Procjena toksičnosti vode rijeke Save pomoću modelnih organizama

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Toxicity assessment of the water from Sava River using algae, plants and fish

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TOXICITY ASSESSMENT OF THE WATER FROM SAVA RIVER USING ALGAE, PLANTS AND FISH

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With the rapid expansion of modern society in both industrial and technological aspect, it is becoming important to assess the risk of compounds which are released daily in our environment in enormous quantities. For this reason, a broad spectrum of ecotoxicological assays with various model organisms has been developed. In this study, toxicity of the Sava River has been assessed by using different model organisms: freshwater algae *Chlorella vulgaris*, clover *Trifolium repens*, common wheat *Triticum aestivum* and zebrafish *Danio rerio* embryos. Model organisms have been exposed to filtered samples of the Sava River from five different locations (Jesenice, Jankomir, Hrušćica, Rugvica, and Lukavec). Pollution profiles of samples were assessed by chemical analysis. Exposure of *Chlorella vulgaris* to samples caused growth inhibition and decreased chlorophyll a concentration. Root and/or sprout elongation of *Trifolium repens* and *Triticum aestivum* was observed to have lowest rates after exposure to same samples in both model organisms. Exposing the zebrafish embryos to samples resulted in minor mortality rate and sublethal effects. Activity of ABC transporters was inhibited in all samples.

The use of these ecotoxicological bioassays, combined with chemical analysis, has resulted in comprehensive toxicity assessment of water from five different sites in Sava River, offering basis for monitoring of lentic and lotic waters.

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PROCJENA TOKSIČNOSTI VODE RIJEKE SAVE POMOĆU MODELNIH ORGANIZAMA

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Uzevši u obzir brzo širenje modernog društva u industrijskom i tehnološkom aspektu, postaje nužno procijeniti rizik tvari koje se svakodnevno ispuštaju u naš okoliš u velikim količinama. U tu svrhu, razvijen je široki spektar ekotoksikoloških testova na različitim modelnim organizmima. Unutar ovog rada, istraživana je toksičnost rijeke Save upotrebom različitih modelnih organizama: slatkovodne alge *Chlorella vulgaris*, djeteline *Trifolium repens*, pšenice *Triticum aestivum* i embrija ribe zebrice Danio rerio. Modelni organizmi izlagani su filtriranim uzorcima rijeke Save s pet lokacija (Jesenice, Jankomir, Hrušćica, Rugvica i Lukavec). Prisutnost toksikanata na ovim postajama utvrđena je kemijskom analizom vode. Izloženost alge *Chlorella vulgaris* uzorcima uzrokovala je inhibiciju njihovog rasta i smanjenu koncentraciju klorofila a. Produljenje korijenja i/ili izdanka kod vrsta *Trifolium repens* i *Triticum aestivum* imalo je najnižu stopu nakon izloženosti istim uzorcima kod oba modelna organizma. Izlaganje embrija zebrica uzorcima rezultiralo je niskom stopom mortaliteta i subletalnih efekata. Aktivnost ABC transportera kod zebrica inhibirana je prilikom izlaganja svim ispitivanim uzorcima.

Ovakva upotreba ekotoksikoloških testova u kombinaciji s kemijskom analizom, omogućila je sveobuhvatnu procjenu toksičnosti uzoraka rijeke Save s pet različitih postaja, ali i pružila temelj za praćenje stanja tekućica i stajaćica.

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Abbreviations

ABC	ATP-binding cassette
ATP	Adenosine triphosphate
BBM	Bold's Basal Medium
BCRP	Breast Cancer Resistance Protein
CA	Cyclosporine A
DDT	Dichlorodiphenyltrichloroethane
Dpf	Days post fertilization
HALMED	Agency for Medicinal Products and Medical Devices of Croatia
HBCDD	Hexabromocyclododecane
Hpf	Hours post fertilization
MDR	Multidrug resistance
MXR	Multixenobiotic resistance
NP1EO	Nonylphenol monoethoxylate
NP2EO	Nonylphenol diethoxylate
OECD	The Organisation for Economic Cooperation and Development
OP2EO	Octylphenol diethoxylate
PBC	Polychlorinated biphenyl
PTFE	Polytetrafluoroethylene
Pgp	P-glycoprotein
PPCPs	Pharmaceuticals and personal care products
RB	Rhodamine B
TG	Test Guidelines
UHPLC–MS/MS	Ultra performance liquid chromatography - tandem mass spectrometer
ZET	Zebrafish embryotoxicity test

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CURRICULUM VITAE

1 INTRODUCTION

The rapid industrial and technological development of modern society results in the release of various chemicals in the environment, and the aquatic environments usually act as the final recipient. These chemicals can present a health risk for both humans and the wildlife by causing a wide range of negative effects: teratogenicity, neurotoxicity, genotoxicity, endocrine disruption, immunosuppression, etc. (Hollert and Keiter, 2015). Also, pollutants can interact and may cause effects that are different from the effect of the individual chemical (Hollert and Keiter, 2015; Petrie et al., 2015). These adverse effects of pollutants on ecosystems can be acute or chronic (sublethal effects on the organism's ability to grow, develop and reproduce) (Cooney, 1995). Compounds present in the ecosystems may also eventually accumulate in the food chain, representing a risk to various trophic levels, as well as to humans through fish and water consumption (Babić et al., 2017).

Considering that water is one of the most important natural resources, it is becoming critical to assess the toxic effects of those compounds in order to monitor the quality of the water and protect the biodiversity of aquatic environments. Current legislation, therefore, requires a detailed data on risk assessment for the registration of chemicals. Furthermore, in European Union, national water agencies that follow EU policy and the requirements of the EU Water Framework implement regular water monitoring with the aim to control and prevent pollution (Milačič et al., 2017).

In this thesis, the tested water samples were collected from the Sava River (945 km), which is the largest tributary of the River Danube. It flows through Slovenia, Croatia, Bosnia and Herzegovina and Serbia. Its basin covers a catchment area of 95,719 km^2, which represents the source of more than 80% of the total available freshwater in the area (Källqvist et al., 2008). The river receives a wide range of organic and inorganic substances from various industrial activities that are located along the river. Several studies have reported that there is an increased load of nutrients, metals and low-molecular weight organic compounds (pesticides, plasticizers, pharmaceuticals, personal care products, etc.) that lead to alterations of river flow regimes and sediment pollution (Källqvist et al., 2008). Many of them are known to have, or are suspected to have, toxic effects or may disrupt endocrine system at trace levels (Antonijevic et al., 2014). Since the drinking water supply in the Sava River basin relies on the resources of

high-quality groundwater that are under the influence of the Sava River, the assessment of possible negative effects of pollution is rather important (Smital and Ahel, 2015).

The qualification and quantification of pollutants using chemical analyses is able to specify the levels of contaminants present in the ecosystem. However, these pollutants rarely occur alone and are usually found to be in complex mixtures and can be combined with nonchemical stressors (Eggen et al., 2004; Sauvant et al., 1997). Therefore, the real problem within ecotoxicological studies is predicting the impact and the adverse effects of these pollutants on the health of living organisms that inhabit studied ecosystems (Sauvant et al., 1997).

For that reason, various model organisms and assays have been developed for ecotoxicological studies (Sauvant et al., 1997). Methods that are based on small-scale or in vitro bioassays have greatly improved the ability to monitor water quality. They are usually based on cellular components, cells, organs or small animals or plants, and have multiple advantages: they are highly sensitive, rapid, reproducible and require minute amounts of samples, which makes them well suited for screening large numbers of samples (Källqvist et al., 2008). These screening methods are rather useful due to the fact they are able to integrate the toxicological activity of multiple contaminants that may act through a common mechanism, making it possible to assess the potentially negative effects of complex environmental mixtures of pollutants in greater details (Källqvist et al., 2008).

2 THE AIM OF THE RESEARCH

In this study, water samples were collected from the Sava River at five different locations with presumably different pollution burden: Jesenice, Jankomir, Hrušćica, Rugvica and Lukavec. The aim of the research was to assess the toxicity of water pollution at each sampling site by the application of four different test organisms for the determination and comparison of toxicity.

In order to give a detailed toxicity assessment of the water from the chosen locations of the Sava River, following steps were taken:

i) Chemical analysis of water from each sampling site was done to determine concentrations of 571 different organic pollutants.

ii) Toxicity was assessed using following methods:

- algae (*Chlorella vulgaris* Beijerinck, 1890): growth inhibition (OECD 201, 2011) and chlorophyll a concentration
- plants (*Trifolium repens* L. and *Triticum aestivum* L.): seed germination inhibition and root and sprout elongation
- fish [*Danio rerio* (Hamilton, 1822)]: zebrafish embryotoxicity test (ZET; OECD 236, 2013) and transport activity of ABC proteins involved in multixenobiotic resistance mechanism (MXR).

3 LITERATURE OVERVIEW

3.1 Classification of water pollutants

In modern society, quality of freshwaters is threatened by various sources of pollution, such as sewage and domestic wastes, agricultural discharges, detergents, siltation, radioactive materials, industrial effluents, fertilizers, toxic metals and thermal pollutants (Sharma, 2005). The overview of the most important water pollutants released from those sources has been given in Table 1.

Table 1. Classification of water pollutants (Ibáñez et al., 2008; Sharma, 2005).

	Type of pollutants	Examples
1	Oxygen demanding wastes	Human, animal waste, decaying vegetation.
2	Infectious agents	Bacteria and viruses.
3	Organic molecules	Detergents, oils, pesticides, pharmaceuticals.
4	Plant nutrients	Nitrates, phosphates.
5	Inorganic chemicals	Hg, Cd^{2+}, Pb^{2+} etc.
6	Heat	Water used for cooling in industry.
7	Suspended materials	Silt from land erosion.
8	Radioactive substances	Fallout products, radioactive waste, etc.

The most common form of water pollution is due to organic compounds, which enter the aquatic ecosystem through domestic sewage, industrial wastes, waste from the meat packing plants, food processing plants, plant nutrients, detergents, biocides, runoff from crop plants and decomposition products of organic matter (Sharma, 2005).

These organic pollutants may be classified as follows (Sharma, 2005):

i. *Carbohydrates and proteins* – mostly contributed by household wastes and faecal matter.

ii. *Oils* – originated while petroleum drilling. They can spread easily over the surface, inhibiting diffusion of oxygen and reaeration of the water.

iii. *Aldehydes* – acetaldehyde, benzaldehyde, furfural, formaldehyde, vanillin etc. Cause odour in the water, inhibit algal growth and are toxic to aquatic animals. Their release is caused by the thermal decomposition of fats, oils and glycerol.

iv. *Polychlorinated biphenyl (PCBs)* – used in dielectrics, lubricants and plasticizers. Similar to DDT and can cause several physiological disturbances in humans. In animals, they affect the central nervous system and respiratory tract.

v. *Phenolic compounds* – they enter into the water system through trade waters. Low concentration causes a bitter taste in water. Higher levels result in pain, renal irritation, severe shock and possibly death.

vi. *Aromatic hydrocarbons* – create carcinogenic effects on humans and animals. Benzene like compounds cause odour pollution in water.

vii. *Other compounds* – carbon tetrachloride and chloroform, both used for making fluorocarbons for refrigerants and propellants, as well as polyvinyl chloride, have carcinogenic effects on rats, mice and other animals. The main sources of hydrocarbons such as benzene, benzopyrene, etc. are automobiles that use petrol. Benzopyrene is considered to be the most potent cancer inducing hydrocarbon.

Main groups of organic pollutants

In this study, the main focus was on three groups of organic pollutants due to their increasing threat to freshwater ecosystems: pharmaceuticals/drugs, pesticides and industrial chemicals.

Pharmaceuticals are defined as chemical compounds that are used or administered to help diagnose, treat, cure, mitigate or prevent diseases or other abnormal conditions (http://www.pharmacistspharmajournal.org). They are produced and used in large quantities and their use and diversity is increasing every year (Bound and Voulvoulis, 2004). Their presence in waterways, including surface water, groundwater and drinking water, has been established for over 40 years as a result of manufacturing processes, improper disposal or metabolic excretion (Bound and Voulvoulis, 2004; Hernando et al., 2006; Jones et al., 2002). The reason why pharmaceutical substances may be interesting in ecotoxicological context, is that they are developed with the intention of performing a biological effect. They often have the same type of physio-chemical behaviour e.g. are lipophilic in order to be able to pass membranes, are persistent in order to avoid the substance to be inactive before having a curing

effect, as other harmful xenobiotics. This means that these medical substances have many of the necessary properties to bioaccumulate and provoke effects in the non-target aquatic or terrestrial organisms (Halling-Sørensen et al., 1998).

Pesticides are chemicals used to kill, repel, or regulate the growth of biological organisms (Laetz et al., 2009). This diverse group includes subgroups such as insecticides, herbicides and fungicides that were involved in this study, as well as nematicides, acaricides, rodenticides, avicides, wood preservatives, and antifoulants (Laetz et al., 2009). Nowadays, with the important role of agriculture in modern society, pesticides are used widely and may cause significant negative effects on the environment, especially on aquatic ecosystems where they are extremely toxic to non-target organisms like fish which they affect through impairment of metabolism, sometimes leading to mortality (Fochtman et al., 2000; Sabra and Mehana, 2015).

Industrial chemicals are compounds that are used in industrial activities. A wide spectrum of chemicals within this group are used for many different purposes: perfluoroalkyl acids are used as organic surfactants in a variety of products; common applied as repellents in textiles, fire retardants and food packaging, while bisphenol A is used in plastics applications. (Alexander et al., 1988; Ulhaq et al., 2013). Furthermore, benzotriazoles are components of hydraulic brake fluids, intermediates for colours, fungicides and pharmaceuticals and UV stabilizer on plastic packages, while nonylphenol ethoxylates are used widely as detergents, emulsifiers, wetting and dispersing agents, antistatic agents, demulsifiers and solubilisers (Seeland et al., 2012; Soares et al., 2008). Many of them are stable in the environment and resist hydrolysis, photolysis and biodegradation and, therefore, have a tendency to bioaccumulate and to magnify in the food chain (e.g. perfluorinated compounds) (Bossi et al., 2008). Furthermore, some of them have the capability for long-range transport through the atmosphere and water (Ahrens and Bundschuh, 2014).

3.2 Toxicity assessment

Chemical and physical analyses alone do not have the ability to detect all potentially toxic substances in the water (Mankiewicz-Boczek et al., 2008). They usually do not include the products that are the result of interactions between individual chemicals, and also only determine the amount of a single contaminant, but not the total pollution of water. By applying bioassays with different model organisms, it is possible to indicate general stress which is

caused by mixtures of pollutants present in the analysed environmental sample, and obtain data about potential hazard to aquatic life (Mankiewicz-Boczek et al., 2008).

It seems necessary to use a battery of bioassays with different organisms, because each tested organism can have different sensitivity to chemicals or their mixture in the tested sample. A battery of bioassays, therefore, gives the opportunity to treat obtained data as information about the whole aquatic ecosystem (Mankiewicz-Boczek et al., 2008). This kind of thorough analysis is important in order to ensure good ecological and chemical status of water, as required by the Water Framework Directive of the European Union 2000/60/EC (Mankiewicz-Boczek et al., 2008).

For a proper and detailed assessment of the toxicity of a chemical, it is necessary to define the endpoints of toxicity and their dose-response correlation, elucidate the mechanisms of toxicity and determine the toxicodynamics of the chemical. In addition to detailed toxicological investigations of a single chemical, the need for large-scale screening for toxicity of several hundred chemicals at a time has also been recognized (Hill et al., 2005).

3.3 Model organisms

The sensitivity of a model organism to toxic compounds is a complex issue, since it involves not only the species of model organism, but also types of toxicants studied, environmental conditions, test methods, life stage of the model organism, and other factors (Tuikka et al., 2011; Wang and Freemark, 1995).

In order to thoroughly assess the toxicity of water, tests with different species of organisms are needed, since each one may have a different sensitivity (Silva et al., 2009). In this thesis, phylogenetically distant model organisms have been used for the assessment of toxicity of the Sava river: algae, plants, and fish.

3.3.1 Algae – *Chlorella vulgaris*

3.3.1.1 Taxonomy and biology of *Chlorella vulgaris*

Table 2. Taxonomical classification of *Chlorella vulgaris*.

Kingdom	Plantae
Subkingdom	Viridiplantae
Infrakingdom	Chlorophyta
Division	Chlorophyta
Subdivision	Chlorophytina
Class	Trebouxiophyceae
Order	Chlorellales
Family	Oocystaceae
Genus	*Chlorella* Beijerinck, 1890
Species	*Chlorella vulgaris* Beijerinck, 1890

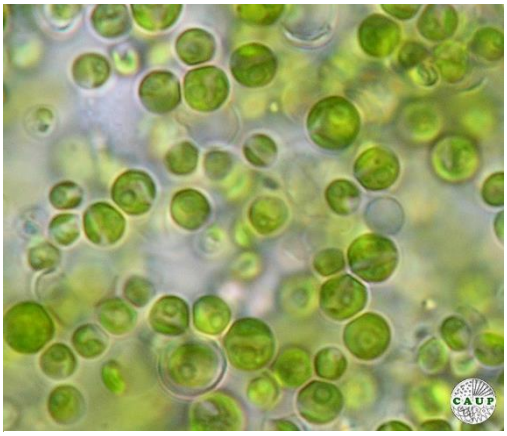

Figure 1. Test organism *Chlorella vulgaris* (https://botany.natur.cuni.cz)

Chlorella vulgaris is a unicellular green microalga (Fig. 1) that grows in freshwater and has been present on Earth since the pre-Cambrian period 2.5 billion years ago. Since then, its genetic integrity has remained constant. It is a spherical microscopic cell with 2–10 μm diameter and has many structural elements similar to plants. *Chlorella vulgaris* is a non-motile reproductive cell that reproduces asexually and at a rapid rate. Grown in optimal conditions, it multiplies by autosporulation, which is the most common asexual reproduction in algae (Safi et al., 2014).

Other than in algal bioassays for toxicity assessment of river water and sediments, *Chlorella vulgaris* has a great potential in several other areas such as bioremediation of textile wastewater, as well as production of biofuel due to many advantages, such as its robustness, high content of oil, mixotrophic culturing condition, high growth rate under various environmental conditions and tolerance to high levels of heavy metals (Rioboo et al., 2002; Serra-Maia et al., 2016; Znad et al., 2018). It is also used as medicinal food and nutritional supplement and was shown to help in cancer prevention and immune system support (Serra-Maia et al., 2016).

3.3.1.2 The use of algae in scientific research

Algae present good model organism in ecotoxicological studies for multiple reasons. Algae, as primary producers, are a key functional group of organisms. Alterations of the of the phytoplankton community as a result of toxic stress may change the structure and functioning of the whole aquatic ecosystem (Nyholm and Källqvist, 1989). Due to their short generation time, microalgae respond rapidly to changes in the environment, allowing the evaluation of toxic effects over several generations, and can, therefore, report impacts on higher organisms, which would respond on a longer timescale (Rioboo et al., 2002; Silva et al., 2009). An additional advantage of using *Chlorella vulgaris* in this study is the fact that tests with unicellular model organisms show a greater reproducibility, reliability, and robustness than ones with multicellular model organisms (Silva et al., 2009).

Algal growth inhibition test (OECD, 2011) presents one of the most commonly used aquatic ecotoxicological tests. This test uses the *in vivo* phytotoxic effects of sample matrixes, such as porewater and organic extracts of sediments and water (Källqvist et al., 2008). In comparison to acute and chronic toxicity tests with other aquatic species, such as fish and crustaceans, the algal growth inhibition test had frequently been proven to be the most sensitive test (Källqvist et al., 2008). Some of the advantages of this test are its high sensitivity, good reproducibility, compatibility with different environmental sampling techniques, rapidness and low-cost effectiveness (Källqvist et al., 2008; Rioboo et al., 2002).

Environmental stress factors can affect photosynthesis negatively and damage algal growth and function. The inhibition of photosynthesis or biochemical processes that are connected to photosynthesis may present the physiological state of the algae. Therefore the measurement of chlorophyll concentration – and thereby photosynthesis, can also be used as an indicator of toxicological stress (Popović et al., 2003).

Due to listed advantages, algal bioassays are often included in test batteries for toxicity assessment of river water and sediments, as well as in toxicity assessment of industrial wastewaters and leachates from waste deposits (Källqvist et al., 2008; Rioboo et al., 2002). Algal toxicity tests of chemicals are mandatory tests for the classification of chemicals in the European Union (Rioboo et al., 2002).

3.3.2 Plants – *Trifolium repens* and *Triticum aestivum*

3.3.2.1 Taxonomy and ecology of *Trifolium repens*

Table 3: Taxonomical classification of *Trifolium repens*.

Kingdom	Plantae
Subkingdom	Viridiplantae
Infrakingdom	Streptophyta
Superdivision	Embryophyta
Division	Tracheophyta
Subdivision	Spermatophytina
Class	Magnoliopsida
Superorder	Rosanae
Order	Fabales
Family	Fabaceae
Genus	*Trifolium* L.
Species	*Trifolium repens* L.

