with the aquatic environment (i.e., gills and skin), as well as other affected vital organs (such as liver, gonads and kidneys). Histopathological analyses contribute to the understanding of pollutant activity mechanisms, or of the activity of a pollutant mixture. They also take into account specific sensitivity of various fish species to pollution, depending of their ecological niche or life history. Finally, since fish also represent a model of vertebrate organisms, detected effects of pollution can be extrapolated to mammals.

Histopathology is considered as a reliable bioindicator of aquatic pollution (16). Nevertheless, it also possesses certain drawbacks: histological changes are not pollutant specific; although the cost of the assessment procedure is relatively low, it requires specific equipment and trained staff; procedure is also time consuming, with considerable laboratory work needed for the preparation of samples; certain subjectivity of the method should be also emphasized, since

it depends substantially on the proficiency of the researcher; it is a descriptive method and quantitation is rather challenging. In their extended review, van der Oost et al. (17) discussed the relevance of histopathology as a biomarker of fish health status, giving it a score of 3.5 out of 5. The authors claimed that it is a relevant method, with high toxicological significance, but with a relatively low ability to detect the specific type of pollution. Other authors pointed out the advantage of histopathological approach in fish toxicology and ecotoxicology, particularly its usefulness in monitoring sublethal chronic effects of chemicals on fish (18), as well as its sensitivity to low levels of environmental contaminants (19).

However, it is not always easy to establish strong relationship between environmental pollution and histopathological alterations. Open water bodies are often exposed to mixtures of different chemicals, such as persistent organic pollutants, pesticides and endocrine-disrupting compounds, which produce diverse effects on fish tissues and organs (20). Moreover, while physical and chemical characteristics of water play an important role in the activation and fate of these substances (21), they can also impact organ morphology themselves (22). Other factors, such as the presence of heavy metals, parasites or cyanotoxins could produce negative effects on fish homeostasis and cause histopathological changes in various organs. Histopathological changes represent a general cumulative effect of all the stressors affecting the fish organism. Therefore, it is necessary to include more than a single organ in histological assessments, since some chemicals can induce changes only in a single organ, while the other organs of the same fish could remain unaffected.

Histopathology is subjective and often depends on the proficiency of a researcher. In the majority of manuscripts, an extent of a tissue alteration is usually categorized as either mild, moderate or a severe change (23), or as a percentage of the altered tissue (24). This can be avoided to an extent by using morphometric or stereological approaches (25, 26, 27, 28), which are, however, often time-consuming. Therefore, researchers are commonly assessing fish organs using semi-quantitative and/or scoring systems (29). Currently, we are using two scoring systems for histopathological analyses of fish tissues and organs. One of them is based solely on gill histology

(30), while the second one takes into account four vital organs: gills, kidney, liver, and skin (1). Both systems are based on the same principle: besides the extent of detected alterations, there is also an importance factor of the type/severity of change, which multiplies the score. The sum of all detected changes is calculated separately for each organ, while the total index value represents a sum of scores for all assessed organs. The basic difference between two systems is the principle of describing the lesions: while Poleksic and Mitrovic-Tutundzic's system is taking into account specific gill changes (i.e. lifting of the epithelium, rupture and peeling of lamellar epithelium, telangiectasis, etc.), Bernet's system defines certain patterns of tissue response to pollution. Those reaction patterns are classified as: circulatory, regressive, progressive, inflammatory, and neoplastic, and each has subcategories, depending on the place where lesion occurs: epithelium or supporting tissue. This allows the researcher to use Bernet's scoring for different organs in the fish organism, because reaction patterns are universal to the tissues. The importance factor of both systems is in the same range: from 1 to 3, meaning that all lesions do not have the same importance/severity in the evaluation process. These scoring systems enable quantification and comparison between two or more polluted sites, or with the reference site. Scoring systems are commonly used in assessments of streams (31), rivers (32), lakes (33) and fish farms (34).

Genotoxicity testing

Water quality monitoring is frequently restricted to the measurement of physical and chemical parameters. However, established alarm thresholds for these parameters are related to toxic levels of polluting substances, and they usually do not take into consideration risks posed by chronic exposures at low pollutant concentrations, which are frequently present in complex mixtures and can produce genotoxic effects (35). Consequently, it is necessary to employ additional tests that are able to detect changes at the molecular level, especially those that can result in genetic mutations. Genotoxicity testing is considered as one of the most valuable fish biomarkers, and as such it should become an integral part within environmental risk assessments (17).

