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Oxidative stress indices and histopathological effects of the nonsteroidal antiinflammatory drug naproxen in adult zebrafish (*Danio rerio*)

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Abstract

OBJECTIVES: The aim of this study was to investigate the effects of the nonsteroidal antiinflammatory drug naproxen on adult zebrafish (*Danio rerio*).
METHODS: Three months old zebrafish (*Danio rerio*) were exposed to naproxen at concentrations of 0.001, 0.1 and 5 $mg.L^{-1}$. We focused on the changes in oxidative stress indices during and at the end of the experiment and histopathological examination of tissues after a two week long exposure period.
RESULTS: We found that a 3 day long exposure to naproxen causes mild oxidative stress and affects detoxification in zebrafish, which is demonstrated by the increased activity of glutathione peroxidase and glutathione S-transferase at 0.001 and 0.1 $mg.L^{-1}$ of naproxen, respectively. After a 7 day long exposure to 0.1 and 5 $mg.L^{-1}$, more potent effects on enzymes occur. However, these effects are only short lasting. At the end of the experiment, the activities of the target enzymes recover back to homeostatic baseline levels. Except catalase, which is induced only after a two week long exposure to the environmental concentration of naproxen. Despite the fact that naproxen causes mild oxidative stress in zebrafish, exposure to this drug does not result in lipid peroxidation. Histopathological examination revealed obvious changes to the gills and liver even at exposure to the environmental concentration of naproxen.
CONCLUSION: This study demonstrates that the environmental concentration of naproxen can slightly influence both the antioxidant defense system and histopathology of non-target fish.

ORIGINAL ARTICLE

Abbreviations

CAT - catalase
GPx - glutathione peroxidase
GR - glutathione reductase
GST - glutathione S-transferase
LPO - lipid peroxidation level
LC-ESI-MS/MS - high performance liquid chromatography coupled with electrospray ionization – tandem mass spectrometry
MS-222 - tricaine methanesulfonate
NPX - naproxen
NSAID - nonsteroidal antiinflammatory drug
ROS - reactive oxygen species
TBARS - thiobarbituric acid reactive substances

INTRODUCTION

Pharmaceutical concentration data for surface waters in the Czech Republic are currently not available. However, these compounds generally occur at low concentrations (ng–μg.L^{-1}) in the aquatic environment (Pal *et al.* 2010). Since most pharmaceuticals are designed to be biologically active at low concentrations, there is a risk that these compounds may affect aquatic wildlife. Because of the continuous input of these, due to wastewater treatment plant effluent, to the aquatic environment, fish are chronically exposed to these substances (Thibaut *et al.* 2006).

Nonsteroidal antiinflammatory drug (NSAID) naproxen (NPX) is one of the most frequently detected pharmaceuticals in the aquatic environment. This drug was found in Canadian waste water treatment plant effluents at a maximum level of up to 33.9 μg.L^{-1} (Metcalfe *et al.* 2003) and the highest measured value in Spanish Turia River water was 7.189 μg.L^{-1} (Carmona *et al.* 2014).

Phototransformation appears to be the main abiotic elimination process in the environment for NPX (Grenni *et al.* 2013). However, the photoproducts are more toxic than the parent compounds (Isidori *et al.* 2005). Biodegradability also plays an important role in maintaining exposure concentration in surface waters. The half-lifetime of NPX was found to be about 27 days in microbiologically active water, while under sterile conditions no significant decrease in concentration was observed in the dark over 44 days (Grenni *et al.* 2013).

Our research team has previously found that drugs can cause oxidative stress in the susceptible early life stages of common carp (*Cyprinus carpio*) and the juvenile development stages of zebrafish exposed for a period of one month (Zivna *et al.* 2013; Plhalova *et al.* 2014; Stancova *et al.* 2015). In our experiment we decided to reveal, if naproxen affects the antioxidant defense system, lipid peroxidation level and histopathology of adult zebrafish after a two week long exposure. At the same time, we focused on changes in oxidative stress indices during the experiment. We exposed adult zebrafish to 0.001, 0.1 and 5 mg.L^{-1} of NPX. The lowest concentration corresponded to the environmental concentration, the higher referred to the heavily polluted wastewater and the highest concentration was set to reveal, if any dose dependency occurs in the indices investigated.

