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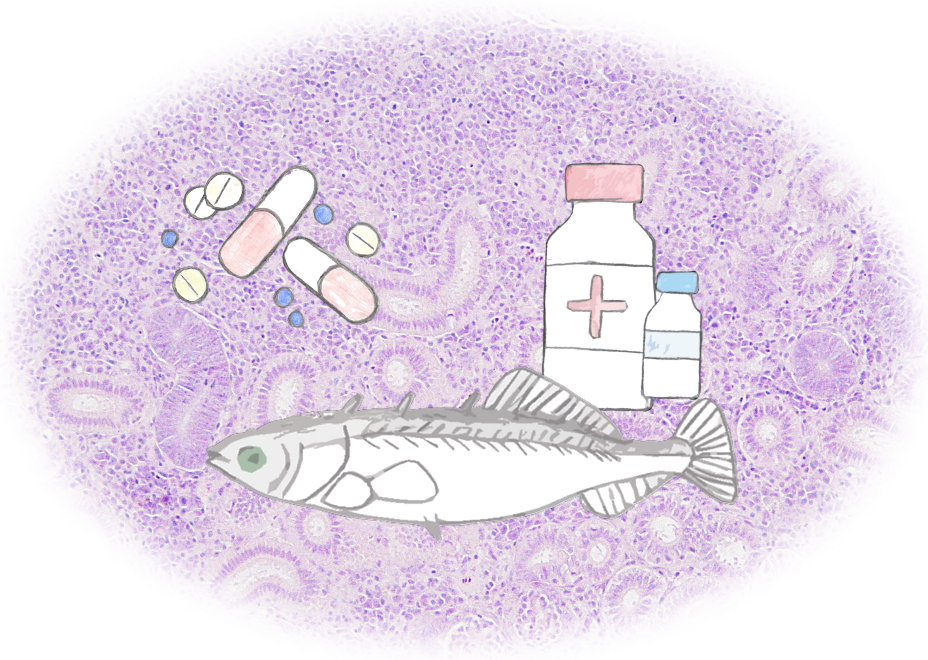
Pharmaceuticals in the environment:

Histopathological effects of non-steroidal anti-inflammatory drugs (NSAIDs) in fish

Johanna Näslund



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Cover: Composite image of a histological micrograph, showing renal hematopoietic hyperplasia (photo J. Näslund), with illustrative drawings of pharmaceuticals and a three-spined stickleback (*Gasterosteus aculeatus*) by Freja and Linnea Larsson.

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Abstract

Pharmaceutical residues have been detected in the environment for several decades. Concerns that diclofenac, a commonly used non-steroidal anti-inflammatory drug (NSAID), could cause histopathological and other effects in aquatic organisms have triggered different regulatory initiatives. However, the scientific basis for understanding risks to fish is inconclusive with large discrepancies between studies. The aim of this thesis was to further evaluate the effects of NSAIDs, focusing on histopathological effects in the kidney and liver using the three-spined stickleback (*Gasterosteus aculeatus*) as a model organism. This was complemented by a review of reported histopathological effects in fish after exposure to NSAIDs or treated municipal wastewater.

Two laboratory exposure studies showed that renal hematopoietic hyperplasia occurred after exposure to diclofenac already at the lowest tested concentration of 4.6 µg/L, but only at 299 µg/L or higher for naproxen. Mortality, decreased condition factor and jaw lesions were also observed, but only at high concentrations. In the review, few studies fulfilled the inclusion criteria, and the evidence for overlapping histopathological effects was weak. A third experimental study exposed sticklebacks to treated municipal wastewater using a matched experimental design to facilitate comparisons with previous NSAID studies. No overlapping histopathological or apical effects were detected, but the results highlighted the importance of including sex as a factor in the analysis.

In conclusion, NSAIDs cause histopathological effects in fish, but only at concentrations rarely reported in the aquatic environment. Diclofenac is also more potent than naproxen. There is currently no clear evidence that NSAIDs cause histopathological effects in wild fish, although the possibility of more sensitive species or effects in other organs cannot be excluded. This thesis also highlights the importance of robust methodology and transparent data interpretation.

Keywords: Non-steroidal anti-inflammatory drugs (NSAIDs), diclofenac, naproxen, three-spined stickleback, ecotoxicology, histopathology, wastewater, environmental risk assessment

Läkemedel i miljön: Histopatologiska effekter av icke-steroida anti-inflammatoriska läkemedel (NSAID) i fisk

Sammanfattning

Läkemedelsrester har påvisats i miljön under flera decennier. Ett läkemedel som misstänkts påverka vattenlevande organismer är diklofenak, ett vanligt icke-steroidalt antiinflammatoriskt läkemedel (NSAID). Detta har lett till olika regulatoriska initiativ. Det finns dock luckor i det vetenskapliga underlaget, och forskningsresultaten skiljer sig mellan studier. Syftet med denna avhandling var därför att vidare undersöka effekter av olika NSAID med fokus på histopatologiska effekter i njure och lever. Detta undersöktes i tre experimentella studier med storspigg (*Gasterosteus aculeatus*) som modellorganism, samt genom en litteraturstudie av rapporterade histopatologiska effekter hos fisk efter exponering för NSAID eller behandlat kommunalt avloppsvatten.

Två laboratoriestudier visade att hematopoetisk hyperplasi i njuren uppstår efter exponering för diklofenak redan vid den lägsta testade koncentrationen på 4,6 µg/L, medan motsvarande effekt för naproxen observerades först vid 299 µg/L. Dödlighet, minskad konditionsfaktor och käkskador observerades också, men endast vid höga koncentrationer. I litteraturöversikten uppfyllde få studier inklusionskriterierna, och evidensen för överlappande effekter var svag. I en tredje studie exponerades storspigg för behandlat kommunalt avloppsvatten med hjälp av en matchad experimentell design för att möjliggöra direkta jämförelser med tidigare NSAID-studier. Inga överlappande histopatologiska eller apikala effekter påvisades, men resultaten tydliggjorde vikten av att inkludera kön i analysen.

Sammanfattningsvis orsakar NSAID histopatologiska effekter hos fisk, men endast vid koncentrationer som sällan rapporteras i den akvatiska miljön. Diklofenak är dessutom mer potent än naproxen. Det finns i dagsläget inga tydliga bevis för att NSAID orsakar histopatologiska effekter i vild fisk, även om effekter hos känsligare arter eller i andra organ inte kan uteslutas. Avhandlingen belyser också vikten av robust metodik och transparent tolkning av data.

