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Campus Vesalius

Metal bioaccumulation and biomarker responses in the liver of Prussian carp (*Carassius gibelio* Bloch, 1782) from the Croatian river Ilova

Thieu De Coninck

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Academic year 2017-2018

Biomedical laboratory technology

Pharmaceutical and Biological laboratory technology

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Deze bachelorproef is gemaakt door Thieu De Coninck, Arnaud Nobels en Irene Vanheertum, studenten aan de Hogeschool Gent, ter voltooiing van de bacheloropleiding biomedische laboratoriumtechnologie. De standpunten die in deze bachelorproef zijn verwoord, zijn louter het persoonlijke standpunt van de individuele auteur en reflecteren niet noodzakelijkerwijs de mening, het officiële standpunt of het beleid van de Hogeschool Gent.

Acknowledgement

We came to Zagreb not knowing anything about the culture or the people. In the four months of our stay, we were pleasantly surprised by the hospitality of the country. When we arrived at Ruđer Bošković Institute we felt very welcome and this lifted a weight from our shoulders.

First we would like to thank our Promotor Dr. Zrinka Dragun. She helped and guided us so much and whenever we had questions she was always ready to answer them. Thanks to her, the content of this thesis is professional and consistent. Second we want to thank Dr. Brita Muyssen for her support and guidance. She made sure that we were feeling okay abroad and gave helpful advice. Thirdly we would like to thank our parents, for letting us have this unique experience of an internship abroad.

Since this thesis was based on the subject of Irene's internship, and she worked the closest with the scientists at the Laboratory for Biological Effects of Metals, she would like to thank some more people personally.

> I would like to thank Nesrete Krasnići and Dr. Dušica Ivanković together with Dr. Zrinka Dragun for guiding me with the lab work. They taught me everything I needed to know to be able to work independently in the laboratories. I would also like to thank the people from Laboratory for Aquaculture and Pathology of Aquatic Organisms (situated in IRB) and Dr. Marijana Erk for letting me join on a field trip to the two sites at the river Ilova: Ilova Village and Trebez Village. Lastly, I would like to thank Tatjana Mijošek , Dr. Vlatka Filipović Marijić, Zuzana Redžović and everyone I already named before, for being so kind to me and making me feel at home in this foreign country where I knew so little of. Thank you for everything, and I wish you all the best.

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Abbreviations

AChE	Acetylcholin esterase
CAT	Catalase
DTNB	5,5-dithio-bis-(2-nitrobenzoic acid)
EDTA	Ethylenediaminetetraacetic acid
ETS	Electron transfer system
FCI	Fulton condition index
GSH	Glutathione (reduced glutathione)
GSI	Gonadosomatic index
GSR	Glutathione reductase
GSSG	Glutathione disulfide (oxidized glutathione)
GST	Glutathione S-transferase
HSI	Hepatosomatic index
HR ICP-MS	High resolution inductively coupled plasma mass spectrometry
KPE	Potassium phosphate EDTA
MDA	Malondialdehyde
Mr	Molecular mass
NADPH	Nicotinamide adenine dinucleotide phosphate
PSSG	Glutathionylated proteins
SEC-HPLC	Size exclusion-high-performance liquid chromatography
SSA	5-sulfosalicylic acid
TNB	5-thio(2-nitrobenzoic acid)
TP	Total proteins

Glossary

Bioaccumulation: the accumulation of chemicals in an organism due to faster intake than output of the chemical.
Biotransformation: a chemical modification made by an organism on a chemical compound.
Chelation: a type of bonding, or rather the forming of a complex, the formation of a complex between ions or molecules and metal ions through coordinate bonds between a polydenate, ligand and single central atom.
Gill rakers: the bone or bonelike parts of the gill arch that are involved in filtering tiny food particles out of the water.
Macro elements: chemical elements which are found and needed in high concentrations.
Metalloids: elements that are neither metals, nor nonmetals, or elements that have properties between those of metals and nonmetals.
Peritoneum: epithelial tissue that covers the inside of the abdominal cavity and the outside of intra-abdominal organs.
Trace elements: chemical elements which are found and needed in low concentrations.

Abstract

The study aim was to determine whether the wastewater input from a petrochemical factory into the Ilova River has caused contamination of the river water that could affect the aquatic organisms and the environment. The concentrations of multiple trace and macro elements in the river water and in the liver of Prussian carp (*Carassius gibelio*) were measured and compared between two sites: the reference site Ilova village, upstream of the wastewater input, and the potentially contaminated site Trebež village, downstream of the input. Stress caused by potential trace and macro element bioaccumulation in fish liver was tested by measuring biomarkers (total cytosolic proteins, metallothionein, glutathione and catalase).

The concentrations of dissolved Na, Cs, Cd and Rb in the river-water downstream of the wastewater inflow were generally slightly higher compared to the upstream samples, whereas Mn had higher concentration upstream of wastewater inflow.

The majority of total hepatic concentrations of elements showed comparable bioaccumulation levels in the liver at both sites. Only Na, Rb, and Cs had higher concentrations at Trebež village, which was probably a direct consequence of higher exposure in the river-water. Cytosolic proportions of many elements in Prussian carp liver were very high, and for highly toxic metals Ag, Cd and Cs they were approximately 100%, indicating their high metabolic availability and toxic potential.

Biomarker levels did not differ between two sampling sites, indicating that the effects of slightly increased exposure to several elements in the river-water and their bioaccumulation in the liver downstream of petrochemical factory were still not observable in the fish.

It can be concluded that, despite observed bioaccumulation of several metals in fish liver, there was no observable effects on the fish.

Abstract

Het doel van de studie was bepalen of de instroom van afvalwater van een petrochemische fabriek in de Ilova rivier vervuiling van het rivierwater veroorzaakt, en of dat een invloed heeft op waterorganismen en de omgeving. De concentratie van verschillende spoor- en macro-elementen werd gemeten in het rivierwater en in de lever van de giebel (*Carassius gibelio)* en dan vergeleken tussen twee plaatsen: de referentieplaats Ilova dorp, stroomopwaarts van de instroom van afvalwater, en de mogelijks vervuilde plaats Trebež dorp, stroomafwaarts van de instroom. Stress veroorzaakt door eventuele bioaccumulatie van spoor- en macro-elementen in de lever van de vis werd gemeten aan de hand van biomarkers (totale cytosolische proteïnen, metallothioneïne, gluthathion en katalase).

De concentratie van opgeloste elementen was in het algemeen iets hoger in het rivierwater stroomafwaarts van de instroom van afvalwater, en meer specifiek Na, Cs, Cd en Rb, in tegenstelling hiertoe, had Mn een hogere concentratie stroomopwaarts.

De meeste elementen hadden een gelijkaardig niveau van bioaccumulatie in de lever van de giebel in beide plaatsen. Alleen Na, Rb en Cs hadden een hogere concentratie in Trebež dorp, wat mogelijks een direct gevolg is van een hogere blootstelling in het rivierwater. Cytosolische verhoudingen van verscheidene elementen in de lever van de giebel waren zeer hoog. Voor de sterk toxische metalen Ag, Cd en Cs waren deze ~100%, wat hun hoge metabolische activiteit aanwijst en de mogelijkheid voor toxische effecten.

Er was geen verschil in de concentraties van biomarkers in beide plaatsen, wat aantoont dat een iets verhoogde blootstelling aan verschillende elementen in het rivierwater en hun bioaccumulatie in de lever stroomafwaarts van de petrochemische fabriek, geen effect hadden op het stresslevel van de vissen.

1 Introduction

1.1 River water contamination with metals

1.1.1 Speciation of metals in the water

Knowledge of total concentrations of metals in the river water is generally not sufficient to know if harmful effects will occur. Metals can have different chemical forms and oxidizing conditions in the water. As a consequence, their toxicity changes depending on the chemical form (Odobasic, 2012).

Toxicity, biodegradability, bioaccumulation, mobility, solubility and other important characteristics depend on the physical/chemical form of a metal (Odobasic, 2012). Most studies on toxicity of metals show that a free hydrated metal ion is the most toxic possible form of a metal (Sterrit & Lester, 1980).

Other forms of metals in water are inorganic and organic complexes, metals adsorbed to colloid particles, polymers and pseudocolloids, and metals adsorbed to suspended particles or absorbed by microorganisms. The mobility of metals in water depends on multiple parameters such as the pH of water, the presence of carbonates and phosphates in the water, oxidation conditions, content of organic matter and the presence of sulphide ions.

There are several methods that can be used for measuring the different the speciation of metals, from electrochemical methods, such as voltammetry and potentiometry, to other analytical methods, like extraction, dialysis and ultrafiltration (Odobasic, 2012).

1.1.2 Metal bioavailability

Metals and metalloids are one of the biggest threats for the aquatic environmental health due to their toxicity, persistence and possibility to accumulate in sediment and all living organisms (Dragun *et al*., 2015; Fidan *et al*., 2008; Harte *et al*., 1991). This can eventually become a threat for human society, because fish are consumed by humans as a part of their diet (Barlas, 1999; Holcombe *et al*., 1976).

Bioavailable metals are the portion of total metals in the environment that can be incorporated into organisms. Only a part of the total metal concentration in the water is considered as bioavailable. High exposure level of bioavailable metals can eventually lead to bioaccumulation (John and Leventhal, 1995).

There are two types of metals, according to their biological functions. Some metals, like Zn, Cu, Fe, and Mn, are essential for physiological functions of aquatic organisms. For example, iron (Fe) is a part of the haem group in haemoglobin and thus essential for oxygen transport. Zinc (Zn) has an important role in catalytic reactions, as a cofactor in enzymes, and has structural and regulatory functions (Roohani *et al*., 2013). Aside from its involvement in iron metabolism, copper (Cu) has a key role in other biological processes, such as the immune system and antioxidant defense (Bost *et al*., 2016). Manganese (Mn) is a crucial metal for normal cell functioning and metabolism (Tuschl *et al*., 2013). The

aquatic organisms take all these metals up by a combination of water and food. These metals can also be toxic for the organisms, but only when the concentration reaches a critical point, and this critical point differs from metal to metal.

There are also metals with no clear biological function, like Cd, Ag, and Hg. These metals can already be toxic at low concentrations. Cadmium (Cd) is known to induce tissue damage through oxidative stress, it also has an influence on the transport pathways, inhibiting or upregulating them (Bernhoft, 2013). The toxicity of mercury (Hg) depends on the form, the rate of exposure and on the dose. The organic form of mercury affects different parts of the body such as the brain, the kidneys or the lungs. Chronic exposure to low doses of elemental mercury cause only mild symptoms (Bernhoft, 2012). Exposure to silver (Ag) through inhalations can cause irritation to the respiratory system, both upper and lower tract. Silver can also accumulate in the other organs, where it can bind to the thiol groups on glutathione and thereby disturb its functionality (Drake and Hazelwood, 2005).

1.1.3 Sources of metal contamination

Metals are naturally found in the earth's crust and therefore they can also be found in the river water. The natural contamination of water can occur due to the effects of metal corrosion, volcanic eruptions, soil erosion, atmospheric deposition, etc. (Tchounwou *et al*., 2012)(Figure 1). There are many natural sources of water pollution and the most prevalent metals from those sources are Zn, As, Cd, Pb and Hg (Taillefert and Tercier-Waeber, 2008).

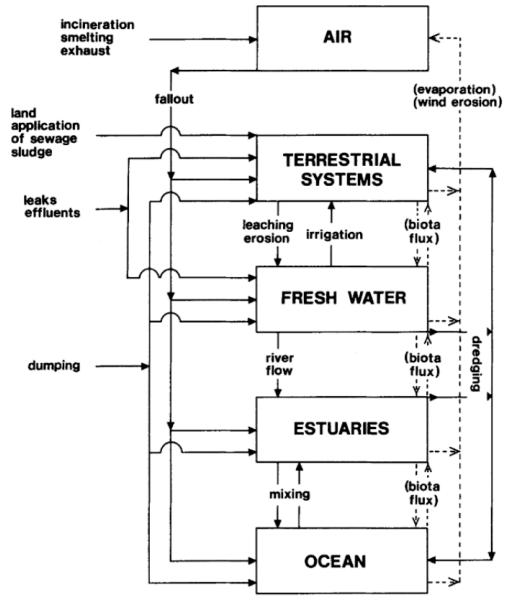

Figure 1: Routes of water pollution (Förstner and Wittmann, 1981)

These are all natural ways of water contamination, but mostly the contamination does not occur naturally but rather due to the activities of humans. These activities, such as mining for ore, smelting the ore, use of metals in industrial processes for production, use of metals for domestic and agricultural purposes, burning coal for power, etc., are the main cause of metal contamination of waters and rivers. It should be noted that metal pollution produced by humans can contaminate the soil and these metals can then percolate into the ground water, thereby polluting the water (Figure 1). By burning waste and using fossil

fuels for driving cars, the air is polluted, which can cause a fallout and thereby polluting the water as well (Figure 1).

So when a water sample is analysed for metal contamination, it has to be considered that it can result from human as well as natural processes.

1.1.4 Metal contamination of the Ilova River

The Ilova river (Figure 2) is situated in central Croatia and is a left tributary of the Sava River. It has a length of 93.4 km from spring to the mouth. The river originates from three springs. The main spring is situated at an altitude of 205 m on the northern slopes of Papuk Mountain and two smaller ones preserve additional water 800 m downstream. There are three locations that can be considered as the river mouth. The first is the point where Ilova River flows into Stari Trebež River and the second one is Pakra River emerging with Ilova River. The third location is considered the actual river mouth of the Ilova River today. It is the point where the Ilova River flows into the Sava River (Plantak *et al*., 2016).

The Ilova River is mainly contaminated through municipal sewage wastewater and industrial and agricultural sources (Radić *et al*., 2013). Radić *et al*. (2013) investigated the level of water contamination at one sampling site of the Ilova River located immediately downstream of Kutina town, which has a fertilizer factory. When they compared the results to the reference site, they found higher values of Fe, Cd, Pb, Cr, Hg, Zn, Cu, and Ni, but only Pb and Hg had values above WHO limits (Radić *et al*., 2013; WHO, 1998).

Figure 2: The Ilova River catchment in Croatia (Plantak *et al.*, 2016)

1.2 Metal bioaccumulation in the fish liver

Fish are sensitive to pollutants such as metals, and therefore they are useful for obtaining the data involving the induction of oxidative stress, carcinogenicity, mutagenicity

and other effects. The liver of fish is used to evaluate all these effects, because it is the main detoxification and storage organ (Dragun *et al*., 2012).

1.2.1 Bioindicators

Bioindicators are living organisms that make it possible to evaluate the environmental health, the level and duration of contamination in a certain region, and indirectly the effects on human society (Khatri and Tyagi, 2015).

By use of bioindicators it is possible to determine, both qualitatively and quantitatively, the response to environmental stress. There are three main functions associated to the use of bioindicators. They are used for monitoring the environment, ecological processes and the biodiversity (Holt and Miller, 2010). The use of bioindicators has several advantages compared to the classic methods for measuring environmental quality (Holt and Miller, 2010):

(1) unlike chemicals, they can give us information about the cumulative effects of different pollutants;
(2) more biologically relevant: it is possible to see the effects directly on the environment;
(3) provide a picture of meaningful levels of pollutants, no matter how low;
(4) give us data about the past, present or future environmental status, while chemicals only tell us something about the time of sampling.

The use of bioindicators also has some disadvantages, such as the natural variability that cannot be controlled (e.g., size, sex, reproduction status, and diseases).

Various organisms can be used as bioindicators, such as plankton, copepods, small water crustaceans or fish (Parmar *et al*., 2016). The characteristics of a good bioindicator are presented above, in Table I.

Table I. Characteristics of good bioindicators (Holt and Miller, 2010)

Good indicator ability	Provides measurable response
	Response reflects the whole ecosystem
	Responds in proportion to the degree of contamination or degradation
Abundant and common	Adequate local population density
	Common, including distribution within area of question
	Relatively stable despite moderate climatic and environmental variability
Well-studied	Ecology and life history well understood
	Taxonomically well documented and stable
	Easy and cheap to survey
Economically/commercially interesting	Species already being harvested for other purposes
	Public interest in or awareness of the species

1.2.2 Fish as a bioindicator

Fish are used as bioindicators because they meet all criteria for conducting biological monitoring programmes. They are sensitive to stressors, the methods to measure certain stressors are standardised and the results are representative of many other aquatic organisms. They are easy to collect and identify, and they meet the requirements for three major types of bioindicators:

1. compliance indicators, for the evaluation of the attainment and maintenance of the environment;
2. diagnostic indicators, for providing the insight in causes of any changes in the environment;
3. early warning indicators, for indication when actions should be taken to preserve the environment (Chovanec *et al*., 2003).

When the water is contaminated, it can cause harm to the fish in multiple ways. In addition to obvious toxicity that can be caused by the contaminants, it can also cause a change in the amount and content of the food and energy source. It can change their habitat

structure, and lower the availability of oxygen and nutrients. Also flow regime and biotic interactions can be influenced through contamination (Chovanec *et al*., 2003).

1.2.3 Prussian carp (*Carassius gibelio* Bloch, 1782)

Prussian carp (*Carassius gibelio* Bloch, 1782) is an abundantly present fish species in the Ilova River, and thus a bioindicator of choice for monitoring the ecological status of that river.

1.2.3.1 Taxonomy

The Prussian carp, *Carassius gibelio* (Bloch, 1782), can be classified in the phylum Chordata, subphylum Vertebrata and class of the Actinopterygii. It belongs to the order Cypriniformes and the family Cyprinidae. The genus is *Carassius* and the species is *Carassius gibelio* (U.S. Fish and Wildlife Service, 2012). Other names for the Prussian carp are the gibel carp or the silver crucian carp (NatureGate, 2013).

Overview of the classification of Prussian carp (U.S. Fish and Wildlife Service, 2012):

Domain: Eukaryota
Kingdom: Metazoa
Phylum: Chordata
Subphylum: Vertebrata
Class: Actinopterygii
Order: Cypriniformes
Family: Cyprinidae
Genus: *Carassius*
Species: *Carassius gibelio*

1.2.3.2 Morphology

Figure 3: *Carassius gibelio* (Picture by Jawad, L.A.) (Froese, 2015)

The Prussian carp (Figure 3) is a medium-sized cyprinid with a deep-bodied and plump shape. It has yellow or light silvery sides and belly. It can be distinguished from the other carps due to its distinctive forked tail fin and light reddish underfins and its black

peritoneum. The dorsal fin and the anal fin of the Prussian carp are heavily serrated. When looking at the gills, you can normally count 37 to 52 gill rakers, which are a way of protecting the gill arch and may also serve as a food-trapping mechanism (Naylor, 2007).

The Prussian carp can grow up to 45 cm, but the most common length is around 20 to 25 cm. It can weigh up to 3 kg (Froese, 2015).

1.2.3.3 Distribution

Originally the Prussian carp has its origin in Asia (Siberia), but it is considered as native to the region from central Europe to Asia. Now it can be found all over Europe, from Spain to Great Britain, in southern as well as in the eastern Europe (Britton, 2011).

1.2.3.4 Habitat and feeding

The Prussian carp inhabits a lot of different water bodies such as shallow lagoons, shallow lakes and pools and slow flowing rivers. As the Prussian carp is a warm water species, it is known that its preferred habitat is shallow and eutrophic, and has a lot of vegetation. It is a freshwater fish, but it can cope with a certain salinity (ranging from 3 to 6 psu) and thus can live in brackish waters. It can also tolerate low oxygen levels and pollution.

The Prussian carp eats plankton, plant material, detritus (dead organic material) and benthic invertebrates (Froese, 2015).

1.2.3.5 Reproduction

The females spawn together with the other fish of the same species. The eggs are sticky and are layed on water plants or on submerged objects. The eggs do not need to be fertilised by a male Prussian carp. Due to the process of gynogenesis or reproduction from unfertilised eggs, most of the populations consist of triploid female fishes only, but in some populations, there are up to 25% of diploid male fishes (Froese, 2015). They spawn in summer in warm water in shallow bays (http1). The older fishes spawn before the younger ones and the ripe females are followed around by the males (Froese, 2015).

1.2.4 Fish liver as a target organ for metal analyses

The liver of fish is used as a target organ because it is the main detoxifying organ of the organism. This means it will transform toxic compounds into other less toxic or non-toxic forms which are more easily excreted from the organism. Toxins and metals tend to accumulate in the liver, where high concentrations of metallothioneins are present, which have a high affinity for binding multiple metals and aid to their excretion (Chovanec *et al*., 2003).

The liver is also chosen as a target organ because its response to short-term fluctuations in metal concentrations in the environment is less evident than in organs which are in direct contact with the surroundings, such as gills or the intestine. This way the liver

is representative for long term exposure even to low concentrations of metals (Dragun *et al*., 2012).

1.3 Biomarkers

Biomarkers are defined as 'any biological response to an environmental chemical at the individual level or below demonstrating a departure from the normal status' (Walker *et al*., 2001). It can be concluded that biochemical, physiological, histological, morphological and behavioural measurements could be considered as biomarkers. Bioindicators cannot be considered as specific biomarkers because they represent biological changes on a higher organizational level such as the population, community and ecosystem (Walker *et al*., 2001). The relationship between biomarkers and bioindicators regarding their specificity and ecological relevance is shown in Figure 4.