MATERIAL AND METHODS

Experimental design

As test organism 3 month old zebrafish were obtained from a commercial fish farm with an average body weight and total body length of 438.53±132.22 mg and 36.88±11.12 mm, respectively. Fish were randomly distributed into twelve tanks (volume 20 L) for acclimatization to experimental conditions (flow-through system, light/dark: 12 h/12 h, aeration). After two weeks acclimatization a 14 – day long experiment was performed. Fish were exposed in triplicates to sub-lethal concentrations of NPX at nominal values of 0.001, 0.1 and 5 mg.L^{-1}. The total amount of fish used in the experiment was 600 individuals. Every experimental and control group consisted of 150 individuals, divided into three separate tanks, thus each tank contained 50 fish. Fish were fed with dried *Artemia salina* without shells *ad libitum*. During the test, the condition of fish was checked twice per day and the temperature, pH, and the oxygen saturation of tap drinking water were recorded daily. Water quality values were measured as follows: temperature 23.6–24.2 °C, oxygen saturation 86–94%, and pH 7.6–8.2.

A stock solution was prepared by dissolving NPX (Sigma Aldrich, Czech Republic) of >98% purity in distilled water and this was stored wrapped in aluminum foil in amber glass vials to avoid the formation of phototransformation products. A stock solution was prepared every second day and stored in a fridge at 4 °C until usage. Third and seventh day of the experiment (15 fish per treatment) and at the end of the test (20 fish per treatment), fish were euthanized by the approved anesthetic MS-222 (250 mg.L^{-1}, tricaine methanesulfonate, Sigma Aldrich, Czech Republic).

Homogenization and determination of antioxidant enzymes activity and lipid peroxidation.

Changes in enzymatic activity in zebrafish were investigated after 3, 7 and 14 days long exposure, while lipid peroxidation level was measured only at the end of the experiment. Whole body samples were thawed, weighed, and after the addition of a phosphate buffer (1 mL of buffer per 0.1 g of sample), homogenized on ice.

The catalytic activities of the enzymes glutathione reductase (GR), glutathione peroxidase (GPx), glutathione-S-transferase (GST), and catalase (CAT) were measured spectrophotometrically according to the methods described by Habig *et al.* (1974), Carlberg & Mannervik (1975), Aebi (1984), and Flohe & Gunzler (1984), respectively. To determine the lipid peroxidation level (LPO) in fish samples, the thiobarbituric acid reactive substances (TBARS) method described by Lushchak *et al.* (2005) was used. The activities of enzymes were

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related to protein content. The concentration of proteins was determined by Bicinchoninic Acid Protein Assay Kit (Sigma-Aldrich, Czech Republic), in which bovine serum albumin was used as a standard (Smith *et al.* 1985). All spectrophotometric measurements were performed using a Varioskan Flash Spectral Scanning Multimode Reader (Thermo Fisher Scientific Inc., USA).

Histology

The fish sampled at the end of the experiment were prepared for histopathological examination, fixed in buffered 10% neutral formalin, dehydrated, embedded in paraffin wax, sectioned (cross-section) on a microtome at 4 μm, and stained with hematoxylin and eosin. The histology of skin, gills, kidney, and liver was examined by light microscopy. Six fish from each experimental and control group were investigated.

Measurement of naproxen in water

Water samples were collected four times (from each aquarium) during the test to assess the real concentrations of NPX in water. The methodology of water sampling was as follows: every sampling time consisted of two separate samplings. The first sample was reached from newly prepared solution of NPX and the second sample was reached from the aquaria after 12 hours. This process was repeated four times. Measurement of NPX in water samples was performed by high performance liquid chromatography coupled with electrospray ionization – tandem mass spectrometry (LC–ESI–MS/MS). Detailed information regarding the determination of naproxen in water samples is provided in our previous published paper (Stancova *et al.* 2015). Since good agreement was found between the nominal and measured concentrations, the subsequent biological effects were evaluated on the basis of nominal concentrations.

