VERSITA

Biologia **69**/11: 1615—1624, 2014
Section Zoology
DOI: 10.2478/s11756-014-0460-y

Biomarkers in European perch (*Perca fluviatilis*) liver from a metal-contaminated dam lake

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Abstract: The present study was carried out in three seasons – spring, summer and autumn in Topolnitsa Dam Lake (Bulgaria) which has been subjected to continuous contamination with trace metals due to copper extraction in the area. We investigated the trace metal levels in surface water and liver samples of European perch (*Perca fluviatilis* L.). We also linked the metal levels we determined with the various histological and biochemical changes which we observed. Lesions in the liver parenchyma were found to be degenerative and necrotic, as well as, they were presented as hyperemia which consequently leads to disturbances in the hepatic blood circulation. Activities of the hepatic enzymes lactate dehydrogenase (LDH), alanine aminotransferase (ALAT) and aspartate aminotransferase (ASAT) were found to be significantly elevated, particularly in summer. Therefore, based on our results we could recommend that the investigated tissue and cell alterations may be successfully applied as reliable biomarkers for monitoring polluted with a mixture of trace metals freshwater ecosystems.

Key words: trace metals; liver; histological alterations; enzymatic activity; European perch

Introduction

Aquatic ecosystems are frequently contaminated with metals through anthropogenic activities, although many of them are naturally present and essential in low but toxic in higher concentrations (Lushchak 2011). Fish are considered among the most indicative organisms in freshwater ecosystems for estimating the effects of trace metal pollution (Moiseenko et al. 2008). Furthermore, fish are at the top of the aquatic food chain and may concentrate some metals from the water often in concentrations several times higher than in the ambient media (Mansour & Sidky 2002). Thus, monitoring sentinel fish species is widely used to assess the degree of trace metal accumulation and the effects on health status (Bervoets & Blust 2003).

Bioaccumulation is a process in which a toxicant is absorbed in an organism by all routes as it occurs in the natural environment, i.e., dietary and ambient environment sources (Arnot & Gobas 2006). Hence, bioaccumulation occurs primarily due to the inability to excrete necessary levels of contaminants and its degree is the result of imbalance between the input rate and the rate of toxicant elimination.

Trace metals are taken up through different organs and tissues of a fish because of the affinity between them. Furthermore, the organ surface serves as a metal-binding ligand and metal bioaccumulation can occur due to positively charged metal species in the water to negatively charged sites on the gills (Teien et al. 2006). In this process, many of these trace metals are concentrated at different levels in different organs of the fish body (Rao & Padmaja 2000). Thus, according to many authors (Cogun et al. 2003; Karadede et al. 2004; Jin et al. 2008; Liu et al. 2008; Monteiro et al. 2009; Oymak et al. 2009; Padmini & Usha Rani 2009) the liver is reported to be the primary organ for bioaccumulation and it has been extensively studied in regards to the toxic effects of trace metals.

Trace metal effects on fish can be studied at different levels of biological organization. Hinton & Laurén (1990), Bernet et al. (1999), Monteiro et al. (2005), Cazenave et al. (2009) and Marchand et al. (2009) consider that the different changes in many biochemical and morphological parameters of fish may be used as successful biomarkers for toxic effects of xenobiotics such as trace metals. Initial effects of metal pollution may be evident only at cellular or tissue levels before significant changes are identified in fish behavior or external appearance. Histological alterations for example have been examined for decades in fish tissues and organs in order to assess the effects of pollutants (Johnson et al. 1993; Stentiford et al. 2003; Au 2004). Various responses of enzymes have been also observed

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in fish exposed to metallic contaminants which indicated an increase or a decrease in the activity depending on the dose, species and route of exposure (Wong & Wong 2000; Lopes et al. 2001; Harikrishnan et al. 2003). Therefore, biomarkers have been proposed as sensitive tools for the early detection of environmental exposure to pollutants and their adverse effects on aquatic organisms (van Der Oost et al. 2003; De La Torre et al. 2005). Biomarkers also serve as links between the environmental contamination (cause) and its effects, providing unique information on the ecosystem health (Maria et al. 2009).

In the present study we hypothesized that the levels of contamination in the studied reservoir were linked to the process of metal bioaccumulation, histological alterations and enzymatic changes in the liver of European perch (*Perca fluviatilis* L., 1758) which could be further used as biomarkers for trace metal contamination. To confirm our hypothesis we sat the main objectives to (i) determine the trace metal concentrations (As, Cd, Cu, Ni, Pb, Zn) in surface water samples; (ii) determine the trace metal concentrations in European perch livers in three seasons (spring, summer and autumn); (iii) investigate the hepatic alterations; and (iv) examine the hepatic activities of the enzymes lactate dehydrogenase (LDH), alanine (ALAT) and aspartate (ASAT) aminotransferases.