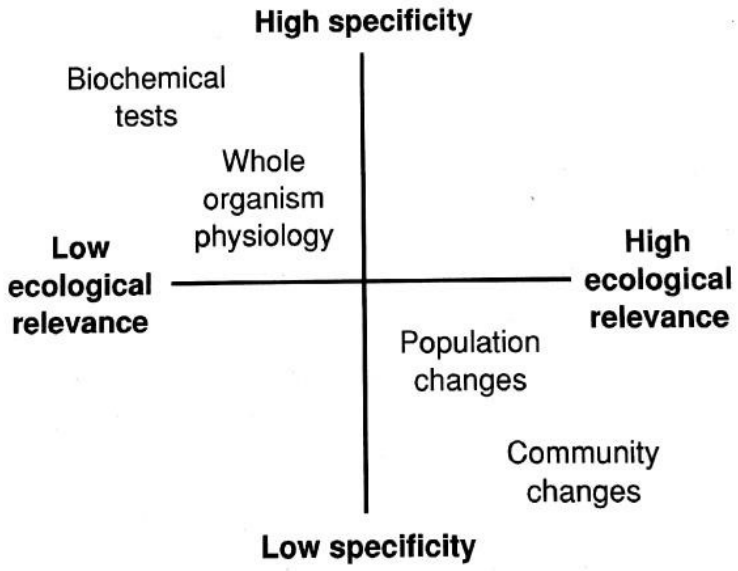

Figure 4: Relationship between biomarkers (left, top) and bioindicators (right, down) regarding their specificity and ecological relevance shown diagrammatically (Walker *et al.*, 2001)

The most widely used classification of biomarkers is dividing them in two groups: biomarkers of exposure and biomarkers of effect. Biomarkers of exposure show us if the organism has been exposed to certain chemicals and biomarkers of effect show us if this exposure has caused any toxic effect on the organism (Kroon *et al*., 2017).

Biomarkers are considered '*early warning*' indicators that have the potential to detect an effect in target biota prior to one being observed at the population, community or ecosystem level (Kroon *et al*., 2017).

1.3.1 Total cytosolic proteins

Total cytosolic proteins are soluble proteins present in the cytosol of the cell. These proteins all fluctuate under the influence of stress. By measuring total cytosolic proteins in hepatic cytosol of fish, the level of general stress this organism was exposed to, is estimated.

Some of those proteins are continuously present in the cytosol and change in concentration under the influence of stress. Others are only present in the cytosol when the organism is under stress (Whitley *et al*., 1999).

1.3.2 Metallothioneins

Metallothioneins or MTs are a family of proteins and oligopeptides which have a low-molecular weight, are cysteine-rich and can bind multiple metals. The binding of metals on MTs occur through the sulfhydryl groups on cysteins (Chovanec *et al*., 2003; Dragun *et al*., 2009a; Huggett *et al*., 1989). The structure of MTs is presented in Figure 5.

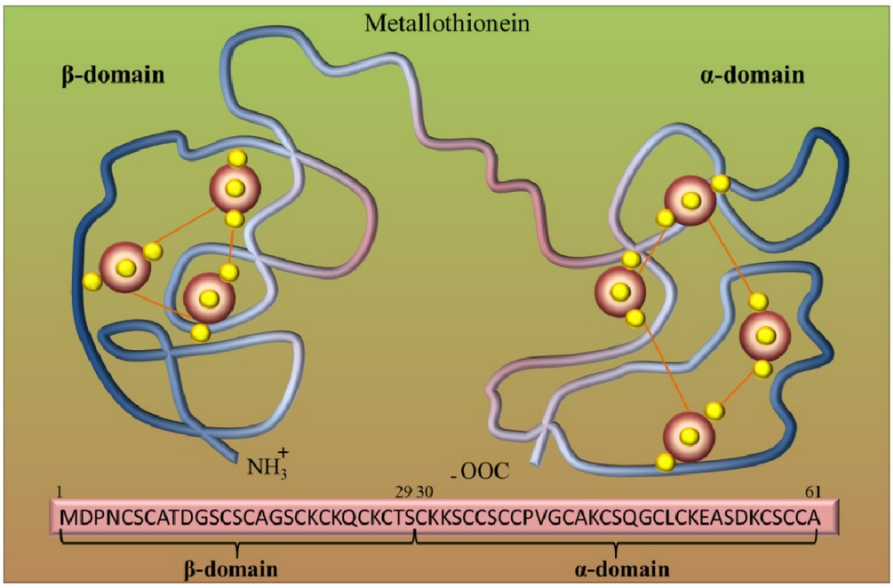

Figure 5: The structure of metallothionein. Two binding sites of metallothionein. Big red beads are metal atoms (e.g. Zn), small yellow beads are sulfur atoms. (Ruttkay-Nedecky et al., 2013).

An increase of bioavailable metals in the environment and their consequent bioaccumulation can result in an increase in the production of MTs. Therefore, MTs could be used as a potential biomarker of metal exposure. However, tissue levels of MTs are also affected by reproduction and stress factors like handling, starvation, anoxia, cold, heat, exercise, and the presence of antibiotics, vitamins or herbicides. Fortunately, the level of induction by those factors is lower than that caused by metals (Chovanec *et al*., 2003; Dragun *et al*., 2009a; Huggett *et al*., 1989).

Fish MTs are found in all tissues, but mostly in the liver and kidney. There they play an important role in the intracellular regulation of the essential metals Zn and Cu. These two metals can be found in mixed-metal clusters on the MTs. After exposure to Cd, the protein may contain Cd, Cu and Zn. Alongside the regulation of Zn and Cu, MTs are thought to be involved in metal detoxification and donation of metals to metalloproteins (Chovanec *et al*., 2003; Huggett *et al*., 1989).

In general, MTs are involved in the homeostasis of essential trace elements, like Zn and Cu, the sequestration of toxic metals, such as Cd and Hg, and the protection against oxidative damage.

Hypothetically, MTs would supply Zn, and possibly Cu, in growing, injured or regenerating tissues for nucleic acid metabolism, protein synthesis and other metabolic processes. It has also been shown that MTs are effective in scavenging free-radicals, which is very important to maintain normal cellular metabolism (Huggett *et al*., 1989).

1.3.3 Catalase

Catalase is a hematin containing enzyme found in nearly all living organisms that are exposed to oxygen. It is present in almost every organ, with particularly high concentrations in the liver. The function of this enzyme is to catalyse the decomposition of hydrogen peroxide by the following reaction (Gaetani *et al*., 1996):

$$2\ H_2O_2\ _{(aq)} \rightarrow 2\ H_2O\ _{(aq)} + O_2\ _{(g)}$$

Catalase is usually located in the peroxisomes. Peroxisomes are involved in the catabolism of several biomolecules (Fahimi and Sies, 1987). During this catabolism, hydrogen peroxide is produced by the following reaction:

$$R{-}CH_2{-}CH_2{-}CO{-}SCoA + O_2 \xrightarrow{FAD} R{-}CH{=}CH{-}CO{-}SCoA + H_2O_2$$

FAD = Flavin Adenosine Dinucleotide

Hydrogen peroxide is a reactive oxygen species, which can cause damage to all parts of the cell including lipids, proteins and DNA. To prevent damage, hydrogen peroxide must be quickly converted into less dangerous substances. This process can occur thanks to catalase.

Catalase has one of the highest turnover numbers of all enzymes: millions of molecules of hydrogen peroxide can be converted to water and oxygen with only one molecule of catalase (Goodsell, 2004).

The concentration of catalase is correlated with the level of oxidative stress that the organism has undergone, and therefore we can use it as a biomarker.

1.3.4 Total glutathione

Glutathione is a peptide that consists of three amino acids: cysteine, glutamic acid and glycine (Figure 6). This tripeptide is present in the tissue of most of the animals, but also in plants, fungi and even in some bacteria and archaea. The most important function of glutathione is its ability to work as an antioxidant or detoxifying agent (National Cancer Institute, 2018). Furthermore, it can act as a cofactor for glutathione peroxidase and in the synthesis of leukotrienes, it plays a central role as a chelating agent for metals, it has a role in the cell cycle (Lu, 2013), it has a vital function in the metabolism of iron (Kumar *et al*., 2011) and it regulates the nitric oxide cycle (Ha *et al*., 1999).

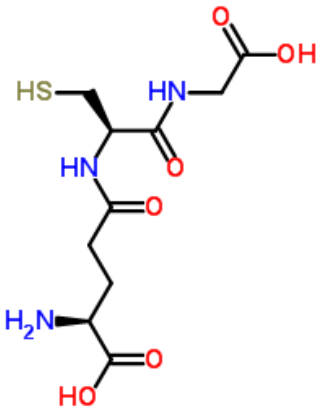

Figure 6: 2-D structure of glutathione (http2)

There are three forms in which glutathione can exist: the reduced state (GSH), the oxidized state (GSSG) and glutathionylated proteins (PSSG). When in the reduced state, the thiol group of the cysteine amino acid can donate a reducing equivalent, an electron and a H^+ to the other molecules, for example to the oxygen radicals, and thus neutralise them. By donating a reducing equivalent, GSH becomes reactive. It then quickly reacts with another reactive glutathione molecule to form GSSG (Kaplowitz, 1981).

Glutathione can only function when it is in its reduced form, therefore GSH has to be regenerated form GSSG by glutathione reductase (GSR) in the presence of NADPH. There are two molecules of GSH regenerated for every molecule of GSSG and NADPH (Couto *et al*., 2013).

Normally, when the tissue and cells are healthy, there is more than 90% of all the glutathione in the GSH form and less than 10% in the GSSG form. When this ratio is altered and there is more than 10% of GSSG, it can be an indication of oxidative stress (Halprin and Ohkawara, 1967).

Due to the presence of the thiol group on the cysteine amino acid, metals can be easily chelated, because they have a high affinity for thiol. One such interaction between GSH and Ag ion is presented in the Figure 7. It is also tasked to form and maintain disulfide bonds and has a role in transport of amino acid across the cell membranes (Jozefczak *et al*., 2012).

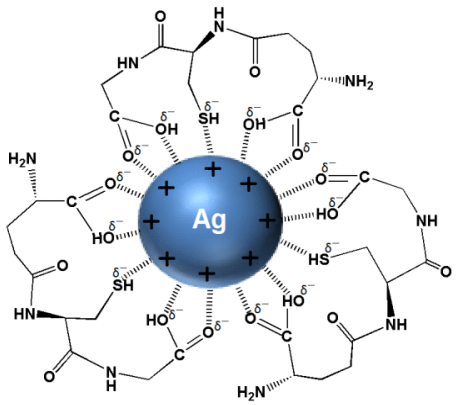

Figure 7: Interaction of glutathione and a silver ion (Balavandy *et al.*, 2014)

1.4 Aim of the study

The aim of this study was to investigate if the lowland Ilova River in Croatia is polluted as a consequence of the inflow of municipal wastewaters and wastewaters of a petrochemical factory. The purpose was to know if the wastewater inflow has influenced the river water quality, the quality of life and health of fish. This was achieved by determining the concentrations of multiple trace and macro elements, essential and non-essential to fish, in the river water and in the liver of the selected bioindicator fish species, the Prussian carp (*Carassius gibelio*). The study was performed at two sites in the Ilova River, one near the Ilova village, upstream of the wastewater input, and the other near the Trebež village, downstream of the wastewater input. Additonally, the effect of water contamination on the stress level of the fish was studied by measuring four biomarkers in the liver of *C. gibelio* to determine (i) the level of oxidative stress (by measuring total glutathione (tGSH) and catalase (CAT)) and (ii) the level of general stress (by measuring total cytosolic proteins (TP) and metallothioneins (MT)).

2 Materials and methods

2.1 Sampling

2.1.1 Study area and period

The river water and fish sampling were performed at two sites at the Ilova River (Figure 8).

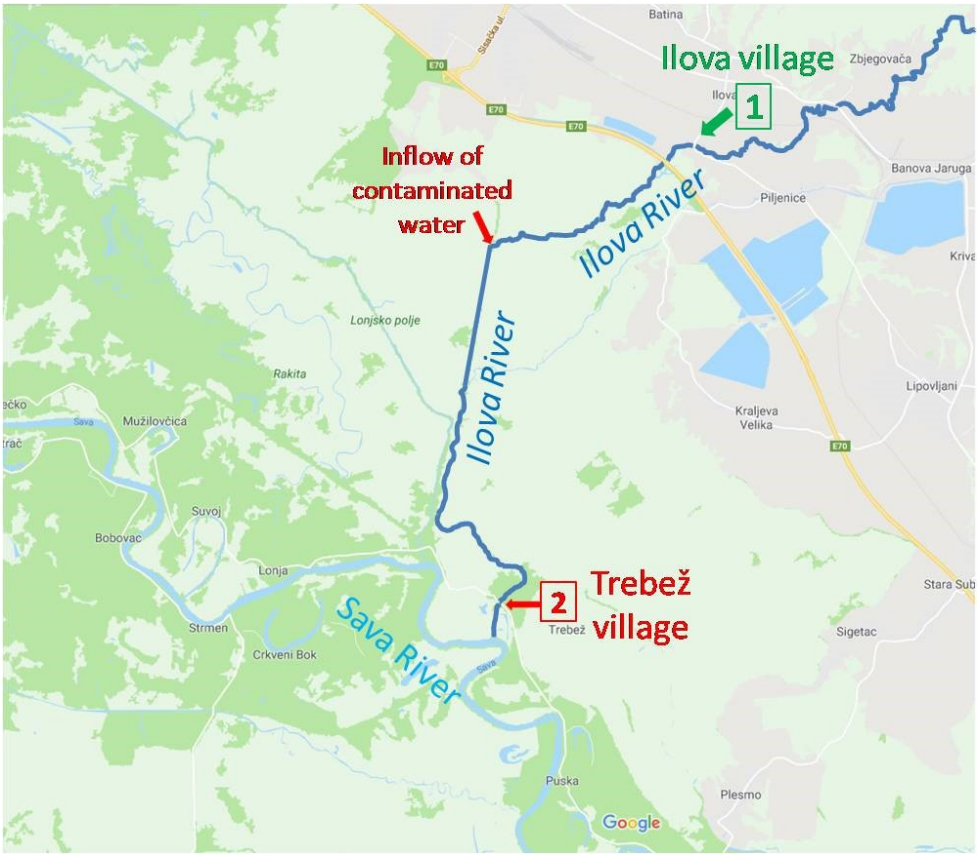

Figure 8. A map showing two sampling sites: site near the Ilova village as a reference site and site near the Trebež village as a contaminated site.

The first site was situated near the Ilova village (Figure 9), in the vicinity of the bridge crossing the Ilova River. That site was considered as a reference site, since it is located upstream of known sources of pollution, such as municipal and industrial wastewater outlets. The second site was situated 16 km downstream from the reference site, and approximately 10 km downstream of the site where the Kutinica River flows into the Ilova River (Figure 8). The Kutinica River is potentially contaminated with the municipal wastewaters of the Kutina town and industrial wastewaters of the petrochemical factory. The second sampling site, thus considered as potentially contaminated, was located near the Trebež village (Figure 10) in the vicinity of the bridge crossing the Ilova River and close to the Ilova River mouth into the Sava River.

Figure 9. Sampling site near the Ilova village.

Figure 10. Sampling site near the Trebež village.

The sampling was performed on October 5th 2017, to obtain the information characteristic for the autumn period, i.e. for the after-spawning period of Prussian carp (*C. gibelio*).

2.1.2 Fish sampling

The selected bioindicator fish species for this study was Prussian carp (*C. gibelio*) (Figure 11). Forty specimens of this fish, 20 at each sampling site, have been caught by electrofishing as stated in the Croatian standard HRN EN 14011 (2005). After capturing, the fish were put in aerated water, before relocation to the lab for dissection.

Figure 11. The Prussian carp (*Carassius gibelio*) from the Ilova River near Trebež village.

To euthanize the fish, a freshly made batch of the anaesthetic tricaine methane sulphonate (MS 222, Sigma Aldrich) was added to the water. This procedure is necessary to conform to the Ordinance on the protection of animals used for scientific purpose (NN 55/2013). Before dissection, the mass and length of the fish were measured. The liver was dissected, weighed and then stored at -80°C awaiting further analysis. The gonad tissue (reproductive organs) was used for the determination of the fish sex on macroscopic level (Figure 12).

Later on, biometric indices were calculated as described by Dragun *et al*. (2018). The ratios of the mass of the liver and of the gonads to total mass of *C. gibelio* are respectively called the hepatosomatic index (HSI) and the gonadosomatic index (GSI).

Furthermore, using the equation: [(mass in grams x 100) / (length in centimetres)^3], the Fulton condition index (FCI) was calculated according to Rätz and Lloret (2003).

Figure 12. The female of Prussian carp (*Carassius gibelio*) with visible gonads.

2.1.3 Water sampling

The river water was collected in triplicates in acid-cleaned (10% v/v nitric acid, p.a. Kemika, Croatia) polyethylene plastic bottles for dissolved trace element analyses. The water samples were filtered immediately after collection through a 0.45 μm pore diameter cellulose acetate filter (Sartorius, Germany) mounted on syringes. The aliquots of filtered samples which were used later for the analyses were transferred into acid pre-cleaned 20 mL polyethylene bottles and 400 μL of concentrated nitric acid (Suprapur, Merck, Germany) was added. The bottles were stored at +4°C (Filipović Marijić *et al*., 2018).

2.2 Analyses in the river water samples

2.2.1 Sample preparation

To measure trace elements, the filtrated and acidified river water samples were used undiluted. For the measurements of macro elements, the filtrated and acidified river water samples were 10 times diluted with Milli-Q water (Millipore Corporation).

2.2.2 Measurements of trace and macro element concentrations in the river water

Measurements of trace and macro elements in the river water were performed by the same procedure as will be described below, in the section 2.3.3 Measurement of trace and macro elements in the hepatic cytosols and homogenates.

Limits of detection (LODs) for measurements of trace elements in the river water were calculated by taking three times the standard deviation of trace elements measured in ten blank samples (filtered and acidified Milli-Q water). Limits of detection for trace elements in the filtered river water (μg/L) were: Ag, 0.064; As, 0.028; Cd, 0.002; Co, 0.019; Cs, 0.001; Cu, 0.401; Fe, 0.624; Mn, 0.050; Mo, 0.011; Rb, 0.003; Se, 0.059; Sr, 0.182; and Zn, 7.34.

2.3 Assessment of bioaccumulation of trace and macro elements in the Prussian carp liver

2.3.1 Homogenisation of hepatic tissues and isolation of soluble cytosolic fractions

In Figure 13, the protocol is presented for liver homogenization and isolation of hepatic cytosol. The frozen samples of the hepatic tissue (Figure 14) were cut into smaller pieces. One piece of hepatic tissue was always set aside for GSH determination and one piece for ETS (electron transport system) measurements.

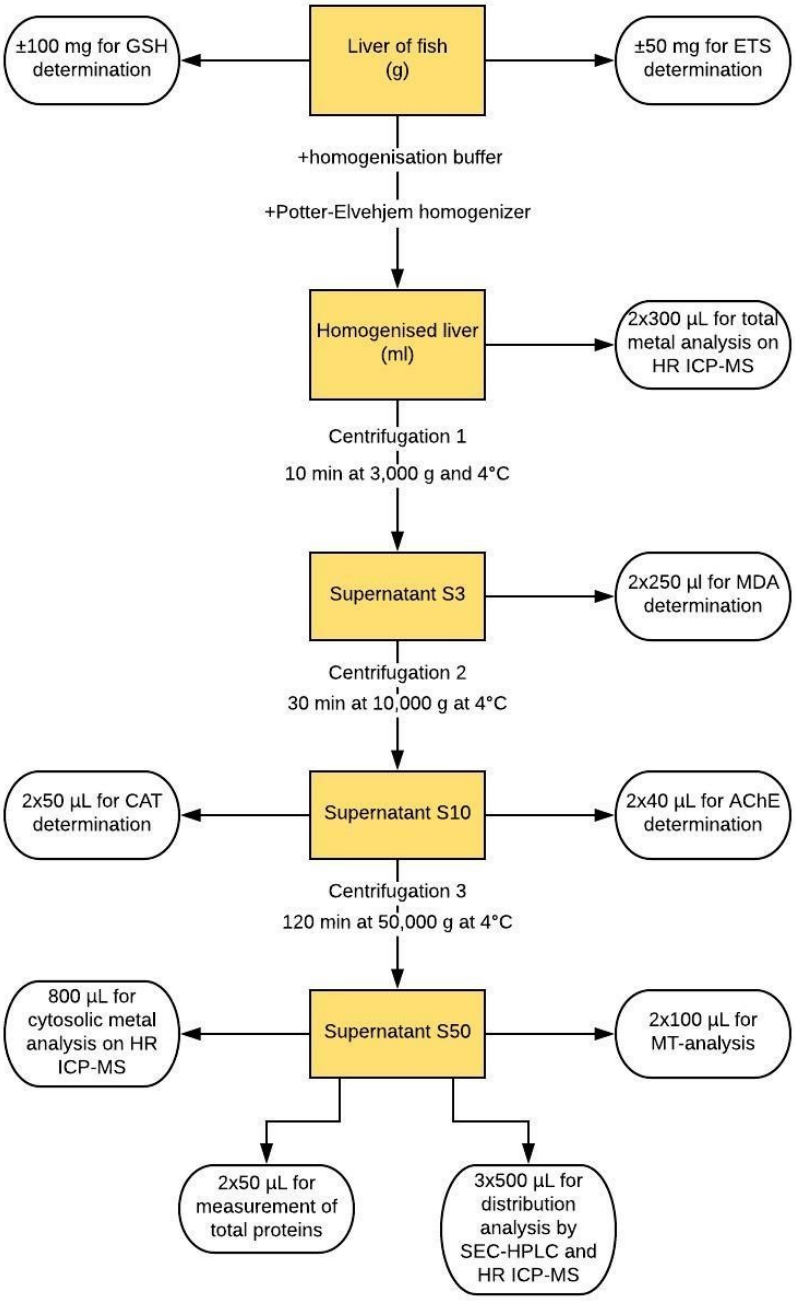

Figure 13. Flowchart of the protocol for liver homogenization and isolation of hepatic cytosols.

In Figure 13, the protocol is presented for liver homogenization and isolation of hepatic cytosol. The frozen samples of the hepatic tissue (Figure 14) were cut into smaller pieces. One piece of hepatic tissue was always set aside for GSH determination and one piece for ETS (electron transport system) measurements.

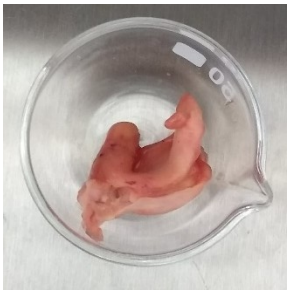

Figure 14. The frozen liver of Prussian carp (*C. gibelio*) prior to homogenization.