Statistical analysis

Data are presented as mean ± standard error of mean. Statistical analyses of activity of enzymes and lipid peroxidation were performed using Unistat for Excel 5.6 software. Data were tested for criteria of normality using Kolmogorov–Smirnov test. If data were normally distributed, the effects of NPX on oxidative stress indices were determined by testing for the homogeneity of variance across groups, an analysis of variance and the differences among test groups were assessed with the Tukey's test. For non-normally distributed data Kruskal-Wallis test and Dunn's Multiple Comparison test was used. In our experiment the difference between groups was assessed to be significant at $p<0.05$ and highly significant at $p<0.01$.

Ethical Statement

All experimental procedures were approved by the institutional committee and performed in compliance with institutional guidelines and national legislation (Act No. 246/1992 Coll., on the Protection of Animals Against Cruelty, as amended).

RESULTS

Mortality and growth

The mortality in any experimental and control group did not exceed 2% and no statistically significant difference was found between the mortality in the experimental groups investigated. Similarly, no difference in either weight or length was found in fish exposed to NPX in comparison to control fish (Table 1).

Oxidative stress indices

The changes in the activity of antioxidant enzymes after 3 (A), 7 (B) and 14 (C) days long exposure to naproxen (0.001, 0.1 and 5 $mg.L^{-1}$) are depicted in Figure 1. Three days long exposure to NPX induced activity of GPx and GST. However, after seven days long exposure there was observed more potent impact on antioxidant enzymes. At the end of the experiment, only slight changes of enzymes activity were investigated.

Lipid peroxidation

Non-significant changes in the lipid peroxidation level after two weeks long exposure to different concentrations of naproxen are depicted in Table 2.

Tab. 1. Mean body wet weight and total body length ± standard error of mean of zebrafish (n=20) in control and experimental groups after 14 days long exposure to naproxen at 0.001, 0.1 and 5 $mg.L^{-1}$.

	Control	**0.001 [$mg.L^{-1}$]**	**0.1 [$mg.L^{-1}$]**	**5 [$mg.L^{-1}$]**
Body weight [mg]	499.12±16.86	507.34±17.68	508.09±19.25	480.11±25.11
Total length [mm]	37.29±0.40	38.16±0.36	37.65±0.34	37.10±0.48

Tab. 2. Mean lipid peroxidation level (nmol.g ww $tissue^{-1}$) ± standard error of mean of zebrafish in control and experimental groups after 14 days long exposure to naproxen at 0.001, 0.1 and 5 $mg.L^{-1}$ (n=20).

Exposure period	**Control**	**0.001 [$mg.L^{-1}$]**	**0.1 [$mg.L^{-1}$]**	**5 [$mg.L^{-1}$]**
14 days	37.18±4.42	49.27±7.04	53.65±5.25	59.23±8.90

