Aquatic Toxicology 227 (2020) 105583

Contents lists available at ScienceDirect

Aquatic Toxicology

journal homepage: www.elsevier.com/locate/aqtox

Naproxen affects multiple organs in fish but is still an environmentally better alternative to diclofenac

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ARTICLE INFO

Keywords:
Naproxen
Diclofenac
Fish
Histology
Kidney
Quantitative PCR

ABSTRACT

The presence of diclofenac in the aquatic environment and the risks for aquatic wildlife, especially fish, have been raised in several studies. One way to manage risks without enforcing improved wastewater treatment would be to substitute diclofenac (when suitable from a clinical perspective) with another non-steroidal anti-inflammatory drug (NSAID) associated with less environmental risk. While there are many ecotoxicity-studies of different NSAIDs, they vary extensively in set-up, species studied, endpoints and reporting format, making direct comparisons difficult. We previously published a comprehensive study on the effects of diclofenac in the three-spined stickleback *(Gasterosteus aculeatus)*. Our present aim was to generate relevant effect data for another NSAID (naproxen) using a very similar setup, which also allowed direct comparisons with diclofenac regarding hazards and risks. Sticklebacks were therefore exposed to naproxen in flow-through systems for 27 days. Triplicate aquaria with 20 fish per aquarium were used for each concentration (0, 18, 70, 299 or 1232 µg/L). We investigated bioconcentration, hepatic gene expression, jaw lesions, kidney and liver histology. On day 21, mortalities in the highest exposure concentration group unexpectedly reached ≥ 25 % in all three replicate aquaria, leading us to terminate and sample that group the same day. On the last day (day 27), the mortality was also significantly increased in the second highest exposure concentration group. Increased renal hematopoietic hyperplasia was observed in fish exposed to 299 and 1232 µg/L. This represents considerably higher concentrations than those expected in surface waters as a result of naproxen use. Such effects were observed already at 4.6 µg/L in the experiment with diclofenac (lowest tested concentration). Similar to the responses to diclofenac, a concentration-dependent increase in both relative hepatic gene expression of *c7* (complement component 7) and jaw lesions were observed, again at concentrations considerably higher than expected in surface waters. Naproxen bioconcentrated less than diclofenac, in line with the observed effect data. An analysis of recent sales data and reported concentrations in treated sewage effluent in Sweden suggest that despite higher dosages used for naproxen, a complete substitution would only be expected to double naproxen emissions. In summary, naproxen and diclofenac produce highly similar effects in fish but the environmental hazards and risks are clearly lower for naproxen. Hence, if there are concerns for environmental risks to fish with diclofenac, a substitution would be advisable when naproxen presents an adequate alternative from a clinical point-of-view.

1. Introduction

In the last decades, there has been an increasing concern about pharmaceuticals in the environment and the effects they can have on non-target organisms. Diclofenac, a commonly used non-steroidal anti-inflammatory drug (NSAID) has received much attention for being the culprit in the near extinction of several vulture species in Pakistan (Oaks et al., 2004; Prakash et al., 2012). Effects of diclofenac on a range of aquatic organisms have been studied as well (Cleuvers, 2003; Schwaiger et al., 2004; Ericson et al., 2010; Näslund et al., 2017; Yokota et al., 2018).

Cytological effects in kidneys and other organs of salmonid fish have

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https://doi.org/10.1016/j.aquatox.2020.105583
Received 24 February 2020; Received in revised form 17 July 2020; Accepted 22 July 2020
Available online 28 July 2020

been reported at concentrations down to 1 μg/L (Triebskorn et al., 2004). Diclofenac concentrations in treated sewage effluents are generally below (Loos et al., 2013) or around 1 μg/L (Brown et al., 2007; Fick et al., 2010a; Meyer et al., 2016) and similar concentrations have been reported in surface water (Lacina et al., 2013; Marsik et al., 2017). Together, these findings have led to concerns for effects on wild fish and the inclusion of diclofenac as one of the first pharmaceuticals on the watch list of priority substances within the European Water Framework Directive (EU, 2013). However, some studies, funded by a company marketing diclofenac, has challenged the initial reports (Memmert et al., 2013; Wolf et al., 2014). These studies reported a No Observed Effect Concentration (NOEC) of 320 μg/L on fish. Independently, we conducted a study showing that diclofenac caused histological changes in the three-spined stickleback already at the lowest tested concentration (4.6 μg/L) following a clear concentration-response pattern, and with significant mortality occurring at 271 μg/L (Näslund et al., 2017). The European Commission recently concluded that diclofenac should be removed from the watch list due to "sufficient high-quality monitoring data" (EU, 2018). There is still no official information whether or not diclofenac will be included on the priority substances list in the Water Framework Directive. If an Environmental Quality Standard is set for diclofenac, large investments may be required to reduce emissions from wastewater treatment plants (WWTPs).

An alternative or additional way to decrease the concentration of diclofenac in effluents that does not involve upgrading wastewater treatment is to reduce incoming amounts of diclofenac. As several NSAIDs with largely similar effects are available, it is plausible that some of these could be used as clinically equivalent alternatives to diclofenac in many situations. In fact, different countries tend to use different NSAIDs in very different proportions (Kookana et al., 2014). A "replacement drug", should not only provide the desired effects on the patient without increasing risks for side effects. Evidence should also exist that the risks for adverse environmental effects indeed would be reduced. Naproxen is a frequently used over-the-counter drug, and it is the first-line NSAID treatment of e.g. nociceptive pain and inflammatory joint diseases in Sweden (Janusinfo Region Stockholm, 2020). Naproxen could probably quite often be a realistic substitute to diclofenac from a clinical point of view (Coxib and traditional NSAID Trialists' (CNT) Collaboration, 2013; van Walsem et al., 2015; Schmidt et al., 2018). However, it should be noted that NSAIDs differ in both potency and side effects, but in practice, they are often marketed and used for the same indications, particularly with regards to over-the-counter use. Fish appears to be the most sensitive aquatic organism to NSAIDs, but the effects of naproxen are sparsely investigated (Stancova et al., 2015a; Li et al., 2016; Sehonova et al., 2017; Kwak et al., 2018; Xu et al., 2019). The data on histopathological effects is even more limited with only three published studies to the best of our knowledge (Stancova et al., 2015a; Li et al., 2016; Sehonova et al., 2017). This incompleteness of data makes the risk evaluation for fish exposed to naproxen difficult. Other NSAIDs may also pose lower environmental risks than diclofenac, but a thorough evaluation of these is beyond the scope of this article.

The primary aim of the present study was to generate relevant effect data on naproxen in fish and to do so in a way that allows a direct comparison with diclofenac regarding their environmental hazards and risks. Based on existing studies, it is difficult to draw firm conclusions on their relative potency due to different endpoints analyzed in different ways, in different labs, in different species and under highly variable exposure conditions. To facilitate a more direct comparison, we therefore used a very similar study set-up with the three-spined stickleback as in our previous study on diclofenac (Näslund et al., 2017). Specifically, we investigated bioconcentration, growth, hepatic gene expression, macroscopic lesions and kidney and liver histology.

2. Material and methods

2.1. Animals

Wild three-spined sticklebacks with no external signs of disease were collected by ring nets in Öresund on the Swedish southwest coast in February 2014. The fish was approximately 8–9 months old (considered juveniles). They were transported in well aerated tubs to the Aquatic Facility at Swedish University of Agricultural Sciences (SLU) in Uppsala where they were acclimatized to laboratory conditions by gradually replacing the water to carbon filtrated tap water and adjusting the temperature. A few fish from the batch were used for a parasitological investigation. Scrapings from gills, skin and fins were investigated via light microscopy and occasional protozoan parasites were found in some individuals. All fish were then treated with a dilute formaldehyde bath for one hour once to remove any external parasites. (2.5 mL 37 % formaldehyde to 10 L of water). A new parasitological investigation was performed afterwards in the same manner as previously described. No parasites were detected during the second investigation. The fish were kept in 100-L filtrated well-aerated glass aquaria with continuous flow-through of new carbon-filtrated tap water. The temperature was approximately 13–14 °C and the fish were fed frozen bloodworms 1–2 times a day. The photoperiod was set to 8 h light and 16 h dark to keep the sticklebacks reproductively inactive. Fish were held under these conditions for approximately 2 months before the experiment started. An animal ethics permit was given in advance by the Uppsala Ethical Committee on Animal Research (C198/12).

2.2. Test chemical

Naproxen sodium salt (CAS: 26159-34-2, purity 98.0–102.0 %) was purchased from Sigma–Aldrich (Steinheim, Germany).

