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Lead concentration increase in the hepatic and gill soluble fractions of European chub (*Squalius cephalus*) - an indicator of increased Pb exposure from the river water

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Abstract

Purpose: To examine if chronic exposure of feral fish to elevated Pb concentrations in the river water (up to 1 μg L^{-1}), which are still lower than European recommendations for dissolved Pb in surface waters (7.2 μg L^{-1}; EPCEU 2008), would result in Pb accumulation in selected fish tissues.

Methods: Lead concentrations were determined by use of HR ICP-MS in the gill and hepatic soluble fractions of European chub (*Squalius cephalus*) caught in the Sutla River (Croatia-Slovenia).

Results: At the site with increased dissolved Pb in the river water soluble gill Pb levels (17.3 μg L^{-1}) were approximately 20 times higher compared to uncontaminated sites (0.85 μg L^{-1}), whereas the ratio between contaminated (18.1 μg L^{-1}) and uncontaminated sites (1.17 μg L^{-1}) was lower for liver (15.5). Physiological variability of basal Pb concentrations in soluble gill and hepatic fractions associated to fish size, condition, sex or age was not observed, excluding the possibility that Pb increase in chub tissues at contaminated site could be the consequence of studied biotic parameters. However, in both tissues of Pb-exposed specimens, females accumulated somewhat more Pb than males, making female chubs potentially more susceptible to possible toxic effects.

Conclusions: The fact that Pb increase in gill and hepatic soluble fractions of the European chub was not caused by biotic factors and was spatially restricted to one site with increased dissolved Pb concentration in the river water points to the applicability of this parameter as early indicator of Pb exposure in monitoring of natural waters.

Key words: European chub, liver, gills, soluble fraction, lead, the Sutla River

1. Introduction

In the aquatic environment, metals present one of the major contamination problems and a permanent threat to health of both aquatic organisms and eventually humans, due to their high toxicity, persistence, and tendency to accumulate in sediment and living organisms (Has-Schön et al. 2006). Lead is one of four metals that have the most damaging health effects (Permyakov 2009), and therefore is included in the list of priority toxic pollutants within the European Water Framework Directive (EU WFD; EPCEU 2008). The interaction of Pb with proteins represents a fundamental mechanism by which Pb exerts its toxicity (Goering 1993). One of the best documented targets for Pb is the second enzyme in the heme biosynthetic pathway, a zinc enzyme δ-aminolevulinic acid dehydratase (ALAD; Magyar et al. 2005). Lead is also known to decrease specific activity of acetyl cholinesterase and cause neurotoxic effects (Reddy et al. 2007).

Lead toxicity was often reported for the aquatic organisms living in highly contaminated environments. For example, elevated blood Pb concentrations accompanied by decrease of ALAD activity were documented in stoneroller, sunfish, and hog sucker from mining-impacted sites (Schmitt et al. 2007a; Schmitt et al. 2007b). The bioavailability and biochemical activity of Pb released from smelters to waterways was also confirmed by findings of increased Pb concentrations in blood, carcass, or liver of various fish species, as well as reduced ALAD activity (Schmitt et al. 2002). With the aim to prevent the toxic effects on the aquatic biota and preserve biodiversity, various environmental agencies have defined the acceptable concentrations for dissolved Pb in natural waters. The recommended value in the EU WFD (EPCEU 2008) for the annual average dissolved Pb concentration in surface freshwater is 7.2 μg L^{-1}, whereas the US Environmental Protection Agency (US EPA 2006) has recommended even lower concentrations for Criterion Continuous Concentration (CCC) of Pb (2.5 μg L^{-1}). The CCC is an estimate of the highest concentration of a material in surface water to which an aquatic community can be exposed indefinitely without resulting in an unacceptable effect (US EPA 2006).

However, even within the acceptable concentration range, Pb concentrations can vary considerably. In regular monitoring of natural freshwaters, it is common practice to only define whether the concentrations of dissolved Pb are below or above the recommended threshold. This kind of approach neither enables spotting the increasing trend of Pb concentrations in the water before they surpass the acceptable limits, nor implementation of adequate

remediation measures before the appearance of negative effects. Therefore, it is essential to evaluate whether any changes occur after long-term exposure of aquatic organisms to increased Pb concentrations even within "the environmentally acceptable range".