Next, 5 volumes of a cooled homogenization buffer consisting of 100 mM Tris-HCl/Base (Sigma, pH 8.1 at 4 °C) supplemented with a reducing agent (1 mM dithiothreitol, Sigma) were added to the remaining hepatic tissue (v/w: 5/1). The mixture of hepatic tissue and buffer was homogenized in an ice cold tube with a Potter-Elvehjem homogenizer (Glas-Col, USA) by moving it up and down 10 times at 6000 rpm (Figure 15).

Figure 15. The homogenization of Prussian carp (*C. gibelio*) liver.

After the homogenization, aliquots of homogenates were taken for measurement of total metal concentrations, which was followed by several cycles of centrifugation of the remaining homogenates (Figure 16). Homogenates (Figure 16a) were centrifuged for the first time in an Avanti J-E centrifuge (Beckman Coulter) at 3000×g for 10 min at 4°C. Aliquots of supernatants (S3; Figure 16b) were removed for MDA (malondialdehyde) determination. The second centrifugation lasted 30 min at 10000×g and 4°C. After that, aliquots of supernatants (S10; Figure 16c) were taken for AChE (acetylcholine esterase) and CAT analyses. After the third centrifugation cycle of 120 min at 50000×g and 4°C, aliquots of supernatants (S50; Figure 16d) were removed for measurements of MTs, total proteins, cytosolic metal concentrations and analyses by size exclusion high performance liquid chromatography (SEC-HPLC) and high resolution inductively coupled plasma mass

spectrometry (HR ICP-MS) (Dragun *et al*., 2018). Supernatants S50 or cytosolic fractions of Prussian carp liver contained cytosolic biomolecules, lysosomes and microsomes, while the pellets contained cell membranes, nuclei, mitochondria and granules (Bonneris *et al*., 2005; Dragun *et al*., 2013a,b; 2018; Podrug *et al*., 2009).

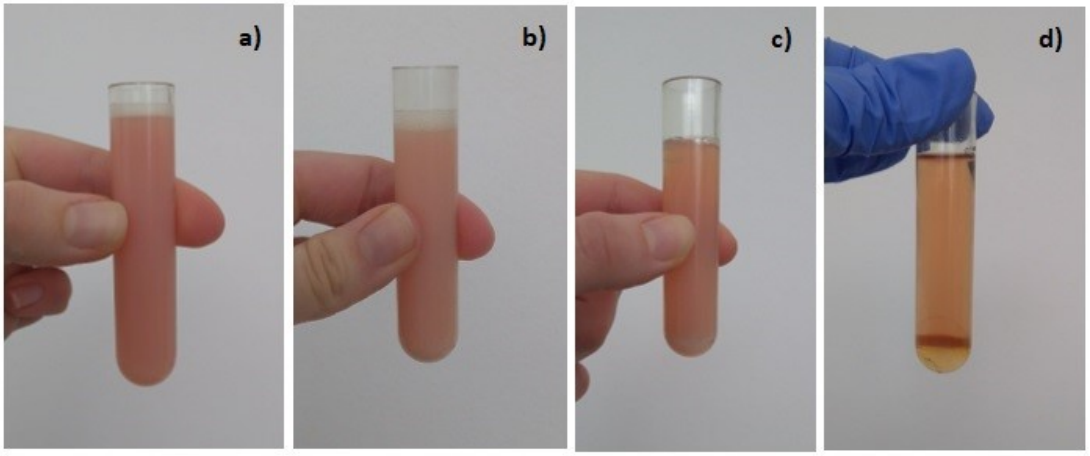

Figure 16. The centrifugation of hepatic homogenates of Prussian carp (*C. gibelio*): a) homogenate before centrifugation; b) after centrifugation at 3000×g; c) after centrifugation at 10000×g; d) after centrifugation at 50000×g.

2.3.2 Digestion of hepatic homogenates and cytosols

While homogenizing the liver, we took an aliquot of each homogenate for a subsequent digestion. The procedure for the digestion was adapted from a previously described procedure (Dragun *et al*., 2013a; Filipović Marijić *et al*., 2013). The digestion of the hepatic homogenates was done in duplicate by adding oxidation mixture (v/v 1:3), containing concentrated HNO_3 (Rotipuran® Supra 69%, Carl Roth GmbH + Co. KG, Germany) and 30% H_2O_2 (Suprapur®, Merck, Germany) (v/v 3:1). All the digestions took place at 85°C in the laboratory dry oven for 3.5 hours.

Apart from the hepatic homogenates, the cytosolic fractions were also digested. The digestion of these fractions was also done in duplicate by adding oxidation mixture (v/v 1:1). This oxidation mixture also contained concentrated HNO_3 (Rotipuran® Supra 69%, Carl Roth GmbH + Co. KG, Germany) and 30% H_2O_2 (Suprapur®, Merck, Germany) (v/v 3:1). Analogous to the digestion of the hepatic homogenates, the digestion of the cytosolic fractions was done in the laboratory dry oven at 85°C for 3.5 hours.

After the digestion of both the homogenates and the cytosolic fractions, the samples were diluted before analyses. They were diluted five times for the analyses of calcium (Ca) and trace elements, and 20 times for the analyses of sodium (Na), potassium (K) and magnesium (Mg) (Dragun *et al*., 2018).

2.3.3 Measurement of trace and macro elements

There were 17 trace and macro elements that were analysed using HR ICP-MS (Element 2, Thermo Finnigan, Germany). This HR ICP-MS was equipped with an autosampler SC-2 DX FAST (Elemental Scientific, USA) and a sample introduction kit which consisted of a Seaspray nebulizer and a cyclonic spray chamber Twister. Indium (1µg/L; indium atomic spectroscopy standard solution, Fluka, Germany) was used as an internal standard in all samples (Fiket *et al*., 2007). Several elements, ^{82}Se, ^{85}Rb, ^{98}Mo, ^{109}Ag, ^{111}Cd and ^{133}Cs, were measured in low resolution mode; ^{23}Na, ^{24}Mg, ^{42}Ca, ^{55}Mn, ^{56}Fe, ^{59}Co, ^{63}Cu, ^{66}Zn, and ^{86}Sr were measured in medium resolution mode; and ^{39}K and ^{75}As were measured in high resolution mode. Three standards were used for external calibration:

1. a multi-element standard for macro elements containing Na, K, Mg and Ca (Fluka, Germany),
2. a standard containing Ag (Fluka, Germany), and
3. a multielement standard solution for trace elements (Analytika, Czech Republic) supplemented with Rb (Sigma-Aldrich, Germany) and Cs (Fluka, Germany).

Indium (1µg/L; Fluka, Germany) was added after preparation of all the standards in 1.3% HNO_3 (Suprapur, Merck, Germany). All measurements were performed in duplicate (Dragun *et al*., 2018).

Quality control samples acquired from UNEP/GEMS (QC trace metals, catalogue no. 8072, lot no. 146142–146143; QC minerals, catalogue no. 8052, lot no. 146138–146139; Burlington, Canada) were used to check the accuracy of HR ICP-MS measurements. In general the acquired values were close to the certified values when looking at the following recoveries (%): Ag (91.1±7.5), As (98.0±5.1), Ca (95.2±2.4), Cd (95.7±1.9), Co (94.1±2.9), Cu (98.4±5.1), Fe (94.7±3.4), K (94.8±5.1), Mg (92.6±1.7), Mn (93.5±3.2), Mo (93.9±1.9), Na (96.1±3.8), Se (99.5±6.3), Sr (97.1±2.2), and Zn (107.3±12.2).

Total trace and macro element concentrations in fish were obtained based on the measurements in digested homogenates, whereas soluble/cytosolic trace and macro element concentrations were obtained from measurements in cytosolic fractions of liver. The concentrations obtained are presented as ng/g or µg/g of wet hepatic tissue.

Limits of detection (LOD) for cytosols were calculated by taking three times the standard deviation of trace and macro elements measured in ten blank samples (100 mM Tris-HCl/Base, 1 mM dithiothreitol) which were digested using the same procedure as cytosolic samples. Limits of detection for macro elements in cytosols (µg/g) were: Ca, 1.07; K, 0.112; Mg, 0.024; and Na, 0.320. Limits of detection for trace elements in cytosols (ng/g) were: Ag, 0.255; As, 6.72; Cd, 0.430; Co, 0.266; Cs, 0.102; Cu, 13.5; Fe, 141; Mn, 0.810; Mo, 0.680; Rb, 0.339; Se, 2.93; Sr, 1.09; and Zn, 635 (Dragun *et al*., 2018). According to the applied digestion procedure, LODs for trace and macro element concentrations in the homogenates were two times higher than the LODs for trace and macro elements in the cytosols (Dragun *et al*., 2018).

The proportions of trace and macro elements in the soluble cytosolic fractions of *C. gibelio* liver were calculated as the ratios of the cytosolic to total trace and macro element

concentrations in *C. gibelio* liver, multiplied by 100, and expressed in percentages (Dragun *et al*., 2018).

2.4 Analyses of biomarkers in Prussian carp liver

2.4.1 Analysis of total cytosolic protein (TP) concentrations

Total cytosolic protein concentrations were measured according to Lowry *et al*. (1951) with the Bio-Rad DC Protein Assay, which was applied according to the instructions of the manufacturer. This is a colorimetric assay which uses two reagents. Reagent A is an alkaline copper tartrate solution, and reagent B contains a diluted Folin reagent. We needed to perform two steps to get colour development (Figure 17).

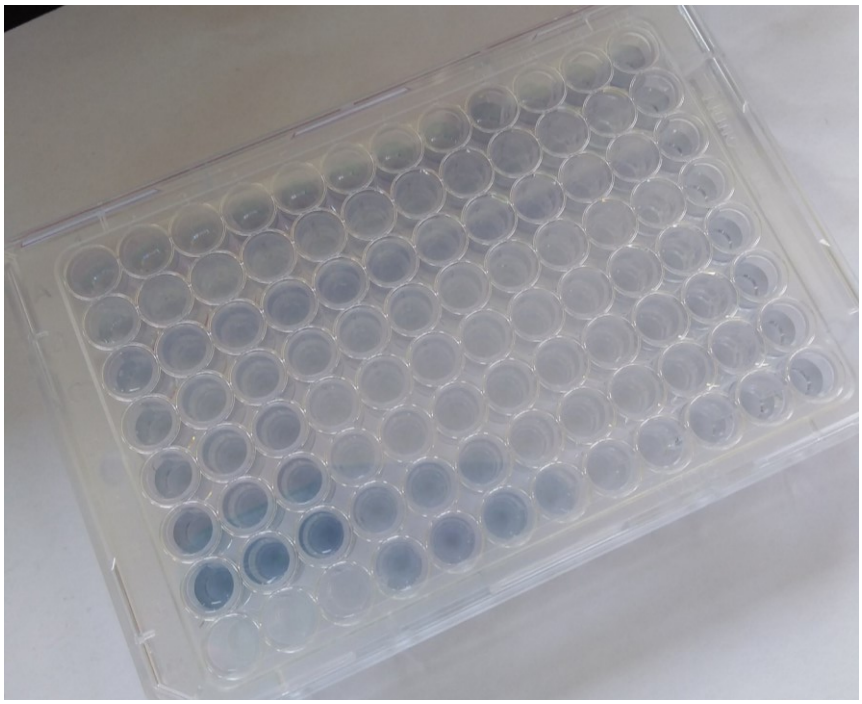

Figure 17. Example of a Bio-Rad DC Protein Assay performed according to Lowry to measure total protein concentrations.

In the first step, there was a reaction between the copper in reagent A and the protein. In the second step, the copper-treated protein induced reduction of the Folin reagent by one, two or three oxygen atoms. The characteristic blue colour of these reduced species of Folin reagent was measured with the photometer Microplate Reader HT3 (Anthos, Austria) at a wavelength of 750 nm. Five different concentrations (0.25-2.0 mg/mL) of bovine serum albumin (Serva, Germany) dissolved in the homogenization buffer were used to construct the calibration curve (Figure 18) (Dragun *et al*., 2013b; BIO-RAD).

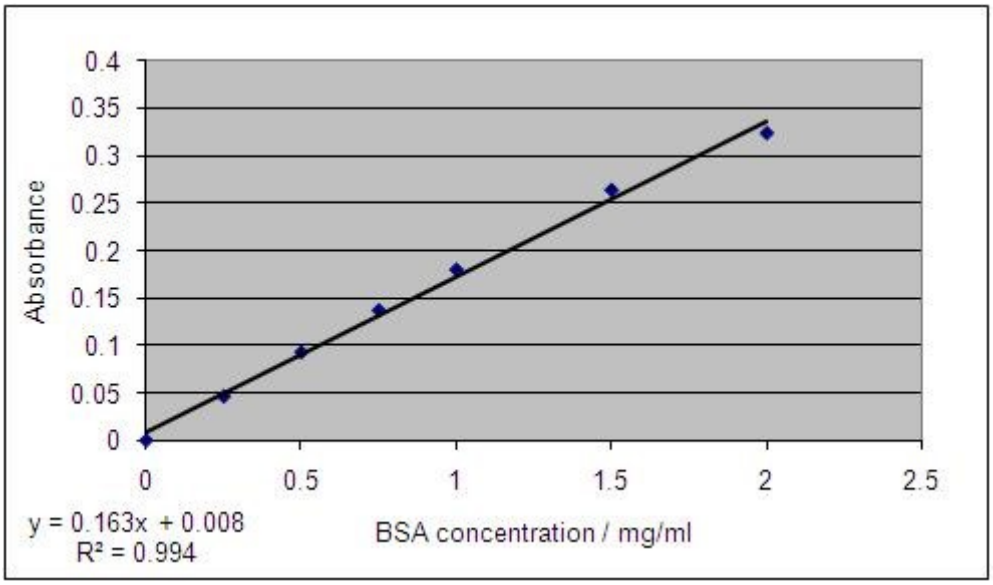

Figure 18. Calibration line for measurement of total cytosolic protein concentrations.

2.4.2 Analysis of metallothionein (MT) concentrations

To measure MTs, cytosolic fractions (S50) that were purified by heat-treatment were used. The heat-treatment was necessary to denature high molecular mass proteins which could interfere with the electrochemical MT determination (Erk *et al*., 2002). First the cytosolic fraction was diluted 10 times with 0.9% NaCl (Suprapur, Merck), then it was heat treated for 10 min at 85 °C in The Dri Block (Techne), and subsequently it was placed on ice for 30 min. Afterwards the heat treated cytosol was centrifuged at 10000×g for 15 min at 4°C in Biofuge Fresco centrifuge (Kendro, USA). The supernatant (HT S50) was separated from the pellet and stored at -80°C. The MT concentrations in HT S50 were measured by differential pulse voltammetry (DPV) followed by the modified Brdička procedure (Raspor *et al*., 2001) using 797 VA Computrace (Metrohm, Switzerland) with a three-electrode system (hanging mercury drop electrode, HMDE, with a surface area of 0.40 mm^2 as a working electrode, an Ag/AgCl/saturated KCl reference electrode and a platinum counter electrode).

Measurements were performed in 10 ml of supporting electrolyte solution (5 ml 2M NH_4Cl/NH_4OH and 5 ml 1.2×10^{-3} M $Co(NH_3)_6Cl_3$; pH = 9.5) at a temperature of 20°C, deaerated with extra pure nitrogen. The volume of 20-40 µl of hepatic HT S50 was added for measurement. DPV had the following instrumental parameters: potential scan from −0.9 V to −1.65 V; scan rate 0.005 V s^{-1}; voltage pulse amplitude 0.025 V; duration of the pulse application 0.057 s; and a clock time 0.5 s.

A calibration curve for deriving MT concentrations (µg/ml) was constructed by using the commercially available, ≥95% pure, rabbit liver Zn_7-MT2 (Enzo Life Sciences, USA) dissolved in 0.25 M NaCl. The final results were expressed as mg of MTs per g of hepatic tissue (wet mass), obtained by multiplying the measured MT concentrations with the dilution factors (Dragun *et al*., 2009a; Dragun *et al*., 2013b).

2.4.3 Analysis of catalase (CAT) activities

The activity of the CAT (rate of H_2O_2 decomposition) was measured spectrophotometrically by registering the changes in H_2O_2 absorbance during 90 seconds. The reaction was registered by absorbance decreases at a wavelength of 240 nm. Activity of catalase was measured in supernatants obtained after centrifugation of hepatic homogenates at 10000×g for 30 minutes (S10) (Khessiba *et al*., 2005).

The reaction mixture for measuring the catalase activity consisted of 50 mM phosphate buffer (Kemika, Zagreb; pH=7.0 at room temperature) and 15 mM H_2O_2 (*Suprapur*, Merck, Germany).

The protocol consisted of the following steps:

1) preparation of 100 mL of 50 mM phosphate buffer in MilliQ water, pH=7.0 at room temperature;

2) preparation of 15 mM H_2O_2 (*Suprapur*, Merck, Germany) in freshly prepared 50 mM phosphate buffer;

3) 50 times dilution of hepatic supernatants S10 in 50 mM phosphate buffer;

4) measurement of CAT activity in microplates (Figure 19)

- addition of 15 µl of diluted samples (supernatants S10) into the wells of microplate;
- addition of 285 µL of 15 mM H_2O_2 into microplate wells containing diluted samples;
- measurement of absorbance decrease (ε = 40 mM^{-1} cm^{-1}) at a wavelength of 240 nm (Figure 20)
  ➔ repeating the last step 9 times, measuring every 10 seconds;

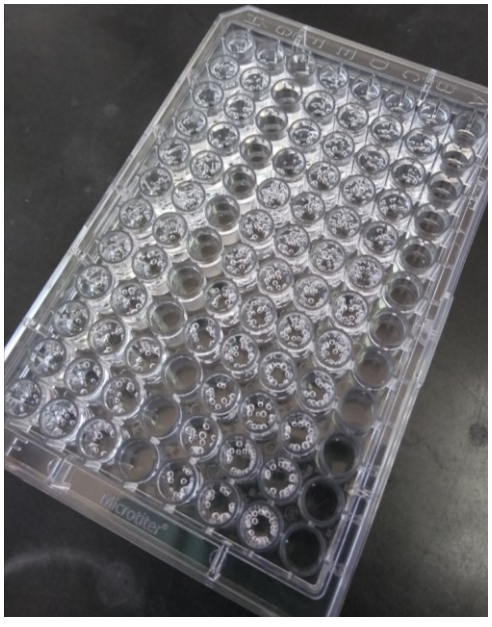

Figure 19. Example of 96-well plate in which the catalase test has been performed on samples.

5) determination of protein concentrations in supernatants S10, according to Lowry *et al*. (1951); supernatants should be diluted 20 times in 100 mM Tris buffer (Tris-HCl/Base, Sigma, pH 7.4 at room temperature), prior to measurement.

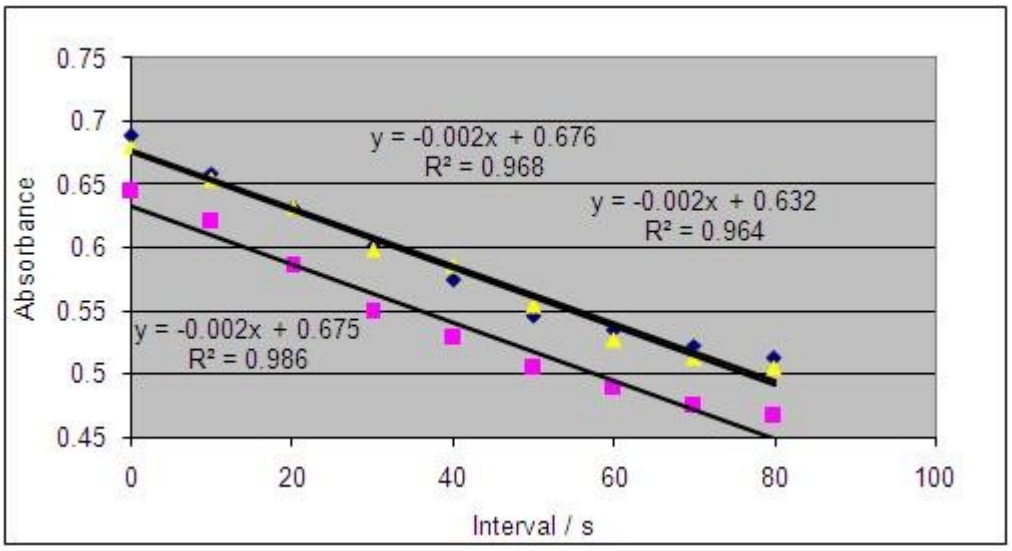

Figure 20. Example of absorbance decrease during the measurement of catalase activity.

The activity of catalase is expressed in μmol of decomposed H_2O_2 per minute per mg of S10 proteins, i.e. in catalytic units per mg of S10 proteins (U/mg).

2.4.4 Analysis of total glutathione (tGSH) concentrations

Concentrations of tGSH were determined using a modification of Tietze`s recycling assay (Tieze, 1969) described in Akerboom nad Sies (1981) and adapted to the microplate reader in the Laboratory for biological effect of metals. The assay is based on the reaction of GSH and DTNB (5,5'-dithiobis(2-nitrobenzoic acid)) with the formation of a chromophore and an oxidized glutathione-TNB (GS-TNB) complex. This chromophore, TNB, has an absorbance in the visible light spectrum at 412 nm. The amount of TNB measured is proportional to the amount of GSH in the sample. The oxidized GS-TNB complex is reduced in the presence of NADPH by glutathione reductase (GR), thereby forming GSH that can be reused in the reaction. Due to the fact that GR reduces one molecule of GSSG to two molecules of GSH, the total amount of glutathione is the sum of the reduced and the oxidized glutathione in the sample (as shown in the equation below).

$$[GSH]_{total} = [GSH] + 2\times[GSSG]$$

The rate by which the absorbance changes (ΔA_{412nm} / min) is linearly proportional to the total amount of glutathione. When determining the concentration of an unknown sample, we use the linear equation we obtained using the regression curve which was generated from the reaction rates of known concentrations of GSH standards.