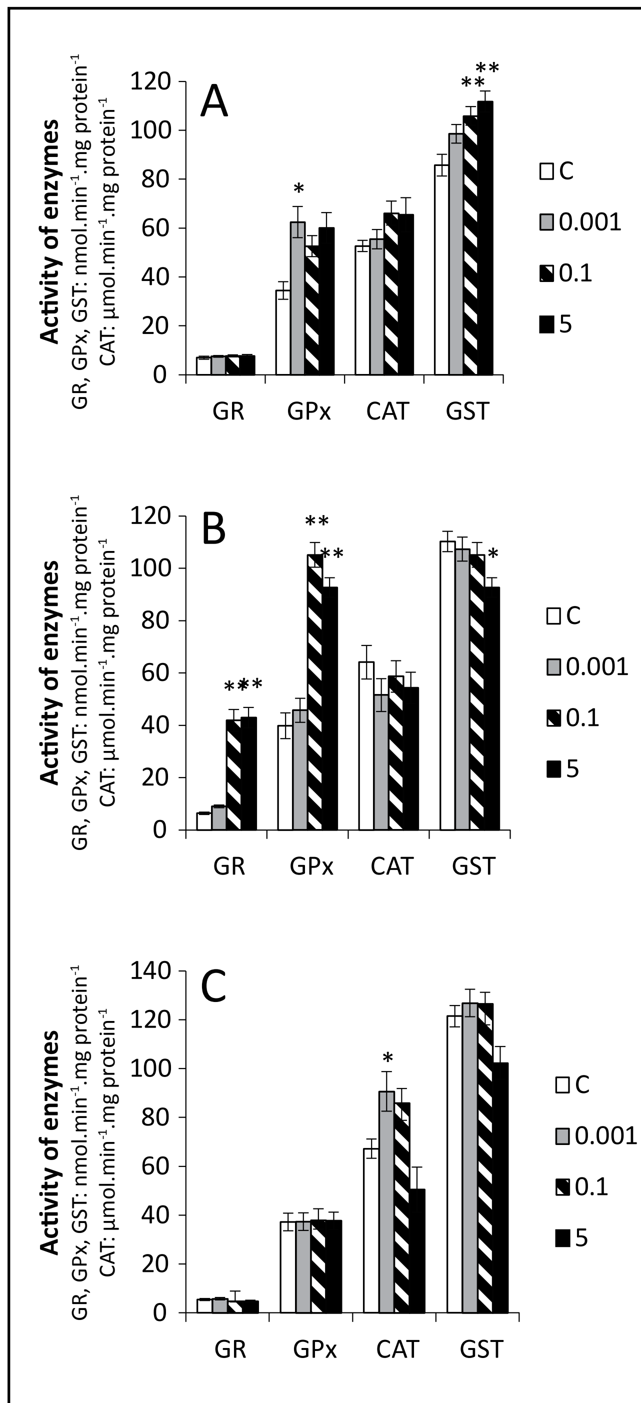

Fig. 1. Comparison of the activity (mean ± standard error of mean) of glutathione reductase (GR), glutathione peroxidase (GPx), catalase (CAT) and glutathion-S-transferase after 3 (A, n=15), 7 (B, n=15) and 14 (C, n=20) days long exposure to narpoxen. (C – control group, 0.001, 0.1, 5 – experimental groups exposed to naproxen at concentration of 0.001, 0.1 and 5 mg.L^{-1}; * statistically significant difference at p<0.05, ** high statistically significant difference at p<0.01).