Material and methods

Description of the study site

Topolnitsa Dam Lake, Bulgaria (42°25′90″ N, 23°59′38″ E) is built across Topolnitsa River in Bulgaria and is located in a region which is anthropogenically loaded with copper mines, metallurgy plants and mine tailings that have been left after the metals of interest have been extracted from the mineral rocks (Georgieva et al. 2014). In addition, the reservoir serves as a final sink for all types of contaminants which are carried with the river and its tributaries. Even though these circumstances call for a full investigation and monitoring, no data have been published over the last few decades on metal levels and their effects on fish from this artificial lake (Yancheva et al. 2014).

Water sampling

Water samples for trace element analysis were collected (ISO 5667-4:1987) using a boat once every season – spring (May), summer (July) and autumn (September). Prewashed double caped polyethylene bottles were used. They were rinsed three times with the water to be sampled prior to sampling. Samples were acidified with 1% HNO_3 (Merck Group, Darmstadt, Germany) and stored on ice for as short time as possible to minimize the changes of metals physicochemical characteristics before analysis. During the field trip, pH, temperature (°C) and conductivity (μS cm^{-1}) were recorded, simultaneously, using a field kit-meter (Multi 340i, WTW).

Fish sampling

European perch (51.8 ± 15.12 g; 14.3 ± 1.7 cm) was selected as an indicator species because it is one of the most abundant fish in the reservoir. Perch is a species that is typically found in both pristine and metal-contaminated lakes and it reflects the contamination of the sites where it is sampled (Eastwood & Couture 2002; Giguère et al. 2004). Therefore, perch is used as a test species in many ecotoxicological studies. In Sweden, for example biomarkers in perch have been used for more than 25 years to evaluate the environmental impact of pulp mills, metal industry, landfills and large population centres (Hansson et al. 2006).

During the present study thirty individuals in total (ten each season) were caught using fishing nets and a boat. All fish samples were collected according to the international standard procedures for determination of metal accumulation given in the EMERGE Protocol (Rosseland et al. 2001). Liver of each fish was dissected out and divided in three pieces for different analyses. For trace metal analysis the samples were kept on ice in the field and then frozen (–20 °C) until analysis. For histological analysis the samples were stored in formalin in the field and then processed for examination. For enzymatic assay the samples were frozen in liquid nitrogen in the field and then the sample preparation was continued in the laboratory.

Ten healthy European perch (52 ± 13.2 g; 13.9 ± 1.9 cm) were obtained from the Institute of Fisheries and Aquaculture in Plovdiv, Bulgaria and used as reference material. All experimental work was conducted in accordance with national and international guidelines of the European Parliament and the Council on the protection of animals used for scientific purposes (Directive 2010/63/EU).

Chemical analyses

Water analysis

Water analysis (ISO 17294-2:2003) was carried out at the regional laboratory of the Executive Environment Agency in Plovdiv, Bulgaria. Trace metal content in water was analyzed by using ICP-MS (Agilent 7500ce, Japan) and reported as mg L^{-1}. Certified reference standard for trace metals in waters SRM 1643ĺ (National Institute of Standards and Technology, USA) was used. Agreement with the standard was within 10%.

Fish analysis

Fish analysis was carried out at the regional laboratory of the Executive Environment Agency in Plovdiv, Bulgaria and reported as μg kg^{-1} wet weight. Prior to the actual assay approximately 1 g of each liver sample was mineralized wet using a microwave digestion system (Milestone Ethos Plus, Italy). Digestion solution was prepared with 6 mL of 65% HNO_3 and 2 mL of 30% H_2O_2 at 200 °C (Merck Group, Darmstadt, Germany). After mineralization the samples were brought up to 25 mL by adding ultra-pure water (Merck Group, Darmstadt, Germany) and underwent trace metal analysis. Metal content was analyzed by using ICP-MS (Agilent 7500ce, Japan) and certified reference standard DORM-3 (National Research Council Canada, Ottawa, Ontario, Canada) was used. Agreement with the standard was within 10%.

Histological analysis

All samples were placed in vials with 10% neutrally buffered formaldehyde solution (pH 7) for 12 h. They were rinsed in tap water, dehydrated in a graded series of ethanol concentrations, cleared in xylene, embedded in paraffin wax with melting point of 54–56 °C, sectioned to a thickness of 5–7 μm using a rotary microtome (model 840, American Optical Corp.) and mounted on sterilized glass slides. Sections were then deparaffinised, stained with hematoxylin and eosin (H&E) for histological examinations and prepared for light microscopy analysis (Takashima & Hibiya 1995).