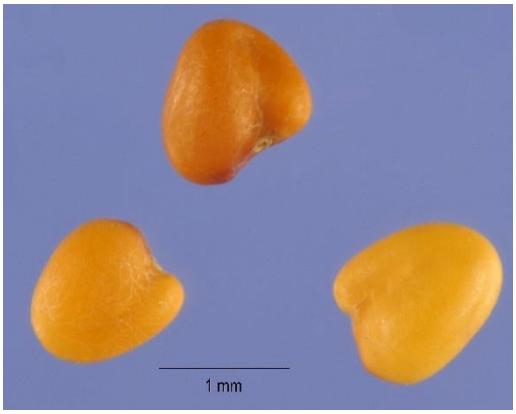

Figure 2. Seeds of *Trifolium repens* (adapted from https://plants.sc.egov.usda.gov/).

Figure 3. Test organism *Trifolium repens* (http://www.e-herbar.net/).

White clover (*Trifolium repens*; Fig. 3) is plant that is native to Europe, the Middle East and northern Africa. It produces embryos with two cotyledons, which makes it a dicotyledon. This plant grows 10 to 30 cm long, hairless and rooting at the nodes. Its flowers are white or sometimes yellowish or pinkish and usually, more than 20 flowers can be found in globular heads. White clover is mostly found as a weed in lawns, cultivated areas and waste ground, and can also indicate freshwater habitats (http://vro.agriculture.vic.gov.au). White clover harbours microbes in its root system that fix nitrogen, converting atmospheric nitrogen into a form that can be used by plants (http://eol.org/).

3.3.2.2 Taxonomy and ecology of *Triticum aestivum*

Table 4: Taxonomical classification of *Triticum aestivum*.

Kingdom	Plantae
Subkingdom	Viridiplantae
Infrakingdom	Streptophyta
Superdivision	Embryophyta
Division	Tracheophyta
Subdivision	Spermatophytina
Class	Magnoliopsida
Superorder	Lilianae
Order	Poales
Family	Poaceae
Genus	*Triticum* L.
Species	*Triticum aestivum* L.

Figure 4. The seeds of *Triticum aestivum* (adapted from http://fsi.colostate.edu/).

Figure 5. Test organism *Triticum aestivum* (http://www.agroatlas.ru/).

Common wheat (*Triticum aestivum*; Fig. 4) is a plant that grows 60 to 180 cm tall. It is a monocotyledon that is extremely polymorphous, with winter and intermediate varieties. Originally from the Mediterranean region and southwest Asia, wheat's habitat now covers all agricultural areas of the world, making it a leading crop worldwide (http://www.agroatlas.ru/). Wheat is one of the most ancient domesticated crops, with archaeological evidence of the cultivation of various species dating back to 9,600 B.C. (http://eol.org).

3.3.2.3 The use of plant seeds in scientific research

Plants are essential for the processes of nutrient cycling and soil stabilization. The toxic effect on plants can directly affect the structure and function of the ecosystem, which may result in oxygen depletion, decreased primary productivity, increased surface runoff and soil erosion, as well as degradation of wildlife habitat (Wang and Freemark, 1995). Consequently, it is important for plants to be included in environmental monitoring and risk assessment, which is why they nowadays have an important role in effluent biomonitoring, regulation of hazardous waste and pesticide regulation (Wang and Freemark, 1995).

Phytotoxicity is defined as a toxic effect of a substance or growing condition on plant growth and development. It includes the delay of seed germination, inhibition of plant growth or any other adverse effect on plants (Blok et al., 2008). Generally, phytotoxicity assays can be employed using seed germination or root and/or sprout elongation as endpoints. Initially developed to ascertain the toxicity of polluted liquid samples on seeds, seed germination and sprout and root elongation tests are one of the simplest methods in environmental biomonitoring and have several advantages (Wang and Keturi, 1990).

Numerous dry plant seeds remain a viable source as they possess a long shelf life, and therefore are low-cost and easy to maintain. This also means they can be activated instantaneously, which gives the test permanent stand-by status. These tests are rather simple and inexpensive and do not require major equipment. Additionally, a small volume of test sample is required, which minimizes the disposal of a potentially toxic sample (Wang and Keturi, 1990). In the case of water sample with low dissolved oxygen, no aeration is required, which prevents compromised sample integrity. Additionally, water samples with high turbidity do not require filtration. Toxicity to plants can be tested using the renewal or flow-through method (Wang and Freemark, 1995).

Inhibition of root elongation, as well as seed germination inhibition, presents a valid and a sensitive indicator of environmental toxicity. It was reported that the seed plants were not as sensitive to heavy metals and insecticides as algae and daphnids were, but they were far more superior when it came to sensitivity to herbicides (Wang and Keturi, 1990).

The plants chosen in this thesis are the most common cultivars used in Croatia and are often exposed to floods after the winter months. Particularly *Trifolium repens* and *Triticum aestivum* were chosen for a better understanding of the sensitivity of monocotyledons and dicotyledons to the water pollution.

3.3.3 Zebrafish – *Danio rerio*

3.3.3.1 Taxonomy and ecology of *Danio rerio*

Table 5: Taxonomical classification of *Danio rerio*.

Kingdom	Animalia
Subkingdom	Bilateria
Infrakingdom	Deuterostomia
Phylum	Chordata
Subphylum	Vertebrata
Infraphylum	Gnathostomata
Superclass	Actinopterygii
Class	Teleostei
Superorder	Ostariophysi
Order	Cypriniformes
Superfamily	Cyprinoidea
Family	Cyprinidae
Genus	*Danio* Hamilton, 1822
Species	*Danio rerio* (Hamilton, 1822)

Figure 6. Comparison of the adult male and female zebrafish *Danio rerio* (Holtzman et al, 2016).

The zebrafish (*Danio rerio*; Fig. 6) belongs to the Cyprinidae family, which is composed of more than 2,000 species. Formerly named *Brachydanio rerio*, the species name was changed to *Danio rerio* in 1981 (Ribas and Piferrer, 2014).

The zebrafish is a freshwater fish that evolved in South Africa around 320 million years ago. Its habitat includes mainly India, Bangladesh and Nepal, but it can also be found in Pakistan, Myanmar, Sri Lanka and in the rivers draining in the Arabian sea. They usually inhabit rivers, small streams and stagnant or slow-moving pools near streams and rice paddies, and are considered euthermic species, tolerating a wide range of temperatures, pH and various amounts of dissolved salts (Ribas and Piferrer, 2014).

The weight of an adult zebrafish typically reaches up to 0.9 g, and the length of an adult individual ranges from 22 to 38 mm. They exhibit sexual growth dimorphism that favours

females and is easy to spot when fish reach adulthood. Males are smaller with more elongated body, often with gold and blue stripes. Females are bigger, rounder, with a whitish stomach, and they exhibit a small genital papilla in front of the anal fin (Ribas and Piferrer, 2014) (Fig. 6).

Although the zebrafish reach sexual maturity in 10 to 12 weeks (Fig. 7), the breeding fish should be between 7 and 18 months of age for maximum embryo production. Under laboratory conditions, eggs can be spawned through the entire year, every 4 to 7 days (Ribas and Piferrer, 2014). Spawning in domesticated zebrafish is influenced by photoperiod and coincides within half an hour after the exposure to light after a period of darkness (Spence et al., 2007). On average, 150-200 embryos per female can be generated (Hollert et al., 2003).

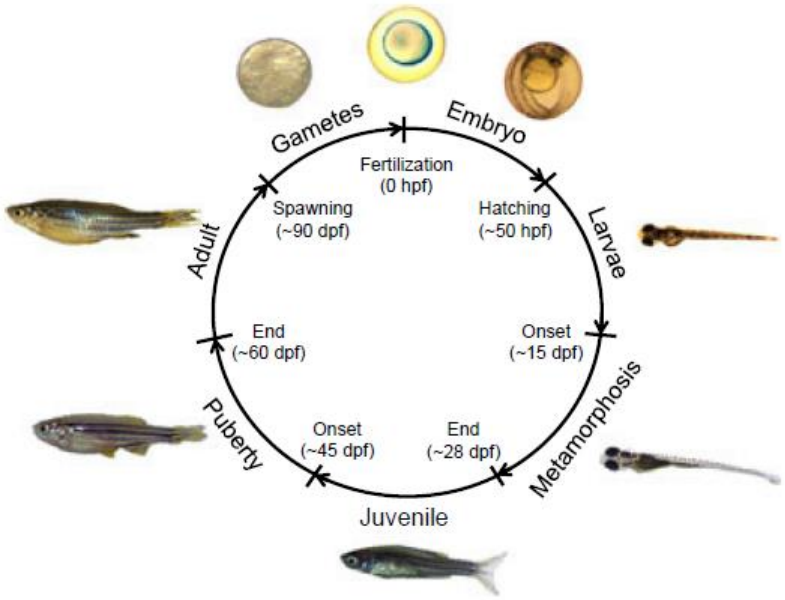

Figure 7. The life cycle of the zebrafish, with representative events and different life stages illustrated (Ribas and Piferrer, 2014).

This species is valuable as a model organism due to their high fecundity and transparent embryos, and development that has been well characterized. Their eggs are transparent during the whole embryonic development which allows easy observation of the main morphological changes up and beyond pharyngulation. Embryonic development is very fast and is completely done within 72 hours post fertilization (hpf; Fig. 8). Furthermore, embryonic development can be observed in live individuals using non-invasive techniques due to *ex utero* fertilization (Hill et al., 2005).

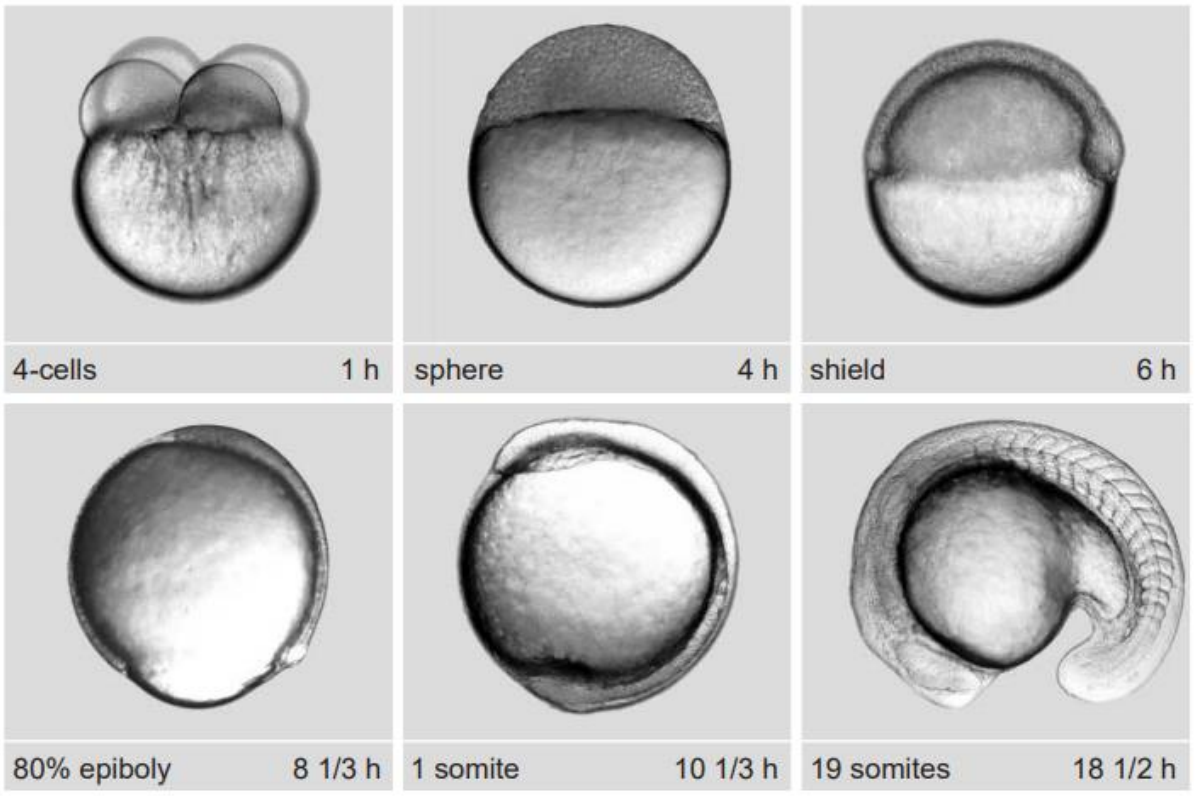

Figure 8. Relevant stages of the embryonic development of the zebrafish during the first 24 hours after fertilization (adapted from Haffter et al., 1996).

3.3.3.2 The use of the zebrafish embryos in scientific research

The use of fish as research models goes back almost 200 years. A good fish model organism fulfils the following criteria: a) has the basic biological features possessed by the most cultured species, b) has similar physiological responses to cultured fish, c) short life cycle, d) easy and inexpensive breeding and, e) has many resources that facilitate research in most areas (Ribas and Piferrer, 2014). The zebrafish fulfils those conditions and therefore presents a perfect model organism for ecotoxicological studies that has been acknowledged as one of the major model organisms within the OECD Test Guidelines.

Around 450 million years of divergent evolution separate humans and zebrafish, but many of its critical pathways that regulate vertebrate development are highly conserved when it comes to human ones (Ribas and Piferrer, 2014). Approximately 70% of all genes associated with diseases in humans have functional homologs in zebrafish, which makes it an ideal model organism for human research (Hollert and Keiter, 2015). Additionally, based on comparison

with effective blood doses, teratogenic effects in zebrafish embryos were found to be concentrations relevant to human exposure data (Braunbeck et al., 2015). Furthermore, the zebrafish are easily obtainable, maintainable and inexpensive (Hollert and Keiter, 2015).

3.3.3.2.1 Zebrafish embryotoxicity test

ZET was originally designed as an alternative for the acute fish toxicity testing and has been adopted as OECD 236 TG as a tool to access toxicity of fish embryos (Braunbeck et al., 2015). Its principle is based on exposing newly fertilized zebrafish embryos to the test sample for up to 96 hours in transparent well plates (Fig. 9). Through that period, various lethality and developmental abnormality endpoints are observed (Table 7) during exposure to environmental samples like effluents from wastewater treatment plants (Babić et al., 2017) and pharmaceutical industries (Bielen et al., 2017), but also to heavy metals (Ansari et al., 2015), nanoparticles (Asharani et al., 2011), and newly synthetized compounds (Carlsson and Norrgren, 2004; Farooq et al., 2012; Ruyra et al., 2015).

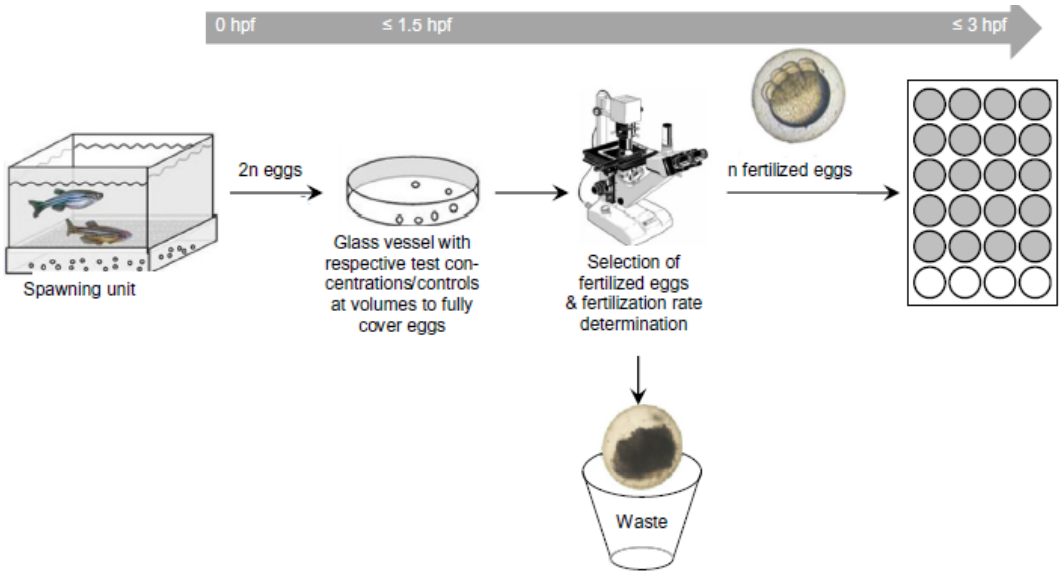

Figure 9. Principle of the zebrafish embryotoxicity test (adapted and modified from Braunbeck et al., 2015).

The use of zebrafish embryos is increasingly gaining attention due to the fact that they are considered to be a replacement method for animal experiments (Braunbeck et al., 2015). Furthermore, according to EU Directive 2010/63/EU (2010), the earliest life stages of fish, characterised by a lack of independent feeding, do not define as protected and therefore do not fall into regulations dealing with animal experiments. A license is required for regulated procedures on fish from the time they are capable of independent eating – for the zebrafish, that is 120 hpf. Life stages before 120 hpf are considered to be not sufficiently aware that they will suffer or otherwise have a poor welfare when a procedure is carried out on them (Braunbeck et al, 2015). They also allow the analysis of multiple endpoints that range from the determination of acute and developmental toxicity to complex functional genetic and physiological analysis (Hollert and Keiter, 2015). Due to the small size, costs through low quantities of dosing solutions are also minimalized, and thereby the quantity of waste for disposal and labware is lowered. In addition, small embryos allow reasonable sample sizes to be tested together using well plates or Petri dishes. Optimum breeding and maintenance conditions have also been well determined (Hill et al., 2005).

Another advantage of this assay is the fact it follows the 3R principle, which has been a long-term goal in mammalian toxicity testing (Embry et al., 2010):

Replacement – the usage of methods that avoid or replace the use of animals. This includes absolute (inanimate systems, such as computer programs) and relative replacements (using animals that have a significantly lower level of pain perception, such as some invertebrates, to replace more sentient animals).

Reduction – any strategy that results in fewer animals being used to obtain sufficient data, or in maximizing the information obtained per animal, which limits or avoids the use of additional animals.

Refinement – the modification of husbandry or experimental procedures in order to minimize pain and distress, and to enhance the welfare of an animal used in the experiment from the time it is born (Fenwick et al., 2009).

Despite its many advantages, ZET does have a few limitations (Braunbeck et al., 2015):

i. Toxic substances with a very high molecular weight (larger than 3 kDa), such as polymers, are unable to pass the chorion before hatching or experimental dechorionation.
ii. Certain neurotoxic substances are proven to be less toxic in the embryo than in adult fish.

iii. There are some minor evidence of limitations when it comes to biotransformation capacity of the early embryonic stages.

However, it has been demonstrated that ZET can be used as a screening tool to prioritize compounds and could contribute to the reduction of animal experiments in the field of toxicology (Braunbeck et al., 2015).

3.3.3.2.2 Multixenobiotic resistance in zebrafish embryos

During the period of embryonic development of the zebrafish, its organs have not yet fully developed. Therefore, in order to survive in a potentially contaminated environment, the zebrafish embryos have developed an effective cellular defence mechanism (Babić et al., 2017). After the passive uptake of hydrophobic compounds by epithelial cells, ATP-binding cassette (ABC) proteins represent the first line of defence against pollutants. They have the role of pumping out hydrophobic substrates, and therefore reducing concentrations of various toxic compounds within the cytoplasm (Babić et al., 2017; Fischer et al., 2013). In the aquatic organisms, such transport is known under the term of the multixenobiotic resistance (MXR) mechanism (Kurelec, 1997; Luckenbach et al., 2014).

ABC proteins, in general, are one of the largest families encoded in the human genome and were first described as a factor in multidrug resistance (MDR) in cancer, showing an important role in the resistance of tumour cells against structurally and functionally unrelated drugs (Epel et al., 2008; Luckenbach et al., 2014). ABC proteins are known to have many functions: some act as ion channels or receptors and factors involved in transcription and translation, while others play an important role in drug transport, which is of a particular toxicological significance (Luckenbach et al., 2014). All eukaryotic ABC drug transporters act as active efflux pumps, where translocation occurs from the cytosol or the cell membrane towards the extracellular space, and is energetically mediated by the cleavage of ATP, which enables substrate transport against the concentration gradient (Luckenbach et al., 2014).

There are three ABC subfamilies (ABCB/Abcb, ABCC/Abcc and ABCG/Abcg) that are known to contain drug transporters. These proteins show different levels of expression in different tissues. In humans, the most important ABC transporters are ABCB1 (MDR1 or P-glycoprotein) – the best studied ABC transporter thus far, ABCC1 (MRP1), ABCC2 (MRP2 and cMOAT) and ABCG2 (BCRP) (Fischer et al., 2013; Luckenbach et al., 2014)

In the zebrafish in particular, 52 ABC transporter genes were identified, including all 48 transcribed genes of human ABC transporters including 2 Pgp/MDR1 transporter genes (ABCB1a and ABCB1b), MDR, ABCB1a and ABCB1b, and four paralogue copies of ABCG2 (ABCG2a, b, c and d) (Park et al., 2008). Transcripts of ABC transporters were found in the early embryonic stages of the zebrafish development, which suggests that they are maternally transferred to the embryo and possess an important function in embryo protection in this stage (Fischer et al., 2013; Luckenbach et al., 2014).