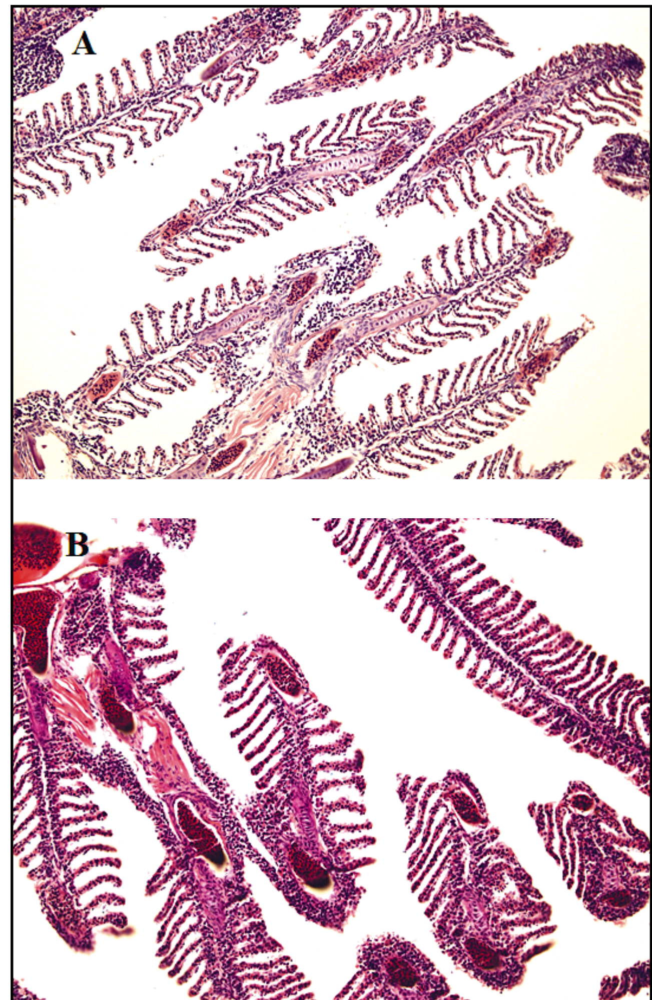

Fig. 2. Results of histopathological examination of gill samples (hematoxylin and eosin, 200×). **A** – gills of adult zebrafish from the control group. **B** – pathological changes (hyperemia, widening of leaflet`s apex and desquamation of leaflet`s epithelium) in the gills exposed to naproxen at a concentration of 0.001 mg.L^{-1} after 14 day.

Histopathology

Histopathology examination of kidney, skin and intestine did not reveal any impact on exposed tissues when compared to the tissues of control fish. Apparent changes occurred in gills and liver. Even at environmental concentration (Figure 2B) the gills showed hyperemia, as well as a widening of leaflet`s apex and desquamation of leaflet`s epithelium in comparison with the control (Figure 2A). A similar finding reoccurs at higher concentration levels. A separation of hepatocytic trabeculae, hyperemia and edema was found in liver parenchyme exposed to an environmental concentration (Figure 3B) in comparison to the control (Figure 3A). In addition, steatosis has been found in liver parenchyme exposed to concentration 0.1 mg.L^{-1}. A mixed form of dystrophy, steatotic and glycogenous, was found in the liver parenchyme exposed to naproxen at a concentration of 5 mg.L^{-1}.

DISCUSSION

The antipyretic, analgesic and antiinflammatory drug naproxen is registered for use in humans and animals and is widely detected in the aquatic environment world-

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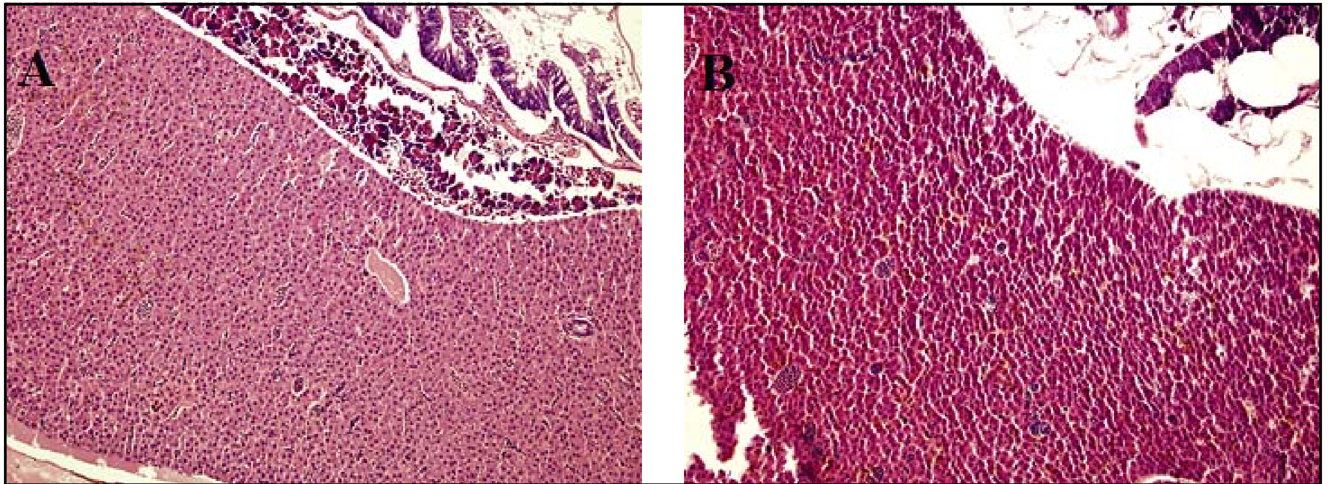