During the water quality monitoring of the Sutla River in Croatia, it was established that, contrary to low Pb concentrations at the majority of sampling sites (≤ 0.1 μg L^{-1}), at one restricted area dissolved Pb concentration was higher, amounting approximately to 1 μg L^{-1} (Dragun et al. 2011b). A key process in environmental risk assessment is coupling the concentration of pollutants found in the environment to the concentrations in the tissues of exposed organisms (Shinn et al. 2009). With the aim to define if exposure to increased, but still environmentally acceptable, Pb concentrations, results in the increase of Pb accumulation in tissues of exposed organisms, we have performed a study on the European chub (*Squalius cephalus*) from the Sutla River. Chub was selected as an important bioindicator, due to the fact that it is an omnivorous fish species, wide spread in European freshwaters, and therefore suitable for monitoring purposes. Lead concentrations were determined in two target organs: gills, due to their direct contact with the ambient water; and liver, as the main detoxification and storage organ. Unlike the majority of environmental studies, Pb concentrations were not measured in the whole digested tissues, but rather in the soluble tissue fractions.

The aim of this approach was to obtain the information on the portion of metal which is presumably available for the interactions with vital cell components and consequently could cause toxic effects. It has been described for some fish species that Pb is mainly located in the non-cytosolic fraction which is considered as detoxified (Linde et al. 1999), and by the measurement of the total Pb load, the information on variations of small, but possibly toxic Pb fractions could be disguised. The specific aim of our study, therefore, was to define whether long-term exposure of chub to dissolved Pb concentration of 1 μg L^{-1} in the river water results in increased Pb concentrations in the soluble fractions of chub gills and liver. In addition, the physiological variability of Pb concentrations in the soluble tissue fractions was assessed, to examine the possibility of its use as an early indicator of increased Pb exposure in the regular monitoring of natural freshwaters.

2. Materials and methods

2.1 Study area and period

The study was carried out on the Sutla River, a 91 km long river flowing along Croatian and Slovenian state border, with a water discharge between 0.73 and 68.8 m^3 s^{-1} and a catchment area of 581 km^2 (Dragun et al. 2011b). It is a tributary to the Sava River, a major river in Croatia. Five locations (Fig. 1) covering the entire course of the river were selected for fish sampling to enable the assessment of the influence of different anthropogenic pressures on the river water quality (medium industrial facility, household and thermal bath discharges, agricultural runoff; Dragun et al., 2011b). The fish were sampled in the late summer of 2009, from September 14th to 16th, to avoid the influence of the reproductive period on the analyzed parameters, as suggested by Podrug and Raspor (2009).

2.2 Fish sampling

Fifteen specimens of European chub (*Squalius cephalus* L.) were caught per site. The sampling was performed by electro fishing in accordance with the Croatian standard HRN EN 14011:2005. The captured fish were kept alive in opaque plastic tank with aerated river water till further processing in the laboratory. Individual fish were anesthetized with Clove oil (Sigma Aldrich) and sacrificed, and biometric data was recorded: total mass, total length, mass of liver, gills, and gonads. The gills and liver were stored at -80°C until further analyses. For sex determination we have compared the results from field observations and results obtained in the laboratory through both macroscopic and microscopic examination of gonads. For microscopic identification of sex, a section of gonad tissue from each fish was placed on a glass microscope slide. The slides were viewed under a 40 and 100 times amplification using optical microscope (BH-2 Olympus). Hepatosomatic (HSI, %) and gonadosomatic (GSI, %) indices were calculated as ratios between tissue mass (liver and gonads, respectively) and total body mass (g), multiplied with 100 (Şaşi, 2004). Fulton condition indices (FCI) were calculated in accordance with Rätz and Lloret (2003). A few scales (5-10) were taken from below the dorsal fin, and stored for subsequent age determination. The age was determined by counting growth annuli using an optical microscope (BH-2 Olympus; 40 times amplification) (Ognev and Fink 1956, Treer et al. 1995).

2.3 Isolation of S50 tissue fraction

The gill and liver tissue samples were first diluted 6 times with cooled homogenization buffer (20 mM Tris-HCl/Base (Sigma), pH 8.6 at 4°C) supplemented with reducing agent (2 mM dithiotreitol (Sigma)), and then homogenized by 10 strokes of Potter-Elvehjem homogenizer (Glas-Col, USA) in an ice-cooled tube at 6,000 rpm.

