# Ecotoxicological Characterization of the Sava River: Biomarker Responses and Biological Assays

**Tvrtko Smital and Marijan Ahel**

**Abstract** Driving forces related to settlements, agriculture, and release of contaminated untreated effluents from municipalities and industrial facilities that are greatly dominated by old and environmentally unfriendly technologies have always been considered as key elements that exert significant pressure on the ecological status of the Sava River. Despite such an unfavorable situation, the biological monitoring activities and chemical identification capabilities in most of the countries of the region have been traditionally restricted to a very limited number of biological markers and potentially hazardous contaminants, respectively. Nevertheless, the biomarker approach for the detection of hazardous chemical contamination in the Sava River was applied early in the 1980s, and the research studies that followed in subsequent decades introduced various biomarkers measured in various freshwater species. The use of the small-scale or in vitro bioassays has been more frequently used only from the late 1990s and culminated more recently with the investigations carried out within the related international research projects. In this chapter we present an overview of the research that has been done so far on the ecotoxicological evaluation of the Sava River using ecotoxicological biomarkers and bioassays, summarize the described evidence, and offer a general evaluation of the present ecotoxicological status of the Sava River.

**Keywords** Sava River • Ecotoxicological evaluation • Biomarkers • Bioassays

## List of Abbreviations

| | |
|---|---|
| APEO | Alkylphenol polyethoxylates |
| B[*a*]PMO | Benzo[*a*]pyrene monooxygenase |
| CYP1A | Cytochrome P4501A |
| EDA | Effects-directed analysis |
| EROD | 7-Ethoxyresorufin-*O*-deethylase |
| GC/MS | Gas chromatography/mass spectrometry |
| GST | Glutathione S-transferase |

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|---|---|
| HPLC | High-performance liquid chromatography |
| LAS | Linear alkylbenzenesulfonates |
| LC-QToF-MS | Liquid chromatography/quadrupole time-of-flight mass spectrometry |
| MXR | Multixenobiotic resistance |
| PAHs | Polycyclic aromatic hydrocarbons |
| PCBs | Polychlorinated biphenyls |
| RQ | Risk quotient |
| WFD | European Union Water Framework Directive |
| WWTP | Wastewater treatment plant |

## 1 Introduction

The Sava River basin is the major drainage basin of the Southeastern Europe covering the total area of approximately 97,700 km$^2$. The Sava River was the biggest national river of the former Socialist Federal Republic of Yugoslavia and was often considered as the life artery of the state. After the dissolution of former Yugoslavia in the early 1990s, it has become an international river of recognized importance. As the largest by discharge and the third longest tributary to the Danube, on its way from Slovenian Alps until its mouth to the Danube in Belgrade, the Sava River now connects the four states and the three capitals (Ljubljana, Zagreb, and Belgrade). The large complex of preserved alluvial wetlands in the middle of the basin, called Central Posavina, makes the Sava River basin unique for the outstanding biological and landscape diversity. Nevertheless, driving forces related to settlements, industry, agriculture, and waste management have always been considered as key elements that exert significant pressure on surface water bodies [1]. Furthermore, as compared to the situation in Western Europe, the key environmental problem, which is common for all transition countries in the Sava River basin, is the release of contaminated untreated effluents from municipalities and industrial facilities that are greatly dominated by old and environmentally unfriendly technologies. Since the drinking water supply in the Sava River basin relies almost exclusively on the rich resources of high-quality groundwater, which are under direct influence of the Sava River, the assessment of possible adverse effects of hazardous chemical contamination is of great importance. Despite such an unfavorable situation, the monitoring activities and identification capabilities in most of the countries of the region have been traditionally restricted to a very limited number of biological markers and potentially hazardous contaminants.

Considering the overall level of industrial activities and economy of former Yugoslavia in general, the mid-1980s represented the peak pollution pressure to the Sava River. The first comprehensive characterization of organic pollution in the Sava River was performed in 1985 by Ahel and Giger [2] using gas chromatography/mass spectrometry (GC/MS) technique. The study indicated the presence of numerous specific organic contaminants, which were not regulated by the national ordinance on the maximum allowable concentrations. It turned out that some of the compounds, identified in the analyzed samples, belonged to the compound classes that 15 years later became prominent candidates of the so-called emerging contaminants. For example, Croatia was one of the first countries that introduced water quality criteria for nonylphenol; some 15 years before it was accepted as a priority pollutant in the European Union Water Framework Directive (WFD).

Likewise, the biomarker approach for the detection of hazardous chemical contamination in the Sava River was applied very early. In order to assess the biological effects of substances being discharged in the Sava River, in the early 1980s, the ecotoxicology group from Ruđer Bošković Institute (RBI) in Zagreb performed the first large-scale biomarker studies. They measured early toxic effects, the induction of benzo[*a*]pyrene monooxygenase (B[*a*]PMO) in feral fish populations, and the late, ultimate toxic effects, the appearance of tumors in fish. In addition, the mutagenic capacity of the surface water extracts was determined by the Ames test [3–6]. The studies that followed in subsequent decades introduced additional biomarkers measured in various freshwater species. However, apart from the Ames test determinations, the use of the small-scale or in vitro bioassays as tools for the determination of ecotoxic potential of the Sava River surface water or sediments samples has been more frequently used only from the late 1990s and culminated more recently with the investigations carried out within the related EU FP6 projects EMCO and SARIB [7, 8] and the NATO Science for Peace and Security Programme [9].

In this chapter we present an overview of the research that has been done so far on the ecotoxicological evaluation of the Sava River using ecotoxicological biomarkers and bioassays. The first section is dedicated to biomarker responses in biota. The second one addresses data on the determination of the ecotoxicological potential of the Sava River complex environmental samples, obtained utilizing various bioassays and different end points. Finally, we close this chapter with an attempt to summarize the described evidence and offer a general evaluation of the present ecotoxicological status of the Sava River.

## 2 Biomarker Responses in the Sava River Biota

The early 1980s marked the beginning of ambitious field studies directed to the evaluation of biomarker responses in various indicator species inhabiting the Sava River. The biomarker studies that resulted with relevant publications in peer-reviewed scientific journals are chronologically enlisted in Table 1. A few important observations should be pointed out before considering the mentioned studies in more detail.

First, although the Sava River is some 990 km long, most of the studies have been carried out on the Croatian part of the Sava River or the section of the river shared between Croatia and Bosnia and Herzegovina. Furthermore, a 150 km long