Fig. 3. Results of histopathological examination of liver samples (hematoxylin and eosin, 200×). **A** – liver of adult zebrafish from the control group. **B** – pathological changes (separation of hepatocytic trabeculae) in the liver exposed to naproxen at a concentration of 0.001 mg.L^{-1} after 14 days.

wide. This pharmaceutical acts by blocking the synthesis of prostaglandins by the non-selective inhibition of cyclooxygenases (COX-1, COX-2), similarly like diclofenac, ibuprofen or acetylsalicylic acid, which are included in the same therapeutic class NSAID (Shah *et al.* 2001).

As we expected, NPX did not have any impact on the mortality or growth in our experiment. On the other hand, interesting findings were observed regarding oxidative stress indices. Oxidative stress is a situation when the steady-state reactive oxygen species (ROS) concentration is enhanced in the organism. Exposure to environmental contaminants e. g. pharmaceuticals may enhance ROS-mediated oxidative stress. The evaluation of oxidative stress is commonly based on the measurement of the activity of antioxidant enzymes, which many authors consider as biomarkers of oxidative stress (Gómez-Oliván *et al.* 2014). After a 3 day long exposure changes in enzyme activity were found. A significant increase in activity was observed in glutathione peroxidase and glutathione S-transferase. GPx metabolizes hydroperoxides and GST is the most important phase II biotransformation enzyme, which decreases the toxicity of pollutants by catalyzing the conjugation of glutathione to these compounds (He *et al.* 2011). Increased activity of these enzymes points out a pro-oxidant condition in cells. In our experiment, prolonged exposure (7 days) to NPX led to a more noticeable impact on enzymes activity. A significant increase in both GPx and GR activity is the next sign of mild oxidative stress as GR is involved in the recycling of glutathione forms by reducing the oxidized form of glutathione into its reduced type. Glutathione acts as a catalyst in the reduction of peroxides. Increased activity of GST after a 3 day long exposure was followed by its decreased activity after a 7 day exposure period. Such suppression is due to strong oxidative damage. Surprisingly it was showed to be not permanent. Probably the antioxidant defence system adapted to the stable occurrence of NPX in the water environment. At the end of the experiment an equalization of the enzymatic activity of all enzymes among fish from experimental and control groups except catalase happened, where activity was increased only after a 14 day long exposure to NPX. Catalase metabolizes hydrogen peroxides to molecular oxygen and water. A significant elevation of catalase's activity points out a higher presence of peroxides. Similarly, Gonzalez-Rey & Bebianno (2012) found that ibuprofen induced significant but only transitory antioxidant defense responses in the digestive gland of the mussel *Mytilus galloprovincialis*.

Changes in the activity of antioxidant enzymes indicated mild oxidative stress in zebrafish during our experiment, nevertheless we did not find any increased peroxidation of lipids in exposed fish in comparison to the control at the end of the exposure period. Similarly, a 30 day long exposure to ibuprofen did not increased lipid peroxidation level in zebrafish (Bartoskova *et al.* 2013). However, these studies did not reveal the negative impact of pharmaceuticals on lipid, impairment of the antioxidant system balance, which can lead also to changes in proteins, DNA strand breaks and cellular damage (Hellou *et al.* 2012; Gómez-Oliván *et al.* 2014).