Nyckelord: Icke-steroida antiinflammatoriska läkemedel (NSAID), diklofenak, naproxen, storspigg, ekotoxikologi, histopatologi, avloppsvatten, miljöriskbedömning

Dedication

To my beloved family, both near and far ♥

“Absence of evidence is not evidence of absence”
/Martin Rees, Carl Sagan and others

Contents

List of publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I. Näslund, J., Fick, J., Asker, N., Ekman, E., Larsson, D. G. J. & Norrgren, L. (2017). Diclofenac affects kidney histology in the three-spined stickleback (*Gasterosteus aculeatus*) at low µg/L concentrations. Aquatic Toxicology, 189, 87-96. https://doi.org/10.1016/j.aquatox.2017.05.017

II. Näslund, J., Asker, N., Fick, J., Larsson, D. G. J. & Norrgren, L. (2020). Naproxen affects multiple organs in fish but is still an environmentally better alternative to diclofenac. Aquatic Toxicology, 227, 105583 https://doi.org/10.1016/j.aquatox.2020.105583

III. Näslund, J., Norrgren, L. & Larsson, D. G. J. (2026). No clear evidence of histopathological effects linked to NSAIDs in the kidney or liver of fish exposed to treated municipal wastewaters. Toxicologic Pathology, 0(0) https://doi.org/10.1177/01926233261423895

IV. Näslund, J., Fick, J., Björlenius, B., Larsson, D. G. J. & Norrgren, L. Histopathological effects in three-spined stickleback (*Gasterosteus aculeatus*) exposed to treated municipal wastewater do not mimic those of non-steroidal anti-inflammatory drugs (NSAIDs) (manuscript)

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Abbreviations

AA-QS	annual average quality standard
API	active pharmaceutical ingredient
BC	basophilic cluster
CEC	critical environmental concentration
COX	cyclooxygenase
DCF	diclofenac
DDD	defined daily dose
DDT	dichloro-diphenyl-trichloroethane
DN	developing nephron
E2	17β-estradiol
EC10	effect concentration for 10 % of test organisms
EC50	effect concentration for 50 % of test organisms
EDC	endocrine disrupting chemicals
EE2	17α-ethinylestradiol
EMA	European Medicines Agency
EQS	environmental quality standards
ERA	environmental risk assessment
$F_{SS}PC$	fish steady state plasma concentration
GFR	glomerular filtration rate
H&E	hematoxylin and eosin
H_TPC	human therapeutic plasma concentration
IBU	ibuprofen
KET	ketoprofen
LC50	lethal concentration for 50 % of test organisms

LC-MS/MS	liquid chromatography-tandem mass spectrometry
LNG	levonorgestrel
LOEC	lowest observed effect concentration
MAV	mean assessment values
NOEC	no observed effect concentration
NPX	naproxen
NSAID	non-steroidal anti-inflammatory drug
PAS	periodic acid-Schiff
PCBs	polychlorinated biphenyls
PEC	predicted environmental concentration
PMA	pigmented macrophage aggregates
PNEC	predicted no effect concentration
POPs	persistent organic pollutants
qPCR	quantitative polymerase chain reaction
UBA	Umweltbundesamt (the Federal Environmental Agency in Germany)
VTG	vitellogenin
WFD	Water Framework Directive
WWTP	wastewater treatment plant

AI declaration

During the preparation of the thesis, ChatGPT (OpenAI) was used as a complementary tool for literature search, assistance with outlining and structuring of the text and language editing. All output were manually verified and revised by the author. AI was not used in the preparation of Papers I–IV.

1. Introduction

Pharmaceuticals are widely used to prevent and treat disease, improving the quality of life of millions of humans and animals every day. The overwhelming majority contain biologically active substances designed to affect specific biological systems at low concentrations. To ensure effectiveness, they are also intentionally relatively stable. Molecular targets for most pharmaceuticals are typically conserved across vertebrates and sometimes to much more distant species (Gunnarsson et al., 2008). Exactly these features may, unintentionally, also enable effects in non-target species when exposed to excreted pharmaceuticals. The number of laboratory studies reporting adverse effects on different species after exposure to pharmaceuticals is increasing, but only a few substances have been confirmed to cause effects in the environment. The most well-documented example is the collapse of several vulture species in India and Pakistan, where diclofenac, a common non-steroidal anti-inflammatory drug (NSAID), was identified as the cause (Oaks et al., 2004). Together with studies reporting feminization of fish caused by estrogens (Jobling et al., 2002; Jobling et al., 1998; Kidd et al., 2007; Larsson et al., 1999; Purdom et al., 1994; Örn et al., 2003), this likely contributed to the initiation of regulatory efforts to include pharmaceuticals among other environmental contaminants (Boxall et al., 2012).

1.1 Pharmaceuticals in the environment

1.1.1 History

In 1962, the book Silent Spring by Rachel Carson was published, marking one of the starting points for increased awareness of unwanted substances in the environment and their effects on wildlife (Carson, 1962). At that time, persistent organic pollutants (POPs) such as dichloro-diphenyl-trichloroethane (DDT) were in focus. Properties like bioaccumulation in organisms and biomagnification at higher trophic levels enabled long-term effects.

Although the interest in ecotoxicology increased, pharmaceuticals were not recognized as potential environmental contaminants until later. The first analytical data from environmental samples were published in the 1970s

where a metabolite from clofibrate, a lipid-lowering agent, was detected in municipal effluent in the United States (Garrison, 1977). However, no connection to obvious ecological effects was apparent, and the finding remained primarily of analytical interest.

Substances such as polychlorinated biphenyls (PCBs) or DDT are both persistent and bioaccumulative and have thus often been detected at relatively high concentrations in environmental biota, even far from points of emission. Their presence was linked to various adverse effects, including reproductive disorders (Cade et al., 1971; Helle et al., 1976). In contrast to compounds such as DDT, pharmaceuticals are typically released continuously from widespread point sources, resulting in so-called pseudopersistence (Daughton, 2003). However, concentrations of pharmaceuticals in the environment are typically low although exceptions occur.

Advances in analytical technologies played an important role in recognizing the potential risk of pharmaceuticals in the environment. The development of more sensitive methods, such as liquid chromatography coupled with mass spectrometry, increased the number of detectable pharmaceuticals. Over the years, detection limits improved substantially, with typical limits of quantification at low ng/L levels. Several types of matrices, including water, biota and sediment could also be analysed, greatly improving detection capabilities.