**Table 1** Chronological list of relevant field studies focused on biomarker responses in freshwater fish, crayfish, or plant species inhabiting the Sava River

| Year | Authors | End point(s) | Species | Ref. no. |
|---|---|---|---|---|
| 1980 | Kezić et al. | Carcinogenicity (neoplasia frequency), CYP1A induction (B[*a*]PMO) | 21 fish species | [3] |
| 1981 | Kurelec et al. | CYP1A induction (B[*a*]PMO), carcinogenicity (neoplasia frequency), bioactivation potential (Ames test) | 21 fish species | [4] |
| 1983 | Kezić et al. | CYP1A induction (B[*a*]PMO) | European chub (*Squalius cephalus*), carp (*Cyprinus carpio*), barbel (*Barbus barbus*), nase (*Chondrostoma nasus*) | [5] |
| 1984 | Kurelec et al. | CYP1A induction (B[*a*]PMO) | European chub (*Squalius cephalus*), barbel (*Barbus barbus*), nase (*Chondrostoma nasus*) | [6] |
| 1989 | Kurelec et al. | Genotoxicity (DNA adducts) | European chub (*Squalius cephalus*), carp (*Cyprinus carpio*), barbel (*Barbus barbus*), bream (*Abramis brama*) | [10] |
| 1993 | Britvić et al. | CYP1A induction (B[*a*]PMO), bioactivation potential (Ames test), bile fluorescence | European chub (*Squalius cephalus*), carp (*Cyprinus carpio*), barbel (*Barbus barbus*), roach (*Rutilus rutilus*), *Rutilus pigus virgo*, bream (*Abramis brama*), bleak (*Alburnus alburnus*) | [11] |
| 1999 | Kolak et al. | Genotoxicity (micronucleus test) | European chub (*Squalius cephalus*) | [12] |
| 2003 | Klobučar et al. | Genotoxicity (comet assay, micronucleus test) | Zebra mussel (*Dreissena polymorpha*) | [13] |
| 2003 | Smital et al. | MXR (P-glycoprotein activity) | Zebra mussel (*Dreissena polymorpha*) | [14] |
| 2007 | Krča et al. | CYP1A induction (EROD), GST induction; bioactivation potential (Ames test); OH-PAH bile metabolites | European chub (*Squalius cephalus*) | [15] |
| 2007 | Dragun et al. | Cytosolic concentrations of metals and proteins in the gills | European chub (*Squalius cephalus*) | [16] |
| 2008 | Kopjar et al. | Genotoxicity (comet assay) | Balkan loach (*Cobitis elongata*) | [20] |
| 2009 | Podrug et al. | Cytosolic total protein, metallothionein (MT), and metal concentrations | European chub (*Squalius cephalus*) | [17] |

(continued)

**Table 1** (continued)

| Year | Authors | End point(s) | Species | Ref. no. |
|---|---|---|---|---|
| 2009 | Dragun et al. | Gill metallothionein (MT) | European chub (*Squalius cephalus*) | [18] |
| 2011 | Radić et al. | Peroxidase activity, lipid peroxidation, genotoxicity (comet assay) | Duckweed (*Lemna minor*) | [23] |
| 2011 | Pavlica et al. | Genotoxicity (comet assay; micronucleus test) | European chub (*Squalius cephalus*) | [21] |
| 2012 | Klobučar et al. | Genotoxicity (comet assay; micronucleus test) | Crayfish (*Astacus leptodactylus*) | [22] |
| 2012 | Marijić and Raspor | Trace metal concentrations, tissue metallothionein (MT) | European chub (*Squalius cephalus*) | [19] |

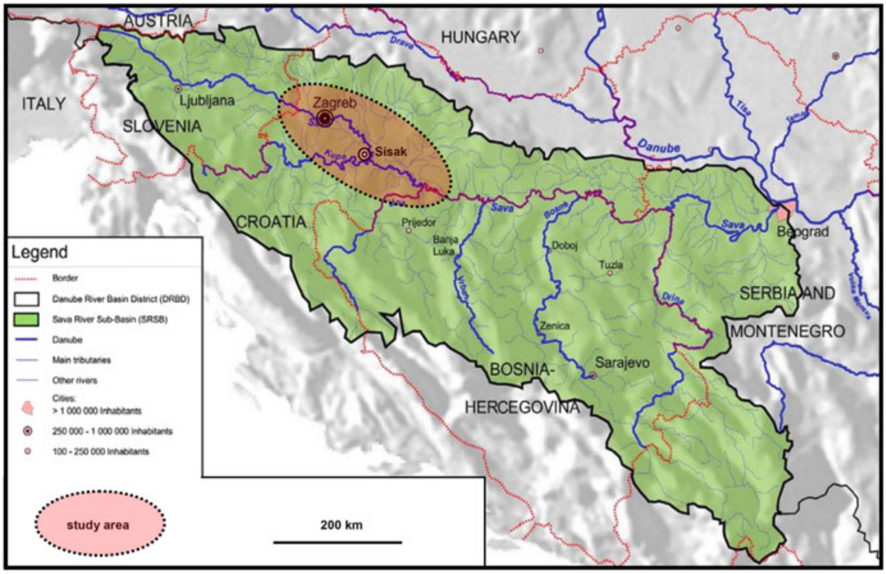

**Fig. 1** Map of the Sava River basin. The most frequently studied section of the river, stretching from the Slovenian–Croatian border over the Zagreb City area to the confluence of the Una River, is encircled in red (study area)

section of the river starting at the Slovenian–Croatian border up to the confluence of the Una River has been by far the most studied part of the river (Fig. 1).

This section was often selected due to the well-defined gradient of pollution, ranging from low-to-moderately polluted sites before the city of Zagreb (1 mil. inhabitants, heavily industrialized) to the sites situated downstream from the

Zagreb and Sisak City areas, which are characterized by the enhanced pollution loads. The rest of the Sava River, however, has been much less studied.

Secondly, almost all of the studies utilized fish as indicator species (Table 1). Two studies were performed on a bivalve, one study on a native crayfish species, and only one study utilized a plant as indicator species.

Finally, less than 20 peer-reviewed articles were published on the subject in the course of over 30 years. All of these studies were not result of national monitoring programs but were rather carried out as integral parts of various national and international research projects. Therefore, although results of all these investigations represent a valuable and relatively solid data set, one has to be aware that there was no any systematic, long-term, scientifically sound biomonitoring program of the ecotoxicological status of the Sava River.

Fish are well known as species at the top of the food chain in aquatic ecosystems. They accumulate and bioconcentrate xenobiotics available in the water column or in the sediments. This line of reasoning was the base for the first large-scale biomarker studies in the Sava River watershed, performed in the early 1980s by the Kurelec group from the RBI in Zagreb. In their first attempt, focused on monitoring tumor frequencies in native fish populations as a proxy for detecting the effects of mutagenic/carcinogenic substances present in the heavily polluted Sava River, they did a massive scale work—almost 200,000 specimens belonging to 21 fish species were examined [3]. Data were collected by fish pathologists by direct observation of catches during official fishing competitions, and some competitions were even intentionally organized on certain heavily polluted stretches of the river contaminated by known quantity and type of contaminants. As a result, some 5.56 % specimens were necropsied, and most of the diseases observed were the consequence of either viral, bacterial, or helminth parasite infections. Surprisingly, however, there were no neoplasms detected. Five out of 21 fish species, caught at the most polluted locations downstream of the Zagreb City main wastewater outlet, were then chosen for determination of their liver B[*a*]PMO activities. The measured B[*a*]PMO activity of those fish species was invariably high. For example, B[*a*]PMO activity in wild carps from the Sava River was over ten times higher on average than in control carp specimens from local fish farms, clearly indicating a highly significant exposure to pollutants able to induce cytochrome P4501A (CYP1A)-dependent (phase I) liver detoxification enzymes. In the follow-up study [4], the same group introduced a few additional end points: (1) B[*a*]PMO activity was determined in the caged carps exposed from 5 to 140 days at polluted locations and compared with control specimens held in laboratory conditions, (2) B[*a*]PMO induction was measured in carps i.p. treated with hexane extracts of the Sava River collected at several locations (the so-called induct test), (3) the concentration of the B[*a*]PMO inhibitors in corresponding hexane extracts was evaluated in vitro, and finally (4) testing of the mutagenic potential of the Sava River extracts was performed by the Ames test. Overall, there were a good correlation between the level of pollution and B[*a*]PMO activity as determined both in the native and the caged specimens, but no correlation between the low water quality and frequency of neoplasia in native fish populations. Although the hexane extracts of a few liters of the Sava River surface water at some locations contained sufficient concentrations of mutagenic substances to yield significant increase in the number of revertants in the Ames test, the presence of these harmful substances neither affected the reproduction status of the fish nor increased the neoplasia frequency. Therefore, the authors concluded that monitoring of the fish tumor frequency for evaluation of the health hazard from waterborne mutagens/carcinogens does not appear to be a promising approach.