This way of analysing is quick, simple, sensitive, accurate and it has minimal interference from the other non-specific thiol groups and minimal loss of GSH as a result of the metabolic usage in the GSH-requiring processes. The method consists of the removal of

protein precipitates when working with proteinaceous samples, the mixing of the sample with the DTNB + GR reagent, the addition of NADPH and the measurement of the absorbance at 412 nm for five minutes with a one minute interval. The reagents required for this analysis are listed in Table II.

Table II. Reagents for GSH determination

KH_2PO_4 (Potassium dihydrogen orthophosphate)	M_r 136.09	Kemika
K_2HPO_4 (Dipotassium hydrogen orthophosphate)	M_r 174.2	Kemika
EDTA sodium salt	M_r 372.24	Merck
SSA (Sulfosalicylic acid)	M_r 254.2	Kemika
DTNB (5,5'-Dithiobis(2-Nitrobenzoic acid)) **Store at room temperature. Protect from light.**	M_r 396.3	Sigma
β-NADPH **Store at 4°C.**	M_r 833.4	
Glutathione reductase **Store at 4°C.**	500 units/ml	Sigma G-3664
Glutathione (reduced form), GSH, **Store at 4°C.**	M_r 307.3	Sigma

Solutions required for sample preparation and GSH analysis

- 0.1 M potassium phosphate buffer with 1 mM EDTA disodium salt, pH 7.5 (KPE)
- 5% 5-sulfosalicylic acid (SSA) solution
- 0.5% SSA solution

Stock solutions

- DTNB stock solution (3.79 mM)
  → 1.5 mg of DTNB in 1 ml of KPE
- NADPH stock solution (1.92 mM)
  → 1.6 mg of β-NADPH in 1 ml of KPE
- GSH stock solution (the primary stock, 10 mM GSH)
  → 3.073 mg of GSH in 1 ml of 0.5% SSA
- glutathione reductase (GR) stock solution (6 U/mL)
  → 9 µL of GR (500 U/ml) in 741 µL of KPE

Working solutions

The working solutions should be freshly prepared

- DTNB+GR working solution
  → 228 µL GR stock solution / 228 µL DTNB stock solution / 8 ml KPE
- NADPH working solution (0.192 mM)
  → dilution of NADPH stock solution (1.92 mM) 10 times with KPE

Protocol

Sample preparation

1. Weighing the tissue samples (30-40 mg is sufficient for analysis) and homogenizing them in ice-cold 5% SSA. The ratio of tissue to SSA has to be 1:5.
2. Transferring the homogenates into cooled 1.5 ml Eppendorf tubes and keeping them on ice.
3. Centrifuging the homogenates at 10000×g for 10 minutes at 4°C.
4. Transferring the supernatants into cooled Eppendorf tubes. When not used directly, the samples should be stored at -80°C.

Preparation of GSH standards

1. Making a series of GSH solutions with following concentrations:
   a. **Primary stock** (stock 1)**: 10 mM GSH**;
   b. **Secondary stock** (stock 2)**: 1 mM GSH**;
   c. **Tertiary stock** (stock 3): **100 µM GSH**.

2. Making an additional series of standards by making a twofold serial dilution from the tertiary GSH stock. The concentration of the standard series will range from 50 – 3.125 nM/ml. The procedure to make this series is shown in Table III.

Table III. GSH standard series

	Standard 1	Standard 2	Standard 3	Standard 4	Standard 5
GSH standards (nM/mL)	50	25	12.5	6.25	3.125
GSH (µL)	250 (from tertiary stock)	250 (from standard 1)	250 (from standard 2)	250 (from standard 3)	250 (from standard 4)
0.5% SSA (µl)	250	250	250	250	250

A volume of 250 µl should be removed from standard 5 to have equal volumes in all five standards.

GSH assay: measurement

1) This step starts by diluting the samples ten times with Milli-Q water and if it is necessary, diluting them further with 0.5% SSA.

2) Setting a plate reader to the wavelength of 412 nm and programming the reader with a kinetic read for five minutes with one minute intervals (six readings), shaking for ten seconds and letting it settle for three seconds.

3) Making a reaction scheme with the following steps:

   a) adding 10 µl in each well; doing the test in triplicate, for blank (0.5% SSA), GSH standard and for the unknown sample;
   b) adding 150 µl of DTNB+GR working solution to every well; mixing it and letting it incubate for five minutes at room temperature; covering the plate with aluminium foil;
   c) adding 50 µl of NADPH working solution to each well;
   d) measuring the absorbance at 412 nm for five minutes with a one minute intervals.

4) Calculating the GSH concentrations

   a) calculating the blank, the standards and the sample rate with linear regression (i.e. slopes represent reaction rates used in GSH calculations);
   b) subtracting the value of the reagent blank form the measurements of each standard and sample (adjusted rates);
   c) making a graph with the known nmol/ml concentrations of the GSH standards on the x axis and their adjusted rates (slope) on the y axis (Figure 21);
   d) using linear regression to generate the equation of the standard curve;
   e) using that equation to calculate the GSH concentration of each sample;
   f) multiplying the GSH concentrations of the samples by the supernatant dilution factor and by a factor of six to convert the nmol/ml to nmol of GSH/g of wet tissue mass.

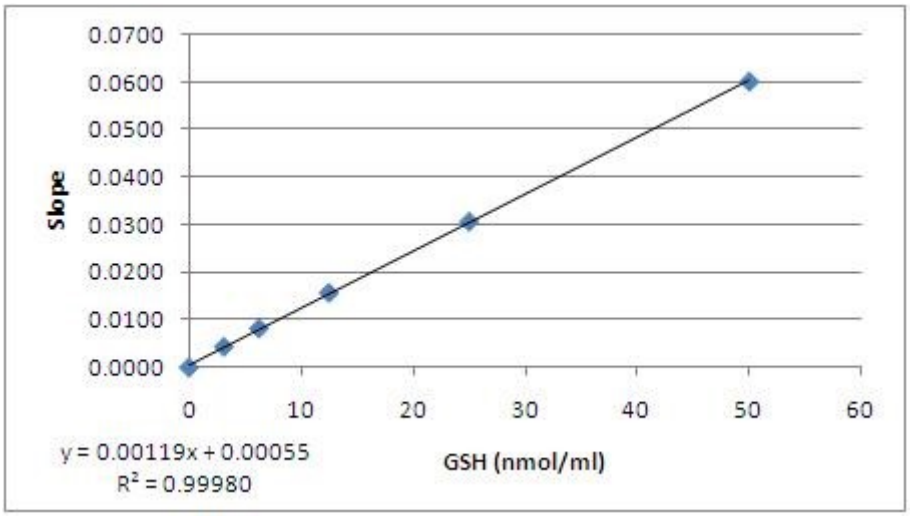

Figure 21. Calibration line for measurement of GSH concentrations.

2.5 Data processing and statistical analysis

Microsoft Excel 2007 was used for performing the basic calculations. SigmaPlot 11.0 for Windows was used for statistical analysis and creating the graphs. Nonparametric statistical tests have been used because expectations of normality and homogeneity of variance were not always met. The level of significance was set at 95% ($p < 0.05$) (Dragun *et al*., 2018).

3 Results

3.1 Concentrations of dissolved trace and macro elements in the river water

Most of the studied elements had resembling concentrations at both sites (Table IV). The highest spatial difference was found for Cs. Caesium was 90 times higher than the LOD at the Trebež site, whereas it had values below LOD at the Ilova village site.

Table IV. Dissolved trace and macro element concentrations in the river water, expressed as average ± standard deviation, and limits of detection (LOD) of each analysed element.

Element	Ilova village	Trebež village	LOD (µg/L)
Na (mg/L)	9.91 ± 0.359	26.5 ± 0.171	5.60
Mg (mg/L)	15.1 ± 0.556	16.9 ± 0.189	6.04
K (mg/L)	2.82 ± 1.70	4.58 ± 0.065	1.98
Ca (mg/L)	47.3 ± 1.78	57.0 ± 0.829	21.2
Se (µg/L)	0.786 ± 0.019	1.01 ± 0.112	0.059
Rb (µg/L)	0.644 ± 0.008	3.74 ± 0.251	0.003
Mo (µg/L)	0.561 ± 0.027	0.981 ± 0.062	0.011
Ag (µg/L)	<LOD	<LOD	0.001
Cd (µg/L)	0.011 ± 0.006	0.053 ± 0.003	0.002
Cs (µg/L)	<LOD	0.090 ± 0.007	0.001
Mn (µg/L)	93.2 ± 1.13	18.4 ± 0.918	0.050
Fe (µg/L)	17.9 ± 2.17	21.6 ± 1.52	0.624
Co (µg/L)	0.137 ± 0.005	0.121 ± 0.011	0.019
Cu (µg/L)	<LOD	0.716 ± 0.030	0.401
Zn (µg/L)	<LOD	<LOD	7.340
Sr (µg/L)	123.1 ± 1.03	150.4 ± 11.9	0.182
As (µg/L)	2.10 ± 0.126	4.47 ± 0.684	0.028

The other differences were found for metals Rb and Cd. They both had five to six times higher concentrations at the contaminated Trebež site than at the reference Ilova village site, whereas Na, K and As had approximately twice higher concentrations at Trebež. Manganese surprisingly had five times higher concentrations at the reference site. It also has to be noted that the values of Zn and Ag were so low at both of the studied sites, they could not be detected by applied methodology.

3.2 Biometry of fish

Twenty specimens of Prussian carp (*C. gibelio*) were caught at each sampling site. Their basic biometric characteristics are presented in Table V. Total lengths, as well as total masses of fish from the Trebež village were significantly higher than of the ones from the Ilova village (Figures 22 and 23). Comparing the medians, there is a statistically significant increase of ~20% in total length (Figure 22, Table V) and ~70% in total mass (Figure 23, Table V) of Prussian carp caught at the Trebež village in comparison to the Ilova village.

Table V. Biometric characteristics of *C. gibelio* expressed as average ± standard deviation with median within brackets.

Biometry	**Ilova village**	**Trebež village**
Total length (cm)	16.2 ± 1.62 (16.0)	18.8 ± 2.91 (18.7)
Total mass (g)	69.8 ± 23.172 (67.1)	122.3 ± 58.1 (112.2)
FCI (%)	1.59 ± 0.085 (1.59)	1.69 ± 0.119 (1.69)
HSI (%)	5.87 ± 1.78 (5.74)	5.44 ± 1.52 (5.58)
GSI (%)	3.11 ± 1.44 (2.59)	4.67 ± 2.68 (4.53)
Sex	F: 13/20 M: 7/20	F: 12/20 M: 8/20

Concerning the FCI (Figure 24, Table V) we can conclude that it was significantly higher at Trebež by 10%. The other two indices, GSI and HSI, (Figures 25 and 26, respectively, Table V) were comparable at both sites, but the difference between the lowest and highest GSI was more pronounced at the Trebež village than at the Ilova village. Though there was no significant difference, it still pointed to a higher variation of gonad

size in fish caught at the Trebež village. Sex composition was similar at both sites, with 60-65% of females within the sampled populations (Table V).

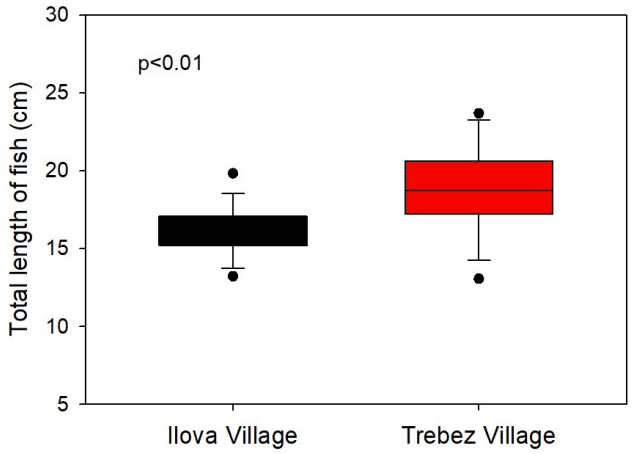

Figure 22. Boxplots of total lengths of *C. gibelio* at the reference site (Ilova village) and the potentially contaminated site (Trebež village).

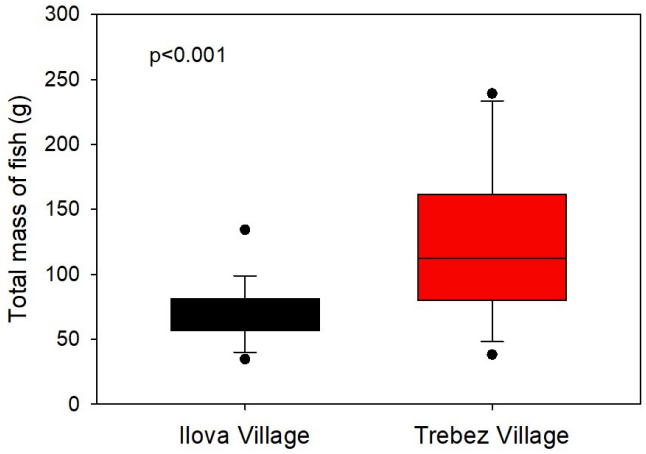

Figure 23. Boxplots of total masses of *C. gibelio* at the reference site (Ilova village) and the potentially contaminated site (Trebež village).

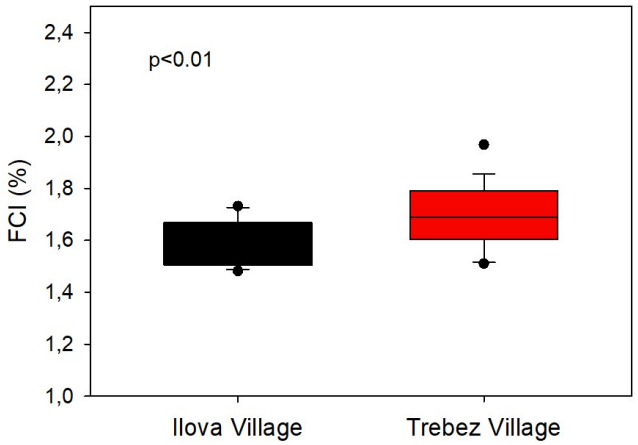

Figure 24. Boxplots of Fulton condition indices of *C. gibelio* at the reference site (Ilova village) and the potentially contaminated site (Trebež village).

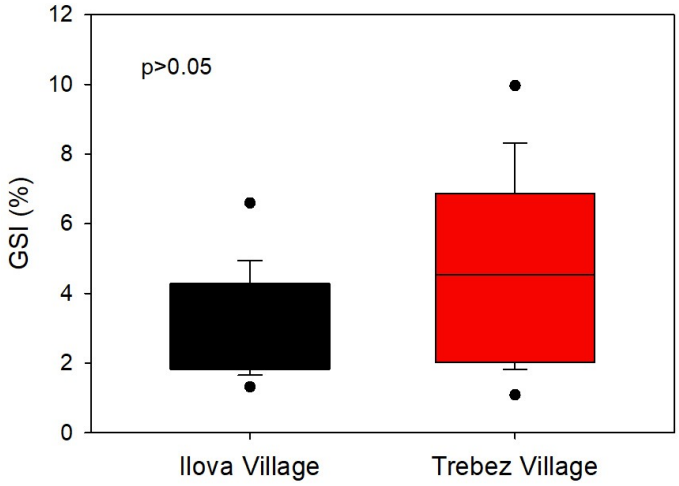

Figure 25. Boxplots of gonadosomatic indices of *C. gibelio* at the reference site (Ilova village) and the potentially contaminated site (Trebež village)

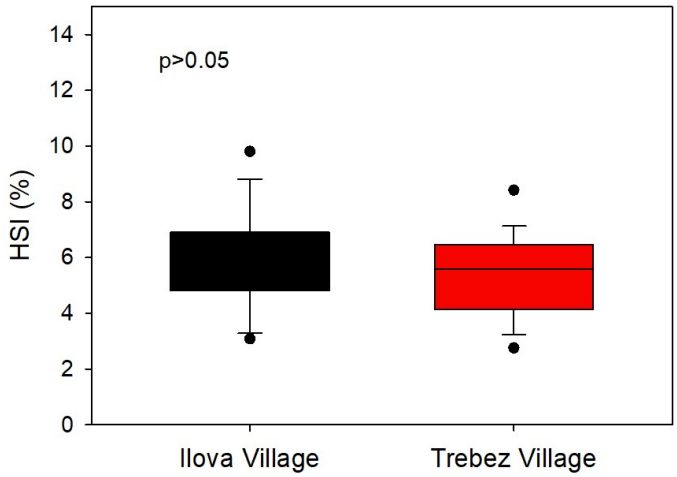

Figure 26. Boxplots of hepatosomatic indices of *C. gibelio* at the reference site (Ilova village) and the potentially contaminated site (Trebež village)

3.3 Concentrations of trace and macro elements bioaccumulated in the liver of Prussian carp

In the course of this study the total and cytosolic concentrations of 17 trace and macro elements in the liver of Prussian carp (*C. gibelio*) from the reference site near the Ilova village and potentially contaminated site near the Trebež village were analysed, and the results are presented in Tables VI and VII, respectively. The majority of analysed elements (Na, Mg, K, Se, Mo, Cd, Mn, Fe, Co, Cu, Zn, and As) had comparable concentrations, both total and cytosolic, at the two studied sites. Significantly higher values of either total or cytosolic concentrations, or both, near the Trebež village was observed only for Rb and Cs, whereas Ca, Ag and Sr had significantly higher values at the reference site, near the Ilova village.

Table VI. Total trace and macro element concentrations in hepatic tissue of *C. gibelio*, expressed as average ± standard deviation with median within brackets, and limits of detection (LOD) of each analysed element in digested hepatic homogenate (Dragun *et al.*, 2018).

Element	Ilova village	Trebež village	LOD
Na (µg/g)	447.4 ± 54.8 (444.1)	516.1 ± 91.5 (493.7)	0.640 µg/g
Mg (µg/g)	122.7 ± 8.40 (122.9)	131.2 ± 17.3 (130.2)	0.048 µg/g
K (µg/g)	2952 ± 125.1 (2960)	2974 ± 282.1 (2996)	0.224 µg/g
Ca (µg/g)	16.7 ± 5.97 (15.4)	15.3 ± 6.59 (12.84)	2.14 µg/g
Se (ng/g)	437.4 ± 90.1 (440.5)	463.2 ± 103.0 (419.0)	5.86 ng/g
Rb (µg/g)	0.971 ± 0.331 (0.900)	1.17 ± 0.329 (1.08)	0.678 ng/g
Mo (ng/g)	70.8 ± 23.5 (65.1)	70.9 ± 21.1 (63.1)	1.36 ng/g
Ag (ng/g)	37.5 ± 19.7 (29.9)	25.7 ± 16.4 (20.7)	0.510 ng/g
Cd (ng/g)	147.8 ± 229.7 (84.7)	132.8 ± 182.6 (59.0)	0.860 ng/g
Cs (ng/g)	1.64 ± 0.556 (1.62)	2.54 ± 0.968 (2.37)	0.204 ng/g
Mn (ng/g)	490.7 ± 122.6 (458.5)	456.4 ± 150.6 (413.9)	1.62 ng/g
Fe (µg/g)	60.1 ± 66.0 (36.7)	49.0 ± 27.5 (38.3)	282 ng/g
Co (ng/g)	7.03 ± 1.48 (6.90)	7.02 ± 1.96 (6.81)	0.532 ng/g
Cu (µg/g)	6.89 ± 3.76 (5.86)	6.35 ± 3.45 (6.17)	27.0 ng/g
Zn (µg/g)	9.38 ± 1.96 (9.95)	9.76 ± 3.12 (9.14)	1270 ng/g
Sr (ng/g)	34.7 ± 12.6 (31.2)	24.0 ± 10.0 (18.9)	2.18 ng/g
As (ng/g)	23.1 ± 7.42 (19.5)	25.5 ± 7.13 (23.8)	13.4 ng/g

Table VII. Cytosolic trace and macro element concentrations in hepatic tissue of *C. gibelio*, expressed as average ± standard deviation with median within brackets, and limits of detection (LOD) of each analysed element in digested hepatic cytosol (Dragun *et al.*, 2018).

Element	Ilova village	Trebež village	LOD
Na (µg/g)	453.9 ± 55.6 (452.5)	542.8 ± 95.3 (521.0)	0.320 µg/g
Mg (µg/g)	117.7 ± 10.45 (117.3)	115.5 ± 12.5 (118.2)	0.024 µg/g
K (µg/g)	2962 ± 215.5 (2950)	2931 ± 262.1 (3019)	0.112 µg/g
Ca (µg/g)	10.9 ± 3.60 (10.5)	8.472 ± 3.39 (6.95)	1.07 µg/g
Se (ng/g)	517.4 ± 131.9 (507.1)	544.3 ± 121.7 (495.6)	2.93 ng/g
Rb (µg/g)	0.971 ± 0.325 (0.891)	1.22 ± 0.379 (1.14)	0.339 ng/g
Mo (ng/g)	52.0 ± 16.4 (49.2)	48.2 ± 12.6 (45.7)	0.680 ng/g
Ag (ng/g)	33.0 ± 20.0 (25.4)	24.7 ± 16.3 (21.0)	0.255 ng/g
Cd (ng/g)	169.7 ± 262.7 (95.1)	155.1 ± 217.2 (67.9)	0.430 ng/g
Cs (ng/g)	1.54 ± 0.605 (1.51)	2.54 ± 1.00 (2.32)	0.102 ng/g
Mn (ng/g)	437.5 ± 101.5 (415.9)	414.3 ± 132.4 (376.0)	0.810 ng/g
Fe (µg/g)	43.2 ± 40.7 (30.5)	36.5 ± 16.4 (31.1)	141 ng/g
Co (ng/g)	6.91 ± 1.60 (6.82)	6.98 ± 2.02 (6.87)	0.266 ng/g
Cu (µg/g)	7.61 ± 4.26 (6.70)	7.03 ± 3.74 (6.92)	13.5 ng/g
Zn (µg/g)	9.63 ± 2.33 (9.89)	10.2 ± 3.45 (9.45)	635 ng/g
Sr (ng/g)	24.7 ± 7.71 (22.8)	16.4 ± 5.54 (14.7)	1.09 ng/g
As (ng/g)	18.5 ± 6.78 (15.9)	19.2 ± 6.95 (18.4)	6.72 ng/g

Furthermore, the percentage of trace and macro elements present within the hepatic cytosol of *C. gibelio* (compared to the total metal concentration) were calculated, which is a fraction presumably more available for metabolic requirements and possible toxic effects. The results of this analysis are presented in Table VIII. Percentages of almost 100% in the cytosol were observed for Se, Cd, Cu, Na, K, Rb, and Zn, The other elements were also

partly present in the other parts of the cell (cell membranes, organelles, granules), so their percentages within the cytosol were lower: Mg, Cs and Co (90-100%), Ag and Mn (80-90%), Mo, Fe, Sr and As (70-80%) and Ca (50-70%).