In 1998, Halling-Sørensen et al. (1998) published a central review on the occurrence, fate and effects of pharmaceuticals in the environment. This is sometimes considered the first broader recognition of pharmaceuticals as potential environmental risks. Taken together, although the first pharmaceuticals were detected in the environment long ago, they were not recognized as potentially important environmental contaminants until much later. This was likely due to properties that differed markedly from those of many classical pollutants.

1.1.1. Sources

Pharmaceuticals can end up in the environment in several ways with both direct and indirect sources (Figure 1). Pharmaceutical manufacturing has been identified as a notable source of environmental contamination (Fick et al., 2009; Larsson, 2014; Larsson et al., 2007; WHO, 2024) where exceptionally high concentrations have been detected in discharged

wastewaters and receiving waters. Major point sources include not only effluents released directly from pharmaceutical industries, but also illegally dumped waste, as well as effluents from common wastewater treatment plants (WWTPs) receiving discharge from manufacturing facilities and other sources.

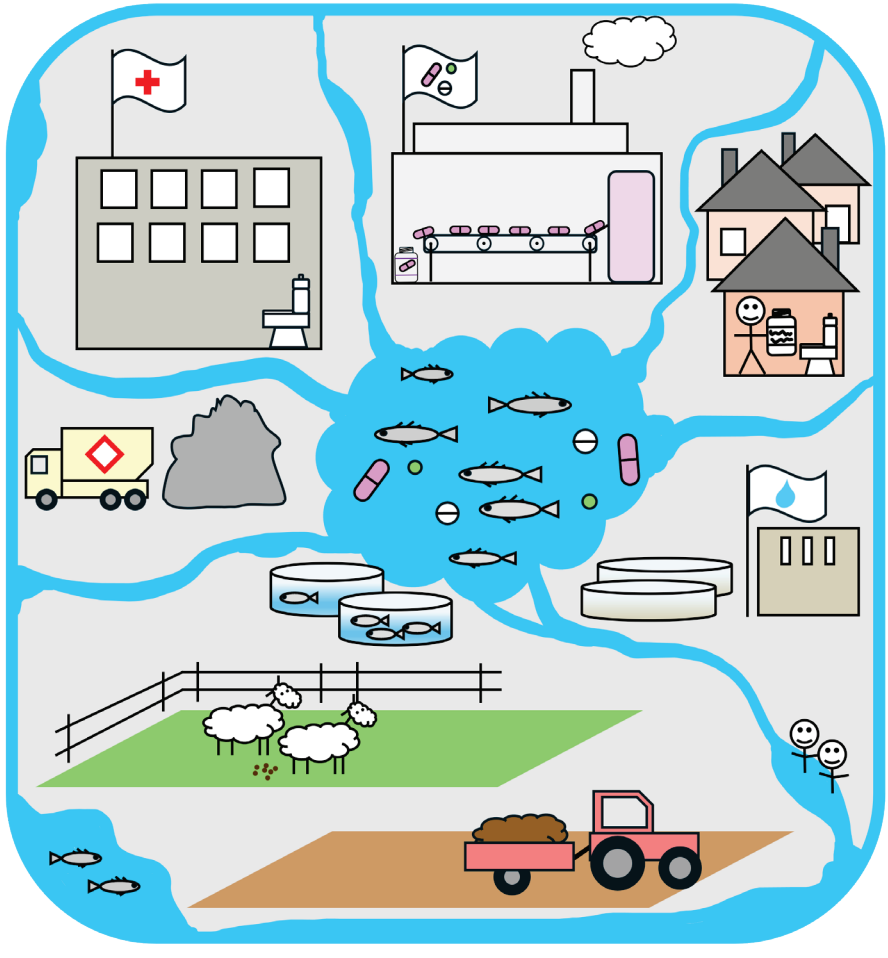

Figure 1. Direct and indirect sources of pharmaceuticals to the environment.

In contrast to local emissions from the manufacturing industry, human use provides a more diffuse and widespread source. There are several types of dosage forms, including injections, tablets, gels and creams, all of which can

affect how much of a substance that ultimately reaches the environment. Pharmaceuticals administered orally or parenterally are partly metabolised and then excreted through urine or faeces. Not all active substance is absorbed through the skin and residues can easily be washed off during bathing (Davies & Anderson, 1997; Vieno & Sillanpää, 2014). Both hospitals and households produce wastewater containing pharmaceuticals, which typically ends up at municipal WWTPs. Since WWTPs are typically designed to remove human waste of natural origin, pharmaceuticals are often only partly eliminated by conventional treatment methods (Daughton & Ternes, 1999). Effluents are normally diluted in the receiving waters, but effluent-dominated rivers may at certain times of the year consist almost entirely of treated wastewater (Brooks et al., 2006). From a global perspective, one should note that several hundred million people still practice open defecation (UNICEF & WHO, 2023), allowing pharmaceutical residues to enter the environment without prior wastewater treatment. Pharmaceuticals can also be adsorbed to sludge in WWTPs. Sludge is commonly spread on agricultural fields, which is another possible source of pharmaceutical pollution (Rutgersson et al., 2020; Topp et al., 2008).

Expired or leftover pharmaceuticals can also be a source of environmental contamination. In Sweden, pharmacies have been obliged for several decades to accept returns of unused medicines and incinerate them. The original reason for this practice was to prevent unauthorized access to medicines via, for example, outdoor garbage bins. Today, environmental reasons are typically emphasized when Swedish pharmacies campaign for the return of unused medicines. Almost all Swedish household waste that is not recycled (excluding compost) is incinerated, and disposing of pharmaceuticals in regular household waste may therefore in most cases be equally effective from an environmental perspective. If the alternative is to flush them down the toilet, return and incineration is preferable. However, using the toilet as a disposal route for medicines has never been recommended practice in Sweden. In countries with other traditions regarding disposal of medicines and/or treatment of household waste through landfill disposal, collection of leftover pharmaceuticals is more strongly motivated from an environmental contamination standpoint. A review by Tong et al. (2011) highlighted the variability of pharmaceutical disposal practices worldwide, with toilets and sinks being widespread disposal routes in addition to garbage bins.

Veterinary pharmaceuticals are also widely used and contribute to several emission pathways. Although a strong simplification, environmental exposure to veterinary medicines typically involves contamination of soil to a greater extent than emissions of human pharmaceuticals, which mainly contaminate waterways. The spreading of urine and faecal waste (manure) from livestock as soil fertiliser, as well as deposition on pastures across the world, contributes to this pathway. Besides direct effects on terrestrial organisms, as exemplified by the effects of anthelmintic drugs on dung beetles (Wall & Strong, 1987), runoff from these areas has the potential to transport residual pharmaceutical substances to the aquatic environment (Garric et al., 2007). Aquaculture has also been identified as a potential source of pharmaceutical contamination (Boxall et al., 2004).