The potential of B[*a*]PMO determinations as an effective biomarker of exposure was then exploited in two studies that further evaluated putative correlation between the liver B[*a*]PMO activity and pollution load of the Sava River and some smaller, much less polluted rivers (Krka and Kupa) in the same area [5, 6]. Again, the RBI group examined B[*a*]PMO activity in the three chosen native species (European chub, barbel, and nase). Based on these initial data, 10- and 20-day cage exposure experiments with carps were performed at the three typical segments of the Sava River and one each in the Kupa and Krka Rivers. The obtained activity levels were compared with the domestic and industrial load of these rivers derived from data obtained from the Water Management Authorities in Slovenia and Croatia. The determined B[*a*]PMO activities in nonmigratory fish populations were highly correlated with the recent pollution history for the particular part of the river and were highly correlated with the pollution load as expressed in population equivalents. The very same set of data clearly revealed that the pollution of the Sava River, especially in the Zagreb City area, resulted in much higher biomarker (B[*a*]PMO) response in comparison to the responses measured in fish inhabiting less polluted Rivers Krka and Kupa. Therefore, the measurement of liver B[*a*]PMO activity in natural fish populations proved to be a useful tool both for detecting the presence and estimation of the quantity of xenobiotics in water. Furthermore, the use of caged experimental fish offered the same predictive validity as that of wild fish populations, with significant practical advantages that were frequently exploited in subsequent studies in the Sava River watershed.

In 1989 the ecotoxicology group from RBI published an interesting study that focused on the application of the measurement of specific DNA adduct concentration in target tissues of the Sava River fish as a key biological end point of exposure to environmental carcinogens [10]. Using a highly sensitive assay based on the $^{32}$P-postlabeling technique, they found that natural populations of freshwater fish species (European chub, barbel, bream, and carp) from the Sava River revealed the presence of four to nine qualitatively similar adducts, irrespective of whether they were caught from unpolluted or polluted waters. No significant differences were observed between the adduct levels of fish from the unpolluted waters and those of fish from the polluted waters, and a dominant feature of the fish DNA adducts was species specificity. The finding that a vast majority of DNA modifications in fish were obviously caused by natural factors rather than by exposure to man-made contaminants offered a basis for a more realistic view in assessing the genotoxic risks in the Sava River basin.

Unfortunately, the warfare in the region started in the early 1990s, and the following postwar situation caused difficulties in the organization of any meaningful field studies, especially considering the fact that the Sava River basin became shared between four independent countries. As a result, almost no biomonitoring studies had been carried out in the 1990s. The exception was the study reported by Britvić and colleagues in 1993 [11]. This study was based on the data obtained by chemical determination of metabolites of compounds to which fish were exposed. As some of these compounds may cause profound biological effects in fish, the authors studied the correlation between the increase in bile fluorescence caused by petroleum hydrocarbon metabolites, the induction of liver B[*a*]PMO activity, and the increase in the liver potential for the bioactivation of promutagenic benzo[*a*]pyrene to *Salmonella typhimurium* TA100 mutagens. Seven fish species caught at polluted locations along the Sava River showed several-fold increase in the levels of all three parameters, as compared with their levels in fish living in the reference Korana River or with the responses determined in control carp specimens held in laboratory. These results offered qualitatively new support to the idea of using simple measurements of fluorescence of diluted bile as a rapid and cheap complementary investigative tool for monitoring and assessment studies.

The end of the 1990s denoted revitalization of biomarker studies in the Sava River basin, as well as the inclusion of new indicator species and new ecotoxicological biomarkers. Genotoxicity/mutagenicity determinations were updated with new methods, like micronucleus test as one of the most successful and reliable assays for detecting aneugenic and clastogenic genotoxicants or the detection of DNA damage at the level of the single cells using the comet assay. Kolak and colleagues [12] were the first to determine genotoxicity of the Sava River by the measurement of the micronuclei frequencies in European chub erythrocytes. The fish were caught at different seasons at three locations in Croatia and compared with data on chub caught from the unpolluted river Kupčina. Although there were no seasonal differences, the average frequency of micronuclei in erythrocytes from the Sava River specimens (0.89–0.93‰) was twice higher than in the controls (0.42‰). The fish in the laboratory were further i.p. injected with benzo[*a*]pyrene, and the results showed that the determination of micronuclei frequency in fish erythrocytes could serve as a useful and reliable part of genotoxic biomonitoring programs in the Sava River basin.

Then, the very first biomarker study done on non-fish species was the work published in 2003 by Klobučar and colleagues, who monitored genotoxicity of the Sava River using micronucleus test and comet assay on the mussel *Dreissena polymorpha* hemocytes [13]. Caged mussels were exposed for 30 days at four monitoring sites of different pollution intensity. The baseline level of micronuclei frequencies in the hemocytes of mussels from the reference site (River Drava) was 0.5‰. No increase in micronuclei frequency was found in mussels from the medium-polluted site while other, more polluted sites showed higher frequencies ranging from 2.7 to 5.2‰. Results from the comet assay showed concordance with micronucleus test, indicating higher intensity of DNA damage at polluted locations.

Again using the zebra mussel as indicator species, Smital and colleagues introduced in 2003 a new ecotoxicological end point, inducibility of the so-called multixenobiotic resistance (MXR) mechanism primarily mediated by the efflux activity of the P-glycoprotein as the phase III of cellular detoxification machinery [14]. The main goal of the study was to ascertain the rate-dynamic level as well as the possible usability of MXR in environmental biomonitoring. Since the primary result of MXR induction should be the decrease in intracellular accumulation of xenobiotics, the determination of MXR induction was performed using the measurement of P-glycoprotein transport activity. The authors measured the accumulation or the efflux rate of the model P-glycoprotein substrate rhodamine B in the gills of mussels previously exposed to polluted versus reference locations in the Sava River area. The results obtained showed that the P-glycoprotein transport activity was induced according to the level of pollution and that only a 4-day period was already long enough for the significant induction and deinduction of MXR activity. However, the inducibility of Pgp transport activity was significantly limited—the maximal level of induction obtained in this study resulted in 50–60 % lower rhodamine B accumulation in the gills of induced specimens when compared with control, non-induced animals, indicating that the use of the MXR as a relevant biomarker should be measured along with the determination of DNA, mRNA, and/or related protein expression.