2.3. Experimental design

Before the start of the experiment, 300 sticklebacks were randomly distributed in 15 glass aquaria (size 54 L with 44 L of water, n = 20 fish/aquarium). There was a continuous flow-through of carbon-filtrated, aerated tap water delivered by a multi-channel peristaltic pump (Ismatec®, Wertheim, Germany) with PharMed Ismaprene tubing in the pump and silicone tubing as extensions. Each aquarium was enriched with a glass jar made non-transparent by sandblasting and all aquaria had airstones to ensure appropriate oxygen levels in the water. For chemical characteristics of the tap water used, see supplementary data.

Naproxen sodium was mixed with MilliQ water on a magnetic stirrer approximately 1–2 h in total darkness. No solvents were used. This created a 'superstock' with the concentration of 1280 mg/L of naproxen. Aliquots from the superstock were diluted with MilliQ water to produce four different stock solutions (8 mg/L, 32 mg/L, 128 mg/L and 512 mg/L) with three replicates for each concentration so each aquarium had its own stock solution bottle. The control aquaria had only MilliQ water in their stock solution bottles. A multi-channel peristaltic pump (Ismatec®, Wertheim, Germany) with PharMed Ismaprene tubing in the pump and polytetrafluoroethylene (PTFE) tubing as extensions were used to deliver the stock solutions to the aquaria. The nominal concentrations in the aquaria were set to be 0 μg/L, 20 μg/L, 80 μg/L, 320 μg/L and 1280 μg/L, with 3 replicate aquaria for each concentration. This created four different exposure concentration groups and one control group, each group containing three replicate aquaria (placed in mixed order). The superstock and stock solutions were renewed once a week. The concentrations were chosen so that there would be an overlap with the nominal concentrations used in our previous study on diclofenac (Näslund et al., 2017). The

concentration range, however, was shifted up one dilution step as the fish plasma model suggested lower potency of naproxen (Fick et al., 2010b). The highest nominal concentration was more than 500 times lower than the reported LC_{50} in fish (Rodriguez et al., 1992) to reduce risks for direct, drug-induced mortalities.

At the onset of the experiment (day zero), the pump delivering stock solutions was started and different volumes of the superstock were added manually to all but the control aquaria to immediately reach the target concentrations. The fish were fed frozen bloodworms 1–2 times daily and fecal matter was removed by siphoning two times a week. Temperature (15.0–16.6 °C) and oxygen level (≥ 97.1 %) were measured two times a week, and pH (8.11–8.25) on day 6 and 20. Water samples for determination of actual exposure concentrations were taken 2–3 times a week and stored at −20 °C until analysis.

The fish were checked daily and dead fish were removed. Moribund fish (included in mortality counts) were also removed and euthanized by decapitation followed by rapid destruction of the brain, in line with the approved ethical permit. Any external symptoms on the removed fish were noted. The experiment was planned to last 27 days.

2.4. Sampling

On day 21, the mortality had unexpectedly reached ≥25 % in all aquaria in the highest exposure concentration group (1280 µg/L, nominal) and that fish were sampled the same day due to animal welfare reasons. All of the other treatment groups were sampled as planned on day 27. The sampling was done as described in detail in paragraph 2.4 in Näslund et al. (2017) but due to a higher number of fish in each aquarium in the present study, four fish from each aquarium, were used for analysis of whole-body naproxen concentration.

2.5. Chemical analysis

Chemical analysis is described in full detail in Näslund et al. (2017) and Grabic et al. (2012) using D3-naproxen as the internal standard. Briefly, naproxen concentration in water samples and in stickleback (whole-body) were measured using a triple stage quadrupole tandem mass spectrometry (MS/MS) TSQ Quantum Ultra EMR (Thermo Fisher Scientific, San Jose, CA, USA). Two pumps were used in the analytical system, an Accela and a Surveyor LC pump (Thermo Fisher Scientific, San Jose, CA, USA), and these were coupled with a PAL HTC autosampler (CTC Analytics AG, Zwingen, Switzerland). Concentrations were reported per wet weight.

2.6. Bioconcentration and condition factors

Bioconcentration factors (BCFs) were calculated by dividing the average naproxen concentration of the four analyzed fish in each aquarium (whole-body; ng/g; wet weight (ww)) with the mean naproxen concentration in the water of the same aquarium (µg/L). Fulton´s condition factor was calculated by dividing the total weight (g) of the fish by the cube of the total length (cm) and multiplying the result with 100.

2.7. Histological analysis

The formalin-fixated fish were trimmed before sectioning. The head was removed caudally to the gills and the tail caudally to the anus. The spines on the back and the abdomen were cut away to facilitate sectioning and the swim bladder was punctured to ensure paraffin penetration. All abdominal organs were left *in situ*. The remaining body of the fish was put on its lateral side in plastic cassettes followed by routine processing and imbedding. The sectioning and staining procedure were the same as in our previous study (Näslund et al., 2017). Presence of hematopoietic hyperplasia, tubular necrosis, pigmented macrophage aggregates (PMA), tubular regeneration, tubular hyaline degeneration/droplets and parasites were graded in the kidney. Presence of hepatocellular vacuolation, inflammatory cell foci, pigmented macrophage aggregates (PMA), hepatocellular necrosis and parasites were graded in the liver. A 4-point grading system was used with 0 = Not present, 1 = Minimal, 2 = Mild, 3 = Moderate and 4 = Severe. All slides were coded, leaving the pathologist (JN) unaware of the treatment. The section with the largest part of the kidney was chosen for both the kidney and the liver assessment. Liver from a minimum of six fish and kidney from a minimum of eight fish from each aquarium were investigated. The different numbers were due to mortalities or insufficient tissue for grading. For full details, see supplementary data. Before the start of the grading, a subset (20) of histological slides from stickleback kidney from Näslund et al. (2017) was reexamined blindly regarding renal hematopoietic hyperplasia (the only histological lesion with statistically significant differences between the exposure concentration groups and the control group from that study). This was done to ensure a comparable grading between the investigations. Ninety percent of the slides were assigned the same grade as the previous grading, which was considered acceptable.

2.8. Quantitative real-time PCR

Total RNA was extracted from livers from four individual stickleback per aquaria (two females and two males) using an RNeasy® Plus Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's recommendations. RNA quantity and quality was checked using the NanoDrop 2000c spectrophotometer (Thermo Scientific, Gothenburg, Sweden) and an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Extracted RNA (1 µg) was reverse-transcribed to cDNA using the iScript® cDNA Synthesis Kit from Bio-Rad Laboratories, Inc. (Hercules, CA, USA) The following genes were selected: cytochrome P450 1A *(cyp1A),* complement component 7 *(c7),* vitellogenin *(vtg),* glutathione reductase *(gr)* and superoxide dismutase 1 *(sod-1)*. The qPCR analyses were performed using SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad Laboratories, Inc., Hercules, CA, USA) with 10 ng of cDNA (in duplicates) in a reaction volume of 10 µL. The primer concentration and annealing temperature were set to obtain efficiency between 95–105 %. A protocol of melting (95 °C, 10 s), annealing and elongation (60 °C, 30 s) was repeated for 40 cycles and followed by a final melting step to verify a single PCR product. No-template controls (NTC) and no-reverse transcriptase controls (NoRT) from random samples were used to check for primer dimer formation or DNA contamination. The mean C_T of the duplicates were used. Quantitative PCR data were analyzed using $2_T^{-\Delta\Delta C}$ method (Livak and Schmittgen, 2001) using β-actin *(β-act)* as reference gene, which was stable across treatment groups. For full details, see supplementary data.

2.9. Statistical analysis

The variation between fish within the same aquaria as well as the variation between aquaria within the same treatment was taken into account in the statistical analysis. Calculations were done in Stata (StataCorp. 2017. Stata Statistical Software: Release 15. College Station, TX: StataCorp LLC). Continuous data (length, weight, condition factor and normalized hepatic mRNA levels (ΔC_T)) were analyzed by a mixed multilevel model with nominal naproxen aquarium concentration as a fixed factor and aquaria as a random factor. A mixed ordered logistic model was used for ordinal data (histological grading), again with nominal naproxen aquarium concentration as a fixed factor and aquaria as a random factor. Multiple comparisons were done according to Dunnett´s test with $\alpha = 0.05$. Jaw lesions were analyzed by a mixed logistic model and a Poisson model was used for analysis of mortality data. A p-value of < 0.05 was considered significant.

Table 1
Average measured naproxen concentrations in water (µg/L) and whole fish (ng/g ww).

	Naproxen concentration				
Nominal aquaria concentration (µg/L)	0	20	80	320	1280
Measured concentration in water (µg/L; mean ± S.D.) (n[1])	< LOQ[2] (27)	18 ± 2 (27)	70 ± 3 (27)	299 ± 15 (27)	1232 ± 67 (21)
Measured concentration in fish (ng/g ww; mean ± S.D.) (n[1])	< LOQ[3] (12)	1.7 ± 0.6 (11)	4.6 ± 2.7 (12)	20 ± 18 (10)	90 ± 37 (11)

[1] n = number of samples.
[2] LOQ = Limit of quantification 5 ng/L.
[3] LOQ = Limit of quantification 0.1 ng/g.