For better separation, the homogenates were centrifuged twice (Avanti J-E centrifuge; Beckman Coulter) at 50,000×g for 2 h at 4°C. The supernatant (S50) obtained from the second centrifugation, which corresponds to the water-soluble tissue fraction, was separated and stored at -20°C for subsequent metal analyses.

2.4 Determination of Pb concentrations in the soluble fractions of chub gill and liver

Sample preparation for Pb determination in S50 fractions of chub gill and liver comprised of a simple S50 dilution with Milli-Q water (1:9) and addition of 20 µL of 65% HNO_3 (*Suprapur*, Merck, Germany) per 2 mL of diluted S50 prior to measurement (Dragun et al. 2011a). The measurement was performed on high resolution inductively coupled plasma mass spectrometry (HR ICP-MS, Element 2, Thermo Finnigan, Bremen, Germany), equipped with a double focusing mass analyzer using reverse Nier-Johnson geometry. An autosampler (ASX 510, Cetac Technologies, USA) and sample introduction kit consisting of a SeaSpray nebulizer and cyclonic spray chamber Twister were employed to transport the analytes into the plasma of the HR ICP-MS. Measurements of ^{208}Pb were operated in low resolution mode. Indium (1 µg L^{-1}; Indium Atomic Spectroscopy Standard Solution, Fluka, Germany) was added to samples as an internal standard (Dautović 2006) thus enabling the use of external calibration with the standards prepared in 2% HNO_3 (S*uprapur*, Merck, Germany) by appropriate dilutions of 100 mg L^{-1} multielement stock standard solution (Analytika, Prague, Czech Republic).

2.5 Analytical quality control

The samples were prepared in duplicate for Pb measurements. A quality control sample (QC for trace metals, UNEP GEMS/Water PE Study No. 7, Canada) was used for checking the accuracy of Pb measurements by HR ICP-MS. A generally good agreement was observed between our data and the certified values. Limit of detection (LOD) and limit of quantification (LOQ) were determined based on three and ten standard deviations, respectively, of ten consecutive Pb determinations in the blank sample (2 mM Tris-HCl/Base, 0.2 mM dithiotreitol, 0.65% HNO_3). The LOD and LOQ amounted to 0.010 and 0.034 µg L^{-1}, respectively. Taking in consideration that measurements were performed in ten times diluted S50 hepatic and gill fractions, the lowest Pb concentrations that could be reliably detected and quantified amounted to 0.10 and 0.34 µg L^{-1}, respectively.

2.6 Statistical analyses

Statistical analyses were performed using SAS for Windows, SAS 9.1.3 Service Pack 4. One-way ANOVA was applied to compare the levels of measured parameters at the five sampling sites, as well as Pb concentrations in S50 fractions of different age groups. Tukey *post-hoc* pair-wise comparisons were then performed. For comparison of the levels of measured parameters at contaminated and uncontaminated sites, as well as Pb concentrations in S50 fractions of females and males, t-tests were applied. The level of significance was set at $p<0.05$. If necessary, data were transformed to obtain normal distribution which was confirmed by Shapiro-Wilk or Kolmogorov-Smirnov test. The correlation analysis was performed by use of Spearman correlation coefficient.

3. Results and discussion

3.1 Association of Pb concentrations in the hepatic and gill S50 fractions with dissolved Pb levels in the water

Simultaneously with fish sampling in September 2009, dissolved Pb concentrations in the river water were determined by Dragun et al. (2011b) at the same five sites where chubs were caught (Fig. 1). At four sites (sites 1 and 3-5) dissolved Pb concentrations were either below or equal to 0.1 μg L^{-1}, thus those sites could be defined as uncontaminated with Pb. At only one location (site 2), Pb concentration was prominently increased (0.910±0.056 μg L^{-1}), singling out that site as Pb-contaminated (Dragun et al., 2011b). The observed Pb concentration was well below the environmental quality standard set in the WFD (EPCEU 2008), recommending 7.2 μg L^{-1} as acceptable annual average dissolved Pb concentration in the river water, as well as bellow the CCC of 2.5 μg L^{-1} recommended by US EPA (2006). Based on that fact, and despite the obvious water contamination with Pb, the increase at site 2 would be overlooked in regular monitoring of natural water, and the water categorized as having good chemical status. However, if Pb accumulates in the tissues of aquatic organisms already at this level of contamination, it could possibly have detrimental effects on their health. By measuring the Pb concentrations in the soluble fractions of gills and liver of European chub from differently contaminated sites of the Sutla River, we aimed at establishing whether the accumulation of Pb in the tissues of this selected bioindicator organism actually occurs even within the acceptable limits for dissolved Pb in the river water.