The most ambitious biomarker study done recently in the Sava River basin was the extensive work accomplished within the EU FP6 project SARIB [8]. Considering biomarker determinations, the most significant contribution was published in 2007 by Krča et al. [15], reporting hepatic biomarker responses in European chub that was selected as indicator fish species within the SARIB project. In an attempt to first determine the species-specific physiological range of selected biomarkers (minimal and maximal responses) in European chub, juvenile specimens caught in the Sava River were laboratory-exposed to various (0.25–50 mg/kg) doses of either model polycyclic aromatic hydrocarbon (PAH) promutagen benzo[*a*]pyrene (BaP) or a well-known model CYP1A inducer β-naphthoflavone (β-NF) for 3–5 days. The responses of several hepatic biomarkers were determined in the exposed fish: 7-ethoxyresorufin-*O*-deethylase (EROD) activity, CYP1A content, glutathione S-transferase (GST) activity, liver bioactivation potential, and finally the amount of hydroxylated polycyclic aromatic hydrocarbon bile metabolites determined by the fixed wavelength fluorescence and the high-performance liquid chromatography (HPLC) technique. The relevance of determined biomarker responses has been analyzed further and cross-correlated with the same set of biomarkers, as well as with tissue concentrations of PAHs and polychlorinated biphenyls (PCBs), determined in European chub specimens collected simultaneously at five different polluted locations along the Sava River. The species-specific upper and lower limits in responses of studied biomarkers were determined and the obtained ranges successfully evaluated in field situation. With the exception of the GST activity, all other biomarkers determined in European chub proved to be valuable indicators of environmental pollution, clearly reflecting higher pollution in locations downstream of Zagreb City and at the sites downstream of the oil refinery of the town of Sisak. Furthermore, these data for the first time showed that even at the most polluted locations, the determined hepatic biomarker responses in feral chub specimens were well below maximal, species-specific physiological response. In the

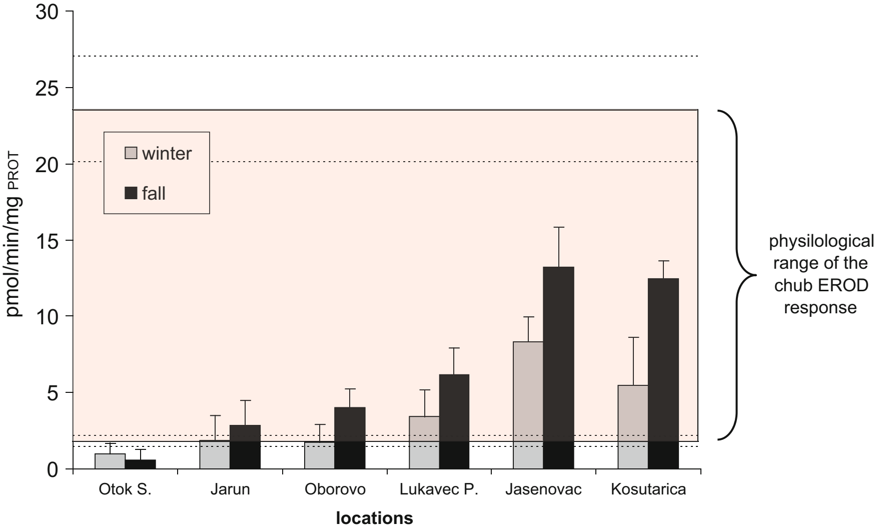

**Fig. 2** Seasonal variability in the liver 7-ethoxyresorufin-*O*-deethylase (EROD) activity in chub specimens caught at denoted locations along the Sava River in the winter (February) and fall (September) of 2005. The upper and/or lower limits of the chub, species-specific EROD responses, as were determined in the β-naphthoflavone (25 mg/kg) laboratory exposure experiments, are given as additional information (*solid lines*, means; *dashed lines*, SDs)

follow-up investigations, the RBI group analyzed the possible influence of seasonal differences on selected biomarker responses. Most of the hepatic biomarkers determined in chub showed no significant variation in response, with the exception of the EROD (phase I) and GST (phase II) activities that were elevated in chubs caught in the fall (September) versus those analyzed in the winter months (February) (Figs. 2 and 3).

Additional important results of the SARIB project were investigations that were directed to the analysis of the concentration of metals, total cytosolic proteins, and metallothioneins—specific metal-binding proteins—in European chub gill tissue. In the first study published in 2007 by Dragun and colleagues [16], the authors analyzed the influence of the season and the biotic factors (age and gill mass) on metal and protein levels in the juvenile European chub gill tissue. Five metals were addressed (Zn, Fe, Cu, Mn, and Cd), and a clear, seasonally dependent influence of the gill mass on both the protein and the metal levels was observed. The proposed explanation for the different dependence of metal levels on the gill mass in autumn and spring was the seasonal difference in feeding intensity and metabolic rate, with presumably faster metabolism and water filtration through gills in spring. In the next study, the same group focused on the assessment of metal accumulation in the liver as a target organ [17]. The metallothionein concentrations did not differ between the study sites, and the authors suggested the main reason for this observation was relatively the low dissolved and labile concentrations of metals known as metallothionein inducers (Zn, Cu, and especially Cd) in the Sava River water

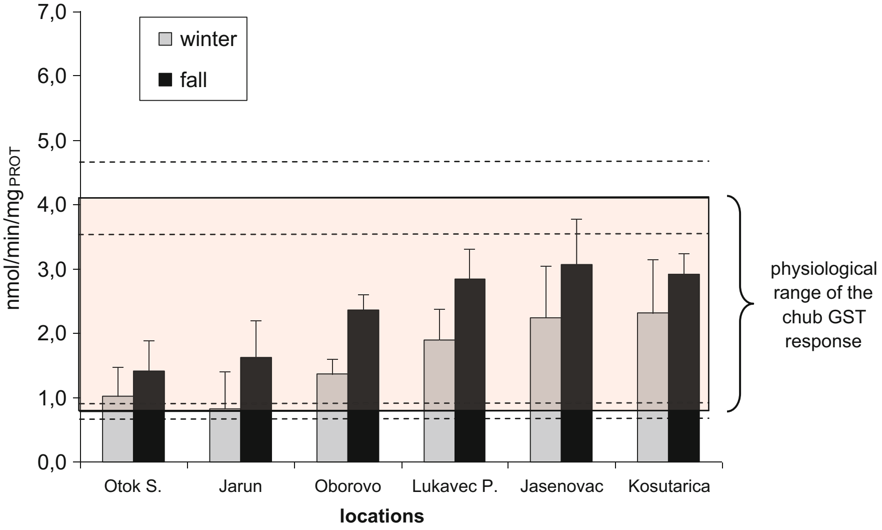

**Fig. 3** Seasonal variability in the liver glutathione *S*-transferase (GST) activity (EROD) in chub specimens caught at denoted locations along the Sava River in the winter (February) and fall (September) of 2005. The upper and/or lower limits of the chub, species-specific GST responses, as were determined in the β-naphthoflavone (25 mg/kg) laboratory exposure experiments, are given as additional information (*solid lines*, means; *dashed lines*, SDs)

column. However, hepatic cytosol concentrations of Cd showed statistically significant increase from the less polluted sites upstream of Zagreb City towards more affected locations downstream of Zagreb City and the town of Sisak, respectively. Therefore, it has been suggested that Cd concentrations in hepatic cytosol of European chub can be recommended as an early-warning marker of fish chronic exposure to Cd from combined sources, both the water and ingested food.