Table VIII. Percentages of trace and macro elements in the hepatic cytosols of *C. gibelio*, calculated as ratios between cytosolic and total element concentrations in hepatic tissue multiplied by 100, expressed as average ± standard deviation with median within brackets.

Metal	Ilova village	Trebež village
Na (%)	102.2 ± 8.67 (102.8)	105.2 ± 3.75 (105.1)
Mg (%)	95.9 ± 4.91 (96.5)	88.4 ± 5.17 (87.7)
K (%)	100.4 ± 5.98 (100.9)	98.7 ± 4.70 (98.1)
Ca (%)	66.6 ± 13.8 (69.0)	56.7 ± 10.6 (57.1)
Se (%)	117.4 ± 8.79 (117.5)	117.5 ± 5.44 (117.2)
Rb (%)	100.3 ± 5.72 (99.6)	104.0 ± 4.65 (104.5)
Mo (%)	74.2 ± 8.39 (77.7)	68.8 ± 7.93 (66.9)
Ag (%)	83.8 ± 14.5 (87.4)	92.9 ± 11.5 (93.3)
Cd (%)	115.0 ± 8.50 (114.1)	117.6 ± 5.63 (117.2)
Cs (%)	92.5 ± 8.05 (90.0)	99.4 ± 4.17 (100.0)
Mn (%)	89.8 ± 9.40 (89.4)	91.2 ± 4.84 (91.2)
Fe (%)	79.9 ± 15.9 (81.4)	78.8 ± 10.2 (79.9)
Co (%)	98.3 ± 7.54 (98.5)	99.7 ± 6.07 (99.5)
Cu (%)	109.5 ± 6.52 (109.3)	110.5 ± 5.06 (111.4)
Zn (%)	102.2 ± 7.08 (100.7)	104.4 ± 5.47 (105.3)
Sr (%)	72.7 ± 10.4 (71.8)	71.7 ± 12.8 (70.8)
As (%)	79.5 ± 11.5 (75.1)	75.3 ± 16.6 (78.4)

3.3.1 Sodium (Na)

For hepatic Na statistically significant differences were found between two sites, with higher concentrations of both total and cytosolic Na in the liver of Prussian carp from the Trebež village (Figure 27 a,b, Tables VI and VII).

Total hepatic Na was ~10% higher in the fish from the Trebež village compared to the Ilova village (Figure 27a, Table VI), whereas cytosolic Na was 15% higher in the fish from that same site (Figure 27b, Table VII). Based on the comparison between total and cytosolic Na concentrations, we have found that approximately 100% of Na is present within the hepatic cytosol of *C. gibelio*, and the percentages obtained at two sites were not significantly different (Figure 28, Table VIII).

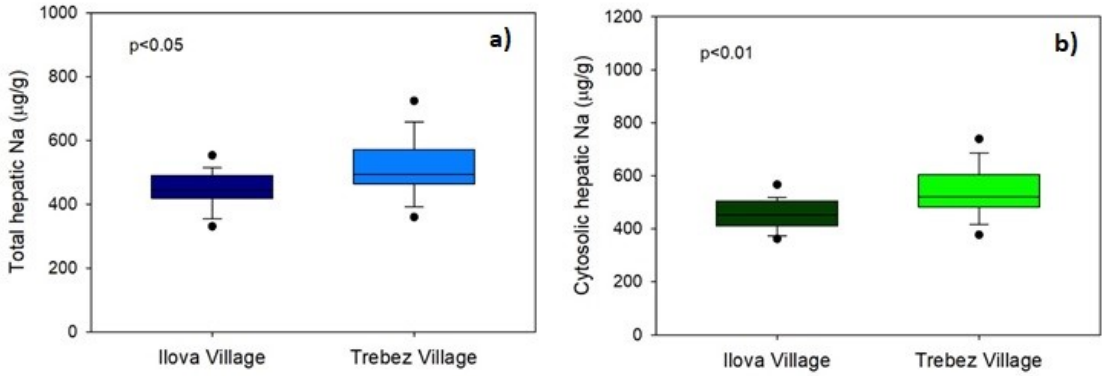

Figure 27. Total (a) and cytosolic (b) Na concentrations in the liver of *C. gibelio* at the reference site (Ilova village) and possibly contaminated site (Trebež village)

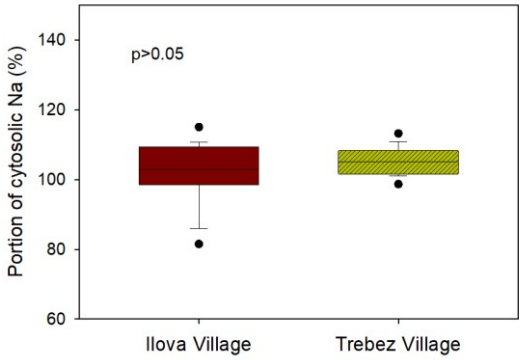

Figure 28. Ratio of cytosolic Na to total Na in the liver of *C. gibelio* expressed in percentages

3.3.2 Magnesium (Mg)

Total hepatic Mg concentrations (Figure 29a, Table VI) and cytosolic hepatic Mg concentrations (Figure 29b, Table VII) of the Prussian carp both showed no statistically significant difference between the reference site and the contaminated site. There was however a statistically significant difference between the proportions of cytosolic Mg in Prussian carp liver calculated for two sites (Figure 30, Table VIII). There was a difference of ~10% between the Ilova village and the Trebež village, with the lower value (~87%)

found at the Trebež village, which suggested that there was more Mg found in the nonsoluble part of the cells at the contaminated site than at the reference site.

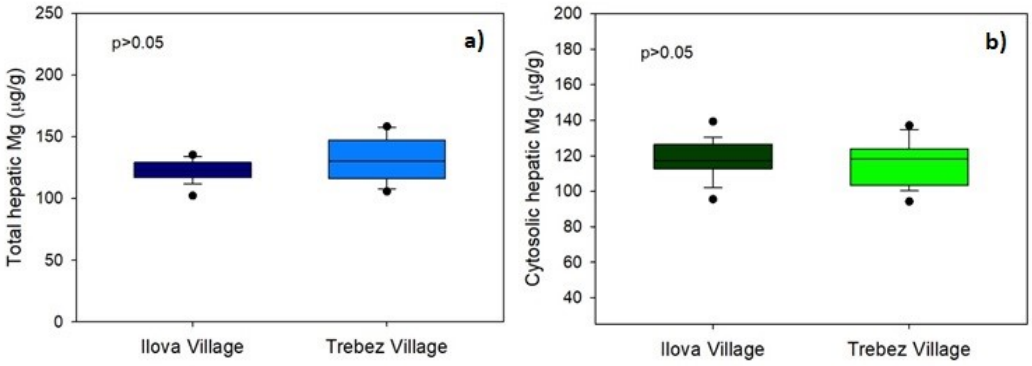

Figure 29. Total (a) and cytosolic (b) Mg concentrations in the liver of *C. gibelio* at the reference site (Ilova village) and possibly contaminated site (Trebež village)

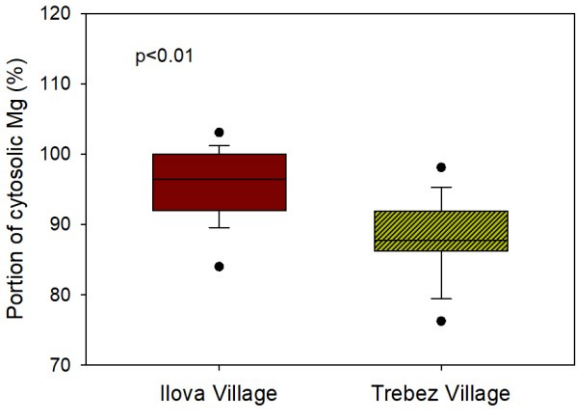

Figure 30. Ratio of cytosolic Mg to total Mg in the liver of *C. gibelio* expressed in percentages

3.3.3 Potassium (K)

No statistically significant difference was found for total hepatic K concentration and cytosolic hepatic K concentration of Prussian carp between the Ilova village and the Trebež village (Figure 31 a,b, Tables VI and VII). Also, for the portion of cytosolic K in Prussian carp liver no significant difference was found between sites (Figure 32, Table VIII). They were both ~100%.

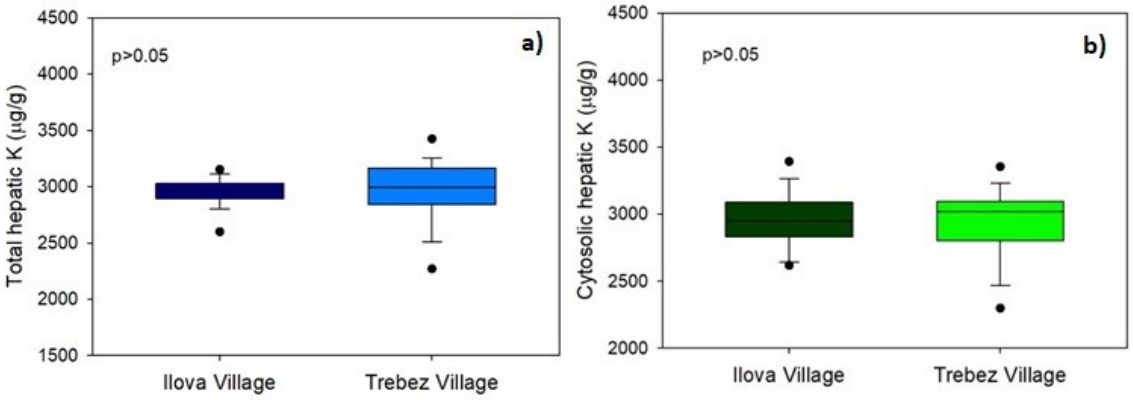

Figure 31. Total (a) and cytosolic (b) K concentrations in the liver of *C. gibelio* at the reference site (Ilova village) and possibly contaminated site (Trebež village)

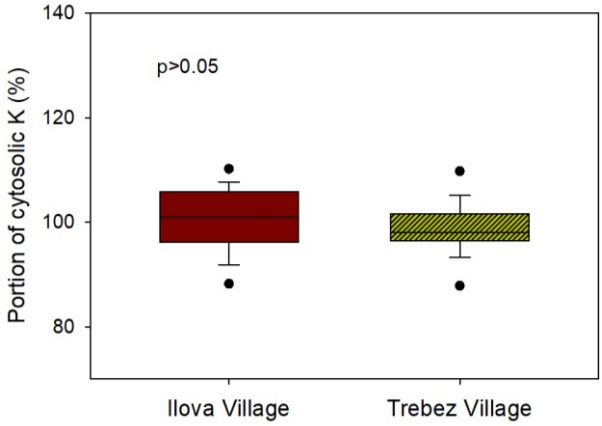

Figure 32. Ratio of cytosolic K to total K in the liver of Prussian carp expressed in percentages

3.3.4 Calcium (Ca)

There was no statistically significant difference found between fish caught at two sites regarding total hepatic Ca levels (Figure 33a, Table VI), but did found a difference regarding the cytosolic Ca level (Figure 33b, Table VI). There was a raise of ~50% of cytosolic hepatic Ca concentrations in Prussian carp from the Ilova village compared to fish from the Trebež village.

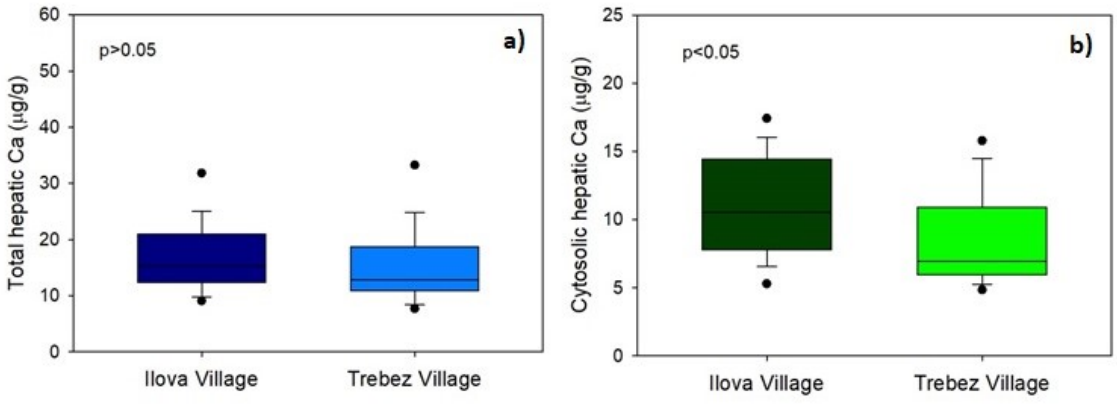

Figure 33. Total (a) and cytosolic (b) Ca concentrations in the liver of *C. gibelio* at the reference site (Ilova village) and possibly contaminated site (Trebež village)

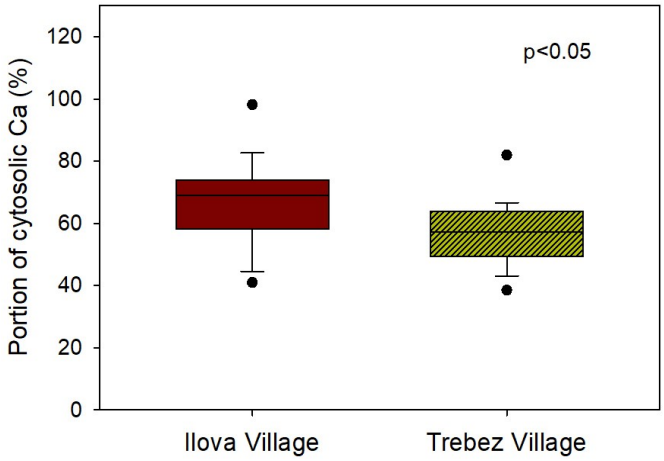

Figure 34. Ratio of cytosolic Ca to total Ca in the liver of Prussian carp expressed in percentages

Accordingly, the portion of cytosolic Ca in total Ca was only ~60% in fish from the Trebež village and ~70% in fish from the Ilova village (Figure 34, Table VIII). This difference was significant, and amounted to ~10%, similar as observed for Mg (Figure 30, Table VIII).

3.3.5 Selenium (Se)

Comparable results between two sites for both total hepatic Se and cytosolic hepatic Se of Prussian carp were found(Figure 35 a,b, Table VI and VII). The portions of cytosolic Se were almost identical at two sites and the difference was not statistically significant. A very high percentage of cytosolic concentration (~100%) was noticeable (Figure 36, Table VIII).

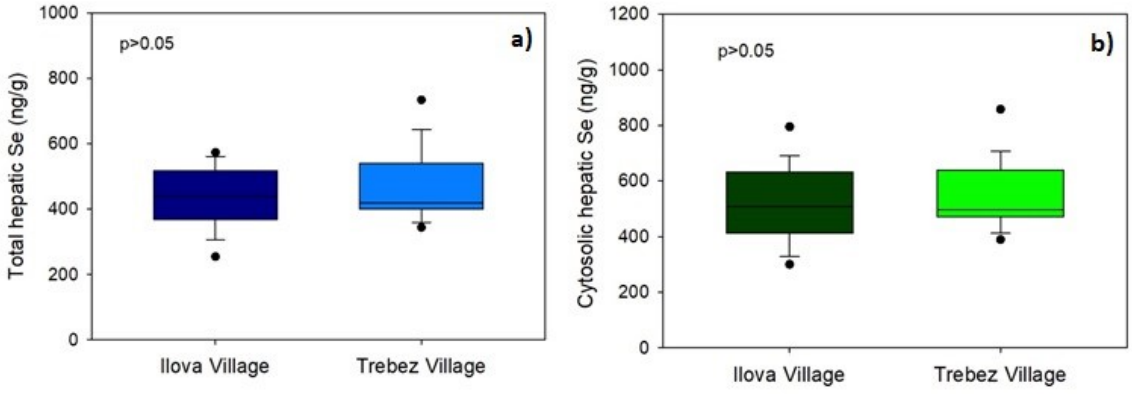

Figure 35. Total (a) and cytosolic (b) Se concentrations in the liver of *C. gibelio* at the reference site (Ilova village) and possibly contaminated site (Trebež village)

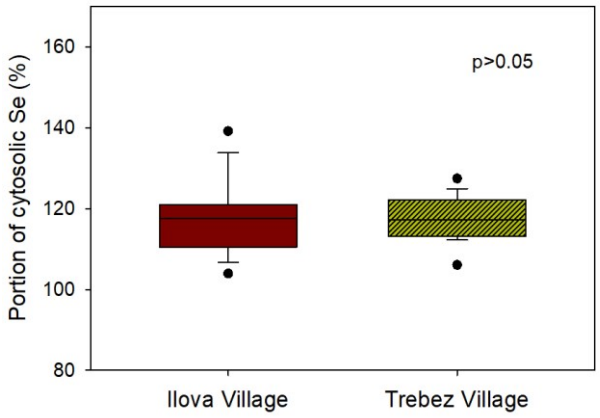

Figure 36. Ratio of cytosolic Se to total Se in the liver of Prussian carp expressed in percentages

3.3.6 Rubidium (Rb)

Rubidium has shown a statistically significant difference between two sites for both total hepatic concentrations and cytosolic hepatic concentrations in Prussian carp. The total hepatic Rb concentrations for fish at the Trebež village were 20% higher than at the Ilova village (Figure 37a, Table VI). For cytosolic hepatic Rb concentrations the difference was even higher, and amounted to ~30%, with higher values again observed at the Trebež village (Figure 37b, Table VII).

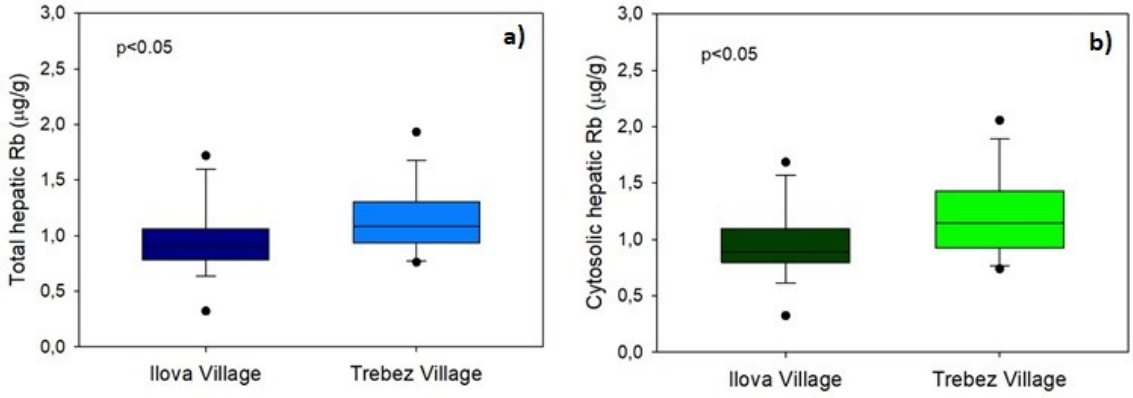

Figure 37. Total (a) and cytosolic (b) Rb concentrations in the liver of *C. gibelio* at the reference site (Ilova village) and possibly contaminated site (Trebež village)

When looking at the proportion of cytosolic hepatic Rb, a statistically significantly higher percentage (~5%) at the Trebež village compared to the Ilova village, with the lower percentage being ~100% (Figure 38, Table VIII) was noticed, meaning that somewhat more Rb was found in the cytosol of the liver from fish caught at the contaminated site than at the reference site.

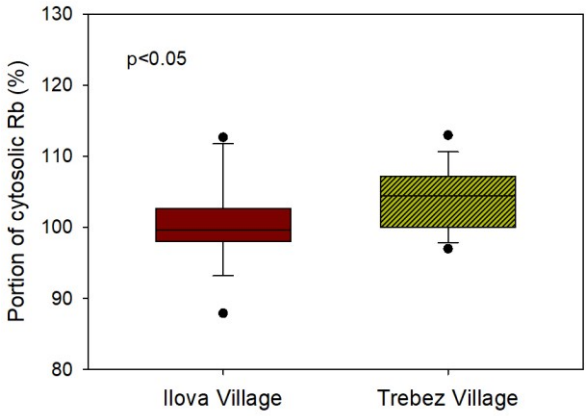

Figure 38. Ratio of cytosolic Rb to total Rb in the liver of Prussian carp expressed in percentages

3.3.7 Molybdenum (Mo)

Total and cytosolic Mo concentrations in the liver of *C. gibelio* were not statistically significantly different between the contaminated and the reference site (Figure 39 a,b, Tables VI and VII). A wider range of values for cytosolic hepatic Mo at the reference site compared to the contaminated site can be noticed, though the difference between sites was not statistically significant. A significant difference was perceived when comparing the ratios of cytosolic Mo to total hepatic Mo between fish caught at the Ilova village (~80%) and fish caught at the Trebež village (~70%) (Figure 40, Table VIII), similar to findings for Mg and Ca (Figures 30 and 34, Table VIII).

