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Biological effects of anthropogenic pollutants in recipients of treated sewage water

Biologický vliv antropogenních polutantů přítomných v recipientech komunálních odpadních vod

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CONTENT

CHAPTER 1

GENERAL INTRODUCTION

Anthropogenic pollutants present in sewage treatment plant (STP) effluents negatively affect aquatic organisms, including fish and other microorganisms, and consequently can affect the aquatic ecosystem as a result of disruption in fish reproduction and food chain relationships in the aquatic environment. In this thesis, the effects of STP effluent discharge water on aquatic ecosystems was investigated in two common effluent recipients: (1) a biological pond (with static water) and (2) a small stream (with running water) - under real conditions using the two native fish species (common carp and brown trout).

1.1. SEWAGE TREATMENT PLANT EFFLUENT DISCHARGE – A RISK FOR AQUATIC RECIPIENTS?

Rapid development of highly sensitive and automated analytical instruments have made possible to discover an enormous number of substances in the aquatic environment, which were previously undetectable. Many of those substances were proven to have originated from STP's effluents. They typically occur in trace concentrations but pose risks to aquatic organisms due to their potent bioactivity. Recently, STP effluents have been considered as the common source of anthropogenic pollutants entering aquatic environments. STP effluents have been shown to contain a wide range of chemicals, including pharmaceuticals and personal care products (PPCPs), pesticides, solvents, heavy metals, and other contaminants that originate from household, commerce, and industrial sources (Al-Musharafi et al., 2013; Campo et al., 2013; Yang et al., 2017). In general, those pollutants are present in STP effluents at low concentrations (from ng/l to μg/l), which is mostly below the threshold toxicity level for aquatic organisms (Ebele et al., 2017; Kim and Farnazo, 2017; Petrie et al., 2015). However, concern is growing over STP effluent contamination because of its constant discharge and the risk of toxic effect combinations. STP effluent discharge has major detrimental effects on the aquatic ecosystems, including disturbance in the nutrient balance of recipients in addition to the health of aquatic organisms at both individual and population levels (Diniz et al., 2005; Drury et al., 2013). The increased nutrient load can lead to eutrophication (Björn Gücker, 2006) and temporary oxygen deficits (Rueda et al., 2002) and alter energy flow/ energy relationships in the stream. Contamination can directly or indirectly impact aquatic organisms and then disrupt biotic community structures and functions. It has been shown that STP effluent discharge disturbed benthic invertebrate (Ginebreda et al., 2010; Marcogliese et al., 2015; Munoz et al., 2009), benthic bacteria consortia (Drury et al., 2013; Wakelin et al., 2008) and fish (Liney et al., 2006) living in recipients receiving effluent water. The concentration of contaminants in STP-affected aquatic environment significantly depends on the concentration's dilution factor. Therefore, the effect of STP effluent discharge on aquatic environment depends on the proportion of discharged water in the recipient. This indicates that the smaller receiving rivers/streams have higher toxicity risk for aquatic organism. In the central European scenario, STP effluent is commonly discharged directly into a stream/river or through a biological pond. The effect of STP effluent discharge on the aquatic organisms in these two systems may be different due to dilution and hydrological factors. The effects of STP effluent on aquatic ecosystems of a receiving recipient (river) has been recent subject of attention (Gillis et al., 2017; Hu et al., 2018; Kalčíková et al., 2017). However, to the best of our knowledge, less information is available about the effects of STP effluent on the aquatic environment of a small stream or biological pond.

In order to understand the effects of STP effluents on the aquatic ecosystem, it is important to understand the pollution type and levels. The presence of contaminants in STP effluents have been studied over the course of the last few decades. However, this is still a hot topic in aquatic ecotoxicology. Recently, a list of common contaminants in STP effluents was expanded

by the addition of new contaminants, especially the emerging contaminant group (Blum et al., 2018; Vidal-Dorsch et al., 2012). The investigation of pollutants found in STP effluents not only depends on analytical instruments but also on the sampling method, especially, for investigation of polar compounds such as pharmaceuticals and pesticides (Bolong et al., 2009; Söderström et al., 2009). As practice has shown, identification of all substances dissolved in water is difficult due to low concentration levels. The classical monitoring strategies have some disadvantages; for example, simple water grab may give relatively high detection limits due to the low amount of water sample. Therefore passive sampling is a promising innovative monitoring tool for time-integrated identification of bioavailable substances in water and sediment (Martinez Bueno et al. 2009). Polar organic chemical integrative sampler (POCIS) devices are widely used as monitoring tools for priority and emerging pollutants in water (Togola and Budzinski, 2007). These devices are reliable, robust, and cost-effective and can be used in field monitoring programs. A further advantage of POCIS is the ability to mimic biological uptake precluding the use of aquatic organisms for biomonitoring (Alvarez et al. 2005; Kot et al. 2000).

Previous studies have indicated the deleterious effects of different contaminating groups such as heavy metals (Runck, 2007), pesticides (Beketov, 2013), and organic chlorine compounds (PCB) (Wang et al., 2007) present in STP effluent on aquatic organisms. However, less is understood about effect of PPCPs on fish. As an important group among emerging environmental contaminants, concerns about PPCPs have been raised in the scientific community. PPCPs are highly consumed in the modern society, especially in industrialized countries. Their residues are carried to STPs and end up in the aquatic environment via discharge of the treated effluents to surface waters. Pharmaceuticals are different from other emerging contaminants because they have been designed to be biologically active, and their biological activities may persist even after they have been eliminated from the target organism. Biological effects of PPCPs and other pollutants may differ greatly between species. These differences are highly dependent on exposure time, uptake routes, metabolism following uptake, rates of accumulation, and target organ sensitivity (Logan, 2007; Norrgren, 2012). Chronic and subtle effects are expected in cases in which aquatic organisms are exposed long-term to pseudo-persistent, persistent, and accumulative PPCPs. However, it is difficult to predict the effects because pharmacological actions on nontarget organisms are not commonly known. PPCPs are ordinarily detected in STP effluents as a complex mixture of compounds with diverse chemical structures, properties, persistence, specificities, and biological activities (Fent et al., 2006; Lopez-Serna et al., 2012). There are concerns regarding the capability of PPCPs mixture to induce endocrine disruption and genetic mutations in aquatic animals (Anway et al., 2005). It is challenging to assess the effects of a complex mixture of PPCPs (Rajapakse et al., 2004). Effects of mixtures cannot be calculated by simply adding the effects of the mixture components when applied alone, especially if the components have differently shaped dose-response curves (Payne et al., 2000). For example, estrogenic substances concentrations are rather unlikely to cause estrogenic effects with the exception of 17α-ethinylestradiol (EE2) and 17β-estradiol (E2). However, when acting together they can pose a hazard, which may be underestimated by focusing on individual compounds alone (Sumpter and Johnson, 2005). The joint ecotoxicity of such chemical cocktails is typically higher than the toxicity of each individual compound (Kortenkamp, 2009). In particular, even if the compounds of a mixture are present only below their respective toxicity threshold, a joint toxic effect cannot be ruled out. Hence, ignoring possible mixture effects might cause an underestimation of the actual impact of PPCPs on the environment; these effects would depend on the number of compounds involved in addition to their concentrations and ecotoxicological profiles.

At least three experimental approaches have been applied to investigate the effects induced by water discharged from STP effluent: (1) laboratory *in vivo* experiments that simulate native environmental conditions (Daouk et al., 2011; Liney et al., 2006), (2), *in situ* cage studies (Bernet et al., 2000; Liu et al., 2015; Vincze et al., 2015); and (3) *in situ* field experiments (Bruneau et al., 2016). The advantage of *in situ* experiments over the other methods is their greater relevance to native situations, which can mimic the actual effects of the contamination scenario on the native environment. In cases of fish caging experiments, the studies may have disadvantages, which can lead to fish stress and underestimation of bioaccumulation and biomagnification factors due to restricted natural nutrition and interactions with other vertebrates. The method of randomly collecting fish from up and downstream sites may reflect more native conditions (Bruneau et al., 2016). However, the disadvantage of this method occurs when compared fish may be not in the same initial conditions as they were prior to the start of the experiment. Moreover, there is also a risk of obtaining fish that have migrated from other sites. Therefore, it is necessary to find a holistic approach that can overcome the above-mentioned disadvantages in order to reflect the effects of STP effluent on the existing native conditions.

1.2. ECOTOXICOLOGY AND BIOMARKERS

STP effluent discharge water contains a wide range of pollutants which may impact aquatic ecosystem at all trophic levels. Fish is an important organism in aquatic ecosystem. Common carp and brown trout have been commonly used as model organisms in ecotoxicological studies (Bolis et al., 2001; Carvan, 2007; García-Medina et al., 2017). Several studies have proven that these fish are suitable for investigation PPCP effects in aquatic environments because of their sensitivity to contaminants, well-established study methods and biophysical responses (Bolis et al., 2001; Brown et al., 2014). Moreover, both carp and trout are widespread freshwater species native to the investigated area. Therefore, carp and trout were chosen as representatives for fish living in static water and running water, respectively. Physical and biochemical changes in fish's organs have been reported to be linked to the changes in aquatic environment. Thus, the liver is an important target organ related to important metabolic and detoxification mechanisms (Ayas et al., 2007). Gills are in permanent contact with water and represent an important target organ for any pollutants dissolved in the surrounding water. This organ is essential for gas exchange and osmotic regulation. The gonads have an important role in reproduction, which can be affected by both pollutants and nutritional factors. Other organs such as kidney, intestine, and muscle are frequently studied in ecotoxicology. Several studies have reported the effects of STP effluent water on fish health, have indicated biochemical and physical disturbance in blood, gills, liver, and muscle (Bernet et al., 2000; Bruneau et al., 2016; Grabicova et al., 2014).

Pollutants present in STP effluents are usually at low environmental concentrations but have a wide range of actions. It is important to select a relevant and suitable set of biomarkers that can reflect the presence of the pollutants. On the other hand, the selected biomarkers should reflect both subtle and evident changes.

In next paragraphs, several of the most relevant biomarkers for field studies are described.

1.2.1. Morphological bioindicators

Morphological indices, including condition factor (CF), hepatosomatic index (HSI), and gonadsomatic index (GSI) are indicatives of metabolic fish conditions that may provide information about water quality (Shibatta, 2005) and have been proposed as index of exposure

to environmental contaminants (Whyte et al., 2000). CF is an indicator of the overall fish condition that reflects fish shape and energy reserves and often is used to evaluate fish stress (Lohner et al., 2001). CF, which assumes that heavier fish of a given length demonstrate better physiological conditions, is capable of indicating fish fitness under pollution-induced stress as a metabolic trade-off is required to deal with detoxification; thus the energy available for growth may be reduced (Fang et al., 2009). Some reports have demonstrated that CF declined in fish exposed to environmental pollutants such as copper, petroleum, and pharmaceuticals (Khan, 2003; Li et al., 2011a; Roussel et al., 2007). HSI reflects relative liver size and is linked to the hepatic detoxification-related enzyme activities, which indicate exposure to pollutants (Li et al., 2010b; Yeom et al., 2007). The HSI is associated with detoxification activities in response to the presence of toxic compounds. This index can increase or decrease in the presence of such compound (de Souza Pereira and Kuch, 2005; Kopecka-Pilarczyk and Correia, 2009; Pereira et al., 1993; Liu et al., 2015). The GSI has been widely used as a biomarker in aquatic organisms for exposure to estrogenic environments. Correlations have been established in male fish between the inhibition of testicular growth and the potency of estrogenic compounds (Gimeno et al., 1997; Li et al., 2009).

1.2.2. Blood hematological and biochemical parameters

Hematological and biochemical profiles of blood can provide important information about the internal environment of an organism. The evaluation of hematological and biochemical characteristics in fish has become an important means of understanding normal and pathological processes and toxicological impacts of certain compounds (Borges et al., 2007; Sudova et al., 2009; Velisek et al., 2011). Hematological indices such as: the erythrocyte count (RBC), haemoglobin (Hb), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), leukocyte count (Leuko), and differential leukocyte count in addition to biochemical indices, including glucose (GLU), total protein (TP), triacylglycerols (TRIG), alanine aminotransferase (ALT), creatine kinase (CK), aspartate aminotransferase (AST), and lactate (LAC) are widely used to investigate the state of the fishes' health.

Fish hematological blood parameters are widely used in toxicological research for the evaluation of the immune toxic effects of pharmaceuticals (Burkina et al., 2016; Li et al., 2011b; Steinbach et al., 2014; Steinbach et al., 2016). The reduction of PCV, Hb, RBC, and Leuko count (Li et al., 2011a) and elevation of MCH, and MCV (Saravanan et al., 2011) were observed in fish exposed to a single pharmaceutical compound. Long-term exposure to verapamil resulted in increase in levels of GLU, ammonia (NH_3), TP, LAC, and LDH, ALT, and AST activities (Li et al., 2011a). Only a small amount of information about the effects of PPCP mixtures is available.

The changes in blood parameters can be linked to alterations in the internal environment that are induced by pollution. Decreases in Hb concentration, RBC count, and PCV levels have been suggested as indicators of anemia, while changes in differential leukocyte count have been recognized as sensitive indicators of environmental stress (Li et al., 2011a). Elevated levels of NH_3 concentration in plasma can indicate that detoxifying mechanisms were unable to convert the toxic ammonia to less harmful substances. While elevated blood glucose level indicates metabolic stress (Li et al., 2010a). Decreases in the LAC of exposed fish may indicate a decrease in glycolytic processes due to lower metabolic rates. LDH is a tetrameric enzyme that has been recognized as a potential marker for assessing a chemical's toxicity. Activities of the plasma LDH enzyme and the transaminases (ALT and AST) have been shown to be relevant stress indicators (Ishikawa et al., 2007). Elevated LDH levels in haemolymphs might be due

to the release of isozymes from the destroyed tissues (Mishra and Shukla, 2003), while an increase in ALT and AST activities indicate amplified transamination processes.

Many anthropogenic pollutants found in STP effluents have been shown to have effects on the immune system (Liney at al., 2006; Vos, 1977). These pollutants may directly or indirectly induce immunosuppression and therefore cause aquatic organisms to become susceptible to disease. Many chemical pollutants that are capable of suppressing the human immune system have been found to modify fish immune responses via similar mechanisms to those found in humans (Galloway and Handy, 2003). This phenomenon not only occurs as a result of actions of xenobiotics such as PAHs, pesticides, aromatic amines, heavy metals, solvents but also with endogenous hormones, which can indirectly suppress immune functions (Zelikoff et al., 1994). STP effluents have been shown to significantly suppress the immune system in goldfish (*Carassius auratus*) (Kakuta and Murachi, 1997). The total number and lymphocyte and granulocyte functions were found to have decreased which may have increased the fish's susceptibility to infection (Kakuta and Murachi, 1997). Secombes et al. (1991) found that the effects of STP effluent on the immune system of dab (*Limanda limanda*) caused a decrease in the thrombocyte circulation after 12 weeks of exposure. It has been suggested that different species may exhibit different susceptibilities for immune suppressive under effects of STPs (Barber et al., 2006). The fish's life stage may be more important than the concentration and duration of exposure with respect to immunosuppressive effects (Milston et al., 2003).

1.2.3. Oxidative stress and antioxidant enzymes

Oxidative stress is defined as a condition in which production of reactive oxygen species (ROS) is greater than antioxidant production, leading to potential oxidative damage to cellular molecules (Sies, 1997). Exposure to anthropogenic compounds can increase ROS production within a cell. A well-studied example of non-enzymatic antioxidant defense is the glutathione (GSH)-based system. GSH provides reducing equivalents for enzymes such as glutathione peroxidase (GPx) so that it can function properly in reducing lipid peroxides, hence preventing oxidation-chain reactions. The reduced form of GSH becomes oxidized, and the ratio between the oxidized forms of glutathione (GSSG) and reduced GSH levels is an important factor in the redox balance of a cell in addition to having a role in cell signal transduction pathways (Sies, 1999). GSH levels in the cell are maintained by a set of enzymes, including glutathione reductase (GR), g-glutamylcysteine synthetase (GCS), and glutathione synthetase (GS) (Seelig and Meister, 1985). Other examples of antioxidant enzymes include superoxide dismutase (SOD), which reduces the superoxide anion, and catalase (CAT), which reduces hydrogen peroxide to water and molecular oxygen. Glutathione S-transferase (GST) is another antioxidant enzyme, which is involved in both harmful electrophilic endogenous and exogenous compound detoxification. By conjugating glutathione with toxic electrophilic substrates, the resulting molecules generally become less reactive and more soluble, thus facilitating their excretion from cells and the organism. In protein molecules, methylation is widely used as a technique to investigate oxidative damage.

Measurements of antioxidant defence systems, both enzymatic and molecular, have been used in field and laboratory studies (Stephensen et al., 2002; Sturve et al., 2005) in order to study the effects of pro-oxidants in fish. In recent years, measurement of oxidative damage products in aquatic organisms has received more attention (Almroth et al., 2005; McDonagh et al., 2005). It has been suggested that it is important to incorporate measurements of antioxidant defences in addition to oxidative damage in order to gain a complete understanding of xenobitics' effects on exposed fish (Carney Almroth et al., 2008; Regoli et al., 2002). Anthropogenic pollutants may be harmful to organisms because of their ability to

form reactive oxygen species (ROS) and cause oxidative stress. Many of these pollutants have been documented to induce oxidative stress in fish and have ability to cause sub-lethal effects such as oxidative damage to fish proteins and lipids (Livingstone, 2001). Sturve et al. (2008) indicated that STP effluents contain pro-oxidants that affect fish. This induced consistent oxidative damage and elevation of GR and CAT in rainbow trout after five days of exposure. Additionally, lipid peroxides were found to have increased in fish caged at the STP site (Carney Almroth et al., 2008). Even oxidative stress parameters in field toxicological studies have been widely known to be related to antioxidant defenses systems, which consist of low molecular weight scavengers and antioxidant enzymes (Regoli et al., 2002; Valavanidis et al., 2006; van der Oost et al., 2003). However, there are large gaps in researchers' knowledge about how complex mixtures of chemicals affect oxidative stress in aquatic organisms.

1.2.4. Histopathology

Histopathology is considered as a robust and efficient method for detection of acute and chronic adverse effects in fish, which may express the state of the exposed individuals' health and their aquatic ecosystem (Ayas et al., 2007; Miranda et al., 2008). Histological investigations within the fish toxicology field have focused on general pathology including externally visible disease, skin structure, and lesions, necrosis, and apoptosis in addition to inflammatory reactions and tumor incidence (Dick Vethaak, 1992; Wahli et al., 2002). Chemical contaminants can induce lesions in different fish target organs, especially in the liver (Miranda et al., 2008), gills (Benli et al., 2008), heart, and reproductive organs (Steinbach et al., 2014). Histopathological changes in gills may represent adaptive strategies for conservation of some physiological functions or endpoints to evaluate acute or chronic exposure to chemicals present in water and sediment (Tkatcheva et al., 2004). Histopathological analysis can be used to reveal the impact of toxicants on fish as this method can provide direct translation of toxic xenobiotic effects on vital anatomical functions. Histological analysis appears to be a very sensitive parameter and is crucial for determining cellular changes. Histological examination of fish liver could serve as a model for studying the interactions between stress factors, including bio-toxins, parasites, infectious germs, physicochemical parameters, and pollutants. Pathogens produce pathological changes in fish such as necrosis in liver, tubular damage of kidney, and gill lamellar abnormalities. Therefore, histopathological studies are necessary for the description and evaluation of potential lesions in aquatic animals exposed to various infections and toxicants.

1.2.5. Fish endocrine status

STPs' effluent discharges contain a complex mixture of synthetic and biogenic exogenous endocrine-active chemicals (EACs) (Golovko et al., 2018; Johnson et al., 2005; Vega-Morales et al., 2013). Surface waters that are the recipients of STP effluent have been found to have higher-than-normal hormone levels, including steroidal and non-steroidal estrogens (Johnson et al., 2005; Kolpin et al., 2002), androgens (Kolodziej et al., 2003), and PPCPs (such as antidepressants) which can act as endocrine disruptors (Schultz et al., 2010) and UV filters (Wang et al., 2016). EACs can interact directly with the endocrine regulatory systems of exposed organisms, thus leading to the disruption of reproductive development and function in these organisms(McLachlan, 2001). EACs have also been shown to be structurally and functionally identical to endogenous steroid hormones that can destabilize vertebrate endocrine signals when these compounds are added to endogenous hormone loads. Estrogen-contamination (such as EE2) has been documented to cause adverse effects on fish reproduction which may

reduce fecundity, fertility, and intersex and skewed sex ratios at low concentrations (Mills and Chichester, 2005).

Endocrine disruption occurs when EACs interact with internal endocrine signaling pathways in nontarget organisms (Cheek et al., 1998). EACs affect fish development and reproduction by interfering the normal synthesis, storage, release, transport, metabolism, binding, and actions or elimination of endogenous hormones (Kavlock et al., 1996). EACs have the potential to induce endocrine disruption because they are frequently present in the aquatic environment; concurrently, some of them are persistent and show the potential for bioaccumulation (Tyler et al., 1998). EACs may affect the sensitive hormone pathways that regulate reproductive functions, and thus, lead to a reduction in fish egg production and/or fertility (Arcand-Hoy and Benson, 1998). Researchers have used various indicators to investigate EACs' effects on piscine reproductive systems, including an increase in intersexuality in reproductive adults and the presence of the female phospholipoprotein vitellogenin (VTG) in male fish at high concentration in addition to the ability of a chemical to bind to hormone receptors in *in vitro* assays. Fish species vary widely in both their sensitivity to induction of VTG upon exposure to EACs and the magnitude of their response (Jobling et al., 1998; Thompson et al., 2006). Other factors may also contribute to variable vitellogenic responses, including water temperature (Purdom et al., 1994), migratory behavior (Kirby et al., 2004), and prior history and type of EAC exposure (Pait and Nelson, 2003; Panter et al., 2002).

Vajda et al. (2008) found that when white sucker male fish (*Catostomus commersoni*) were exposed to STP effluents, adverse effects on reproductive system including gonadal intersex, reduced sperm abundance, and elevated plasma VTG level were observed. Increases in hepatic *vtg* expression in male fish (4-fold) and high VTG levels in plasma (1.5 mg/ml) were detected in fathead minnow and rainbow trout exposed to STP effluents (Jasinska et al., 2015; Larsson et al., 1999).

1.2.6. Enzyme activities of CYP

Cytochrome P450 (CYP) is a superfamily of hemoproteins. Their roles include cholesterol synthesis and vitamin D metabolism, which are metabolites of potentially toxic compounds. In mammals, pharmaceuticals are mainly metabolized by the CYP superfamily, particularly members of the CYP1/P3 families, which are predominantly expressed in the liver (Hukkanen et al., 2002). CYP1A- and 3A-like CYPs are the most studied fish isoforms in connection with the metabolism of various substances, mainly PPCP, and both isoforms are widely used as biomarkers of fish exposure to environmental contaminations (Guengerich, 1999; Hegelund and Celander, 2003; Lewis, 2004; Nebert and Russell, 2002; Uno et al., 2012).

Recently, CYP1A- and 3A-like CYPs have been widely studied in both laboratory and field toxicological studies (Burkina et al., 2016; Kroon et al., 2017; M. Nilsen et al., 1998). Induction of both hepatic CYP1A and 3A were found in fathead minnows, carp, and sunshine bass exposed to STP effluents (Jasinska et al., 2015; Liu et al., 2015; McArdle et al., 2000).

1.2.7. Fatty acid composition

Recently, fatty acid composition has been used as a biomarker in ecotoxicological studies. Change in fatty acid composition can reflect physical and biochemical changes in fish, which may be linked to environmental changes such as temperature, salinity, diet, and presence of contaminants. It has been proven that occurrence of pollutants in the diet and water environment can impact the fatty acid composition of fish's muscle tissue (Carnevali et al., 2017; Cheng et al. 2016). Lipid-lowering compounds such as bezafibrate and clofibric acid

have been documented to affect fatty acid synthesis (Weston et al., 2009). A decrease in omega-3 and saturated fatty acids were seen in fish fed contaminated diets (Cheng et al., 2016) or exposed to STP effluents (Sakalli et al., 2018). The ratio of n-3/n-6 and total fat content also are indicators for orgnaisms' energy statuses (Arab, 2003; simopoulos, 2003). Moreover, fatty acid composition in fish muscle is also an important indicator of nutritional value for human consumption.

1.2.8. Bacterial consortia

Bacteria are abundant in aquatic environments. Any changes in environmental conditions may result in bacterial composition changes in lotic environments and in aquatic organisms (Menz et al., 2017; Xue et al., 2017). Both healthy and unhealthy fish may contain bacteria. They do not occur only on external organs but also in internal organs of healthy fish, including intestine, liver, kidney, and spleen. Bacteria play an important role in degradation of complex molecules that are potentially beneficial in fish nutrition. They are the indispensable components of fish, which benefit not only nutrition but also other processes needed to maintain normal fish life (Austin, 2002). The composition of the fish's bacterial population (in fish skin, gills, and digestive tracts) correlates with the bacterial community in their aquatic environments (Austin, 2002). It is clear that fish are continuously exposed to bacteria in water and sediment and are influenced by them. Following that, the microflora on external surfaces of fish, including gills, are directly affected by these bacteria. Similarly, the digestive tract, which is the recipient of water and food, is also affected by external microorganisms. Colonization of bacteria in fish may start at an early stage (egg or larval stage) and then continue during the fish's development (Olafsen, 2001). Any changes in the bacterial consortia in the environment due to contamination also indirectly or directly affects the fish, indicating that the fish's bacterial composition can reflect environmental changes. Chronic exposure fish to anthropogenic pollution also results in an increase in the occurrence of bacterial species exhibiting acquired resistance to various veterinary and human antibiotics (Navinier, 2006). There are only a few studies concerning the effects of anthropogenic pollutants on fish through the bacterial consortia. The analyses of fish-associated microorganisms have mainly focused on cultivable bacteria. The employment of high throughput molecular biology methods is promising for yielding a more complex view of the bacterial consortia composition in fish and its dynamics over time and as a function of anthropogenic pollution.