Nevertheless, as the determined concentration of metallothioneins was highly variable among sampling campaigns and seasons, the possible causes of this variability were studied in more detail and resulting data published in 2009 by Dragun et al. [18]. Apart from the putative influence of metabolic activity on metallothionein levels, the correlation analysis indicated a significant association between metallothioneins and the fish size. Differences between males and females, as well as between mature and non-mature fish, were not observed in juvenile specimens, even in the spring reproductive season. Based on the analysis of the site-specific metallothionein variability, the authors concluded that, under the conditions of low dissolved metal concentrations in the river water (as was reported for the Sava River), the metal-binding proteins seem to be more affected by different biotic factors than by metal exposure. Therefore, the measured concentrations of metallothioneins were rather considered as the constitutive levels that differ between the season of lower metabolic rate (autumn) and the season of higher metabolic activity (spring). This assumption was further confirmed in a recent comprehensive field survey on the site-specific variability of trace metal concentrations in the gut content, gastrointestinal tissue, and two gastrointestinal subcellular fractions (defined as metal-sensitive fraction and metal-detoxified fraction, respectively) [19]. At five sampling sites along the Sava River, 1- to 5-year-old European chub specimens were caught in the post-spawning period (September) in order to estimate if metal concentrations in fish intestines are related to their levels in the gut content or fish age. Clear difference in metal abundance between the gut content and gastrointestinal tissue was observed, implying a selective metal absorption in fish intestines. Relationship among metal concentrations in the gastrointestinal tissue and two subcellular fractions was significant for all analyzed metals. Site-specific differences indicated the age-related increase of gastrointestinal Cu, Mn, and Cd concentrations towards more polluted sites, while significant correlation between metal concentrations in the gut content and fish age exists only for Mn. In the subcellular gastrointestinal fractions, site-specific differences were not recorded on total water-soluble protein and metallothionein concentrations, which was ascribed to the constitutional, basal metallothionein concentrations, as hypothesized in the previous study from the same group [17].

Several additional studies have been recently published on the assessment of the genotoxic effects in plant and animal species inhabiting the section of the Sava River in or close to the Zagreb City area. One new indicator fish species, the Balkan loach (*Cobitis elongata*) was introduced in the study reported in 2008 by Kopjar and colleagues [20]. The amount of DNA damage in the erythrocytes was estimated using the alkaline comet assay in loach specimens from the Sava River and the reference Kupa River. The obtained data revealed modest genotoxic damage in fish from the Sava River and demonstrated significantly lower levels of DNA damage in fish from the Kupa River. However, although a good DNA damage determination pattern was obtained for Balkan loach, due to its global and regional conservation status, only restricted use of a small number of specimens per sampling site was suggested. Another follow-up study of the SARIB project was published in 2011 by Pavlica et al. [21], again in native European chub specimens caught in different seasons at several locations that followed the pollution gradient of the Sava River. The extent of genotoxic damage was addressed by the comet assay and micronucleus test carried out on fish erythrocytes. The results of the comet assay showed the lowest genotoxic influence at the least polluted site, while higher DNA damage was observed at the polluted sites. Although the basal levels of DNA damage were also elevated, a clear gradation of DNA damage was found due to pollution intensity in all sampling periods. Likewise, the lowest cytogenetic damage as revealed by the micronucleus test was observed at the least polluted site. High variations in micronuclei frequency were observed between sampling periods, although the number of micronucleated erythrocytes was consistently the highest one at the most polluted site. The comet assay as a biomarker of genotoxic effect exhibited higher sensitivity in discriminating the genotoxic capacity of studied polluted sites while the micronucleus assay appeared to be less sensitive. However, the study demonstrated that in optimal biomonitoring programs, both tests should be used together as they can reveal different aspects of DNA damage.

As can be seen from this overview, most of the biomarker studies on the Sava River were traditionally performed on fish. However, apart from rather scarce studies that utilized mussels, two recent studies addressed genotoxic potential of the Sava River using new taxa. In 2012 Klobučar and colleagues assessed the genotoxicity by measuring DNA damage in hemocytes of the caged freshwater crayfish *Astacus leptodactylus* by means of comet assay and micronucleus test, integrated with the measurements of physiological (total protein concentration) and immunological (total hemocyte count) hemolymph parameters as additional stress biomarkers [22]. Crayfish were collected at the reference site (River Mreznica) and exposed in cages for 1 week at three polluted sites along the Sava River. The long-term pollution status of these locations was confirmed by chemical analyses of sediments. Statistically significant increase in DNA damage measured by the comet assay was observed at all three polluted sites comparing to the crayfish from the reference site. In addition, native crayfish from the mildly polluted site (Krapje) cage-exposed on another polluted site (Zagreb) showed lower DNA damage than crayfish from the reference site exposed at the same location, indicating adaptation and acclimatization of crayfish to lower levels of pollution. Micronuclei induction showed similar gradient of DNA damage as the comet assay. The observed increase in total hemocyte count and total protein content in crayfish from polluted sites also confirmed stress caused by exposure to pollution. The results of this study have proved the applicability of caging exposure of freshwater crayfish in environmental genotoxicity monitoring in the Sava River basin.

## 3 Evaluation of the Ecotoxicological Status of the Sava River Using Small-Scale or In Vitro Bioassays

Complementing biomonitoring programs traditionally based on the determinations of biomarker responses, our ability to monitor water quality has been additionally improved in recent decades through the use of ecotoxicological test methods based on the so-called small-scale or in vitro bioassays. Contrary to biomarker responses typically measured in biota collected from or exposed in situ to various environmental pressures in real environmental conditions, the bioassays are in aquatic toxicology mostly based on determinations of biological responses of various cellular components, cells, organs, or small animals that are laboratory-exposed to row environmental samples or more often to various chemical extracts of complex environmental samples [24, 25]. The use of these methods has the advantage of being highly sensitive, rapid, and reproducible. Furthermore, they require minute amounts of sample material and are thus well suited for screening large amounts of samples. These screening methods also have the advantage of being able to integrate the toxicological activity of multiple contaminants that act through a common toxic mechanism and making it possible to assess the total potential for a biological effect in complex samples. There are also disadvantages, however, as cell/tissue/organism-specific factors may result in data that are not applicable to other species or effects observed at the doses tested might not be environmentally relevant, making the ecological significance of bioassay data lower in comparison to biomarker responses.

Bioassays have rarely been used in the monitoring of the Sava River before the late 1990s, with the exception of the Ames test as a method of choice for detecting mutagenic/genotoxic potential. A more intensive application of bioassays actually started a decade ago, again mostly fostered by recent EU or other international research projects focused on chemical and ecological characterization of the Sava River basin. The first bioassay study performed after the war activities in the early 1990s was the study published in 1997 on the determination of MXR inhibitory potential of river water in the Sava River basin [26]. In this chapter we showed that the effect of MXR inhibitors present in water can be directly demonstrated in differently affected natural waters using the measurements of the rhodamine B accumulation in the gills of mussels exposed to either natural water samples or XAD-7 extracts of corresponding river waters. The sensitivity of direct measurement of MXR inhibitors in natural waters enabled the identification of the most significant point sources of contaminants within the stretch of the Sava River along the Zagreb City area. Water from the Sava River collected downstream of the inlet of municipal wastewaters had a higher MXR-inhibiting potential than water from the Sava River collected upstream of the inlet, even after a fivefold dilution. Furthermore, concentration of MXR inhibitors in the most polluted part of the Sava River appeared to be 3.6- and 5-fold higher than in the less polluted rivers Dobra and Korana, respectively.