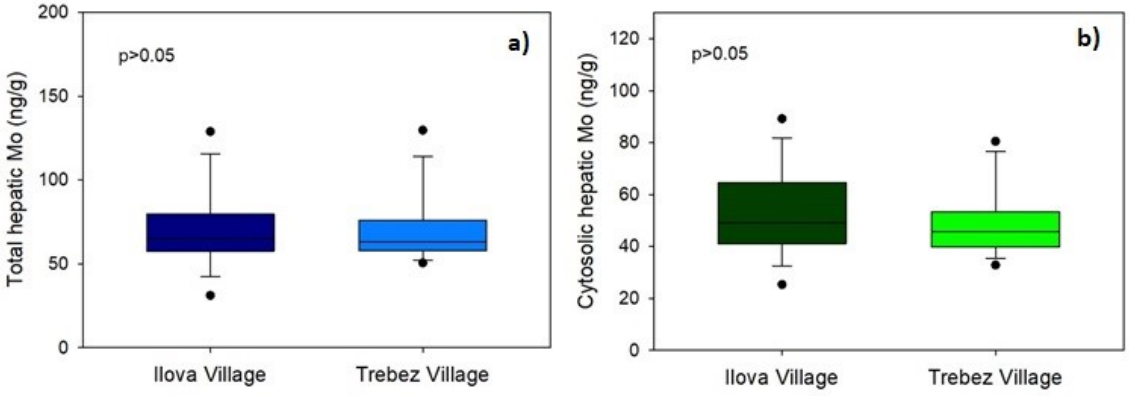

Figure 39. Total (a) and cytosolic (b) Mo concentrations in the liver of *C. gibelio* at the reference site (Ilova village) and possibly contaminated site (Trebež village)

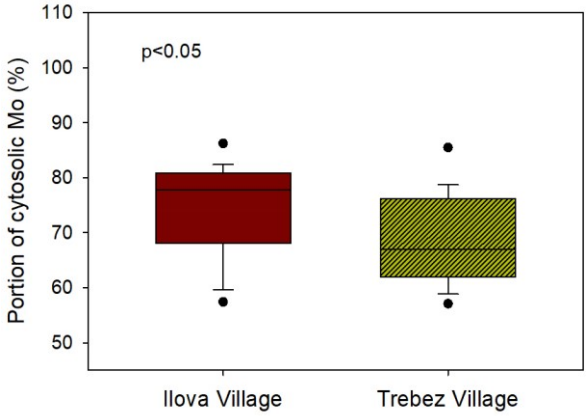

Figure 40. Ratio of cytosolic Mo to total Mo in the liver of Prussian carp expressed in percentages

3.3.8 Silver (Ag)

Total hepatic Ag concentrations in fish caught at the Ilova village and at the Trebež village were statistically significantly different (Figure 41a, Table VI). There was an increase of ~45% in total hepatic Ag concentrations at the Ilova village compared to the Trebež village. The cytosolic hepatic Ag concentrations were comparable at two sites (Figure 41b, Table VII).

Accordingly, there was a statistically significant difference between the portions of cytosolic Ag at the Ilova village and the Trebež village (Figure 42, Table VIII). The portion of cytosolic Ag in Prussian carp liver was ~87% of total hepatic Ag at the Ilova village, and ~93% at the Trebež village, similar as found for Rb (Figure 38, Table VIII).

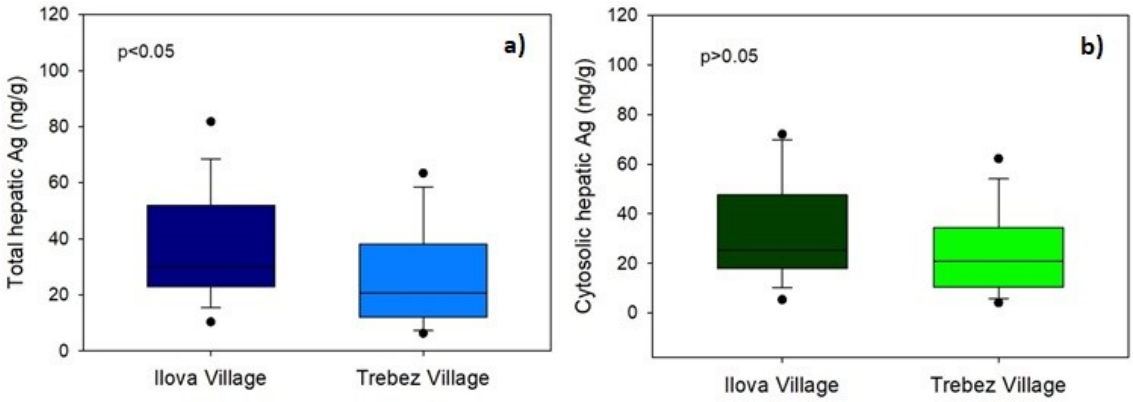

Figure 41. Total (a) and cytosolic (b) Ag concentrations in the liver of *C. gibelio* at the reference site (Ilova village) and possibly contaminated site (Trebež village)

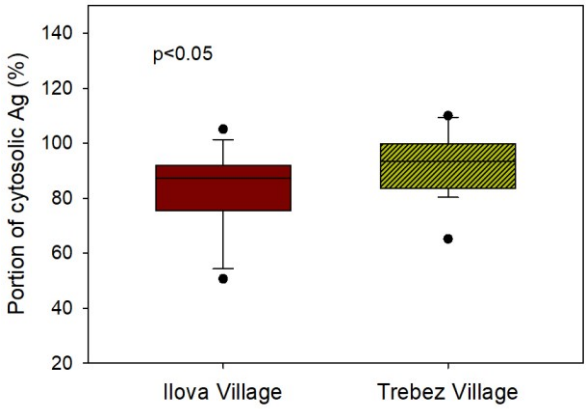

Figure 42. Ratio of cytosolic Ag to total Ag in the liver of Prussian carp expressed in percentages

3.3.9 Cadmium (Cd)

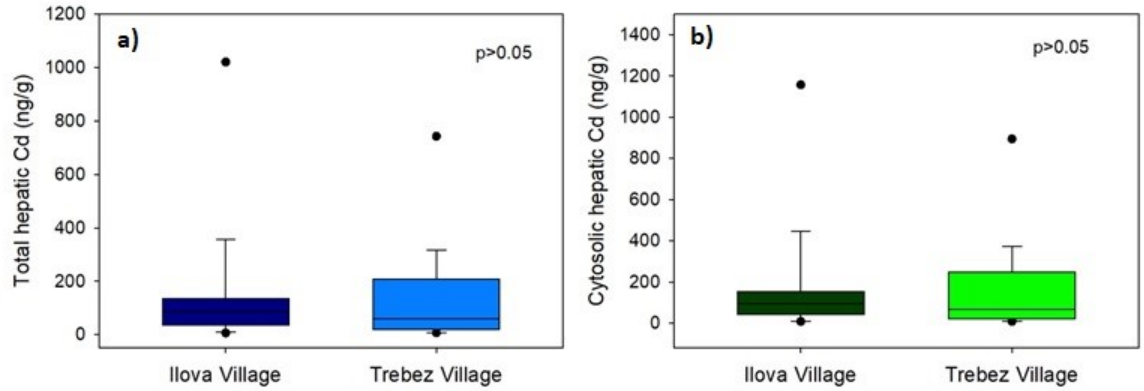

Figure 43. Total (a) and cytosolic (b) Cd concentrations in the liver of *C. gibelio* at the reference site (Ilova village) and possibly contaminated site (Trebež village)

There was no statistically significant difference found for either total or cytosolic concentrations of Cd in the liver of Prussian carp between the reference site and the contaminated site (Figure 43 a,b, Tables VI and VII). It was neither found between the portions of Cd present in the cytosols of the liver of Prussian carp at two sites (Figure 44, Table VIII).

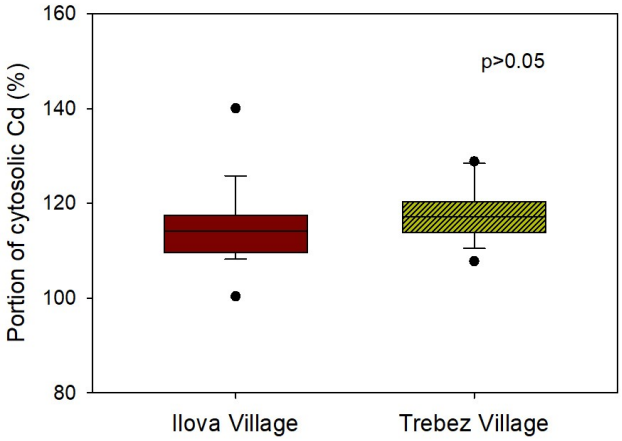

Figure 44. Ratio of cytosolic Cd to total Cd in the liver of Prussian carp expressed in percentages

3.3.10 Caesium (Cs)

All differences between two sites were statistically significant ($p<0.05$). We could also see that in all three figures the higher values were found at the Trebež village. The total hepatic Cs concentrations of Prussian carp caught in the Ilova River at the Trebež village were ~45% higher than the concentrations found at the Ilova village (Figure 45a, Table VI). For the cytosolic hepatic concentrations of Cs the difference amounted to ~50% (Figure 45b, Table VII).

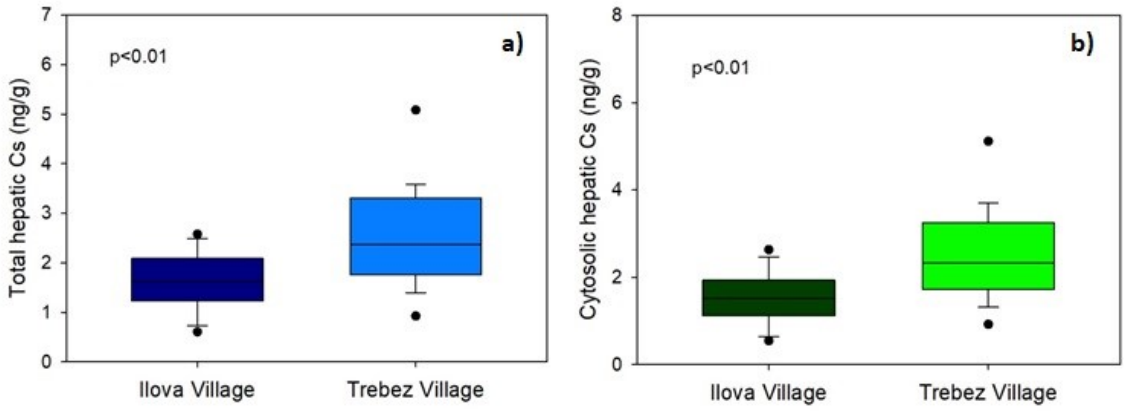

Figure 45. Total (a) and cytosolic (b) Cs concentrations in the liver of *C. gibelio* at the reference site (Ilova village) and possibly contaminated site (Trebež village)

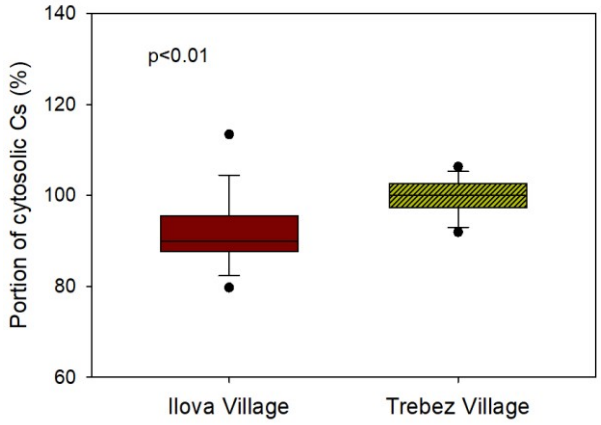

Figure 46. Ratio of cytosolic Cs to total Cs in the liver of Prussian carp expressed in percentages

The portion of cytosolic hepatic Cs concentrations in total hepatic Cs concentrations found in fish of the Trebež village was 100% while the ratio found at the Ilova village was 90%. The difference was significant and amounted to 10% (Figure 46, Table VIII).

3.3.11 Manganese (Mn)

When looking at Figures 47 a and b and Tables VI and VII, it was clear that there were no statistically significant differences between Mn concentrations found in fish at the reference site and at the contaminated site (p>0.05), concerning both total and cytosolic Mn concentrations in the liver of Prussian carp. The portions of Mn in the cytosol were also comparable at two sites, with a value of ~90% (Figure 48, Table VIII).

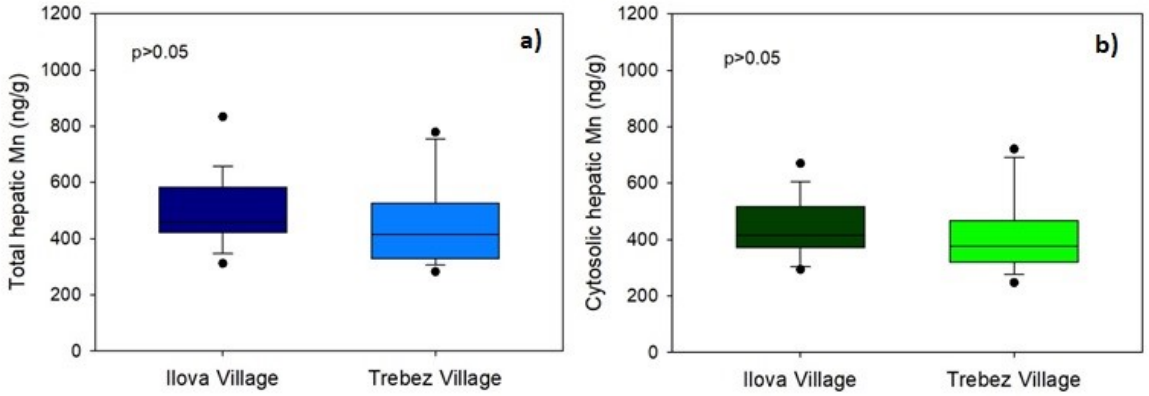

Figure 47. Total (a) and cytosolic (b) Mn concentrations in the liver of *C. gibelio* at the reference site (Ilova village) and possibly contaminated site (Trebež village)

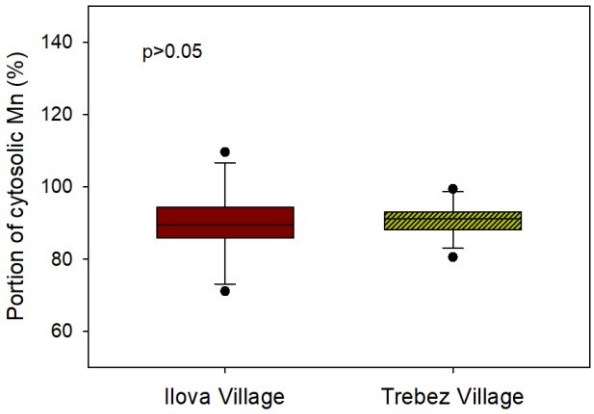

Figure 48. Ratio of cytosolic Mn to total Mn in the liver of Prussian carp expressed in percentages

3.3.12 Iron (Fe)

There were no statistically significant differences between two sites concerning either total hepatic Fe or cytosolic hepatic Fe concentrations in Prussian carp (Figure 49 a,b, Tables VI and VII). Also, the portions of cytosolic hepatic Fe concentrations in total hepatic Fe concentrations were at both sites around 80% (Figure 50, Table VIII).

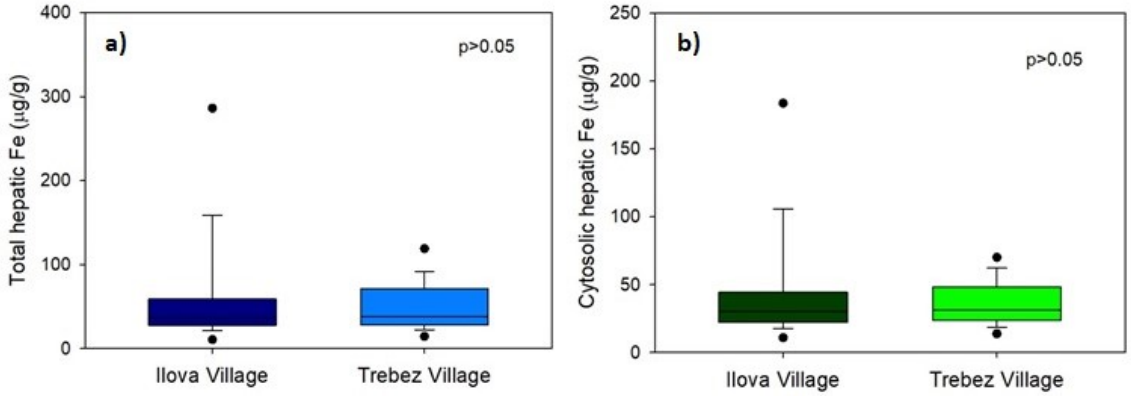

Figure 49. Total (a) and cytosolic (b) Fe concentrations in the liver of *C. gibelio* at the reference site (Ilova village) and possibly contaminated site (Trebež village)

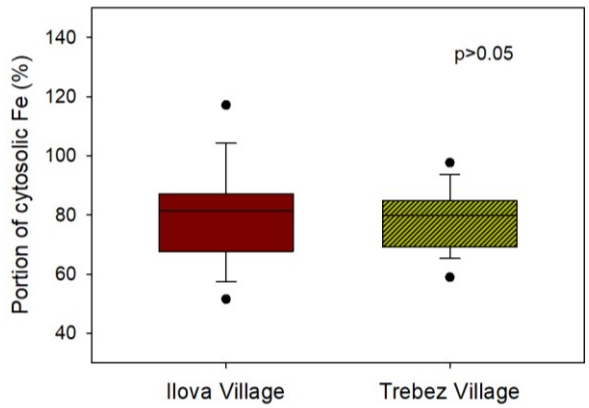

Figure 50. Ratio of cytosolic Fe to total Fe in the liver of Prussian carp expressed in percentages

3.3.13 Cobalt (Co)

There were no statistically significant differences between two sites in any of the investigated parameters concerning Co in the liver of Prussian carp (Figure 51 a,b, Tables VI and VII). The portions of Co in the cytosol of the liver of Prussian carp were at both sites close to 100% (Figure 52, Table VIII).

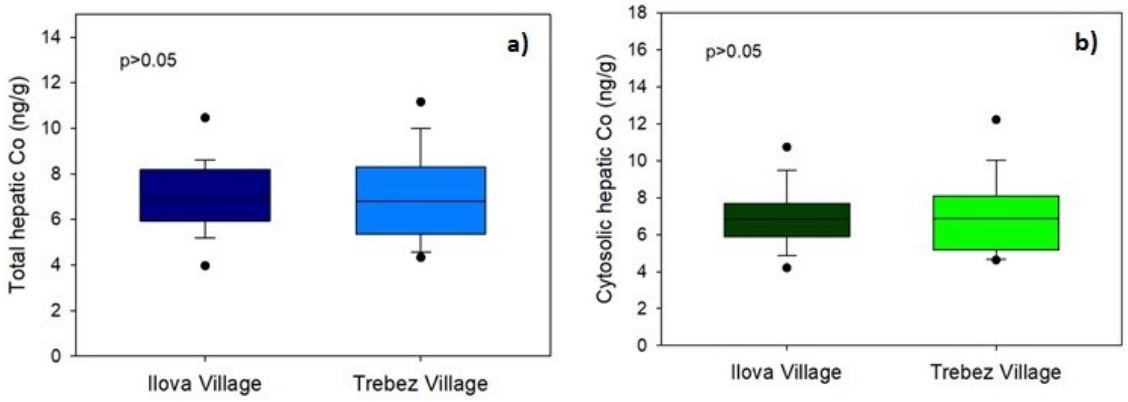

Figure 51. Total (a) and cytosolic (b) Co concentrations in the liver of *C. gibelio* at the reference site (Ilova village) and possibly contaminated site (Trebež village)

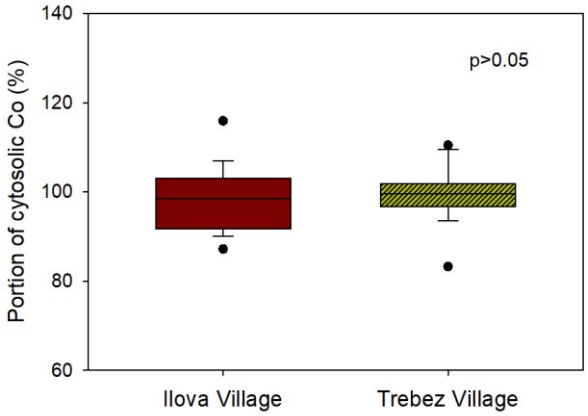

Figure 52. Ratio of cytosolic Co to total Co in the liver of Prussian carp expressed in percentages

3.3.14 Copper (Cu)

There were no statistically significant differences between two sites regarding total and cytosolic Cu concentrations in the liver of Prussian carp, as well as regarding Cu portion within the hepatic cytosol (Figures 53 a,b and 40, Tables VI, VII and VIII). We observed that the portion of cytosolic Cu in total hepatic Cu was equal to ~110% at both sites (Figure 54, Table VIII).