All sources mentioned above can contribute to pharmaceuticals reaching surface water and potentially affecting organisms. Besides water, pharmaceuticals have been detected in sediment, soil, and biota (Katsikaros & Chrysikopoulos, 2021; Kinney et al., 2006).

1.1.2 Occurrence and effects in the environment

Concentrations found in the environment vary greatly depending on the matrices and pharmaceuticals analysed. Umweltbundesamt (UBA), the Federal Environmental Agency in Germany, has compiled almost 300 000 environmental measurements of pharmaceuticals, their metabolites and transformation products from close to 100 countries in a database. Data from all kinds of waterways, from groundwater and sewage, from wastewater and sludge, and from soil and sediment are represented. Residues have also been identified in biota, with concentrations in both vertebrates and invertebrates primarily from aquatic environments, but also in birds and terrestrial mammals (UBA, 2026).

Although pharmaceuticals are frequently reported in the environment, to date, only a few pharmaceuticals with documented environmental effects have been identified. Ethinylestradiol (EE2) is one of the most well-established examples where levels around 1 ng/L can induce feminization of male fish, alter gonad development, reduce fertility and cause population-level effects (Jobling et al., 2002; Jobling et al., 1998; Kidd et al., 2007; Larsson et al., 1999; Purdom et al., 1994; Örn et al., 2003). However, the relative contribution from ethinylestradiol compared to natural estrogens excreted from women is somewhat unclear. Perhaps the clearest example of

an effect in the environment caused by pharmaceutical exposure is due to the non-steroidal anti-inflammatory drug (NSAID) diclofenac (Oaks et al., 2004). It was identified as the cause of the near eradication of several vulture species in India and Pakistan. The birds were exposed when feeding on carcasses of diclofenac-treated cattle and died due to kidney failure followed by visceral gout. Another example relates to ivermectin, an antiparasitic drug primarily for veterinary use. It is excreted through faeces largely unchanged, which facilitated exposure to dung dwelling insects. Increased mortality in larvae and reduced reproduction in dung beetles were among the reported effects (Wall & Strong, 1987). An additional clear example is the selection of antibiotic-resistant bacteria exposed to wastewater from antibiotic production facilities (Fick et al., 2009; Flach et al., 2015; Karkman et al., 2019; Kristiansson et al., 2011). Although this may have direct local effects on bacterial communities downstream from production plants, the main concern is the development of multidrug-resistant pathogens, which could have devastating consequences for the ability to prevent and treat bacterial infections all over the world (Larsson & Flach, 2022; UN General Assembly, 2024; WHO, 2024).

Pharmaceuticals with plausible effects in the environment include anti-anxiety benzodiazepines such as oxazepam and clobazam, for which both laboratory and field studies have demonstrated behavioural changes in fish at concentrations that may occur in the environment (Brand et al., 2025; Brodin et al., 2013). The synthetic hormone levonorgestrel (LNG) is a synthetic gestagen (progestin) found in contraceptives that can activate both mammalian and fish androgen receptors. Laboratory studies have identified reproductive effects (reduced egg-laying) in fish after exposure to as little as 0.8 ng/L of LNG (Zeilinger et al., 2009). Several studies on the NSAID diclofenac have reported effects in fish at low µg/L (Hoeger et al., 2005; Mehinto et al., 2010; Schwaiger et al., 2004; Triebskorn et al., 2004). Concentrations in the same range have occasionally been reported in treated municipal effluents (Brown et al., 2007; Fick et al., 2010a; Meyer et al., 2016; UBA, 2026). However, none of the examples above have demonstrated that the corresponding effects occur in the environment.

1.1.3 Risk assessment and management

In the 1980's, the National Research Council in the USA published the "Red Book" forming the basis of modern risk assessment (National Research

Council, 1983), which later was incorporated in environmental frameworks across the world. The main principle was to separate risk assessment (i.e., the scientific analysis in which a risk is characterized) from risk management (the actions and decisions following the risk assessment). Risk assessment was formalized with four steps: hazard identification, dose-response assessment, exposure assessment and risk characterization, a system still used today. Risk management was defined as "the process of weighing policy alternatives and selecting the most appropriate regulatory action, integrating the results of risk assessment with engineering data and with social, economic, and political concerns". While the risk assessment should be based on science, risk management is the policy work dealing with the results from the risk assessment together with handling conflicts of interest. In practice, the risk management of classical environmental pollutants was mainly phasing out and banning, to protect the environment. However, banning human pharmaceuticals will have a direct negative effect on public health and is therefore rarely a desired or realistic option.

In the EU, Directive 2001/83/EC regulates the approval of new pharmaceuticals for human use, which requires an environmental risk assessment (ERA) (European Union, 2001). However, the results of the ERA do not influence the approval decision. Instead, measures to reduce identified risks are introduced. The European Medicines Agency (EMA) has adopted a guidance document that describes how environmental risks from pharmaceuticals should be identified, assessed and reported (EMA, 2024). The document consists of two phases, where Phase I assesses the expected concentrations in the environment by calculating the predicted environmental concentration (PEC) based on dosage and the estimated use. Pharmaceuticals with PECs above a certain threshold level continue to Phase II where a more extensive assessment of the substance is required. This includes ecotoxicological testing on three trophic levels (algae, crustaceans and fish) and identifies concentrations causing effects in 10 % of tested organisms (EC10). Further, the no observed effect concentration (NOEC) is determined to facilitate the derivation of the predicted no effect concentration (PNEC) in the environment. Pharmaceuticals already on the market may receive a somewhat more realistic PEC based on sales data, and with the possibility to consider expected reduction of levels in WWTPs and during excretion. For human pharmaceuticals, patient benefit is prioritised,

whereas veterinary pharmaceuticals face the risk of non-approval after an unacceptable ERA (European Medicines Agency, 2026).

In 2000, the EU launched the Water Framework Directive (WFD) with the overall goal of protecting all water bodies and achieving "good status", both ecological and chemical, for all surface waters and groundwater (European Union, 2000). The WFD established a list of priority substances that Member States are required to monitor in surface waters. Initially, it consisted of 33 substances including industrial chemicals, pesticides, metals and POPs. Environmental Quality Standards (EQS) were defined for each substance, setting the allowed concentrations in water bodies. Member States were obliged to act if any substances exceeded the EQS. After an update in 2013, three pharmaceuticals (diclofenac, ethinylestradiol (EE2) and estradiol (E2)) were added to a "watch list" with the aim of pointing out substances of concern for which a sufficient risk assessment was lacking (EU, 2013). This required the Member States to monitor the occurrence of these substances in water bodies to assess the exposure and relate this to effect data. Depending on the results, substances on the watch list later continue to the priority list or are removed without further regulation in the WFD.