3. Results

3.1. Chemical analysis

Nine water samples were analyzed from each aquarium except in the highest exposure concentration group where only seven samples were included due to the pre-termination of that group. Fifty-six fish samples were analyzed (four samples were lost during the preparation). Naproxen was not detected in any of the water or fish samples from the control group. The average measured naproxen concentrations per exposure concentration group (calculated as the average of all analyzed samples within the same exposure concentration group) can be found in Table 1. The overall average bioconcentration factor was 0.07 and it was relatively stable across the entire range of exposure concentrations (Fig. 1; see supplementary data for full details).

3.2. Mortality

Eight moribund fish in six different aquaria were euthanized in advance during the experiment and are hereafter counted as mortalities. Fish in the two highest exposure concentration groups (299 µg/L and 1232 µg/L, measured concentration) appeared to eat slower and even leave some food after approximately one week of exposure. After three weeks of exposure (day 21), the total mortality had unexpectedly reached ≥ 25 % in all three replicate aquaria in the highest exposure concentration group (1232 µg/L, measured). We therefore decided to terminate this group in advance due to animal welfare reasons, and the remaining fish were sampled the same day. The mortality was significantly increased in that group compared to the control group ($p < 0.001$) (Fig. 2). At the end of the experiment (day 27), the second highest exposure concentration group (299 µg/L, measured), also reached a significantly increased mortality compared to the control group ($p = 0.04$) (Fig. 2) Full details can be found in the supplementary data.

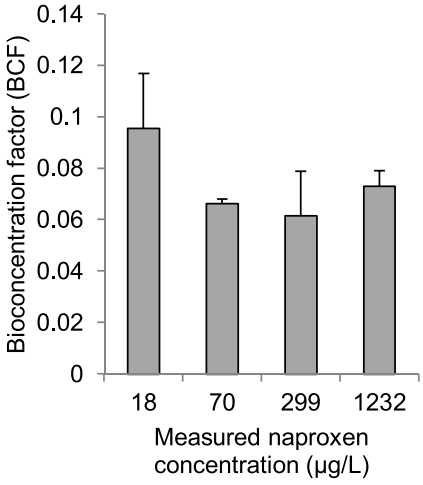

Fig. 1. Bioconcentration factor (BCF; whole-body to water) in stickleback exposed to naproxen. Grey bars show the average BCF of three aquarium replicates + S.D.

3.3. Jaw lesions

Jaw lesions (Fig. 3) were observed among some of the dead or moribund fish removed during the experiment and at the sampling. It should be noted that none of the fish had any signs of jaw lesions before the start of the experiment. The overall prevalence was 22 % (67/300). In the two highest exposure concentration groups (299 µg/L and 1232 µg/L), more than half of the fish had jaw lesions (Table 2). This was statistically significant compared to the control group ($p < 0.001$ for both groups, one-tailed test). Lesions were not observed in fish from any of the control aquaria and very few fish were affected in the 18 µg/L and 70 µg/L exposure concentration groups. For full details, see supplementary data.

3.4. Length, weight and condition factor

Five fish were excluded from the statistical calculations for length, weight and condition factor due to caudal fin rot (four fish) or severe jaw lesion (one fish) resulting in non-comparable length estimates. There were no statistical significant differences in lengths between the different treatment groups. The weights of fish in the 299 µg/L and the 1232 µg/L exposure concentration groups were lower compared to the control group ($p = 0.042$ and $p < 0.001$, respectively; two-tailed test; see supplementary data, Table S1). The fish in the highest exposure concentration group (1232 µg/L) had a lower condition factor compared to the control group ($p < 0.001$; two-tailed test; see supplementary data, Table S1). For full details, see supplementary data.

3.5. Histology

3.5.1. Kidney

The kidneys of 191 sticklebacks were examined histologically (8–16/aquarium). Renal hematopoietic hyperplasia was more extensive in fish from the 299 µg/L and the 1232 µg/L exposure concentration groups compared to the control group ($p = 0.001$ and $p = 0.011$, respectively; one-tailed test based on previous literature (Schwaiger et al., 2004; Näslund et al., 2017); Fig. 4). Note that fish exposed to the highest concentration were exposed for a shorter time. Micrographs of the different grades can be found in Fig. 5. Six fish (four in the control group, one in 18 µg/L group and one in the 70 µg/L group) had a moderate inflammation in their back musculature and one fish in the control group had a severe protozoan infection in the kidney. As such lesions probably could affect the renal hematopoietic hyperplasia and hence receive a higher grade (the median and mode grade for these seven fish were 3) one could argue that those fish should be removed from the statistical comparison. We performed an additional statistical analysis where these seven fish were excluded which resulted in an even stronger significant difference between the two highest exposure concentration groups and the control group ($p < 0.001$ and $p = 0.001$ for 299 µg/L and 1232 µg/L respectively). Tubular necrosis was not found in any of the examined samples. None of the other graded renal

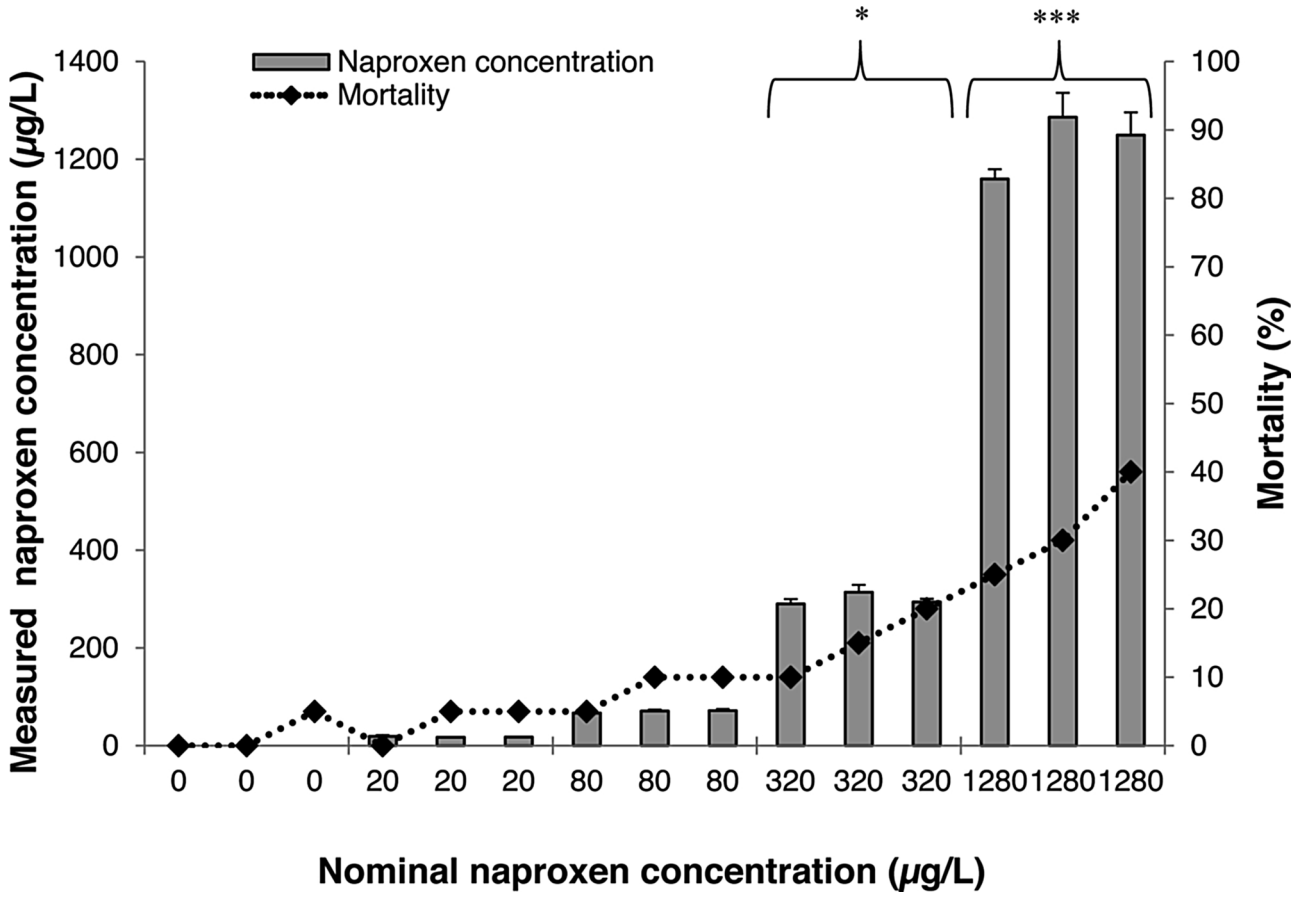

Fig. 2. Measured naproxen concentration and mortality of exposed stickleback. Each grey bar represents the average naproxen concentration in one aquarium replicate + S.D. Asterisks indicate significant differences in mortality to the control group, * $p = 0.04$ (d. 27) and *** $p < 0.001$ (d. 21).