In ecotoxicology, the bacterial community can be used as an indicator for investigating both water quality and fish health. The occurrence of *pleuroscapsa* and *chamaesiphon* are typical in clean water (Loza et al., 2013), while occurrence of some actinobacterium, bacteroidetes are indicators for anthropogenically polluted water (Myung et al., 2015). STP effluent discharges frequently contain a wide range of chemicals, especially antibiotics, which may impact the microorganisms present in aquatic environment and fish´s organs (Menz et al., 2017; Roberts et al., 2016; Xu et al., 2015). It is well known that STP effluents can change the bacterial consortia in the recipient. However, the effects of these changes on fish is not well understood.

1.2.9. Integrated biomarker response

In ecotoxicology, it is necessary to investigate both a wide range and suitable set of biomarkers (Forbes et al., 2006). Recently, several biomarkers have been used to investigate fish health. However, each biomarker may have a different response to environmental changes. Therefore, sometimes it is challenging to draw a clear conclusion based on biomarker response. To overcome this issue, the integrated biomarker response (IBR) index has been suggested

as a useful tool to yield a general view of this index effect in ecotoxicological studies (Beliaeff and Burgeot, 2002). This method can be used to summarize the biomarker responses and visualize the results using a star plot. The IBR value can then be computed as the star plot area. The improved version (IBR version 2 - IBRv2) was introduced by Sanchez et al. in 2013. Eventually, IBRv2 was recommended for data visualization of upstream/downstream investigations. The IBRv2 is based on the reference deviation concept. The advantage of this method over IBR is clearance of sampling site discrimination and visualization of both up- and down-regulation-associated responses. Currently, IBR and IBRv2 have been frequently used in aquatic ecotoxicology (Broeg and Lehtonen, 2006; Li et al., 2011a). A combination of IBR and statistical methods can be used as an aid in order to derive the main conclusions.

The main aim of present thesis was to investigate the effects of anthropogenic pollutants discharged from STP effluents on aquatic ecosystems. A unique experimental set up was applied in order to overcome the disadvantages of classical methods. In this setup, native fish of the same origin were tagged and exposed to STP-affected and control sites under real conditions. The undesirable factors that induce fish stress and consequently mislead the experimental data evaluation (such as those events that happen in laboratory or caging experiments) were minimized. Two water systems that represent common recipients of STP effluents in a European scenario were selected for investigation:

- Running water system: a small stream (Zivny Stream) that receives 25% of its water from STP effluents
- Static water system: a biological pond (Cezarka Pond) that receives 100% of its water from STP effluents

The level of pollutants in STP effluent recipients was investigated with special focus on PPCPs using POCISs.

The biological effects of STP effluents were investigated in fish using a wide set of biomarkers and by evaluation of lotic microbiota.

The results of these two experiments are presented in PAPERS I and II in the subsequent chapters.

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CHAPTER 2

BIOLOGICAL EFFECTS OF STP EFFLUENT ON COMMON CARP LIVING IN A BIOLOGICAL POND

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Effects of Multi-Component Mixtures from Sewage Treatment Plant Effluent on Common Carp (*Cyprinus carpio*) under Fully Realistic Condition

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Abstract This study characterized changes in biomarker responses in common carp (*Cyprinus carpio*) upon exposure to effluent water discharged from a sewage treatment plant (STP) under real conditions. Fish were exposed to contamination in Cezarka pond, which receives all of its water input from the STP in the town of Vodnany, Czech Republic. Five sampling events were performed at day 0, 30, 90, 180, and 360 starting in April 2015. In total, 62 pharmaceutical and personal care products (PPCPs) were detected in the polar organic chemical integrative sampler. Compared to a control pond, the total concentration of PPCPs was 45, 16, 7, and 7 times higher in Cezarka pond at day 30, 90, 180, and 360, respectively. The result of oxidative stress and antioxidant enzyme biomarkers indicated alterations in the liver and intestine tissues of fish from Cezarka pond at day 30 and 360, respectively. High plasma vitellogenin levels were observed in both exposed females (180 and 360 days) and males (360 days) compared with their respective controls. However, only exposed female fish had higher vitellogenin mRNA expression than the control fish in these periods. Exposed female fish showed irregular structure of the ovary with scattered oocytes, which further developed to a vitellogenic stage at day 360. Low white blood cell levels were indicated in all exposed fish. Despite numerous alterations in exposed fish, favorable ecological conditions including high availability of food resulted in a better overall condition of the exposed fish after 1 year of exposure compared to the controls.

Keywords Biological pond · Biological effects · Endocrine disruption · Integrate biomarker response

Abbreviations

ALT	Alanine aminotransferase
ALB	Albumins
ALP	Alkaline phosphatase
NH^3	Ammonia
AST	Aspartate aminotransferase
Ca^{2+}	Calcium
CAT	Catalase
CK	Creatine kinase
RBC	Red blood cell
FFPW	Faculty of Fisheries and Protection of Water
GLU	Glucose
GR	Glutathione reductase
GST	Glutathione S-tranferase
GPx	Glutathione peroxidase
PCV	Hematocrit value
Hb	Hemoglobin concentration
PHOS	Inorganic phosphate
LACT	Lactate
LDH	Lactate dehydrogenase

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The original version of this article was revised: The authors' given and family name was corrected.

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WBC	White blood cell
Mg	Magnesium
MCH	Mean corpuscular hemoglobin
MCHC	Mean corpuscular hemoglobin concentration
MCV	Mean erythrocyte volume
NSAIDs	Non-steroidal anti-inflammatory drugs
POCIS	Polar organic chemical integrative sampler
STPs	Sewage treatment plants
PPCPs	Pharmaceutical and personal care products
SOD	Superoxide dismutase
TBARS	Thiobarbituric acid reactive substances
TP	Total proteins
TRIG	Triglycerides
VTG	Vitellogenin

Introduction

Sewage treatment plant (STP) effluents have been shown to contain a wide spectrum of chemical contaminants, such as pharmaceutical and personal care products (PPCPs), pesticides, and other contaminants originating from households, industry, and agriculture (Calisto and Esteves 2009; Halling-Sørensen et al. 1998; Köck-Schulmeyer et al. 2012). The discharge of effluents from STPs has several detrimental effects on the health of aquatic ecosystems, including the aquatic environment and aquatic organisms. These effects include nutrient imbalance (Björn Gücker 2006), behavioral changes (Garcia-Reyero et al. 2011; Schoenfuss et al. 2002), and disruption of the endocrine pathways of aquatic organisms (Anway et al. 2005; Mills and Chichester 2005).

The presence of PPCPs and their impact on the aquatic environment have received increasing concern (Zenobio et al. 2015). Environmentally relevant concentrations of PPCPs are much lower than the corresponding therapeutic doses used for medical treatments, and acute toxicity tests have often failed to detect the subtle action elicited by drugs at low dosage (Fent et al. 2006). Furthermore, PPCPs are regularly detected in the complex mixtures of unrelated molecules with diverse chemical structures, persistence, specificity, and biological activity (Fent et al. 2006; Lopez-Serna et al. 2012). The joint ecotoxicity of such chemical cocktails is typically higher than the toxicity of each individual compound (Cleuvers 2003; Eguchi et al. 2004; Kortenkamp 2009). In particular, even if the levels of compounds in the mixture are only present below their respective toxicity thresholds, a joint toxic effect cannot be ruled out. Therefore, it is challenging to predict the effects of such mixtures on fish health. Neglecting the potential impacts of chemical cocktails could possibly result in underestimating the actual effect of PPCPs under real conditions, which depend on the concentration, number of compounds, and their modes of action.

There is increasing evidence of the presence, distribution, and effects of PPCPs (Ebele et al. 2017). However, a significant proportion of the data are derived from laboratory experiments. To the best of our knowledge, data from laboratories are properly used as reference data. However, these data may fail to reflect the effects in environmental conditions due to underestimation of the additive, synergistic, or antagonistic interactions during chronic exposure to mixtures of hundreds of compounds. Their interactions with other stressors such as temperature or pH are also unclear.

Several approaches have been applied in toxicological studies to understand the effect of pollutants mixtures in environmental conditions, such as field and cage studies. However, each method has its own disadvantages. In fact, information about the bioaccumulation and biomagnification of toxic compounds may be impossible to gather in cage studies because the organisms do not consume natural food. Furthermore, randomly caught fish in field studies may yield invalid results because of the migration of fish. Without information about the origin and history of fish, it is almost impossible to determine the effects and their time variations.

In this study, an experiment was performed in a biological pond that receives input from only STP effluent under fully realistic conditions. This scenario represents a common condition in central Europe, where treated communal wastewater is released to a recipient pond via biological and production ponds. The fish were stocked in an STP recipient pond in order avoid the stress of caging and to ensure natural feeding conditions as well as environmental interactions.

The common carp (*Cyprinus carpio*) is one of the most economically important freshwater species in Europe (Bostock et al. 2016). This specie has been widely used as a model to monitor the effects of pollutant compounds and water quality in both laboratory and field conditions (Dobsikova et al. 2006; Gungordu 2011; Witeska and Wakulska 2007; Zivna et al. 2016). The gills, liver (Gonzalez-Gonzalez et al. 2014; Thibaut et al. 2006), and blood (Islas-Flores et al. 2013) have high xenobiotic metabolizing. Biochemical changes in these tissues have been used as effective indicators of pollutant exposure in fish.

To describe sequential responses, the focus was on the early biomarker signals (oxidative stress), later responding parameters (blood parameters, endocrine disruption) and chronic effects (histology). The response of enzyme activities and ionoregulatory has previously been shown to change in the gills, liver, and blood of common carp exposed to PPCPs (Gonzalez-Gonzalez et al. 2014; Saravanan et al. 2011). Despite low environmental

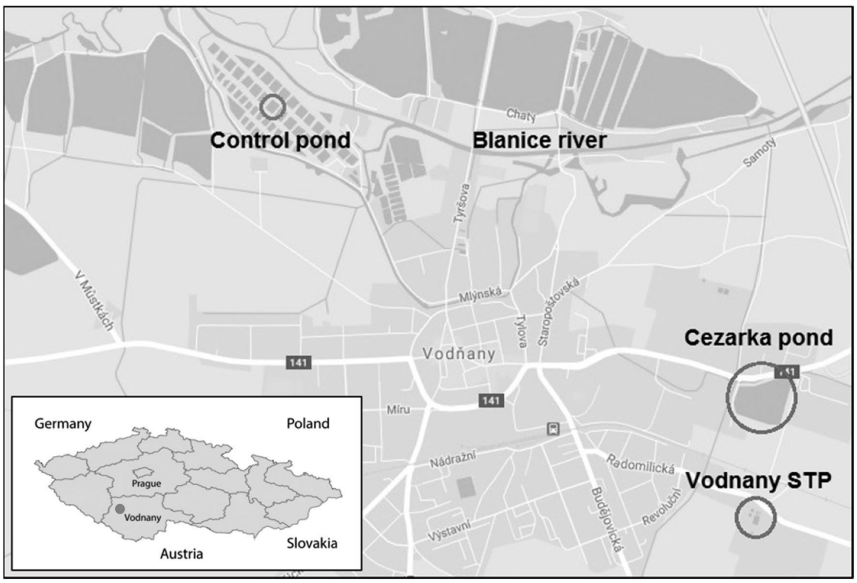

Fig. 1 Map of the Vodnany sewage treatment plant, control, and Cezarka ponds

concentrations, PPCPs have a wide range of modes of action. Therefore, a large set of biochemical responses that can reflect both subtle and evident changes is needed to investigate the effect of PPCP mixtures on fish (He et al. 2011; van der Oost et al. 2003).

Oxidative stress and antioxidant enzymes are among the sensitive parameters that change in the presence of PPCPs. The induction of lipid peroxidation (LPO) and glutathione S-tranferase (GST) has been detected in the gills (Li et al. 2011b) and digestive tissues (Brandao et al. 2013) of fish exposed to carbamazepine. Changes in superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) activities were detected in the brain, gills, and liver of common carp exposed to a mixture of diclofenac and acetaminophen (Nava-Álvarez et al. 2014).

Hematological and biochemical plasma parameters are important indices for assessing the physiological status of fish and toxicological symptoms (Rao 2006a; Velisek et al. 2011). Vitellogenin (VTG) is an important and effective endocrine disruption biomarker (Hansen et al. 1998) that has been proven to have increase in fish exposed to certain pollutants (Paraso et al. 2017; Petrovic et al. 2002). Histology has commonly been used to define the toxicological effects and is considered as a gold standard (Kilty et al. 2007).

This study investigated the effects of a complex mixture of chemicals predominated by PPCPs from STP effluent. Common carp was exclusively exposed to the effluent of an STP for a period of 360 days in a biological pond to assess the effects in realistic conditions.

Material and Methods

Standard and Reagents

Liquid chromatography–mass spectrometry (LC/MS)-grade acetonitrile and methanol (Lichrosolv, Hypergrade) were obtained from Merck (Darmstadt, Germany). Formic acid (LC/MS grade) was obtained from Fisher Scientific (USA). All other chemicals were obtained from Sigma-Aldrich (Europe).

Experimental Area

The study areas were Cezarka pond and a control pond (Fig. 1). Cezarka pond (2.6 ha) is a biological pond designed for the retention of treated effluent from the Vodnany STP in the Czech Republic. Vodnany (population 7000) is a town that is adjacent to the Blanice River in South Bohemia. The commercial activity in Vodnany consists of light industry (poultry slaughter, manufacturing of agricultural machinery) along with intensive agriculture and horticulture in the surrounding area. Cezarka belongs to a cascade of aquaculture ponds that are connected to the

Blanice River. Moreover, the pond is suitable for the breeding of common carp. Sewage water treatment in the STP facility involves primary mechanical filtration and sedimentation followed by activated sludge treatment.

The control pond (0.12 ha) was selected from the pond system of the Faculty of Fisheries and Protection of Water (FFPW), University of South Bohemia, Vodnany, Czech Republic. The pond was chosen as an ecological representative for the water bodies in the region. The control pond is about 2 km away from Cezarka pond and is in the same range of geography and weather. Although it was different in size from the Cezarka pond, the depth and fish density were kept similar. Similar to other water bodies in the region, the control pond receives water from the Blanice River upstream from the town.

Sampling Sites and Field Deployment of the Polar Organic Chemical Integrated Sampler (POCIS)

Water pollutants in the control and Cezarka ponds were monitored using both grab and passive samplers. POCISs were deployed for a period of 10 days prior to each fish sampling event. Passive and grab water samplers were collected at three locations in Cezarka (near the inlet, near the outlet, and in a middle location) and in one location in the control pond (in a middle location). The analyses of polar compounds from pesticide configuration of POCIS-Pest were performed according to the procedures of Grabic et al. (2010, 2012). Briefly, exposed samplers were cleaned and disassembled, and the sorbent was transferred to glass chromatographic columns.

The analytes targets were eluted with 50 ml of methanol, dichloromethane, and toluene (1:8:1 v/v/v). The extraction solvent was then changed to methanol, and samples were analyzed using LC-MS/MS. The target analytes were separated and detected using a TSQ Quantum triple-stage quadrupole mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA) coupled with an Accela 1250 LC pump (Thermo Fisher Scientific, San Jose, CA, USA) and HTS XT-CTC autosamplers (CTC Analytics AG, Zwingen, Switzerland). An analytical Hypersil GOLD aQ column (50 mm length, 2.1 mm i.d, 5-µm particles; Thermo Fisher Scientific) was used to chromatographically separate the target analytes. The dates of sampling events and the sampling points are shown in Supplementary Material 1.

Fish

Both male and female common carp were obtained from a local facility (66 ± 3 g body weight and 170 ± 0.3 mm in length). The carp were randomly stocked in the control and experimental ponds with similar relative stock density (0.14 fish/m^2) in April 2015. Both ponds were harvested, and all of the remaining fish were removed prior to stocking. The fish ate only natural food in both ponds. The trophic level of the control pond is typical for an average aquaculture pond in the region.

Sampling took place after 0, 30, 90, 180, and 360 days of exposure. Additional information about the sampling times is shown in Supplementary Material 1. The fish were collected from both ponds by electrofishing. The fish were handled according to the national and institutional guidelines for the protection of human subjects and animal welfare and the Law Against Animal Cruelty (082/2002-V2). Approval was obtained from Czech National Directive No. 419/2012 for the protection of experimental animals.

Twelve individual fish from each pond were sampled at each time point. Blood samples were taken from each fish by puncturing the caudal vein using a syringe with heparin as an anticoagulant at a concentration of 5000 IU/ml of heparin sodium salt. The fish were then sacrificed by severing the spinal cord. Their weight and length were then measured. Organs including the gills, gonads, liver, intestine, and white muscle were quickly removed. Blood plasma was obtained by centrifuging the blood samples in a cooled centrifuge (4 °C, 10,000 × g, 10 min) and stored at −80 °C until analysis. A small volume of blood was immediately used to determine hematological variables (Svobodova et al. 2012). The liver, muscle, intestine, and gills were dissected and stored at −80 °C for biochemical analysis. At each sampling point, samples of the kidneys, livers, gills, and gonads of fish from both ponds were also fixed in 10% formalin for histological examination.

Morphological Indices

The body, liver, and gonad weights were recorded for each fish, in addition to each animal's length. The condition factor (CF), hepatosomatic index (HSI), and gonadal somatic index (GSI) were calculated for each fish according to the literature (White and Fletcher 1985):

$$\mathrm{CF} = \frac{b}{L^3} \times 100 \qquad \mathrm{HSI} = \frac{1}{b} \times 100 \qquad \mathrm{GSI} = \frac{g}{b}\mathrm{x}100,$$

where b is the body weight (g), L is the total length (cm), l is the liver weight (g), and g is the gonad weight (g).

Histological Examination

Fixed samples of the gills, kidney, liver, spleen, and gonads of exposed and control fish were embedded in paraffin and cut with a microtome into 4-µm sections for histology. The sections were stained with haematoxylin-eosin (H&E) and examined by light microscopy. The organs were excised and placed longitudinally in a capsule to ensure the largest possible cut section for each organ. Due to the relatively

small size of samples, one cut section per fish and organ was examined, and the lesions were assumed to be distributed equally throughout the tissue. Pathological changes were graded as 0 (none), 1 (minimal), 2 (mild), 3 (mild to moderate), 4 (moderate), 5 (moderate to severe), or 6 (severe) relative to the normal structures described in healthy animals. After a first screening, the following criteria were selected for semiquantitative evaluation: gills: epithelial hyperplasia, lamellar fusion, parasitic infestation; liver: hepatocyte vacuolation, pericholangiar inflammation, perivascular inflammation, granuloma, vessel wall degeneration; gonads: sex, differentiation stage, uniformity of germ cells, oocyte degeneration.

Biochemical Assays of Fish Tissues

The post-mitochondrial supernatant (PMS) was obtained as described by Howcroft et al. (2009). GST activity was determined using 1-chloro-2,4-dinitrobenzene as a substrate according to the method of Habig et al. (1974), which was adapted for a microplate reader by Frasco and Guilhermino (2002). CAT activity was determined using the method of Claiborne (1985) by measuring the decrease in hydrogen peroxide in a 96-well flat-bottom ultraviolet-transparent microtiter plate.

SOD activity was determined using the method of Nishikimi et al. (1972). This assay relies on the ability of the enzyme to inhibit the phenazine methosulfate-mediated reduction of nitro blue tetrazolium (NBT) dye. Glutathione reductase (GR) activity was determined using the method of Cribb et al. (1989) with some modifications using 50 µl of PMS (approx. 0.2 mg/ml) and 150 µl of reaction solution. GPx activity was measured using the method of Mohandas et al. (1984). Oxidative damage was assessed by determining the level of LPO, which was measured as thiobarbituric acid reactive substances (TBARS). This was carried out using the methodology of Ohkawa et al. (1979). The details of each method are presented in Supplementary Material 2.1.

Biochemical Assays in Fish Blood Plasma

A VETTEST 8008 analyzer (IDEXX Laboratories Inc., USA) was used according to the manufacturer's instructions to determine biochemical indices, including glucose (GLU), total proteins (TP), ammonia (NH_3), aspartate aminotransferase (AST), alanine aminotransferase (ALT), albumin (ALB), creatine (CREA), lactate dehydrogenase (LDH), creatine kinase (CK), lactate (LACT), phosphorous (PHOS), magnesium (Mg), triglyceride (Trig), alkaline phosphatase (ALP), and calcium (Ca^{2+}).

Vitellogenin

Gene expression of VTG in liver tissue

RNA isolation was carried out using the Trizol method (Rio et al. 2010). The isolated RNA was converted to cDNA by reverse transcription using an iScript cDNA synthesis kit (Bio-Rad, Canada), according to the manufacturer's instructions. cDNA was diluted in RNAase free water and stored at 20 °C until further use. Semi-quantitative PCR was conducted according to the method of Rasmussen et al. (2011) using TaqMan probes. Primers and TaqMan probes were designed with Primer Express 3.0.1 using common-carp-specific sequences of genomic DNA. The primers and probes are shown in Supplementary Material 2.2.1.

The relative mRNA expression was calculated by relating the obtained values for threshold cycles to a standard curve obtained by running a serial dilution of one cDNA sample. The mRNA expression was normalized to the mRNA expression of beta actin and expressed as arbitrary units. The expression of beta actin did not significantly differ between the experimental and control groups. The average of the control groups according to exposure time was arbitrarily set to 1, and the experiment groups were expressed relative to the corresponding control group. The details of this method are presented in Supplementary Material 2.2.

Concentration of VTG in blood plasma

The concentration of VTG in blood plasma was determined using a Biosense Elisa test kit for carp (Biosense, Norway), according to the instructions of the manufacturer. Principally, the test utilizes specific binding between antibodies and VTG to quantify the VTG concentration in samples. The wells of microplates were pre-coated with a specific capture antibody that binds to VTG in standards and samples added to the wells. A different VTG-specific detecting antibody was added to create a sandwich of VTG and antibody, which was detected by an enzyme-labeled secondary antibody. The enzyme activity was determined by adding a substrate that yields a colored product, and the color intensity was directly proportional to the amount of VTG present.

Hematological Parameters

Transformation solution (0.1 g of potassium ferricyanide, 0.025 g of potassium cyanide, 0.07 g of potassium dihydrogenphosphate, and up to 0.5 l of distilled water) was used to determine the hemoglobin (Hb) concentration. The indices tested were determined by methods described by Svobodova et al. (2012) and included red blood cells

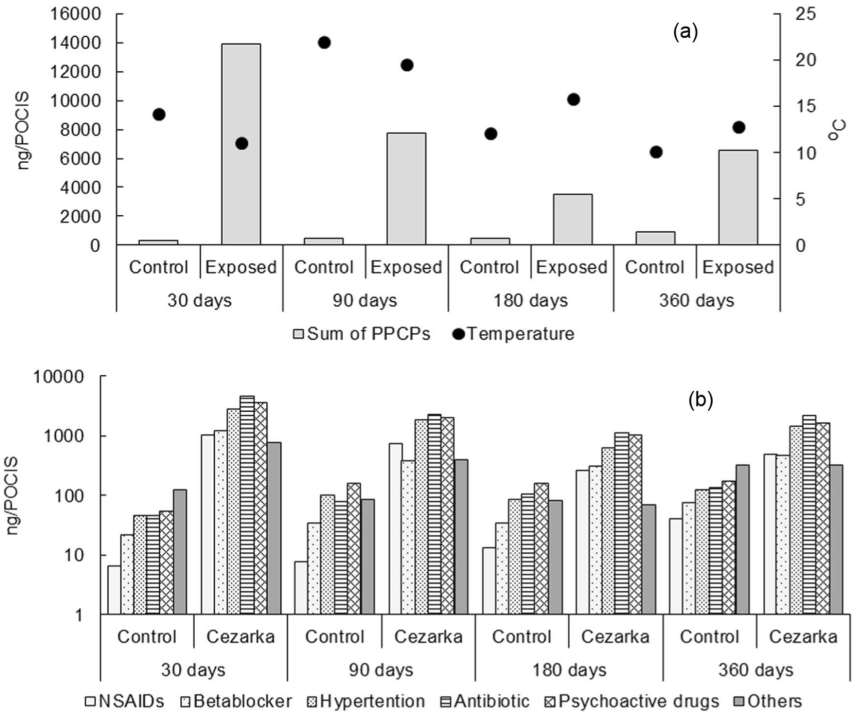

Fig. 2 Sum of pharmaceutical and personal care products (PPCPs) in polar organic compounds integrated samplers (POCISs) (column) ($n = 3$) and average temperature (dot) (**a**); and the total PPCPs according to groups—NSAIDs (Non-steroidal anti-inflammatory drugs), beta blocker, hypertension, psychoactive compound, antibiotic, others (fibrate, azole, antihistamine)—in the control and Cezarka ponds (**b**)

(RBCs), hematocrit (PCV), mean erythrocyte volume (MCV), mean erythrocyte hemoglobin (MCH), and mean color concentration (MCHC). The level of Hb was determined spectrophotometrically at 540 nm (Helios Epsilon, UNICAM). The MCV and MCHC values were obtained from blood count analysis as conventional biomarkers. The procedures were based on unified methods for hematological examinations of fish (Svobodova et al. 2012).