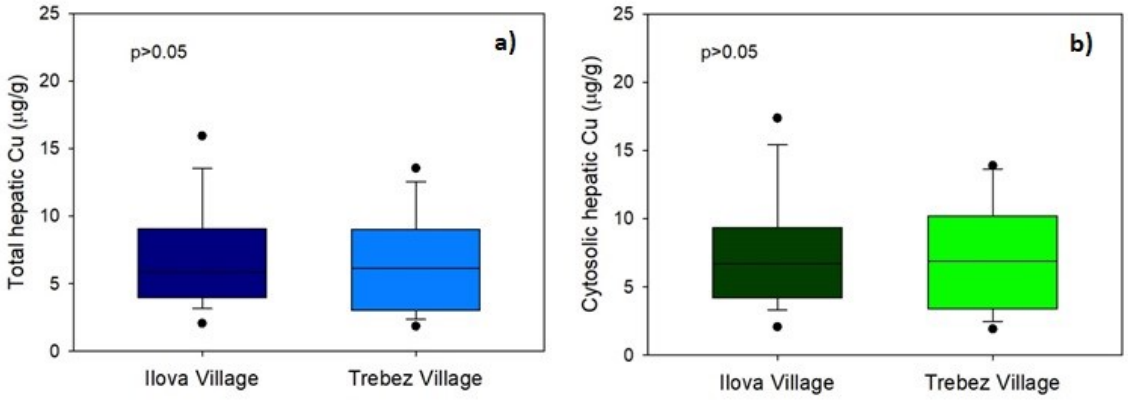

Figure 53. Total (a) and cytosolic (b) Cu concentrations in the liver of *C. gibelio* at the reference site (Ilova village) and possibly contaminated site (Trebež village)

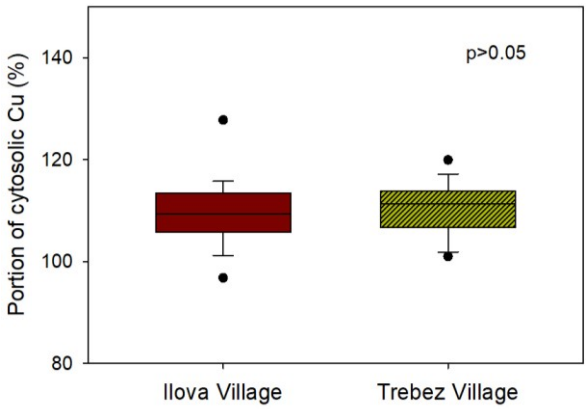

Figure 54. Ratio of cytosolic Cu to total Cu in the liver of Prussian carp expressed in percentages

3.3.15 Zinc (Zn)

The same results are seen here as for several of the previously presented elements, with no statistically significant differences found between the Zn concentrations measured in Prussian carp liver caught in the Ilova River at the Trebež village and at the Ilova village, regarding both total (Figure 55a, Table VI) and cytosolic levels (Figure 55b, Table VII). The percentages of cytosolic Zn in total Zn concentrations at the the Ilova village were ~100% and at the Trebež village ~105% (Figure 56, Table VIII).

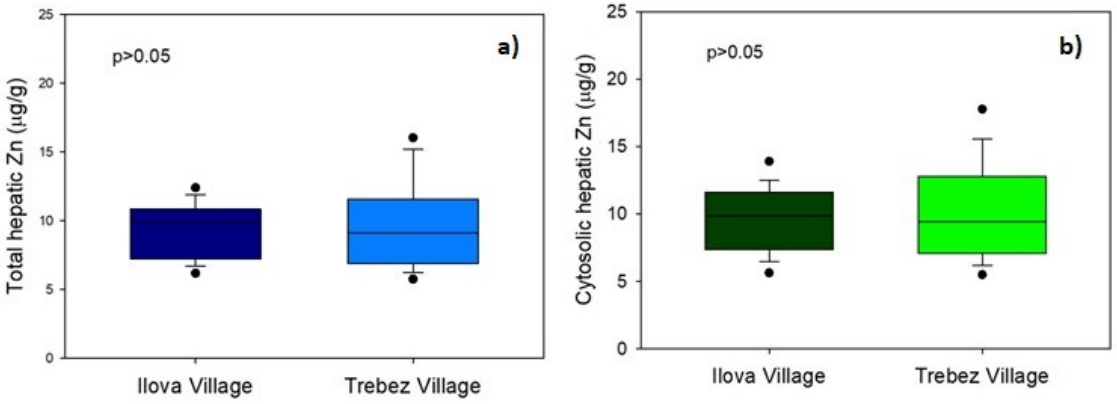

Figure 55. Total (a) and cytosolic (b) Zn concentrations in the liver of *C. gibelio* at the reference site (Ilova village) and possibly contaminated site (Trebež village)

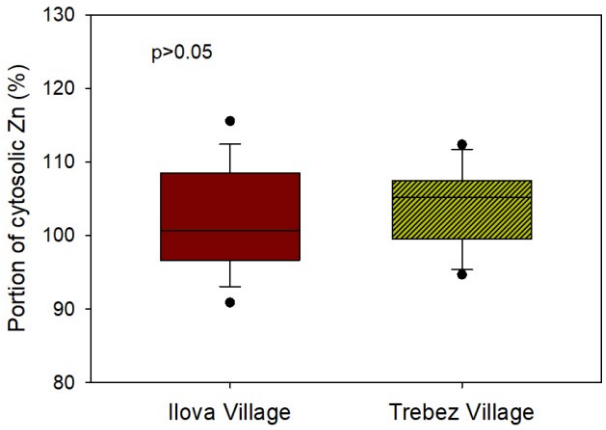

Figure 56. Ratio of cytosolic Zn to total Zn in the liver of Prussian carp expressed in percentages

3.3.16 Strontium (Sr)

Total hepatic Sr concentrations and the cytosolic hepatic Sr concentrations showed statistically significant differences between two sites. Prussian carp caught at the reference site, the Ilova village, had values that were 65% higher for total Sr concentrations than the values of those caught at the Trebež village (Figure 57a, Table VI). The cytosolic Sr concentrations in fish caught at the Ilova village were 55% higher than the values at Trebež village (Figure 57b, Table VII). The ratio of cytosolic Sr to total Sr was comparable at both sites and close to 70% (Figure 58, Table VIII).

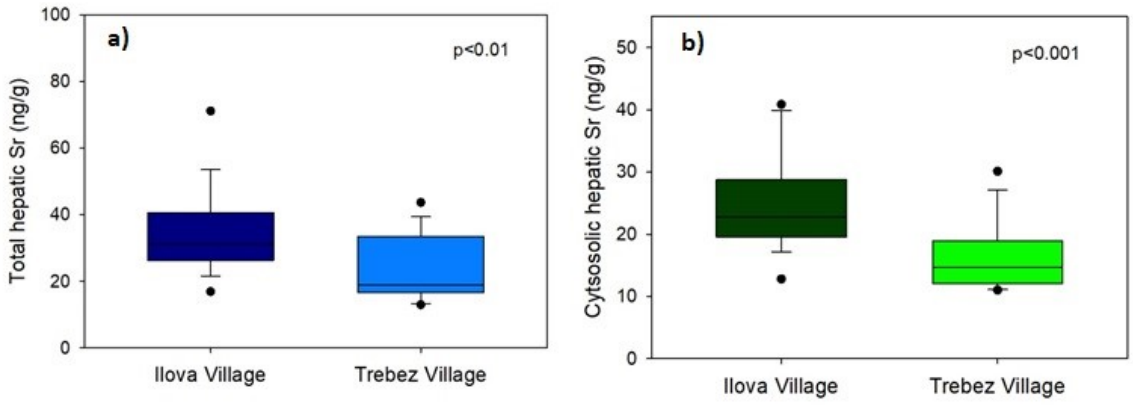

Figure 57. Total (a) and cytosolic (b) Sr concentrations in the liver of *C. gibelio* at the reference site (Ilova village) and possibly contaminated site (Trebež village)

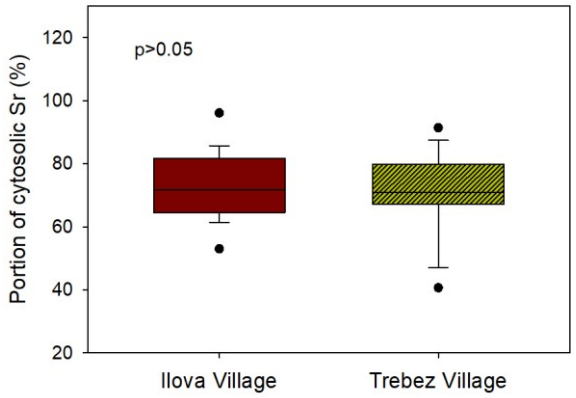

Figure 58. Ratio of cytosolic Sr to total Sr in the liver of Prussian carp expressed in percentages

3.3.17 Arsenic (As)

Both total and cytosolic concentrations of As were comparable at both sites and the observed differences were not statistically significant (Figure 59 a,b, Tables VI and VII). The ratios of cytosolic As to total hepatic As were around 75% at both sites (Figure 60, Table VIII).

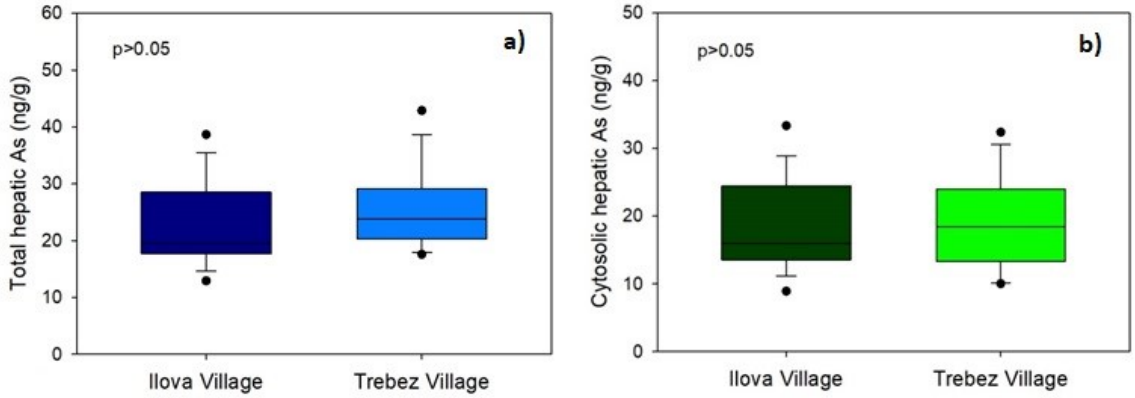

Figure 59. Total (a) and cytosolic (b) As concentrations in the liver of *C. gibelio* at the reference site (Ilova village) and possibly contaminated site (Trebež village)

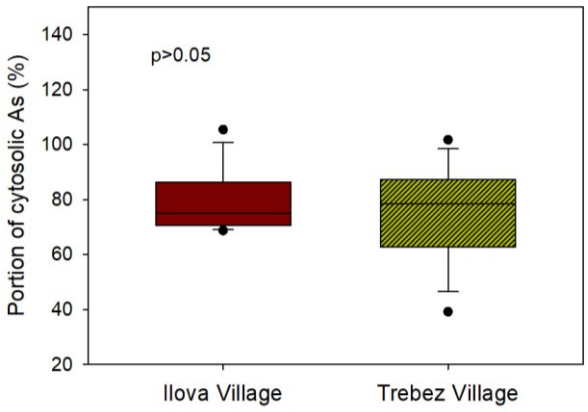

Figure 60. Ratio of cytosolic As to total As in the liver of Prussian carp expressed in percentages

3.4 Assessment of biomarkers in the Prussian carp liver

Four biomarkers were measured within this study, one biomarker of general stress (total cytosolic proteins), one biomarker of metal exposure (metallothioneins) and two biomarkers of oxidative stress (catalase and glutathione).Their values are presented in Table IX. There were no statistically significant differences between the reference and the contaminated site for any of the measured biomarkers. The values were generally comparable and had differences less than 10% between two sites for total proteins (Figure 61, Table IX), metallothionein (Figure 62, Table IX), catalase (Figure 63, Table IX), and glutathione (Figure 64, Table IX). Although the differences were small and not significant, it was interesting to notice that for all four biomarkers higher values were found at the reference site, the Ilova village. We have also noticed that there was a higher variation for glutathione values at the Ilova village than at the Trebež village, though the difference was not statistically significant.

Table IX. Results of biomarker concentrations in hepatic cytosol of *C. gibelio* expressed as average ± standard deviation with median within brackets

Biomarker	**Ilova village**	**Trebež village**
Total proteins (mg/g)	51.6 ± 10.2 (51.3)	49.4 ± 14.5 (47.1)
Metallothioneins (mg/g)	0.577 ± 0.146 (0.620)	0.536 ± 0.168 (0.503)
Catalase (U/mg of proteins)	562.9 ± 100.5 (579.0)	512.3 ± 100.8 (531.2)
Glutathione (nmol/g)	1779 ± 295.2 (1818)	1753 ± 263.1 (1764)

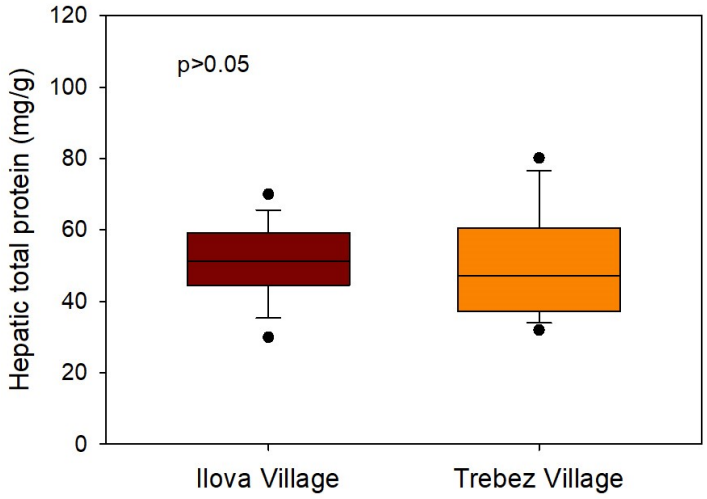

Figure 61. Total cytosolic protein concentrations in the liver of *C. gibelio*

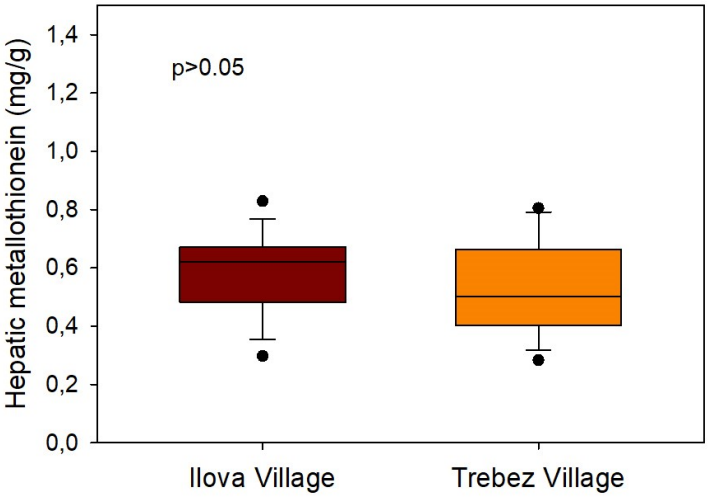

Figure 62. Metallothionein concentrations in the liver of *C. gibelio*

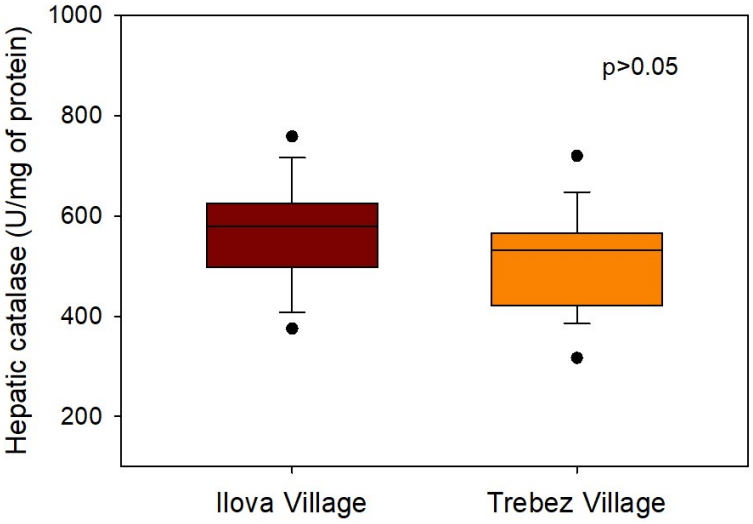

Figure 63. Catalase activity in the liver of *C. gibelio*

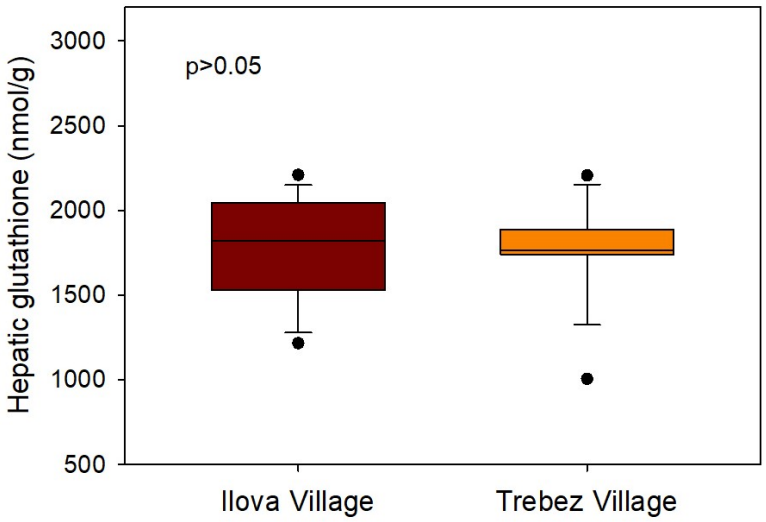

Figure 64. Glutathione concentrations in the liver of *C. gibelio*

4 Discussion

The aim was to investigate the effects of the instream of wastewater from the town of Kutina and the nearby petrochemical factory on the water quality and the fish in the Ilova River. Contamination of metals and organic material by the instream of municipal and industrial wastewaters was suspencted. To determine the quality status of the Ilova River and the possible effects of the instream of wastewater, the results were compared to those from the other researchers.

4.1 The Ilova River water analysis

To determine the water quality of the Ilova River, the dissolved concentrations of large number of trace and macro elements in the river water were measured. Those elements, when present in the river water, can be roughly categorized into two groups: the dissolved metal fraction and the particulate metal fraction. The particulate metal fraction can be defined as the fraction that remains on the filter after filtering the river water through a 0.45-µm pore diameter filter. The dissolved metal fraction is the fraction that goes through the filter. This fraction contains free metal ions (e.g. Na^+, K^+, Mg^{2+}, etc.), unstable complexes of metals with organic and inorganic substances and inert organic metal complexes (Dragun *et al*., 2009b).

Of those two fractions, the dissolved metal fraction is the one that contains the metals which can be regarded as more bioavailable to fish and the other aquatic organisms. This is due to the fact that dissolved metals are more easily absorbed by the organisms. Since the European Union's Water Framework Directive (WFD) defines the maximum recommendable concentration of dissolved metals in the surface waters, the total dissolved metal concentrations measured in our study were compared with the Environmental Quality Standards (EQS) defined by WFD (Dragun *et al*., 2009b).

When looking at the results measured at two sites in the Ilova River (Table IV), it is clear that both sites had mostly resembling metal concentrations in the river water. Overall, the concentrations were slightly higher near the Trebež village. When examining the results more closely there are several elements which had higher concentrations at the Trebež sampling site. Those metals were Na, Cs, Rb and Cd. The concentration of Cs was much higher in the Ilova village compared to Trebež village. Cadmium and Rb both had five times higher concentrations at the contaminated site than at the reference site, whereas the concentration of Na was two and a half times higher at the Trebež village than at the reference site. Contrary, it has to be mentioned that Mn had a five times higher concentration at the Ilova sampling site compared to Trebež. The concentrations of the metals Zn and Ag in the water samples were so low at both sites that they could not be detected. Slightly higher concentrations of Na, Cs, Rb and Cd possibly could be associated to the known sources of pollution, whereas higher Mn concentrations in the river water of the reference site can be perhaps a consequence of agricultural activities in that area.

When comparing the results obtained for the Ilova River with values published for the Sava River (urban and industrially contaminated, but with much higher dilution

capacity), the Sutla River (medium size river, with both clean and industrially contaminated sites) and the Krka River (pristine site and site contaminated with municipal and industrial wastewaters), a general conclusion can be made that the Ilova River is a relatively clean river (Table X). Most of the values for analysed elements were around the values of the known pristine part of the Krka River in the Krka National park (Filipović Marijić *et al*., 2018) and of the reference site of the Sutla River (Dragun *et al*., 2011). There were, however, big differences between some metal concentrations. For example, the values of Mn at the Ilova sampling site were much higher even compared to the contaminated part of the Sutla River and the Sava River (Table X).

When looking at the values of the EQS (CEC, 2006; Crane *et al*., 2007; EPCEU 2008) determined by the European Union's Water Framework Directive and suggested by environmental scientists, it has to be noted that the concentration for Fe exceeds this value at both of the sampling sites of the Ilova River, but not as much as the Sutla River at its two sampling sites. However, it should be emphasized that the suggested Fe concentration is by opinion of many scientists very strict and is not yet accepted as recommendable (Crane *et al*., 2007). The other concentrations were below the so far recommended EQS-values, provided only for Cd, Pb, Ni and Hg by WFD, and suggested for Cu, Fe and Zn (Crane *et al*., 2007).

We can conclude that the Ilova River is a relatively clean river and can be placed between the pristine and clean Krka River and the moderately contaminated Sutla River. The wastewater from the town of Kutina and the nearby factory gave, resulted in? slightly higher metal concentrations than the ones at the reference site with the exception of Mn. Metals that should be monitored in the river water of the Ilova River are Cd and Cs, as highly toxic metals, due to their several times higher concentrations at the Trebež site compared to the reference site, as well as the fact that the Cd concentration has approached the limit of 80 ng/L recommended by WFD.

Table X. The comparison of total dissolved concentrations of 17 metals measured in the Ilova River at two sites (near the Ilova village and near the Trebež village) with the concentrations published for the other rivers in Croatia and with the EQS.