The ERA for pharmaceuticals in the EU addresses aspects including persistence, bioaccumulation and environmental toxicity (EMA, 2024). Little attention is paid to the specific modes of action of pharmaceuticals or to drug target conservation across species. Long-term test data in fish, such as full life-cycle tests, is rarely available, despite that genetic comparisons often indicate a lack of conserved drug targets in non-vertebrates (Gunnarsson et al., 2008). Sub-lethal effects at the molecular or enzymatic level, histopathological effects or behavioural changes are generally not considered in the risk assessment (European Commission, 2018).

Risk management may consist of many different measures; all aimed at reducing environmental exposure. A reduction in usage can be achieved by appropriate dosing and duration of treatment but must be implemented without compromising patient safety. Replacing the medicine with an environmentally safer alternative is another way to achieve a reduction. Information aimed at promoting safe disposal of unused medicines, including avoiding the use of landfills, may further contribute to reduced environmental exposure. Pharmaceutical-containing wastewater from manufacturing facilities has been proven to be a substantial source of environmental contamination (Larsson, 2014). The need for applying

standards for risk assessments and management of waste streams from manufacturing is therefore broadly recognized (UN General Assembly, 2024; WHO, 2024). Most WWTPs today are not designed to remove pharmaceuticals, and the costs for upgrading them across Europe would be substantial. However, risks associated with pharmaceutical emissions have led to the recent adoption of a revised Urban Wastewater Treatment Directive that stipulates all larger WWTPs across the EU must install advanced treatment for pharmaceutical removal in the coming decades (European Commission, 2024). The most realistic alternatives for reducing pharmaceutical concentrations, already adopted in full scale at some sites, include ozonation and treatment with activated carbon, either as granules or as addition of powdered carbon. Notably, these technologies remove many types of microcontaminants, not only pharmaceuticals. Hence, the value of treatment goes beyond reducing risks associated with pharmaceuticals in the environment.

On a global scale, however, the situation is very different. UN-Water reports that 42 % of household wastewater is not safely treated. The figures from industrial wastewater are even lower with data for only 8 % of the global population, of which 73 % is not safely treated (The United Nations Human Settlements Programme (UN-Habitat) and the World Health Organization (WHO), 2024). Hence, the installation of basic wastewater treatment worldwide is a more urgent step that should be prioritized before the addition of more advanced treatment targeting pharmaceuticals.

1.2 Non-steroidal anti-inflammatory drugs (NSAIDs)

1.2.1 Mechanism of action

NSAIDs are used to treat inflammation, pain and fever. Prostaglandins play a significant role in the inflammatory response by increasing vasodilatation and oedema. They also elicit pain by sensitizing nociceptors to inflammatory mediators and induce fever by affecting the temperature regulation in the hypothalamus (Rang et al., 2003). The mechanism of action of NSAIDs is the inhibition of the enzyme cyclooxygenase (COX) and hence suppression of prostaglandin and thromboxane synthesis. This leads to decreased inflammation, pain and fever (Rang et al., 2003). There are two COX enzymes, COX-1, which is involved in physiological processes and COX-2,

which is generally induced in tissue damage, inflammation and stress. However, assigning their function as purely homeostatic (COX-1) or pathophysiological (COX-2) is an oversimplification (Rouzer & Marnett, 2009). The cyclooxygenase selectivity of different NSAIDs varies greatly with naproxen and ibuprofen being relatively COX-1 selective and diclofenac showing higher affinity for COX-2 (Rang et al., 2003). As cyclooxygenases are evolutionarily well conserved (Gunnarsson et al., 2008), it is reasonable to assume that NSAID concentrations below those identified as causing acute toxic effects may still interact with cyclooxygenases and produce other effects in non-target vertebrates, including fish.

1.2.2 Usage

NSAIDs are commonly used worldwide and the relative use of NSAIDs varies between countries (Kookana et al., 2014). Diclofenac and ibuprofen are the most frequently used (McGettigan & Henry, 2013) and they are available both over the counter and by prescription. The defined daily dose (DDD) is the assumed average maintenance dose per day for a drug used for its main indication in adults (WHO Collaborating Centre for Drug Statistics Methodology, 2026). This makes it possible to compare the relative usage of specific drugs between populations and over time. The concept of DDDs can also be valuable in understanding the potential changes in environmental concentrations and hence risks, when replacing one active pharmaceutical ingredient (API) with another. In the Nordic countries, NSAID consumption in 2016 ranged from 32 DDDs per 1,000 inhabitants per day in Denmark and 74 in Iceland and Finland, with Sweden and Norway falling in between. Comparing data from 2005 with 2015, the proportion of total sales increased for ibuprofen and naproxen in all Nordic countries, whereas diclofenac sales decreased (Kristensen et al., 2019).

1.2.3 Uptake, metabolism and excretion

Oral administration is the major route for NSAIDs. However, topical administration has increased over the last decades. This introduced a new entry route to the environment as substances were removed by showering, bathing or swimming. After oral administration, absorption occurs in the gastrointestinal tract and NSAIDs are mainly metabolized by e.g. oxidation and conjugation in the liver and the metabolites formed, together with the

parent compound, are excreted via urine and faeces. The proportion of metabolised and unmetabolised substance and the stability of the metabolites in the environment differ between different NSAIDs (Osena et al., 2025). Topical administration, on the other hand, is not fully absorbed through the skin and therefore the unmetabolised compound may be released into the environment. Conjugated pharmaceuticals are typically less biologically active, but some conjugates (e.g., glucuronides) may be deconjugated in WWTPs, sediment or aquatic environments, resulting in reformation of the parent compound (Ternes, 1998).