Integrated Biomarker Response (IBR)

For better understanding of the overall effect of STP effluent on fish, the IBR was calculated according to Beliaeff and Burgeot (2002). The final IBR values were calculated by dividing the number of biomarkers (n) based on the suggestion of Broeg and Lehtonen (2006). The necessary results of the data standardization procedure for the IBR calculation are presented in star plots. The IBR index was calculated using the results of LPO (measured as TBARs), SOD, CAT, GPx, GR, and GST.

Statistical Analysis

Differences within the sampling period were tested between the exposed group and the corresponding control group in CF, HSI, GSI, mRNA expression, VTG, oxidative stress and antioxidant enzymes, blood parameters, and histopathology. First, the data were tested for normality using the Shapiro–Wilk test. If the normality condition was satisfied, the Student's unpaired t-test was used to determine whether there were any significant differences between the control and exposed groups within sampling period. If the normality condition was not satisfied, a nonparametric Mann–Whitney U-test was used. A significant difference was recognized when $P < 0.05$ was found. All of statistical analyses were performed using SPSS statistical software (version 23, IBM Corp.) for Windows.

Results

At the day 0, 30, and 90, the fish were juveniles. Therefore, all parameters were investigated without considering sex. At 180 and 360 days, the fish were separated into males and females to see the sex differences in the respective parameters.

Occurrence of Selected Compounds in Water

Figure 2a shows the results of water temperature and total PPCPs identified using POCIS in the control and Cezarka ponds. At 30, 90, 180, and 360 days, the water temperature in Cezarka pond was 3.1 °C lower, 2.4 °C lower, 3.6 °C higher, and 2.6 °C higher than in the control pond, respectively. The total concentrations of 62 detected PPCPs ranged from 305 to 880 ng/POCIS in the control pond and from 3490 to 14000 ng/POCIS in Cezarka pond. The total PPCPs increased slightly over time in the control pond, and a sinusoidal trend was noted in the Cezarka pond. The total PPCPs concentration was highest after 30 days. After that point, it constantly decreased until 180 days before suddenly spiking up by 360 days. Non-steroidal anti-inflammatory drugs (NSAIDs) (6.6–1020 ng/POCIS), beta-blockers (22–1210 ng/POCIS), hypertension drugs (47–2770 ng/POCIS), antibiotics (48–4590 ng/POCIS), and psychoactive drugs (55–3580 ng/POCIS) were the most abundant drug compounds in both ponds (Fig. 2b). The pharmaceuticals irbesartan, telmisartan, carbamazepine, and tramadol were found in relatively high concentrations in all of the sampling events in Cezarka pond. The average concentrations of these compounds were 1990, 1440, 512, and 422 ng/POCIS, respectively. The full list of measured concentrations of analyzed compounds is presented in Supplementary Material 4.

Morphological Indices

The morphological indices of common carp from the control and Cezarka ponds are shown in Table 1. Information about the mean full length and body weight of the fish are presented in Supplementary Material 5. Within a year, the CF value was highest at 90 days with 66 and 92% increases compared with day 0 in the control and Cezarka ponds, respectively (Table 1). The relationship between fish body weight and total body length was 25, 34, and 52% higher in fish from Cezarka pond compared with fish in the control pond at 30, 90, and 360 days.

In the control pond, the mean HSI was higher at day 30 compared with day 0, and we noted a decreasing trend of HSI at other time points. Significant differences between the control and exposed fish were observed in the sampled fish at 30 (33), 90 (57), and 180 (20%) days. Although both male and female-exposed fish had lower HSI than the

Table 1 Effect of long-term exposure to STP effluent on CF, HSI, and GSI of common carp in control and Cezarka pond

Time	0 days	30 days	90 days	180 days			360 days		
Fish	Juvenile ($n = 12$)	Juvenile ($n = 12$)	Juvenile ($n = 12$)	♀ ($n = 5$)	♂ ($n = 7$)	♀ and ♂ ($n = 12$)	♀ ($n = 6$)	♂ ($n = 6$)	♀ and ♂ ($n = 12$)
Condition factor									
Control	1.31 ± 0.01	1.94 ± 0.08	2.17 ± 0.07	2.25 ± 0.26	2.10 ± 0.13	2.16 ± 0.13	1.94 ± 0.52	1.92 ± 0.36	1.93 ± 0.04
Exposed		2.19 ± 0.06*	2.51 ± 0.05*	2.10 ± 0.07	2.26 ± 0.84	2.18 ± 0.06	2.36 ± 0.13*	2.52 ± 0.58*	2.45 ± 0.06*
Hepatosomatic index									
Control	4.32 ± 0.21	5.97 ± 0.25	2.59 ± 0.10	2.75 ± 0.13	2.77 ± 0.12	2.76 ± 0.09	3.87 ± 0.09	4.29 ± 0.33	4.12 ± 0.20
Exposed		3.95 ± 0.15*	4.09 ± 0.34*	2.54 ± 0.11	1.85 ± 0.20*	2.20 ± 0.15*	3.34 ± 0.50	3.61 ± 0.32	3.50 ± 0.27
Gonad somatic index									
Control	NA	0.16 ± 0.03	0.13 ± 0.03	0.50 ± 0.13	3.13 ± 0.59	2.17 ± 0.56	0.37 ± 0.08	2.64 ± 0.49	1.72 ± 0.44
Exposed		0.25 ± 0.03	1.21 ± 0.47*	2.17 ± 0.85*	6.61 ± 1.90	4.67 ± 1.24	2.29 ± 0.47*	9.41 ± 0.77*	6.44 ± 1.21*

NA not available

Note: Data are presented as mean ± SEM; an asterisk corresponds to significant differences compared to control value (*$P < 0.05$)

Table 2 Effect of STP effluent on the level of TBARS (μmol g of wet tissue^{-1}), GST (μmol min^{-1} mg protein^{-1}), CAT (μmol min^{-1} mg protein^{-1}), SOD (μmol NBT min^{-1} mg protein^{-1}), GR (nmol min^{-1} mg protein^{-1}), and GPx (nmol min^{-1} mg protein^{-1}) in the liver, gill, intestine and muscle of common carp tissues

Time points		0 days	30 days	90 days	180 days			360 days		
Fish		Juvenile ($n = 12$)	Juvenile ($n = 12$)	Juvenile ($n = 12$)	♀ ($n = 5$)	♂ ($n = 7$)	♀ and ♂ ($n = 12$)	♀ ($n = 6$)	♂ ($n = 6$)	♀ and ♂ ($n = 12$)
Liver										
CAT	Control	2.16 ± 0.20	1.61 ± 0.20	0.54 ± 0.07	0.98 ± 0.07	0.98 ± 0.13	0.98 ± 0.08	1.55 ± 0.06	1.47 ± 0.08	1.50 ± 0.05
	Exposed		0.74 ± 0.04*	0.80 ± 0.13	1.07 ± 0.15	0.56 ± 0.10*	0.82 ± 0.12	1.32 ± 0.06	1.44 ± 0.07	1.39 ± 0.05
SOD	Control	2.29 ± 0.17	2.53 ± 0.74	6.60 ± 0.10	4.13 ± 0.30	3.89 ± 0.42	3.99 ± 0.27	3.94 ± 0.31	3.99 ± 0.39	3.97 ± 0.25
	Exposed		4.40 ± 0.67*	5.65 ± 1.23	2.87 ± 0.56*	4.68 ± 0.75	3.78 ± 0.52	3.90 ± 0.26	3.54 ± 0.35	3.69 ± 0.23
GPx	Control	0.38 ± 0.03	0.51 ± 0.06	0.17 ± 0.02	0.29 ± 0.04	0.34 ± 0.07	0.32 ± 0.04	0.26 ± 0.04	0.22 ± 0.03	0.24 ± 0.03
	Exposed		0.23 ± 0.03*	0.25 ± 0.04	0.38 ± 0.06	0.21 ± 0.04	0.30 ± 0.04	0.17 ± 0.04	0.21 ± 0.05	0.19 ± 0.03
GR	Control	0.07 ± 0.01	0.08 ± 0.01	0.02 ± 0.00	0.03 ± 0.00	0.03 ± 0.00	0.03 ± 0.00	0.03 ± 0.00	0.04 ± 0.00	0.04 ± 0.00
	Exposed		0.03 ± 0.00*	0.03 ± 0.00	0.04 ± 0.01	0.06 ± 0.00	0.04 ± 0.01	0.04 ± 0.01	0.06 ± 0.00*	0.05 ± 0.00*
GST	Control	1.96 ± 0.19	1.66 ± 0.21	0.42 ± 0.07	1.29 ± 0.13	1.51 ± 0.26	1.42 ± 0.16	3.08 ± 0.23	2.62 ± 0.13	2.81 ± 0.14
	Exposed		0.65 ± 0.07*	1.44 ± 0.29*	1.84 ± 0.26	1.29 ± 0.34	1.56 ± 0.22	3.22 ± 0.43	4.20 ± 0.17*	3.79 ± 0.29*
TBARs	Control	7.67 ± 0.69	7.98 ± 2.01	30.95 ± 3.97	15.95 ± 1.21	17.64 ± 2.22	16.94 ± 1.36	6.10 ± 1.00	6.53 ± 0.43	6.35 ± 0.46
	Exposed		8.58 ± 1.84*	36.26 ± 11.8	13.41 ± 2.02	23.14 ± 5.58	18.28 ± 1.84	8.67 ± 3.88	7.04 ± 1.21	7.72 ± 1.68
Gill										
CAT	Control	0.32 ± 0.02	0.32 ± 0.04	0.29 ± 0.02	0.26 ± 0.01	0.31 ± 0.05	0.30 ± 0.03	0.15 ± 0.06	0.22 ± 0.04	0.19 ± 0.03
	Exposed		0.30 ± 0.02	0.25 ± 0.02	0.22 ± 0.02	0.18 ± 0.02	0.20 ± 0.02*	0.24 ± 0.07	0.27 ± 0.06	0.24 ± 0.04
SOD	Control	2.03 ± 0.07	1.53 ± 0.08	1.08 ± 0.09	0.95 ± 0.30	1.41 ± 0.17	1.22 ± 0.17	4.64 ± 0.23	5.13 ± 0.69	4.93 ± 0.37
	Exposed		1.68 ± 0.09	1.75 ± 0.06*	2.08 ± 0.31	1.91 ± 0.40	2.00 ± 0.24*	5.42 ± 0.69	4.43 ± 0.30	4.84 ± 0.35
GPx	Control	0.30 ± 0.01	0.27 ± 0.01	0.36 ± 0.02	0.27 ± 0.03	0.28 ± 0.01	0.28 ± 0.01	0.16 ± 0.04	0.10 ± 0.02	0.13 ± 0.02
	Exposed		0.32 ± 0.02*	0.28 ± 0.01*	0.22 ± 0.01	0.21 ± 0.01*	0.21 ± 0.01*	0.12 ± 0.02	0.09 ± 0.02	0.11 ± 0.01
GR	Control	0.53 ± 0.02	0.41 ± 0.03	0.28 ± 0.02	0.26 ± 0.03	0.25 ± 0.02	0.25 ± 0.02	0.10 ± 0.01	0.14 ± 0.02	0.12 ± 0.01
	Exposed		0.35 ± 0.02	0.24 ± 0.01	0.20 ± 0.02	0.20 ± 0.02	0.20 ± 0.01*	0.13 ± 0.02	0.14 ± 0.01	0.14 ± 0.01
GST	Control	1.97 ± 0.08	1.63 ± 0.09	1.04 ± 0.06	0.93 ± 0.05	1.06 ± 0.03	1.00 ± 0.03	2.41 ± 0.11	2.13 ± 0.03	2.25 ± 0.09
	Exposed		1.56 ± 0.07	1.48 ± 0.06*	1.29 ± 0.09	0.91 ± 0.05	1.10 ± 0.08	2.20 ± 0.09	2.11 ± 0.07	2.15 ± 0.05
TBARs	Control	6.22 ± 0.74	9.62 ± 1.15	11.17 ± 1.55	11.09 ± 1.26	14.1 ± 4.04	12.84 ± 2.37	0.42 ± 0.13	0.88 ± 0.21	0.69 ± 0.15
	Exposed		8.18 ± 1.24	8.23 ± 0.89	10.07 ± 2.79	8.51 ± 2.08	9.29 ± 1.67	1.49 ± 0.28*	3.34 ± 1.08*	2.57 ± 0.68*
Intestine										
CAT	Control	0.71 ± 0.04	0.56 ± 0.07	0.61 ± 0.07	0.30 ± 0.04	0.38 ± 0.04	0.34 ± 0.04	0.44 ± 0.04	0.41 ± 0.03	0.43 ± 0.02
	Exposed		0.84 ± 0.08*	0.53 ± 0.05	0.34 ± 0.04	0.32 ± 0.05	0.33 ± 0.03	0.30 ± 0.06	0.26 ± 0.06*	0.28 ± 0.04*
SOD	Control	3.11 ± 0.30	1.25 ± 0.15	1.84 ± 0.23	1.97 ± 0.38	2.01 ± 0.29	1.99 ± 0.22	4.18 ± 1.68	6.31 ± 0.45	5.43 ± 0.77
	Exposed		1.30 ± 0.23	1.88 ± 0.19	2.32 ± 0.38	2.21 ± 0.23	2.26 ± 0.21	5.99 ± 2.03	5.66 ± 1.11	5.81 ± 1.04
GPx	Control	0.27 ± 0.23	0.20 ± 0.04	0.16 ± 0.01	0.14 ± 0.02	0.18 ± 0.03	0.17 ± 0.02	0.96 ± 0.21	0.46 ± 0.21	0.67 ± 0.16

Table 2 continued

Time points		0 days	30 days	90 days	180 days			360 days		
Fish		Juvenile ($n = 12$)	Juvenile ($n = 12$)	Juvenile ($n = 12$)	♀ ($n = 5$)	♂ ($n = 7$)	♀ and ♂ ($n = 12$)	♀ ($n = 6$)	♂ ($n = 6$)	♀ and ♂ ($n = 12$)
	Exposed		0.17 ± 0.02	0.15 ± 0.02	0.13 ± 0.01	0.18 ± 0.02	0.15 ± 0.01	0.17 ± 0.06*	0.16 ± 0.05	0.17 ± 0.04*
GR	Control	0.47 ± 0.10	0.52 ± 0.05	0.27 ± 0.02	0.21 ± 0.02	0.27 ± 0.02	0.25 ± 0.02	0.01 ± 0.00	0.00	0.00
	Exposed		0.41 ± 0.02	0.29 ± 0.02	0.28 ± 0.01	0.28 ± 0.04	0.28 ± 0.02	0.00	0.00	0.00
GST	Control	1.93 ± 0.15	1.60 ± 0.18	1.83 ± 0.16	1.34 ± 0.07	1.54 ± 0.09	1.46 ± 0.06	0.81 ± 0.11	1.05 ± 0.14	0.95 ± 0.10
	Exposed		1.36 ± 0.15	2.03 ± 0.26	1.74 ± 0.08*	1.8 ± 0.23	1.80 ± 0.12*	0.96 ± 0.24	0.96 ± 0.09	0.96 ± 0.11
TBARs	Control	4.47 ± 1.25	4.38 ± 0.49	2.73 ± 0.39	2.18 ± 0.44	2.97 ± 0.40	2.64 ± 0.31	3.52 ± 0.50	4.50 ± 0.86	3.76 ± 0.36
	Exposed		5.37 ± 1.38	4.07 ± 0.48	4.53 ± 1.09	4.63 ± 0.74	4.58 ± 0.63	3.93 ± 0.51	5.02 ± 0.49	4.80 ± 0.44
Muscle										
CAT	Control	0.37 ± 0.05	0.35 ± 0.05	0.28 ± 0.03	0.26 ± 0.06	0.26 ± 0.04	0.26 ± 0.03	0.08 ± 0.03	0.17 ± 0.04	0.13 ± 0.03
	Exposed		0.27 ± 0.03	0.23 ± 0.05	0.24 ± 0.04	0.39 ± 0.06	0.31 ± 0.04	0.11 ± 0.05	0.09 ± 0.01	0.10 ± 0.02
SOD	Control	0.96 ± 0.08	1.00 ± 0.15	1.10 ± 0.06	1.99 ± 0.25	1.36 ± 0.15	1.62 ± 0.12	3.66 ± 0.24	3.89 ± 0.11	3.80 ± 0.63
	Exposed		1.54 ± 0.18*	1.38 ± 0.18	1.78 ± 0.20	1.25 ± 0.14	1.52 ± 0.14	3.27 ± 0.98	5.76 ± 2.10	4.72 ± 1.30
GPx	Control	0.31 ± 0.01	0.33 ± 0.01	0.32 ± 0.02	0.28 ± 0.01	0.35 ± 0.01	0.32 ± 0.01	1.04 ± 0.66	0.85 ± 0.54	0.93 ± 0.40
	Exposed		0.33 ± 0.01	0.34 ± 0.01	0.34 ± 0.02	0.37 ± 0.02	0.35 ± 0.01	1.48 ± 0.97	0.55 ± 0.39	0.94 ± 0.46
GR	Control	0.12 ± 0.01	0.16 ± 0.02	0.09 ± 0.01	0.07 ± 0.01	0.09 ± 0.01	0.08 ± 0.01	0.05 ± 0.01	0.06 ± 0.01	0.06 ± 0.01
	Exposed		0.12 ± 0.01	0.12 ± 0.03	0.08 ± 0.01	0.11 ± 0.02	0.09 ± 0.01	0.05 ± 0.01	0.05 ± 0.01	0.05 ± 0.01
GST	Control	0.74 ± 0.04	1.06 ± 0.15	0.98 ± 0.15	0.80 ± 0.11	1.04 ± 0.13	0.94 ± 0.09	0.23 ± 0.05	0.21 ± 0.08	0.22 ± 0.05
	Exposed		0.97 ± 0.04	1.30 ± 0.35	0.78 ± 0.11	1.10 ± 0.10	0.94 ± 0.09	0.51 ± 0.04*	0.58 ± 0.11*	0.55 ± 0.07*
TBARs	Control	2.21 ± 0.48	1.86 ± 0.56	1.58 ± 0.34	1.04 ± 0.16	1.10 ± 0.15	1.07 ± 0.11	5.12 ± 0.90	5.17 ± 0.42	5.15 ± 0.42
	Exposed		1.60 ± 0.20	0.99 ± 0.12*	1.97 ± 1.08	1.43 ± 0.17	1.70 ± 0.53*	6.06 ± 0.46	5.03 ± 0.67	5.46 ± 0.44

Note: Data are means ± SEM. An asterisk corresponds to significant differences between exposed and corresponding control group (*$P < 0.05$)

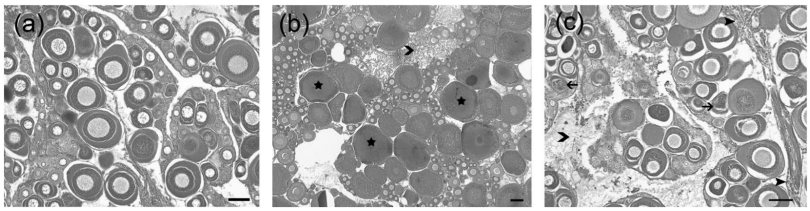

Fig. 3 Histopathology (H&E stain) of fish ovaries in the control (**a**) and the Cezarka ponds (**b, c**) after 360 days of exposure. **a** Ovary of a control female, oocytes up to previtellogenic stage; **b** ovary of an exposed female, oocytes further developed up to vitellogenic stage (stars), multifocal interstitial edema (open arrowhead); **c** ovary of an exposed female, irregular structure with interstitial edema (open arrowheads) and multifocal infiltration with lymphocytes and macrophages (closed arrowheads), multiple oocytes degenerated (arrow). The scale bar corresponds to 50 µm

control fish at 180 days, the reduction was significant in only male fish.

The GSI of fish from Cezarka pond was significantly higher than in the control group at 90 and 360 days. The GSIs of both males and females in the control pond decreased over the period of 180–360 days, and the GSI of fish exposed to STP effluent discharge constantly increased in both sexes. Although the GSIs of both male and female-exposed fish were higher than control at 180 days, the elevation was significant in only females, resulting in no significant difference when including both sexes. At 360 days, the GSI levels in both male and female-exposed fish were higher than in control fish.

Oxidative Stress and Antioxidant Responses

LPO levels (measured as TBARs) and four antioxidant enzymatic reactions (CAT, SOD, GR, and GPx) were measured in the liver, gills, intestine, and muscle tissues. Changes in these levels are summarized in Table 2 for each tissue type. In the liver, the levels of TBARs, CAT, SOD, GR, and GPx in fish exposed to STP effluent were significantly different from those of control fish after 30 days. STP effluent-exposed carp showed a significant increase in hepatic TBARs level and SOD activities (by 2.3-fold and 1.7-fold, respectively) and a decline in hepatic CAT, GR, and GPx (by 2.2-fold, 3.2-fold, and 2.2-fold, respectively). Additionally, GR activity in the exposed fish was significantly higher than in the control fish at day 360. Although both male and female-exposed fish had higher GR levels than control fish at this sampling time, the elevation was significant in only male fish.

In the gills, only the GPx level was significantly elevated in fish exposed in Cezarka pond for 30 days. At day 90, significantly lower GPx and higher SOD activities were observed in the gills of exposed fish compared with the control fish. Significantly lower levels of activity of CAT, GR, and GPx (by 1.4-fold, 1.2-fold, and 1.3-fold, respectively) and higher levels of SOD (by 1.6-fold) in the gills of exposed fish were observed when compared with the control fish at day 180. The TBAR level in the gills was significantly higher at 360 days of exposure. However, no significant differences were found in terms of antioxidant enzyme activities in this period. At 180 days, both male and female-exposed fish had higher levels of SOD and lower levels of GPx, GR, and CAT than control fish. However, the only significant differences were in the elevation of SOD in females and the reduction of GPx in males (Table 2).

In the intestine, a significantly higher level of CAT activity was found in Cezarka fish on day 30, but the level was lower after 360 days of STP exposure. The activity of GPx was significantly lower on day 360 of exposure. At 360 days, both male and female-exposed fish had lower levels of CAT and GPx, but the reductions were significant in only males for CAT and females for GPx (Table 2).

In the muscles, only SOD activity was significantly elevated in exposed fish on day 30. The TBAR levels exhibited significantly larger differences from the controls on day 90 and 180.

Glutathione-S-Transferase Activity

Table 2 shows the changes in the GST activity in different fish tissues after long-term exposure to STP effluent. Hepatic GST activity exhibited a bi-phasic variation in exposed fish, indicating a significant decrease in GST activity at 30 days and an increase at 90 and 360 days. The GST activity in the gills, intestine, and muscle tissues of effluent-exposed fish was significantly higher than in the control fish at 90 (by 1.4-fold), 180 (by 1.24-fold), and 360 (by 2.6-fold) days.