The concentrations of Pb in the soluble fractions of both liver and gills of European chub from the Sutla River (Fig. 2a and b) followed the spatial distribution of dissolved Pb in the river water (Dragun et al., 2011b). The similar

pattern of Pb variability in both tissues was confirmed by high, statistically significant, positive correlation between Pb concentrations in the hepatic and gill S50 fractions ($r=0.706$; $p<0.0001$; $n=72$). The highest Pb levels were obtained at the sampling site 2, indicating Pb accumulation in the fish tissues already after exposure in the river water weakly contaminated with Pb. This can be explained by the known fact that metal accumulation in the tissues of aquatic animals is dependent not only on the exposure concentration, but also on the duration of exposure (Ay et al. 1999), and confirms once again that regulation of Pb was not characteristic for fish (Heath 1987). For further statistical comparison, data obtained at four sites with low Pb level were pooled together and designated as uncontaminated, whereas data obtained at the site 2 were designated as contaminated. Comparison of Pb levels in the hepatic and gill S50 at uncontaminated sites revealed slightly higher hepatic (1.17 μg L^{-1}) than gill (0.85 μg L^{-1}) Pb concentrations (38%), whereas they were comparable at the contaminated site (hepatic: 18.1 μg L^{-1}; gill: 17.3 μg L^{-1}). On average, Pb levels in the gill S50 were 20.4 times higher at contaminated compared to uncontaminated sites, whereas the ratio between contaminated and uncontaminated sites was lower for liver (15.5). However, the differences between uncontaminated and contaminated sites were statistically significant in both cases ($p<0.0001$). Although Pb accumulation was obvious in both tissues, it seemed to be somewhat more pronounced in the gills. Our results are in agreement with the report of Atli and Canli (2008), suggesting both gills and liver as equally eligible for Pb accumulation in *Oreochromis niloticus* after waterborne Pb exposure. However, more pronounced Pb accumulation in the gills than in the liver has been reported for the freshwater fish *Labeo umbratus* (Nussey et al. 2000), as well as for *Tilapia zillii* at higher level of Pb exposure (1-4 mg L^{-1}; Ay et al. 1999). Similar to our results, a study on fathead minnow (*Pimephales promelas*) revealed higher Pb concentrations in the viscera than gills of unexposed fish, contrary to more evident accumulation in the gills after exposure to sublethal Pb levels (Spokas et al. 2006). Experimental bioaccumulation studies in carp (*Cyprinus carpio*) also revealed higher Pb concentrations in the gills than liver, as opposed to the higher Pb levels in the liver than gills in the unexposed fish (Nishihara et al. 1985). This may be explained by the fact that blood Pb is mainly carried by erythrocytes (Eisler 1988), so the high concentrations of Pb in the gills may reflect uptake from the blood passing through (Spokas et al. 2006). Furthermore, increased Pb concentrations in the gill tissue could be the consequence of increased mucus excretion from gills in Pb exposed fish, as described for *Oncorhynchus kisutch* (Varanasi and Markey 1978) and *O. niloticus* (Cicik et al. 2004).

3.2 Physiological variability of Pb concentrations in the chub hepatic and gill S50 fractions

To be able to use a specific parameter, such as metal concentrations in the fish tissues, as an indicator of anthropogenic influence on fish, it is necessary to exclude the possibility of its changes due to natural, physiological causes, for instance as a result of differences in size, condition, sex or age. In the cases when such variability is established, the specimens of minimal variability in biometric parameters should be used for monitoring purposes, to prevent false interpretation of collected data. In order to examine the physiologically driven variability of Pb concentrations in the chub hepatic and gill S50 fractions, we tested their dependence on the total chub mass, FCI, HSI, GSI, sex and age. The fact that chubs sampled during this study were of variable mass (33.0-400.5 g), age (1-4) and of both sexes (Table 1), made such analysis unavoidable. The analyses were conducted separately for uncontaminated and contaminated sites. Lead concentrations measured in the chub hepatic S50 fraction during this study at uncontaminated sites (1.89±2.17 μg L^{-1}) fell within the previously defined basal range for the chub hepatic S50 fraction based on a former study performed on the Sava River in Croatia (0.97-5.93 μg L^{-1}; Dragun et al., 2011a). Therefore, analysis of the data from uncontaminated sites provided insight on the physiological variability of basal Pb levels, whereas analysis of the data from the contaminated site indicated the physiologically-induced differences of Pb accumulation in the tissues of exposed organisms.