Taking the rather substantial role of the ABC proteins into consideration, there is a concern that their activity might be affected by environmental chemicals – chemosensitizers (Scholz et al., 2008). The competition or inhibition of these proteins, also known as chemosensitization, can leave the organism unprotected on a cellular level in a polluted environment and can therefore lead to increased accumulation of toxins in cells (Babić et al., 2017; Epel et al., 2008) (Figure 10). This may cause DNA damage, embryo mortality, impairment of reproduction and various mutations (Babić et al., 2017; Kurelec, 1997).

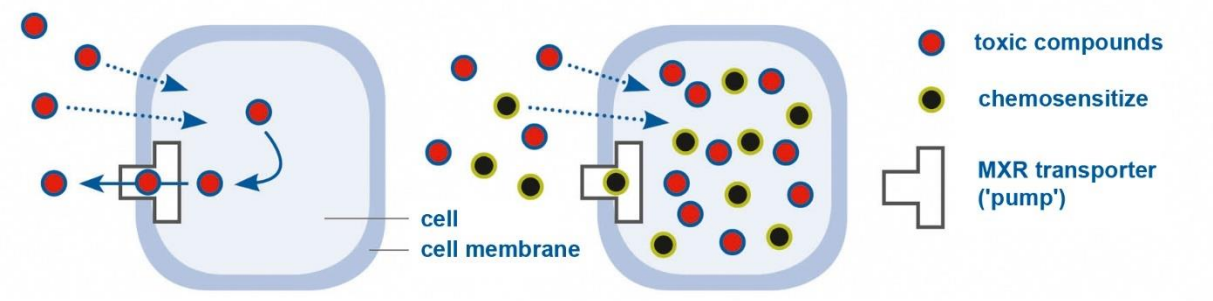

Figure 10. Left cell: Normal MXR transport. MXR transporter pumps toxic chemicals out of the cell. Right cell: The MXR transport is disturbed by chemosensitizers (inhibitors) that block the transporter. Toxic chemicals can't be pumped out (https://www.ufz.de/).

3.3.3.2.2.1 Functional ecotoxicological test for MXR

Considering that the MXR phenomenon is constitutive to aquatic organisms, and is inducible as a response to specific environmental pollutants of both natural and anthropogenic origin (also known as MXR inhibitors or chemosensitizers), it is evident that it can be used as a sensitive tool for monitoring of the environmental pollution (Babić et al., 2015; Sauerborn Klobučar et al., 2010).

The principle of the functional ecotoxicological test of MXR activity is based on the fact that the efflux activity in living cells can be measured by the quantification of substrate uptake into cells in the absence and presence of specific transporter inhibitors. In case of an uninterrupted transporter activity, the substrate uptake will be minor. If there is an inhibitor of the transporter added, the uptake will be increased. This means that the ratio of substrate accumulation in the cell in the absence and presence of inhibitors can provide a measure of transport activity (Epel et al., 2008).

In order to measure the inhibition of the transport activity, it is necessary to use test substrates. They can be either radioactive (^{14}C vinblastine) or fluorescent (rhodamine or calcein-AM). For positive controls in the test, model inhibitors are used, such as cyclosporin, verapamil or MK571 (Epel et al., 2008).

4 MATERIALS AND METHODS

4.1 Locations, sampling and sample preparation

Samples of river water from five sampling sites were collected: Jesenice, Jankomir, Hrušćica, Rugvica, and Lukavec (Fig. 11). Samples were collected in May of 2018. The sites were chosen according available information on pollution intensity in Sava River (Babić et al., 2018).

Jesenice site is placed approximately 13 km upstream from Zagreb and represented a low to a moderately polluted site. Jankomir site is located in suburb area of Zagreb. Hrušćica site is placed approximately hundred meters downstream from the main drainage canal of Central wastewater treatment plant Zagreb. Rugvica site is located approximately 10 km downstream from the effluent outlet of wastewater treatment plant and therefore receives municipal and industrial wastewaters from Zagreb. Lukavec site is approximately 10 km downstream from the city of Sisak, that is inhabited by 20 times fewer inhabitants in comparison to Zagreb, and receives industrial effluents of pesticide production facility, ironworks, an oil refinery and urban runoff, but in lesser extent (Babić et al., 2018).

Samples were collected in polycarbonate bottles (250 mL), which were better suited for water sampling and storage than glass bottles, as some organic compounds absorb on glass (Stipaničev et al., 2017). Water samples were then thawed and filtrated on 0.2 μ PTFE filters.

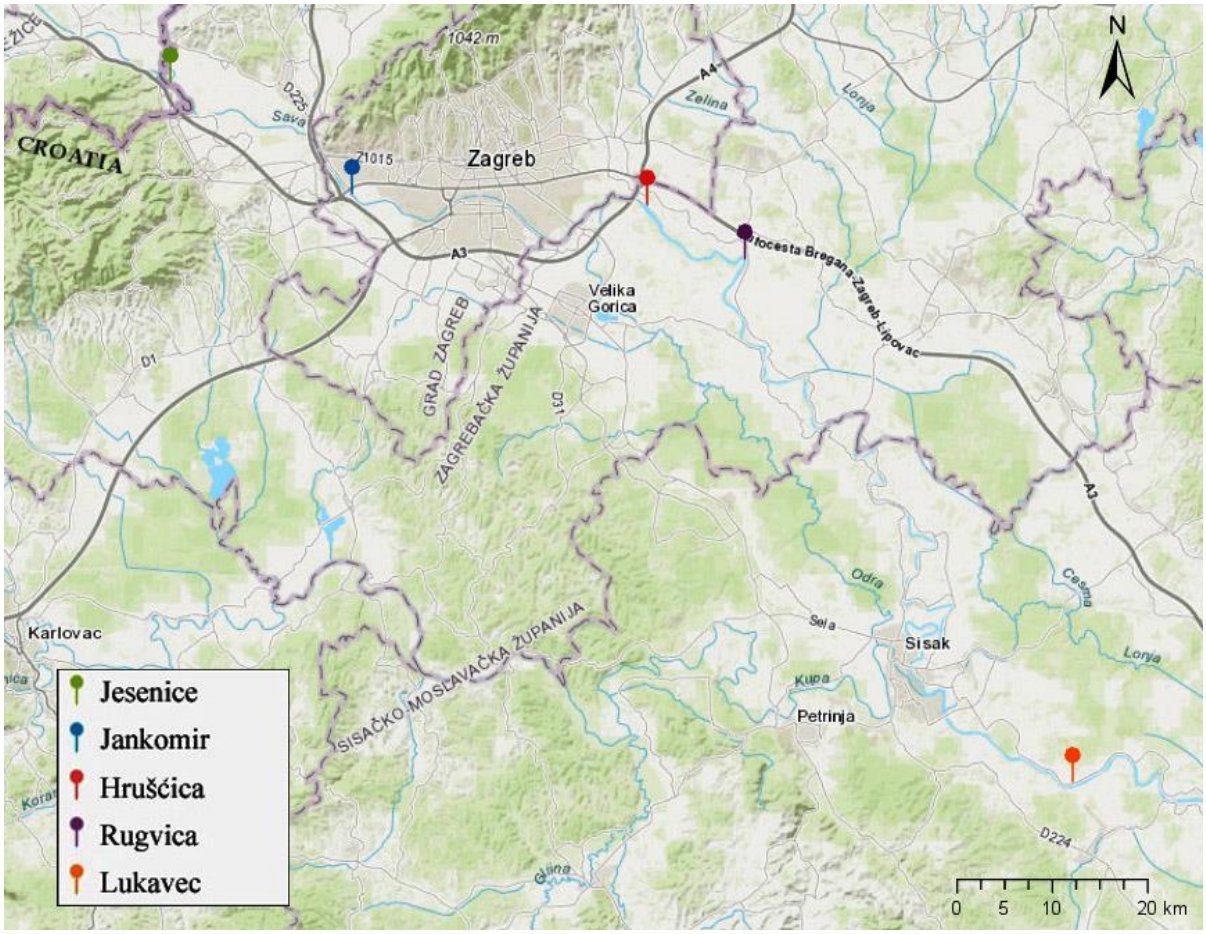

Figure 11. A map of the sampling locations. Generated with ArcGIS Pro software.

4.2 Water analysis

A wide range of organic contaminants in samples of the water from the Sava River was determined by liquid chromatography tandem mass spectrometry (8060 UHPLC/MS/MS Shimadzu, Japan) in the Central Water Management Laboratory of Croatian Waters. The method was based on injecting 100 µL (200 µL for acidic pesticides) of a water sample that was filtered through 0.2 µm PTFE filter into the UHPLC/MS/MS system. This method was used to determine the following organic compounds: pesticides (carbamates, organophosphates, triazines, imidazolinones, chloroacetanilides, sulfoanilides, urea, benzimidazoles, diphenyl ethers, phenoxyacetic, dinitrophenol, phenoxypropionic, unclassified and residual compounds), fluorosefactanates, hormones, pharmaceuticals, UV-blockers, antibiotics and personal care compounds, according to Stipaničev et al. (2017).

4.3 Toxicity assessment

4.3.1 Growth inhibition of *Chlorella vulgaris*

The test was carried out based on the OECD 201 Guideline (2011), and was performed with three replicates for each sample. BBM was used as negative control (Table 6). Due to the possibility of degradation within water samples, an additional control of tested water with no algae was used. The pH of the medium and samples was adjusted to 6.8 using HCl (Agilent Technologies 3200 EA pH meter equipped with P3211 pH Combination Electrode, USA).

Table 6. Composition of Bold's Basal Medium for algae.

Stocks	**per 400 mL**
1. $NaNO_3$	10.00 g
2. $MgSO_4 \cdot 7H_2O$	3.00 g
3. NaCl	1.00 g
4. K_2HPO_4	3.00 g
5. KH_2PO_4	7.00 g
6. $CaCl_2 \cdot 2H_2O$	1.00 g
	per litre
7. Trace elements solution (autoclave to dissolve)	
$ZnSO_4 \cdot 7H_2O$	8.82 g
$MnCl_2 \cdot 4H_2O$	1.44 g
MoO_3	0.71 g
$CuSO_4 \cdot 5H_2O$	1.57 g
$Co(NO_3)_2 \cdot 6H_2O$	0.49 g
8. H_3BO_3	11.42 g
9. EDTA	50.00 g
KOH	31.00 g
10. $FeSO_4 \cdot 7H_2O$	4.98 g
H_2SO_4 (conc)	1.00 mL

Stock solutions 1 – 6: 10 mL, stock solution 7: 2 mL, stock solutions 8 – 10: 1 mL; made up to 1 litre with distilled water.

The population of exponentially growing algae was collected by centrifugation for 10 minutes at 2,000 rpm (Eppendorf Centrifuge 5804 R, Germany). Algae cultures were inoculated in 40 mL of each tested sample and grew in autoclaved (121 °C, 30 min; Tuttnauer 2540 EKA, Netherlands) Erlenmeyer flasks (Fig. 11). The respective negative controls on BBM were run in parallel. Initial cell density for each sample was 10^5 cells/mL. Microalgal

cultures were maintained at 25±1°C and continuous light (7,000 lux, photoperiod of 24 hours) over 72 hours, with frequent shaking. Absorbance was measured in order to determine algal growth rate every 24 hours at the wavelength of 680 nm using spectrophotofluorimeter (Tecan Infinite M200 PRO, Switzerland, equipped with Magelan software version V 7.2).

Figure 11. Set up experiment for the growth inhibition test of *Chlorella vulgaris*.

In order to interpret results, it was necessary to make a calibration curve that defined relation between cell density and absorbance at 680 nm. For that reason, at the beginning of the experiment, range of algae solutions was prepared and absorbance at 680 nm was measured. Cell density was determined by direct counting of algae in each dilution using a Bürker-Türk chamber (Olympus BX51, Japan). Considering measured absorbances and cell densities for each of prepared dilutions, calibration curve was done.

4.3.2 Chlorophyll a concentration in *Chlorella vulgaris*

Chlorophyll a concentration was measured according to the protocol adapted from the ESS Method 150.1 (ESS Method 150.1: Chlorophyll-Spectrophotometric, 1991) and was with slight modification applied for the determination of chlorophyll a concentration in *Chlorella*

vulgaris. It was necessary for this experiment to be completed in the dark, due to the photosensitivity of chlorophyll a.

At 0 (beginning of the experiment) and 72 hours of the experiment, 6 mL of each sample was centrifuged in Falcon tube at 4,200 rpm for 40 minutes (Eppendorf Centrifuge 5804 R, Germany). As much supernatant as possible was poured off with a plastic Pasteur pipette, and 8 mL of 95% ethanol was added. Samples were ultrasonicated for 30 seconds (Bandelin sonorex, Germany), and then heated at 60°C in a water bath (Bandelin sonorex, Germany). Tubes had been shaken several times during the heating process. Samples were ultrasonicated again for 30 seconds, and then centrifuged twice (4,000 rpm, 10 minutes). Since green residue remained at the bottom of the tubes, the samples were shaken and left overnight in the fridge at 4°C.

The next day, samples were ultrasonicated for 30 seconds, centrifuged (4,000 rpm, 10 minutes), and then the absorbance was measured at two wavelengths – 664 and 649 nm (Tecan Infinite M200 PRO, Switzerland, equipped with Magelan software version V 7.2). For the calculation of chlorophyll a concentration, the following equation was used:

$$c\left(\frac{\mu g}{mL}\right) = (13.36 \times A_{664} - 5.19 \times A_{649}) \times \frac{V_{EtOH}}{V_{sample} \times l_{cuvette}}$$

where V_{EtOH} = 8 mL, V_{sample} = 6 mL and $l_{cuvette}$ = 1 cm.

4.3.3 Seed germination and root and sprout elongation of *Trifolium repens* and *Triticum aestivum*

Seed germination test was performed according to Visioli et al. (2014) with slight modification. Filter paper was placed on a Petri dish (100x15 mm) and moistened with 2 mL of tested sample. For the control group, 2 mL of distilled water was used. 8 seeds of *Triticum aestivum* and 10 seeds of *Trifolium repens* were chosen randomly and placed on a Petri dishes. Petri dishes were covered and sealed with Parafilm to prevent evaporation and cross contamination. The germination assay was performed using three replicates per sample. During the whole experiment, Petri dishes were kept in the dark and dry conditions, at room temperature (25±1°C) (Fig. 12). After 120 hours of incubation, seeds were examined to determine the germination rate and to measure sprout and root elongation. As the operational definition of germination, 1 mm sprout was used.

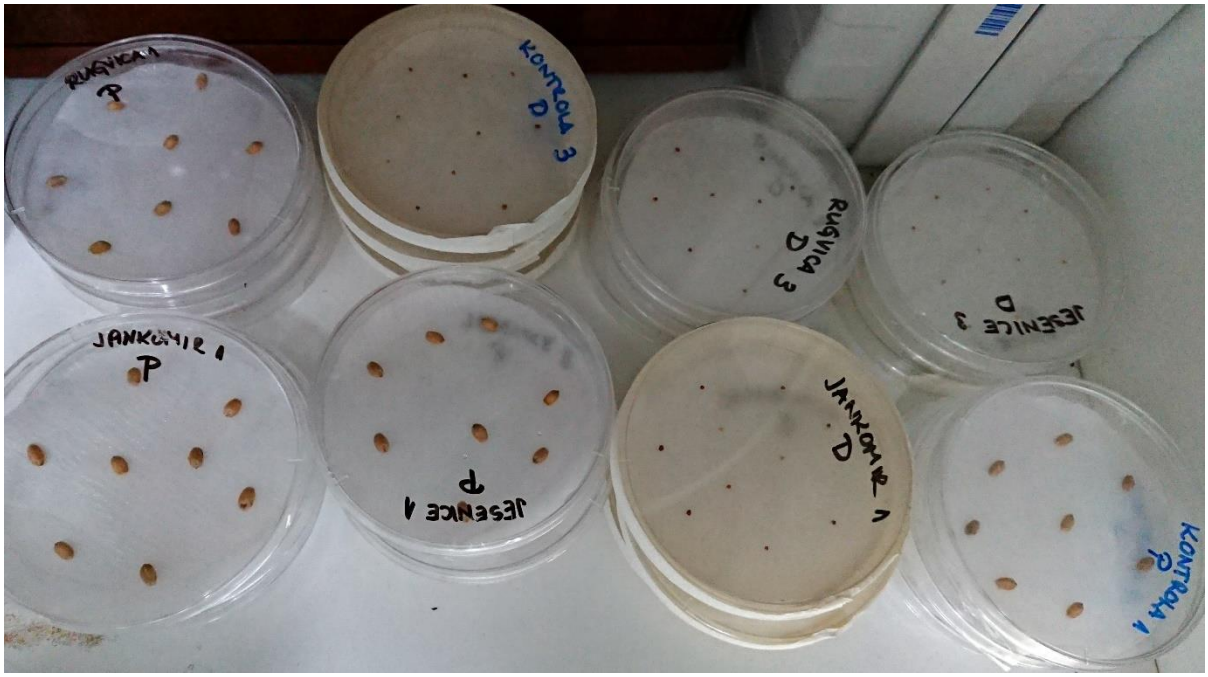

Figure 12. Experimental set up for germination inhibition and sprout and root elongation determination of clover *Trifolium repens* and wheat *Triticum aestivum.*

4.3.4 The Zebrafish embryotoxicity test

ZET was done accordingly to OECD 236 (2013). Animal housing and spawning were performed in aquaria units within Laboratory for Biotechnology in Aquaculture, Ruđer Bošković Institute, approved by the Croatian Ministry of Agriculture and according to the Directive 2010/63/EU (2010).

4.3.4.1 Fish maintenance and egg production

A breeding stock of zebrafish aged ~18 months with no visible diseases or deformations was used for egg production. Females and males were kept together in a glass aquarium (50 L) in controlled conditions (temperature: 27±0.5°C, pH: 7.5±0.3, conductivity: 500 μS/cm, oxygen saturation ≥ 80%, water hardness: 250 mg/l $CaCO_3$). Lighting was controlled by a timer to provide a 14:10 h light:dark cycle. Adults were fed with dry food (Zebrafish diet standard 05.08, Mucedola, Italy) twice daily and additionally with brine shrimp *Artemia*.

In order to optimize fecundity, three days prior to spawning, males and females were housed separately and fed only with *Artemia*. Due to the possibility of breeding groups of

zebrafish failing to spawn, two spawning boxes were used. On the evening before spawning, males and females (2:1) were placed into divided spawning boxes with egg traps to prevent egg predation (Fig. 13).

The next morning, spawning and fertilization took place within 15 to 30 minutes after the onset of light and removal of the divider. Eggs were collected using 800 μm mesh and washed with artificial water in order to remove debris. Artificial water was prepared by dissolving 294.0 mg/L $CaCO_2 \cdot 2H_2O$, 123.3 mg/L $MgSO_4 \cdot 7H_2O$, 63.0 mg/L $NaHCO_3$, and 5.5 mg/L KCl in distilled water (ISO, 1996), followed by pH adjustment to 7.0. Fertilization rate of approximately 80% was set as the minimum to proceed with the further experiment.

Figure 13. Separated female (red dash) and males (blue dash).

4.3.4.2 Embryotoxicity test

All eggs were placed into a Petri dish containing artificial water (ISO, 1996; Fig. 14) and then, in order to minimize dilution error, transferred into Petri dishes containing the tested sample. Afterward, a stereo microscope (PRO-LUX, Croatia) was used to separate fertilized eggs from nonfertilized, damaged and abnormal ones. Eggs from the 4 to 64-cell stage (Kimmel et al., 1995) were transferred into a transparent polyethylene 24-well plates (NEST Scientific,

USA) containing 1 ml of tested sample. Each sample was tested with 10 embryos in four replicates, amounting to a total of 40 embryos. For negative control, artificial water was used. Four wells of each plate were used as an internal negative plate control, and one additional plate was used for external negative control.

Over the period of 96 hours, plates were incubated (New Brunswick Innova® 42, USA) at 28±0.5°C with regulated 14:10 h light:dark photoperiod (Fig. 15). Every 24 hours, 30% of the tested sample (30 µL) in each well was replaced with a previously aerated and incubated sample.

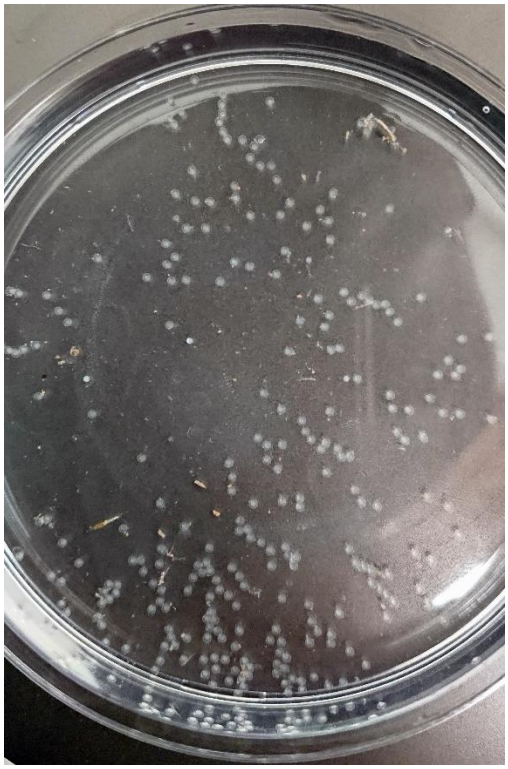

Figure 14. Unselected eggs after fertilization.