Histopathology

The results of the histopathological examination are shown in Fig. 3. The gonads of control and effluent-exposed male fish were in the same developmental stage (up to sperms) after 360 days of exposure. In contrast, the ovaries of STP effluent-exposed females were more advanced compared

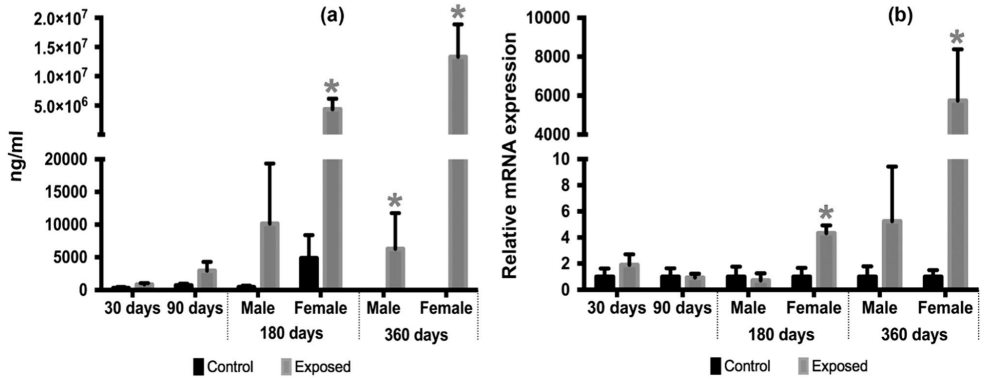

Fig. 4 Effect of STP effluent on the concentration of vitellogenin (VTG) in blood plasma ($n = 12$) (**a**) and VTG relative mRNA expression in liver (**b**) ($n = 10$). Data are means ± SEM, $n = 12$. An asterisk corresponds to significant differences between exposed and corresponding control group ($^*P < 0.05$)

Table 3 Effect of STP effluent on hematological parameters in fish at day 90, 180, and 360 from the control and Cezarka ponds

Time points	90 days	180 days			360 days		
Fish	Juvenile ($n = 12$)	♀ ($n = 5$)	♂ ($n = 7$)	♀ and ♂ ($n = 12$)	♀ ($n = 6$)	♂ ($n = 6$)	♀ and ♂ ($n = 12$)
PCV (l/l)							
Control	0.40 ± 0.01	0.31 ± 0.10	0.33 ± 0.20	0.33 ± 0.01	0.26 ± 0.1	0.32 ± 0.20	0.29 ± 0.01
Exposed	0.33 ± 0.02*	0.32 ± 0.10	0.42 ± 0.2*	0.37 ± 0.02	0.30 ± 0.20	0.36 ± 0.10	0.34 ± 0.01*
Hb (g/l)							
Control	84.1 ± 2.8	57.7 ± 1.6	60.0 ± 3.2	59.0 ± 2.0	50.5 ± 2.5	63.4 ± 3.7	58 ± 3.0
Exposed	71.1 ± 4.4*	57.4 ± 1.3	72.9 ± 4.3*	65.1 ± 3.2	55.7 ± 3.5	71.4 ± 2.9	64.8 ± 3.1
RBC (T/l)							
Control	1.31 ± 0.03	1.48 ± 0.04	1.45 ± 0.06	1.46 ± 0.04	1.23 ± 0.07	1.49 ± 0.06	1.38 ± 0.06
Exposed	1.14 ± 0.06*	1.11 ± 0.05*	1.47 ± 0.10	1.29 ± 0.08	1.27 ± 0.08	1.39 ± 0.06	1.34 ± 0.05
WBC (G/l)							
Control	185.3 ± 20.1	116.0 ± 20.4	91.4 ± 14.8	101.7 ± 12.1	100.9 ± 9.4	78.9 ± 11.0	88.1 ± 7.9
Exposed	84.3 ± 7.8*	42.6 ± 4.1*	37.3 ± 5.1*	39.9 ± 3.2*	28.9 ± 5.6*	18.7 ± 2.5*	23.0 ± 2.9*
MCV (fl)							
Control	302.7 ± 8.9	210.8 ± 6.4	231.1 ± 11.6	222.6 ± 7.6	213.0 ± 11.2	212.6 ± 6.7	212.8 ± 5.8
Exposed	296.6 ± 16.4	293.4 ± 13.5*	292.1 ± 24.8	292.8 ± 13.4*	239.1 ± 3.9	261.9 ± 8.1*	252.4 ± 5.9*
MCH (pg)							
Control	64.8 ± 3.2	39.0 ± 1.7	41.5 ± 2.0	40.4 ± 1.3	41.2 ± 1.6	42.6 ± 1.6	42.0 ± 1.1
Exposed	63.1 ± 3.5	52.0 ± 2.3*	50.7 ± 3.7*	51.3 ± 2.1*	43.8 ± 1.4	51.5 ± 1.8*	48.3 ± 1.6
MCHC (l/l)							
Control	0.21 ± 0.03	0.18 ± 0.01	0.18 ± 0.00	0.18 ± 0.01	0.19 ± 0.01	0.20 ± 0.00	0.20 ± 0.00
Exposed	0.21 ± 0.01	0.18 ± 0.00	0.17 ± 0.01	0.18 ± 0.01	0.18 ± 0.00	0.20 ± 0.00	0.19 ± 0.00

Erythrocyte count (RBC), hematocrit value (PCV), hemoglobin concentration (Hb), leukocyte count (WBC), mean erythrocyte volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC)

Note: Data are presented as mean ± SEM; an asterisk corresponds to significant differences between the exposed and corresponding control group, $^*P < 0.05$

with the control fish. The control females uniformly exhibited primary oocytes only, but in exposed fish scattered oocytes in the vitellogenic stage were visible.

Additionally, the structure of the ovaries in exposed females was more irregular compared with that of the controls, showing interstitial edema, higher amounts of

Table 4 Effect of STP effluent on biochemical blood plasma parameters in fish from control and Cezarka pond at 30 days

Biochemical blood parameters at 30 days for control and exposed fish		
Parameters	Control	Exposed
ALB (g/l)	7.0 ± 0.7	8.2 ± 0.3
ALT (U/l)	36.8 ± 8.6	26.5 ± 3.3
AST (U/l)	125 ± 13	188 ± 25*
Ca^{2+} (mmol/l)	2.66 ± 0.07	2.78 ± 0.04
LDH (u/l)	2277 ± 399	4826 ± 1263*
NH_3 (mmol/l)	609 ± 49	704 ± 59
CREA (mmol/l)	40.6 ± 6.9	78.8 ± 8.6*
TP (g/l)	22.4 ± 1.7	26.1 ± 1.3
PHOS (mmol/l)	5.2 ± 0.4	5.0 ± 0.2
Mg (mmol/l)	1.9 ± 0.1	2.0 ± 0.1
TRIG (mmol/l)	5.0 ± 0.8	2.0 ± 0.2*
LAC (mmol/l)	9.8 ± 0.6	16.1 ± 0.7
GLU (mmol/l)	4.18 ± 0.57	2.72 ± 0.33*
CK (U/l)	1173 ± 288	2212 ± 1016
ALP (U/l)	13.3 ± 1.3	8.8 ± 0.7*

Glucose (GLU), total proteins (TP), albumins (ALB), ammonia (NH_3), triglycerides (TRIG), aspartate aminotransferase (AST), alanine aminotransferase (ALT), lactate dehydrogenase (LDH), creatine kinase (CK), lactate (LACT), alkaline phosphatase (ALP), calcium (Ca^{2+}), magnesium (Mg), and inorganic phosphate (PHOS)

Note: Data are presented as mean ± SEM; $n = 12$; an asterisk corresponds to significant differences between the exposed and corresponding control group, $*P < 0.05$

atretic oocytes, and inflammation with mainly macrophages and lymphocytes (Fig. 3). The pathology observations in the liver and gills were not significantly different between the control and exposed fish. However, in three Cezarka-exposed individuals, there was moderate infiltration with lymphocytes and macrophages (mainly perivascular) in the liver. No histopathology changes were evident in the kidneys or spleens.

Vitellogenin

Results of plasma VTG protein concentrations and relative hepatic mRNA expression exhibited the same trends (Fig. 4). The level of plasma VTG was generally higher in males and females from Cezarka pond. However, significant differences were registered in only females after 180 days and in both males and females after 360 days of exposure. The same results were observed in mRNA expression level in the liver. However, due to large fluctuation in the hepatic relative mRNA expression between individuals, the values were not significantly different in males. The highest levels of plasma VTG in males and females were 9.2 and 5600 μg/ml in exposed fish, respectively. The lowest level observed in control fish was 0 μg/ml for both sexes at 360 days.

Hematological Parameters and Biochemical Blood Plasma Parameters

The hematological properties of common carp in the Cezarka pond are shown in Table 3. The levels of Hb and RBC in effluent-exposed fish were significantly lower than in control fish after 90 days (by 13 and 16%, respectively). The numbers of WBC constantly decreased in the exposed group on day 90 (55), 180 (61), and 360 (74%) compared with the control fish. At 180 days of exposure, the levels of MCH in the exposed fish were significantly higher than in the control fish (by 27%). MCV were significantly higher in the exposed animals after 180 and 360 days of exposure. Although both male and female-exposed fish had higher levels of MCV, the elevations were significant in only females at 180 days and males at 360 days.

The plasma biochemical parameters of the carp exposed to STP discharges were investigated at day 30 only, and the results are shown in Table 4. After 30 days, AST, LDH, and CREA levels were significantly higher (by 1.5-fold, 2.1-fold, and 1.9-fold, respectively) in STP effluent-exposed fish. In contrast, the TRIG, GLU, and ALP concentrations were significantly lower (by 2.5-fold, 1.5-fold, and 1.5-fold, respectively) in STP effluent-exposed fish compared with the control fish. No significant changes in ALB, ALT, Ca, NH_3, TP, PHOS, Mg, LAC, or CK levels were found in the blood plasma of the fish.

Integrated Biomarker Response

The results of IBR and star plots are presented in Fig. 5. The IBR was calculated according to the time points that include the score of each parameter in each tissue. A IBR indicates to a high response. The IBR of exposed fish was much higher than in the control fish in the liver at 30 days (3.15 and 1.57, respectively) and in the intestine at 360 days (3.90 and 2.04). Additionally, the IBR was higher in the gills of exposed fish at 90 days and 180 days and in the livers at 360 days compared with the control fish. The star plots show the response of each biomarker for each tissue and time point (Fig. 5a). Different responses were observed for all of the biomarkers in the liver at 30 days. Furthermore, high responses were noted for GST and GR in the liver at 90 days, as well as SOD, GPx, and CAT in the intestine at 360 days.

Discussion

Occurrence of PPCPs

The highest concentration of PPCPs was detected in spring (30 days of exposure in May), and the lowest was detected

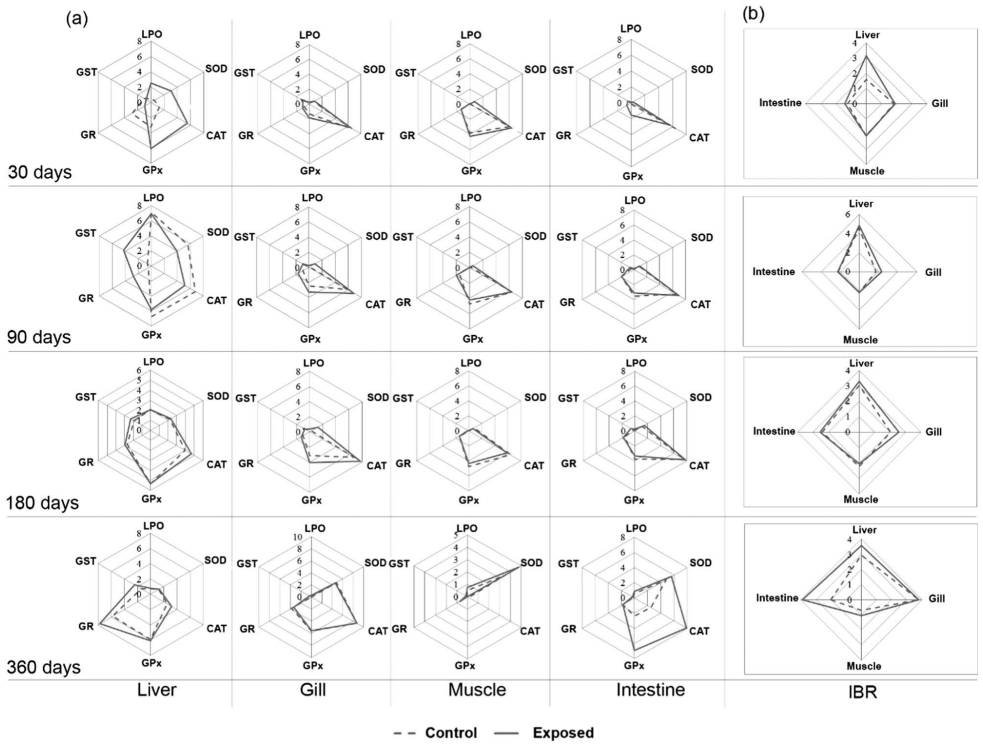

Fig. 5 Star plot for each sampling time (30, 90, 180, and 360 days) in each tissue (liver, gill, intestine, muscle) (**a**); and integrated biomarker response of all six biomarkers, including glutathione S-tranferase (GST), lipid peroxidation (LPO), superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and glutathione reductase (GR) at each sampling time point (**b**)

in autumn (180 days of exposure). The PPCP concentrations measured in Cezarka pond, which receives water from only STP effluent, differed by one to two orders of magnitude compared with the control pond, which receives surface water from the Blanice River. These results are in agreement with literature findings that STPs are a major source of PPCP pollutants in aquatic environments (Zheng and Li 2013).

Irbesartan, telmisartan, tramadol, carbamazepine, and its metabolite trans-dihydro-dixydroxy carbamazepine were the dominant contaminating compounds in Cezarka pond. The variation in PPCP concentrations can be attributed to increased human consumption of PPCPs during winter and spring. Furthermore, the low temperature during this period often results in less efficient removal of compounds during STP treatment (Golovko et al. 2014). Our results are consistent with previous findings of high PPCP concentrations detected in winter compared with summer (Golovko et al. 2014; Koba et al. 2017). Unfortunately, the control pond was not absolutely free of PPCPs due to the practically ubiquitous contamination of surface water by pharmaceutically active substances. However, the total level of PPCPs was low and can be referred to as a background concentration under central European conditions (Ebele et al. 2017).

Morphological Indices

Fish living in polluted environments are believed to reduce their CF because they have to expend energy for detoxification (Fang et al. 2009). It has been demonstrated that the CF declines in fish exposed to environmental pollutants (Khan 2003; Roussel et al. 2007). Fish exposed to STP effluents in Cezarka pond had higher CFs than those from the control pond. This can be explained by the abundance of STP-related nutrients in Cezarka pond compared with the control pond (Supplementary Material 3), resulting in highly fertile conditions with increased phytoplankton

productivity serving as dietary components for fish. This result is consistent with previous studies indicating that STP led to increased water nutrient loads (Björn Gücker 2006) and CF (Tetreault et al. 2013) for downstream fish.

In contrast to Cezarka, the trophic conditions in the control pond were representative of average aquaculture ponds, with an average natural weight gain of 0.5 kg (without artificial feeding) per season for different sizes of carp. Extreme growth intensity in Cezarka pond led to a high total fish biomass at 90 days. High feeding pressure in this period caused a significant reduction of available natural food. In the following period, high competition for food resulted in a dramatic decrease of CF, reaching comparable levels to the control pond at 180 days. The effect of STP effluent on CF was not different between sexes (Table 1).

The HSI reflects the relative liver size and is linked to the hepatic enzyme activity for the detoxification of pollutant compounds (Li et al. 2010; Yeom et al. 2007). An elevation of HSI was observed in carp that had been captured in a metal-contaminated site (Bervoets et al. 2009; Ozmen et al. 2006). Other studies found elevated HSI values in fish from sites contaminated with PCBs and PAHs compared to fish from uncontaminated sites (Pinkney et al. 2001). However, several natural factors might significantly affect the HSI of fish as well, such as nutrient levels and feeding strategies (Turano et al. 2007).

Significantly lower HSIs were observed in the exposed group with an exception of the results at 90 days. The reduction in liver size is likely linked to glycogen depletion due to the fish expending energy for detoxification activities. This assumption is supported by the low level of GLU in the blood plasma of the fish. The results of plasma biochemical parameters (AST, ALP), oxidative stress, and antioxidant enzymes (all parameters) might suggest damage to the liver tissue at 30 days. The histopathological examination after 360 days of exposure revealed a slight but non-significant decrease in the amount of glycogen vacuoles in hepatocytes in exposed fish compared to controls. The higher HSI in exposed fish compared with controls at 90 days might be explained by the enormous excess of natural food, which allow for deposition of excess energy in the liver. This result is consistent with the highest CF value observed at 90 days in fish from Cezarka pond. The same trend of HSI was observed in both male and female fish from Cezarka pond (Table 1).

The GSI has been used as a biomarker in aquatic organisms for exposure to environmental estrogens. Correlations have been established between the inhibition of testicular growth and the potency of estrogenic compounds in male fish (Gimeno et al. 1997). Field studies have reported that estrogenic chemicals decrease the GSI of exposed fish (Kukkonen et al. 1999). The GSI was lower among fish exposed to river water with high concentrations of estrogenic compounds, such as nonylphenol, bisphenol A, and 17β-estradiol (Hassanin et al. 2002). However, in this study, GSIs were higher in exposed female (at 180 and 360 days) and male fish (at 360 days) from Cezarka pond than in control fish (Table 1).

These results might indicate that the nutrient-rich environment in Cezarka pond forced exposed fish to grow larger gonads or to reproduce earlier compared to fish in the control pond. To confirm this hypothesis, fish gonad histology, VTG expression, and its concentration level were assessed (see section "Gonad histology and VTG biomarkers"). The decrease of GSI in control fish at 360 days can be explained by the expense of energy for the winter period. In Cezarka pond, warmer water from the STP effluent helped the fish to grow. The same trend of GSI was observed between male and female fish from Cezarka pond.

Gonad Histology and VTG Biomarkers

Previous studies have demonstrated an increase in VTG concentration in female common carp in streams receiving effluent water from STPs (Petrovic et al. 2002). In the present study, the gonads of exposed females were more developed compared with fish in the control group. VTG levels of females in the effluent-exposed group were higher at days 180 and 360 than those in the control group. The highly elevated level of VTG in exposed female fish and irregular ovary structures suggest a synergistic effect of xenoestrogens (Rankouhi et al. 2002). In addition, the high concentration of VTG in males is definitely linked to the action of the PPCP mixture with eventual estrogenic action, even if there were no structural changes visible in male gonads. Early gonad maturation of fish in the pond receiving water from the STP might lead to changes in the spawning success. Additional studies are necessary to examine regarding the reproduction success of fish under long-term exposure to a biological pond receiving only STP effluent.

Oxidative Stress and Antioxidant Enzyme Biomarkers

Considering the trend of each biomarker for all tissues and sampling time points, the effluent-exposed fish showed a general elevation of TBARs and SOD and a decline in CAT, GPx, and GR compared with control fish. SOD is known to be the first line of defense in response to the conversion of superoxide anion radicals to molecular oxygen and hydrogen peroxide (Fridovich 1989). The following steps are completed by other enzymes, such as CAT, GPx, and GR. The decline of CAT, GPx, and GR indicates that the fish's abilities to protect cells from hydrogen peroxide were reduced. The inhibition of CAT, GPx, and GR was reported in fish exposed to carbamazepine (Li et al.

2009), resulting in increased H_2O_2 in the cells (Ahmad et al. 2000). This result is consistent with hypotheses that elevated SOD activities can be combined with a decrease in CAT and GPx (Huang et al. 2007; Pandey et al. 2003; Stanic et al. 2006).

Different intensities of oxidative stress and antioxidant enzymes for different tissues and exposure periods have been indicated. The largest amount of PPCPs detected at 30 days obviously induced changes in oxidative stress and the system responses of antioxidant enzymes in the fish livers. The liver was the most responsive tissue in this period, and significant differences were noted between the control and effluent-exposed fish in all of the measured biomarkers.

At 180 days, most effects were evident in the gill tissue, which followed the same trend as the liver (an elevation in SOD and a decline in CAT, GPx, and GR). Interestingly, significant changes in SOD, CAT, GPx, and GR antioxidant enzymes did not lead to oxidative damage investigated according to TBAR levels. The opposite situation occurred at day 360 with increased TBARs but no respective alteration of enzyme activities in the gills. One potential explanation for this finding is that the antioxidant system of fish reacts to early exposure, and then oxidative damage follows. After 180 days of exposure, antioxidant enzymes were activated, but no damage occurred after the activation. In a later period, while the antioxidant system stabilized, oxidative damage caused by previous disturbances was evident.

Fewer changes were observed in intestine and muscle tissues. A significant reduction in the GPx level was detected at 360 days of exposure in combination with increasing CAT, which may be related to the damage induced by a high density of parasites (tapeworm—*Caryophyllaeus* sp.) detected in the intestines in this period (Supplementary Material 6). The decrease of GPx level has been observed in intestinal parasitic infections in humans (Mahittikorn et al. 2014).

Phase II Detoxification Enzyme (GST) in Liver, Gill, Intestine, and Muscle

The liver was the most responsive tissue in terms of GST activity. Considering the changes in GST for all tissues and sampling times, in most cases, high levels of GST were observed in exposed fish compared with the control fish. This result is consistent with previous studies of goldfish (Kubrak et al. 2012) and common carp (Schmidt et al. 2004) exposed to cobalt and polychlorinated biphenyl. Interestingly, low levels of GST were observed in the exposed fish livers at 30 days of exposure compared with the control fish. The result suggests an inhibition of GST due to the highest concentration of PPCPs detected in this period. A previous study also detected the inhibition of GST in the liver of common carp exposed to the herbicide quinclorac (Cavalheiro de Menezes et al. 2012). The lack of differences observed at day 180 can be explained by the lowest concentration of PPCPs detected in Cezarka pond at this time point.

Hematological Parameters and Biochemical Blood Plasma Parameters

The same trend was observed between male and female fish for most of changes in hematological parameters in Cezarka pond. The reduction of Hb and RBC at only day 90 could be linked with the variation of total PPCPs, which was higher in this period compared with later periods in Cezarka and the control pond. Unfortunately, hematological parameters at day 30 were not investigated due to the limited amount of blood samples of juvenile fish. Several studies have described a decrease in RBC and Hb in carp (Sudova et al. 2009), rainbow trout (Li et al. 2011a), and striped catfish (*Mystus vittatus*) (John 2007) exposed to contaminated environments.

Decreases in Hb concentration and RBC count levels are linked to anemia (Li et al. 2011a). Changes in WBC are recognized as a sensitive indicator of environmental stress (Cole et al. 2001). Declines in WBC detected at 90, 180, and 360 days reflect the constant disturbance of the immune system. Additionally, the increases in MCV and MCH (180 days) and PCV and MCV (360 days) suggest changes in the internal equilibrium of exposed fish. The effect of 62 PPCPs occurring in water may induce both a synergistic effect and an antagonistic effect. Therefore, the variation of MCV and MCH may not exactly reflect the trend of the total PPCP concentration in each sampling event, which was higher at 90 days. These changes may be caused by other factors, such as the occurrence of parasites detected at 360 days.

Due to numerous significant changes detected at day 30, the biochemical parameters in blood plasma were additionally investigated. The increase in AST and decrease in ALP may be linked to alterations in the liver. The increase in AST activity in plasma may be due to liver damage, which results in the liberation of intercellular enzymes and elevated plasma aminotransferase levels (Rao 2006b). Elevated AST has been observed in common carp exposed to trifluralin (Poleksić and Karan 1999) and deltamethrin (Velíšek et al. 2006) and was proposed as a biomarker of acute hepatic damage (Abdel-Tawwab et al. 2013).

ALP is produced by cells lining the small bile ducts in the liver. The decline in ALP may be linked to a decrease of this function in the liver. Previous studies have indicated a decrease in ALP level in the blood plasma of common carp exposed to chemical stress conditions (Dobsikova et al.

2006). In addition, the increasing level of LDH in the present study could indicate tissue damage, hypoxic conditions, and a switch to anaerobic metabolism (Nemcsok and Benedeczky 1990; Saravanan et al. 2011).

LDH is a tetramer of anaerobic glycoses. It is crucial for muscle physiology, particularly under conditions of chemical stress when a high level of energy may be required over short periods of time (Monteiro et al. 2007). Other changes suggest disturbances in kidney function (CRE) and energy metabolism (GLU, TRIG). Creatine is mainly removed from the blood by the kidneys. Thus, increased CRE levels indicate that pollution affects kidney function (Abdel-Tawwab et al. 2013). Decreases in GLU and TRIG were also observed in common carp exposed to antimicrobial peptide (Dong et al. 2015).

Integrated Biomarker Response

The IBR index is often used to describe general effects and to assess ecological risk by combining the results of a wide set of biomarkers (Beliaeff and Burgeot 2002). The usage and efficiency of this index have been demonstrated in many studies (Ferreira et al. 2015; Li et al. 2011b). In the present study, the IBR values clearly reflected a high response in the liver during the first period of exposure, when fish were small (early stage) and sensitive to the polluted environment. The liver is the most important organ for metabolizing PPCPs.

It is obvious that high PPCP concentrations in Cezarka pond affected the response of the fish livers. After this period, a small effect was observed on the gills. The adaptation of fish can be explained by warm weather periods (summer and autumn), when the growth conditions of fish were optimal and there were seasonally low PPCPs in Cezarka pond. A strong response was noted in the intestine at 360 days of exposure. This finding could be related to the high density of parasites detected in the intestine.

Conclusion

Our data have demonstrated that the biological pond Cezarka contained significantly higher PPCP concentrations compared with the control pond. Seasonal variations of PPCPs were observed, with the highest concentrations occurring in spring. Several effects of the treated effluent on exposed fish were observed in this scenario. The greatest effects were found in fish in the early stages of exposure, with rapid changes observed in numerous parameters. The observations of oxidative stress, antioxidant enzymes, and biochemical blood plasma parameters might indicate alterations in liver metabolism. The initial rapid response to the effluent environment was followed by a weakening biomarker response, which corresponds to a decrease of PPCP load. However, indications of endocrine disruption and oxidative stress were noted at later stages.

Despite the numerous physiological alterations observed in exposed fish, the fish growth was greater in the exposed pond than the control pond. The effects of pollutants and consequent physiological alterations in fish organs were probably compensated by the high nutrient content with a high availability of natural food. At the end of the experiment, the exposed fish were in good condition. However, their reproduction ability remains to be tested.

Although modest negative effects were observed in fish after long-term exposure in the STP effluent reservoir, the impact of the effluent after retention in the biological pond cannot be ignored in the receiving ecosystem. The effects of STP effluent on natural water bodies are highly dependent on the dilution factor, which must be considered in low-flow receiving streams. To minimize the impact, longer retention of water in biological ponds and balanced microbial, macrophyte, plankton, and fish communities must be maintained to achieve maximum degradation and removal capacity.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no competing interests.

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CHAPTER 3

BIOLOGICAL EFFECTS OF STP EFFLUENT ON BROWN TROUT LIVING IN AN STP EFFLUENT DOMINATED STREAM

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Biomarker response, health indicators, and intestinal microbiome composition in wild brown trout (*Salmo trutta* m. *fario* L.) exposed to a sewage treatment plant effluent-dominated stream

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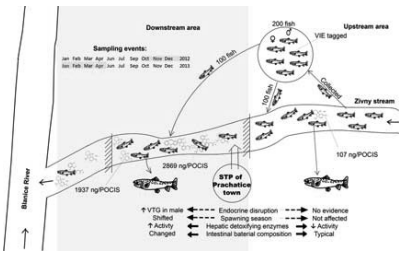
HIGHLIGHTS

- Tagged native/wild trout from an unpolluted site were exposed to STP effluent.
- Elevated hepatic BFCOD activity was linked to the total concentration of 53 PPCPs.
- Elevated plasma VTG level in males and shifted the spawning season in females.
- Downstream fish had higher fat content but lower total ω-3 acid in muscle tissue.
- Bacteria taxa typical of activated sludge were detected in trout intestinal microbiome.