For basal Pb concentrations, dependence on biometric parameters was not observed in this study (Tables 2 and 3). The correlations with biometric parameters were low and insignificant (Table 2), and the levels measured for males and females, as well as for the different age groups (Table 3) were comparable, presenting no significant differences in either tissue. A somewhat different situation was observed in the case of chubs from the contaminated site. Although the correlations with fish mass and biometric indices were not significant, possibly due to small dataset (n=15) within Pb-exposed group, positive associations between hepatic Pb level and total fish mass and GSI were obtained, whereas gill Pb level correlated positively only with GSI (Table 2). In both tissues, females had higher Pb concentrations than males, with a statistically significant difference in the liver (Table 3). Additional comparison for Pb-exposed chubs revealed that females at sampling site 2 also had significantly ($p<0.01$) higher total mass and GSI (average: 93.5 g and 2.24%, respectively) compared to males (average: 58.4 g and 1.32%, respectively), which may be the explanation of positive correlation of cytosolic Pb with mass and GSI (Table 2). Differences between age-groups were not observed (Table 3). Contrary to our findings, dependence on the fish age was reported for total Pb load in the gills and liver of *Abramis brama*, starting with a concentration increase up to 4 years of age, followed by a decrease up to 8 years of age (Farkas et al. 2003). Similarly, Nussey et al. (2000) found

Pb accumulation decrease in the tissues of the moggel (*Labeo umbratus*) in correlation with the increase in fish length. The finding that Pb concentrations in the soluble tissue fractions of European chub do not seem to depend on fish age and size, together with the fact that they provide information on the Pb portion which presumably could cause toxic effects, is a noteworthy advantage of this parameter over measuring the total metal load inside the fish tissues which is common practice in environmental monitoring. Therefore, it can be concluded that increase of Pb concentrations in the soluble tissue fractions of unexposed organisms solely due to physiological causes is not expected, making this parameter potentially a good indicator of chronic environmental exposure to Pb even in weakly contaminated water, such as the water at sampling site 2. However, there is an indication, which should be further explored, that under the conditions of increased exposure to Pb, females might be more susceptible to Pb accumulation in their tissues than males.

In addition, it should be emphasized that obvious Pb accumulation observed in the liver and gills of European chub already after chronic exposure to 1 μg L^{-1} of dissolved Pb in the water is a good early indicator of exposure, but gives no information about the possible toxic effects. The soluble tissue fractions also contain metallothioneins (MT), specific metal binding proteins, which represent an important mode of metal detoxification (Olsson et al., 1998). Binding on these proteins would prevent Pb association with some vital enzymes (Roesijadi and Robinson 1994), meaning that Pb could be present in the cytosol in the detoxified form. On the other hand, labile binding of Pb to MT (Huang et al. 2007), or even absence of Pb-binding MT-proteins has been frequently reported in the literature (Atli and Canli 2003; Reichert et al. 1979; Roesijadi and Robinson 1994). For example, after exposure of *O. niloticus* to as much as 4 mg L^{-1} of Pb, Atli and Canli (2008) described a significant Pb accumulation in the liver which was not reflected in adequate MT increase. Campana et al. (2003) hypothesized that Pb is a weak MT inducer relative to other metals because of its comparatively low sulfhydryl binding affinity. Therefore, possible toxicity as a result of Pb increase in the soluble tissue fractions should also be considered. Heier et al. (2009) reported that 15-45 μg L^{-1} of total Pb in water can cause ALAD suppression, a specific and extremely sensitive indicator of environmental Pb exposure (Schmitt et al. 2007b), in brown trout (*Salmo trutta*) under ecologically relevant exposure scenarios. In rainbow trout (*Oncorhynchus mykiss*) as little as 10 μg L^{-1} of Pb may significantly inhibit blood ALAD already after two weeks of exposure (Hodson et al. 1977), confirming previous findings that ALAD inhibition is not equally sensitive to Pb in all species (Campana et al. 2003; Schmitt et al. 2005). In addition, behavioural effects have been induced in mirror carp (*Cyprinus carpio*) at ≤50 μg L^{-1} (Shafiq-ur-Rehman 2003), as

well as in rainbow trout (*Oncorhynchus mykiss*) already at 29 μg L^{-1} of waterborne Pb (Burden et al. 1998). For this reason it would be essential to direct future studies towards establishing whether or not observed Pb accumulation in fish tissues due to exposure to Pb concentrations lower than acceptable limit for freshwaters could also be accompanied with corresponding toxic effects and disorders.