Table 1. Topolnitsa Dam surface water quality.

Season	pH	T (℃)	Conductivity (μS cm^{-1})	Dissolved oxygen (mg L^{-1})
Spring (March)	8.2	5.3	640	7.5
Summer (July)	9	23.2	309	8.9
Autumn (September)	8.18	5.3	600	7.5
Average ± SD	8.46 ± 0.5	11.26 ± 10.3	530 ± 190	7.96 ± 0.81

Histological changes in the liver were observed and photographed by using microscope model SE (Nikon, Japan) mounted with a digital camera DCE-2, AMCAP software version 1.0.2. Liver histology of all specimens, including reference fish livers were appraised individually and semi-quantitatively by using the grading system of Peebua et al. (2006) which we slightly modified. Evaluation of the histological changes was carried out and presented as an average value in percentages. Each grade represents specific histological characteristics and is categorized as follows: no histological alterations – (–); mild histological alterations – (+/–); moderate histological alterations – (+); severe histological alterations – (++); and very severe histological alterations – (+++) in the hepatic architecture.

Enzyme assay

Sample preparation

Livers (pooled wet mass of each two individuals) were rapidly thawed on ice and manually homogenized, using a Potter Elvehjem homogenizer fitted with a Teflon pestle in chilled phosphate buffer (50 mM, 300 mM NaCl, pH 7.4). Homogenates were subjected to centrifugation at 9000 rpm for 15 min in a cooling centrifuge (MPW 351 R) at 4 ℃. Supernatant fractions were aliquotted, transferred in new eppendorf tubes and stored at –80 ℃ for further enzyme assays. All biochemical assays were measured spectrophotometrically (Beckman Coulter Spectrophotometer DU 800) at 25 ℃. Chemicals used for these analyses were purchased from Sigma-Aldrich Chemical Co. and were of analytical grade.

Lactate dehydrogenase

Lactate dehydrogenase (LDH, E.C. 1.1.1.27) activity was assayed in 100 mM potassium phosphate buffer (pH 7.4), 1 mM pyruvate, and 0.14 mM NADH lactate dehydrogenase and determined by measuring the amount of pyruvate consumed due to NADH oxidation at 340 nm (backward reaction) according to Vassault (1983).

Alanine and aspartate aminotransferase

Alanine aminotransferase (ALAT, E.C. 2.6.1.2) and aspartate aminotransferase (ASAT, E.C. 2.6.1.1) activities were determined by the method of Reitman & Frankel (1957) as described by Bergmeyer et al. (1986) using commercially available kits (Merck Group, Darmstadt, Germany). Briefly, ALAT was assayed in 1 mL containing phosphate buffer (100 μmol, pH 7.4), DL-alanine (100 μmol, pH 7.4), Ł-ketoglutaric acid (2 μmol, pH 7.4), and 0.2 mL of freshly prepared homogenate. Reaction mixture was incubated at 37 ℃ for 30 min. ASAT was assayed in 1 mL medium containing phosphate buffer (100 μmol, pH 7.4), L-aspartic acid (100 μmol, pH 7.4), L-ketoglutaric acid (2 μmol, pH 7.4), and 0.2 mL of freshly prepared homogenate. Reaction mixture was incubated at 37 ℃ for 1 h.

Protein analysis

Protein levels were measured by the Bradford (1976) method with Coomassie Brilliant Blue G-250 using bovine serum albumin as standard. Absorbance of samples was detected at 595 nm and expressed as milligram protein per milliliter homogenate.

One unit of LDH is defined as the amount of the enzyme that consumes 1 mol L^{-1} of substrate or generates 1 mol L^{-1} of product per min. Activity was expressed in international units per milligram of protein. ALAT and ASAT activity were expressed in international units per litre.

Data analysis

Statistical analyses on trace metal concentrations in water and liver samples, and enzyme activities, respectively were undertaken using the software program STATISTICA (version 7.0 for Windows, StatSoft, 2004). Differences between the individual variables were tested for significance using the Student's t-test ($P < 0.05$) and one-way analysis of variance (ANOVA). Relationships between the contents of elements in the collected water and liver samples were tested using Pearson correlation coefficient and Principle components and Classification analysis ($P < 0.05$). Data was reported as mean ± SD.

Results and discussion

Trace metals in water

Investigation of the fresh water quality aimed to indicate if it reflected significantly the trace metal accumulation in the analyzed fish organs. General freshwater chemistry data on Topolnitsa Dam Lake is presented in Table 1. Trace metal concentrations are presented in Table 2.