GRAPHICAL ABSTRACT



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ABSTRACT

Concerns about the effect of sewage treatment plant (STP) effluent on the health of freshwater ecosystems have increased. In this study, a unique approach was designed to show the effect of an STP effluent-dominated stream on native wild brown trout (*Salmo trutta* L.) exposed under fully natural conditions. Zivny stream is located in South Bohemia, Czech Republic. The downstream site of Zivny stream is an STP-affected site, which receives 25% of its water from Prachatice STP effluent. Upstream, however, is a minimally polluted water site and it is considered to be the control site. Native fish were collected from the upstream site, tagged, and distributed to both upstream and downstream sites. After 30, 90, and 180 days, fish were recaptured from both sites to determine whether the downstream site of the Zivny stream is associated with the effects of environmental pollution. Several biomarkers indicating the oxidative stress and antioxidant enzyme activities, cytochrome P450 activity, xenoestrogenic effects, bacterial composition, and lipid composition were investigated. Additionally, polar chemical contaminants (pharmaceuticals and personal care products (PPCPs)) were quantified using polar organic chemical integrative samplers (POCIS). Fifty-three PPCPs were detected in the downstream site; 36 of those were constantly present during the 180-day investigation period. Elevated hepatic 7-benzyloxy-4-trifluoromethylcoumarin-O-debenzyloxylase (BFCOD) (after 90 days) and blood plasma vitellogenin concentrations in males were detected in fish downstream of the STP effluent during all sampling events. An increase in the

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fishes' total fat content was also observed, but with low levels of ω-3 fatty acid in muscle tissue. Two bacterial taxa related to activated sludge were found in the intestines of fish from downstream. Our results show that Prachatice STP is a major source of PPCPs in the Zivny stream, which has biological consequences on fish physiology.

1. Introduction

Pharmaceuticals and personal care products (PPCPs) and their transformation products are regularly released by sewage treatment plants (STPs) into surface waters (Calisto and Esteves, 2009; Halling-Sørensen et al., 1998). The abundance of PPCPs and other pollutants in the aquatic environment has increased concerns about their potential adverse effects on non-target species (Anway et al., 2005; Garcia-Reyero et al., 2011; Schoenfuss et al., 2002). The total mass load of PPCPs in STPs highly depends on the season (Golovko et al., 2014a; Sun et al., 2016) as well as the urbanization level of the town served by the STP (Subedi et al., 2017). The removal efficiency of each STP does not allow complete cleaning of active compounds from water, even with advanced technology (Rozman et al., 2017; Yang et al., 2013), and trace levels of chemicals are released into the aquatic environment. For example, the average removal efficiency of diclofenac and carbamazepine was 0% in four large STPs in south Bohemia (2009–2011) and about 60% and 4% in the STP in central Bohemia (2015), respectively (Rozman et al., 2017). In the UK, the PPCP removal efficiency of the Cilfynydd and Coslec STPs was about 70% and 85%, respectively, and this contributes about 3 and 1 kg of PPCPs daily to the Taff and Ely Rivers, respectively (Kasprzyk-Hordern et al., 2009). The final environmentally relevant concentrations of PPCPs from municipal wastewater are highly dependent on the dilution factor in the respective recipient. Thus, small streams (low dilution factor) that are receiving STP water discharges can be assumed to be the worst-case scenario for evaluating responses of aquatic organisms to PPCP contaminants (Brooks et al., 2006).

A wide range of PPCP groups have been reported to be present in STP discharge, such as antibiotics (Golovko et al., 2014a, 2014b), psychoactive compounds (Grabicova et al., 2017), fibrate (Jelic et al., 2011), and estrogen compounds (Sun et al., 2014). Thus, the risks of disturbances in behavior, endocrine system, intestinal bacteria flora, and fatty acid composition may be expected in fish exposed to a PPCP-contaminated environment. Recently, cytochrome (CYP)1A, CYP3A-like enzymes and a complex that varies in response to oxidative stress and antioxidant parameters such as lipid peroxidation (LPO), catalase (CAT), superoxide dismutase (SOD), glutathione reductase (GR), glutathione peroxidase (GPx), glutathione S-transferase (GST), have been frequently used to study detoxification and oxidative stress status in fish impacted by PPCPs exposure (Li et al., 2011a; Nava-Álvarez et al., 2014; Thibaut et al., 2006). Vitellogenin (VTG) is an effective indicator for endocrine disturbance linked to the occurrence of estrogenic compounds (Hansen et al., 1998). Changes in fatty acid composition in tissues are considered as suitable biomarker for the occurrence of fibrate compounds (Olivares-Rubio and Vega-López, 2016). While changes of bacterial types in both the lotic environment (Drury et al., 2013) and in fish intestine are useful indicators for occurrence of antimicrobial compounds.

Several studies have investigated the consequences of STP effluent that can occur in fish (Bruneau et al., 2016; Grabicova et al., 2014; Hapke et al., 2016; Jiang et al., 2014). A large number of observations have been obtained from chronic and acute toxicity tests performed under laboratory conditions, where a single, or at most a few, different compounds can be studied simultaneously. Some laboratory experiments attempt to mimic environmental situations via feed supplements (Daouk et al., 2011), water sampled from STP effluent (Harding et al., 2016), or caging experiments up- and downstream of an STP (Vincze et al., 2015). However, these studies do not entirely reflect the real situation with wild fish living close to STP effluent, where the possible mixture toxicity can occur. Moreover, any spatial, feeding or behavioral restrictions in traditional experiments may lead to misinterpretation of collected data. The method of randomly collecting fish from investigated sites may be applied (Bruneau et al., 2016). However, the disadvantage of this method is that the compared fish are not at the same initial condition. Moreover, there is also a risk of obtaining immigrant fish.

In the present study, a new approach was used to determine the effect of STP effluent on wild fish in a small stream under real conditions. Zivny stream is a small stream located in the urbanized area of Prachatice town in the Czech Republic. Recently, the occurrence of industrial and cosmetic chemicals (e.g. PCB and DDT and their degradation products) and PPCPs has been observed in surface water, sediment, and biota in Zivny stream (Fick et al., 2017; Grabicova et al., 2015; Grabicova et al., 2017; Li et al., 2011b). Psychoactive compounds and industrial chemicals were found to bioconcentrate in fish muscle tissue (Grabicova et al., 2017; Li et al., 2011b) and benthic organisms (*Hydropsyche* and *Erpobdella octoculata*) (Grabicova et al., 2015) in Zivny stream downstream of the STP discharge. The first aim of the present study was to characterize the current Zivny stream PPCP contamination level, and the second aim was to evaluate the effect of pollution from STP effluent on brown trout in selected locations along Zivny stream. Native fish (brown trout, *Salmo trutta* m. *fario* L.) were collected from the upstream site that had a very low pollution level (Grabicova et al., 2015; Li et al., 2011b), tagged, and redistributed at both upstream and downstream sites. Using this approach, we believe, that most of the living conditions for exposed fish remain the same as for fish in the control group, except for those affected by the STP effluent. Therefore, the above-mentioned disadvantages of traditional study methods are substantially reduced.

2. Materials and methods

The information on instruments and all used chemicals in the present study is listed in Supplementary Material 1.

2.1. Experimental setup

The Prachatice STP serves a population of 11,000 (population data from 2012) in South Bohemia, Czech Republic. Sewage water treatment involves primary mechanical filtration and sedimentation followed by activated sludge treatment (Prachatice STP, 2017). The commercial activity consists of light industry (food, manufacturing of machinery, and electronics), municipal hospital, along with agriculture in the surrounding area. The STP effluent contributes approximately 25% of the water in the Zivny stream (a tributary of the Blanice River). The stream is 13 km long, and the average depth and width are 30 cm and 3 m, respectively. Its flow rate varies between 0.150 and 0.600 m^3/s.

The location of Prachatice STP and sampling localities are shown in Fig. 1. The upstream site (approximately 1 km long, 3 km upstream from the STP) was used as a control site because of the low contamination level (Grabicova et al., 2015). The downstream site (approximately 5 km long, 0.1 km starting from the STP discharge point and ending at the barrier before to the confluence with the Blanice River) was used as the exposure site because it receives the discharged water from STP effluent. At both sites, concrete cross barriers (from the bottom of the river and about 1 m above the water surface) are present preventing fish from escaping from the downstream site and the Blanice River to

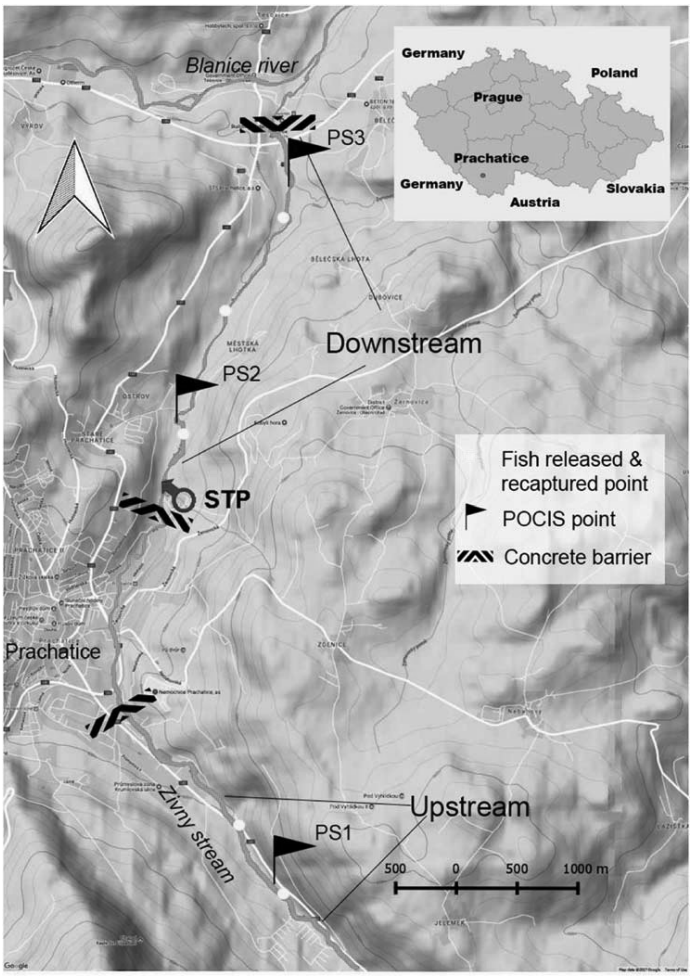

Fig. 1. Map showing the location of Prachatice town in the Czech Republic and the Zivny stream. Fish were distributed and recaptured at two sites: upstream and downstream. PS_{n}, site of passive sampler installation. The map was created using QGIS v2.6.

the control site (Fig. 1). Two hundred experimental fish were captured by electrofishing at the control site, and tagged on the head using visible implant elastomer tags (Northwest Marine Technology, Inc.). Within 1 h of handling, 200 fish were equally redistributed directly into each experimental site (Fig. 1) (Grabicova et al., 2017). The number of tagged fish distributed into both sites is expected not to exceed the resident capacity of each site. Before observation, 12 fish (22 ± 2 cm, 99 ± 32 g) were captured at the control site to determine their initial health status (day 0). The experiment started at the end of October 2012 and continued until the end of April 2013, with three additional sampling events on days 30 (in November), 90 (in January), and 180 (in April) from both the control and downstream sites. There were 36% and 47% of tagged fish that were recaptured after three sampling events at the control and downstream sites, respectively. Twelve tagged fish were recaptured by electrofishing at the control site in each sampling event, while, 24, 11, and 15 fish were collected at the downstream at 30, 90, and 180 days, respectively. The sex of recaptured fish is listed in Table 1. Initially, 24 fish were planned to be collected at the downstream site during each sampling event. However, because of the variable efficiency of electrofishing and loss of fish to predators, the number of collected fish was reduced during subsequent sampling events.

2.2. Passive samplers deployment, extraction, and analysis

2.2.1. Passive samplers deployment

Chemical analyses of surface water from control and downstream sites were performed using Polar organic chemical integrative samplers (POCIS) extracts. Overall, 12 POCIS were installed in the stream at three

Table 1
The information sampling events and water temperature in upstream and downstream sites of Zivny stream over 180 days. The water temperature in the downstream area presented as the average temperature at PS2 and PS3 sites. Data are presented as the mean ± S.E.

Site	Upstream/control site (1 km long, 3 km from STP)				Downstream/STP affected site (5 km long, 0.1 km from STP)		
Sampling events (day)	0	30	90	180	30	90	180
Sampling time	October 2012	November 2012	January 2013	April 2013	November 2012	January 2013	April 2013
Number of collected fish	$n = 12$	$n = 12$	$n = 12$	$n = 12$	$n = 24$	$n = 11$	$n = 15$
Sex	6M/6F	6M/6F	6M/6F	4M/8F	12M/12F	6M/5F	5M/10F
CF, HSI, GSI, CAT, SOD, GR, GPx, GST, TBARs, EROD, BFCOD, VTG	✓	✓	✓	✓	✓	✓	✓
Lotic microbiome	✓	–	–	✓	–	–	✓
Intestinal microbiome	–	–	✓	✓	–	✓	✓
Temperature (°C)	6.9 ± 2.2		1.3 ± 0.8	9.8 ± 1.3	9.2 ± 2.1	4.9 ± 1.0	10.8 ± 1.4

Note: Sewage treatment plant (STP), condition factor (CF), hepatosomatic index (HSI), gonadosomatic index (GSI), catalase (CAT), superoxide dismutase (SOD), glutathione reductase (GR), glutathione peroxidase (GPx), glutathione S-transferase (GST), thiobarbituric acid reactive substances (TBARs), 7-ethoxyresorufin-O-deethylase (EROD), 7-benzyloxy-4-trifluoromethylcoumarin-O-debenzylase (BFCOD).

sites (one upstream as control site [PS1], and two downstream as the STP effluent discharge-affected sites [PS2 and PS3, 4 km away from each other]) (Fig. 1), for 14 days during October, November, January, and April. The sampling period of 14 days was set to achieve a linear uptake of a broad suite of hydrophilic compounds and a representative overview over a longer time span. The passive samplers were installed for 14 days and collected when fish were sampled at days 0, 30, 90, and 180. After the accumulation period, samplers were removed from the canisters and racks, cleaned with ultrapure water, placed in airtight bags, transported to laboratory, and stored at − 18 °C until analyses.

Thermal sensors (Datalogger Minikin T, SN: 10031604) were included with POCIS canisters at the three sites (PS1, PS2, and PS3) during each installation event. Water temperature was recorded twice per day.

2.2.2. POCIS extraction and analysis

POCIS extraction was performed according to the standardized procedure (Fedorova et al., 2014a). Details of extraction, analysis, and analytical method performance are given by Fedorova et al. (2014b) and Alvarez et al. (2004). Briefly, a triple stage quadrupole MS/MS TSQ Quantum Ultra Mass Spectrometer (Thermo Fisher Scientific, San Jose, CA, USA) coupled to an Accela 1250 LC pump (Thermo Fisher Scientific) and an HTS XT-CTC autosampler (CTC Analytics AG, Zwingen, Switzerland) was used for analysis. A Hypersil GOLD phenyl column (50 mm × 2.1 mm ID × 3 μm particles; Thermo Fisher Scientific, San Jose, CA, USA) and a Cogent Bidentate column (50 mm × 2.1 mm ID × 4 μm particles; Microsolv Technology Corporation, Eatontown, NJ, USA) were used for the separation of target analytes. Heated electrospray (HESI-II) in positive and negative ion modes was used for the ionization of the target compounds.

2.3. Fish tissue sampling

Body, gonad and liver weights and full length of fish were recorded (Supplementary Material 2). The condition factor (CF), hepato and gonado-somatic indexes (HSI and GSI) for each fish were calculated according to White and Fletcher (1985). Fish were sacrificed by severing the spinal cord. Liver tissue (approximately 2 g), gills from both sides and muscle samples were taken and stored at − 80 °C for further analysis. Blood was taken via a caudal vein puncture using a heparinized syringe. An aqueous solution of heparin sodium salt (5000 IU/mL) at 0.01 mL/mL blood was used to stabilize the samples. Blood samples were centrifuged (837 *g*, 10 min, 4 °C) to obtain plasma, which was stored at − 80 °C to determine the total plasma VTG concentration. When all fish samples were collected after exposure periods (180 days), all biomarkers were analyzed at one time.

2.4. Lipid content and fatty acid analysis

Fatty acid and lipid content analysis were determined after 90 days of exposure in six randomly chosen fish from each site. The lipid extraction was performed in duplicate as described by Hara and Radin (1978) and lipid content was quantified gravimetrically. For fatty acid analyses, methylation of total lipids was performed with BF_3 (Sampels and Pickova, 2011). Fatty acid composition was analyzed using gas chromatography (Trace Ultra FID; Thermo Scientific, Milan, Italy) on a BPX-70 50-m fused silica capillary column (i.d., 0.22 mm; film thickness, 0.25 μm; SGE, USA). The peaks were identified by comparing sample retention times to those of the standard mixture GLC-68-A (Nu-Chek Prep, Elysian, USA) and other authentic standards (Nu-Chek Prep, Elysian, USA).

2.5. Biomarkers

2.5.1. Preparation of post-mitochondrial supernatant and microsomal fraction

The post-mitochondrial supernatant (PMS) was obtained as described by Howcroft et al. (2009). Liver (~0.15 g) and gill (~0.2 g) samples of each fish were individually homogenized (1.5 mL K-phosphate 0.1 M buffer, pH 7.4). From the homogenate, 100 μL were taken for LPO determination. The remaining tissue homogenate (1.4 mL) was centrifuged at 10,000*g* for 20 min at 4 °C to isolate the PMS.

Fish hepatic microsomes were prepared by differential centrifugation (Burkina et al., 2013). Microsomal fractions were immediately frozen and stored at − 80 °C for analysis. The protein levels were estimated spectrophotometrically using the method described by Smith et al. (1985), with bovine serum albumin as the standard. The microsomes were diluted to obtain a protein concentration of 5 mg/mL, and the post-mitochondrial supernatant was diluted to obtain a protein concentration of 10 mg/mL.

2.5.2. Biochemical assays

2.5.2.1. Microsomal enzyme activities. Hepatic CYP activity was determined using fluorescent-based catalytic assays for two substrates. CYP1A1 activity was estimated using 7-ethoxyresorufin (ER) as specific substrates (Kennedy and Jones, 1994). A 7-benzyloxy-4-trifluoromethylcoumarin-O-debenzylase (BFCOD) reaction was performed to identify CYP3A-like activity (Renwick et al., 2001). The final reaction volume was 250 μL. The reaction mixture contained potassium phosphate buffer (50 mM, pH 7.4), hepatic microsomal protein (0.2 mg), BFC (12.5 μM), and NADPH (1 mM).

The fluorescence detector (Infinite 200 – Photometer TECAN) was used for detection of resorufin (excitation/emission 544/590 nm) and HFC (excitation/emission 410/538 nm). Enzymatic activities were expressed as pmol of resorufin or HFC formed per min and per mg of microsomal proteins (limits of detection were 2 and 1 pmol/min for resorufin and HFC, respectively).

2.5.2.2. Oxidative stress and antioxidant parameters. The LPO assay (measured as thiobarbituric acid-reactive substances; TBARs) was based on

the methods described by Ohkawa et al. (1979) by measuring malondialdehyde (MDA) at 535 nm.

The PMS was used to measure enzyme activities of SOD, CAT, GR, GPx, and GST. SOD activity was determined based on the method described by Ewing and Janero (1995), and was assessed spectrophotometrically at 420 nm. The CAT activity assay, using the spectrophotometric measurement of H_2O_2 breakdown, measured at 240 nm, was performed following the method of Claiborne (1985). The GST activity was determined based on the method described by Habig et al. (1974), and adapted to the microplate reader by Frasco and Guilhermino (2002). The GR and GPx activity was determined based on the method described by Cribb et al. (1989) and Mohandas et al. (1984), respectively. The enzyme activity was quantified by measuring the disappearance of NADPH at 340 nm. Details on the concentration of substrate, protein, and preparation of reaction mixtures are given in Supplementary Material 3.

Preparations for measuring of antioxidant enzyme parameters were carried out on ice and measured at 20 °C. Reactions were performed in 96-well plates with a total volume of the reaction mixture of 300 μL. A microplate reader (Infinite 200 – Photometer TECAN) was used for determination of all selected microsomal enzyme activities and antioxidant enzymes activities in PMS. The rate of increase/decrease in absorbance at each well was linear over time.

2.5.2.3. Enzyme-linked immunosorbant assay procedure. The total concentration of plasma VTG in brown trout was individually measured using an enzyme-linked immunosorbent assay (ELISA) kit. Blood plasma samples were diluted according to ELISA manual recommendation and incubated in pre-coated 96-well microtiter plates, according to the manufacturer's instructions (No.: V01004402). The standards were prepared by diluting VTG to the desired concentrations using dilution buffer (from 200 to 0.39 ng/mL; $R^2 = 0.996$) and 100 μL was added to the assay, which was conducted in duplicate. The standard curve fitting (from 0.054 ng/mL to 18 mg/mL) provided an opportunity to calculate the VTG concentration in the plasma. Absorbance was measured using a microplate reader (Infinite 200 – Photometer TECAN) at 405 nm.

2.6. Lotic microbiome composition and taxonomic characterization of fish intestinal microbiome

2.6.1. Lotic microbiome composition

For taxonomic characterization of the lotic microbiome, the samples were taken at the beginning of the experiment and at day 180 from the surface of 10 randomly selected pebbles found at sampling sites (PS1, PS2, and PS3). The natural lotic biofilm samples were scraped from the whole surface of approximate egg size pebbles rinsed previously using sterile water and transported frozen at −20 °C for further processing.

2.6.2. Taxonomic characterization of fish intestinal microbiome

For taxonomic characterization of the fish intestinal microbiome, intestinal contents (approximately 0.25 g) from the middle part of intestine were collected on days 90 and 180 and frozen at −20 °C for further processing.

2.6.2.1. DNA extraction. Total DNA was extracted from the scraped pebble samples and from the intestine contents (0.25 g) using PowerBiofilm® DNA Isolation Kit and PowerFecal® DNA Isolation Kit (MO BIO laboratories), respectively, according to manufacturer's instructions and used as a template for 16S rDNA amplicon library preparation.

2.6.2.2. Library preparation and sequencing. The PCR amplification was performed in two steps as described by Baldrian et al. (2012). The universal primers 515F (5′-GTGCCAGCMGCCGCGGTAA-3′) (Turner et al., 1999) and 907R (5′-CCGTCAATTCCTTTRAGTTT-3′) (Muyzer et al., 1995) were used to amplify the V4–V5 region of 16S rDNA from isolated total DNA samples in the first PCR (reaction composition and cycling conditions are shown Supplementary Material 4). The composite primers (Supplementary Material 5) containing sample tags, separated from primers by the spacer (Parameswaran et al., 2007) were used in the second PCR. The gel-purified second PCR products were additionally concentrated using the MinElute PCR Purification Kit (QIAGEN, Germany). The DNA concentration was measured on Qubit 2.0 Fluorometer (Life Technologies, USA). An equimolar mix of all samples was subjected to sequencing using the Illumina MiSeq platform (paired-end reads, 2 × 250 bp).

The total DNA was isolated and respective 16S rDNA sequences were obtained from intestinal samples of 44 brown trout captured from control and downstream sites at 90 days (10 and 12 fish, respectively) and 180 days (11 and 11 fish, respectively). Dataset of 16S rDNA sequences for environment assessment was obtained by sequencing of the DNA library prepared from lotic biofilm samples scraped from the surface of pebbles control and downstream the STP effluent at the start of the experiment (day 0) and at day 180. Sequencing data have been deposited in the MG-RAST public database (http://metagenomics.anl.gov/under accession numbers mgp81691). After filtration and processing, the results were expressed as the relative abundance values of individual taxa per each sample. Taxa exceeding the relative abundance value of 0.5% in at least one sample were considered to be significant and were included in further evaluation (Supplementary Material 6).

2.7. Statistical analysis

Statistical analyses were performed using SAS version 9.3 (SAS Institute Inc., Cary, NC, USA). Assumptions of normality and homogeneity of variances of the parameters studied were tested using the Shapiro–Wilks/Kolmogorov-Smirnov test and Levene's test, respectively. Logarithmic transformation was applied to the non-normally distributed variables to improve normality before further analysis. Treatment effects were evaluated using the mixed model analysis of variance (ANOVA). The model included fixed effects of experimental sites and sampling occasion, and random effect of sex. Least squares means (LS-means) of fixed effects were used to determine differences between groups. Back transformed LS-means and confidence interval are presented in the results. For the comparison of fatty acid composition in fish from control and downstream sites, the Student's unpaired *t*-test was used if the normality was satisfied. If not, the Mann-Whitney *U* test was used. The effect of treatment was considered significant if $p < 0.05$. Additionally, data from individual fish were subjected to a multivariate analysis. The principal component analysis (PCA) was used to assess an overall variance. The correlation matrix of the data was constructed using SAS version 9.4 (SAS Institute Inc., Cary, NC, USA). A stepwise discriminant analysis was used to select a subset of variables for use in discriminating between locations with high (above 1000 ng/POCIS) and low (below 200 ng/POCIS) levels of contamination.