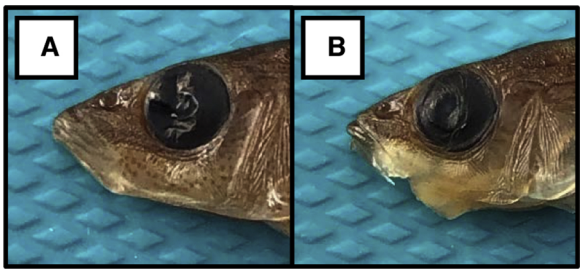

Fig. 3. Formalin-fixated stickleback. **A:** Fish from control group with no signs of jaw lesions. **B:** Fish exposed to naproxen (1232 µg/L) with lesions on the lower jaw.

Table 2
Frequencies of jaw lesions in stickleback exposed to naproxen. Asterisks indicate significant differences to the control group, *** $p < 0.001$.

Treatment group (µg/L)	Fish with jaw lesions
0	0/60 (0 %)
18	1/60 (2 %)
70	2/60 (3 %)
299	31/60 (52 %) ***
1232	33/60 (55 %) ***

lesions (pigmented macrophage aggregates, tubular regeneration, hyaline degeneration/droplets or parasites) showed any statistical significant differences between the treatment groups. For full details, see supplementary data.

3.5.2. Liver

A total of 172 livers were examined (6–15/aquarium). Fish in the highest exposure concentration group (1232 µg/L) had a decreased hepatocellular vacuolation ($p = 0.014$, two-tailed test; Fig. 6). If the fish with the dorsal muscular inflammation mentioned above are removed, the statistical significance of decreased hepatocellular vacuolation is even stronger in the highest exposure concentration group ($p < 0.001$). Micrographs of the different grades can be found in Fig. 7. Note that the level of hepatocellular vacuolation often differ between species, gender, reproductive and nutritional status (Wolf and Wolfe, 2005). Vacuolation can be very pronounced, especially in captive fish (Wolf and Wolfe, 2005) and hence, a basal level of grade 4, as found here, was considered normal. None of the other lesions examined (inflammatory cell foci, pigmented macrophage aggregates, hepatocellular necrosis and parasites) were statistically different between the treatment groups. One fish from the highest exposure concentration group (1232 µg/L) was the only one that showed signs of hepatocellular necrosis. Since a nematode was found in the abdomen of that specific fish, the hepatocellular necrosis could perhaps be due to previous parasite migration in the liver. For that reason, and due to the low prevalence, we interpret it as an incidental finding not related to the naproxen exposure. For full details, see supplementary data.

3.6. Quantitative PCR

Fifty-nine samples were analyzed for hepatic mRNA expression (one sample were lost during preparation in the group exposed to 18 µg/L). None of the previous mentioned sticklebacks with back inflammation were used. It was not possible to generate C_T values for *vtg* in five samples (one from each treatment group), hence only 54 samples were

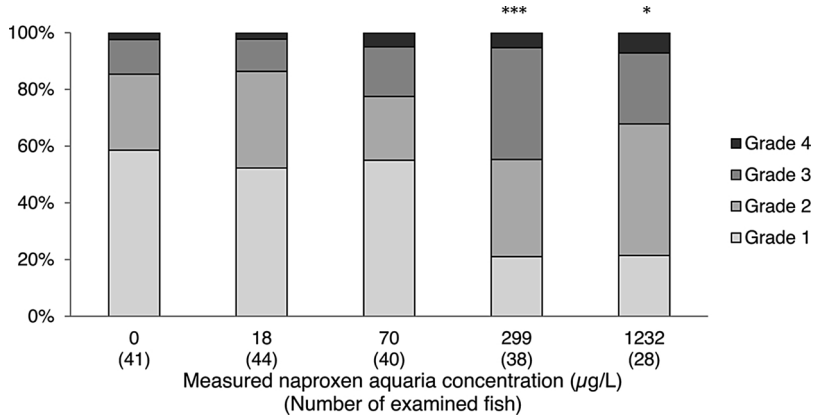

Fig. 4. Grading of renal hematopoietic hyperplasia in stickleback exposed to naproxen, where grade 4 is the most severe hyperplasia. Asterisks indicate significant differences to the control group,* p = 0.011; *** p = 0.001.

included in the statistical comparison for that gene. The normalized hepatic mRNA expression (ΔC_T) of *c7* was higher in the two highest exposure concentration groups (299 μg/L and 1232 μg/L) compared to the control group (Fig. 8). The expression of *cyp1A* (Fig. 8), and *sod-1* (Fig. 8) were significantly lower in the highest exposure concentration group (1232 μg/L). The expression of *gr* was also significantly lower but only in the second highest exposure concentration group (299 μg/L; Fig. 8). One could argue that the lack of definite concentration-response for that gene makes the finding less reliable. However, it should be kept in mind that the fish in the highest exposure concentration group were exposed only 21 days compared to 27 days for all other treatment groups. Furthermore, there was a similar trend (p = 0.097) also in the highest exposure concentration group. There was no statistical difference between the exposure concentration groups and the control group for *vtg* (see supplementary data, Fig. S1). For full details, see supplementary data.

3.7. Comparison of diclofenac and naproxen

Data on pharmacodynamic and pharmacokinetic properties as well as data relating to exposure in Swedish waters have been compiled for both diclofenac and naproxen (Table 3). In order to provide an

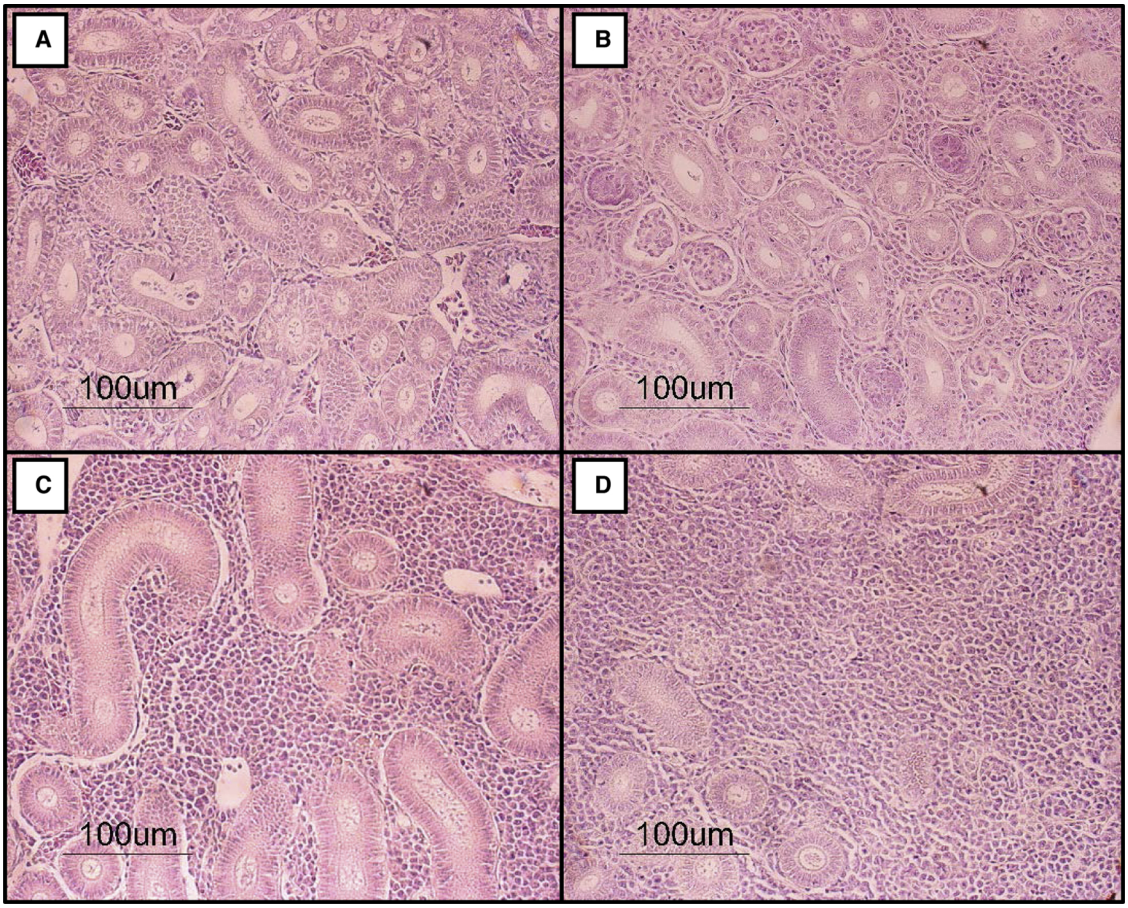

Fig. 5. Grading of renal hematopoietic hyperplasia in stickleback exposed to naproxen (magnification 200x). **A:** Grade 1, fish exposed to 0 μg/L; **B:** Grade 2, fish exposed to 18 μg/L; **C:** Grade 3, fish exposed to 299 μg/L; **D:** Grade 4, fish exposed to 1232 μg/L.