Trace metal concentrations in aquatic ecosystems are usually monitored by measuring their concentrations in the water where they generally exist in low levels but they attain considerable concentrations in the sediments and biota (Camusso et al. 1995). We compared the obtained trace metal concentrations in this study with the maximum permissible levels by the Bulgarian legislation based on Directive 2013/39/EU and the guidelines of WHO (1993) and EPA (2002). In general all trace metal levels were lower than the maximum permissible levels set by law except for arsenic which concentrations in spring were higher (Table 2).

Concentrations of cadmium, nickel, lead and zinc were less than the detection limit of the instrument in autumn and lead concentrations were less than detection limit in all three seasons. However, it seems that the toxic arsenic and copper are constantly present in

Table 2. Concentrations of trace metals in Topolnitsa Dam Lake fresh water, mg L^{-1}.

	Element (average ± SD)		
	Spring (May)	Summer (July)	Autumn (September)
As	0.023 ± 0.01	0.005 ± 0.001	0.012 ± 0.006
Cd	0.003 ± 0.01	<0.00005*	<0.00005*
Cu	0.016 ± 0.0005	0.024 ± 0.01	0.011 ± 0.0007
Ni	0.003 ± 0.01	0.001 ± 0.0005	<0.0005*
Pb	<0.001*	<0.001*	<0.001*
Zn	0.004 ± 0.001	<0.001*	<0.001*

Explanations: *less than the detection limit.

Table 3. Concentrations of trace metals in European perch liver from Topolnitsa Dam Lake ($n = 10$ for each season), mg kg^{-1} (wet weight).

	Element (average ± SD)		
	Spring (May)	Summer (July)	Autumn (September)
As	0.29 ± 0.5	0.26 ± 0.5	0.92 ± 0.1
Cd	3.67 ± 0.1	5.92 ± 0.3	6.09 ± 0.5
Cu	8.75 ± 0.3	45.6 ± 1.2	19.8 ± 1.3
Ni	0.14 ± 0.005	0.07 ± 0.005	0.07 ± 0.005
Pb	0.06 ± 0.01	0.05 ± 0.01	0.07 ± 0.003
Zn	31.8 ± 1.5	44.4 ± 1.5	42.6 ± 2.4

the surface water. Therefore, we consider that the pollution with these two metals is chronic but the presence of the rest of the metals is due to their background levels. Statistical analysis showed a significant difference ($P < 0.05$) only for cadmium and copper levels, and copper and lead levels in all three seasons. In addition, correlation between cadmium and zinc ($r = 1$) in spring, summer and autumn was established. Similarly to Dallas & Day (1993) we also consider that these two metals are present usually together in natural ecosystems.

Trace metals in fish

Bioaccumulation of trace metals from the water was assessed by measuring their levels in the fish livers. Trace metal levels in this organ represent the storage of metals from the water where the fish species live (Karadede et al. 2004) and the liver is considered to be a vulnerable target during prolonged metal exposures, both from waterborne and dietary sources (Olsvik et al. 2000). Trace metal concentrations in the reference fish livers were less than the detection limits (As < 0.01, Cd < 0.001, Cu < 0.01, Ni < 0.01, Pb < 0.03, Zn < 0.03). However, trace metal concentrations in European perch livers from Topolnitsa Dam Lake were significantly higher (Table 3). We determined highest concentrations for cadmium, copper and zinc in the livers in all three seasons and lowest concentrations were those of lead. Statistical processing of the data did not show any correlation between the metal content in the water and their corresponding levels in the perch livers. Therefore, we considered that the fish could receive most of the trace metal load through food since it is a predator species, rather than directly from water.

Statistical analysis proved that the arsenic levels in the livers in all three seasons were significantly different ($P < 0.05$) than those of cadmium and zinc. Furthermore, the cadmium levels were significantly different than those of nickel, lead and zinc. These results suggest that the studied metals show a various deposition degree in the liver. Thus, most pronounced was that of arsenic, following that by cadmium, lead and zinc. Statistical analyses also showed a negative correlation ($r = –1.00$) only between the hepatic levels of arsenic and nickel, which means that the presence of arsenic seriously hampers that of nickel. PCA and Factorial ANOVA analysis showed that for the trace metal accumulation the liver itself as a qualitative factor had stronger influence (83.31%) rather that the factor "season" (5.11%) (Fig. 1).

Furthermore, the element species as a factor had a stronger influence (52%) on the trace metal concentrations in water and livers than the factor "season" (48%) (Fig. 2).

Multiple regression analysis showed that the factor "element" was more important for the trace metal concentrations in water and livers ($P = 0.0004$), than the combined effect of the two factors "element" and "season" ($P = 0.04$). Overall, statistical analysis showed that the levels of the investigated trace metals in the European perch liver were higher in summer than in spring and they consequently decreased in autumn.