2.7.1. Integrated biomarker response

To integrate all results from different biomarkers for a better understanding of the overall effect of STP effluent on fish, the integrated biomarker response version 2 (IBRv2) was calculated according to Sanchez et al. (2013). The results of the data standardization procedure necessary for IBR calculation are presented in star plots. In this study, the results of six biomarkers including TBARs, SOD, CAT, GPx, GR, and GST were used to calculate the IBR index in gills. For the liver, two additional biomarkers ethoxyresorufin-O-deethylase (EROD) and BFCOD were included.

2.7.2. Sequencing data processing

The sequencing data were processed as described previously (Zifcakova et al., 2016). Consensus sequences were constructed for each cluster and the closest hits at a genus level (best hit similarity

≥97%) were identified using blastn (Altschul et al., 1990) and compared against the Ribosomal Database Project (RCoreTeam R, 2016) and the SILVA Update Release 123.1 (Quast et al., 2013; Yilmaz et al., 2014) databases. The closest higher taxon was used when the genus assignment was not possible. Nonbacterial and low-coverage sequences (coverage <90% to the reference) were discarded. The bacterial genome count estimates were calculated for each cluster based on the 16S copy numbers in the closest available sequenced genome as described previously by Vetrovsky and Baldrian (2013). The clusters assigned to identical genera were merged. The number of reads was converted to the relative abundance value for further analysis. Non-metric multidimensional scaling (nMDS) based on the Bray-Curtis distance matrix was used for the visualization of the results in program PAST (PAleontological STatistics version 3.14) (Hammer et al., 2001).

3. Results

3.1. Water temperature and concentrations of pollutants in stream

The water temperature results from the control and downstream sites are shown in Table 1. The mean values of water temperature in the downstream site were 2.3, 3.6, and 1 °C higher than the control site at the 30, 90, and 180 days, respectively.

Concentrations of 155 PPCPs and 16 perfluorinated compounds were analyzed in 12 POCIS over a period of 6 months (at 0, 30, 90 and 180 days) and the results are presented in Supplementary Material 7. Overall, 20, 53, and 50 PPCPs were detected in POCIS at the PS1, PS2, and PS3 sites, respectively (Fig. 1). Ten PPCPs (caffeine, carbamazepine, cotinine, irbesartan, oxazepam, 2-phenylbenzimidazole-5-sulfonic acid (PBS), sulfapyridine, telmisartan, tramadol, and trimethoprim) were detected at all three sites. Levels of all 16 perfluorinated compounds were below the limit of quantitation (LOQ) at the control site, while five perfluorinated compounds (perfluorodecanoic acid (PFDA), perfluoroheptanoic acid (PFHpA), perfluorononanonic acid (PFNA), perfluorooctanoic acid (PFOA), and perfluorooctanesulfonate (PFOS)) were detected at two other selected sites. PPCP concentrations at the PS2 site were between 1.1 and 1.7-fold higher than at the PS3 site. The average total PPCP concentrations were 107 (71–125) ng/POCIS at PS1, 2644 (1675–3860) ng/POCIS at PS2 and 1831 (1518–2294) ng/POCIS at PS3 sites. Average total concentrations of perfluorinated compounds were 2.9 (1.2–4.1) ng/POCIS and 1.7 (1.3–2.4) ng/POCIS at PS2 and PS3 sites, respectively. Among the detected compounds, caffeine (273 ng/POCIS), metaprolol (153 ng/POCIS), telmisartan (294 ng/POCIS), clarithromycin (195 ng/POCIS), tramadol (188 ng/POCIS), carbamazepine (173 ng/POCIS), and sulfamethoxazole (167 ng/POCIS) were found in relatively high average concentrations at the site nearest to the STP effluent discharge (PS2 site). Cardiovascular group compounds, antibiotics/fungicides, analgesics, antiepileptic, and anticonvulsants were detected at the three sites, with the amounts accumulated in POCIS listed in Table 2. Among these groups, the concentration of antibiotics, fungicides, analgesics, and antiepileptics were highest at 90 days and lowest at 180 days. The highest concentration of anticonvulsant compounds and three compounds in the cardiovascular group (irbesartan, telmisartan, and metoprolol) were detected at 30 days and decreased towards day 180. Anti-Alzheimer disease, allergic rhinitis, and opioid groups of PPCP were constantly detected at the downstream site but not at the control site. Additionally, benzophenone-4 (BP-4), furosemide, and diclofenac were detected at high average concentrations at the downstream, but not at control, sites.

3.2. Fish health status

After 30 and 180 days of exposure to water discharged from the STP, there was a significantly higher ($p < 0.05$) CF compared with the relative control groups in the upstream site. HSI was significantly higher ($p < 0.05$) in fish from the downstream site after 30 and 90 days, compared with the fish from the control site (Table 3).

In this study, a significantly higher ($p < 0.05$) GSI was observed in the fish during the spawning period at both sites compared with the other periods (Table 3). After 30 days of living in the downstream site, female fish showed a significantly higher GSI compared with fish from the control site, while no significant change was observed in male fish. On day 30, the GSI of male fish from the downstream site was significantly lower than on day 0, while no significant change was seen in fish from the control site. No significant difference in GSI was seen in up- and downstream fish at 90 and 180 days.

3.3. Fatty acid composition

The approximate fatty acid composition of muscle tissues in brown trout collected after 90 days of exposure is presented in Table 4. A total of 18 major fatty acids, including five saturated fatty acids (SFAs), four monounsaturated fatty acids (MUFAs), and nine polyunsaturated fatty acids (PUFAs) were identified and quantified. Fish from the downstream site showed a significantly higher fat content than fish from the control site.

The most abundant SFAs in brown trout were palmitic acid (16:0) and stearic acid (18:0). Fish from the downstream site showed a significantly higher levels of myristic (C14:0) and arachidic (C20:0) acid (1.7 and 1.4-folds, respectively), but no changes in total SFA were observed in fish from the downstream site after 90 days.

Palmitoleic (C16:1) and oleic (C18:1 ω-9) acid were the most represented MUFA in trout and their levels were significantly higher in fish from the downstream site compared with those from the control site. Additionally, the percentage of total MUFA content was significantly higher (1.6-fold) in fish from the downstream site.

Total tissue PUFA in fish was significantly lower ($p < 0.05$) in fish captured in the downstream site compared with those captured in the control site. Docosahexaenoic acid (C22:6ω-3) was the most abundant ω-3 fatty acid in fish muscle. The ω-3 fatty acids 18:3ω-3, 20:3ω-3, and C22:6ω-3 were significantly lower in fish from the downstream site, while 20:5ω-3, 22:5ω-3 and the major ω-6 fatty acids (18:2ω-6 and 20:4ω-6) and the sum of ω-6 in muscle of the fish were not significantly different ($p < 0.05$) in the control and downstream sites after 90 days.

3.4. Biomarkers

Changes in antioxidant enzyme activity in the liver and gill tissues of brown trout are shown in Fig. 2.

Catalase activity was significantly lower ($p < 0.05$) in gills tissue from fish in the downstream area than from those control at 30 days. The glutathione-related antioxidant defense system was modulated in liver and gill tissues. A significant increase in GR activity was observed in the liver after 90 and 180 days in fish from the downstream site, while a significant decrease (by 0.7-fold, $p < 0.05$) in GR activity was observed in gills after 30 days in fish from the downstream site. Hepatic GPx activity was significantly ($p < 0.05$) inhibited at 180 days in fish from the downstream site.

GST activity in liver was significantly ($p < 0.05$) lower in brown trout from the downstream site at 30 days compared with the control site (Fig. 3), while in the gills, it showed a significantly higher level ($p < 0.01$).

Lipid peroxidation, measured as TBARs, in hepatic tissue, was significantly ($p < 0.05$) lower at 180 days in fish from the downstream site (Fig. 4) compared with control. Significantly higher levels of TBARs were found in the gills after 90 days in fish from the downstream site.

Hepatic EROD activity showed no significant differences at any time point (Fig. 5A). However, on day 90, EROD activity in fish from the downstream was slightly increased. Measurements of BFCOD

Table 2
Concentrations of pharmaceutical and personal care products, and perfluorinated compounds in passive samplers from Zivny stream per sampling location (ng/POCIS).

Chemicals	Upstream (PS1)				Effluent STP (PS2)				Downstream (PS3)				LOQ
	0	30	90	180	0	30	90	180	0	30	90	180	
UV-filters													
PBS	8.4	3.2	2.4	6.7	28	35	50	33	38	44	30	45	2.4
BP-1	<LOQ	6.4	<LOQ	<LOQ	8.2	6.3	<LOQ	<LOQ	6.3	<LOQ	<LOQ	4.4	5.1
BP-3	<LOQ	<LOQ	<LOQ	4	3.4	7.2	18	6.2	4.2	<LOQ	9.3	<LOQ	1.7
BP-4	<LOQ	<LOQ	<LOQ	<LOQ	97	140	181	33	73	125	71	28	5.2
Anticonvulsants													
Carbamazepine	3.1	1.6	1.1	2.8	203	198	165	124	166	171	104	124	0.29
Alprazolam	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	0.95	<LOQ	1.2	<LOQ	0.92	0.67	<LOQ	0.57
Clonazepam	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	1.3	<LOQ	0.71	<LOQ	1.6	<LOQ	<LOQ	0.53
Oxazepam	0.35	0.52	0.45	0.56	19	26	21	11	12	15	12	11	0.26
Antidepressants													
Cotinine	8.1	5.5	6.2	6.7	21	19	53	17	13	18	32	19	0.29
Mirtazapine	<LOQ	<LOQ	<LOQ	<LOQ	11	11	9	3.8	6	7.2	4.2	3.4	0.53
Venlafaxine	<LOQ	<LOQ	1.5	0.83	58	66	75	37	42	45	46	32	0.27
Citalopram	<LOQ	<LOQ	<LOQ	<LOQ	48	39	38	20	18	22	19	17	0.98
Sertaline	<LOQ	<LOQ	<LOQ	<LOQ	20	22	14	8.4	5.3	5.3	6	4.2	0.37
Mianserin	<LOQ	<LOQ	<LOQ	<LOQ	0.88	0.73	0.48	0.51	<LOQ	<LOQ	0.41	0.29	0.23
Cardiovascular													
Sotalol	<LOQ	<LOQ	<LOQ	<LOQ	19	28	29	6.7	13	19	22	7.3	0.61
Valsartan	3.6	4.7	7.4	<LOQ	12	41	152	117	8	30	115	96	1.3
Atenolol	<LOQ	<LOQ	0.64	1.6	59	73	91	43	36	54	52	37	0.47
Irbesartan	12	6.4	5.1	3.3	68	154	101	57	81	136	88	69	0.82
Telmisartan	5.3	5.4	3.9	3.2	282	276	252	149	121	177	126	97	0.29
Bisoprolol	<LOQ	<LOQ	<LOQ	<LOQ	47	63	63	36	30	37	41	29	1.2
Furosemide	<LOQ	<LOQ	<LOQ	<LOQ	97	100	218	110	39	77	101	60	3.09
Metoprolol	3.3	<LOQ	2.3	4.9	277	296	258	153	179	180	150	131	1.00
Diltiazem	<LOQ	<LOQ	<LOQ	<LOQ	1.6	1.8	2.2	0.52	1.2	1.2	1.2	0.53	0.39
Antibiotics/fungicides													
Clarithromycin	1.6	<LOQ	6.1	1.8	143	183	379	75	98	149	243	85	0.94
Clindamycine	<LOQ	<LOQ	<LOQ	<LOQ	11	6	11	6.4	15	12	22	18	1.5
Azithromycin	<LOQ	<LOQ	<LOQ	<LOQ	18	11	21	7.5	3.9	5.3	13	4.5	1.0
Sulfapyridine	40	15	21	11	40	62	85	46	40	53	52	47	0.63
Trimethoprim	1.6	0.47	0.52	0.58	117	129	154	59	67	81	90	52	0.37
Fluconazole	<LOQ	<LOQ	<LOQ	<LOQ	9.1	7.5	16	6.4	8.1	4.6	11	4.6	0.41
Sulfamethoxazole	6.4	<LOQ	2.5	<LOQ	155	191	265	58	98	133	156	46	0.59
Norfloxacin	<LOQ	<LOQ	<LOQ	<LOQ	4.9	2	3.8	4.8	3	2.7	2.6	<LOQ	1.7
Penicillin_V	<LOQ	<LOQ	<LOQ	13	<LOQ	6.3	41	12	<LOQ	1.9	16	5.5	0.69
Analgesics													
Diclofenac	<LOQ	<LOQ	<LOQ	<LOQ	61	100	104	59	43	52	68	49	1.7
Codeine	<LOQ	<LOQ	<LOQ	<LOQ	6.1	15	43	22	5.8	9.7	24	21	2.03
Tramadol	3.9	2	4.2	2.5	199	224	208	122	146	177	147	153	0.45
Stimulant													
Caffeine	27	20	42	59	125	144	655	167	89	109	372	163	0.40
Anti-Alzheimer disease													
Memantine	<LOQ	<LOQ	<LOQ	<LOQ	0.87	0.59	1.2	1	0.71	0.68	0.92	1	0.33
Seasonal allergic rhinitis													
Fexofenadine	<LOQ	<LOQ	<LOQ	<LOQ	1.2	1.9	3.2	5	1.6	3	2.7	4.3	0.52
Diphenhydramine	<LOQ	<LOQ	<LOQ	0.43	0.97	1.1	0.55	0.4	0.33	0.65	0.28	0.33	0.17
Opoids													
Oxycodone	<LOQ	<LOQ	<LOQ	<LOQ	2.7	3.1	5.7	3.6	2.3	2.7	2.8	3.9	0.46
Methamphetamine	<LOQ	<LOQ	<LOQ	<LOQ	29	20	25	17	14	14	21	26	0.57
Benzoylecgonine	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	1.1	1.2	1.3	<LOQ	0.58	0.77	1.3	0.38
Total concentration of PPCPs	125	71	107	123	2311	2734	3864	1678	1531	1983	2296	1519	
Perfluorinated compounds													
PFDA	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	0.4	0.5	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	0.46
PFHpA	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	0.51	0.68	<LOQ	<LOQ	<LOQ	0.42	<LOQ	0.59
PFNA	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	0.57	<LOQ	<LOQ	<LOQ	<LOQ	0.52
PFOA	<LOQ	<LOQ	<LOQ	<LOQ	1.2	1.9	2.3	1.4	1.3	1.3	1.3	1.8	0.64
PFOS	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	0.74	0.61	<LOQ	<LOQ	<LOQ	0.65	<LOQ	0.64
PFUdA	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	0.62	<LOQ	<LOQ	<LOQ	<LOQ	0.62
Total concentration of perfluorinated compounds	<LOQ	<LOQ	<LOQ	<LOQ	1.2	4.5	4.1	2.6	1.3	1.3	2.4	1.8	

PS – sites of passive sampler installation. LOQ- limit of quantification.

activity in the contaminated downstream site of Zivny stream showed that the activity was significantly ($p < 0.05$) upregulated at 90 days (Fig. 5B).

Female brown trout collected during the spawning period (October (day 0) to November (day 30)) had significantly higher VTG concentrations in blood plasma, compared with January (day 90) and April (day

Table 3
Health status characteristics of brown trout from selected sites. Data are presented as the mean ± S.D., $n = 11–24$. Means with different superscripts are significantly different ($p < 0.05$).

Site		Upstream (control site)				Downstream (polluted site)		
Sampling time		0 day	30 days	90 days	180 days	30 days	90 days	180 days
CF, g/cm^3		0.90 ± 0.08^{b}	0.96 ± 0.11^{b}	0.94 ± 0.12^{b}	0.94 ± 0.11^{b}	1.08 ± 0.09^{a}	1.00 ± 0.13^{b}	1.12 ± 0.14^{a}
GSI,%	Male	1.60 ± 0.31^{a}	1.35 ± 0.57^{ab}	0.50 ± 0.37^{c}	0.20 ± 0.147^{c}	1.12 ± 0.33^{b}	0.34 ± 0.10^{c}	0.14 ± 0.04^{c}
	Female	16.04 ± 5.81^{a}	4.47 ± 5.30^{b}	1.09 ± 0.21^{c}	0.92 ± 0.22^{c}	11.11 ± 6.95^{a}	0.74 ± 0.23^{c}	0.73 ± 0.15^{c}
HSI, %		1.24 ± 0.45^{abcd}	1.13 ± 0.24^{cd}	0.94 ± 0.22^{d}	1.55 ± 0.19^{abc}	1.53 ± 0.32^{ab}	1.19 ± 0.33^{bc}	1.70 ± 0.43^{a}

Note: CF- conditional factor; GSI- gonadosomatic index; HSI - hepatosomatic index.
CF = [body weight (g) / fork lengt^3 (cm)] × 100; GSI = [gonad weight (g) / total tissue weight (g)] × 100; HSI = [liver weight (g) / body weight (g)] × 100.

180) sampling events (Fig. 6A). VTG levels in male fish from the control site were below the level of quantitation (LOQ; according to the manufacturer instructions, the LOQ is 0.054 ng/mL). However, high VTG levels were detected in male fish from the downstream site at all sampling events (Fig. 6B). The highest VTG concentration was observed in male fish during the spawning period (day 30). Male VTG plasma concentration significantly decreased just after the spawning period (23110- and 3.1-fold at days 90 and 180, respectively).

Star plots and IBRv2 values are presented in Fig. 7. The IBRv2 was calculated according to the time point that includes the score of each parameter in each tissue. A high value of IBRv2 refers to a high response. The IBRv2 value was highest at day 30 in gills (5.93), and at 90 day in liver (6.45).

3.5. The lotic microbiome composition and taxonomic characterization of fish intestinal microbiome

3.5.1. The lotic microbiome composition in stream and STP effluent

Taxa relative abundance in lotic biofilm is summarized in Supplementary Material 6. When the number of identified taxa are compared with the median relative abundance >0.5%, much higher taxonomic variability was observed downstream of STP than in the control site, and also at the end of the warm season (time 0) compared with in the spring (time 180). The Bray-Curtis distance matrix analysis of the lotic microbiome composition clearly distinguished both between two sampling seasons (time 0 and 180) and between the control site (PS1) and the STP affected sites (PS2 and PS3) (Supplementary Material 8). The sampled environments differed mainly in the abundance of three Cyanobacteria taxa: unclassified Nostocales, *Tychonema*, and *Pleurocapsa*. The sum of their relative abundance was >10% at the PS1 site in both seasons, while in the polluted water downstream sites PS2 and PS3, this sum did not exceed 1%. The relative abundance of several taxa, such as unclassified Acidimicrobiales Candidatus *Microthrix* (Actinobacterium), unclassified Chitinophagaceae similar to *Ferruginibacter* and *Terrimonas* (Bacteroidetes), and *Thermomonas* (Gammaproteobacteria), increased at downstream site compared with the control site.

Table 4
Fatty acids composition (higher than 0.5%) of the total identified fatty acids of the muscle in brown trout from selected sites after 3 months of exposure. Data are presented as the mean ± S.D., $n = 6$ in each group (3 male, 3 female). Asterisk indicates significant differences ($p < 0.05$, t-test) in the downstream fish group compared with upstream fish.

Common acid name	Fatty acid (ω-n)	Upstream, %	Downstream, %
Myristic	C14:0	0.75 ± 0.32	1.3 ± 0.42*
Palmitic	C16:0	16 ± 3.80	17 ± 1.71
Palmitoleic	C16:1	2.9 ± 0.85	5.8 ± 1.28*
Stearic	C18:0	4.7 ± 0.23	4.8 ± 0.40
Oleic	C18:1ω-9	9.4 ± 2.46	15 ± 3.34*
Vaccenic	C18:1ω-7	3.3 ± 1.13	4.6 ± 0.58*
Linoleic	C18:2ω-6	7.4 ± 1.99	9.3 ± 1.37
α-linolenic	C18:3ω-3	4.8 ± 0.56	2.9 ± 0.51*
Arachidic	C20:0	0.16 ± 0.03	0.23 ± 0.07*
Gondoic	C20:1ω-9	0.22 ± 0.07	0.42 ± 0.13*
Eicosadienoic	C20:2ω-6	0.44 ± 0.07	0.6 ± 0.04*
Arachidonic	C20:4ω-6	5.1 ± 0.67	4.3 ± 0.85
Eicosatrienoic	C20:3ω-3	0.44 ± 0.07	0.22 ± 0.08*
Eicosapentaenoic	C20:5ω-3	9.8 ± 1.93	8.6 ± 1.98
Docosapentaenoic	C22:5ω-6	0.49 ± 0.33	0.05 ± 0.07*
Docosapentaenoic	C22:5ω-3	3.5 ± 0.98	3.2 ± 0.7
Docosahexaenoic	C22:6ω-3	30 ± 3.53	22 ± 3.11*
Lignoceric	C24:0	0.13 ± 0.09	0.36 ± 0.15
SFA		21 ± 3.56	24 ± 1.87
MUFA		16 ± 3.47	26 ± 4.58*
PUFA		62 ± 2.43	51 ± 5.04*
ω-3		49 ± 3.60	37 ± 5.60*
ω-6		13 ± 1.89	14 ± 1.18
ω-3/ω-6		3.7 ± 0.73	2.7 ± 0.62*
Total fat content		0.95 ± 0.19	1.5 ± 0.3*

SFA – saturated fatty acids, MUFA- monounsaturated fatty acids, PUFA- polyunsaturated fatty acids.

3.5.2. Taxonomic characterization of fish intestinal microbiome

At 90 days (January) when the food intake was low, the intestinal microbiome was drastically reduced in fish from both biotopes (control and downstream sites) exhibiting a single dominant taxon of the family Enterobacteriaceae. Using 16S rDNA V4-V5 region-based taxonomy, a closer assignment of the identified taxon was not possible because all the "best hit" genera from family Enterobacteriaceae have an identical 16S rDNA sequence in this specific region. Such an extreme composition of the intestinal microbiome was identified mainly in fish captured at the downstream site, where the relative abundance of this dominant taxon ranged from 62 to 92% in 11 out of 12 fish. In one fish, a relative abundance of 39% was identified for the dominant taxon. The same taxon was, however, predominant also in 7 out of 10 fish captured in the control site: its relative abundance ranged from 44 to 95%. Besides Gammaproteobacteria (the above-mentioned predominant Enterobacteriaceae and the unclassified Pseudomonadaceae or *Halomonas*), the intestinal microbiome of all tested fish (day 90) also comprised unclassified Planctomycetaceae, and in the control location, Firmicutes (unclassified Erysipelotrichaceae and unclassified Bacillales or *Hespellia*) was also present.

At day 180, the water temperature was rising and food intake was increasing, and the intestinal microbiome diversity also remarkably increased. The overall number of taxa with a median relative abundance ≥0.5% was almost identical at the control and downstream sites (23 and 24 taxa, respectively), but only 14 taxa were identified to be common to both tested locations. The effect of the environment on the composition of the brown trout intestinal microbiome is thus evident (Fig. 8, the Bray-Curtis distance matrix-based nMDS analysis of intestinal microbiome composition at day 180). The above-mentioned most abundant taxon from family Enterobacteriaceae still predominated in fish from the downstream site, but its relative abundance decreased (median 8%). In the control site, it was also among the most abundant taxa, but with a median relative abundance of 5.5%, it moved into third place following unclassified Bacillales (18%) and unclassified Erysipelotrichaceae (6%). The unclassified Nakamurellaceae (*Nakamurella*), unclassified Oscillatoriales (*Arthronema*), and unclassified Acidimicrobiales (Candidatus *Microthrix*) were identified only in the fish captured in the polluted water downstream site (with median relative abundance 4.08%, 3.88%, and 1.24%, respectively), but not control. The unclassified Oscillatoriales (*Arthronema*) were not identified in the lotic biofilm samples in the downstream site, and unclassified

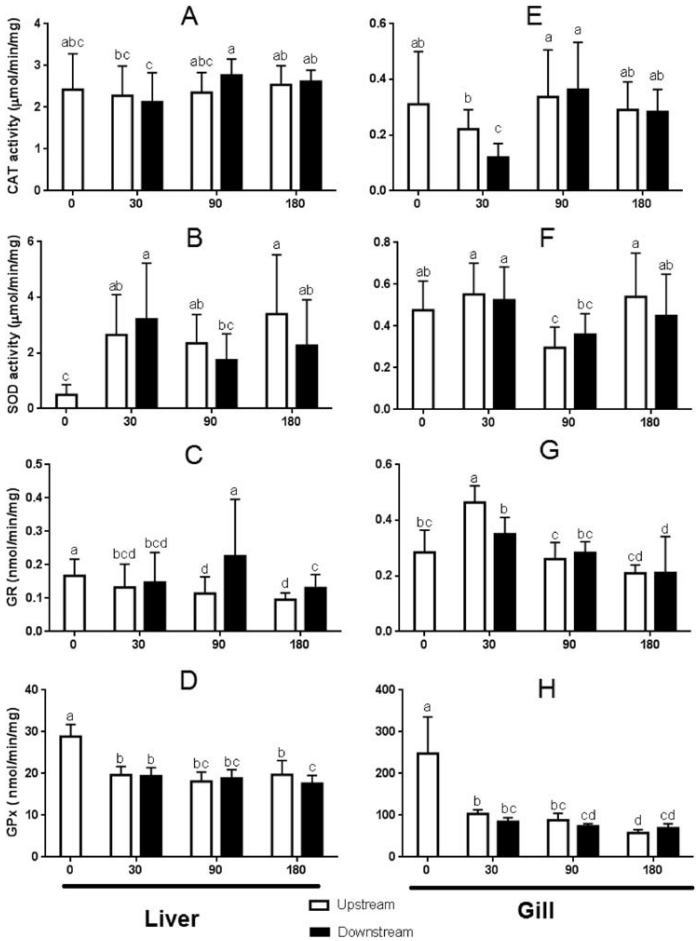

Fig. 2. Antioxidant responses in liver (panel 2A, 2B, 2C, 2D) and gill (panel 2E, 2F, 2G, 2H) tissues in brown trout from selected sites. Data are presented as the mean ± S.D., $n = 11–24$. Means with different superscripts are significantly different ($p < 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

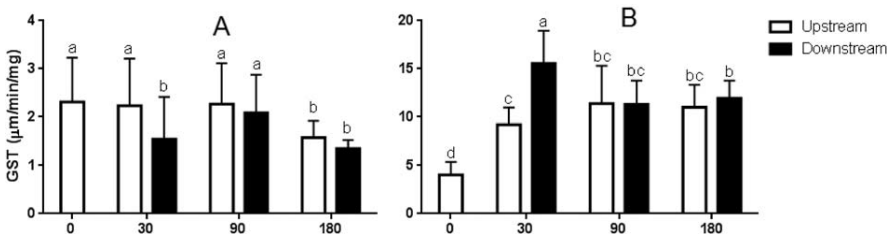

Fig. 3. Glutathione-S-transferase activity in liver (panel 3A) and gill (panel 3B) tissue in brown trout from selected sites. Data are presented as the mean ± S.D., $n = 11–24$. Means with different superscripts are significantly different ($p < 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

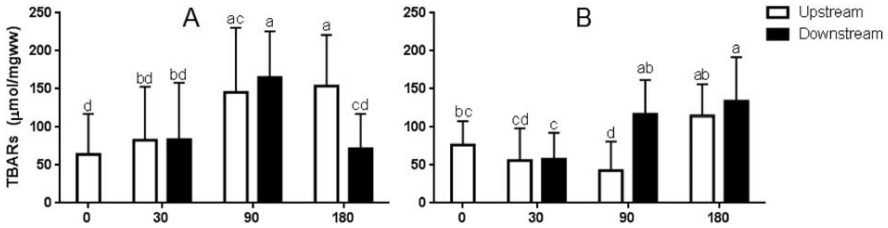

Fig. 4. Level of thiobarbituric acid reactive substances (TBARs) in liver (panel 4A) and gill (panel 4B) tissue in brown trout from selected sites. Data are presented as the mean ± S.D., n = 11–24. Means with different superscripts are significantly different ($p < 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Nakamurellaceae (*Nakamurella*) and unclassified Acidimicrobiales (Candidatus *Microthrix*) were identified only in very low abundance.