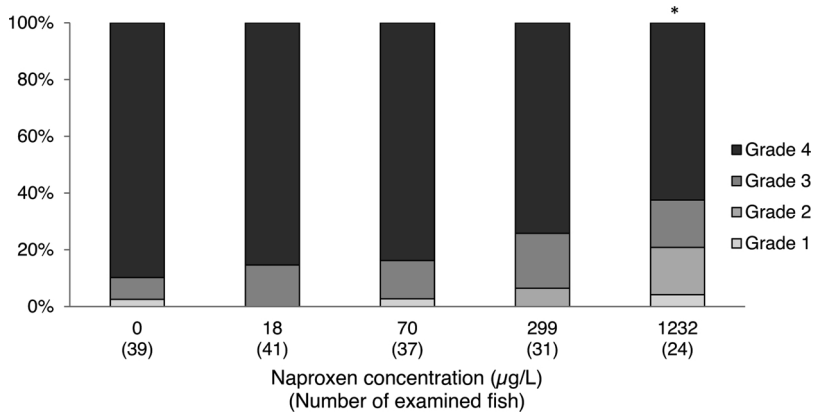

Fig. 6. Grading of hepatocellular vacuolation in stickleback exposed to naproxen where grade 4 is the most extensive vacuolation and in this study considered "normal". Asterisks indicate significant differences to the control group, * p = 0.014.

overview and be able to compare the results in this study with those in our previously published diclofenac study (Näslund et al., 2017), experimental design, lowest observed effect concentration (LOEC) and BCF for both NSAIDs are compiled in Table 4. Overall, the experimental designs were similar with exception of a higher number of fish in each aquarium in the naproxen study, and the concentration range tested was higher. The LOECs for naproxen were similar or higher for all endpoints. The most sensitive endpoint for diclofenac, renal hematopoietic hyperplasia, was significantly affected first at 65 times higher concentrations of naproxen.

4. Discussion

We show here that naproxen affects kidney histology and hepatic gene expression, and induces jaw lesions in fish in a similar way as diclofenac does. This is likely a reflection of both NSAIDs acting via a shared mode of action. That, in turn, suggest that the effects of diclofenac and naproxen are expected to be additive in the case of co-exposure (Backhaus, 2014). The most sensitive endpoint for diclofenac in our previous study (renal hematopoietic hyperplasia) was affected already at 4.6 µg/L - the lowest concentration tested (Näslund et al., 2017). In the present study, naproxen caused similar changes, but first

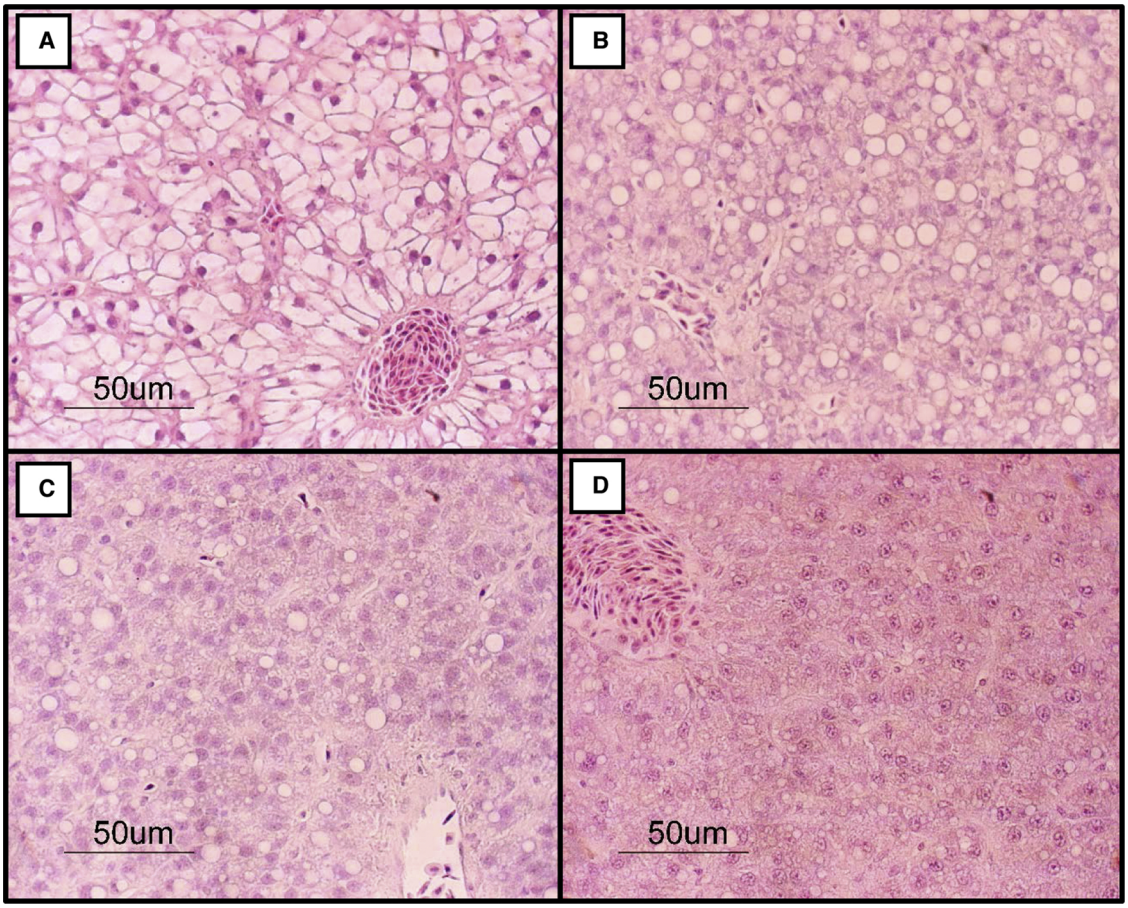

Fig. 7. Grading of hepatocellular vacuolation in fish exposed to naproxen (magnification 400x). **A:** Grade 4, fish exposed to 0 µg/L; **B:** Grade 3, fish exposed to 299 µg/L; **C:** Grade 2, fish exposed to 1232 µg/L; **D:** Grade 1, fish exposed to 1232 µg/L.