Moreover, in summer the hepatic copper concentration was highest from all investigated metal concentrations. We could link this fact with the higher fish metabolic activity in this particular season. Nonetheless, it is well known that the rates of metal uptake and accumulation increase with increasing the temperature

Table 4. Histological alterations in European perch livers from Topolnitsa Dam Lake ($n = 10$ for each season).

Hepatic alterations	Spring	Summer	Autumn
Cellular swelling and granular degeneration	+	++	+
Ballooning and hydropic degeneration	+/–	+	+/–
Fatty degeneration	+/–	+/–	+/–
Necrtoic alterations:			
– karyopyknosis	+/–	+/–	+/–
– karyorrhexis	+/–	+/–	+/–
– karyolysis	+/–	+	+/–
Hyperaemia	+/–	+	+/–

Explanations: no histological alterations – (–); mild histological alterations – (+/–); moderate histological alterations – (+); severe histological alterations – (++); and very severe histological alterations – (+++) in the hepatic architecture.

in ectothermic organisms (Velcheva 2006; Sokolova & Lannig 2008). Increase in metabolic rates at elevated temperatures may contribute to metal accumulation in ectotherms due to a higher energy demand, which results in elevated ventilation (Pörtner 2002).

Histological alterations

Exposure to trace metals may cause histological changes in the liver and a histological investigation of exposed specimens may therefore produce meaningful results (van Dyk et al. 2007; Marchand et al. 2009). The present study demonstrates that the reference fish liver generally exhibited a normal architecture with a typical parenchymatous appearance and there were no pathological abnormalities. Parenchyma itself was primarily composed of hepatocytes typically with a large central nucleus and homogenous cytoplasm. Hepatocytes were located among blood capillaries called sinusoids forming a cord-like structure known as hepatic cell cords. Lumen of sinusoids contained mainly erythrocytes. Venous blood entered the liver caudally from the intestine via the hepatic portal veins and branches into capillaries known as sinusoids. Sinusoids were lined with reticuloendothelial cells which were in turn surrounded by hepatocytes (Figueiredo-Fernandes et al. 2007). However, there were remarkable histological alterations in the liver tissue of the fish from Topolnitsa Dam Lake (Table 4).

In general, histological analysis showed a degenerative and necrotic change which indicates disturbances in the hepatic blood circulation (Fig. 3). Hepatocellular damages which were more frequently observed were presented in various forms of degeneration (Fig. 3A). Furthermore, these hepatic changes were detected in highest degree in the fish caught in summer which was also the season when we determined highest trace metal levels in the studied organ. We determined cellular swelling which was expressed in increasing the hepatocyte size, as well as lightening in color and small fine-grained granules in the cytoplasm (Fig. 3A).

In addition, we observed hepatocytes with more severe degenerative alterations such as ballooning degeneration (cytoplasmic swelling without vacuolization) and hydropic degeneration (water absorption) (Fig. 3B). Fatty degeneration was present in lipid deposits in single areas of the liver parenchyma (Fig. 3B).

Hence, hepatocytes were morphologically altered and resembled typical adipose cells with flattened nucleus located on the periphery. Such histological changes in liver were also observed in other fish species but following sublethal metal exposure (Monteiro et al. 2005).

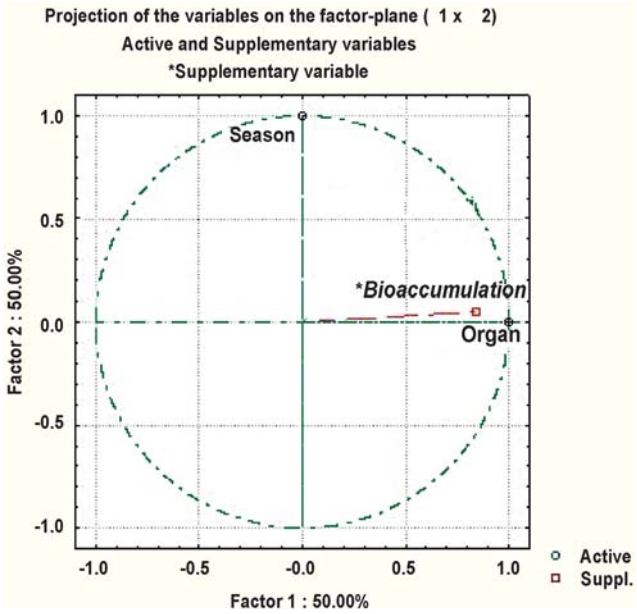

Fig. 1. PCA analysis shows which qualitative factor has more influence on metal bioaccumulation in the European perch liver. Factor 1 – "organ", Factor 2 – "season".