4. Discussion

The occurrence of psychoactive compounds in water and their accumulation in the liver and kidney in fish were confirmed by previous study in the same area of this study (Grabicova et al., 2017). However, no relationship between PPCPs occurrence and physiological effects on aquatic organisms were investigated in this locality. The Zivny stream represents the common scenario for STP discharge into a small stream, resulting in a low dilution of treated sewage water. This experimental design allows us to study the impact of a common mixture of biologically active compounds occurring in treated sewage water. In the present study, the use of wild/native and free range fish reflect the real exposure scenario with minimal disturbing interventions. Fish that originate from the control site were restocked to the control and STP downstream sites. The use of native fish minimized the stress of the transfer to an unfamiliar environment, and therefore the response reflects only the changes of environmental conditions that were induced by the STP effluent.

4.1. Concentrations of pollutants in stream

The low concentration of PPCPs in the control site reflects the common scenario of unpolluted/minimally polluted water bodies in the Czech Republic (300 ng/POCIS) (Giang et al., 2017) and is in agreement with a previous study, which showed that the site can be used as a control site (Grabicova et al., 2015). In the present study, cardiovascular, antibiotic/fungicides, analgesics, and antiepileptic/anticonvulsant classes of drugs were the most abundant compounds at downstream sites. Our results are consistent with previous studies, which demonstrated that STP effluent discharge from Prachatice town causes the presence of PPCPs in the downstream site of Zivny stream, the studies also detected high tramadol and metoprolol concentrations in the downstream site (Fick et al., 2017; Grabicova et al., 2015; Li et al., 2011b). In this study, caffeine, carbamazepine, termisartan and metoprolol were constantly detected at high concentrations in all investigated periods. Seasonal-dependent fluctuation of PPCPs was seen in the downstream site. The highest concentrations of most detected PPCP groups were seen in winter/January (cardiovascular group compounds, antibiotics/fungicides, analgesics, and antiepileptics) and autumn/November (anticonvulsants), while a lower concentration was detected in late spring/April. This finding was consistent with the study of Golovko et al. (2014a, 2014b). The low degradation and/or low removal efficiency of STP effluent during the cold season of the year is often associated with an elevated concentration of PPCPs in recipients of treated municipal sewage water in winter compared to other seasons (Golovko et al., 2014a, 2014b; Vieno et al., 2005). However, the variation of each PPCP group may be associated with the seasonal consumption of PPCPs. A higher concentration of antibiotic (McArdell et al., 2003), cardiovascular and antiepileptic compounds (Valcarcel et al., 2013) were detected in surface water in winter compared with the summer season in the European area condition.

4.2. Fish health status

The important aspect of this *in situ* experiment is that fish can swim freely and interact with biotic and abiotic characteristics of the water body. Fish maintained their feeding habits at the selected sites, and using this information, the fish CF, which reflects the physical and biological status, can be sufficiently estimated. CF values >1 show the well-being of fish (Datta et al., 2013). In the present study, a CF > 1 g/cm^3 was recorded only for fish from the downstream site. The water temperature in this area was 1.0–3.6 °C higher than at the control site, which may affect biochemical and physiological processes in fish, including feeding activities, metabolism, and efficiency of energy transformation. Watz and Piccolo (2011) indicated that the prey-capturing efficiency of brown trout was reduced by about 43% when the

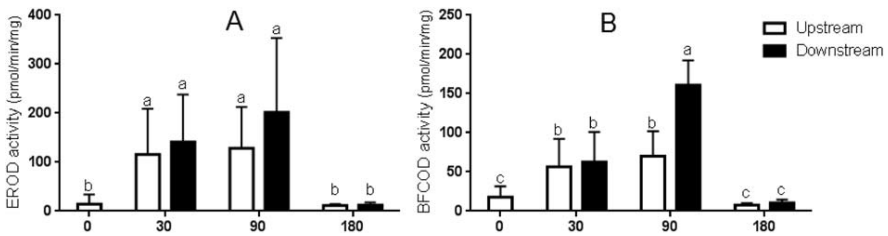

Fig. 5. Hepatic 7-ethoxyresorufin-O-deethylase (EROD, panel 5A) and 7-benzyloxy-4-trifluoromethylcoumarin-O-debenzyloxylase (BFCOD, panel 5B) activity of the brown trout from selected sites. Data are presented as the mean ± S.D., n = 11–24. Means with different superscripts are significantly different ($p < 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

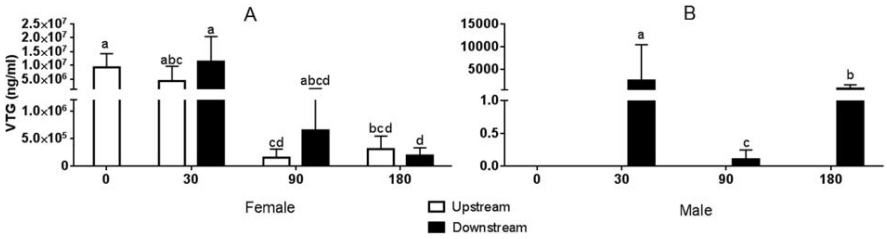

Fig. 6. Levels of vitellogenin (VTG) in blood plasma of female (panel 6A) and male (panel 6B) fish from selected sites. Data are presented as the mean ± S.D., $n = 11–24$. Means with different superscripts are significantly different ($p < 0.05$).

temperature decreased from 14 °C to 6 °C. Additionally, a higher temperature and nutrient-rich environment in the downstream site leads to favorable conditions for brown trout with respect to the availability of natural food. Additionally, higher levels of antibiotic and fungicide pharmaceuticals in the downstream site (approximately 10–30 times higher) might prevent infections in the fish and other organisms in the food chain, which could result in more prey available for the trout. However, the occurrence of bioactive compounds may interfere with the capacity of primary production. The results of the present study are consistent with previous research on the common carp exposed to a biological pond receiving only STP effluent, which assumes that the favorable conditions for natural food and temperature may help fish overcome the negative impacts induced by pollution factors (Giang et al., 2017).

The brown trout reproductive season can vary by geographic area, in the present experimental area, this season starts in mid-October and lasts until the end of November. The GSI is used to estimate fish reproductive conditions. The spawning capability of male and female brown trout are assigned when GSI > 1% and 10%, respectively (Billard, 1987). Both female and male brown trout were able to complete final maturation during mid-October at the control site (time 0). At the end of the spawning period (November), the GSI in female fish downstream was still over 10%. Additionally, 58% of female fish had GSI higher than 10%, while in the control site, only 33% of fish had GSI > 10%. In contrast to these results, the GSI of male fish from the downstream site decreased faster than in those at the control site after 30 days of exposure compared with day 0. These results suggest that the different conditions at the downstream site, including chemical exposure may postpone the spawning period in females, but not in males. A limitation of this study is that the histological examination, sperm quality and reproductive success of fish living in water with STP discharge was not assessed and thus further investigation is required.

A seasonal dependence of HSI fluctuation was obvious in fish from both sites, with the lowest values in the winter (at day 90) and the highest in the spring (at day 180). The variation of HSI was documented, and it depends on nutrient factors (Turano et al., 2007). Reduced food availability in the winter may result in expendature of stored energy in the liver and significantly reduced HSI in fish from both control and downstream sites. In this study, we found a negative correlation between HSI and detoxifying enzymes including GST, CAT, BFCOD, and EROD (Supplementary Material 9). The loss of energy on detoxifying activities and during winter season results in rapid decrease of HSI in fish

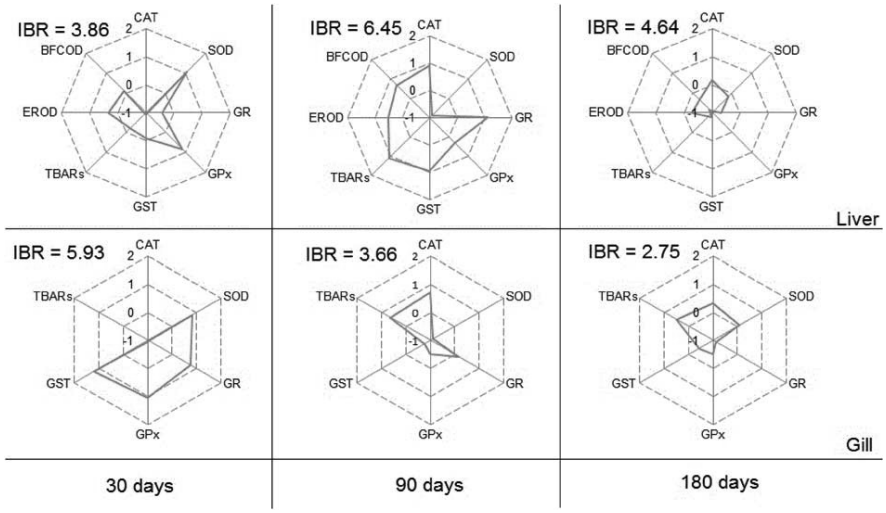

Fig. 7. Star plots for each sampling time (30, 90, and 180 days) in each tissue (liver and gills); and integrated biomarker response version 2 (IBRv2) of all biomarkers, including glutathione S-transferase (GST), thiobarbituric acid reactive substances (TBARs), superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and glutathione reductase (GR), and for hepatic 7-ethoxyresorufin-O-deethylase (EROD) and 7-benzyloxy-4-trifluoromethylcoumarin-O-debenzyloxylase (BFCOD) in each tissue.

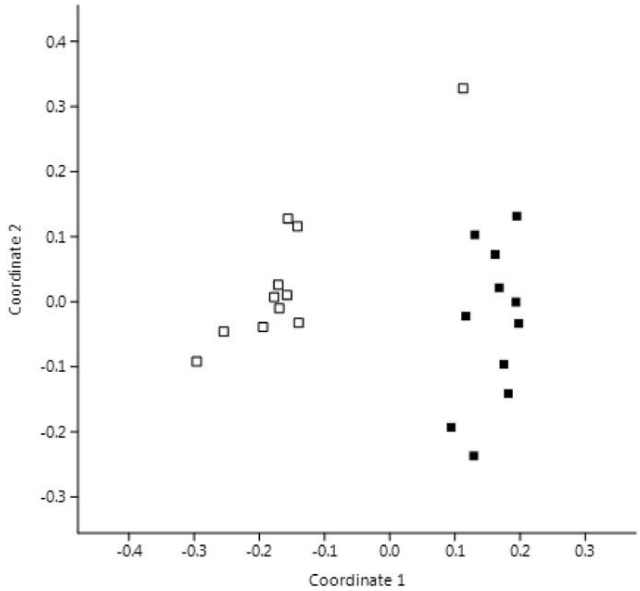

Fig. 8. Bray-Curtis distance matrix-based nMDS analysis plot of the intestinal microbiome composition of fish at day 180; empty squares correspond to the unpolluted location upstream of the STP, and the filled squares correspond to location downstream of the STP.

from downstream site at 90 days. The increased HSI in fish from downstream site (27–35%) compared with the control site might be related to nutrient factors rather than associated with increased hepatic detoxification.

4.3. Fatty acid composition

Generally, the feed fatty acid composition is minored in fish tissues (Pettersson et al., 2009; Zajic et al., 2016) and is one of the main factors affecting fatty acid composition. In the present study, the fatty acid composition of muscle of fish from downstream was changed compared with fish from control in the direction of increasing fat content and the total percentage of MUFA, but decreasing of PUFA (ω-3). The higher water temperature (~3.6 °C) and nutrient rich environment might result in more available food at the downstream site, which may affect feed uptake and cause subsequently higher fat deposition in fish. Storage fat is built by triacylglycerols, which are usually higher in SFA and MUFA (Henderson and Douglas, 1987), which could explain part of the difference in the fatty acid composition. An additional aspect of the altered fatty acid composition could be an effect of chemicals present in the effluent discharge on the direction of lipid metabolism. Lipid-lowering compounds (statins or fibrates) are potent modulators of fatty acid metabolism (Pahan, 2006). Thus, the occurrence of these compounds may disrupt proportions of fatty acids in chronically exposed nontarget organisms, including fish (Weston et al., 2009). In this study, fenofibrate was only detected in the downstream site (Supplementary Material 7). It has been shown that feed contaminated with xenobiotic compounds, including PPCPs, affects lipid metabolism in fish (Carnevali et al., 2017; Cheng et al., 2016; Guo et al., 2015; Maradonna et al., 2015; Weston et al., 2009). Previous studies indicated the accumulation of PPCPs and industrial compounds in fish tissue and benthic organisms in downstream Zivny (Grabicova et al., 2015; Grabicova et al., 2017; Li et al., 2011b), which is another significant exposure pathway deserving consideration. The previous study indicated that fish fed with contaminated feed resulted in a lower ω-3 and SFA percentage (Cheng et al., 2016). However, until now, little information about the relationship of PPCPs contaminated feed and fatty acid composition in fish was known.

4.4. Biomarkers

Reactive oxygen species (ROS) are generated during mitochondrial oxidative metabolism as well as in cellular response to xenobiotics, which are present in water. As the ROS levels increase, the biological system develops a first line defense mechanism by modulating the activities of antioxidants such as SOD, CAT, and glutathione-related enzymes. In the present study, the response of antioxidant enzyme activity was tissue specific. Changes in six investigated biomarkers between fish from the up- and downstream site were more visible in gills at the first sampling and liver tissues at the last sampling event, respectively. However, no clear trend was recorded for the variation of each biomarker in each tissue based on the sampling events. The opposite trend was seen in the results of TBARs, GST, and GR between gill and liver tissue. In this study, fish were exposed under real conditions and showed mixture toxicity. The effect of 53 PPCPs occurring in water may induce both a synergistic effect and an antagonistic effect. Therefore, the result of oxidative stress and antioxidant enzyme biomarkers in each tissue may not exactly reflect the variation of total PPCPs concentration in each sampling event, which was highest at the 90-day sampling event. However, the visible effect in gills and liver tissue at day 30 and 180 can be explained by the first sensitive exposure period and incremental effect after long-term exposure to PPCPs, respectively. The oxidative-based effects of compounds such as UV-filters (BP-1, BP-2, BP-3, BP-4 (Liu et al., 2015)); anticonvulsants (carbamazepine, (He et al., 2011; Li et al., 2009)); and non-steroidal anti-inflammatory drugs (diclofenac, (Praskova et al., 2014)) on fish have been demonstrated.

However, the number of reports relating to the effect of real exposure to mixture toxicity on oxidative stress in aquatic organisms is still limited.

Measurement of CYP1A1-associated EROD activity is considered to be an important biomarker of exposure to degraded environmental conditions (Goksoyr, 1995). The amino acid sequences of Salmonidae CYP3A-like is approximately 50% homologous to human CYP3A4 (Lee et al., 1998; McArthur et al., 2003), which is responsible for metabolizing at least 434 drugs (Preissner et al., 2013). In this study, elevation of BFCOD activity in fish exposed to STP discharged water at day 90 confirmed the presence of a high quantity of PPCPs, which are metabolized through the CYP3A-pathway. This might be connected to remobilization of a wide spectrum of lipophilic PPCPs in lipid-rich tissues during a starvation period (Quabius et al., 2001). Additionally, an elevated level of BFCOD might support the elevation of TBARs levels in fish after 90 days of exposure in the downstream site. Seasonal variation of hepatic EROD and BFCOD activities were observed. These levels were higher during the winter compared with the spring months, which is in agreement with Behrens and Segner's (2005) observations. The similar variation trend between EROD, BFCOD, and the total PPCPs concentration suggests that these biomarkers can be considered to be good indicators for investigating the PPCP mixture toxicity.

The integrated biomarker response index is a useful tool to generally describe the overall effect by combining a set of biomarker signals (Beliaeff and Burgeot, 2002; Broeg and Lehtonen, 2006). IBRv2 was recommended for investigations comparing differences in numerous endpoints between upstream and downstream conditions (Sanchez et al., 2013). In this study, the IBRv2 result confirms the tissue dependence for the defense system response. The diversity of star plot shapes at each time point and in each tissue reflects a dissimilar pattern response to contamination. The IBRv2 index suggest that liver tissue was highly affected at 90 days, which is linked to the highest PPCP concentration. The effect in gill tissue was detected at earlier stage, indicating that gill as a sensitive organ that responds at an earlier time. After this period, fish may adapt to the STP downstream environment and the IBRv2 value in the gill declined gradually. The previous study also indicated a high IBR value in downstream fish compared with Zivny upstream fish, related to xenobiotic compounds, using a set of five oxidative stress and antioxidant enzyme biomarkers (Li et al., 2011b).

The PCA and multivariate regression analysis were further analyzed to identify the effective biomarkers (Table 5). CF, HSI, and biomakers in liver (BFCOD, TBARs, GR, and GST) and in gill (TBARs, SOD, CAT, and GST) were important variables to distinguish between locations with high (above 1000 ng/POCIS) and low (below 200 ng/POCIS) contamination levels.

VTG protein has been widely documented as a biomarker of exposure to xenoestrogens (Hansen et al., 1998). In the reproductive period, a high level of VTG in females is considered to be a normal biological response. However, the occurrence of VTG in male fish is linked with the presence of endocrine-disrupting compounds. In this study, the VTG levels were high in spawning season and correlated with GSIs in female fish at both sites. Previous studies indicated that fish living in areas downstream of STP inflow showed endocrine disruption symptoms, such as alteration of gonad development and induction of vitellogenin (Bjerregaard et al., 2008; Harding et al., 2016). Moreover, feminization of male fish in polluted areas such as near an STP water discharge has been repeatedly reported (Liney et al., 2006; Tarrant et al., 2008). In the present study, STP effluents significantly elevated VTG levels in male fish from the downstream area. This might indicate that vitellogenin synthesis was induced in brown trout males by the presence of estrogenic compounds in the water discharged from STP. The presence of UV filter compounds (Kunz and Fent, 2009; Weisbrod et al., 2007) and perfluorinated compounds (Kim et al., 2010) have been suggested to trigger induction of VTG. In this study, the concentrations of UV filter and perfluorinated compounds in POCISs were low, but the co-occurrence with others detected compounds in water may induce a mixture effect, which could result in high VTG levels in male fish. Unfortunately, the presence of other estrogenic compounds was not investigated in this study. The fluctuation of VTG levels in male fish from the downstream site may be explained by a seasonal presence of estrogenic compounds (Ribeiro et al., 2009). Additionally, the temperature relationship of estrogenic compounds may also impact the VTG level. It has been indicated that an increase in the temperature in a contaminated estrogenic environment induces an increase in VTG in blood plasma in exposed brown trout (Körner et al., 2008).

4.5. The lotic microbiome composition and taxonomic characterization of fish intestinal microbiome

The increased abundance of several taxa such as unclassified Acidimicrobiales Candidatus *Microthrix* (Actinobacterium), unclassified Chitinophagaceae similar to *Ferruginibacter* and *Terrimonas* (Bacteroidetes), and *Thermomonas* (Gammaproteobacteria) at the downstream site could be considered as a biomarker for anthropogenic pollution, because all these genera have been previously associated with activated sludge from wastewater treatment plants (McIlroy et al., 2013; McIlroy et al., 2016; Myung et al., 2015).

Similar to aquatic biofilm, the composition of the intestinal microbiome of the tested fish exhibited a seasonal dependence. The taxonomic composition of both the environmental (lotic) microbiome as well as the brown trout intestinal microbiome clearly differs among the samples from the control location upstream of the STP effluent and the polluted water located downstream. In the environmental samples, the most distinguishing were three Cyanobacteria taxa (unclassified Nostocales, *Tychonema*, and *Pleurocapsa*), which were highly abundant upstream but rare or absent at the downstream site. This is in agreement with findings of Loza et al. (2013), who documented

Table 5
The STEPDISC procedure results defined by a group of indexes and biomarkers, including the condition factor (CF), hepatosomatic index (HSI), gonadosomatic index (GSI), glutathione S-transferase (GST), thiobarbituric acid reactive substances (TBARs), superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR), EROD, BFCOD in liver (L) or gill (G) tissues and vitellogenin (VTG) in plasma (P).

Stepwise selection summary											
Step	Number in	Entered	Removed	Label	Partial R-Square	F value	Pr > F	Wilks' lambda	Pr < lambda	ASCC	Pr > ASCC
1	1	GST G		GSTG	0.3076	40.42	<0.0001	0.6924	<0.0001	0.3076	<0.0001
2	2	CF		CF	0.1788	19.59	<0.0001	0.5687	<0.0001	0.4313	<0.0001
3	3	BFCOD L		BFCODL	0.0917	8.98	0.0035	0.5165	<0.0001	0.4835	<0.0001
4	4	HSI		HSI	0.1368	13.95	0.0003	0.4459	<0.0001	0.5541	<0.0001
5	5	TBARs L		TBARSL	0.0555	5.11	0.0262	0.4211	<0.0001	0.5789	<0.0001
6	6	GR L		GRL	0.0539	4.9	0.0295	0.3984	<0.0001	0.6016	<0.0001
7	7	TBARs G		TBARSG	0.0638	5.8	0.0182	0.3730	<0.0001	0.6270	<0.0001
8	8	SOD G		SODG	0.0505	4.47	0.0374	0.3541	<0.0001	0.6459	<0.0001
9	9	CAT G		CATG	0.0825	7.46	0.0077	0.3249	<0.0001	0.6751	<0.0001
10	10	GST L		GSTL	0.0303	2.56	0.1135	0.3151	<0.0001	0.6849	<0.0001
11	9		HSI	HSI	0.025	2.11	0.1506	0.3232	<0.0001	0.6768	<0.0001

ASCC- average squared canonical correlation.

that the diversity and abundance of Cyanobacteria dramatically decrease in water that is rich in nutrients affected by anthropogenic pollution. Additionally, the unicellular Cyanobacteria genera (for example *Pleurocapsa* or *Chamaesiphon*) that is typical in clean water (Loza et al., 2013) were detected in significant abundance levels control site but their abundance downstream dramatically decreased.

The presence of several genera previously isolated from activated sludge (unclassified Acidimicrobiales related to Candidatus *Microthrix*, unclassified Chitinophagaceae related to *Ferruginibacter* and *Terrimonas*, and *Thermomonas*) in the environmental samples downstream site was not surprising. However, two such taxa that were also generally associated with activated sludge, unclassified Nakamurellaceae (*Nakamurella*) and unclassified Oscillatoriales (*Arthronema*) (Mcllroy et al., 2013; Tice et al., 2010), were identified in significantly high levels in the intestinal microbiome of brown trout captured at the downstream site, while they were only rare in the environmental samples from the same time and location. The acceptance of these taxa with the intake of sludge-contaminated food cannot be excluded, and an additional study is necessary to confirm this assumption. The analysis of microbiome composition, however, confirmed the effect of the Prachatice STP on the ecology downstream the STP effluent.