In recent years, the Comet assay and the micronucleus test have been increasingly used within ecogenotoxicology and for monitoring purposes. The main advantage of the Comet assay, when compared with the micronucleus test, is that it does not require the presence of cells that are undergoing mitosis, as well as its higher sensitivity to low concentrations of genotoxic substances. The Comet assay, also referred to as the single cell gel electrophoresis assay (SCG or SCGE), is a rapid, visual, and quantitative technique for measuring DNA damage in eukaryotic cells (36). Under alkaline conditions (pH >13), the assay is able to detect single and double-stranded breaks, incomplete repair sites, alkali-labile sites, and possibly also DNA–protein and DNA–DNA cross-links, in virtually any eukaryotic cell population that can be obtained as a single cell suspension. The Comet assay has found a wide application as a simple and sensitive method for evaluating DNA damage in fish exposed to various xenobiotics in the aquatic environment (37, 38). There are three different parameters used within the Comet assay to quantify the level of DNA damage: tail length, tail moment (Olive tail moment) and tail intensity (the percentage of the DNA located in tail). Olive tail moment is calculated as a product of two factors, the tail intensity and the distance between the intensity centroids (centers of gravity) of the head and the tail along the main axis of the comet. When using derived measurements (e.g. tail moment), data on primary measurements (i.e., tail length and tail intensity) should also be presented in the analyses (39). As there is a number of different parameters produced by this method, there is still a certain lack of consensus within the scientific community with regard to the most suitable and reliable Comet assay parameter. This could be resolved by employing other methods that would evaluate and compare parameters, and indicate those with the greatest sensitivity and reliability. One of such methods is the sum of ranking differences (SRD), which can be used to compare parameters and tissue combinations (40). In our previous study (41), we have applied SRD to evaluate nine different genotoxicity parameter/tissue combinations - tail length, intensity and moment in three different cell types: erythrocytes, liver cells and gill cells. The study indicated that the Olive tail moment and tail intensity represent equally reliable parameters.

Nevertheless, Comet assay study design and

data analysis still require further investigation, improvement and standardization. This is especially important for scoring methods (visual or computerized), DNA damage quantification parameters and experimental conditions, all of which intensely vary between laboratories (42). Although the Comet assay has a generally straightforward methodology, the image analysis is substantially more complex. Two approaches, visual analysis and computerized image analysis, are commonly used to measure DNA breakage in the Comet assay. In the visual analysis, comets are classified based on their morphology, either by grading their size or by measuring tail lengths (43), and the DNA damage is evaluated as an increase in the percentage of cells with comets (44, 45, 46). It is a relatively reliable and fast approach (47), but the results rely to a certain extent on subjective decisions made by the investigator. Computerized image analysis provides additional measurement criteria as compared with the visual approach, including those for tail length, moment and intensity. Since different laboratory conditions (such as incubation time, electrophoresis intensity and duration, gels and agarose concentration, etc.) can result in significantly different results, laboratories should conduct specific tests to obtain optimal resolution, and the parameters given above should always be specified as part of the experimental conditions of a Comet assay (48).

In our previous research (4, 41, 49), the Comet assay was employed to acquire information through the analysis of DNA damage *in vivo* in blood, liver and gills of European chub (*Squalius cephalus*) and in blood of barbel (*Barbus barbus*) from rivers and reservoirs in Serbia, which are under various degrees and types of pollution. Microscopic images of comets were scored using Comet IV Computer Software (Perceptive Instruments, UK). Gills gave the best response as compared to other tissues. Gills may be more prone to injury than other tissues, due to a high respiratory blood flow and permanent contact with the water environment. Blood was less sensitive in comparison to other tissues. This might be due to regular cycles of change of blood cells in the bloodstream, which indicates that blood could be used as a biomarker only for acute contaminations. Tissue specific responses are expected because of variations in alkali-labile sites and cell types with different background DNA single-strand break levels, due to variations in excision repair activity, metabolic

activity, antioxidant concentrations, or other factors (49). However, although the blood gave the lowest response to DNA damage compared to other tissues, it was still possible to observe the significant difference between the rivers with different intensity of anthropogenic influence (41). Our results confirmed that fish represent a good model system for genotoxicity testing within water pollution monitoring, as well as that evaluated genotoxicity biomarkers are sensitive and suitable for this type of research.

Biomathematics and statistics

Integrated use of different analytical techniques in water pollution monitoring commonly produces a large amount of data. Effective data analysis and the interpretation of results require specific data processing tools, such as chemometrics, which are able to provide optimization of the experimental design, data processing, clustering and pattern recognition, calibration, quality control and the organization of analytical processes. In our research we used principal component analysis (PCA) as a method suitable for data assessment and to illustrate group clustering (7), as well as

canonical discriminant analysis (CDA) in cases when we had predefined groups to produce components, or variables, along which differences between groups are maximized while those within a group are minimized (3). Bearing in mind the known weaknesses of CDA for the evaluation of statistical analysis, post-hoc tests are also used as the most precise method for evaluating group differences (50).