4. Conclusions

The study on the Pb accumulation in the soluble fractions of the gills and liver of the European chub was conducted in a weakly contaminated aquatic environment, with Pb concentrations varying below recommended limits for natural freshwaters. The results revealed clear Pb accumulation in both selected tissues of this bioindicator species at the site with increased dissolved Pb level, with physiological variability being excluded as the possible cause. Only in the group of Pb-exposed chubs, females seemed to accumulate somewhat more Pb in their tissues compared to males, which could eventually lead to their higher susceptibility to possible toxic effects. Since Pb concentrations in the soluble fractions of two selected tissues of European chub unambiguously pointed to the increase of Pb bioavailability in the ambient water, they should be considered as an additional early indicator of increased Pb exposure in environmental monitoring studies.

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Figure captions

Fig 1 The map of the Sutla River with marked sampling sites (1-5). The arrows indicate direction of the river flow.

Fig 2 Lead concentrations at five sampling sites of the Sutla River in September 2009: a) boxplots of Pb in the hepatic S50 fraction of European chub; b) boxplots of Pb in the gill S50 fraction of European chub; the boundaries of boxplot indicate 25^{th} and 75^{th} percentiles; a line within the box marks the median value; whiskers above and below the box indicate 10^{th} and 90^{th} percentiles, whereas dots indicate 5^{th} and 95^{th} percentiles; S50, water soluble tissue fraction (see Materials and Methods for details). The sites with statistically significantly different Pb concentrations in S50 fraction according to *post-hoc* Tukey's test ($p<0.05$) are indicated with different letters (a, b, c).

Fig 1


N
1 - Hum
2 - Donje Brezno
river Sutla
3-Kumrovec
4-Klanjec
SLOVENIA
CROATIA
river Sutla
river Sava
5 - Drenje Brdovečko
river Sava
Zagreb
0
14.5 km

Fig 2a

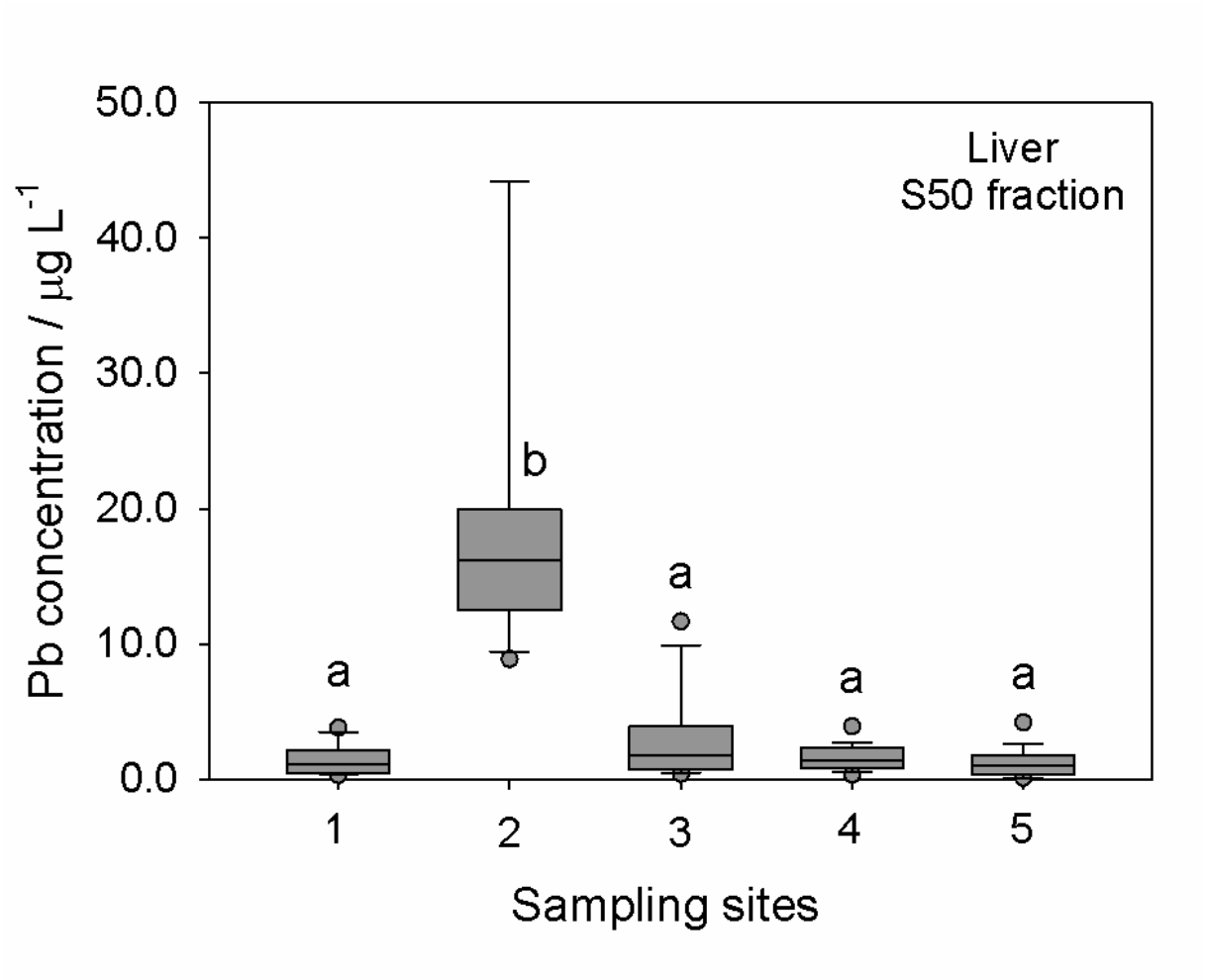
Liver
S50 fraction
Pb concentration / μg L-1
50.0
40.0
30.0
20.0
10.0
0.0
a
b
a
a
a
1
2
3
4
5
Sampling sites