Figure 15. Incubated plates with embryos.

Every day, the following parameters were measured: mortality rate, abnormality rate, heartbeat rate, hatching rate, pigmentation formation and behavioural changes, using an inverted microscope (LEICA DMIL LED, equipped with LEICA EC3 camera and LAS EZ 3 software version 2.0, Germany).

The following observations were considered indicators of lethality: a) coagulation of the embryo, b) non-detachment of the tail bud from the yolk sac, c) non-formation of somites and d) lack of heartbeat (Braunbeck et al., 2015) (Table 7).

Table 7. Endpoints observed in the zebrafish at 24, 48, 72 and 96 hours after fertilization (Beekhuijzen et al., 2015).

	Physiological/dysmorphogenic effect	24 hpf	48 hpf	72 hpf	96 hpf
Lethal endpoint	Coagulation of embryos	•	•	•	•
	Non-deatachment of tail	•	•	•	•
	Non-formation of somites	•	•	•	•
	Lack of heartbeat		•	•	•
Teratogenic endpoint	Malformation of the head	•	•	•	•
	Malformation of the otoliths/sacculi		•	•	•
	Malformation of the tail	•	•	•	•
	Malformation of the heart	•	•	•	•
	Deformed body shape	•	•	•	•
	Yolk deformation	•	•	•	•
	Growth retardation	•	•	•	•

A detailed description of these teratogenic endpoints is given below (Beekhuijzen et al., 2015):

i. *Malformation of the head*: missing structures, uneven or small eye size, abnormal shape of eye or head.
ii. *Malformation of the otoliths/sacculi*: one or both structures are missing, duplicated, or have an uneven size or abnormal shape. Enlarged sacculi and/or otoliths, or not well-defined structures.
iii. *Malformation of the tail*: kink seen mostly at the tip of the tail, tail lifted either up or down. Malformed tail-fins.
iv. *Malformation of the heart*: irregular shape due to edema or aplasia, abnormal heartbeat (too slow, too fast or irregular compared to control).
v. *Deformed body shape*: abnormalities in the neural tube; absence, an indistinct or malformed morphology of the notochord or (one or more) somites. Disclosure of the back (rachischisis) or malformed skeleton (scoliosis).
vi. *Yolk deformation*: edema, bulbs and other malformations that interfere with the normal rounded shape.

4.3.5 MXR transporter activity assessment of *Danio rerio* larvae

The amount of RB accumulated in 96 hpf zebrafish larvae after exposure to tested samples was measured according to Babić et al., 2017, with slight modification. At 96 h, after the determination of lethal and sublethal effects of tested samples, larvae were collected from the 24-well plates and transferred into falcon tubes (15 ml) in duplicate. In falcon tubes, 96 h old larvae were exposed to 4 ml of tested sample and 1 μM RB. A negative control was run on the mixture of RB and artificial water, while positive control larvae were exposed to the mixture of RB and model inhibitor cyclosporine A (10 μM CA). Falcon tubes were wrapped in aluminium foil to prevent photodegradation of RB, and incubated at 25±1.0 °C for 90 minutes. After incubation, larvae were rinsed with distilled water three times to remove RB, and ultrasonicated (Bandelin sonorex, Germany) each time before rinsing. All the remaining water was removed and larvae were homogenized in 1.5 ml tubes in 500 μL of distilled water. Homogenization was performed with 30 strokes of Eppendorf autoclave micropestles. After homogenization, tubes were centrifuged for 10 minutes at 9,000 rpm (Eppendorf Centrifuge 5804 R, Germany). 200 μL of supernatant was transferred into a black 96-well plate (Nunc, Thermo Fisher Scientific, Denmark) and fluorescence was measured at excitation/emission – 530/595 nm using spectophotofluorimeter (Tecan Infinite M200 PRO, Switzerland, equipped with Magellan software version V 7.2). Shaking amplitude was set to 3 mm for 10 seconds, with 25 flashes and gain 141. All samples were measured in duplicates.

4.4 Statistical analysis

Results were expressed as means ± SD, and $p \leq 0.05$ was used as a cutoff value of statistical significance throughout the manuscript.

Algae cell density was estimated from the calibration curve correlating absorbance values (680 nm) and cell density, and expressed as cell/mL.

The results for RB accumulation in the whole embryos were expressed as fluorescent units per mg of embryo tissue (FU mg^{-1}) and normalized relative to non-treated controls.

All the statistical analysis was carried out using SigmaStat software version 1.0. A.

One-way analysis of variance (ANOVA) was used for comparison of the results obtained from measuring the chlorophyll a concentrations, RB concentrations, but also *Trifolium repens* and *Triticum aestivum* germination inhibition and sprout and root elongation. Significant

differences between controls and tested Sava River samples, as well as between tested samples in dependence of the day of the exposure were determined using two-way ANOVA for following tests: algal growth inhibition, zebrafish mortality and abnormality rate, as well as heartbeat rate, pigmentation formation and hatching rate. When the assumption for normality was violated the Kruskal-Wallis One-way Analysis of variance on ranks was performed.

5 RESULTS

5.1 Chemical characterization of the water quality

Samples were examined for the presence of 571 organic contaminants that were sorted into five major groups: pharmaceuticals/drugs, pesticides, industrial chemicals, hormones and other (Table 8). Pharmaceuticals/drugs were further categorized in the following subcategories: hypnotics/anticonvulsants/anaesthetics, antibiotics, hallucinogens/stimulants/illicit drugs, antiepileptics/neuroleptics, opioids, analgesics, cardiovascular medicaments, antidepressants, antiparasitics, diuretics, steroidal anti-inflammatory drugs and other (Fig. 17). Pesticides included herbicides, insecticides, and fungicides. Hormones included both synthetic and natural hormones. Chemical analysis determined following substances in this group: androstenedione, testosterone, estriol, equilin, 17-a-ethynylestradiol, estrone, 17b-estradiol and progesterone. Group "Industrial chemicals" consisted of a wide spectrum of perfluoroalkyl and polyfluoroalkyl substances that are used for providing water, oil and stain repellence to textiles, carpets and leather, and in chrome plating, firefighting foams, and other applications (OEHHA, 2015). Furthermore, it also included chemicals such as 1-H benzotriazole, bisphenol A, 4-nitrophenol, 4-para-nonylphenol, 4-tert-octylphenol and hexabromocyclododecane (HBCDD), as well as octylphenol ethoxylates (OP1EO and OP2EO) and nonylphenol ethoxylates (NP1EO and NP2EO), of which most have proven to act as endocrine disruptors (Jensen and Leffers, 2008; Kortner and Arukwe, 2007; Lee et al., 2006; Takayanagi et al., 2006; Zhang et al., 2013). Chemicals such as acesulfame, which is used as a sweetening agent (Mukherjee and Chakrabarti, 1997), octinoxate – an organic UV filter compound (Bratkovics and Sapozhnikova, 2011), iopromide – a nonionic iodinated contrast agent (Mortelé et al., 2005), and metabolites, such as 2-NP-AOZ, 4-acetylaminoantipyrine, 4-formylaminoantipyrine and N-desmethylclozapine, were included in separate group under the name "Other".

A wide range of contaminants has been detected in each sample: Jesenice – 398, Jankomir – 366, Hrušćica – 396, Rugvica – 391 and Lukavec – 386 contaminants (Table 8). Concentrations of tested chemical summed up to 13.27 μg/L at Jesenice site, 9.15 μg/L at Jankomir site, 14.98 μg/L at Hrušćica site, 13.57 μg/L at Rugvica site, followed by 10.94 μg/L at Lukavec site.

Table 8. Number of pollutants detected in tested water of each sampling site. Total presents a total number of chemicals tested for each category.

Pollutant category	Total	Jesenice	Jankomir	Hrušćica	Rugvica	Lukavec
Pharmaceuticals/Drugs	**234**	142	154	171	164	165
Pesticides	**291**	213	170	182	185	179
Industrial chemicals	**31**	28	27	28	27	27
Hormones	**8**	8	8	8	8	8
Other	**7**	7	7	7	7	7
Sum	**571**	**398**	**366**	**396**	**391**	**386**

Organic pollutants that were detected to have the highest concentrations varied for each sampling site. Ten chemicals that were detected at highest concentrations at each site, along with their classification and concentration, are shown in Table 9.

Table 9. Chemicals detected at highest concentrations at each sampling site. Concentrations are presented in μg/L.

Jesenice	Jankomir	Hrušćica	Rugvica	Lukavec
4-para-nonylphenol (industrial chemical) (3.52)	**Nicotine** (stimulants) (1.56)	**Metoprolol** (cardiovascular medicament) (1.21)	**Nicotine** (stimulans) (2.24)	**Phenobarbital** (hypnotic) (1.27)
Fludrocortisone-acetate (steroidal anti-inflammatory drug) (0.79)	**NP1EO** (industrial chemical) (0.71)	**Nicotine** (stimulants) (1.20)	**Phenobarbital** (hypnotic) (1.29)	**Caffeine** (stimulans) (0.98)
Naproxen (analgesic) (0.55)	**OP2EO** (industrial chemical) (0.61)	**Sulfapyridine** (antibiotic) (1.18)	**Caffeine** (stimulans) (1.10)	**Nicotine** (stimulans) (0.85)
4-tert-octylphenol (industrial chemical) (0.54)	**Metoprolol** (cardiovascular medicament) (0.57)	**Phenobarbital** (hypnotic) (1.15)	**Tetracycline HCl** (antibiotic) (0.67)	**NP1EO** (industrial chemical) (0.71)
Nafcillin sodium monohydrate (antibiotic) (0.52)	**Amoxicillin** (antibiotic) (0.57)	**Caffeine** (stimulants) (1.06)	**Sulfapyridine** (antibiotic) (0.53)	**Sulfapyridine** (antibiotic) (0.65)
Ibuprofen (analgesic) (0.45)	**Caffeine** (stimulans) (0.50)	**Amoxicillin** (antibiotic) (0.96)	**Metoprolol** (cardiovascular medicament) (0.50)	**Acetylsalicylic acid** (analgesic) (0.58)
Nicotine (stimulants) (0.43)	**4-para-nonylphenol** (industrial chemical) (0.49)	**OP2EO** (industrial chemical) (0.75)	**Bisphenol A** (industrial chemical) (0.48)	**Metoprolol** (cardiovascular medicament) (0.57)
Bisphenol A (industrial chemical) (0.41)	**NP2EO** (industrial chemical) (0.31)	**Tetracycline HCl** (antibiotic) (0.68)	**Amoxicillin** (antibiotic) (0.45)	**OP2EO** (industrial chemical) (0.52)
Metoprolol (cardiovascular medicament) (0.37)	**Phenobarbital** (hypnotic) (0.25)	**NP1EO** (industrial chemical) (0.56)	**Naproxen** (analgesic) (0.44)	**4-para-nonylphenol** (industrial chemical) (0.34)
Etodolac (analgesic) (0.33)	**1-H benzotriazol** (industrial chemical) (0.20)	**Acesulfame** (artificial sweetener) (0.39)	**4-para-nonylphenol** (industrial chemical) (0.33)	**NP2EO** (industrial chemical) (0.22)

Among the detected groups, pharmaceuticals/drugs were the most dominant one (Fig. 16), ranging from 47.66% at Jesenice site, 56.26% at Jankomir site, 65.67% at Lukavec site, 69.72% at Hrušćica site, to 75.14% at Rugvica site (Fig. 17).

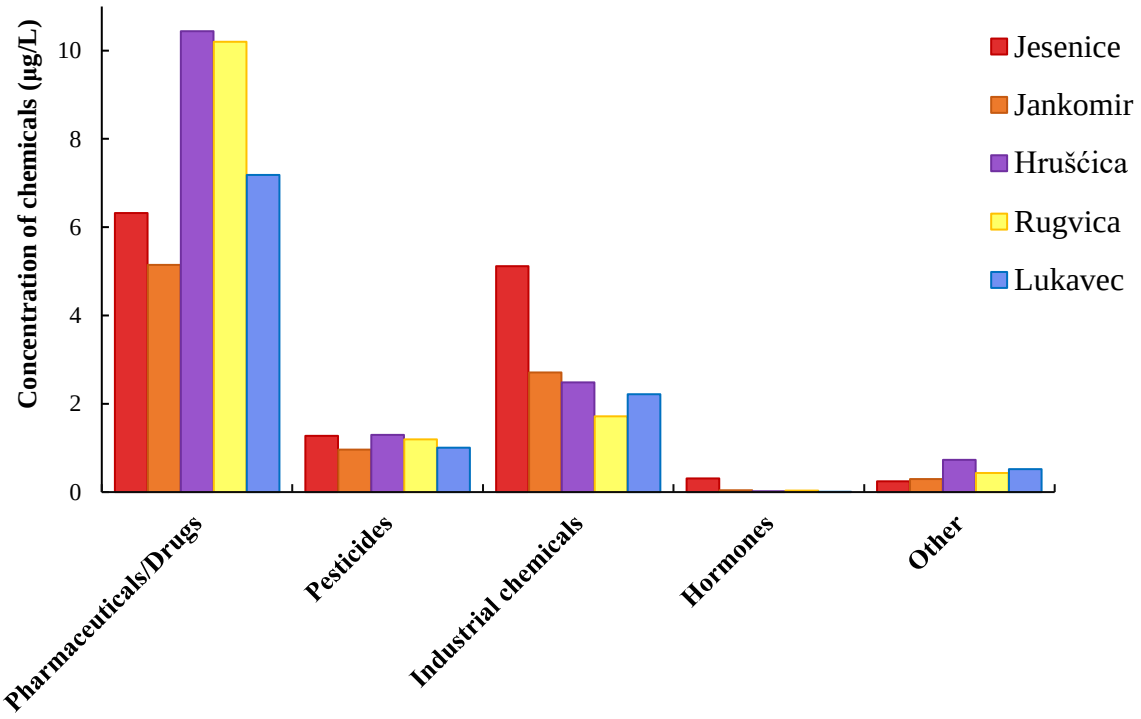

Figure 16. Total concentrations of organic pollutants in tested samples of water from the Sava River by five main categories.

At Jesenice site, the most abundant subcategories in pharmaceuticals/drugs group were antibiotics (27.00%) and analgesics (27.21%), while at Jankomir site, the far most dominant subcategory was hallucinogens/stimulants/illicit drugs (45.46%), as well as at both Rugvica (36.13%) and Lukavec (30.14%). Antibiotics (32.30%) were the most represented subcategory found at Hrušćica site (Fig. 17).

The second most dominant group at Jesenice site was industrial chemicals (38.60%), due to high concentration of 4-para-nonylphenol (3.52 µg/L) that was found, followed by pesticides (9.61%), where most presented subgroup was insecticides (61.21%). At Jankomir site, industrial chemicals also presented second most dominant group (29.58%), while in the third group, pesticides (10.54%), herbicides and insecticides were almost equally represented (44.97%, 45.38%, respectively). At Hrušćica, Rugvica and Lukavec sites, industrial chemicals were the second most represented group as well, though their concentrations were lower (16.61% at Hrušćica site, 12.62% at Rugvica site and 20.29% at Lukavec site). Concentrations of pesticides were similar to ones found at Jesenice and Jankomir site: 8.63% at Hrušćica site, 8.82% at Rugvica site and 9.22% at Lukavec site. At Hrušćica site, herbicides and insecticides subcategory were almost equally represented (36.01% and 39.53%, respectively), while at both

Rugvica and Lukavec, the most copious subcategory was insecticides (51.18% and 55.69%, respectively).

Hormones and separate group “Other” were present in much lower concentrations in all locations (2.31% and 1.82 at Jesenice, 0.41% and 3.21% at Jankomir, 0.14% and 4.89% at Hrušćica, 0.23% and 3.18% at Rugvica, and 0.05% and 4.77% at Lukavec, respectively) (Fig. 17).

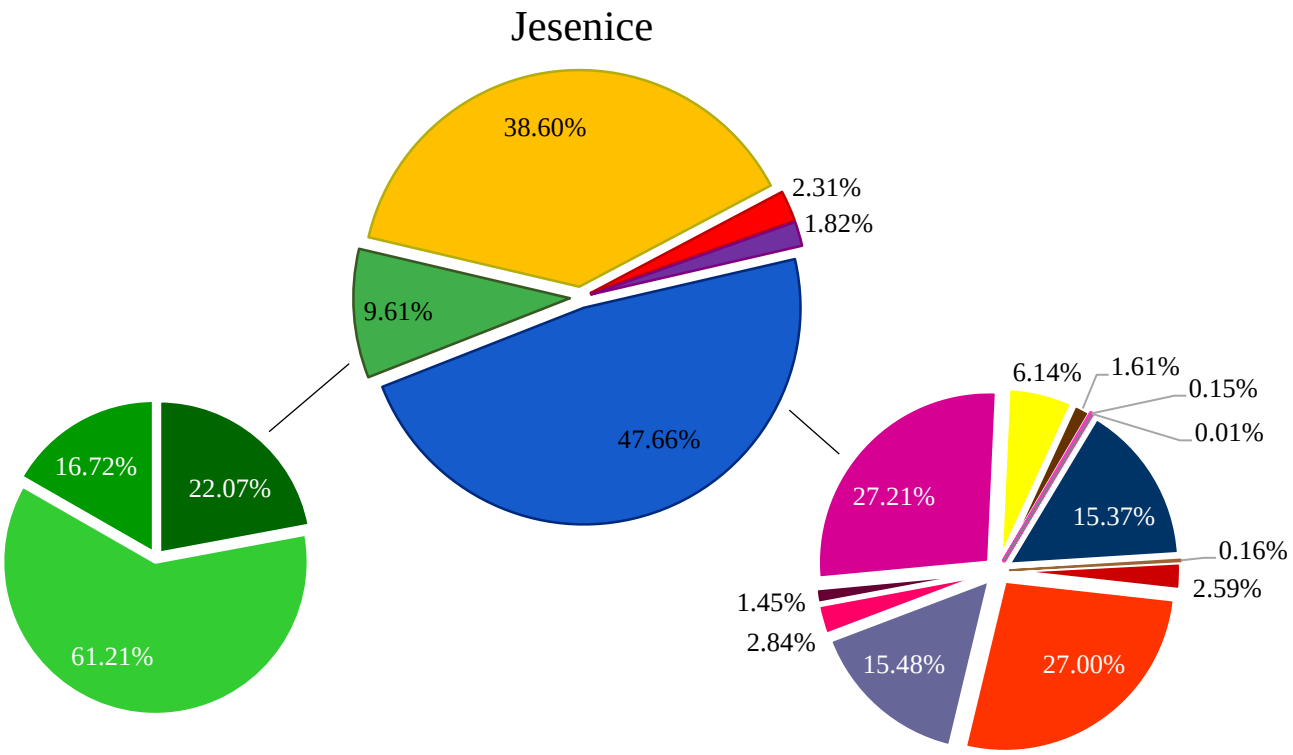

Figure 17. Distribution of the main categories and sub-categories by their cumulative concentration in tested samples of water from the Sava River.