A large-scale bioassay study focused on the evaluation of the chronic toxicity of the Sava River was more recently conducted within the EU FP6 SARIB project [8]. In the study published in 2008 by Källqvist and colleagues [27], the authors presented results on the analysis of the surface water and sediment samples that were in 2006 collected throughout the whole course of the Sava River, with 26 sampling positions selected in the riparian countries Slovenia, Croatia, Bosnia and Herzegovina, and Serbia. The sampling positions were chosen so as to encompass the Sava River basin and to consider the impact on the pollution of the Sava River by its major tributaries (Savinja River, Krka River, Kupa River, Una River, Vrbas River, Bosna River, and Drina River). The final samples were collected at Belgrade, just before the Sava River merges with the Danube. The algal growth inhibition test with the freshwater algae *Pseudokirchneriella subcapitata* was selected as a recommended method of choice for the determination of chronic toxicity of complex mixtures and wastewaters [28]. Although most of the samples were toxic to the algae, large differences in toxic potential were observed. The most toxic samples were up to 18,500 times (sediment extracts) and 32 times (pore water), respectively, more toxic than the least toxic sample. However, organic compounds in the water-soluble and particulate fraction of surface waters from the Sava River were less toxic to the algae. Only four (water-dissolved fraction) and nine (particulate fraction) of the total 21 surface water samples caused chronic toxicity to the algae. The results from this study clearly identified and confirmed several compartment-specific hot spots in the Sava River, and the performed toxicity screening revealed that sediments and river water from the some locations at the Sava River were sufficiently toxic to algae to cause growth inhibition when assessed by established classification and risk-assessment procedures.

As pointed out before, the majority of the studies described confirmed the Zagreb City wastewater treatment plant (WWTP) as the predominant input of pollution into the Sava River. Since Zagreb City has a mixed sewer system, including significant contributions from both domestic and industrial sources, the composition of contaminants in an untreated wastewater is rather complex [2, 25]. Considering this fact, it is especially relevant to discuss available bioassay data obtained after mid-2007, when the full-scale WWTP of Zagreb City becomes operational. According to our knowledge, there are two bioassay studies accomplished following this point, both performed by our group within the NATO Science for Peace and Security Programme directed to the assessment of hazardous chemical contamination in the Sava River basin using the so-called effects-directed analysis (EDA) approach [9]. The EDA protocols are, in principle, laboratory-based studies in which an environmental sample is treated using a variety of analytical chemical procedures and treated and untreated samples are tested for toxicity using various bioassays. EDA approach is today generally accepted as the most efficient way to accurately address problems associated with toxicity in water and sediments, offering a rational tool for risk characterization, toxicity reduction, and the identification of harmful substances in real-world matrices having impacts on aquatic ecosystems [29]. Our first study [30] was focused on the characterization of the Zagreb City wastewater as the major pollution input in the Sava River, using the EDA approach, i.e., a combination of bioassays and chemical analytical methods based on advanced sample preparation and analytical protocols, which allowed the identification of a wide variety of nontarget contaminants. The sampling strategy included analyses of raw wastewater and biologically treated effluents, and special attention was paid to the assessment of the relative importance of contaminants having different polarities. An integral part of the study was evaluation of the efficiency of removal of the observed toxic potential following the advanced WWTP recently established in Zagreb City. Over 100 individual contaminants or closely related contaminant groups were identified by high-resolution GC/MS and liquid chromatography/quadrupole time-of-flight mass spectrometry (LC-QToF-MS). The identified compounds covered a wide range of chemical structures and physicochemical properties, in particular with respect to the chemical compound hydrophobicity and/or polarity. Furthermore, the comparison of their semiquantitatively determined concentrations indicated a large variability of their respective concentration ranges, spanning over five orders of magnitude. Considering the bioassay data, ecotoxicity profiling of the investigated primary and secondary effluent samples, including cytotoxicity, chronic toxicity and EROD activity, inhibition of the multixenobiotic resistance (MXR), and genotoxicity, and estrogenic potential, revealed the most significant contribution of toxic compounds to be present in polar fractions.

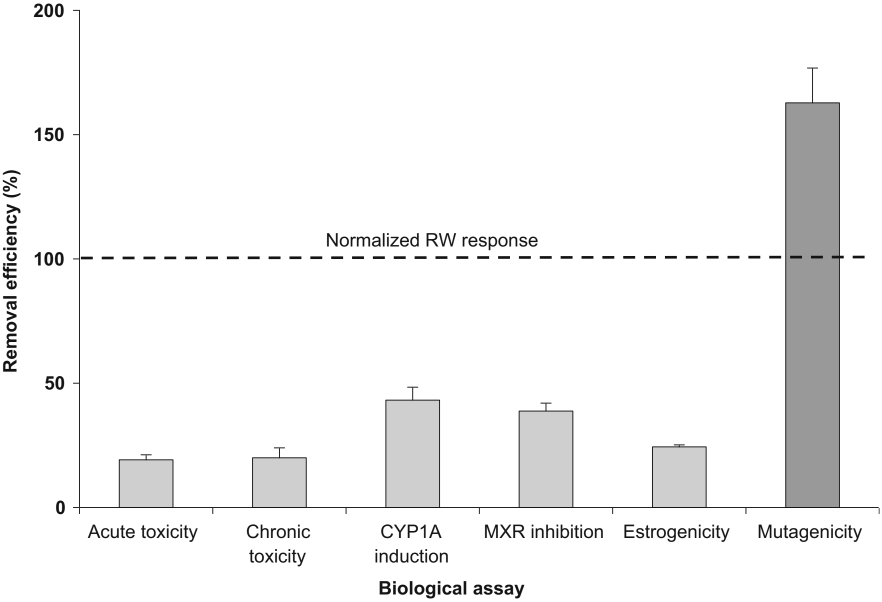

**Fig. 4** Comparative presentation of biologically relevant efficiencies of removal of toxic substances from the wastewater samples collected from the Zagreb City WWTP, as determined using a series of bioassays in the study published in 2011 by Smital and colleagues [30]. Removal efficiency, determined as toxic response in the secondary effluent (SE) sample, is expressed in comparison to the toxic response of the corresponding raw wastewater (RW) sample set at 100 %. Acute toxicity (cytotoxicity) was determined using the MTT assay, chronic toxicity by the algae growth inhibition test, CYP1A induction potential by the EROD assay, MXR inhibition by the calcein-AM assay, estrogenicity by the YES test, and mutagenicity by the Ames test, as described in detail in [30]. Mean ± SDs are shown

Finally, the advanced wastewater treatment using conventional activated sludge process reduced the initial toxicity of raw wastewater to various extents. Although chemical analysis showed that the most efficient toxicity removal was observed for the polar compounds, various bioassay end points used in the study clearly confirmed significant, biologically relevant removal efficiency. Yet, the efficiency varied considerably, ranging from 80 % for acute (cytotoxicity) and chronic (algal) toxicity to 57.2 % decrease in toxicity response for the CYP1A induction (Fig. 4). Mutagenicity determination by the Ames test appeared to be the only exception, as our data indicated possible activation of promutagenic substances that could have been present in the raw wastewater sample. Overall, this study clearly emphasizes the importance of polar organic contaminants in the Sava River. Since the polar fraction, due to analytical limitations, represents the least studied fraction in environmental matrices, further efforts need to be directed towards more detailed analysis of polar environmental contaminants in order to identify novel candidates contributing to different ecotoxicological end points.