5. Conclusion

Assessment of the water quality demonstrated that at least 36 PPCPs were constantly present at the STP downstream site. Using our unique approach, this study reflects the effect of an STP effluent-dominated stream on native fish under fully natural and realistic conditions. Generally, fish from the downstream site show better growth than fish from the control site because of the favorable water conditions including water temperature and nutrients. However, the results from multiple biomarkers such as HSI, GSI, BFCOD, VTG, lipid composition, and the intestinal microbiome show physiological warning signs in brown trout living in water that receives STP discharge. The high VTG concentration in blood plasma of male trout and the shift of the spawning season in female brown trout illustrate the risk of the abnormal reproductive process in fish exposed to STP effluent. As observed in this study, brown trout can be found gathering around the effluent pipes and are exposed to high PPCP concentration that is diluted only a small amount in a small stream. The effect might be serious if fish are exposed from an early life stage, when they undergo a sensitive period of sex formation. Further research using earlier life stages and a histopathology endpoints might reveal possible developmental disturbances. Possible long-term consequences of the changed fatty acid composition on fish health as well as nutritional quality of the fish for human consumption should be investigated in future studies. Moreover, the role and occurrence of two abundant STP-related bacterial taxa detected in the intestine but not in the lotic environment requires further investigation. Future characterization of PPCP concentration in sediments and plants of the Prachatice STP effluent recipient and the genotoxic and immunotoxic effects in wild fish could facilitate the interpretation of the results.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2018.01.020.

Conflict of interest statement

The authors declare no conflict of interest.

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CHAPTER 4

GENERAL DISCUSSION

STPs play important roles in removing contaminants from wastewater for the purpose of ensuring a clean aquatic environment. However, the potential hazard of STP effluent discharge for aquatic ecosystem has been concerned. Some studies already investigated the effect of STP effluent on aquatic ecosystem in the downstream river area. However, there are still some disadvantages in these studies that cannot thoroughly reflect the effect under realistic scenarios. The aim of this thesis was to provide greater insight into the effects of STP effluent discharged water under real conditions in a central European context. A unique experimental setup was applied to overcome the disadvantages of classical experimental setups. Native fish were collected from the experimental area, tagged, and redistributed in control and experiment sites. Using this approach, fish from both sites were under the same initial conditions. Therefore, any changes in fish after they were separated only reflects environmentally-induced differences. In addition to gentle handling during tagging, fish were not stressed by long transportation times and did not need to adapt to a new environment (temperature, water flow, and other factors). By using the tagging technique, fish can swim in free-range conditions, allowing them a free search for food and a safe place. All of these combined factors result in significantly lower stress levels compared to those found in fish under cage experimental conditions. Information concerning bioconcentration, bioaccumulation, and biomagnification patterns are not underestimated as in the laboratory or caged experimental design. This method also ensures that fish are correctly collected with their known history. Therefore, this method eliminates the risk of sampling migrant fish as is done in other *in vivo* studies that randomly collect fish from up and downstream sites. Of course, this novel experimental setup faces several limitations such as repeated fish handling, infection risks, and/or labour demands, but it substantially lessens the disadvantages of the classical methods. The biological effects of STP effluent-discharged water on fish was investigated using a multi-biomarker approach. The selection of biomarkers was based on previous *in situ* studies, in which sensitive endpoints with the potential for reflecting overall fish health condition were identified.

The effects of STP on water quality

It has been frequently reported that the STP effluent discharge affects the quality of aquatic recipients (Aristi et al., 2015). In this thesis, the presence of pollutants and changes in temperature and nutrient elements were observed in two water systems: (1) a small river (Zivny Stream) and (2) a pond (Cezarka Pond), both of which receive discharges from STP effluents. Generally, STP effluents maintain a stable water temperature in aquatic recipients, which are higher in the cold period and lower in the summer season. In general, changes in water temperature affects other factors and hydrological processes in the aquatic environment, including oxygen levels and degradation of organic matter/pollutants followed by changes in the development of different trophic levels.

In order to overcome routine water sampling and analysis limitations, alternative approaches are under continuous development. For example, POCIS was designed to detect contaminants at trace levels. Therefore, this method can be used to observe more compounds than traditional methods such as grab water (Metcalfe et al., 2014). In both experiments, STP effluents released numerous PPCP compounds at high total concentration in the recipients. POCISs have been shown to be linked to bioaccumulation of pollutants in fish tissues and can fulfill the three Rs strategy: (1) replacement; (2) reduction; and (3) refinement in animal testing (Cerveny et al., 2016). Therefore, the results from POCIS experiments not only reflect

the recipients' water quality but also show the hazards of pollutant bioaccumulation in aquatic organisms. The total PPCP concentrations in both recipients are highly dependent on season, which is linked to seasonal consumption of PPCPs and temperature-related STP removal efficiency. The total PPCPs detected in the biological pond (Cezarka Pond, which receives 100% of its water input from STP effluent) was about 3.5-fold higher than in Zivny Stream (that receives 25% of its water input from STP effluent). This difference suggests that PPCPs' concentrations are highly dependent on the dilution factor. Antibiotic compounds were the dominant PPCP group detected in both recipients. However, a different pattern of PPCP composition between the two recipients was observed. Some compounds (such as verapamil and bezafibrate) were observed in Cezarka Pond but not in Zivny Stream, The concentration of most of the detected compounds was higher in the Cezarka pond than in the Zivny stream. However, some compounds such as caffeine (9-fold), diltiazem (2.5-fold), and atorvastatin (2.2-fold) were found at lower levels. The difference in PPCP compositions between the two recipients can be explained by the source input, which mostly depends on the consumption habits in each region and STPs' removal capacities. On the other hand, the hydrological factor in running water may result in different PPCP compositions in these two water systems, which may be related to physical conditions and eventual photo-degradation.

Some PPCPs such as telmisartan, carbamazepine, and tramadol were frequently detected at high concentrations in both recipient systems, which may representative PPCP patterns indicating the presence of STPs in affected aquatic areas in the Czech Republic. Further post-treatment can be applied in order to reduce the level of pollutants in these areas (Bourgin et al., 2018; Krzeminski et al., 2017).

A previous study has suggested that PPCP accumulation in sediment was reduced according to the distance from the inlet point (close to STP effluent discharge) to the outlet point (Koba et al., 2017). Longer retention times, the dimension of biological ponds, and the presence of macrophages and plants in a biological pond may help to reduce pollution levels.

The effect of STP on aquatic organisms

In the two experiments (PAPERS I and II), two native species (common carp and brown trout), which can be representative for aquatic organisms in the studied regions, were used as model organisms. In general, some favorable conditions, including temperature and nutrient factors, resulted in fast growth in the fish in both recipients. Fish in the Cezarka Pond were about 1.8- to 2.8-fold heavier than fish in the control pond while in the Zivny Stream, fish from the STP-affected site ranged from 1.4- to 1.8-fold heavier than control fish. The Cezarka Pond receives only water from STPs, which induce extremely high nutrient conditions resulting in very fast growth of fish in this pond. Additionally, the differences in experimental duration in each study could be the reason for this difference in the fish's growing speeds.

Despite the fast growth, many biomarkers indicated adverse signs in the fish living in these polluted environments. The effects of STP effluent on oxidative stress and antioxidant enzyme activities showed a clear trend in the elevation of lipid peroxidation (LPO), SOD, GST, and a decline in CAT, GR, and GPx in fish from Cezarka Pond but not in fish from Zivny Stream. The different magnitude of total PPCPs concentration in each recipient can be the explanation for this findings. On the other hand, the difference in PPCPs composition in each recipient may induce different effects. Interactions among compounds in their mixtures can induce synergistic or antagonistic effects. Changes in blood biomarkers were found in fish exposed to STP effluents, reflecting disturbances in fish's physiological statuses (Topić Popović et al., 2015). Fish situated in affected areas showed changes in blood biomarkers, including Hb, RCB, PCV, MCV, MCH, AST, ALP, LHD, GLU, TRIS, and CREA. Furthermore, a decrease in WBC

was observed in fish during all exposure periods. The change in these parameters may not only be related to the presence of pollutants but also other factors such as hydrochemical conditions or the presence of parasites in the fish. The induction of CYP1A and 3A enzyme activities have been frequently detected in fish exposed to PPCPs and polycyclic aromatic hydrocarbons (PAH)-polluted environments. Induction of CYP1A and 3A were seen in fish from both Cezarka Pond and Ziny Stream (Sakalli et al., 2018). Also, induction of CYP1A and 3A were consistent with the variations in the total PPCP concentrations, which suggests that these two biomarkers are effective biomarkers for investigating complex PPCP mixtures.

The signs of endocrine disruption were seen in fish from both recipients. Elevations in VTG levels in male fish and occurrence of some irregular structures in female fish were detected. The pilot experiment concerning reproductive success (fertilization rate) was done following endocrine disruption detection. However, no profound effects were seen in reproductive success. It has been shown that fluctuations in VTG levels may impact survival or reproduction in one species (Kidd et al., 2007) but may not show these effects in another one (Pelley, 2003). In this study's experiments, common carp were able to naturally spawn in Cezarka Pond. However, changes in gonad maturation times were investigated in trout in Zivny Stream. In Czearka Pond, the high availability of nutrients and the occurrence of estrogenic compounds may induce faster maturation of the fish's gonads while in Zivny Stream, temperature changes and occurrence of pollutant compounds appear to postpone the spawning season of fish. Although this effect did not seriously impact final spawning success, it should be considered since it was one of the effects of fluctuating VTG levels. Consistent results between VTG concentrations in blood plasma and *vtg* gene expression in liver tissue were observed in fish from both experiments. This study indicated that VTG can be used as a good biomarker for investigation of complex mixtures of pollutant under real conditions.

Fatty acids play an important role in biological processes and construction of biological structures. Changes in fatty acid composition in muscle tissue of exposed fish are not only related to the food intake but also to the perturbation of synthetic processes that may be induced by the presence of pollutants. The reduction of omega-3 fatty acids in muscle tissue of fish from Cezarka Pond and Zivny Stream (Sakalli et al., 2018) needs to be taken into consideration. It suggests that STP effluent discharges may reduce meat quality. This reduction can be explained by different food quality and availability and environmental conditions (temperature, pollutants) between the STP-affected and control sites, which may affect fatty acid synthesis pathways. It has been shown that uptake of pollutants such as fibrates and PCBs can change fatty acid composition (Carnevali et al., 2017; Guo et al., 2015). Moreover, changes in bacterial taxa abundance and composition, especially occurrence of those associated with activated sludge, may impact fatty acid synthesis. This issue needs to be clarified in future studies. Previous studies have detected bioaccumulation of some PPCPs (Grabicova et al., 2017) and PCB (Li et al., 2011) in fish tissue. Further studies of pollutants in muscle tissue may need to investigate nutritional aspects.

Although numerous alterations in biochemical biomarkers were observed that may reflect disturbances in detoxification-associated enzyme activities, oxidative stress, and the endocrine system. However, no clear morphological alterations were seen in the examined tissues, except for some random irregular structures (interstitial edema, atretic oocytes, inflammation) in the exposed fish's gonads. STP effluent-induced effects may be not strong enough to damage tissues such as gills, liver, kidney, and spleen in the exposed fish. The fast growth of fish suggests use of STP effluent-discharged water for aquaculture activities. This method has been applied in some Asian countries (Kumar et al., 2014). However, more studies about the quality of the resulting fish meat are needed in cases in which this method is to be used.

STP effluent discharges can affect bacterial consortia in aquatic recipients (Drury et al., 2013). In this thesis, both bacterial abundance and composition were affected by STP effluents in the downstream lotic environment of Zivny Stream. Three taxa were detected in the downstream, but not upstream locations in Zivny Stream: (1) unclassified Acidimicrobiales Candidatus *Microthrix* (Actinobacterium); (2) unclassified Chitinophagaceae similar to *Ferruginibacter*; and (3) *Terrimonas* (Bacteroidetes), and *Thermomonas* (Gammaproteobacteria). These taxa could be considered as biomarkers for anthropogenic pollution because they were previously proven to be associated with activated STP sludge (McIlroy et al., 2013; McIlroy et al., 2016; Myung et al., 2015). Changes in bacterial composition and abundance may affect aquatic biochemical factors (such as degradation of organic matter) and fish health (such as fish diseases). Additional research may be needed to shed more light into the numerous black corners of this very important ecological field. It has been shown that STP discharge water affects the microorganism population in fish intestines. The occurrence of a high parasite density and bacterial taxa associated with active sludge in fish is definitely affected by the external environment. This occurrence of abnormal microorganisms may have effect on fish health and needs to undergo further investigation.

In both experiments, the integrated biomarker response (IBR) index is a helpful tool to summarize and illustrate a general picture of STP effluent effects. This index can help to clarify the most responsive tissues and sensitive periods of fish exposure. Fish were more sensitive during the early exposure and during their spawning season. Liver and gills were the most responsive tissues. Changes in liver were evidently linked with level of PPCPs, while changes in gills may be related to other hydrological factors. The IBRv2 developed by Sanchez et al. (2013) was applied in PAPER II. This index based on the principles of reference deviation and can be used to overcome the IBR 1st version shortcomings (Beliaeff and Burgeot, 2002) (in PAPER I). IBRv2 can give a more comprehensive summary of results in which it allows visualization of both inhibition and induction responses of biomarkers in the same picture. Use of IBRv2 was successfully applied in our field study to overcome the challenge of presenting a summary of large and variable biomarker data sets.

Although a comprehensive approach was applied, there were still some limitations in both experiments. In the Zivny Stream experiment, using adult fish may have led to underestimation of the impact of exposure because fish are more sensitive during their early life stages. In the Cezarka experiment, the control pond was quite small in comparison with Cezarka Pond. It is difficult to find a control pond that corresponded to size of our experimental biological pond. In this experiment, the control pond was selected in order to approximate the hydrological and ecological conditions as close to Cezarka Pond as possible. Therefore, both ponds were in the same geographical range with a reduction in weather or geological condition differences. Moreover, the water depth and fish density were approximately the same in both ponds. The experimental design allowed fish exposure under fully natural conditions. However, there is a risk of losing fish due to predators. Therefore, future experiments should consider this issue to ensure having enough fish for all sampling events. In both experiments, pollutants were found at the control site; however, most of these were at trace levels. To our best knowledge, it is not possible to find a natural water body with no level of pollution. The level of pollutants at the control sites used in these studies is in the same range as previous studies from other European scenarios, which is acceptable for a control area. A future investigation focusing on specific aspects of natural reproduction success is needed in both experiments.

CONCLUSIONS

In this thesis, two unique experiments were established to help reveal a clear picture of the risks and effects of STP effluents on aquatic ecosystems under real conditions. By applying the passive sampling/mass-spectrometry approach, a total of 95 PPCPs could be detected in the two STP recipients. Exposure of native/wild fish to natural conditions helped to minimize other disturbing interventions and reflects only STP effluent-induced effects. Biomarker responses may be slightly different between the fish from each affected recipient. In general, however, the effects of STP effluent discharges on fish in each recipient showed a similar pattern. Although fish from STP affected sites showed fast growth and good external conditions, several effects, including disturbance in oxidative stress, antioxidant and detoxifying enzyme activities, and endocrine disruption affected the fish's health, cannot be ignored. STP effluent also affected fish muscle tissue by leading to an increase in overall fat content but a reduction in omega-3 fatty acids.

In conclusion, it seems obvious that applications of post-treatment measures are necessary to reduce trace pollutants and hydrochemical effects of STP effluent discharge. Future investigations focusing on numerous ecological consequences, including fish behaviour, population status, trophic relationships, and/or food safety aspects are needed.

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ENGLISH SUMMARY

Biological effects of anthropogenic pollutants in recipients of treated sewage water

Sewage treatment plant (STP) plays an important role in protecting aquatic environments from anthropogenic pollution. Recently, however, STP effluent discharge has been reported to contain a wide range of contaminants. Moreover, emerging contaminants such as pharmaceutical and personal care products (PPCPs) have been continuously detected. These compounds are usually bioactive, and their modes of actions on non-targeted aquatic organisms are unknown, especially when they are present in complex mixtures. Therefore, concern about the effects of STP effluents on aquatic ecosystems has been raised.

In this thesis, a wide range of emerging contaminants was measured with focus on PPCPs using the POCISs and MS/MS approaches. Two native organisms, common carp (*Cyprinus carpio*) and brown trout (*Salmo trutta* L.), which are representative to each receiving aquatic recipients (static water found in Cezarka Pond and running water found in Zivny Stream) were used as models to investigate the effects of STP effluents on aquatic organisms.

In both recipients, STP effluent discharge changed hydrochemical factors such as temperature and nutrients, which consequently had an impact on aquatic organisms. A wide range of PPCPs was detected in each type of recipient. Sixty-two and 53 PPCPs were detected in Cezarka Pond and Zivny Stream, respectively; they belong to different PPCP groups such as antibiotic, psychoactive compound, hypertension drugs, illicit drugs and others. The highest concentration of total PPCPs in Cezarka Pond and Zivny Stream were 14000 ng/POCIS and 3860 ng/POCIS, respectively, which was 45- and 36-fold higher than in each areas' control area, respectively.

Due to the favorable conditions (such as temperature and nutritional factors) fish from STP-affected recipients grew more rapidly than fish from the control sites. However, changes in biomarkers suggest toxic signs in fish from both STP-affected areas. In fact, the increasing detoxification processes were seen with CYP1A and 3A elevations, reflecting the variation of total PPCP concentrations in STP-affected areas. The endocrine disruption signs were investigated in fish from STP-affected sites with elevation of vitellogenin (VTG) concentrations in blood plasma/*vtg* gene expression in hepatic tissue, irregular structures in gonadal tissue, and postponement of spawning season. Despite ongoing natural reproduction, the effects on final spawning success were not sufficiently studied. Additionally, biochemical parameters (oxidative stress parameters) reflecting stress conditions and internal equilibrium (blood biochemical and hematological parameters) also showed that the disturbances in fish health may be linked to pollutants from STP effluents. Moreover, changes in microorganism composition were not only evident in lotic environments but also in the intestines of fish from STP-affected sites. Elevation of total fat content, but lower amounts of omega-3 fatty acids, were observed in the muscles of fish from the affected sites. These findings suggest that further studies related to meat quality are needed.

This thesis reflects the effects of STP effluent discharge on aquatic environments under realistic conditions. Although fish were in good condition externally at the end of the experiment, a wide range of emerging pollutant compounds in water and changes in the investigated biochemical endpoints in the two experiments suggest that more measures need to be applied in order to further reduce the pollutants from STP before it is discharged to natural environments.

CZECH SUMMARY

Biologický vliv antropogenních polutantů přítomných v recipientech komunálních odpadních vod

Čističky odpadních vod jsou považovány za hlavní nástroj, který zajistí vyčištění odpadních vod vypouštěných z čističek odpadních vod (ČOV) do životního prostředí. Bylo však zjištěno, že vyčištěné odpadní vody vypouštěné z ČOV obsahují širokou škálu znečišťujících látek. Kromě toho byl průběžně zjišťován výskyt některých nově identifikovaných polutatnů ze skupiny farmak a prostředků osobní hygieny (PPCP). Tyto sloučeniny mají obvykle bioaktivní účinky, ale jejich způsob působení na necílové vodní organizmy není dostatečně prodstudován, zvláště pokud se vyskytují ve složitých směsích. Tyto skutečnosti vedly ke zvýšeným obavám z vlivu odtoku ČOV na vodní ekosystém. V této práci byla studována široká škála kontaminantů se zaměřením na sledování PPCP při využití pasivního vzorkování a hmotnostní spekrofotometrie. Dva druhy volně se vyskytujících organizmů, kapr obecný (*Cyprinus carpio*) a pstruh potoční (*Salmo trutta* L.), byly využity jako modelové organizmy pro různé typy recipientů (stojatá a tekoucí voda) pro zkoumání vlivu odtoku ČOV na vodní organizmy. U obou typů recipientů ovlivňují výtokové vody ČOV hydrochemické parametry, jako je teplota a obsah živin, které následně ovlivňují život vodních organizmů. V obou typech recipientů byla zároveň zjištěna přítomnost široké škály PPCP. V rybníku Čežarka bylo identifikováno 62 a v Živném potoce 53 PPCP patřících do různých skupin léčiv zahrnujících antibiotika, psychoaktivní látky, antihypertenzních léčiva, zneužívaných drog apod. Nejvyšší koncentrace PPCP dosahovala v Čezarce 14 000 ng/POCIS a Živném potoce 3 860 ng/POCIS, což je 45krát až 36krát více než v kontrolních lokalitách.

Kvůli změněným životním podmínkám, jako je teplota a obsah živin, rostly ryby v prostředí ovlivněném ČOV rychleji než ryby v kontrolních lokalitách. Změny v hodnotách biomarkerů však naznačují nepříznivé biochemické vlivy u ryb v lokalitách ovlivněných ČOV. Zvýšená intenzita detoxikačních procesů byla pozorována v souvislosti s nárůstem CYP1A a CYP3A, jejichž aktivita korelovala s celkovou koncentrací PPCP v lokalitách ovlivněných ČOV. U exponovaných ryb byly pozorovány příznaky narušení endokrinního systému, které se projevily zvýšením koncentrace vitellogeninu v krevní plazmě, změnou exprese genu vitellogeninu v jaterní tkáni, nepravidelnou strukturou tkání gonád a změnou období tření. Navzdory popsaným nepříznivým příznakům byla zaznamenána přirozená reprodukce, avšak vliv na její průběh není dosud dostačně prostudován. Biochemické parametry, které odrážejí stresové podmínky (parametry oxidativního stresu) a vnitřní rovnováhu organizmu (biochemické a hematologické parametry), také naznačují ovlivnění zdraví ryb způsobené znečišťujícími látkami z ČOV. Kromě toho změny ve složení mikroorganizmů nebyly pozorovány pouze ve vodním prostředí, ale také ve střevách ryb žijících v lokalitách ovlivněných ČOV. Zvýšení celkového obsahu tuku, ale zároveň nižší množství omega-3 mastných kyselin bylo pozorováno ve svalech ryb z postižených lokalit. Toto zjištění otvírá prostor pro studie zaměřené na hodnocení vlivu ČOV na kvalitu rybího masa.

Tato práce popisuje vliv vypouštění odpadních vod z ČOV na vodní organizmy v reálném prostředí dvou modelových recipientů. Přestože ryby na konci experimentu vykazovaly dobrý zdravotní stav a kondici, přítomnost široké škály znečišťujících látek ve vodě a změny biochemických parametrů studovaných ve dvou experimentech naznačují, že je třeba provést více opatření ke snížení přítomnosti znečišťujících látek v odpadních vodách před vypuštěním do životního prostředí.

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Žlábek, V., Burkina, V**.,** Sidika, S., Adam, B., Fedorova, G., Koba, O., **Giang P.T.,** Grabic, R., Randák, T., 2015. Responses of antioxidant defense system in liver and gill of rainbow trout (*Oncorhynchus mykiss*), chronically treated with dexamethasone. SETAC Europe 25th Annual Meeting, Barcelona, Catalonia, Spain, 3–7 May 2015.

TRAINING AND SUPERVISION PLAN DURING STUDY

Name	Pham Thai Giang
Research department	Laboratory of Environmental Chemistry and Biochemistry, FFPW USB
Daily supervisor	Viktoriia Burkina, Ph.D., Ganna Fedorova, Ph.D.
Supervisor	Assoc. Prof. Vladimír Žlábek
Period	20th October 2014 until 20th September 2018

Ph.D. courses	**Year**
Pond aquaculture	2015
Applied hydrobiology	2015
Basics of scientific communication	2016
Ichthyology and fish systematics	2016
English language	2016

Scientific seminars	**Year**
Seminar days of RIFCH and FFPW	2015 2016 2016 2017

National conferences	**Year**
Biological effects of sewage treatment plant effluent on common carp (*Cyprinus carpio*) under fully realistic condition. The 18th toxicological conference "Toxicita a biodegradabilita odpadů a látek významných ve vodním prostředí", Vodnany, 23–25 August 2017 (oral presentation)	2017

International conferences	**Year**
Biological effects of anthropogenic pollutants present in pond receiving treated municipal sewage water. SETAC Europe 26th Annual Meeting, Nantes, France, 22–26 May 2016 (Poster presentation)	2016
Effects of multi-component mixtures from sewage treatment plant effluent on common carp under fully realistic condition – a real case study. The 3rd International Conference on Environmental Pollution, Restoration, and Management, 6–10 March 2017 (Poster presentation)	2017

Foreign stays during Ph.D. study at RIFCH and FFPW	**Year**
Aarhus University, Denmark – training in molecular methods (3 months)	2016

Project leader	**Year**
Grant Agency of the University of South Bohemia in Ceske Budejovice: "Metabolic clearance of human pharmaceuticals in fish exposed under real conditions – role of hepatic CYP450 system"	2016
International summer school: "Content of mercury in muscle of fish living in recipient of sewage treatment plant effluent"	2018

CURRICULUM VITAE

PERSONAL INFORMATION

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2012–2014	M.Sc., Szent Istvan University – Godollo, Hungary
2003–2007	B.Sc., Hanoi Agriculture University – Hanoi, Vietnam

RESEARCH INTERESTS: Environmental pollution, aquaculture

Ph.D. courses: Pond aquaculture, Basics of scientific communication, Applied hydrobiology, Ichthyology and fish taxonomy, English language, Czech language

LEADING OF PROJECTS:

Grant Agency of the University of South Bohemia in Ceske Budejovice: "Metabolic clearance of human pharmaceuticals in fish exposed under real conditions – role of hepatic CYP450 system"

International summer school: "Content of mercury in muscle of fish living in recipient of sewage treatment plant effluent"

KNOWLEDGE OF LANGUAGES: Vietnamese (mother tongue), English (IELTS certificate)

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