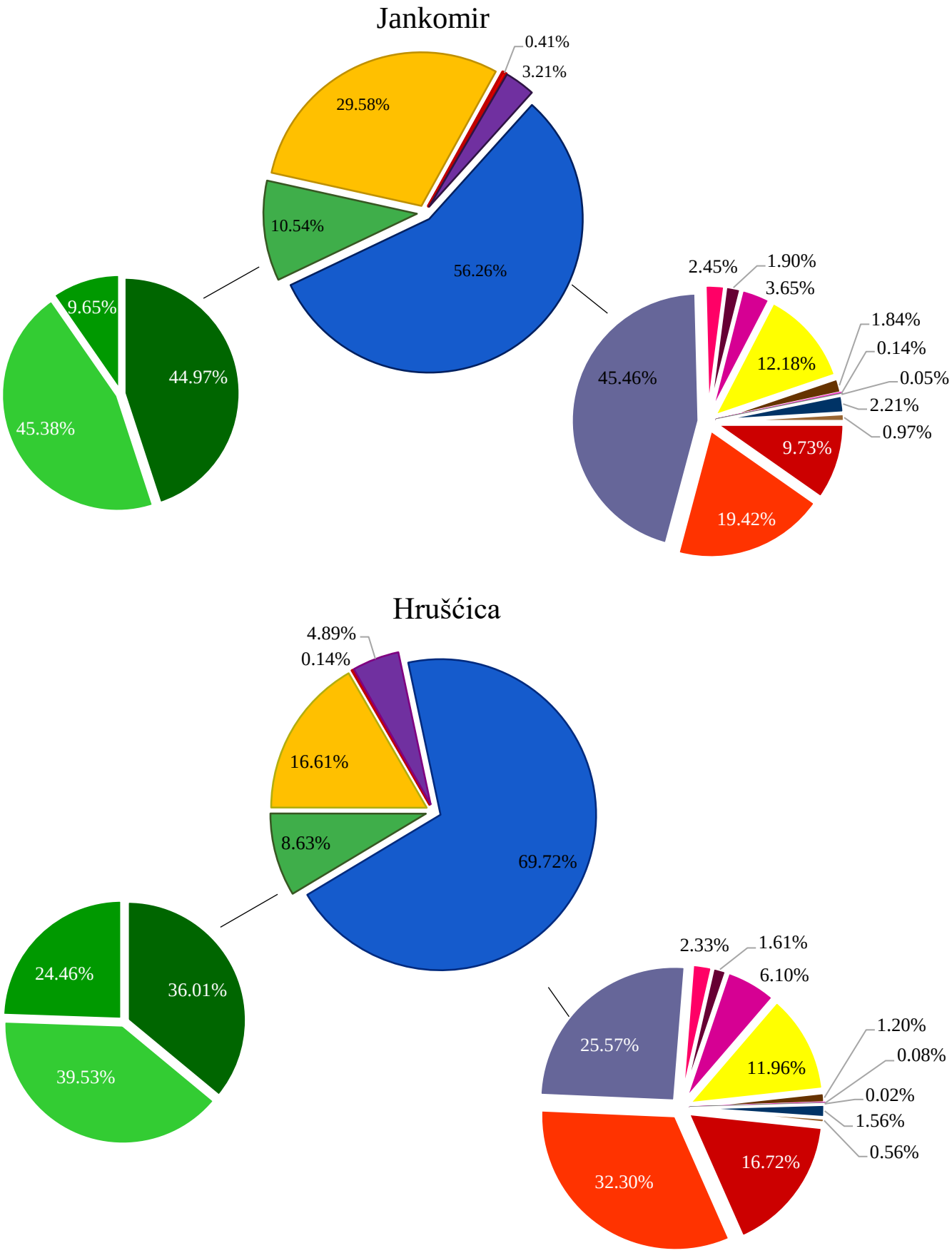

Figure 17. (continued)

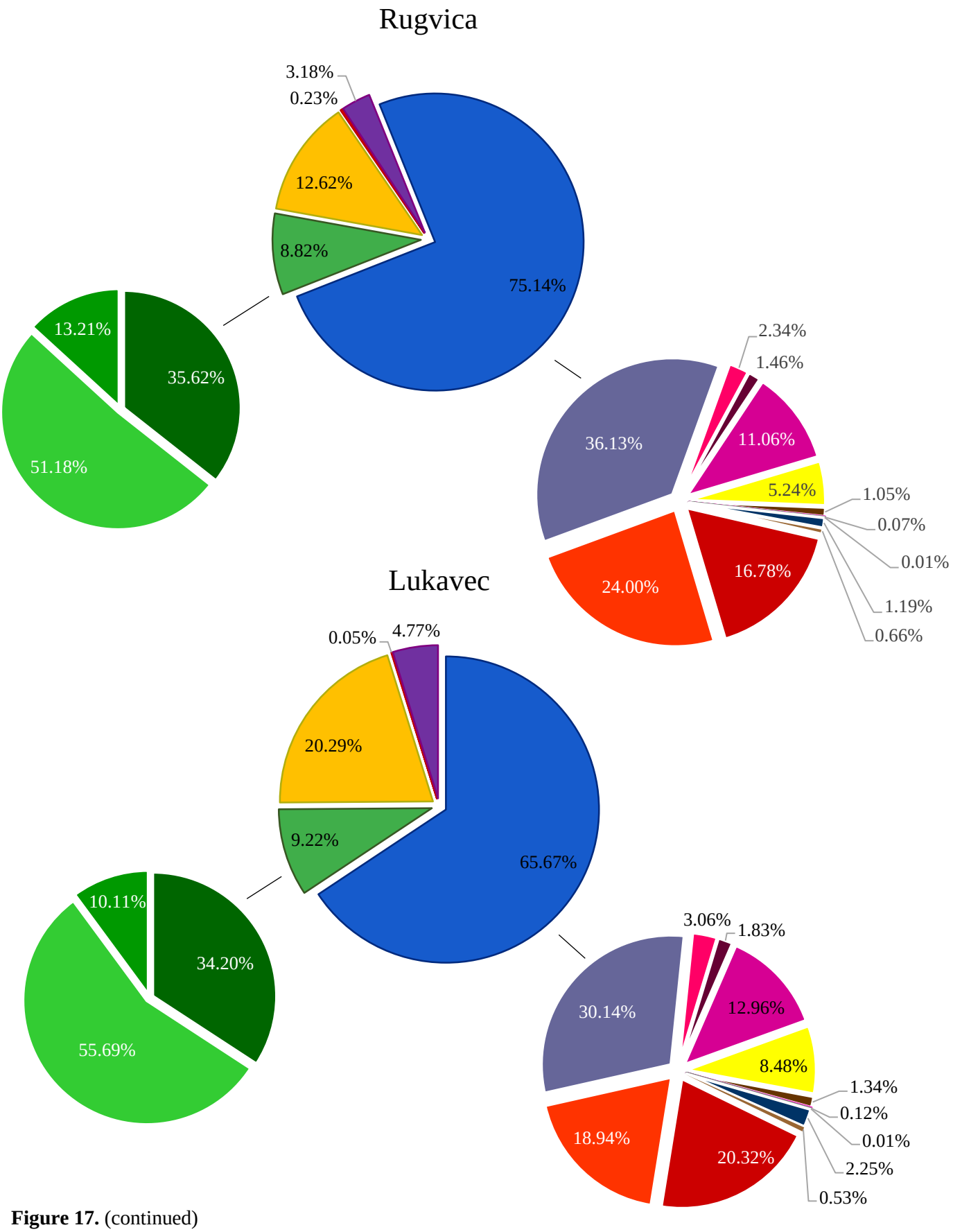

Figure 17. (continued)

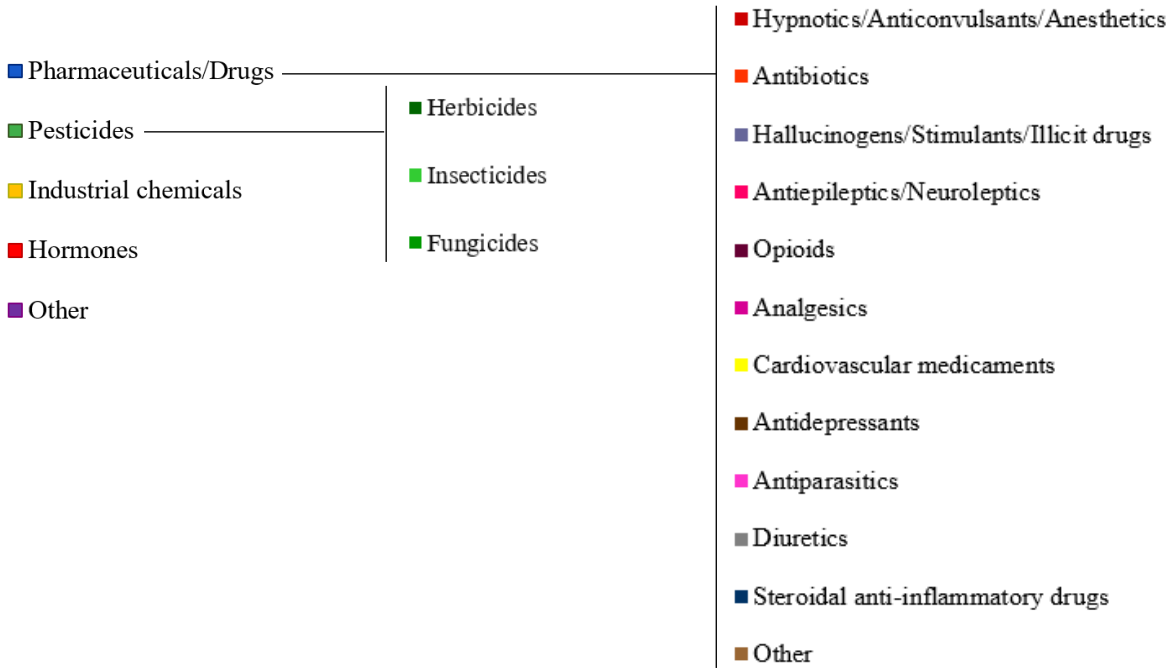

Figure 17. (continued)

5.2 Growth inhibition of *Chlorella vulgaris*

An increase in algae growth in comparison to control has been noticed after 24 hours of exposure. After 48 and 72 hours of exposure to samples, an inhibition of growth has been observed. The significant difference compared to control had been noted in every sample, with the exception of Hrušćica sample upon 48 hours of exposure. Differences in algae growth caused by tested samples during all three days were statistically significant [$F(3.06)= 11.11$, $p < 0.0001$]. There was a significant difference between following samples: Hrušćica – Rugvica, Lukavec – Rugvica, Jankomir – Rugvica, Hrušćica – Lukavec, Hrušćica – Jankomir, Hrušćica – Jesenice, Lukavec – Jesenice, and Jankomir – Jesenice (Fig. 19).

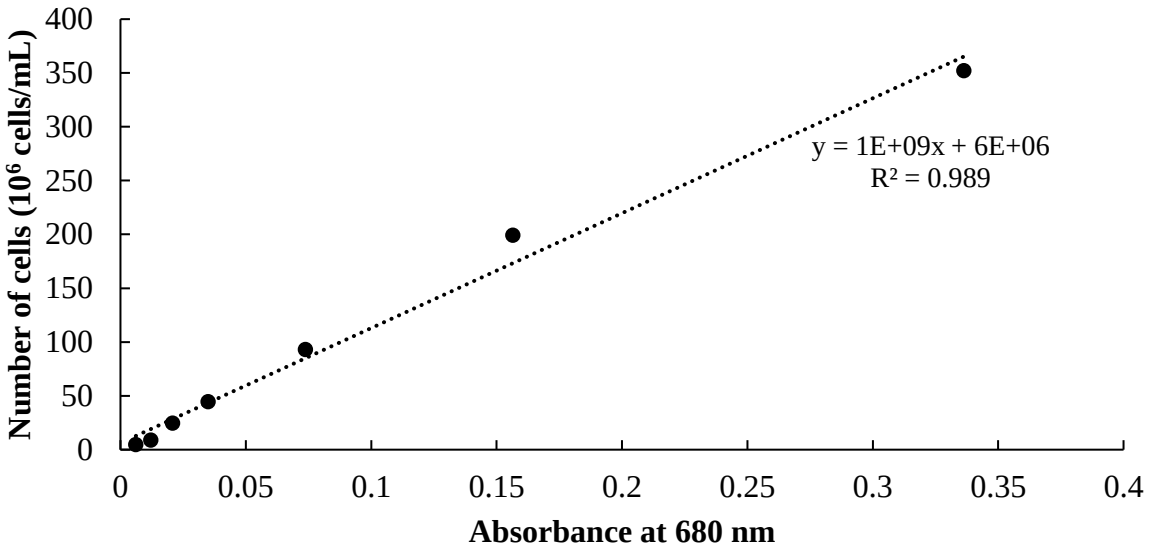

Figure 18. Calibration curve of *Chlorella vulgaris* concentration.

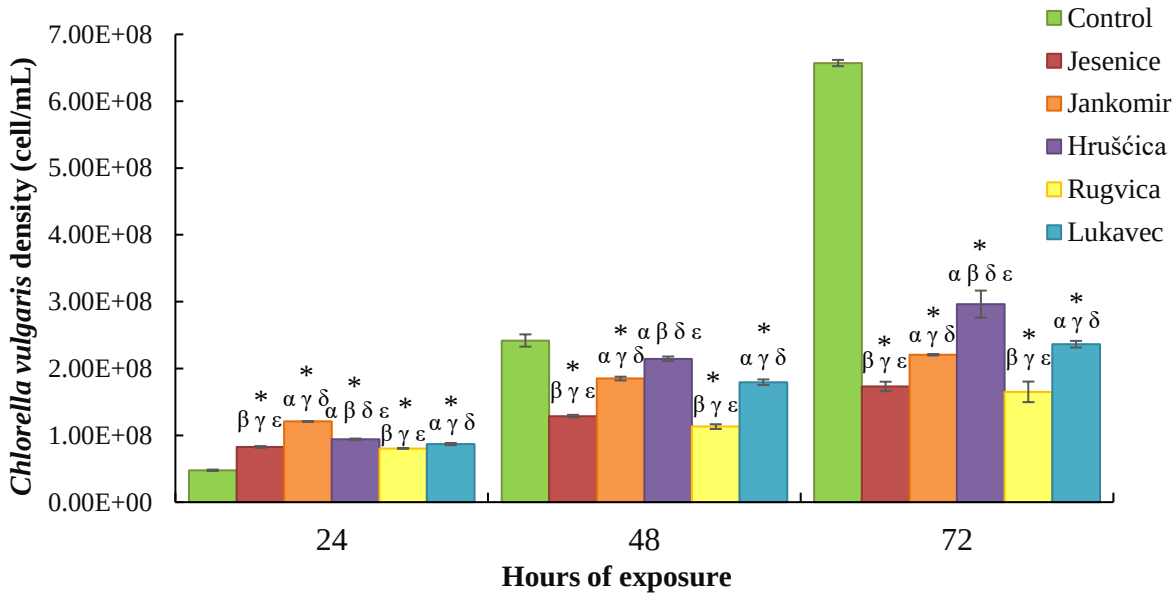

Figure 19. A density of *Chlorella vulgaris* algae (cell/mL) through 72 hours of exposure to control and samples of the water from the Sava River. Results are presented as mean ± SD. The symbol * indicates a significant difference between tested sample and control. Significant differences between sample exposures are marked with letters (α – Jesenice, β – Jankomir, γ – Hrušćica, δ – Rugvica, ε – Lukavec).

5.3 Chlorophyll a concentration in *Chlorella vulgaris*

From the results presented in Table 8. it is evident that there is a statistically significant decrease in its concentration compared to control after 72 hours of exposure to tested samples. The highest decrease in chlorophyll a concentration was recorded in *Chlorella vulgaris* exposed to Rugvica and Jesenice (84.96 and 81.99% lower compared to the control, respectively; Table 10).

Table 10. Chlorophyll a concentration (μg/mL) in *Chlorella vulgaris* at 72 hours of exposure to the samples of water from the Sava River. Results are presented as mean ± SD. The symbol * indicates a significant difference between tested sample and control. Significant differences between sample exposures are marked with letters (γ – Hrušćica, δ – Rugvica).

Control	Jesenice	Jankomir	Hrušćica	Rugvica	Lukavec
4.72 ± 0.43*	0.85 ± 0.18*	1.08 ± 0.05*	1.38 ± 0.25*$^{\gamma}$	0.71 ± 0.13*$^{\delta}$	1.09 ± 0.02*

5.4 Seed germination and root and sprout elongation of *Trifolium repens* and *Triticum aestivum*

After five days of exposure, no significant differences between control group and sample groups were noted in germination of *Trifolium repens* (Fig. 20). While measuring sprout length, a significant difference between Lukavec and control group was noticed (approx. 25% lower compared to control group), as well as between following samples: Rugvica – Hrušćica, Lukavec – Rugvica and Lukavec – Jesenice (Fig. 21).

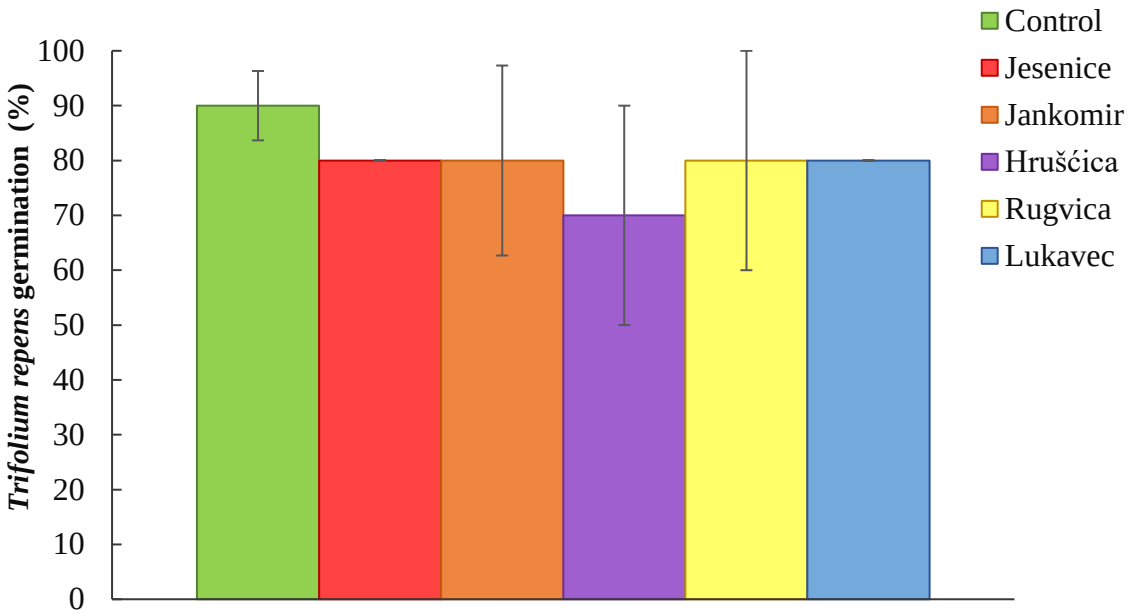

Figure 20. Germination rate (%) of *Trifolium repens* after five days of exposure to tested samples of water from the Sava River. Results are presented as mean ± SD.

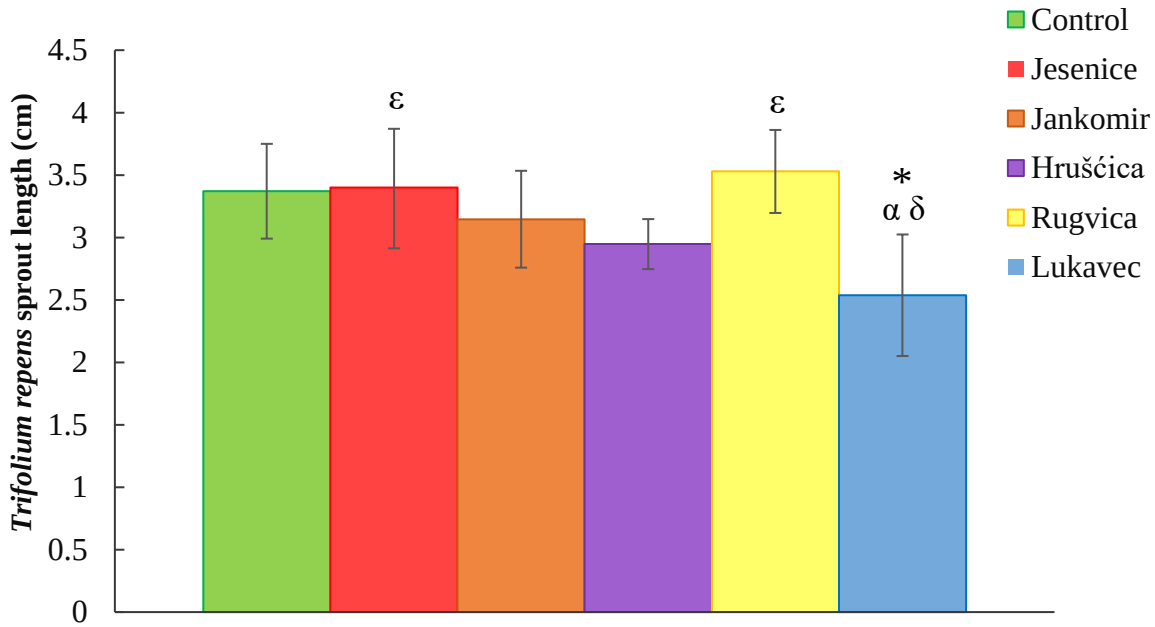

Figure 21. Sprout length (cm) of *Trifolium repens* after five days of exposure to tested samples of water from the Sava River. Results are presented as mean ± SD. The symbol * indicates a significant difference between tested sample and control. Significant differences between sample exposures are marked with letters (α – Jesenice, δ – Rugvica, ε – Lukavec).

Although tested samples had no impact on germination of *Triticum aestivum* (Fig. 22), statistically significant differences were noted in both sprout and root length of *Triticum aestivum* seeds exposed to samples from Lukavec and Hrušćica in comparsion to control group (Fig. 23 and 24). Significant differences were noticed between Rugvica – Hrušćica, Lukavec – Rugvica, Jankomir – Hrušćica, Jesenice – Hrušćica, Lukavec – Jankomir and Lukavec – Jesenice while measuring sprout length, and between Rugvica – Lukavec and Lukavec – Jankomir when measuring root length.