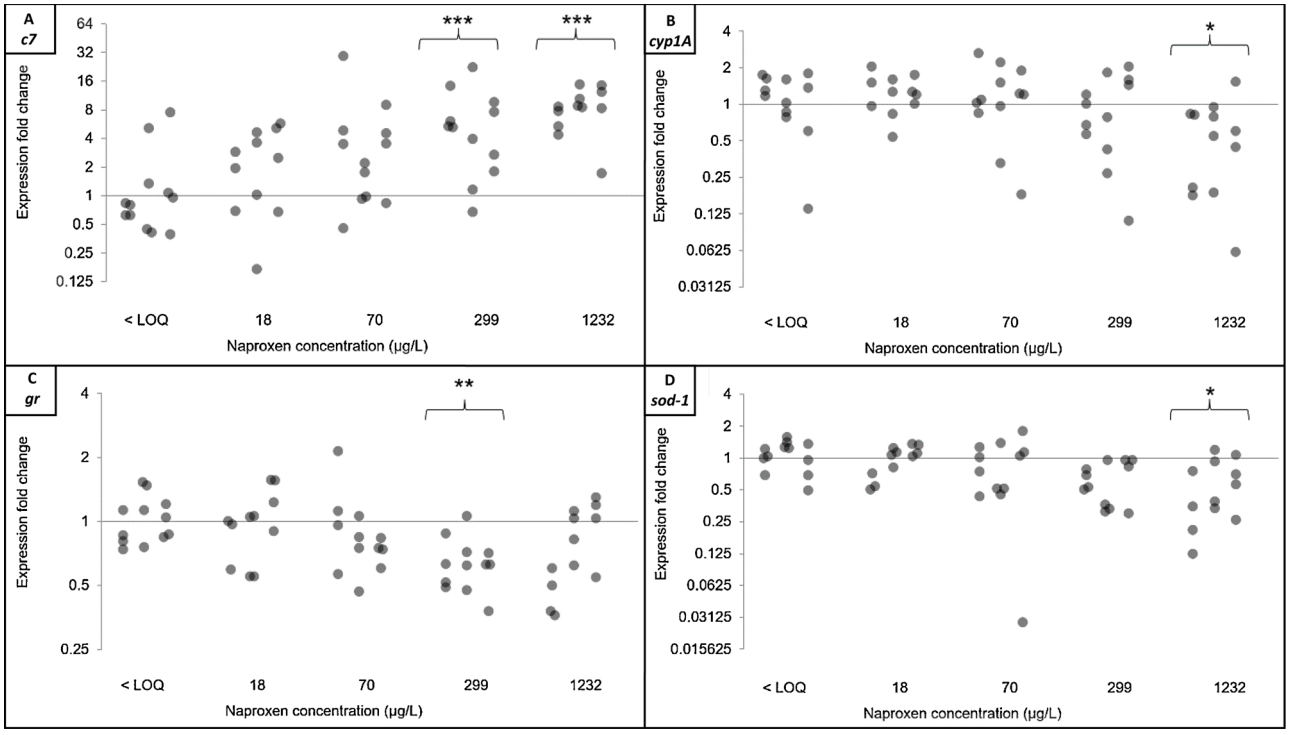

Fig. 8. Expression fold change ($2_T^{-\Delta\Delta C}$) of hepatic *c7*, *cyp1A*, *gr* and *sod-1* mRNA in the different aquaria in relation to the control group. One dot represent one fish and the fish in the same aquarium are depicted on top of each other. Thus, the three aquarium replicates in the same treatment group are clustered beside each other. The statistical analyses are based on ΔC_T-values. Asterisks indicate significant differences to the control group, two-tailed test. **A**(*c7*): *** $p \leq 0.001$; **B**(*cyp1A*): * $p = 0.035$; **C** (*gr*): ** $p = 0.007$; **D** (*sod-1*): * $p = 0.018$.

at 299 µg/L, a 65-fold higher concentration. For other, less sensitive endpoints, the difference was smaller (still large for the change in hepatic mRNA levels of the gene *c7*). It is possible that the effects on e.g. mortality and condition factor is a reflection of additional mechanisms of actions that become relevant only at higher exposure concentrations which are less plausible for fish to encounter. The similar potency in diclofenac and naproxen with regards to jaw lesions, a considerably more specific endpoint than mortality and condition factor, is then perhaps a bit surprising. It should however be pointed out that a recent study by Yokota et al. (2018) demonstrated concentration-response related mandibular defects of diclofenac at 26.5 µg/L in Japanese medaka, which is more than 10 times lower than the LOEC observed here for naproxen. Based on highly comparable experimental setups and analyses between the present study and the study by Näslund et al. (2017), we conclude that while both naproxen and diclofenac cause similar types of effects in fish, the hazards associated with diclofenac exposure in fish appear to be considerably higher than for naproxen.

To interpret the observed differences in effect levels between naproxen and diclofenac, one could also apply a read-across approach, assuming a similar relative potency at the molecular target as in humans (H_TPC, Table 3). This approach has been applied in many studies with pharmaceuticals in fish, since it was first described by Huggett et al. (2003), referred to as "the fish plasma model". As plasma concentrations in the exposed stickleback are not known, this put some limitations for interpretability and an evaluation of how reasonable it is to observe effects at the measured whole body internal concentrations. As the distribution volumes (V_D) in humans are low for both diclofenac and naproxen (0.1 − 0.2 L/kg, (Davies and Anderson, 1997a, b)), it suggests that a relatively large proportion of the drug is present in plasma, at least in humans. Hence, plasma concentrations (which are challenging to analyze in such small fish as sticklebacks) are likely to be higher than the measured whole-body concentrations. This is supported by Brown et al. (2007); Fick et al. (2010a) and Lahti et al. (2011) where all report considerably higher BCFs between water and blood plasma in rainbow trout than the BCF we found between water and whole-body of stickleback for diclofenac. The ratio between the BCF of diclofenac from Näslund et al. (2017) and the BCF of naproxen in our present study (0.3/0.07 ≈ 4.3; water to whole-body) are in very good agreement with the ratio based on their lipophilicity and the theoretic model for bioconcentration (Table 3, 110/24 ≈ 4.6) (Fitzsimmons et al., 2001). In line with our findings, Lahti et al. (2011) also reported a similar BCF ratio between diclofenac and naproxen (4.9/1.4 = 3.5, 5.7/1.6 ≈ 3.6; water to blood plasma in rainbow trout). Together, this supports our observation that diclofenac bioconcentrates more than naproxen does in fish.

The bioconcentration factor from water to whole-body was only 0.07 for naproxen while it was 0.3 for diclofenac in a directly comparable experimental setup (Näslund et al., 2017). Using the human therapeutic plasma concentration (H_TPC) as a measure of potency suggest that diclofenac would be considerably more potent, with a H_TPC 40 times lower than naproxen. Taking both bioconcentration potential and H_TPC into account suggest that diclofenac is over 170 times more potent than naproxen in fish. This is in reasonable agreement with one histopathological endpoint with a LOEC for diclofenac 65 times lower than for naproxen. While difficult to pinpoint exactly how large the difference in potency is, it could even be greater than 65 times, as effects by diclofenac were observed at the lowest concentration tested in our previous study. Additionally, the number of fish in each aquaria was higher in the naproxen study resulting in higher statistical power, still a much higher LOEC was found. Effects on kidney histology were observed at a whole-body concentration corresponding to 0.28 % of the H_TPC for diclofenac and 0.1 % for naproxen.

One of the main findings in this study is increased renal hematopoietic hyperplasia. We used the same classification system as in our previous study (Näslund et al., 2017) and the grading of the findings was made by the same trained fish pathologist (JN). We also used coded slides and the histopathological classification was verified by statistical comparisons. However, this is not the first mentioning of renal effects in

Table 3
Additional data on diclofenac and naproxen.

Characteristics	Diclofenac	Reference	Naproxen	Reference
LC_{50} (96 h) Rainbow trout (*Onchorhynchus mykiss*)	167 mg/L (DCF-Na)	Praskova et al. (2011)	690 mg/L (NPX-Na)	Rodriguez et al. (1992)
LogP (Log $K_{O:W}$)	4.0	Fick et al. (2010b)	3.1	Fick et al. (2010b)
H_TPC (Human therapeutic plasma concentration)	0.5 mg/L	Schulz et al. (2012)	20 mg/L	Schulz et al. (2012)
$T_{½}$ (half-life)	1 – 2 h	Schulz et al. (2012)	10 – 20 h	Schulz et al. (2012)
V_D (Volume of distribution)	0.1 – 0.2 L/kg	Davies and Anderson (1997a)	0.1 – 0.2 L/kg	Davies and Anderson (1997b)
BCF_{Plasma} ($P_{B:W}$; predicted from the" fish plasma model")	110	Fitzsimmons et al. (2001)	24	Fitzsimmons et al. (2001)
$BCF_{Whole\text{-}Body}$ (Measured)	0.3	Näslund et al. (2017)	0.07	This study
Volume sold (Sweden 2016-2017, mean)[1]	4 398 kg	Swedish eHealth Agency (2020)	22 046.5 kg	Swedish eHealth Agency (2020)
DDD (Daily defined dose)	0.1 g	WHO Collaborating Centre for Drug Statistics Methodology (2018)	0.5 g	WHO Collaborating Centre for Drug Statistics Methodology (2018)
Number of sold DDDs (Volume sold/DDD, Sweden 2016-2017, mean)[1]	43 980 000	Swedish eHealth Agency (2020)	44 093 000	Swedish eHealth Agency (2020)
Excreted fraction unchanged/easily hydrolysable pharmaceutical	17 %	Khan and Ongerth (2004)	70 %	Khan and Ongerth (2004)
PEC (Predicted effluent concentration, based on volume sold)[2]	0.6 µg/L	This study	3.0 µg/L	This study
CEC (critical environmental concentration)	4.56 µg/L	Fick et al. (2010b)	828 µg/L	Fick et al. (2010b)
WWTP influent (Sweden 2016-2017, mean)[3]	196 ng/L	Janusinfo Region Stockholm (2019)	1674 ng/L	Janusinfo Region Stockholm (2019)
WWTP effluent (Sweden 2016-2017, mean)[3]	149 ng/L	Janusinfo Region Stockholm (2019)	121 ng/L	Janusinfo Region Stockholm (2019)
WWTP removal rate (Sweden 2016-2017, mean)[3]	16 %	Janusinfo Region Stockholm (2019)	94 %	Janusinfo Region Stockholm (2019)

[1] This includes topical products (such as gels) and combination products. Both lack a DDD but for a rough estimation of the total DDD sold in 2016/2017, they are given the same DDD as for oral use.
[2] PEC (µg/L) = (A * 109 * (100-R))/(365 * P * V * D * 100); A = Volume sold (kg), R = Removal rate (0 %), P = Number of inhabitants in Sweden (9.923 * 106, mean 2016-2017), V = Volume wastewater per capita and day (200L/day), D = Dilution factor (10).
[3] The mean value of three WWTPs (one measurement from each WWTP/year).