Fig 2b.

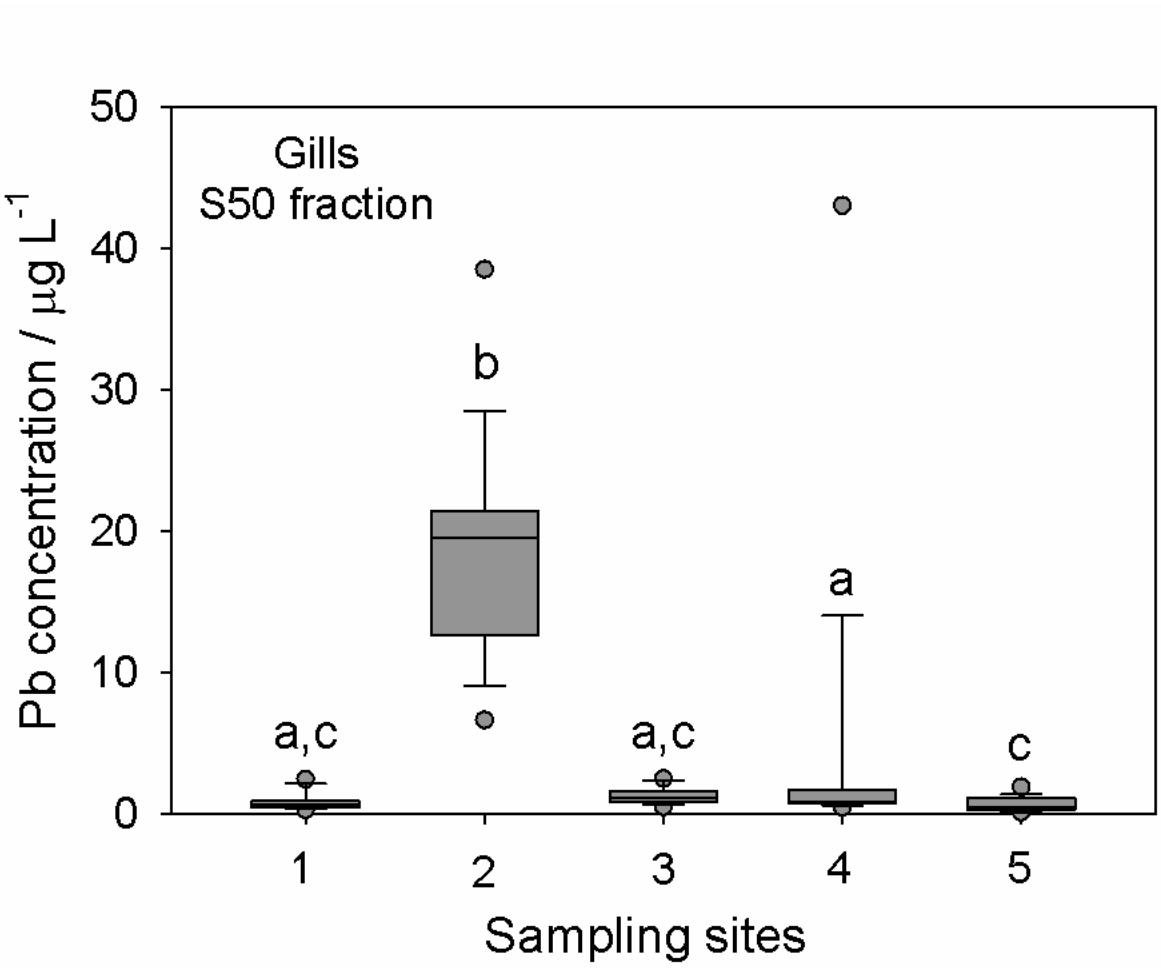
Gills
S50 fraction
Pb concentration / µg L-1
50
40
30
20
10
0
a,c
b
a,c
a
c
1
2
3
4
5
Sampling sites