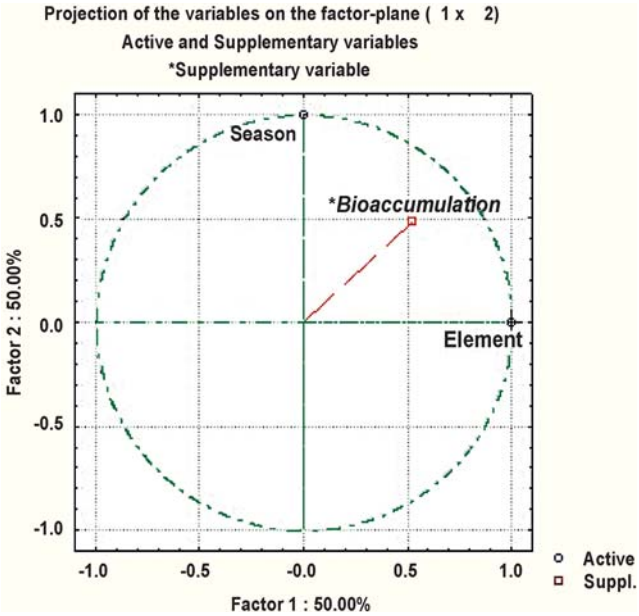

Fig. 2. PCA analysis shows which qualitative factor has more influence on metal bioaccumulation in the European perch liver. Factor 1 – "organ", Factor 2 – "season".

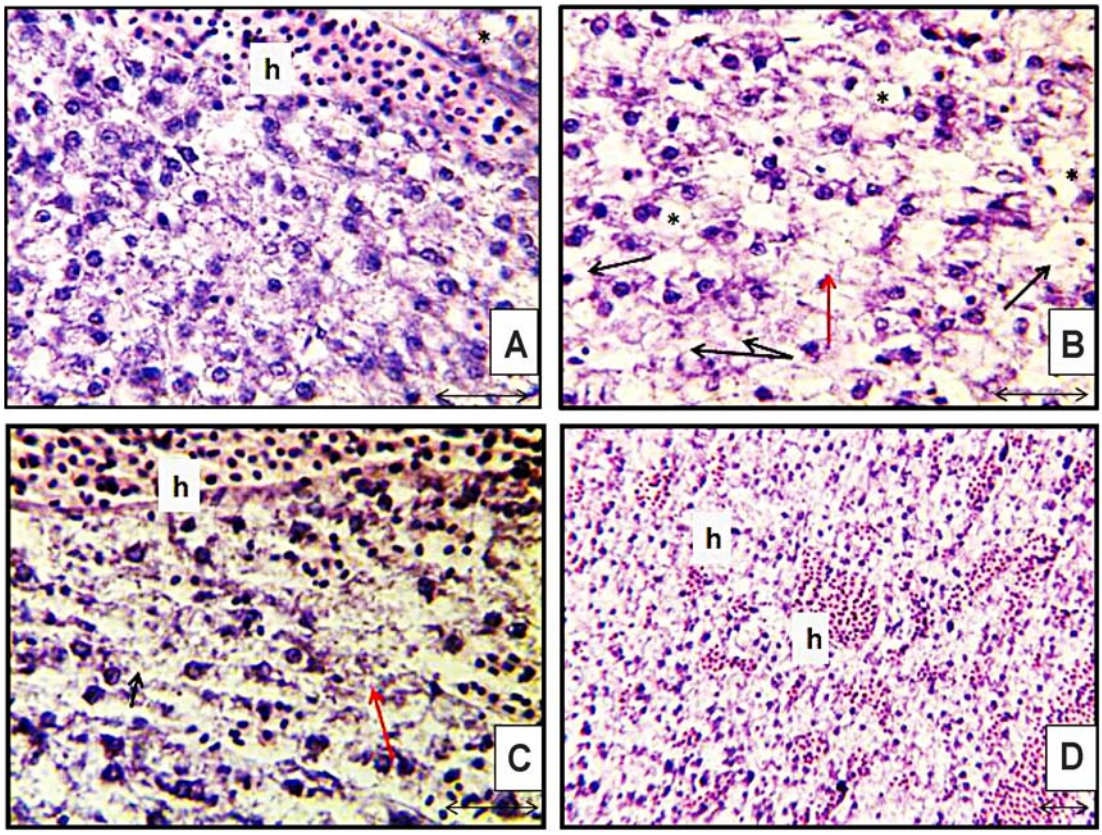

Fig. 3. Histological alterations in the European perch liver from Topolnitsa Dam Lake, H&E. A – Hepatocyte granular degeneration, blood vessel with pronounced hyperaemia (h), ×400. B – Hepatocytes with hydropic, ballooning (black arrow) and fatty (*) degeneration. Area of hepatic cells with karyopyknosis and karyorrhexis (red arrow), ×400. C – Hepatocytes with necrotic alterations – karyolysis (black arrow), karyorrhexis (red arrow), ×400. D – Major and small blood vessels with hyperaemia (h), ×200.