Histopathological examination revealed important changes in the liver and gills at both environmentally relevant and high concentrations of NPX. The gills are in direct contact with the aquatic environment that is why it is commonly influenced by xenobiotics in water. Similarly, the liver is the main organ of detoxification of these compounds. Our earlier published results (Stancova *et al.* 2015) related to the gene expression of antioxidant enzymes in the liver, did not reveal any impact of NPX on these enzymes in zebrafish. Thus, the mechanism causing liver damage does not seem to have anything in common with antioxidant enzymes. Moreover, significant discrepancies exist among the scien-

tific findings in the ultrastructural effects of NSAIDs in fishes. Even different cytological effects on fish organs were found in the range of environmentally relevant concentrations especially for diclofenac (Schwaiger *et al.* 2004; Triebskorn *et al.* 2007; Mehinto *et al.* 2010), although many other studies did not confirm those results (Han *et al.* 2010; Stepanova *et al.* 2013; Stancova *et al.* 2014). Scientists probably have paid more attention to this field of aquatic toxicology and try to reveal the unknown mechanism underlying this cellular damage.

CONCLUSIONS

Our experiment revealed that both the antioxidant defense system and liver and gills structure are affected by the environmental concentration of naproxen in water environment. Despite we found out antioxidant enzymes of zebrafish reacts on presence of naproxen in water, it seems that oxidative stress, which is cause by this pharmaceutical, is only temporary and fishes can adapt to it. On the other hand, important changes in the structure of liver and gills as a consequence of NPX exposure is a field of study which we would investigate in the next experiment.