Subsequently, using the knowledge obtained during the previous study, we recently performed the first regional specific prioritization of organic contaminants in the Sava River, using the described EDA approach. In the recently published study [31], we analyzed ecotoxic potential of surface water and sediment samples collected at four locations covering the already emphasized and well-studied 150-km long river section from the Slovenian–Croatian border to the confluence of the Una River, characterized by well-defined pollution gradients. Total extracts of water and sediment samples were subjected to toxicity screening using a series of small-scale or in vitro bioassays designed to characterize the biological response of hazardous contaminants with different modes of action, as has been done in our previous study. The cytotoxicity of the Sava River water extracts was very low at all locations studied and no significant differences between the individual sampling stations were observed. In contrast, a significant cytotoxicity was detected in all sediment samples, in particular those collected downstream of the Sisak City area, in agreement with the data from bioassay-assisted monitoring of the Sava River using the freshwater algae *Pseudokirchneriella subcapitata* [27], indicating that the effects may be related to industrial effluents from the Sisak City area, in particular those originating from the oil refinery activities. The distribution of EROD induction potential was generally in agreement with the distribution of cytotoxicity. As expected, a significantly enhanced EROD activity was determined in the secondary effluent sample from the Zagreb City WWTP. However, all examined river water samples were characterized by rather low EROD induction potential, with moderately increased activity at the Oborovo location, downstream of the Zagreb City main wastewater outlet. In contrast, high EROD induction potential was determined in the sediment samples, in particular at the locations downstream from the Sisak City, which again probably reflected an additional input of CYP1A inducers such as multi-ring PAHs from the oil refinery. The distribution of MXR inhibitors was significantly different, indicating location-specific differences in compounds causing the bioassay responses that inhibit MXR. The results revealed that these contaminants were primarily associated with the aqueous phase, while their concentrations in analyzed sediments were rather low. The estrogenic potential of both surface water and sediment samples suggested rather modest presence of (xeno) estrogens in the Sava River, most probably reflecting an efficient removal of those substances in the Zagreb City WWTP. Finally, the mutagenic/genotoxic potential of the Sava River samples was generally very low.

Nevertheless, most of the compounds detected in the analyzed water and sediment samples from the Sava River cannot be clearly associated with the specific end points tested. Therefore, we believe it is reasonable to assume that nonspecific biotests, e.g., acute or chronic toxicity determinations, are related to the most abundant compound classes found in the samples, including PAHs, phthalates, sterols, and surfactants. Except for PAHs, the other groups of prominent chemicals identified in the Sava River are not highly toxic. However, although surfactants are only moderately toxic to aquatic life, they should not be neglected when assessing the overall toxic potential since their concentrations in the river water are often 1,000 times higher than the concentrations of the classical hydrophobic contaminants. The observed ratios of measured environmental concentrations (MECs) and predicted no-effect concentrations (PNECs) for moderately toxic chemicals can often be higher than the corresponding ratios of the classical pollutants. That means that even less toxic contaminants may well be responsible for the observed adverse effects, and our preliminary risk-assessment data indicate that this scenario might be correct for the Sava River as well. The risk quotients (RQs) calculated for selected organic contaminants identified in this study revealed that besides PAHs, linear alkylbenzene sulfonates (LAS), cationic surfactants, and alkylphenol polyethoxylates (APEO) may represent the greatest risks for aquatic organisms in the Sava River. It is important to emphasize that surfactants were also the most abundant contaminants in the Sava River sediments. Obviously, their hydrophobic moieties allow an efficient adsorption onto river sediments, warranting the careful monitoring of surfactant contaminants in order to assess the overall indices of water quality. Apart from surfactants, comparatively high RQs were obtained for the personal care products benzophenone and galaxolide, indicating that municipal wastewater is a major source for discharge of pollutants to the Sava River. In addition, a high RQ was obtained for the environmentally ubiquitous plasticizer diethylhexyl phthalate, which even exceeded the EU WFD recommended maximum allowable concentration in the present Sava River water samples.

In addition, a study that for the first time used a plant species (duckweed) for ecotoxicity monitoring of the Sava River has been recently published [23]. In this investigation growth parameters and several additional end points (pigment content, peroxidase activity, lipid peroxidation, and genotoxicity measured by the alkaline comet assay) were used to detect the toxic and genotoxic effects of surface water samples on duckweed plants. The surface waters of different origin and pollutant burdens were collected monthly over a 3-month period at three sampling sites along the Sava River and its confluents. Surface water samples collected from all three stations caused reduction of duckweed growth rates, chlorophylls and carotenoid contents, and peroxidase activity. In contrast, damage to membrane lipids (estimated by malondialdehyde content) and especially to DNA (estimated by tail extent moment) markedly increased in duckweed exposed to industrial wastewater samples. The results from this study demonstrated the potential of the use of a widely available plant species as a sensitive indicator of water quality, further increasing the portfolio of indicator species that may be used in biomonitoring of the Sava River basin.

In conclusion, although it would be premature to use these data for the fully quantitative risk evaluation, the assessment of contaminants in the Sava River watershed clearly emphasizes the possible importance of certain emerging classes of organic contaminants, which are not included in the European and national monitoring strategies. This is particularly true for the most polar fraction. Despite the fact that polar contaminants remain the least studied class in environmental matrices, their bioavailability potential in the aquatic environment is rather high compared to the classical hydrophobic pollutants [32]. Consequently, typical representatives of this class, such as surfactants and pharmaceuticals, should be included in the future region-specific monitoring activities.

## 4 Evaluation of the Current Ecotoxicological Status of the Sava River

We close this chapter in an attempt to do a preliminary evaluation of the current ecotoxicological status of the Sava River. We do it by comparison of relevant analytical chemical determinations and biomarker or bioassay responses determined in monitoring studies performed in the 1980s versus the most recent studies accomplished in the late 2000s.

As mentioned before, the early and mid-1980s were the years with the highest pollution pressure on the Sava River. The industrial and agricultural activities in the former Yugoslavia experienced historical peaks and the use of pharmaceuticals and personal care products in municipalities was relatively high, all of it combined with dominance of environmentally unfriendly technologies and lack of the advanced wastewater treatment practices. After this period, however, the three important factors actually contributed to significant improvement in the chemical and ecological quality of the Sava River: (1) the breakup of Yugoslavia and related decrease in industrial activities during the warfare in the early 1990s, (2) the collapse of many industrial complexes in the postwar period combined with gradual implementation of more advanced production technologies and wastewater treatment practices in Slovenia and Croatia, and (3) the activation of the full-scale wastewater treatment plant of Zagreb City as the most significant point source of pollution along the Sava River. A comprehensive inventory of the current knowledge on hazardous chemical contaminants in the basin, with a special emphasis on wastewaters as their primary source, can be found in several recent studies [30–34].

Therefore, the important question here is whether the available biomarker and bioassay data sets allow any reliable comparison or even evaluation of the past and present ecotoxicological status of the Sava River? And do the biomarker/bioassay data point to any significant improvement? As may be expected, the answers are neither easy nor unambiguous, as both the chemical analytical and ecotoxicological techniques and tools significantly changed over the past decades. The facts that only a relatively short section of the river has been thoroughly studied, that various species have been used in biomarker studies, that bioassay approach has been used only recently, and that a full-scale, systematic monitoring program of the chemical and ecological status of the Sava River has never been established further make a reliable interpretation of data a challenging task. Nevertheless, there are some biological indicators that in part allow a reasonable comparison of past and more recent ecotoxicological status of the river.