1.2.4 The vulture crisis

NSAIDs are also used in veterinary medicine, but the main administration route differs and parenteral administration by injection is common. In Pakistan, diclofenac was available OTC for livestock, which resulted in widespread use (Oaks et al., 2004). In large parts of Southeast Asia, cattle are considered sacred and slaughter or euthanasia is forbidden. This results in sick or old cattle, sometimes recently treated with diclofenac, are left to die. The carcasses are often left out in the open or transported to designated dumping grounds which attracted scavenging birds such as vultures. It was later demonstrated that vultures are extremely sensitive to diclofenac and even low residues in carcasses were lethal to the birds (Oaks et al., 2004). Diclofenac caused altered blood flow to the kidneys which resulted in a decrease in the glomerular filtration rate (GFR), which in turn induced acute kidney failure followed by visceral gout. Population declines of white-rumped vulture (*Gyps bengalensis*) and Indian vulture (*Gyps indicus*) were > 92 % (Prakash et al., 2003). These species are still critically endangered. The vulture collapse may have a great impact on public health as well, as uneaten carcasses can serve as potential sources of pathogens with the potential of contamination of water sources. The decline of vultures has been estimated to result in substantial increases in feral dog populations due to the increased food availability, leading to more dog bites and increased transmission of rabies (Markandya et al., 2008). Because of these effects on vultures, diclofenac was banned for veterinary use and the NSAID meloxicam, which had proven to be a safer alternative for vultures, was recommended instead. However, despite the ban, vultures poisoned by diclofenac were still found years later (Cuthbert et al., 2011).

1.2.5 Occurrence

Table 1 shows the median concentrations of four commonly used NSAIDs in both untreated and treated wastewater across the world and the estimated removal percentage. Measurements from Swedish wastewater treatment plants published by Osena et al. (2025) are added for comparison. An overall conclusion from these data is that all NSAIDs typically occur at concentrations in the low µg/L or a few hundred ng/L range in treated wastewaters. Ibuprofen is almost completely removed in wastewater treatment plants, followed by naproxen with typical removal of around 90 %. The removal of ketoprofen is less effective and diclofenac has the lowest removal with only 16 % in the study by Osena et al. (2025). The number of DDDs sold were similar for diclofenac and naproxen (~ 43 000 000) in Sweden in 2017, while sales of ibuprofen were more than twice as high (~ 103 000 000). For ketoprofen, only about 10 000 000 DDDs were sold (Goodpoint, 2019).

Concentrations in receiving waters are typically lower than reported concentrations in treated wastewaters. This is due both to dilution and to further removal through degradation, and the dilution factor can vary greatly depending on the receiving water body. The Swedish Water & Wastewater Association (Svenskt Vatten) has reported concentrations of diclofenac from rivers and streams up to almost 0.5 µg/L (Svenskt Vatten, 2020). This indicates that sites with the highest concentrations were likely strongly effluent-dominated.

Table 1. Median concentration of four NSAIDs in municipal wastewater globally and in Sweden, and their estimated removal during wastewater treatment. Note that removal in the Swedish dataset is based on matched influent and effluent samples. Abbreviations: DCF=Diclofenac; IBU=Ibuprofen; KET=Ketoprofen; NPX=Naproxen.

	Wastewater UBA[1] (1998-2020)			**Wastewater Osena[2] (2004-2021)**		
	Untreated (µg/L)	Treated (µg/L)	Removal	Untreated (µg/L)	Treated (µg/L)	Removal
DCF	0.360	0.210	42 %	0.333	0.279	16 %
IBU	0.850	0.078	91 %	2.621	0.014	99 %
KET	0.264	0.093	65 %	0.690	0.112	84 %
NPX	1.030	0.127	88 %	2.044	0.156	92 %

[1]UBA (2026)

[2]Osena et al. (2025)

1.2.6 Effects in fish

Oaks et al. (2004) identified diclofenac as the cause of the vulture population crisis. The same year, Schwaiger et al. (2004) published a study where fish were exposed to diclofenac for several weeks and reported histological effects with a lowest observed effect concentration (LOEC) of 5 µg/L. Thereafter, other studies followed with similar reports of mainly histological effects in fish at low µg/L (Hoeger et al., 2005; Mehinto et al., 2010). However, the conclusions from these studies were not without doubt. Schwaiger et al. (2004) only reported "mean assessment values" (MAV) for each organ which made it impossible to evaluate which histological changes were affected by the diclofenac exposure. Hoeger et al. (2005) specified the histopathological diagnoses investigated and clearly showed the frequencies of each grade but unexpectedly claimed no histological findings in the control fish despite almost 20 different endpoints graded in three different organs. In Mehinto et al. (2010), the histopathological effects reported were not supported by the included histological images. Hence, conclusive data linking different levels of diclofenac exposure to specific histopathological effects in fish were lacking.

A process that strongly influences the potential for a substance to affect organisms is its ability to bioconcentrate. Bioconcentration here refers to the relative concentration of a substance in the exposed organism compared to the surrounding water concentration. Factors affecting bioconcentration potential include lipophilicity, but other characteristics such as ionisation and ion trapping and metabolism within exposed aquatic organisms are also involved. The concept of bioconcentration in combination with comparative pharmacology has been used to assess risks for effects of pharmaceuticals in fish. In 2003, Huggett et al. (2003) proposed the so-called "fish plasma model". It predicts steady-state concentrations of pharmaceuticals in fish plasma based on water concentrations and the lipophilicity (demonstrated by log K_{OW}) of the pharmaceutical. The water concentration may either be derived from predicted environmental concentrations (PEC) created from sales data, or from measured environmental concentrations (MEC). The resulting fish steady state plasma concentration ($F_{SS}PC$) is then compared to the human therapeutic plasma concentration (H_TPC). The latter is accessible through the literature for most drugs. If the effect ratio (ER) is < 1, this is interpreted as a probable risk of target receptor activation and effect.

$$Effect\ Ratio\ (ER) = \frac{H_T PC}{F_{SS} PC}$$

Fick et al. (2010b) extended the use of the fish plasma model by calculating a "critical environmental concentration" (CEC) representing the water concentration at which the ratio is expected to be 1. For diclofenac, this is 4.56 µg/L which can be compared with 194.7 µg/L for ibuprofen and 828.0 µg/L for naproxen (Fick et al., 2010b). Concentrations in fish plasma may not only be modelled but also measured, which reduces uncertainty associated with the model (Brown et al., 2007). The bioconcentration of NSAIDs in fish is reported as both stable and highly variable (Brown et al., 2007; Cuklev et al., 2011; Fick et al., 2010a; Schwaiger et al., 2004). These discrepancies warranted further investigations on BCFs of NSAIDs to evaluate the possible effects in fish.