	Na (mg/L)	Mg (mg/L)	K (mg/L)	Ca (mg/L)	Se (µg/L)	Rb (µg/L)	Mo (µg/L)	Ag (µg/L)	Cd (µg/L)	Cs (µg/L)	Mn (µg/L)	Fe (µg/L)	Co (µg/L)	Cu (µg/L)	Zn (µg/L)	Sr (µg/L)	As (µg/L)
Ilova River near the Ilova village	9.91 ± 0.359	15.1 ± 0.556	2.82 ± 1.70	47.3 ± 1.78	0.786 ± 0.019	0.644 ± 0.008	0.561 ± 0.027	<LOD	0.011 ± 0.006	<LOD	93.2 ± 1.13	17.9 ± 2.17	0.137 ± 0.005	<LOD	<LOD	123.1 ± 1.03	2.10 ± 0.126
Ilova River near the Trebež village	26.5 ± 0.171	16.9 ± 0.189	4.58 ± 0.065	57.0 ± 0.829	1.01 ± 0.112	3.74 ± 0.251	0.981 ± 0.062	<LOD	0.053 ± 0.003	0.090 ± 0.007	18.4 ± 0.918	21.6 ± 1.52	0.121 ± 0.011	0.716 ± 0.030	<LOD	150.4 ± 11.9	4.47 ± 0.684
Sutla River – the clean area (Dragun *et al*., 2011)	11.3	18.6	3.79	58.8	-	2.38	0.55	-	0.007	0.002	17.1	36.7	0.068	0.49	-	216.1	0.79
Sutla River - urban and industrially contaminated area (Dragun *et al*., 2011)	88.3	27.1	13.4	77.3	-	9.63	11.96	-	0.117	0.110	51.5	51.8	0.347	0.93	-	416.8	3.83
Sava River - urban and industrially contaminated (Dragun *et al*., 2009b)	10.6	19.5	3.20	91.1	-	-	0.81	-	0.011	-	3.44	12.6	0.064	0.54	2.27	128.0	0.17
Krka River - pristine site (Filipović Marijić *et al*., 2018)	2.1-6.0	11.6-13.5	0.33-0.44	60.3-60.4	0.22-0.32	0.28-0.35	0.38-0.69	0.002-0.02	0.009-0.01	-	0.01-0.06	0.34-2.04	0.004-0.01	0.16-0.22	1.52-3.57	88.4-144.6	0.11-0.12
Krka River – urban and industrially contaminated site (Filipović Marijić *et al*., 2018)	3.2-8.6	11.1-15.0	0.65-0.70	74.4-76.3	0.28-0.31	0.45-0.46	0.51-0.88	0.003	0.008-0.009	-	4.82-6.73	4.66-11.6	0.04-0.11	0.28-0.36	11.5-30.0	186.2-238.5	0.14-0.15
EQS (Dragun *et al*., 2009b)	-	-	-	-	-	-	-	-	0.080	-	-	16.0	-	8.2	7.8	-	-

4.2 Biometry of Prussian carp

When comparing the biometry of the fish from two sampling sites, we have noticed that the fish near Trebež village were 20% bigger and 70% heavier than fish near the Ilova village (Figures 8 and 9, Table V).

The FCI or Fulton condition index was calculated and gave a general idea of the condition of the fish. So, as mentioned above, next to the fact that the fish were bigger and heavier, the fish caught near the Trebež village had a higher FCI for (Figure 10, Table V). This might be explained by the instream of wastewater. It is known that municipal and industrial wastewater contain organic matter which is a source of nutrients for the fish (Dragun et al., 2018). So, more nutrients means more food which can result in the fish growing bigger, and especially heavier.

The hepatosomatic index (HSI) shows the weight of the liver relative to the total weight of the fish and GSI or gonadosomatic index is an index that shows the weight of the gonads as a percentage of the total weight of the fish. When looking at both those values, we can conclude that the values for both sites were comparable (Table V). There was however a larger difference between the lowest and the highest GSI at the Trebež sampling site (Figure 11), meaning that there was a higher variation of gonad size at Trebež, perhaps in some connection to instream of industrial wastewaters that could contain some contaminant affecting the fish reproductive status. However, this should be further investigated.

4.3 Differences in total and cytosolic trace and macroelement concentrations in the liver of Prussian carp between the Ilova village and the Trebež village

Total and cytosolic trace and macro element concentrations were measured in the liver of Prussian carp at two sites of the Ilova River. The Ilova village was selected as the reference site and the Trebež village as the contaminated site. In general, total trace and macro element concentrations were present in the liver of Prussian carp in the following decreasing order: K>Na>Mg>Fe>Ca>Zn>Cu>Rb>Mn>Se>Cd>Mo>Ag>Sr>As>Co>Cs (Table VI), and the cytosolic concentrations were present in almost the same decreasing order (Table VII).

When comparing the concentrations of total and cytosolic trace and macro elements in the liver of fish sampled at two sampling sites, we have noticed three patterns: (1) some elements had comparable concentrations at both sites, (2) some elements were present in higher concentrations at the contaminated site, and (3) some elements had higher concentrations at the reference site (Tables VI and VII).

For the following 12 trace and macro elements there were no statistically significant differences observed for total hepatic concentrations between two sites: Mg, K, Ca, Se, Mo, Cd, Mn, Fe, Co, Cu, Zn, and As. In the same trend lie the following cytosolic trace and macro element concentrations, with no significant differences between two sites: Mg, K, Se,

Mo, Ag, Cd, Mn, Fe, Co, Cu, Zn, As. The only difference between total and cytosolic concentrations referred to Ag and Ca, which both actually showed a trend of higher concentrations at the reference site. However bfor Ag the difference between sites was statistically significant only for total (Table VI) and for Ca for cytosolic metal concentrations (Table VII). These comparable results at two sites correlate well with the little difference in dissolved metal concentrations between sites in the river water (Table IV). There were only two exceptions to this rule. Manganese had five times higher concentration in the river water at the Ilova village, but had no significantly higher total or cytosolic concentrations in Prussian carp liver at that site. A possible explanation could be that the physiological regulation of Mn in the Prussian carp was very efficient, causing low Mn concentration in the fish despite to increased exposure in the water (Dragun *et al*., 2018). The other exception was hepatic Cd, which was also comparable at both sites, but it was increased in the water at the Trebež village (Table IV). Possibly, the exposure was still not high enough to cause the increase in hepatic bioaccumulation, considering that Cd level in the water was still below the level recommended for surface waters by WFD (EPCEU 2008).

To put our results in a wider context, we have compared our results for total hepatic concentrations of Cd, As and Cu with those found in Prussian carp from the freshwaters in Bosnia and Herzegovina, the Svitava Lake and the Neretva River (Table XI). The Svitava Lake is an area with minimum pollution as it is situated in the Nature Park Hutovo Blato where there is almost no traffic and industry, and it lies 30 km away from an area with agriculture (Has-Schön *et al*., 2008). Contrary, the Neretva River is situated in an area with a lot of human activity, traffic and agriculture (Djedjibegovic *et al*., 2012).

Table XI. Comparison of total hepatic metal concentrations of Prussian carp from the Svitava Lake (B&H) (Has-Schön *et al*., 2008), the Neretva River (B&H) (Djedjibegovic *et al*., 2012) and the Ilova River (CRO) for Cd, As and Cu. Values are presented as average ± standard deviation.

Element	Ilova village (ng/g)	Trebež village (ng/g)	Svitava Lake (ng/g)	Neretva River (ng/g)
Cd	147.8 ± 229.7	132.8 ± 182.6	136.0 ± 6.00	15 ± 14
As	23.1 ± 7.42	25.5 ± 7.13	35.0 ± 7.00	
Cu	6890 ± 4260	7030 ± 3740		7980 ± 10600

For Cd, its concentrations at both sites of the Ilova River were in the same range as the concentrations in the pristine Svitava Lake, however much higher than the concentrations in the anthropogenically impacted Neretva river. We can conclude that Cd bioaccumulated in the liver of Prussian carp from the Ilova River was still within the limits

characteristic for clean rivers, and that concentrations reported for the Neretva River reflected the fact that Cd still did not present an ecological problem for that river.

Total hepatic concentrations of As and Cu were lower at both the contaminated and the reference site of the Ilova River compared to the concentrations found in the Svitava Lake and the Neretva River. Since the hepatic As concentrations found in Prussian carp caught in the Ilova River were lower than the As concentrations reported for the liver of Prussian carp caught in the pristine Svitava Lake, we could say that at this point As does not pose a risk for the biota in the Ilova River. Similar conclusions can be made for hepatic Cu in the Prussian carp from the Ilova River, since it was still below the values reported for anthropogenically impacted Neretva River.

Both total and cytosolic concentrations of the following elements in the liver of Prussian carp were statistically significantly higher at the contaminated site: Na, Rb, and Cs (Tables VI and VII). When comparing this to the concentrations of these metals in the river water, we could notice a similar pattern. All three metals had higher concentrations in the water at the Trebež village compared to the Ilova village, although in a different magnitude. Sodium had a concentration two and a half times higher, Rb five times higher and Cs ~90 times higher at the contaminated site. Thus, it could be reasonably presumed that higher concentrations in the water at the Trebež village caused the increase of metal concentrations in the liver, the main bioaccumulation organ, of Prussian carp. In tother words, the higher bioaccumulation level of three metals, Na, Rb and Cs, was very likely the consequence of the higher exposure level of these metals in the river water at the contaminated site of the Ilova River, the Trebež village, downstream of the petrochemical factory.

The following metals showed a trend of higher values at the reference site, the Ilova village: Ca, Sr and Ag. Only for Sr statistically significantly higher values were obtained at the reference site for both total and cytosolic concentrations in the liver of Prussian carp. For the other two metals significant differences were obtained either for total (Ag) or for cytosolic (Ca) concentrations.

The dissolved Ag concentrations in the river water were below LOD at both sampling sites, whereas Ca and Sr concentrations in the river water were comparable between sites. Since fish can also accumulate metals through dietary intake, and not only through the dissolved water phase, the presence of Ag, Ca and Sr in food and sediment at both sites should be investigated (Van Campenhout *et al*., 2009). Filipović Marijić and Raspor (2014) have investigated the presence of metals in gut content of the European chub to demonstrate the relevance of dietary intake of metals, especially of fish found in moderately contaminated waters. Several scientists have suggested to reevaluate whether it is enough to measure only the dissolved metal fractions to establish water quality guidelines (Fisher and Hook, 2002; Hare *et al*., 2003; Lapointe and Couture, 2009; Dragun *et al*., 2018).

4.4 Proportions of trace and macroelements present in the hepatic cytosol of Prussian carp

The proportions of the trace and macro elements in the soluble tissue fraction of Prussian carp liver were calculated by dividing the cytosolic hepatic concentration of each element by the total hepatic concentration. The percentages of the evaluated elements present in the cytosolic hepatic fraction of *C. gibelio* from the reference site Ilova village decreased in the following order: Se, Cd, Cu, Na, K, Zn (>100%) > Rb (99.6%) > Co (98.5%) > Mg (96.5%) > Cs (90.0%) > Mn (89.4%) > Ag (87.4%) > Fe (81.4%) > Mo (77.7%) > As (75.1%) > Sr (71.8%) > Ca (69.0%) (Table VIII). The elements in the cytosolic hepatic fraction of *C. gibelio* from the contaminated site Trebež village decreased in a similar order: Se, Cd, Cu, Zn, Na, Rb, Cs (≥ 100%) > Co (99.5%) > K (98.1%) > Ag (93.3%) > Mn (91.2%) > Mg (87.7%) < Fe (79.9%) > As (78.4%) > Sr (70.8%) > Mo (66.9%) > Ca (57.1%) (Table VIII). For several elements a higher concentrations in the cytosols compared to their total levels was obtained, resulting with cytosolic percentages slightly above 100%, which is probably a consequence of separate digestions of homogenates and cytosols, and multiple steps in the process of sample preparation for analysis, which can lead to slight deviations of the obtained results due to analytical uncertainty.

The elements that were present in the highest percentages in the cytosol can be regarded as completely available for metabolic requirements, and thus also in the case of more toxic elements, available for possible toxic effects. This can be especially worrisome in the case of such elements as Cd, Cs, and Ag, which are highly toxic already in low concentrations and which were present in the cytosolic fraction in high percentages, from 90 to 100%.

There were six elements with statistically significant differences ($p<0.05$) of the ratios when comparing the two sites. The elements whose ratios were significantly higher at the Ilova village, i.e. lower at the Trebež village, were Mg, Ca and Mo. This means that hepatic bioaccumulation of Mg, Ca and Mo at the contaminated Trebež site resulted partly with metal storage in the nonsoluble part of the cell, and thus with their lower presence in the cytosol. The elements with significantly higher proportion in the cytosol at the Trebež village were Cs, Rb and Ag. Since Rb and Cs were also two metals that had higher total hepatic concentrations at the Trebež village, it can be concluded that higher metal bioaccumulation at that site resulted mostly with metal storage in the soluble parts of the cell, thus enabling higher availability of these metals and their potential toxicity.

When comparing the percentages of the cytosolic hepatic fraction of elements found in Prussian carp caught at two sites in the Ilova River with the cytosolic hepatic percentages of the elements found in brown trout from the Krka River (Dragun *et al*., 2018; Table XII), we have seen overall higher proportions at both sites of the Ilova River for the Prussian carp. We can especially point out Cu and Zn with approximately 40% higher proportions in the cytosol, Ag, Mg, Mn and Se around 30% higher and Ca, Cd, Co, Fe and Sr with around 20% higher presence within the cytosol in the liver of Prussian carp in comparison to brown trout. This indicated species specific differences in metal handling strategies between these

two fish species, and possibly higher susceptibility of Prussian carp to metal toxicity, due to higher metal availability in their liver. As a conclusion, the observed differences in metal presence in the cytosolic fractions between brown trout and Prussian carp could be because of different physiological characteristics of these two fish species (Skoric *et al*., 2012).

Table XII. Comparison of the results from Dragun *et al*. (2018) for the hepatic cytosolic portion of trace and macro elements in brown trout (BT) caught in the Krka River with the results of our study for the hepatic cytosolic portion in Prussian carp (PC) caught in the Ilova River, expressed as average ± standard deviation in percentages (%).

	Krka River spring (BT)	**Krka downstream from Knin (BT)**	**Ilova village (PC)**	**Trebež village (PC)**
Ag	58.6 ± 8.0	53.8 ± 11.4	83.8 ± 14.5	92.9 ± 11.5
As	57.5 ± 18.9	80.3 ± 13.9	79.5 ± 11.5	75.3 ± 16.6
Ca	40.2 ± 5.4	41.4 ± 6.2	66.6 ± 13.8	56.7 ± 10.6
Cd	93.0 ± 6.4	87.0 ± 11.3	115.0 ± 8.50	117.6 ± 5.63
Co	86.0 ± 5.0	79.7 ± 12.3	98.3 ± 7.54	99.7 ± 6.07
Cs	87.3 ± 6.0	80.0 ± 10.7	92.5 ± 8.05	99.4 ± 4.17
Cu	63.6 ± 6.2	63.9 ± 7.5	109.5 ± 6.52	110.5 ± 5.06
Fe	59.8 ± 18.1	57.1 ± 12.5	79.9 ± 15.9	78.8 ± 10.2
K	100.1 ± 6.8	102.8 ± 12.1	100.4 ± 5.98	98.7 ± 4.70
Mg	55.2 ± 4.3	57.0 ± 6.8	95.9 ± 4.91	88.4 ± 5.17
Mn	67.5 ± 4.3	62.7 ± 7.9	89.8 ± 9.40	91.2 ± 4.84
Mo	60.0 ± 5.9	60.5 ± 8.4	74.2 ± 8.39	68.8 ± 7.93
Na	120.5 ± 10.5	117.7 ± 11.8	102.2 ± 8.67	105.2 ± 3.75
Rb	94.2 ± 4.5	93.6 ± 8.9	100.3 ± 5.72	104.0 ± 4.65
Se	85.1 ± 9.1	89.1 ± 11.0	117.4 ± 8.79	117.5 ± 5.44
Sr	51.8 ± 8.9	46.7 ± 8.3	72.7 ± 10.4	71.7 ± 12.8
Zn	64.0 ± 4.3	66.7 ± 7.6	102.2 ± 7.08	104.4 ± 5.47

4.5 Assessment of biomarkers in the liver of Prussian carp

Four biomarkers in the liver of Prussian carp at two sites of the Ilova River were measured to determine if any toxic effects have occurred due to exposure to possibly contaminated water. Our results of the biomarker levels showed no statistically significant differences between the reference site Ilova village and the possibly contaminated site Trebež village, indicating that, despite higher values of a few elements found in the river water and bioaccumulated in the liver of Prussian carp at the contaminated Trebež site, the effects in the liver were still not observable. For all four biomarkers, the measured values were even slightly higher at the reference than at the possibly contaminated site, but the difference was negligible (Table IX).

If we compare the results from the Ilova River, with other data published about *C. gibelio* (Table XIII), we can conclude that there was a much higher activity of catalase and even extremely higher concentrations of GSH present in the fish caught in the Ilova River, without any regard to water contamination. A possible reason for this extreme difference between our results and previously published results can be perhaps found in the applied methodology, which often results in different levels of analysed parameters. Some smaller differences could be due to some physiological factors, such as sex, maturity, diet and the season in which the fish were sampled.

A larger part of the population consisted of female fish due to gynogenesis and reproduction from unfertilised eggs, which is characteristic for Prussian carp. Hogstrand *et al*. (1996) described that biochemical parameters can differ between sexes. The variability can also be caused by the maturity of the fish. According to Şaşı (2008), *C. gibelio* becomes mature at the age of three. It is known that levels of MT in the liver could sometimes increase 2-3 times in the reproductive cycle (Dragun *et al*., 2009). A modified diet could also be an influential factor on the level of biomarkers (McCoy *et al*., 1995). All these facts should be considered when the results from different studies are compared, to prevent wrong conclusions about biomarker induction or inhibition.

Table XIII. Comparison of three biomarkers in *C. gibelio* liver reported in different studies.

		Catalase (U/mg)	**Gluthathione (µmol/g)**	**Metallothioneins (mg/g)**
Ilova River	Ilova	579.0	1.818?	0.620
	Trebež	531.2	1.764	0.503
Falfushynska *et al*. (2011)	Zalisci (Reference)		12	0.500
	Borshchiv (Contaminated)		10**	0.420
Falfushynska *et al*. (2013)	Reference		10**	0.250*
De Boeck *et al*. (2003)	Van Stalle Fishfarm (Reference)			0.480
Gavrilovic *et al*. (2014)	Gruza Reservoir Before Bloom	180		
	Gruza Reservoir After Bloom	55		
Tsangaris *et al*. (2011)	Reference	10		
	Desna (Contaminated)	16		

* This value is expressed as MT-SH (µg/g)

** This is quantified by the gluthathione reductase recycling assay

Table XIV. Comparison of the results obtained for Prussian carp in this study with MT and TP levels published previously for European chub from the Sutla and the Sava rivers.

		Metallothioneins (mg/g)	**Total proteins (mg/g)**
European chub			
Sutla River (Dragun *et al*., 2013)	**Upstream**	1.63	117.0
	Downstream	1.23	107.5
Sava River (Dragun *et al*., 2015)	**Contaminated**	1.55	
Prussian carp			
Ilova River	**Ilova**	0.620	51.3
	Trebež	0.503	47.1

The values of MTs measured in the liver of Prussian carp were actually similar to those found in the other articles about Prussian carp liver (Table XIII), both for reference and contaminated sites, again indicating the absence of MT induction in our study as a consequence of increased metal exposure. It means that metal exposure encountered in the Ilova River was still not high enough to cause an additional MT induction. We have further compared MT and TP concentrations in two species, our results for Prussian carp liver with previously published results for the liver of European chub from the Sutla and the Sava rivers (Dragun *et al*., 2013b; Dragun *et al*., 2015). The Sutla River is considered as a moderately contaminated river and the Sava River is considered as somewhat more influenced by anthropogenic activities. Both TP level in the Sutla River and MT levels in the Sava River reported for the liver of European chub were higher compared to values obtained for the liver of Prussian carp from the Ilova River. Total protein levels and MT levels were around two to three times higher in the European chub, but percentage of MTs in TPs in Prussian carp (1.1-1.2%) was comparable to that reported for European chub (1.1-1.4%). Thus, considering that the same methodology was applied in both studies, it can be concluded that those were the differences due to physiological variability of two fish species, which should always be considered in the monitoring studies.

5 Conclusions

The Ilova River is a relatively clean river, it is not as clean as the pristine Krka River, but it is not as contaminated as the Sutla River, which is known to be contaminated by municipal and industrial wastewater at certain sections. The concentrations of the dissolved trace and macro elements in the Ilova River water where, except for Mn, slightly higher downstream of the instream of the wastewaters from the town of Kutina and the nearby factory. For most of the studied elements this was only a slight, almost negligible, increase. However, for Na, Cs, Cd and Rb the increase was noticeably larger. Cadmium, as well as Cs, which are highly toxic metals, should be regularly monitored, since their concentrations were several times higher at the Trebež sampling site, downstream from the petrochemical factory. When comparing the concentrations of the dissolved metals with the EQS, it was found that the concentration of Cd is still acceptable, but relatively close to the recommended limit of 80 ng/L.

The analyses of total hepatic concentrations and cytosolic hepatic concentrations of trace and macro elements in the liver of *C. gibelio* have shown that Na, Rb and Cs were present in high concentrations at the Trebež site, downstream of the sources of pollution. These elements were also present in higher concentrations in the water. The conclusion that higher fish exposure to those elements in the river water has caused an elevation in the bioaccumulated concentrations of those elements in the liver of *C. gibelio* seems plausible. On the other hand, higher concentrations of Mn in the river water at the reference Ilova village had no effect on the concentration in the liver of the fish. Silver, however, was present in low concentration in the water compared to the increased concentrations found bioaccumulated in the liver of the Prussian carp at the reference Ilova village. It is possible that the fish from the reference Ilova village site had obtained higher Ag concentrations in liver through ingestion of sediment and food or because of higher Ag concentrations in the water previous to our sampling moment, but this should be further investigated.

As for the portions of the studied elements present in the cytosolic fractions of Prussian carp liver, we have found very high percentages in the cytosol for the elements Ag, Cd and Cs, which are toxic already in very low concentrations, and their high presence in the cytosol can point to their high metabolic availability and potential for toxic effects. When comparing trace and macro element cytosolic proportions in Prussian carp liver with those reported for brown trout we have concluded that there was a difference in metal handling strategies between two species, with higher presence of metals and the other elements in the cytosol of Prussian carp than in brown trout, and thus possibly higher susceptibility of Prussian carp to metal toxicity.

There were no statistically significant differences found in the levels of four analysed biomarkers (TPs, MTs, tGSH and CAT) in the liver of Prussian carp between two sampling sites, indicating that despite higher exposure levels of several elements in the river water, and their higher bioaccumulation in the liver of *C. gibelio* at the Trebež village, downstream from known sources of pollution, the stress effects were still not observable in the studied fish specimens.

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