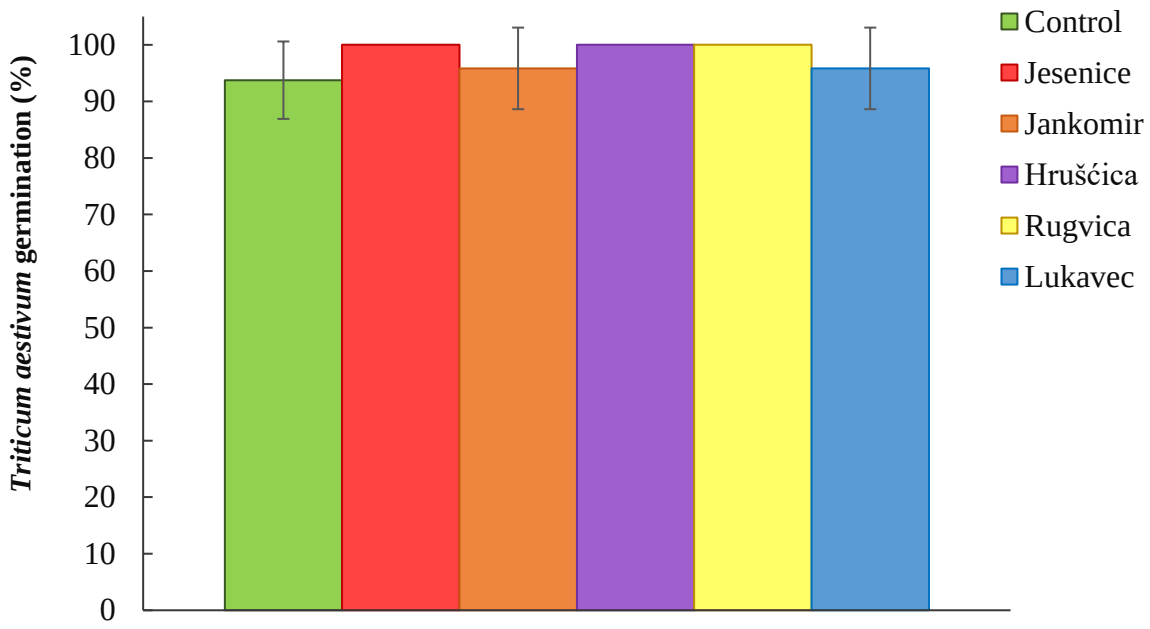

Figure 22. Germination rate (%) of *Triticum aestivum* after five days of exposure to tested samples of water from the Sava River. Results are presented as mean ± SD.

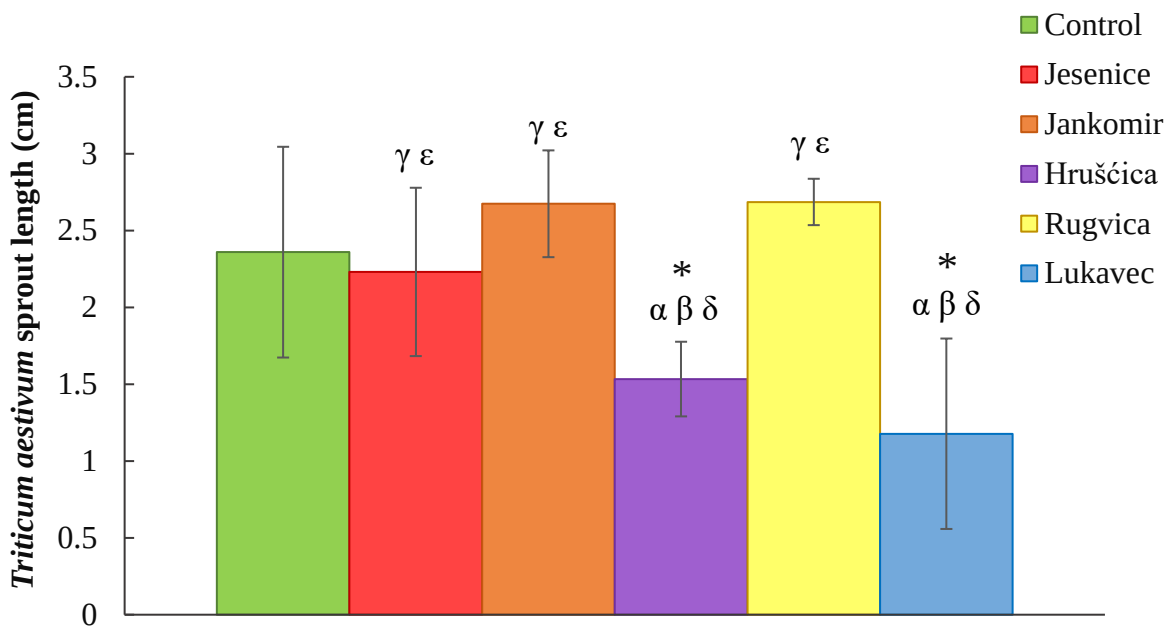

Figure 23. Sprout length (cm) of *Triticum aestivum* after five days of exposure to tested samples of water from the Sava River. Results are presented as mean ± SD. The symbol * indicates a significant difference between tested sample and control. Significant differences between sample exposures are marked with letters (α – Jesenice, β – Jankomir, γ – Hrušćica, δ – Rugvica, ε – Lukavec).

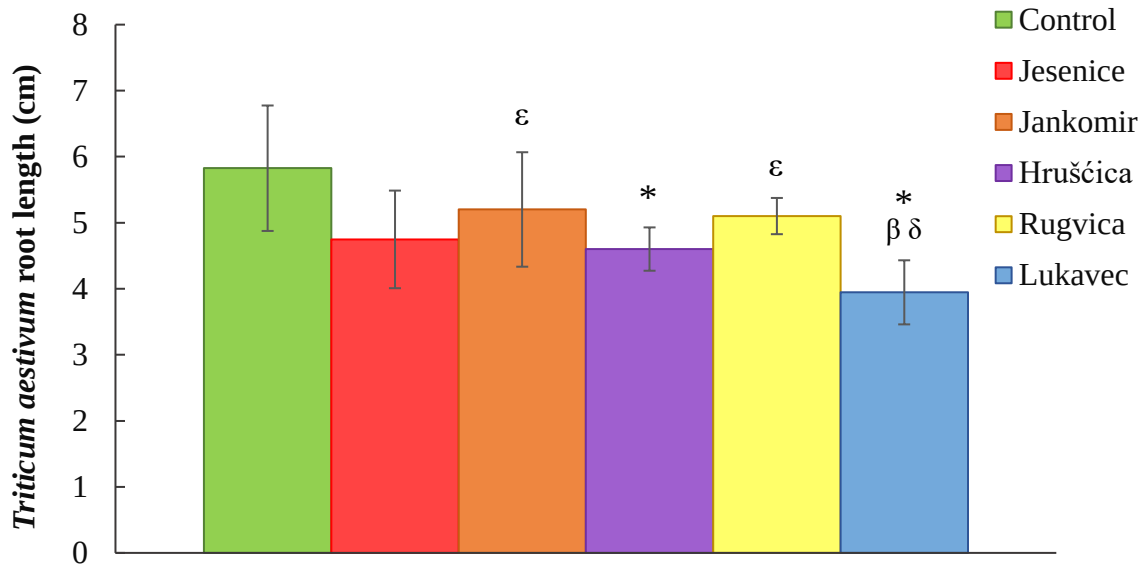

Figure 24. Root length (cm) of *Triticum aestivum* after five days of exposure to tested samples of water from the Sava River. Results are presented as mean ± SD. The symbol * indicates a significant difference between tested sample and control. Significant differences between sample exposures are marked with letters (β – Jankomir, δ – Rugvica, ε – Lukavec).

5.5 The Zebrafish embryotoxicity test

Exposure of *Danio rerio* embryos to collected samples mostly manifested in minor lethal and developmental toxicity effects. Lethality manifested mainly as coagulation of embryos and non-detachment of the tail (OECD 236, 2013). No mortality was recorded during exposure to Jesenice, while exposure to Hrušćica, Rugvica and Lukavec induced <5% of mortality. Mortality rate of Jankomir site was 10%, but was not statistically significant compared to control group due to high standard deviations. Mortality rate of the negative control group was less than 10%.

Abnormalities were observed only in Lukavec and Jankomir samples at 24 hours of exposure to samples, and in larvae during exposure to all samples at 96 hours of exposure (Table 11). Although not statistically significant, the highest abnormality rates were noted in Jankomir and Rugvica samples. Abnormality rate of negative control group was less than 10%.

Table 11. Developmental abnormalities observed in zebrafish *Danio rerio* during 96 hours of exposure to samples of water from the Sava River.

	Toxicological endpoints	**Jesenice**	**Jankomir**	**Hrušćica**	**Rugvica**	**Lukavec**
24 hpf	Coagulation of embryos		†	†	†	†
	Non-deatachment of tail		†		†	†
	Developmental retardation		•			•
	Underdeveloped head region		•			
	Underdeveloped eye					•
48 hpf	Coagulation of embryos			†		
	Scoliosis				•	
	Yolk sac edema		•		•	•
	Pericardial edema				•	•
	Tail deformation		•		•	
	Blood accumulation in the yolk sac region		•		•	•
	Blood accumulation in the brain region		•			
	Blood accumulation in tail region				•	
72 hpf	Scoliosis		•	•	•	•
	Tail deformation		•		•	•
	Blood accumulation in the yolk sac region					•
	Yolk sac edema				•	
	Pericardial edema		•		•	•
96 hpf	Scoliosis	•	•	•	•	•
	Tail deformation	•	•		•	
	Blood accumulation in the yolk sac region					•
	Pericardial edema		•			•

† = lethal endpoint (OECD, 2013)

• = sublethal endpoint (Beekhuijzen et al., 2015; Hollert et al., 2003)

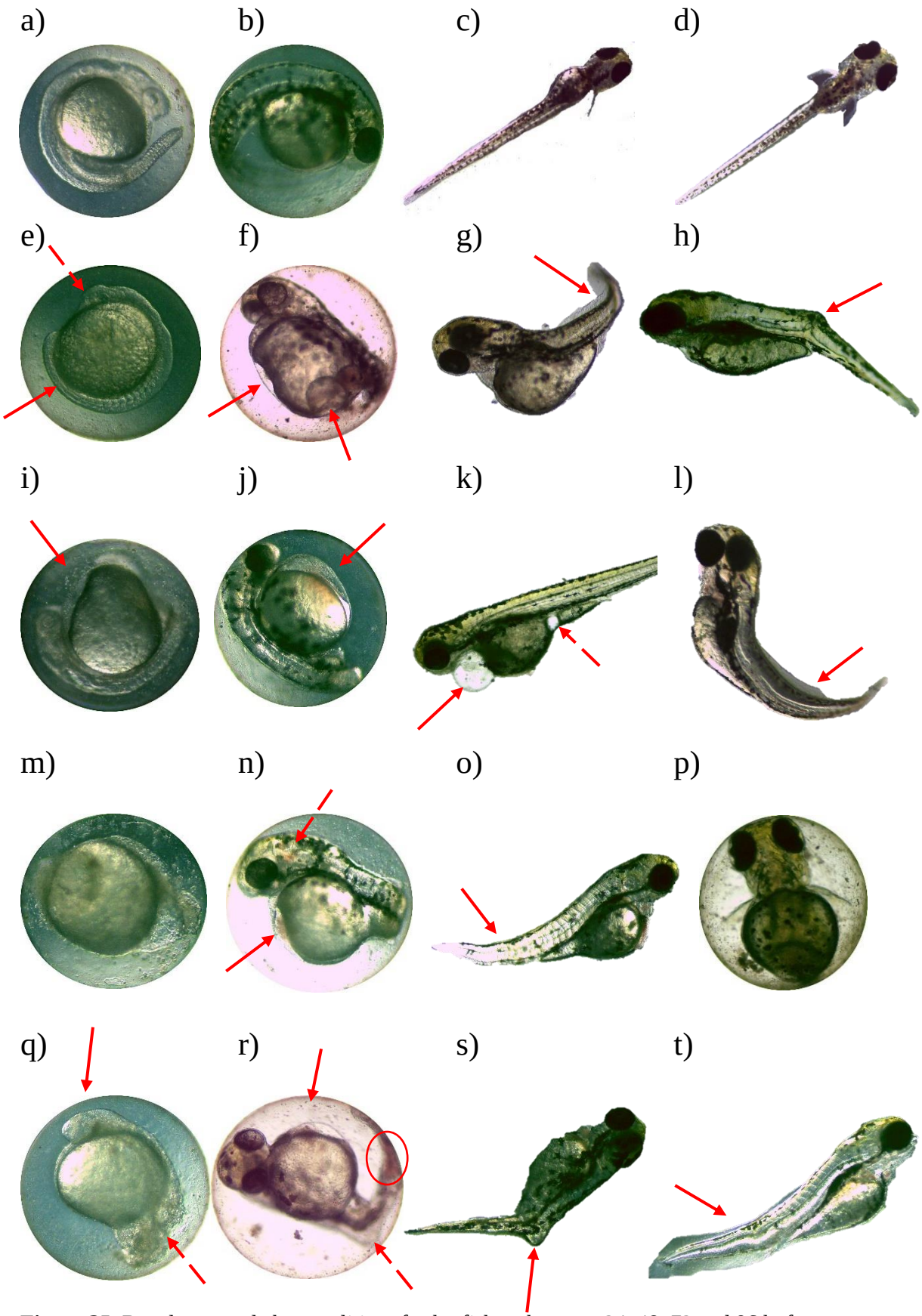

Figure 25. Developmental abnormalities of zebrafish embryos at 24, 48, 72 and 96 hpf.

Figure 25. (continued) a), b), c) and d) normally developed control embryo at 24, 48, 72 and 96 hours after exposure, respectively. 24 hpf embryos exposed to samples: e) developmental retardation, tail not detached from the yolk sac (arrow), underdeveloped head (dashed arrow); i) yolk sac edema; m) whole embryo deformation; q) underdeveloped head (arrow) and spine deformation (dashed arrow). 48 hpf embryos exposed to samples: f) yolk sac edema (arrows); j) yolk sac edema (arrow); n) blood accumulation in the brain region (dashed arrow) and yolk sac region (arrow); r) yolk sac edema (arrow), blood accumulation in tail region (circle), tail malformation (dashed arrow). 72 hpf embryos exposed to samples: g) bent trunk vertebrae (arrow); k) pericardial edema (arrow), yolk sac edema (dashed arrow); o) bent trunk vertebrae (arrow); s) bent tail vertebrae (arrow). 96 hpf embryos exposed to samples: h) bent tail vertebrae; l) bent trunk vertebrae; p) non-hatched fish with delay or anomaly in the absorption of the yolk sac; t) bent trunk vertebrae.

Heart beat rate was measured in 15 seconds long intervals. A decreased heartbeat rate in embryos exposed to samples has been noted, with significant differences compared to control in every sample with the exception of Hrušćica sample at 48 and 72 hours of exposure (Fig. 26). The highest decrease in heartbeat rate was noticed during exposure to Jankomir sample (7.12, 21.04 and 14.83% lower at 24, 48 and 72 hpf, compared to the control, respectively), while the sample of Hrušćica caused the lowest, but still significant at 96 h of exposure, impact on zebrafish heart rate. Differences in heartbeat rate caused by tested samples during all three days were statistically significant only among Hrušćica and Jankomir (Fig. 26).

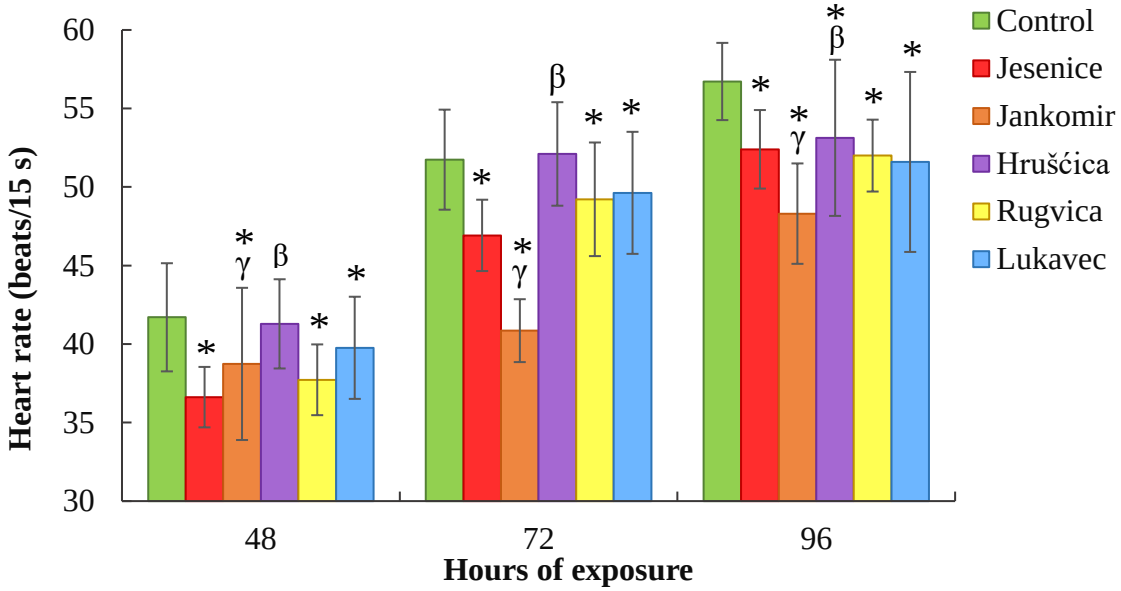

Figure 26. Heartbeat rate (beats *per* 15 seconds) of zebrafish *Danio rerio* through 96 hours of exposure to Sava River samples. Results are presented as mean ± SD. The symbol * indicates a significant difference between tested sample and control. Significant differences between sample exposures are marked with letters (β – Jankomir, γ – Hrušćica).

Hatching had been observed at 72 and 96 hours post fertilization. No significant differences were noted in comparison to control group (Table 12). Furthermore, formation of pigmentation was observed at 48, 72 and 96 hours of exposure to samples, and scored from 0 to 3 (0 – no pigmentation, 3 – fully formed pigmentation) (Table 12).

Table 12. Hatching rate at 72 and 96 hours of exposure to samples (%), and formation of pigmentation at 48, 72 and 96 hours of exposure to samples (scored 0 – 3). The values are given as mean ± SD and $p \leq 0.05$ was used as a cutoff value of statistical significance. The symbol * indicates a significant difference between tested sample and control. Significant differences between sample exposures are marked with letters (α – Jesenice, γ – Hrušćica).

	Hours of exposure	Control	Jesenice	Jankomir	Hrušćica	Rugvica	Lukavec
Hatching rate (%)	**72**	100 ± 0.00	100 ± 0.00	96.88 ± 6.25	100 ± 0.00	100 ± 0.00	100 ± 0.00
	96	100 ± 0.00	100 ± 0.00	100 ± 0.00	100 ± 0.00	100 ± 0.00	100 ± 0.00
Formation of Pigmentation (scored 0 – 3)	**48**	2.48 ± 0.59	2.20 ± 0.56*	2.18 ± 0.65*	2.37 ± 0.58	2.15 ± 0.58*	2.26 ± 0.66
	72	2.73 ± 0.48	2.35 ± 0.70*$^{\gamma}$	2.55 ± 0.72	2.80 ± 0.44$^{\alpha}$	2.43 ± 0.64*	2.87 ± 0.43
	96	2.77 ± 0.43	2.65 ± 0.48	2.84 ± 0.49	2.85 ± 0.36	2.44 ± 0.55	2.77 ± 0.53

5.6 MXR transporter activity assessment of *Danio rerio* larvae

A statistically significant increase in RB accumulation in comparison to negative control has been noticed in zebrafish larvae in all tested samples, except during exposure to Rugvica. The highest increase in accumulation of RB was observed within the samples Jesenice and Lukavec. Comparing with the model MXR inhibitor CYC, RB concentration was significantly higher in larvae exposed to Lukavec. Moreover, a statistically significant difference within the tested samples was also recorded, as follows: Jankomir – Hrušćica, Jankomir – Lukavec, Rugvica – Lukavec, Jesenice – Hrušćica and Hrušćica – Lukavec (Fig. 27).

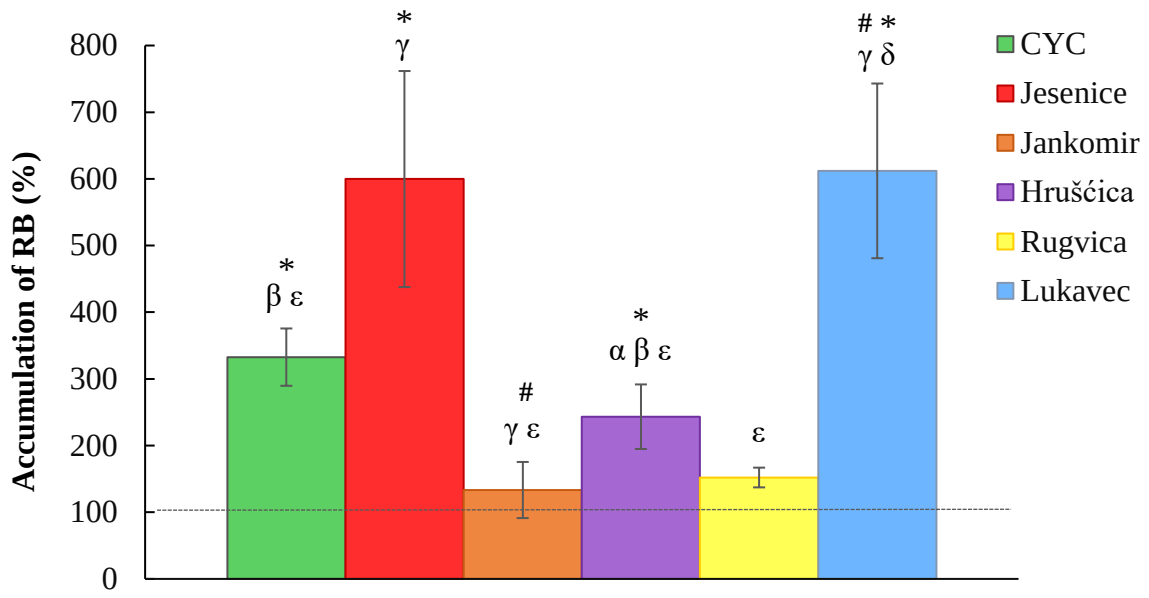

Figure 27. Accumulation of the model fluorescent substrate RB in 96h old zebrafish *Danio rerio* after exposure to model inhibitor CYC and tested Sava River samples. The dashed line indicates the control values. Results are presented as mean ± SD. The symbol * indicates a significant difference between the tested sample and negative control (artificial water), while symbol # indicates that there is a significant difference between the tested sample and positive control (CYC). Significant differences between sample exposures are marked with letters (α – Jesenice, β – Jankomir, γ – Hrušćica, δ – Rugvica, ε – Lukavec).