The fish plasma model is based on evolutionary conservation of targets between species (Gunnarsson et al., 2008). Stimulation of receptors or inhibition of enzymes are also likely to cause effects on the gene expression level. Identification of such regulated genes may not only demonstrate that there has been sufficient exposure to cause some level of effect, but also potentially serve as biomarkers of exposure in a more complex field exposure scenario. Mehinto et al. (2010) investigated effects in rainbow trout exposed to diclofenac and reported a downregulation of cyclooxygenases 1 and 2 expression at exposure concentrations of around 1 µg/L. However, the published primer sequences used in their qPCR analysis targeted cytochrome c oxidase subunit I and II (*cox1* and *cox2*) instead, as also noted by Cuklev et al. (2012). In contrast, the genes encoding the cyclooxygenase enzymes COX-1 and COX-2 are prostaglandin-endoperoxide synthase 1 and 2 (*ptgs1* and *ptgs2*), which likely explains the mix-up in the primer design. Cytochrome oxidase subunits I and II are components in the electron transport chain and have no apparent connection to the known mechanism of action of diclofenac.

The first effect data on diclofenac in fish certainly raised concerns about possible adverse effects at low µg/L and this, together with the widespread occurrence of diclofenac in aquatic environments caused diclofenac to be considered for prioritization in the Water Framework Directive. Further risk assessment based on predicted environmental concentrations and predicted no effect concentrations (PEC/PNEC) further warranted their inclusion.

However, several ambiguities in the available scientific literature made a conclusive assessment of results and associated risks difficult.

Compared to many traditional environmental pollutants, pharmaceuticals are often more tightly linked to the wellbeing of humans. A simple ban to avoid potential environmental effects would therefore often have apparent and rapid negative effects on public health. Replacement of one medicine with another, may however be a feasible option only in some situations. Notably, there are different NSAIDs, and although they have similar mechanisms of action, they have different chemical properties that can be critical for their fate and ability to affect wildlife. Importantly, excretion rates and the ability to withstand degradation in WWTPs (Table 1) differ greatly, as do their predicted and measured abilities to bioconcentrate (Brown et al., 2007; Cuklev et al., 2012). Furthermore, dosages and selectivity for the different COX enzymes could also be associated with different risks of causing adverse effects in the environment. However, assessing the ecotoxicological risks and benefits of replacing one NSAID with another, warrants systematic comparisons. Direct comparisons between studies have proven difficult when neither experimental design nor endpoints are standardised or controlled for. Basing comparisons on matched studies, where experimental design and methodology are standardised, is therefore preferred.

Clear, reproducible effects in laboratory studies at concentrations found in the environment certainly indicate a risk. However, because of numerous uncertainties regarding correctly determined concentrations in complex matrices, maintained bioavailability and absence of important interaction effects, such evidence is not conclusive. Therefore, comparative studies that also expose fish to complex wastewaters or studying fish captured downstream from WWTPs add another dimension to our understanding of risks.

2. Aims of the thesis

To identify and compare effects of different NSAIDs in fish with emphasis on liver and kidney histopathology (Papers I and II).

To identify, evaluate and compare histopathological effects in liver and kidney of fish reported after NSAID or treated municipal wastewater exposure, respectively (Paper III – review).

To identify histopathological effects of treated municipal wastewater in fish (Paper IV) using a matched experimental design from previous studies on NSAIDs (in Papers I and II), thereby facilitating direct comparisons.

3. Methodological considerations

This thesis is based on three different experimental studies (Papers I, II and IV), all approved in advance by a research animal ethics committee, and a review (Paper III). Briefly, in Papers I and II we exposed sticklebacks for four weeks to four concentrations of diclofenac (Paper I) or naproxen (Paper II) in triplicate aquaria with flow-through systems. The setup in Paper IV was very similar, but the fish were exposed to treated municipal wastewater from a tertiary treatment plant. In all these studies, the main endpoints were histopathological changes in kidney and liver. Exposure concentrations of NSAIDs were measured by LC-MS/MS. Paper III is a comprehensive literature review aiming at finding overlapping histopathological effects in kidney and liver of fish exposed to either individual NSAIDs or to treated municipal wastewater. Detailed information about experimental design, workflow and analysis is found in each article including the associated supplementary material. This section thereby complements the individual papers by addressing the overall choices of methods used and discussing both advantages and disadvantages.

3.1 Experimental model - the three-spined stickleback

The three-spined stickleback (*Gasterosteus aculeatus*) was used in all three experiments (Papers I, II and IV). It is commonly found in the northern hemisphere, located in fresh, brackish and salt water. The species is not of economic importance. It is easy to catch and adapts well to laboratory conditions. They quickly learn the location of the food source and gather at the front of the aquarium when feeding approaches.

The stickleback is quite a common research animal and has been extensively studied over several decades. It has a characteristic reproductive behaviour with the male building a nest for the female to lay her eggs in (Katsiadaki, 2006). The kidney in the male stickleback can be considered a "reproductive organ" since spiggin, the nest-building glue, is produced there. This production is androgen-dependent and together with oestrogen-dependent production of vitellogenin (VTG) and their specific reproductive behaviour, the stickleback is considered a good model species in aquatic ecotoxicology, especially regarding endocrine-disrupting chemicals (EDCs) (Katsiadaki, 2006). Further, their whole genome has been sequenced,

facilitating different genetic analyses. However, the generalisability of the effects seen in sticklebacks to effects in other species is not established.

It is a small fish with an adult length around 4–8 cm. It has three prominent spines on the back and two parallel ones ventrally. The latter are perfectly located to be used as handles during dissection of the abdominal cavity. However, because of their hardness, the spines can also introduce artefacts in the histological sections and cause more frequent replacement of microtome blades.

In all three experiments, wild-caught fish were used. This is not always a good idea since they may carry pathogens which may affect the experimental results. It is also impossible to know the complete background of the fish, including previous exposure to contaminants or other conditions that may affect the outcome of the experiments. However, catching all individuals from the same location, removing individuals showing signs of disease, allowing the fish to acclimatize to laboratory conditions and randomly assigning fish to treatments can reduce some of the potential disadvantages associated with wild-caught fish.

3.2 Experimental design

Flow-through exposure was used in all three experiments (Papers I, II and IV) (Figure 2). Compared to semi-static exposure which involves complete or partial renewal of aquarium water at certain intervals, flow-through enables more stable exposure concentrations, at least in theory. Water quality can also be better maintained with new, possibly oxygen-rich water continuously entering the aquaria and replacing old water of less quality. But flow-through can also come with disadvantages such as blockage in the outflow with the following risk of flooding. Mechanical malfunction of the pumps delivering water or exposure media can cause large differences in exposure concentrations between aquaria. Further, exposure to metabolites or transformation products will also be limited due to the constant renewal of water. Degradation will also be difficult to assess but neither that nor exposure to metabolites or transformation products was the intended outcome in these experiments. We chose waterborne exposure to mimic exposure scenarios in the environment. There is also the possibility of exposing fish by injection of pharmaceuticals, but the environmental relevance of that exposure route is low, not the least because it is difficult to

compare injected doses to measured or modelled environmental concentrations of pharmaceuticals.