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REFERENCES

1 Aebi H (1984). Catalase in vitro. Method Enzymol. **105**: 121–126.
2 Bartoskova M, Dobsikova R, Stancova V, Zivna D, Blahova J, Marsalek P, *et al.* (2013). Evaluation of ibuprofen toxicity for zebrafish (*Danio rerio*) targeting on selected biomarkers of oxidative stress. Neuroendocrinol Lett. **34**: 102–108.
3 Carlberg I, Mannervik B (1975). Purification and characterization of the flavoenzyme glutathione reductase from rat liver. J Biol Chem. **250**: 5475–5480.
4 Carmona E, Andreu V, Picó Y (2014). Occurrence of acidic pharmaceuticals and personal care products in Turia River Basin: From waste to drinking water. Sci Total Environ. **484**: 53–63.
5 Flohe L, Gunzler WA (1984). Assays of glutathione peroxidase. Method Enzymol. **105**: 114–121.
6 Gómez-Oliván LM, Neri-Cruz N, Galar-Martínez M, Islas-Flores H, García-Medina S (2014). Binary mixtures of diclofenac with paracetamol, ibuprofen, naproxen, and acetylsalicylic acid and these pharmaceutical in isolated form induce oxidative stress on *Hyalella azteca*. Environ Monit Assess. **186**: 7259–7271.
7 Gonzalez-Rey M, Bebianno, MJ (2012). Does non-steroidal anti-inflammatory (NSAID) ibuprofen induce antioxidant stress and endocrine disruption in musel *Mytilus galloprovincialis*? Environ Toxicol Pharmacol. **33**: 361–371.
8 Grenni P, Patrolecco L, Ademollo N, Tolomei A, Caracciolo AB (2013). Degradation of gemfibrozil and naproxen in a river water ecosystem. Microchem J. **107**: 158–164.
9 Habig WH, Pabst MJ, Jacoby WB (1974). Glutathione *S*-transferases. First enzymatic step in mercapturic acid formation. J Biol Chem. **249**: 7130–7139.
10 Han S, Choi J, Kim J (2010). Endocrine disruption and consequences of chronic exposure to ibuprofen in Japanese medaka (*Oryzias latipes*) and freshwater cladocerans *Daphnia magna* and *Moina macrocopa*. Aquat Toxicol. **98**: 256–264.
11 He X, Nie X, Wang Z, Cheng Z, Li K, Li G, *et al.* (2011). Assessment of typical pollutants in waterborne by combining active biomonitoring and integrated biomarker response. Chemosphere. **84**: 1422–1431.
12 Hellou J, Ross NW, Moon, TW (2012). Glutathione, glutathione S-transferase, and glutathione conjugates, complementary markers of oxidative stress in aquatic biota. Environ Sci Pollut Res. **19**: 2007–2023.
13 Isidori M, Lavorgna M, Nardelli A, Parrella A, Previtera L, Rubino M (2005). Ecotoxicity of naproxen and its phototransformation products. Sci Total Environ. **348**: 93–101.
14 Lushchak VI, Bagnyukova TV, Lushchak OV, Storey JM, Storey KB (2005). Hypoxia and recovery perturb free radical processes and antioxidant potential in common carp (*Cyprinus carpio*) tissues. Int J Biochem Cell B. **37**: 1319–1330.
15 Mehinto AC, Hill EM, Tyler CR (2010). Uptake and biological effects of environmentally relevant concentrations oft he nonsteroidal anti-inflammatory pharmaceutical diclofenac in rainbow trout (*Oncorhynchus mykiss*). Environ Sci Technol. **44**: 2176–2182.
16 Metcalfe ChD, Brenda G, Koenig G., Bennie DT, Servos M, Ternes A, *et al.* (2003). Occurrence of neutral and acidic drugs in the effluents of Canadian sewage treatment plants. Environ Toxicol Chem. **22**: 2872–2880.
17 Pal A, Gin KY-H, Lin AY-Ch, Reinhard M (2010). Impacts of emerging organic contaminants on freshwater resources: Review of reent occurrences, sources, fate and effects. Sci Total Environ. **408**: 6062–6069.
18 Plhalova L, Zivna D, Bartoskova M, Blahova J, Sevcikova M, Skoric M, *et al.* (2014). The effects of subchronic exposure to ciprofloxacin on zebrafish (*Danio rerio*). Neuroendocrinol Lett. **35**: 64–70.
19 Shah AA, Thjodleifsson BT, Murray FE, Kay E, Barry M, Sigthorsson G, *et al.* (2001). Selective inhibition of COX-2 in humans is associated with less gastrointestinal injury: a comparison of nimesulide and naproxen. Gut. **48**: 339–346.
20 Schwaiger J, Ferling H, Mallow U, Wintermayr H, Negele RD (2004). Toxic effects of the non-steroidal anti-inflammatory drug diclofenac, part I: histopathological alterations and bioacumulation in rainbow trout. Aquat Toxicol **68**: 141–150.
21 Smith PK, Krohn RI, Hermanson GT, Mallia AK, Gartner FH, Provenzano MD (1985). Measurement of protein using bicinchoninic acid. Anal Biochem. **150**: 76–85.
22 Stancova V, Plhalova L, Bartoskova M, Zivna D, Prokes M, Marsalek P, *et al.* (2014). Effects of mixture of pharmaceuticals on early life stages of tench (*Tinca tinca*). Biomed Res Int. 2014, Article ID 253468.
23 Stancova V, Zikova A, Svobodova Z, Kloas W (2015). Effects of the non-steroidal anti-inflammatory drug (NSAID) naproxen on gene expression of antioxidant enzymes in zebrafish (*Danio rerio*). Environ Toxicol Pharmacol. **40**: 343–348.
24 Stepanova S, Praskova E, Chromcova L, Plhalova L, Prokes M, Blahova J, *et al.* (2013). The effects of diclofenac on early life stages of common carp (*Cyprinus carpio*). Environ Toxicol Pharmacol. **35**: 454–460.
25 Thibaut R, Schnell S, Porte C (2006). The interference of pharmaceuticals with endogenous and xenobiotic metabolizing enzymes in carp liver: An *in vitro* study. Environ Sci Technol. **40**: 5154–5160.
26 Triebskorn R, Casper H, Scheil V, Schwaiger J (2007). Ultrastructural effects of pharmaceuticals (carbamazepine, clofibric acid, metoprolol, diclofenac) in rainbow trout (*Oncorhynchus mykiss*) and common carp (*Cyprinus carpio*). Anal Bioanal Chem. **387**: 1405–1416.
27 Zivna D, Plhalova L, Praskova E, Stepanova S, Siroka Z, Sevcikova M, *et al.* (2013). Oxidative stress parameters in fish after subchronic exposure to acetylsalicylic acid. Neuroendocrinol Lett. **34**: 116–122.

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