6 DISCUSSION

The Sava River, as well as the Danube basin, is generally assumed to act as a sink for pollutants from various sources, such as industrial wastes, untreated effluents from municipalities and contaminants from agricultural activities (Källqvist et al., 2008; Ščančar et al., 2015). Considering the important role of the Sava basin in the drinking water supply, the need for continuous and thorough water pollution screening is evident (Källqvist et al., 2008). Therefore, detection of the level of organic pollution in the Sava river, as well as its impact on living organisms, was the aim of this study.

Chemical analysis

The number of industrial, agricultural and waste chemicals is continuously increasing with the rapid development of modern society (Livingstone et al., 2000). This release of materials that are potentially toxic has been proven to have negative effects on the aquatic ecosystem: it may deposit large amounts of organic matter, nutrients and pollutants which can result in processes such as eutrophication, oxygen deficits and accumulation of pollutants (Livingstone et al., 2000; Topić Popović et al., 2015). Furthermore, benthic invertebrates and other aquatic organisms may accumulate these toxic substances. If contaminated organisms are eaten by, e.g., fish, the contaminants may eventually reach humans by consumption of contaminated fish (Babić et al., 2017; Hafner et al., 2015).

Although chemical characterization in ecotoxicological studies may give an insight on the variety and quantity of contaminants, alone it is often not sufficient for accurate assessment of toxicity, due to the factors of bioavailability, degradation products, as well as synergistic and antagonistic effects (Babić et al., 2017). Therefore, to offer an accurate assessment of the overall impact of organic pollution on the aquatic environment and living organisms within it, several bioassays have been applied in this study.

In order to determine the extent of organic pollution at each sampling site that was chosen, chemical analysis of water using liquid chromatography tandem mass spectrometry was done. Pollutants in tested samples summed up to 9.15 μg/L at Jankomir site, 10.94 μg/L at Lukavec site, 13.27 μg/L at Jesenice site, 13.57 μg/L at Rugvica site and 14.98 μg/L at Hrušćica site. Interestingly, Jesenice site was more polluted than it was formerly expected. High levels of pollution at Hrušćica and Rugvica sites were according to predictions, as the sample water

of Hrušćica site was taken underneath the main drainage canal of Central wastewater treatment plant Zagreb, and Rugvica site is located 10 km downstream.

The largest portion of detected pollutants in this study were pharmaceuticals and drugs, especially at Hrušćica and Rugvica sites that are under the heaviest influence of Zagreb waste water treatment plant effluents. This was to be expected, considering that in the last decade alone, there was an approximate 60% increase in consumption of pharmaceuticals in Croatia, according to Annual Reports on Drug Utilization from 2005 to 2016 that are provided by HALMED (http://halmed.hr/) (Babić et al., 2018). Nowadays, they are increasingly becoming a concern due to their slow metabolism and persistence in the environment (Brooks et al., 2005; Christen et al., 2010). The primary sources of these pollutants are municipal wastewater treatment plants, agricultural settings, aquaculture, hospitals and pharmaceutical production facilities (Bielen et al., 2017).

Higher concentrations of pharmaceuticals at Hrušćica and Rugvica sites are assumed to be of a consuming nature, which is proven by their position near the Central wastewater treatment plant Zagreb. In both samples, antibiotics and hallucinogens/stimulants/illicit drugs were the most dominant subcategories detected. As the samples were taken in May, these high concentrations of antibiotics may have been residual from their excessive use over the winter months.

Amongst the variety of different pharmaceuticals and drugs, a few of them have been found consistently at almost all sites in high concentrations, such as antibiotics nafcillin sodium monohydrate and amoxicillin, cardiovascular medicament metoprolol and hypnotic phenobarbital. Furthermore, high concentrations of stimulants caffeine and nicotine, accompanied with a moderately high concentration of cotinine (nicotine's metabolite) were also detected in all samples, with the exception of Jesenice site, which indicated the strong influence of urban wastewaters (Babić et al., 2017). High concentration of nicotine is a major concern in aquatic ecosystems, since it can induce changes in some of the most important key groups from different tropic levels of the aquatic compartment, which can compromise the balance of aquatic ecosystem in a short time (Oropesa et al., 2017).

Studies show that nicotine can cause algal growth inhibition (Oropesa et al., 2017). It has also been proven that it can have negative effect on reproductive system of planktonic crustaceans *Daphnia magna* and *Daphnia pulex*, by decreasing the number of juveniles produced by female, and it may act as a weak juvenoid compound of their endocrine system (Oropesa et al., 2017; Savino and Tanabe, 1989). Furthermore, nicotine is known to be toxic to insects and was widely used in the past as an insecticide against aphids, thrips, whiteflies, and other sucking

insects on a range of crops, including fruits, vines, vegetables, and ornamentals (Buerge et al., 2008). Nicotine derivatives may also be harmful, particularly to arthropods (Buerge et al., 2008).

In accordance with reports on drug utilization given by HALMED, the number of pharmaceuticals and drugs that were determined in this study was lower than that of pesticides in each sample, but it has been shown that each sample contained 5–8.6 times higher concentrations of pharmaceuticals/drugs than pesticides (Babić et al., 2018).

Industrial chemicals were second most dominant source of pollution in tested water samples, the highest concentrations found being those of 4-para-nonylphenol, bisphenol A, NP1EO, OP2EO and NP2EO. This presents a major concern, as these chemicals have repeatedly been proven to act as endocrine disruptors (Bonefeld-Jørgensen et al., 2007; Gatidou et al., 2007; Guenther et al., 2002). The highest concentration of an individual pollutant was that of 4-para-nonylphenol (3.52 μg/L , at Jesenice site), a hydrophobic compound that is released from plastics and possesses the ability to accumulate in living organisms (Litwa et al., 2014).

A large number of pesticides has also been detected: Jesenice – 213, Jankomir – 170, Hrušćica – 182, Rugvica – 185 and Lukavec – 179. This was to be expected due to intensive agricultural activities along the area of the Sava river. However, they appeared to be in low concentrations at each site (Jesenice – 1.28 μg/L, Jankomir – 0.96 μg/L, Hrušćica – 1.29 μg/L, Rugvica – 1.17 μg/L and Lukavec – 1.01 μg/L). These low concentrations of detected pesticides could be related to the seasonality of pesticide usage (Babić et al., 2017). Individual pesticides found in the highest concentrations varied between sites: Jesenice – spiromesifen (insecticide), Jankomir – phenmedipham (herbicide), Hrušćica – tolylfluanid (fungicide), Rugvica – chlorpyrifos-methyland (insecticide), and Lukavec – metaflumizone (insecticide). At Jankomir site, herbicides and insecticides were almost equally represented (44.97% and 45.38%, respectively), as well as at Hrušćica site (36.01% and 39.53%, respectively), while at both Rugvica and Lukavec, the most dominant subcategory were insecticides (51.18% and 55.69%, respectively).

Fourth and fifth group, hormones and “Other”, appeared in much lower concentrations in all samples. Interestingly, while concentration of group other summed up to less than 5% in all samples, high concentrations of acesulfame and moderately high concentrations of octinoxate and iopromide were detected. Hormones made less than 1% of total concentration of pollutants in all samples, with the exception of Jesenice site that had 2.31% of hormones, due to higher concentration of progesterone.

Toxicity assays

In order to determine the impact of Sava River pollution on living organisms, various bioassays with different test organisms were used. Model organisms chosen for this study did, indeed, show different sensitivity to different mixtures of pollutants found in tested samples. The cause of this may lay in the fact that different model organisms may be exposed *via* different pathways due to their biological and ecological properties (Schulze-Sylvester et al., 2016).

The growth inhibition and decrease in chlorophyll a concentration of green algae *Chlorella vulgaris* were observed in all samples. Algal growth in samples was increased in comparison to control group during the first day of exposure, most likely due to nutrients that allowed rapid growth. The cell growth decreased during second and third day of exposure to samples, most likely limited with the shortage of nutrients and presence of toxic compounds in the samples, which led to progressive algal cell death (Lau et al., 1996).

Observed growth inhibition could have been caused by various compounds like industrial chemical 4-para-nonylphenol which was detected in all samples in concentrations above 0.33 µg/L. 4-para-nonylphenol was proven to decrease algal growth (He et al., 2016). The highest rate of growth inhibition was found to be in Rugvica and Jesenice samples (approximately 4 and 2.8 times lower compared to the control, after 72 hours of exposure, respectively). At both these samples, high concentrations of bisphenol A were detected, which was already observed to inhibit the growth of algae within the period of five days (Ji et al., 2014). Subsequently, the highest decrease in chlorophyll a concentration was recorded in *Chlorella vulgaris* exposed to same sites (approximately 6.6 and 4.3 times lower compared to the control, respectively).

Two plant model organisms, *Trifolium repens* and *Triticum aestivum,* were chosen to test the sensitivity of monocotyledons and dicotyledons to the water pollution. Another aim of usage of these two model organisms, as they present most commonly used cultivars in Croatia, was to determine possible negative effects on the agricultural activities in the case of floods, such as the cases back in 2010 and 2014 (https://vlada.gov.hr; http://www.voda.hr/). Furthermore, it is important to inspect adverse effects the Sava River water may have on plants in general, since it is used to irrigate certain agricultural areas, such as Bić-Bosut area, through a melioration canal (http://www.voda.hr/).

No significant differences between the control groups and sample groups were noted in germination of *Trifolium repens* and *Triticum aestivum*. However, after exposure to river water samples, a significant difference in length of sprouts of *Trifolium repens* seeds between Lukavec and control group was noticed (approximately 1.3 times lower compared to the control

group). Statistically significant differences were also observed in sprout length of *Triticum aestivum* seeds exposed to samples from Lukavec and Hrušćica (approximately 1.5 and 2 times lower compared to the control group, respectively), as well as root length (approximately 1.3 and 1.4 times lower compared to the control group, respectively). Germination rate of monocotyledons was slightly higher compared to dicotyledons, which is in accordance with other studies focused on herbicide toxicity that confirmed frequently less sensitivity of monocotyledons compared to dicotyledons (Boutin et al., 2004; White and Boutin, 2007). This may be due to differences in uptake (e.g. hairs, nature of waxy cuticle), movement within plants and metabolism (Boutin et al., 2004).

Exposure of zebrafish embryos to water samples from the Sava River resulted in various negative effects, including minor mortality rate, changes in heartbeat rate and minor developmental abnormalities. Unexpectedly, zebrafish mortality rate was not correlated with total concentrations of organic pollutants: the highest mortality rate was observed within embryos exposed to Jankomir sample, while no mortality was noted during exposure to Jesenice. Other samples induced <5% of mortality. This pattern could be caused by the toxicity of contaminants that were not measured, or due to the synergistic effects of organic pollutants (Babić et al., 2017). Embryo coagulation and non-detachment of the tail were most commonly observed causes of death after 24 h of exposure to samples.

The highest abnormality rates were noted in Jankomir and Rugvica samples. Observed developmental abnormalities detected included: developmental retardation, underdeveloped head region and eye, scoliosis, yolk sac and pericardial edema, tail deformation and blood accumulation in the yolk sac, brain and tail region. Both mortality and developmental abnormalities increased in a time-dependent manner.

Considering the variety of compounds detected in tested samples at different concentrations, it is hard for one to speculate the exact cause behind observed endpoints as there are many factors that need to be taken into consideration (metabolites, synergy, bioavailability, pollutants that may not be detected with chemicals analysis). However, multiple other studies have observed the same abnormalities testing chemicals that were found in the highest concentrations in tested samples. Van den Brandhof and Montforts (2010) observed scoliosis, one of most common abnormalities noted in all samples of this study, upon exposure to metoprolol which was detected in high concentrations in all samples. Oliveira et al. (2013) observed that the most frequent malformations due to amoxicillin exposure, also found in high concentrations at all sites except Jesenice, were edemas and tail deformities, which were also frequently noted in this study. Furthermore, Selderslaghs et al. (2009)

observed skeletal deformities as zebrafish response to caffeine, while Parker and Connaughton (2007) noted morphological deformities limited to the body shape in different nicotine treatments.

Decreased heartbeat rate in embryos exposed to all samples was observed. The highest decrease in heartbeat rate was noticed during exposure to Jankomir sample (approximately 1.1, 1.3 and 1.2 times lower at 24, 48 and 72 hpf, compared to the control, respectively), while the sample of Hrušćica caused the most prominent decrease of zebrafish heart rate. No significant differences in hatching rates were noted in comparison to control group at 72 and 96 hpf. Interestingly, significant difference was noted in formation of pigmentation at 48 and 72 hours in embryos exposed to Jesenice and Rugvica sample in comparison to control group. A possible cause for these observations may be that some compounds present in wastewaters can cause lack and/or reduction of pigmentation by inhibiting tyrosinase activity, thus enabling tyrosine to convert into melanin (Babić et al., 2017). Furthermore, increased accumulation of RB in zebrafish embryos confirmed the presence of substances that act as MXR inhibitors in all tested samples. This is in accordance with other studies that showed that some of compounds detected in this study are proven to be chemosensitizers, such as perfluorochemicals and organophosphorus and organochlorine pesticides (Bain and LeBlanc, 1996; Bard, 2000; Epel et al., 2008; Faria et al., 2011; Smital et al., 2004).

Results obtained in this study suggest that there is a broad spectrum of organic contaminants in the Sava river that could potentially have a negative impact on this freshwater ecosystem. This was demonstrated by using a set of ecotoxicological bioassays on different test organisms with various biomarkers. Although this research only included organic pollutants and did not determine any other potentially important groups of contaminants, chemical analysis provided good insight into the general pollution status. Additional use of bioassays with model organisms, along with chemical analysis, took into the account factors such as bioavailability, degradation products and synergy, and therefore offered a useful tool for toxicity assessment.

7 CONCLUSION

Chemical characterization showed that there was a wide range of organic pollutants in water from chosen sampling sites, however, in order to get a more detailed insight in toxic effects they may cause, ecotoxicological bioassays of presumably different sensitivities have been used. This research has showed that our model organisms indeed have different sensitivities to same water samples. Growth inhibition and chlorophyll a concentration in *Chlorella vulgaris* proved to be a cheap, sensitive and rather simple assay, as no special equipment is needed. *Trifolium repens* and *Triticum aestivum*, chosen for better understanding of the sensitivity of monocotyledons and dicotyledons, did not show drastic difference in sensitivity in comparison to each other, other than slightly lower germination rate of dicotyledons, most likely due to different uptake, movement within the plant, as well as metabolism. Root and/or sprout elongation was observed to have lowest rates after exposure to same samples in both model organisms. Exposing the zebrafish embryos to samples resulted in minor mortality rate and sublethal effects, while MXR transporter activity was inhibited in all samples at different rates.

Therefore, the importance of detailed screening of water quality of the Sava river is more than evident, due to its role in the drinking water supply and fishing activities.

8 LITERATURE

Ahrens L, Bundschuh M (2014). Fate and effects of poly- and perfluoroalkyl substances in the aquatic environment: A review. *Environmental Toxicology and Chemistry* **33**: 1921–1929.

Alexander HC, Dill DC, Smith LW, Guiney PD, Dorn P (1988). Bisphenol a: Acute aquatic toxicity. *Environmental Toxicology and Chemistry* **7**: 19–26.

Ansari S, Ansari A, Ansari BA (2015). Effects of Heavy Metals on the Embryo and Larvae of Zebrafish, *Danio rerio* (*Cyprinidae*). *Scholars Academic Journal of Biosciences (SAJB* **3**: 52–56.

Antonijevic M, Arsovic M, Cáslavský J, Cvetkovic V, Dabic P, Franko M, Ilic G, Ivanovic M, Ivanovic N, Kosovac M, Medic D, Najdanovic S, Nikolic M, Novakovic J, Radovanovic T, Ranic D, Sajatovic B, Spijunovic G, Stankov I, et al. (2014). Actual contamination of the Danube and Sava rivers at Belgrade (2013). *Journal of the Serbian Chemical Society* **79**: 1169–1184.

Asharani P V., lianwu Y, Gong Z, Valiyaveettil S (2011). Comparison of the toxicity of silver, gold and platinum nanoparticles in developing zebrafish embryos. *Nanotoxicology* **5**: 43–54.

Babić S, Barišić J, Stipaničev D, Repec S, Lovrić M, Malev O, Martinović-Weigelt D, Čož-Rakovac R, Klobučar G (2018). Assessment of river sediment toxicity: Combining empirical zebrafish embryotoxicity testing with in silico toxicity characterization. *Science of The Total Environment* **643**: 435–450.

Babić S, Barišić J, Višić H, Sauerborn Klobučar R, Topić Popović N, Strunjak-Perović I, Čož-Rakovac R, Klobučar G (2017). Embryotoxic and genotoxic effects of sewage effluents in zebrafish embryo using multiple endpoint testing. *Water Research* **115**: 9–21.

Babić S, Strecker R, Klobučar RS, Čož-Rakovac R (2015). Correlation between MXR inhibitors in wastewater and zebrafish embryotoxicity. at <http://bib.irb.hr/prikazi-rad?rad=776681>.

Bain LJ, LeBlanc GA (1996). Interaction of structurally diverse pesticides with the human MDR1 gene product P-glycoprotein. *Toxicology and Applied Pharmacology* **141**: 288–298.

Bard S (2000). Multixenobiotic resistance as a cellular defense mechanism in aquatic organisms. *Aquatic Toxicology* **48**: 357–389.

Beekhuijzen M, Koning C de, Flores-Guillén M-E, Vries-Buitenweg S de, Tobor-Kaplon M, Waart B van de, Emmen H (2015). From cutting edge to guideline: A first step in harmonization of the zebrafish embryotoxicity test (ZET) by describing the most optimal test conditions and morphology scoring system. *Reproductive Toxicology* **56**: 64–76.

Bielen A, Šimatović A, Kosić-Vukšić J, Senta I, Ahel M, Babić S, Jurina T, González Plaza JJ, Milaković M, Udiković-Kolić N (2017). Negative environmental impacts of antibiotic-contaminated effluents from pharmaceutical industries. *Water Research* **126**: 79–87.

Blok C, Kreij C De, Baas R, Wever G (2008). Analytical methods used in soilless cultivation. *Soilless Culture* 245–289doi:10.1016/B978-044452975-6.50009-5.

Bonefeld-Jørgensen EC, Long M, Hofmeister M V., Vinggaard AM (2007). Endocrine-Disrupting Potential of Bisphenol A, Bisphenol A Dimethacrylate, 4-n-Nonylphenol, and 4-n-Octylphenol in Vitro: New Data and a Brief Review. *Environmental Health Perspectives* **115**: 69–76.

Bossi R, Strand J, Sortkjær O, Larsen MM (2008). Perfluoroalkyl compounds in Danish wastewater treatment plants and aquatic environments. *Environment International* **34**: 443–450.

Bound JP, Voulvoulis N (2004). Pharmaceuticals in the aquatic environment—a comparison of risk assessment strategies. *Chemosphere* **56**: 1143–1155.

Boutin C, Elmegaard N, Kjær C (2004). Toxicity Testing of Fifteen Non-Crop Plant Species with Six Herbicides in a Greenhouse Experiment: Implications for Risk Assessment. *Ecotoxicology* **13**: 349–369.

Brandhof E-J van den, Montforts M (2010). Fish embryo toxicity of carbamazepine, diclofenac and metoprolol. *Ecotoxicology and Environmental Safety* **73**: 1862–1866.

Bratkovics S, Sapozhnikova Y (2011). Determination of seven commonly used organic UV filters in fresh and saline waters by liquid chromatography-tandem mass spectrometry. *Analytical Methods* **3**: 2943.

Braunbeck T, Kais B, Lammer E, Otte J, Schneider K, Stengel D, Strecker R (2015). The fish embryo test (FET): origin, applications, and future. *Environmental Science and Pollution Research **22**:* 16247–16261.

Brooks BW, Kevin Chambliss C, Stanley JK, Ramirez A, Banks KE, Johnson RD, Lewis RJ (2005). Determination of select antidepressants in fish from an effluent-dominated stream. *Environmental Toxicology and Chemistry* **24**: 464.

Buerge IJ, Kahle M, Buser H-R, Müller MD, Poiger T (2008). Nicotine Derivatives in Wastewater and Surface Waters: Application as Chemical Markers for Domestic Wastewater. *Environmental Science & Technology* **42**: 6354–6360.