Table 4
Comparison of experimental designs, LOECs and BCFs for exposure studies with stickleback to either diclofenac (Näslund et al. (2017)) or naproxen (present study).

	Diclofenac	Naproxen
Experimental design		
Exposure concentration (nominal, µg/L)	0, 5, 20, 80, 320	0, 20, 80, 320, 1280
Exposure concentration (measured, µg/L)	0, 4.6, 22, 82, 271	0, 18, 70, 299, 1232
Duration	28 d (21 d for 271 µg/L)	27 d (21 d for 1232 µg/L)
Number of fish/aquaria	12	20
Number of replicate aquaria/ treatment	3	3
Aquaria water temperature	15.7 – 17.6 °C	15.0 – 16.6 °C
LOEC		
Renal hematopoietic hyperplasia[1]	4.6 µg/L	299 µg/L
Hepatic gene expression (*c7*)	22 µg/L	299 µg/L
Condition factor	271 µg/L	1232 µg/L
Mortality	271 µg/L	299 µg/L
Jaw lesions[1]	271 µg/L	299 µg/L
BCF (water to whole-body)	0.3	0.07

[1] One-tailed test applied, otherwise two-tailed test.

fish by naproxen. Górny et al. (2019) recently claimed that naproxen have a negative influence on the kidneys in zebrafish, referring to the study by Ding et al. (2017). But Ding et al. (2017) did not investigate any renal effects in fish, but in turn cited Stancova et al. (2015b) and Chattopadhyay et al. (2016) regarding renal effects in zebrafish. However, neither one of these studies investigated effects of naproxen on fish kidneys, nor do they cite any other studies that do so. Hence, to the best of our knowledge, this is the first histopathological evaluation of the kidney in fish exposed to naproxen. We also observed such effects in sticklebacks exposed to diclofenac, and Schwaiger et al. (2004) showed the same type of effects in rainbow trout exposed to diclofenac although referring to it as "interstitial nephritis" (Schwaiger et al., 2004). It is therefore plausible that the observed renal hematopoietic hyperplasia is a common effect by NSAIDs in fish. We suggest that this terminology should be used rather than "interstitial nephritis" as there is no apparent signs of inflammation (Näslund et al., 2017).

Another shared and rather specific effect of both naproxen and diclofenac in fish are defects on the jaws. Stancova et al. (2014) exposed tench (*Tinca tinca*) larvae to a mixture of diclofenac, ibuprofen and carbamazepine and reported lesions in the lower jaw. As only a mixture was studied, it could not be concluded with certainty if the effects was a consequence of the NSAID or the carbamazepine exposure or a combination effect. We have showed that jaw lesions indeed can be caused by diclofenac exposure in sticklebacks (Näslund et al., 2017). A more comprehensive study regarding mandibular effects of diclofenac on Japanese medaka (*Oryzias latipes*) was recently published by Yokota et al. (2018). Effects were observed on dental bones, hypohyals of the mandible and the premaxillae whereas no visible abnormalities were seen in any other skeletal bones. Although other studies have not investigated the characteristics of jaw lesions in such detail, this appears to be in agreement with both the findings in tench and stickleback exposed to diclofenac and our present findings in sticklebacks exposed to naproxen. We have earlier speculated that both renal hematopoietic hyperplasia and jaw lesions could potentially be a consequence of secondary infections caused by an impaired immune systems due to diclofenac exposure (Näslund et al., 2017). The accumulating observations of specific changes across different studies, laboratories, species and NSAIDs suggest that these rather are direct effects, linked to the shared mode of action of NSAIDs. Yokota et al. (2018) proposed that diclofenac affects bone remodeling in the lower jaws by disrupting osteoclast function, but the mechanism involved are still unknown.

The hepatic gene expression of *c7* also appears to be a quite characteristic response to NSAIDs. It showed a clear concentration-dependent response both to naproxen and diclofenac (Cuklev et al., 2011; Näslund et al., 2017). The c7 protein is a part of the complement system, which is a component of the innate immune system. It forms a membrane attack complex together with other complement component proteins which lead to lysis of foreign cells (Delves and Roitt, 2011). It has been shown that the complement components are connected to the arachidonic acid pathway (Hänsch et al., 1984) and it is therefore reasonable that NSAID exposure could affect *c7*. The observations here supports the analyses of *c7* mRNA as an exposure biomarker for NSAIDs, although specificity should ideally be evaluated further.

In contrast to the more specific responses mentioned above, some of the effects were more unspecific. We observed a decreased hepatocellular vacuolation in naproxen exposed fish. Since the vacuoles can both consist of lipids and/or glycogen with special stains/techniques needed to verify their content, the term vacuolation covers both findings. Decreased vacuolation in fish is a consequence of either direct hepatic toxicity or secondary to stress and/or disease which causes a decreased body condition (Wolf and Wolfe, 2005). Diclofenac has also been shown to cause decreased hepatocellular glycogen in fish (Wolf et al., 2014). However, both in the present study and the study by Wolf et al. (2014), the effect were only significant at a concentration ≥ 1000 µg/L. Hence, hepatocellular vacuolation is not likely to be a response found under realistic field exposure scenarios, and if it is, it could have many other causes.

As discussed above, hepatic gene expression of *c7* seems to be a relatively sensitive marker to NSAID exposure, with at least a plausible mechanistic connection to their mode of action. In contrast, effects on the expression of many other genes have been reported as well, often at higher concentration and without such links to prostaglandin synthesis or inflammatory responses. For example, we found here that the expression of *cyp1A* decreased, an effect not observed for diclofenac (Näslund et al., 2017). One may note that we observed effects only at the highest tested concentration of naproxen and such high concentrations were not tested in the diclofenac experiment. Stancova et al. (2015b) accordingly found no effect on c*yp1A1* (or *sod2*) in zebrafish exposed to 100 µg/L naproxen. Hong et al. (2007) reported increased levels of both *cyp1A*, *p53* and *vtg* in Japanese medaka (Oryzias latipes) at a very low concentration (1 µg/L) of diclofenac. As their conclusion is based solely on the analyses of elevated levels in one single sample of three pooled fish, we think there are good reasons to disregard this report. In a much more well-designed study, Kwak et al. (2018) investigated effects of naproxen on gene expression in Japanese medaka across a range of concentrations, and found effects on vitellogenin (*vtg1*), the estrogen receptor (*erb2*) and *cyp17* but only at concentrations of 500 − 5000 µg/L and higher. Accordingly, we observed no effects on *vtg* in the livers of sticklebacks exposed to up to 1232 µg/L. Taken together, in the genes analyzed so far, only *c7* stands out as a gene that is consistently affected by different NSAIDs in different species and at relatively low concentrations.

A very recent study investigated the effects of naproxen in zebrafish on a large range of endpoints related to thyroid disruption (Xu et al., 2019). The authors motivate their study by referring to Bishnoi et al. (1994), a non-randomized clinical study in humans with NSAID-treated disease. Bishnoi et al. (1994) provide circumstantial, inconclusive evidence for effects of NSAIDs on the thyroid system in humans. Samuels et al. (2003), on the other hand, performed a randomized clinical study in healthy humans and found no evidence that naproxen affect thyroid hormone levels. Having said this, there is support that some other NSAIDs can affect thyroid homeostasis and one could hence not exclude that similar effect could occur after naproxen exposure. In the study on zebrafish, Xu et al. (2019) investigated thyroid hormone levels (total T3 and T4), bioconcentration, gene expression (*cyp1A* and *cyp3A*) and enzyme activities (EROD) potentially involved in/reflecting elimination of naproxen. The expression of a large range of genes specifically related to the hypothalamic-thyroid-axis was also investigated. For a large set of endpoints (*cyp1A*, *cyp3A*, EROD, T4, *dio2*, *nis*, *pax8*, *tg*, *tpo*, *trβ*, *ttr*, *ugt1ab*, TTR) the authors report dose-response related, significant effects already at the lowest concentration tested (0.1 µg/L) although there is some inconsistency between text and figures. This exceptionally low effect concentration is much lower than all other studies investigating the effects on naproxen in fish. The claims of *cyp1A* effects stands in strong contrast to our present study where LOEC for *cyp1A* is 1232 µg/L, i.e. more than a 12 000 times difference in potency and the study by Stancova et al. (2015b) where no significant effects were found on *cyp1A1* in zebrafish – the same species - exposed to naproxen up to 100 µg/L. Furthermore, the bioconcentration data reported by Xu et al. (2019) is unexpected, as exposure to 0.1, 1 and 10 µg/L all resulted in a largely similar whole-body concentration (i.e. the bioconcentration factor decreased more than 50 times as the exposure concentration increased from 0.1 to 10 µg/L). In contrast, we found a stable (and much lower) bioconcentration factor across all exposure concentrations, as did (Lahti et al., 2011). Based on our own experiences and similar studies, we find variances of the gene expression data (for all 14 investigated genes) in the study by Xu et al. (2019) to be very small. Taken together, this led us to contact the authors to ask for clarifications, but without reply. When we involved the editor of the journal, we received the reply from the authors that most of the original data had been lost in a fire accident and could not be provided. We think this is highly unfortunate, as we think an in-depth scrutinization (and independent replication of the experiment) is warranted (Harris et al., 2014) in order to allow an evaluation of the findings before any of these results are incorporated into any risk assessment or management efforts.