Table 1. Biometric parameters (average±standard deviation) of European chub caught in the Sutla River in Croatia, in September 2009. The comparison between the five sampling sites was performed for total body mass, Fulton Condition Index (FCI), Hepatosomatic Index (HSI) and Gonadosomatic Index (GSI) by ANOVA. n, number of individuals; F, female; M, male.

Sampling sites	n	Total body mass g	*FCI %	*HSI %	*GSI %	Sex F:M	Age years
1	15	132.1±63.8	[a]0.96±0.07	[a]1.14±0.22	[a]2.86±0.40	15:0	3.0±0.7
2	15	77.1±23.8	[a]0.95±0.09	[b]1.56±0.22	[b]1.82±0.68	8:7	2.0±0.5
3	15	132.5±112.1	[a]0.99±0.06	[a,b]1.36±0.55	[c]0.86±0.34	15:0	2.5±1.2
4	15	127.6±49.7	[a]1.00±0.06	[a]1.03±0.22	[c,d]0.69±0.32	7:8	2.5±0.5
5	15	122.3±82.0	[b]1.09±0.08	[a]1.21±0.33	[d]0.49±0.13	14:1	2.2±0.6

* statistically significant differences between sites according to ANOVA ($p<0.001$)

[a,b,c,d] sites with statistically significantly differences according to *post-hoc* Tukey's test ($p<0.05$)

Table 2. Spearman correlation coefficients (r) between selected biometric parameters and Pb concentrations in the hepatic and gill S50 fractions of European chub caught in the Sutla River in Croatia, in September 2009. U, uncontaminated; C, contaminated; n, number of individuals; Mass, total body mass; FCI, Fulton Condition Index; HSI, Hepatosomatic Index; GSI, Gonadosomatic Index.

S50 fraction	Sampling sites	n	r			
			Pb:Mass	Pb:FCI	Pb:HSI	Pb:GSI
Liver	U	57	0.074	0.002	-0.022	-0.128
	C	15	0.336	-0.196	0.071	0.393
Gills	U	59	-0.042	0.005	0.060	-0.115
	C	15	0.014	-0.118	0.189	0.507

Table 3. *p*-values and mean Pb concentrations in the hepatic and gill S50 fraction of European chub caught in the Sutla River in Croatia, in September 2009, calculated by t-test (for sex comparison) and ANOVA (for age comparison), and presented separately for females and males, and for four age groups. U, uncontaminated; C, contaminated; F, female; M, male.

S50 fraction	Sampling sites	Sex			Age / years				
		F / µg L^{-1}	M / µg L^{-1}	*p*	1 / µg L^{-1}	2 / µg L^{-1}	3 / µg L^{-1}	4 / µg L^{-1}	*p*
Liver	**U**	1.11	1.56	0.365	1.77	0.90	1.42	1.35	0.365
	C	20.1	12.5	<0.05	12.4	15.9	19.1	-	0.629
Gills	**U**	0.80	1.18	0.267	0.78	0.86	0.88	0.89	0.995
	C	22.0	15.5	0.126	13.8	20.3	16.8	-	0.573