Carlsson G, Norrgren L (2004). Synthetic Musk Toxicity to Early Life Stages of Zebrafish (*Danio rerio*). *Archives of Environmental Contamination and Toxicology* **46**: 102–105.

Christen V, Hickmann S, Rechenberg B, Fent K (2010). Highly active human pharmaceuticals in aquatic systems: A concept for their identification based on their mode of action. *Aquatic Toxicology* **96**: 167–181.

Cooney JD (1995). Freshwater Tests. In: Rand GM (ed) Fundamentals Of Aquatic Toxicology: Effects, Environmental Fate And Risk Assessment. Taylor & Francis, New York: 71

Eggen RIL, Behra R, Burkhardt-Holm P, Escher BI, Schweigert N (2004). Peer Reviewed: Challenges in Ecotoxicology. *Environmental Science & Technology* **38**: 58A–64A.

Embry MR, Belanger SE, Braunbeck TA, Galay-Burgos M, Halder M, Hinton DE, Léonard MA, Lillicrap A, Norberg-King T, Whale G (2010). The fish embryo toxicity test as an animal alternative method in hazard and risk assessment and scientific research. *Aquatic Toxicology* **97**: 79–87.

Epel D, Luckenbach T, Stevenson CN, MacManus-Spencer LA, Hamdoun A, Smital and T (2008). Efflux Transporters: Newly Appreciated Roles in Protection against Pollutants. *Environmental Science & Technology* **42**: 3914–3920.

Faria M, Navarro A, Luckenbach T, Piña B, Barata C (2011). Characterization of the multixenobiotic resistance (MXR) mechanism in embryos and larvae of the zebra mussel (Dreissena polymorpha) and studies on its role in tolerance to single and mixture combinations of toxicants. *Aquatic Toxicology* **101**: 78–87.

Farooq M, El-Faham A, Ahmad M, Sami AS, Wadaan MA (2012). Teratogenic Profile of Valproic Acid and Newly Synthesized Derivatives in Zebrafish Embryos. *International Journal of Agriculture & Biology Int J Agric Biol* **14**: 894–900.

Fenwick N, Griffin G, Gauthier C (2009). The welfare of animals used in science: How the "Three Rs" ethic guides improvements. *The Canadian Veterinary Journal* **50**: 523.

Fischer S, Klüver N, Burkhardt-Medicke K, Pietsch M, Schmidt A-M, Wellner P, Schirmer K, Luckenbach T (2013). Abcb4 acts as multixenobiotic transporter and active barrier against chemical uptake in zebrafish (*Danio rerio*) embryos. *BMC Biology* **11**: 69.

Fochtman P, Raszka A, Nierzedska E (2000). The use of conventional bioassays, microbiotests, and some "rapid" methods in the selection of an optimal test battery for the assessment of pesticides toxicity. *Environmental Toxicology* **15**: 376–384.

Gatidou G, Thomaidis NS, Stasinakis AS, Lekkas TD (2007). Simultaneous determination of the endocrine disrupting compounds nonylphenol, nonylphenol ethoxylates, triclosan and bisphenol A in wastewater and sewage sludge by gas chromatography–mass spectrometry. *Journal of Chromatography A* **1138**: 32–41.

Guenther K, Heinke V, Thiele B, Kleist E, Prast H, Raecker T (2002). Endocrine Disrupting Nonylphenols Are Ubiquitous in Food. *Environmental Science & Technology* **36**: 1676–1680.

Haffter P, Granato M, Brand M, Mullins MC, Hammerschmidt M, Kane DA, Odenthal J, Eeden FJ van, Jiang YJ, Heisenberg CP, Kelsh RN, Furutani-Seiki M, Vogelsang E, Beuchle D, Schach U, Fabian C, Nüsslein-Volhard C (1996). The identification of genes with unique and essential functions in the development of the zebrafish, *Danio rerio*. *Development (Cambridge, England)* **123**: 1–36.

Hafner C, Gartiser S, Garcia-Käufer M, Schiwy S, Hercher C, Meyer W, Achten C, Larsson M, Engwall M, Keiter S, Hollert H (2015). Investigations on sediment toxicity of German rivers applying a standardized bioassay battery. *Environmental Science and Pollution Research* **22**: 16358–16370.

Halling-Sørensen B, Nors Nielsen S, Lanzky PF, Ingerslev F, Holten Lützhøft HC, Jørgensen SE (1998). Occurrence, fate and effects of pharmaceutical substances in the environment-A review. *Chemosphere* **36**: 357–393.

He N, Sun X, Zhong Y, Sun K, Liu W, Duan S (2016). Removal and Biodegradation of Nonylphenol by Four Freshwater Microalgae. *International Journal of Environmental Research and Public Health* **13**: 1239.

Hernando M, Mezcua M, Fernandezalba A, Barcelo D (2006). Environmental risk assessment of pharmaceutical residues in wastewater effluents, surface waters and sediments. *Talanta* **69**: 334–342.

Hill AJ, Teraoka H, Heideman W, Peterson RE (2005). Zebrafish as a Model Vertebrate for Investigating Chemical Toxicity. *Toxicological Sciences* **86**: 6–19.

Hollert H, Keiter S, König N, Rudolf M, Ulrich M, Braunbeck T (2003). A new sediment contact assay to assess particle-bound pollutants using zebrafish (*Danio rerio*) embryos. *Journal of Soils and Sediments* **3**: 197–207.

Hollert H, Keiter SH (2015). *Danio rerio* as a model in aquatic toxicology and sediment research. *Environmental Science and Pollution Research* **22**: 16243–16246.

Ibáñez M, Sancho J V., Hernández F, McMillan D, Rao R (2008). Rapid non-target screening of organic pollutants in water by ultraperformance liquid chromatography coupled to time-of-light mass spectrometry. *TrAC Trends in Analytical Chemistry* **27**: 481–489.

ISO, 1996. Water quality - Determination of the acute lethal toxicity of substances to a freshwaterfish (*Brachydanio rerio* Hamilton-Buchanan (Teleostei, Cyprinidae)). Parts 1-3.

Ji M-K, Kabra AN, Choi J, Hwang J-H, Kim JR, Abou-Shanab RAI, Oh Y-K, Jeon B-H (2014). Biodegradation of bisphenol A by the freshwater microalgae *Chlamydomonas mexicana* and *Chlorella vulgaris*. *Ecological Engineering* **73**: 260–269.

Jensen AA, Leffers H (2008). Emerging endocrine disrupters: perfluoroalkylated substances. *International Journal of Andrology* **31**: 161–169.

Jones O, Voulvoulis N, Lester J (2002). Aquatic environmental assessment of the top 25 English prescription pharmaceuticals. *Water Research* **36**: 5013–5022.

Källqvist T, Milačič R, Smital T, Thomas K V., Vranes S, Tollefsen K-E (2008). Chronic toxicity of the Sava River (SE Europe) sediments and river water to the algae *Pseudokirchneriella subcapitata*. *Water Research* **42**: 2146–2156.

Kimmel CB, Ballard WW, Kimmel SR, Ullmann B, Schilling TF (1995). Stages of embryonic development of the zebrafish. *Developmental Dynamics* **203**: 253–310.

Kortner TM, Arukwe A (2007). The xenoestrogen, 4-nonylphenol, impaired steroidogenesis in previtellogenic oocyte culture of Atlantic cod (Gadus morhua) by targeting the StAR protein and P450scc expressions. *General and Comparative Endocrinology* **150**: 419–429.

Laetz CA, Baldwin DH, Collier TK, Hebert V, Stark JD, Scholz NL (2009). The Synergistic Toxicity of Pesticide Mixtures: Implications for Risk Assessment and the Conservation of Endangered Pacific Salmon. *Environmental Health Perspectives* **117**: 348–353.

Lau PS, Tam NFY, Wong YS (1996). Wastewater Nutrients Removal by *Chlorella vulgaris*: Optimization Through Acclimation. *Environmental Technology* **17**: 183–189.

Lee Y-M, Seo JS, Kim I-C, Yoon Y-D, Lee J-S (2006). Endocrine disrupting chemicals (bisphenol A, 4-nonylphenol, 4-tert-octylphenol) modulate expression of two distinct cytochrome P450 aromatase genes differently in gender types of the hermaphroditic fish Rivulus marmoratus. *Biochemical and Biophysical Research Communications* **345**: 894–903.

Litwa E, Rzemieniec J, Wnuk A, Lason W, Krzeptowski W, Kajta M (2014). Apoptotic and neurotoxic actions of 4-para-nonylphenol are accompanied by activation of retinoid X receptor and impairment of classical estrogen receptor signaling. *The Journal of Steroid Biochemistry and Molecular Biology* **144**: 334–347.

Livingstone DR, Chipman JK, Lowe DM, Minier C, Pipe RK (2000). Development of biomarkers to detect the effects of organic pollution on aquatic invertebrates: recent molecular, genotoxic, cellular and immunological studies on the common mussel (*Mytilus edulis* L.) and other mytilids. *International Journal of Environment and Pollution* **13**: 56.

Luckenbach T, Fischer S, Sturm A (2014). Current advances on ABC drug transporters in fish. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology* **165**: 28–52.

Mankiewicz-Boczek J, Nałęcz-Jawecki G, Drobniewska A, Kaza M, Sumorok B, Izydorczyk K, Zalewski M, Sawicki J (2008). Application of a microbiotests battery for complete toxicity assessment of rivers. *Ecotoxicology and Environmental Safety* **71**: 830–836.

Milačič R, Zuliani T, Vidmar J, Oprčkal P, Ščančar J (2017). Potentially toxic elements in water and sediments of the Sava River under extreme flow events. *Science of The Total Environment* **605–606**: 894–905.

Mortelé KJ, Oliva M-R, Ondategui S, Ros PR, Silverman SG (2005). Universal Use of Nonionic Iodinated Contrast Medium for CT: Evaluation of Safety in a Large Urban Teaching Hospital. *American Journal of Roentgenology* **184**: 31–34.

Mukherjee A, Chakrabarti J (1997). In vivo cytogenetic studies on mice exposed to acesulfame-K—A non-nutritive sweetener. *Food and Chemical Toxicology* **35**: 1177–1179.

Nyholm N, Källqvist T (1989). Methods for growth inhibition toxicity tests with freshwater algae. *Environmental Toxicology and Chemistry* **8**: 689–703.

OECD (2011). Test No. 201: Freshwater Alga and Cyanobacteria, Growth Inhibition Test, *OECD Guidelines for the Testing of Chemicals, Section 2*, OECD Publishing, Paris, https://doi.org/10.1787/9789264069923-en.

OECD (2013). Test No. 236: Fish Embryo Acute Toxicity (FET) Test. *OECD Guidelines for the Testing of Chemicals, Section 2*, OECD Publishing, Paris, https://doi.org/10.1787/9789264203709-en.

Oliveira R, McDonough S, Ladewig JCL, Soares AMVM, Nogueira AJA, Domingues I (2013). Effects of oxytetracycline and amoxicillin on development and biomarkers activities of zebrafish (*Danio rerio*). *Environmental Toxicology and Pharmacology* **36**: 903–912.

Oropesa AL, Floro AM, Palma P (2017). Toxic potential of the emerging contaminant nicotine to the aquatic ecosystem. *Environmental Science and Pollution Research* **24**: 16605–16616.

Park D, Haldi M., Seng, WL, McGrath, P (2008). Zebrafish: A Predictive Model For Assessing Modulators of P-Glycoprotein Efflux. Presented at abcam, Multidrug Resistance and ABC Transporters.

Parker B, Connaughton VP (2007). Effects of Nicotine on Growth and Development in Larval Zebrafish. *Zebrafish* **4**: 59–68.

Petrie B, Barden R, Kasprzyk-Hordern B (2015). A review on emerging contaminants in wastewaters and the environment: Current knowledge, understudied areas and recommendations for future monitoring. *Water Research* **72**: 3–27.

Popovic R., Dewez D., Juneau P. (2003) Applications of Chlorophyll Fluorescence in Ecotoxicology: Heavy Metals, Herbicides, and Air Pollutants. In: DeEll J.R., Toivonen P.M.A. (eds) Practical Applications of Chlorophyll Fluorescence in Plant Biology. Springer, Boston, MA: 152

Ribas L, Piferrer F (2014). The zebrafish (*Danio rerio*) as a model organism, with emphasis on applications for finfish aquaculture research. *Reviews in Aquaculture* **6**: 209–240.

Rioboo C, González O, Herrero C, Cid A (2002). Physiological response of freshwater microalga (*Chlorella vulgaris*) to triazine and phenylurea herbicides. *Aquatic Toxicology* **59**: 225–235.

Ruyra À, Yazdi A, Espín J, Carné-Sánchez A, Roher N, Lorenzo J, Imaz I, Maspoch D (2015). Synthesis, Culture Medium Stability, and In Vitro and In Vivo Zebrafish Embryo Toxicity of Metal-Organic Framework Nanoparticles. *Chemistry - A European Journal* **21**: 2508–2518.

Sabra F, Mehana E (2015). Pesticides Toxicity in Fish with Particular Reference to Insecticides. *Asian Journal of Agriculture and Food Sciences* **3**: 40–60.

Safi C, Zebib B, Merah O, Pontalier P-Y, Vaca-Garcia C (2014). Morphology, composition, production, processing and applications of *Chlorella vulgaris*: A review. *Renewable and Sustainable Energy Reviews* **35**: 265–278.

Sauerborn Klobučar R, Žaja R, Franjević D, Brozović A, Smital T (2010). Presence of Ecotoxicologically Relevant Pgp and MRP Transcripts and Proteins in Cyprinid Fish. *Archives of Industrial Hygiene and Toxicology* **61**: 175–182.

Sauvant MP, Pepin D, Bohatier J, Groliere CA, Guillot J (1997). Toxicity Assessment of 16 Inorganic Environmental Pollutants by Six Bioassays. *Ecotoxicology and Environmental Safety* **37**: 131–140.

Savino JF, Tanabe LL (1989). Sublethal effects of phenanthrene, nicotine, and pinane on Daphnia pulex. *Bulletin of Environmental Contamination and Toxicology* **42**: 778–784.

Scholz S, Fischer S, Gündel U, Küster E, Luckenbach T, Voelker D (2008). The zebrafish embryo model in environmental risk assessment—applications beyond acute toxicity testing. *Environmental Science and Pollution Research* **15**: 394–404.

Schulze-Sylvester M, Heimann W, Maletz S, Seiler T-B, Brinkmann M, Zielke H, Schulz R, Hollert H (2016). Are sediments a risk? An ecotoxicological assessment of sediments from a quarry pond of the Upper Rhine River. *Journal of Soils and Sediments* **16**: 1069–1080.

Seeland A, Oetken M, Kiss A, Fries E, Oehlmann J (2012). Acute and chronic toxicity of benzotriazoles to aquatic organisms. *Environmental Science and Pollution Research* **19**: 1781–1790.

Selderslaghs IWT, Rompay AR Van, Coen W De, Witters HE (2009). Development of a screening assay to identify teratogenic and embryotoxic chemicals using the zebrafish embryo. *Reproductive Toxicology* **28**: 308–320.

Serra-Maia R, Bernard O, Gonçalves A, Bensalem S, Lopes F (2016). Influence of temperature on *Chlorella vulgaris* growth and mortality rates in a photobioreactor. *Algal Research* **18**: 352–359.

Sharma, BK (2005). Water Pollution. Krisha Prakashan Media, Meerut.

Silva A, Figueiredo SA, Sales MG, Delerue-Matos C (2009). Ecotoxicity tests using the green algae *Chlorella vulgaris*—A useful tool in hazardous effluents management. *Journal of Hazardous Materials* **167**: 179–185.

Smital T, Ahel M (2015). Ecotoxicological Characterization of the Sava River: Biomarker Responses and Biological Assays. 177–200doi:10.1007/978-3-662-44034-6_8.

Smital T, Luckenbach T, Sauerborn R, Hamdoun AM, Vega RL, Epel D (2004). Emerging contaminants—pesticides, PPCPs, microbial degradation products and natural substances as inhibitors of multixenobiotic defense in aquatic organisms. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis* **552**: 101–117.

Soares A, Guieysse B, Jefferson B, Cartmell E, Lester JN (2008). Nonylphenol in the environment: A critical review on occurrence, fate, toxicity and treatment in wastewaters. *Environment International* **34**: 1033–1049.

Spence R, Gerlach G, Lawrence C, Smith C (2007). The behaviour and ecology of the zebrafish, *Danio rerio*. *Biological Reviews* **83**: 13–34.

Stipaničev D, Dragun Z, Repec S, Rebok K, Jordanova M (2017). Broad spectrum screening of 463 organic contaminants in rivers in Macedonia. *Ecotoxicology and Environmental Safety* **135**: 48–59.

Ščančar J, Heath E, Zuliani T, Horvat M, Kotnik J, Perko S, Milačič R (2015). Elements and Persistent Organic Pollutants in the Sediments of the Sava River. 95–121doi:10.1007/978-3-662-44034-6_5.

Takayanagi S, Tokunaga T, Liu X, Okada H, Matsushima A, Shimohigashi Y (2006). Endocrine disruptor bisphenol A strongly binds to human estrogen-related receptor γ (ERRγ) with high constitutive activity. *Toxicology Letters* **167**: 95–105.

Topić Popović N, Strunjak-Perović I, Klobučar RS, Barišić J, Babić S, Jadan M, Kepec S, Kazazić SP, Matijatko V, Beer Ljubić B, Car I, Repec S, Stipaničev D, Klobučar GIV, Čož-Rakovac R (2015). Impact of treated wastewater on organismic biosensors at various levels of biological organization. *Science of The Total Environment* **538**: 23–37.

Tuikka AI, Schmitt C, Höss S, Bandow N, Ohe PC von der, Zwart D de, Deckere E de, Streck G, Mothes S, Hattum B van, Kocan A, Brix R, Brack W, Barceló D, Sormunen AJ, Kukkonen JVK (2011). Toxicity assessment of sediments from three European river basins using a sediment contact test battery. *Ecotoxicology and Environmental Safety* **74**: 123–131.

Ulhaq M, Carlsson G, Örn S, Norrgren L (2013). Comparison of developmental toxicity of seven perfluoroalkyl acids to zebrafish embryos. *Environmental Toxicology and Pharmacology* **36**: 423–426.

Visioli G, Conti FD, Gardi C, Menta C (2014). Germination and Root Elongation Bioassays in Six Different Plant Species for Testing Ni Contamination in Soil. *Bulletin of Environmental Contamination and Toxicology* **92**: 490–496.

Wang W, Freemark K (1995). The use of plants for environmental monitoring and assessment. *Ecotoxicology and environmental safety* **30**: 289–301.

Wang W, Keturi PH (1990). Comparative seed germination tests using ten plant species for toxicity assessment of a metal engraving effluent sample. *Water, Air, and Soil Pollution* **52**: 369–376.

White AL, Boutin C (2007). Herbicidal effects on nontarget vegetation: investigating the limitations of current pesticide registration guidelines. *Environmental Toxicology and Chemistry* **26**: 2634–2643.

Zhang Y, Piao Y, Li Y, Song M, Tang P, Li C (2013). 4-Nitrophenol induces Leydig cells hyperplasia, which may contribute to the differential modulation of the androgen receptor

and estrogen receptor-α and -β expression in male rat testes. *Toxicology Letters* **223**: 228–235.

Znad H, Ketife AM. Al, Judd S, AlMomani F, Vuthaluru HB (2018). Bioremediation and nutrient removal from wastewater by *Chlorella vulgaris*. *Ecological Engineering* **110**: 1–7.

***Web sources*:**

http://www.agroatlas.ru/en/content/cultural/Triticum_aestivum_winter_K/ [accessed July 31, 2018]

https://botany.natur.cuni.cz/algo/CAUP/H1998_Chlorella_vulgaris.htm [accessed July 30, 2018]

http://www.e-herbar.net/main.php?g2_itemId=4321 [accessed July 30, 2018]

http://eol.org/pages/1115240/overview [accessed July 31, 2018]

http://eol.org/pages/703436/details [accessed July 31, 2018]

http://fsi.colostate.edu/flour-draft/ [accessed 07, August 2018]

http://halmed.hr/en/Novosti-i-edukacije/Publikacije-i-izvjesca/Izvjesca-o-potrosnji-lijekova [accessed 21, August 2018]

http://www.pharmacistspharmajournal.org/2010/11/definitions-of-drug-radioactive-drug_11.html#.W8XeLGgzbIU [accessed 16, October 2018]

https://plants.sc.egov.usda.gov/core/profile?symbol=TRAE [accessed 07, August 2018]

https://www.ufz.de/index.php?en=35645 [accessed August 01, 2018]

https://vlada.gov.hr/sjednice/82-sjednica-vlade-republike-hrvatske/955 [accessed October 20, 2018]

http://www.voda.hr/hr/novosti/cetiri-godine-nakon-poplava-u-istocnoj-hrvatskoj [accessed October 20, 2018]

http://www.voda.hr/hr/novosti/hrvatske-vode-obiljezile-140-godina-organiziranog-upravljanja-vodama-u-hrvatskoj [accessed October 20, 2018]

http://vro.agriculture.vic.gov.au/dpi/vro/vrosite.nsf/pages/sip_white_clover [accessed July 31, 2018]

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