In general, histopathological examinations of fish exposed to naproxen is sparse, with only three published papers (Stancova et al., 2015a; Li et al., 2016; Sehonova et al., 2017). Stancova et al. (2015a) treated zebrafish with naproxen for 14 days and reported “obvious changes to the gills and liver” at 1 µg/L. There is, however, no quantification of any lesion and it is not clear if the assessment was performed in a blinded manner. Furthermore, it is very difficult to evaluate the provided histological images due to low magnification and poor resolution. The only obvious difference is that the gill sections vary in staining intensity/thickness. None of the stated pathological changes (hyperemia, widening of leaflet’s apex and desquamation of leaflet’s epithelium) can with certainty be identified in the figures. The authors also report separation of hepatocytic trabeculae in the liver, but these are most likely artefacts due thick sections and subsequent cracks. In conclusion, our judgement is that none of the claimed histopathological findings can be verified by the provided data. Stancova et al. (2015a) also report changes in enzyme activities of whole-body homogenates, but these are not consistent over time, nor do they follow clear concentration-response relationships. Li et al. (2016) studied acute toxicity in zebrafish (*Danio rerio*) at 10 − 240 mg/L and reported liver damage. Judging by the figures in the paper, the quality of the slides are poor and only a qualitative assessment was made. The lack of quantification thus precludes statistical comparisons. However, the described histopathological changes seen in the liver may very well be present but the interpretation is challenging. Sehonova et al. (2017) reports histopathological changes in skin and gills of common carp (*Cyprinus carpio*) at exposure concentration and lengths of exposure similar to our present study. While the authors claim there are differences in the number of mucous cells and gill lamella deformations, no quantification is presented. As information lack with regards to histopathological methodology, it is again difficult to interpret the results.

Reported concentration of both naproxen and diclofenac in WWTP effluents vary greatly, even within Sweden. Internationally, naproxen have occasionally been reported at levels up to 33.9 µg/L (Metcalfe et al., 2003) but levels around or below 1 µg/L are much more frequent (Tixier et al., 2003; Lishman et al., 2006; Loos et al., 2013). A Swedish, national surveillance study report naproxen levels of 1.2–1.8 µg/L (Fick

et al., 2010a) but in 2016 and 2017, levels of only 21 – 391 ng/L were detected in the effluent from three large WWTPs in the Stockholm region (Janusinfo Region Stockholm, 2019). Substantial differences have also been reported for diclofenac, with µg/L concentrations reported in some studies (Andreozzi et al., 2003; Stülten et al., 2008; Gros et al., 2010) and low ng/L concentrations in others (Loos et al., 2013; Yu et al., 2013). While all this may reflect real concentrations, the use of different analytical methods, in different labs, variable consumption patterns and other factors that differ between sampling times and sampling points are also likely to contribute to variability. To enable direct comparisons of concentrations, studies that analyze both naproxen and diclofenac in the very same samples in parallel with the same methodology and where the sales volumes are known are therefore preferred. Region Stockholm has analyzed both NSAIDs in influents and effluents of three major WWTPs over several years (Janusinfo Region Stockholm, 2019). Focusing on their recent data from 2016 and 2017, naproxen (mean value of 1674 ng/L) dominated over diclofenac (mean value 196 ng/L) in influents, in accordance with more kilograms sold and a larger proportion of unchanged drug excreted (Table 3). Effluent concentrations were more similar (naproxen mean value of 121 ng/L, diclofenac mean value of 149 ng/L). These data also reveal a much more efficient removal of naproxen and this is in agreement with a range of other studies of NSAIDs in Swedish WWTPs (Falås et al., 2012). Thus, while absolute levels of both naproxen and diclofenac in treated WWTP effluents (and hence surface waters) are difficult to derive from the literature, there is reasonably good support that with current usage and treatment technologies in Sweden, expected concentrations of diclofenac are similar or somewhat higher than naproxen.

It is clear that exposure to naproxen can cause adverse effects in fish if exposure is sufficiently high. However, we only observed effects at concentrations of 299 µg/L and higher. Diclofenac on the other hand had a LOEC of 4.6 µg/L in a directly comparable study (Näslund et al., 2017). Given the discussion above on the exposure from these NSAIDs, the safety margin for naproxen is considerably greater than for diclofenac. However, if surface water concentrations remain below 0.1 µg/L (corresponding to the proposed Environmental Quality Standard for diclofenac under the European Water Framework Directive; (EU, 2012)) the risks is probably low or very low for both.

Controlled exposure studies to individual chemicals, such as the present one, are limited in that they rarely take into account co-exposure to other chemicals, variability in bioavailability that depends on water chemistry or alternative exposure routes (e.g, via the food chain). A way forward to investigate if there indeed are effects of diclofenac, naproxen and/or other similarly acting NSAIDs on fish in the environment, we propose dedicated effect studies of fish exposed to treated sewage effluent in controlled aquaria experiment, in fish caged up-and down-stream from sewage treatment plants and in wild fish, as all three approaches have their pros and cons. As renal hematopoietic hyperplasia, jaw malformations and induction of hepatic c7 expression appears to be rather consistent effects of diclofenac and naproxen exposure, we suggest these effects should be monitored. In combination with analyses of NSAID levels in plasma or tissues, such an approach could add substantially to our understanding of risks.

Although risks are greater for diclofenac given available hazard data and current use of NSAIDs in Sweden, it is critical to investigate what a substitution would mean in terms of environmental exposure levels, particularly as a typical dose of naproxen is five times higher than for diclofenac (Table 3). Sweden provides a good study case, as there is excellent data on total sales and corresponding measured levels in the WWTP effluents. As for 2016 – 2017, sales for naproxen were five times higher in terms of kilogram sold active substance (Table 3). However, counted as sold doses, the two drugs are very similar (Table 3). If all current sales of diclofenac were replaced by naproxen, the sales of naproxen would therefore increase two-fold and actual effluent levels would likely increase proportionally. Reciprocally, if all naproxen were replaced by diclofenac, the sales and the concentrations of diclofenac would be expected to increase by two-fold. The difference in hazard is much larger than two-fold. Hence, based on the hazard data presented here, replacing diclofenac with naproxen would decrease risks to fish.

While the number of ecotoxicological studies reporting effects levels of different pharmaceuticals is increasing, it does not automatically mean that the amount of relevant, reproducible and comparable data is increasing at the same pace. To facilitate a thorough comparison between diclofenac and naproxen we have performed and reported the results of two very similar studies (Näslund et al., 2017, this study). Although naproxen and diclofenac produce similar effects in fish, the environmental hazards and risks are lower for naproxen compared to diclofenac based on available data. A way to manage risks to fish would therefore be to substitute diclofenac with naproxen when it provides an adequate alternative from a clinical point-of-view.

CRediT authorship contribution statement

Johanna Näslund: Conceptualization, Methodology, Formal analysis, Investigation, Resources, Writing - original draft, Writing - review & editing, Visualization. **Noomi Asker:** Methodology, Formal analysis, Resources, Writing - review & editing. **Jerker Fick:** Methodology, Formal analysis, Resources, Writing - review & editing. **D.G. Joakim Larsson:** Conceptualization, Methodology, Resources, Writing - review & editing, Supervision, Funding acquisition. **Leif Norrgren:** Conceptualization, Methodology, Resources, Writing - review & editing, Supervision, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

We are especially grateful to Agneta Boström for excellent histological preparation, Anna Östlund for invaluable assistance in fish sampling, Mikael Svensson for statistical support and Bertil Borg for always being helpful with matters regarding sticklebacks. We also thank the Swedish Research Council (VR) for financial support. The funding agency was not involved in the study design; in the collection, analysis and interpretation of data; in the writing of the report; or in the decision to submit the article for publication.

Appendix A. Supplementary data

Supplementary data related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.aquatox.2020.105583.

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