The first potentially useful comparative biomarker relates to the exposure of fish species to CYP1A inducers. The most commonly used biomarkers are involved in

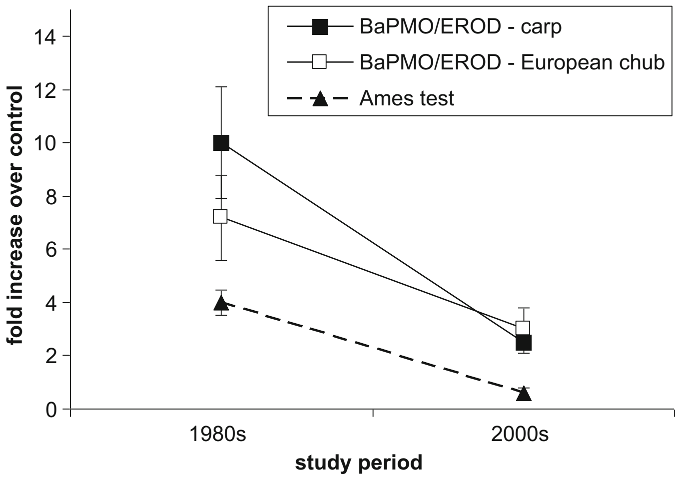

**Fig. 5** Comparison of selected biomarker responses determined in the 1980s versus more recent determinations performed in the 2000s. The B[*a*]PMO, EROD, or Ames test data determined in corresponding study periods are expressed as fold increase in biomarker responses over related controls. These were in the 1980s studies performed in carps from the local fish farm and the European chub specimens caught before Zagreb City [3–6] or in the 2000s studies performed in chub specimens held in laboratory for 3 weeks and carps i.p. treated with XAD-7 extract of the surface water collected before Zagreb City ([15] and our unpublished data, respectively). Controls for the Ames test were the mutagenic potentials of the Sava River surface water samples collected in corresponding periods at locations upstream of Zagreb City [4, 31]

the detoxification of xenobiotics and their metabolites (biotransformation enzymes like CYP1A), and alterations in these enzymes are being used as biomarkers of induction or inhibition. The induction/inhibition of fish CYP1A had been in the 1980s measured as an increase in B[*a*]PMO activity. However, the CYP1A determination had been in the late 1990s improved by the use of another, this time non-promutagenic substrate 7-ethoxyresorufin, and the related liver 7-ethoxyresorufin-*O*-deethylase (EROD) activity is now being used as a gold standard in the determination of the environmental exposure to CYP1A inducers or inhibitors [35]. Therefore, comparison of the B[*a*]PMO activities determined in the 1980s in fish from the Sava River can be used in relation to the EROD activities measured more recently, providing that appropriate controls are available and the comparison is based on the same fish species. Having those prerequisites set, relatively correct comparison of the results is possible. As can be seen in Fig. 5, data from the 1980s clearly showed that native carp and European chub specimens from the Sava River, caught at the most polluted locations within or downstream of the Zagreb City area, had from seven to over ten times higher B[*a*]PMO activities in comparison with the carps from the local fish farms or European chub specimens caught before Zagreb City, respectively. However, data on EROD activities determined in the course of the most extensive biomarker study performed in 2007 in European chub specimens from the Sava River [15] showed only threefold induction of the liver EROD activity in specimens from the most polluted locations, in comparison to the basal EROD level measured in specimens held in laboratory for 3 weeks. Likewise, i.p. treatment of carps with XAD-7 extract of the Sava River surface water collected in 2008 at the location downstream of the Zagreb City wastewater outlet showed only 2.5 induction in comparison to the response determined in carps i.p. treated with XAD-7 extract of the Sava River surface water collected before Zagreb City (Fig. 5, our unpublished data). Therefore, the levels of the liver B[*a*]PMO and EROD activities, respectively, determined in the 1980s and the late 2000s indicate a highly significant decrease in exposure of the Sava River native fish populations to CYP1A inducers.

The second biological parameter of potential comparative value is the measurement of the mutagenic potential of the Sava River surface water samples, as has been in both periods determined by the use of Ames test. In the 1980s, the mutagenic potential of the Sava River water collected downstream of the Zagreb City wastewater outlet resulted in approximately fourfold increase in the number of bacterial revertants (higher mutagenic potential) in comparison to the mutagenic potential of less polluted locations upstream of Zagreb City (Fig. 5). In contrast, no significant differences in mutagenic potential were determined between the same locations in surface water samples collected in the summer of 2008 [31], again indicating marked improvement in comparison to the mutagenic profiles determined in previous decades. This observation is further supported by data on tissue concentration of PAHs and PCBs in chub specimens determined in 2007 in the SARIB project study and reported in related article published by Krča et al. [15]. As the authors reported, the concentrations of the seven PCB congeners and PAHs determined in the muscle and liver tissue of chub specimens sampled in September 2005 at several locations on the Sava River revealed relatively modest increase in tissue concentration of PCBs and PAHs along the pollution gradient from the location upstream of Zagreb City towards locations downstream of Zagreb City and Sisak City areas, respectively.

The observed decrease in intensity of biomarker and/or bioassay responses indicates that fish either acquired a highly effective adaptation of their cellular detoxification machinery to pollution pressure or, more likely, that the recent level of pollution of the Sava River decreased in comparison with the levels experienced in the 1980s. In support of the later scenario, chemical analytical determinations of organic contaminants in the same section of the Sava River reveal the same pattern of decrease in the overall pollution load. Two caveats, however, make the interpretation of chemical analytical data less reliable. Firstly, chemical analytical determinations in the 1980s mostly relied on the GC/MS techniques [2, 36] which did not allow reliable determinations of more polar contaminants that were monitored in recently published studies using the LC/MS methodology [30–34], along with the GC/MS determinations. Secondly, most of the available data from both periods are semiquantitative estimates. Nevertheless, a comparison of estimated concentration ranges of several classes of organic contaminants amenable by the GC/MS approach and determined in the Sava River in the 1980s versus the late 2000s clearly shows 10- to 100-fold decrease in concentrations of contaminants typically used in industrial processes or household activities. An overview of the existing water quality of the Sava River was prepared in 2009 under the framework of the International Sava River Basin Commission and is publicly available [1].

In summary, despite the described historical drawbacks and inadequacies in the biological monitoring of the Sava River basin, we believe it is reasonable to conclude that ecotoxicological status of the Sava River greatly improved in the last two decades. Unfortunately, any comprehensive biomonitoring study has not been performed after 2007, the year when a full-scale mechanical and biological treatment of the Zagreb City wastewater treatment plant actually started. As Zagreb City remains the most important source of pollution of the Sava River, however, it would be interesting to see if, and to which extent, the advanced treatment of wastewaters further improved ecological status of the river. Therefore, considering all of the points discussed in this chapter, a well-defined biomarker and bioassay study coupled with advanced chemical determinations, both in selected indicator species and in wastewater, surface water, and sediment samples, would be highly recommended. In this regard, data from previous studies can and should be used as a highly valuable input critical for a scientifically sound design of future biomonitoring studies in the Sava River basin.

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