Necrotic alterations in the fish liver were associated with presence of karyopyknosis, karyorrhexis and karyolysis (Fig. 3B, C). These morphological changes, however, affected single hepatocytes and small sections of the liver parenchyma. Moreover, in the perch caught in spring and autumn these hepatic changes were presented in milder form, but in the fish caught in summer the morphological impairment was fairly moderate.

Our findings are in agreement with those of Varanka et al. (2001) who suggested out that metal accumulation in the liver of common carp (*Cyprinus carpio* L., 1758) causes hepatocyte lysis, cirrhosis and ultimately death.

Lastly, venous hyperaemia was observed in highest degree in the fish caught in summer. Hence, hyperaemia is linked to disturbances in the hepatic blood circulation (Fig. 3A). It was presented in the major and small blood vessels, as well as, hepatic sinusoids, most likely due to common vein congestion (Fig. 3D). We consider that this inevitably impacts the hepatic blood flow and could lead to hepatic atrophy and necrosis.

Overall, these histological changes identified within the hepatocytes in the present study could have been the result of various biochemical lesions. In scientific literature it is stated that cellular swelling occurs either directly by denaturation of volume-regulating ATPases or indirectly by disruption of the cellular energy transfer processes required for ionic regulation (Hinton & Laurén 1990). In addition, vacuolations of hepatocytes is a common response associated with exposure to many different toxicants. Such histological changes could represent lesions at biochemical level, including inhibition of protein synthesis and energy depletion.

Enzymatic responses

Antioxidant defences are commonly very well developed in the liver compared to other organs as a result of the central role of this organ in detoxifying toxicants and processing many metabolic products for degradation. This could be related to the fact that the liver is the site of multiple oxidative reactions and maximal free radical generation (Gül et al. 2004; Avci et al. 2005). Responses of LDH, ALAT and ASAT are presented in Fig. 4. Enzyme activities were enhanced in the hepatic tissue in all three seasons – spring, summer and autumn compared to the reference fish. Similarly, to Regoli (1998) we also think that the metal concentrations in organs and tissues are likely to change with the season, reflecting variability in the environmental inputs, but also changes in the metabolism. Low temperature reduces the metabolic rates in ectothermic organisms, and hence lower enzymatic activities are in general observed in colder seasons. Moreover, higher temperatures lead to an increase in oxygen consumption and consequently to ROS generation enhancement (Amado et al. 2006).

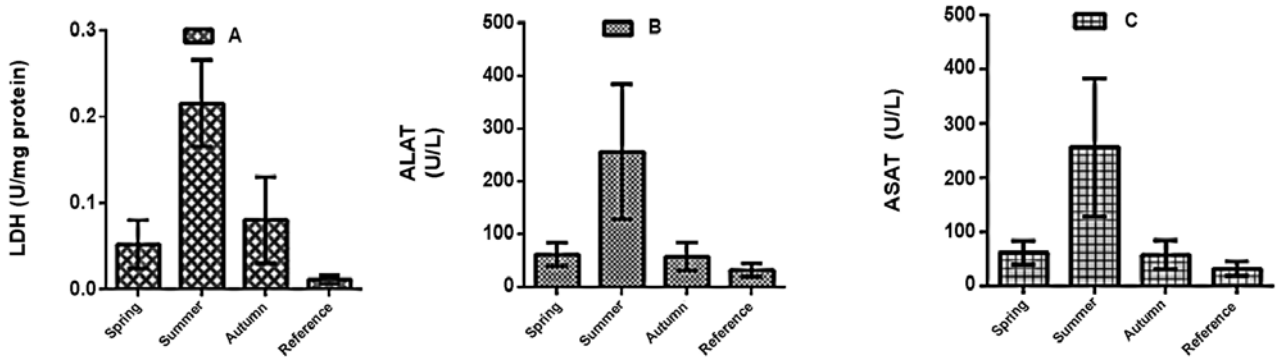

Fig. 4. Activities of LDH, U mg^{-1} protein (A), ALAT U L^{-1} (B), and ASAT, U L^{-1} (C) in the liver of European perch in three seasons (average ± SD), including enzymatic responses in reference fish.