Figure 2 presents a general approach of the integrated use of methods presented in this study, such as acquiring histopathological data and applying scoring systems, genotoxicity testing and measuring DNA damage, as well as detecting concentrations of different elements in fish tissues. Obtained results can be consequently used to distinguish fish species that have the greatest potential as aquatic pollution indicators. Future research should also include assessment of persistent organic pollutants in fish tissues and their pathological and genotoxic effects, enzymes involved in xenobiotic metabolism, blood parameters, as well as the use of discriminated functions and neural networks for data analysis. As a result, the general approach presented in Figure 2 could be enhanced by the inclusion of these methods and research topics.

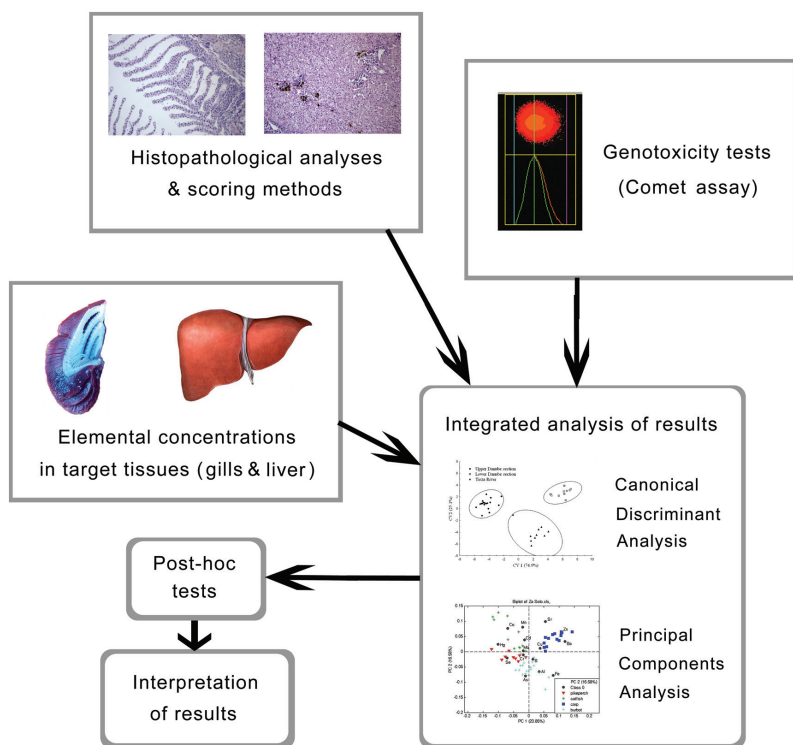


Figure 2: Schematic overview of the integrated use of different toxicological methods in aquatic pollution research and monitoring

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CELOSTNA UPORABA RAZLIČNIH RIBJIH PARAMETROV ZA OCENO STANJA VODA

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Povzetek : V sladke vode prehajajo visoke koncentracije različnih strupenih snovi. Ribe so običajno na vrhu vodne prehranjevalne verige, zato so primerni kazalniki onesnaženosti voda. Ocena prisotnosti toksinov in njihove strupenosti se lahko učinkovito izvede s kombinirano uporabo analizne kemije, bioloških testov in uporabne matematike. V tej raziskavi smo predstavili splošen pristop integrirane uporabe različnih parametrov, povezanih z ribami, za oceno onesnaženosti sladkovodnih voda. V članku predstavljamo lastne izkušnje s področja analitične toksikologije, toksikološke patologije in testiranja toksičnosti kot tudi biomatematičnih in statističnih metod, ki omogočajo integracijo rezultatov, pridobljenih s posameznimi metodami. Poleg tega je govor tudi o prednostih in pomanjkljivostih posameznih metod ter o potrebnih prihodnjih korakih pri njihovem razvoju. Ker se industrijske in komunalne odpadne vode v Srbiji še vedno ne očistijo, preden se spustijo v vodotoke, predstavljajo resno tveganje za vodne ekosisteme in zdravje ljudi. V članku opisani kazalniki onesnaženosti in parametri genotoksičnosti, ki predstavljajo pomembno orodje za učinkovito spremljanje vodnih ekosistemov. Metodološki pristop, predstavljen v članku, bo zanimiv za raziskovalce in menedžerje, ki se ukvarjajo z ekotoksikološkimi raziskavami in spremljanjem sladkovodnih ekosistemov

Ključne besede: sladkovodne ribe; težke kovine; histopatologija; genotoksičnost; PCA; CDA