We determined higher LDH activities in all three seasons compared to LDH activity in the reference fish, with highest LDH activity in summer, respectively (Fig. 4A). Statistical analysis did not show a significant difference among the enzyme responses in different seasons but the enzyme response in reference fish was significantly different ($P < 0.05$) than the enzyme response measured in summer. LDH is an enzyme located at a strategic point between glycolysis and citric acid cycle, which catalyzes the reversible oxidation of lactate to pyruvate, serving in the terminal step of glycolysis (Reddy et al. 2011). Furthermore, elevated lactate dehydrogenase (LDH) activity is a marker for metabolic changes in fish, i.e. glycogen catabolism and a glucose shift towards the formation of lactate, tissue damage in fish (Ramesh et al. 1993), hypoxic conditions (Das et al. 2004), and muscular harm (Balint et al. 1997) and serves as a good diagnostic tool in toxicology. According to the literature trace metal exposure on glycolytic enzymes is controversial. Toth et al. (1996), Carvalho & Fernandes (2008), Liu et al. (2010) and Reddy et al. (2011) found an increased LDH activity in fish exposed to trace metals (copper, cadmium, lead and mercury). In contrast, the exposure of *Sparus aurata* L., 1758 to copper lead to a decrease in hepatic LDH activity (Antognelli et al. 2003). We agree with Reddy et al. (1998) who stated that a LDH increase may favor the anaerobic respiration to meet the energy demand when aerobic oxidations are lowered. In addition, under anoxic conditions the body tries to shift respiratory metabolism to anaerobiosis and conversion of lactate to pyruvate (the end product of glycolysis) in order to mitigate the energy crisis (Calbreath 1992).

Alanine aminotransferase (ALAT) and aspartate aminotransferase (ASAT) are liver specific enzymes and they are more sensitive measures of hepatotoxicity and histopathologic changes and can be assessed within a shorter time (Balint et al., 1997). ALAT is present in high concentrations in the liver and to a lesser extent in the skeletal muscles, kidney and heart. ASAT is mainly located in the liver (liver guiding enzyme) and any change in its activity is suggested as reflecting the functional state of the liver (Van Vuren et al. 1994).

We found elevated ALAT and ASAT activities in the hepatic tissue of European perch from the studied site in all three seasons compared to reference fish (Fig. 4). ALAT and ASAT activities were generally higher in summer and also higher in all seasons compared to the enzyme responses in reference fish (Fig. 4B, C). However, there was a significant difference ($P < 0.05$) only between ASAT activity in summer and its activity in reference fish. Increased ALAT and ASAT activity suggests increased proteolysis, enhanced protein catabolism and hepatocellular damage in the organism. In the present study hepatic damage was confirmed by changes in ALAT and ASAT responses. Thus, our findings are in agreement with other authors (Rajyasree & Neeraja 1989; Oluah 1998, 1999) who stated that any changes in responses of ALAT and ASAT indicate tissue damage in organs such as the liver.

Generally, the trace metal levels in perch liver led to fish responses at tissue and cellular level, respectively. Therefore, we consider that the fish from the investigated reservoir suffers from severe disorders in the liver function which are linked to disorders in the protein and energy synthesis. On one hand, the histological and biochemical alterations which we observed could prove the trace metal stress on perch liver due to the fact that fish lives in a constantly water basin where metals such as copper are constantly present.

On the other hand, these alterations could also allow fish to activate their defense and adaptive mechanisms which might help them to survive in contaminated environment. We therefore suggest that further investigations in this particular field should be carried out to better understand the trace metal effects on hepatic function.

Conclusions

The data from our research which was carried out under field conditions in three different seasons revealed a highly complex link between the trace metal levels in water and European perch livers and the hepatic changes in morphological structure and enzyme activities, respectively. We could conclude that the most significant factor for the process of bioaccumulation is the trace element species and its properties. Furthermore, the trace metal deposition in the liver is enhanced by the season in which the toxicants enter the fish body.

Overall, the elevated trace metals in the livers led to important changes in this organ at tissue and cellular level. Thus, based on our findings we could recommend that the investigated histological and biochemical alterations could be successfully applied as reliable biomarkers for monitoring polluted with a mixture of trace metals freshwater ecosystems.

Acknowledgements

The authors express sincere gratitude to the East Aegean Sea River Directorate, Plovdiv and Ministry of Environment and Water of Bulgaria. We would like to thank all colleagues from Basin Directorate for Water Management – East Aegean Region for helping with water sampling and regional laboratory of the Executive Environment Agency in Plovdiv for helping with metal analysis.

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Received July 22, 2013
Accepted September 29, 2014