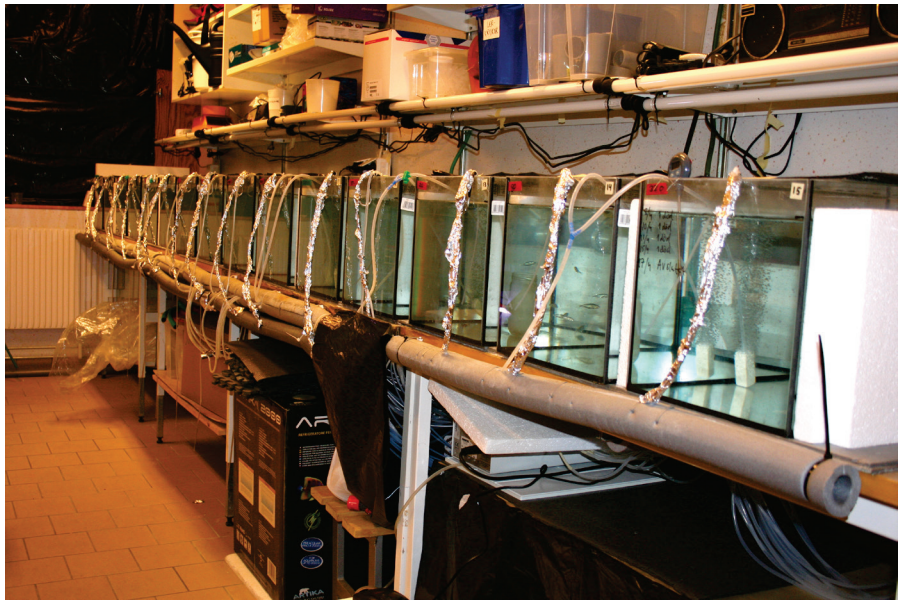

Figure 2. Aquaria setup in Papers I and II.

The chosen diclofenac exposure concentrations in Paper I (5-320 µg/L) were based on previously published papers (Mehinto et al., 2010; Schwaiger et al., 2004). Based on different human therapeutic concentrations between diclofenac and naproxen, exposure concentrations for naproxen in Paper II (20-1280 µg/L) were increased to match the diclofenac concentrations. In both studies, diclofenac sodium salt or naproxen sodium salt (purity ≥ 98 %) were used and hence there was no need to use solvents in the preparation of the exposure solutions. Stock solutions were prepared weekly during the respective studies with the possibilities of errors in the weighing or measuring. The stock solutions were delivered to the aquaria through a peristaltic pumping system (Figure 3) while water was delivered simultaneously by a separate peristaltic pumping system. This enables more stable exposure concentrations over time, but this setup, as previously described, is also vulnerable to malfunctioning of the pumps and subsequent failure to achieve targeted concentrations. A possible reason for not achieving the targeted exposure concentrations includes binding of the test

substance to vials and hoses or other materials. To avoid this, only glass aquaria were used with enrichment also made of glass and the exposure solution was delivered by polytetrafluoroethylene (PTFE) tubing. An additional important aspect is the potential contamination from exposure aquaria to the control aquaria. Separate nets and cleaning equipment were used in all experiments to reduce such risks, but this was also controlled by pharmaceutical analysis of the control aquaria water.

Figure 3. Exposure bottles delivering NSAID via a peristaltic pump.

In the wastewater experiment (Paper IV), the fish were exposed to undiluted treated municipal wastewater in aquaria in a shipping container specifically designed for this study (Figure 4). This setup was chosen to control as many conditions in the experimental study as possible. It facilitates relevant comparisons with the control aquaria (Figure 5) receiving tap water and to assign any observed effects specifically to the wastewater exposure. Other common experimental designs when exposing aquatic organisms to wastewater are the deployment of cages in rivers downstream from WWTPs.

Besides the risk of sabotage or the fish escaping, there are several conditions that may affect the fish that are not correlated to the wastewater per se.

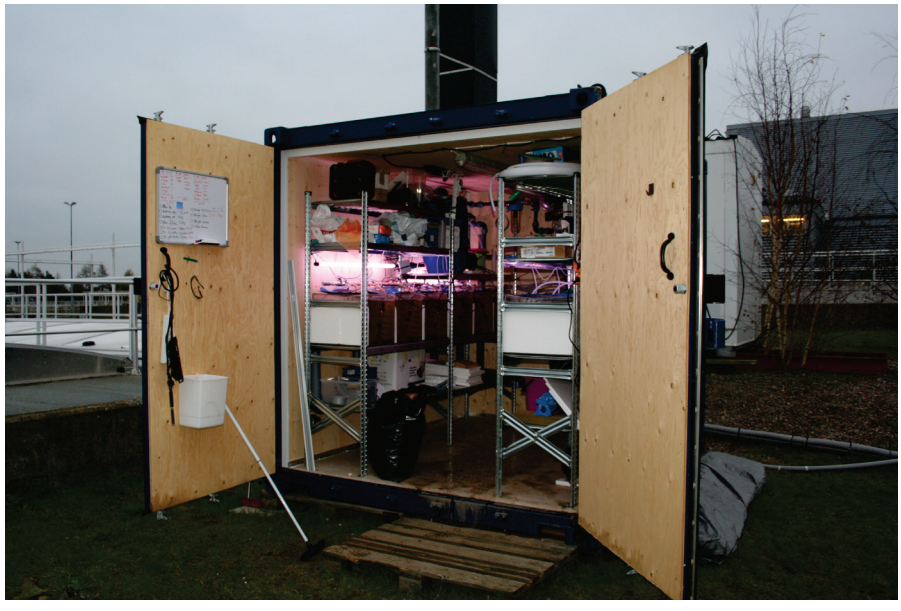

Figure 4. Exposure container placed at Kungsängsverket for the experimental study in Paper IV.

These include variations in food availability, oxygen levels, temperature, wastewater dilution, stress from predators etc. Another possibility to assess the effects of wastewater exposure is to sample free-living wild fish. This approach is more ecologically relevant, but again, it is close to impossible to evaluate the actual exposure since fish might migrate in the discharge area resulting in fluctuating exposure. To evaluate effects in both caged fish and free-living fish, they need to be compared with representative controls. The most suitable control would probably be upstream from the WWTP to include confounding factors in the river water itself, but this will come with the same disadvantages as previously described.

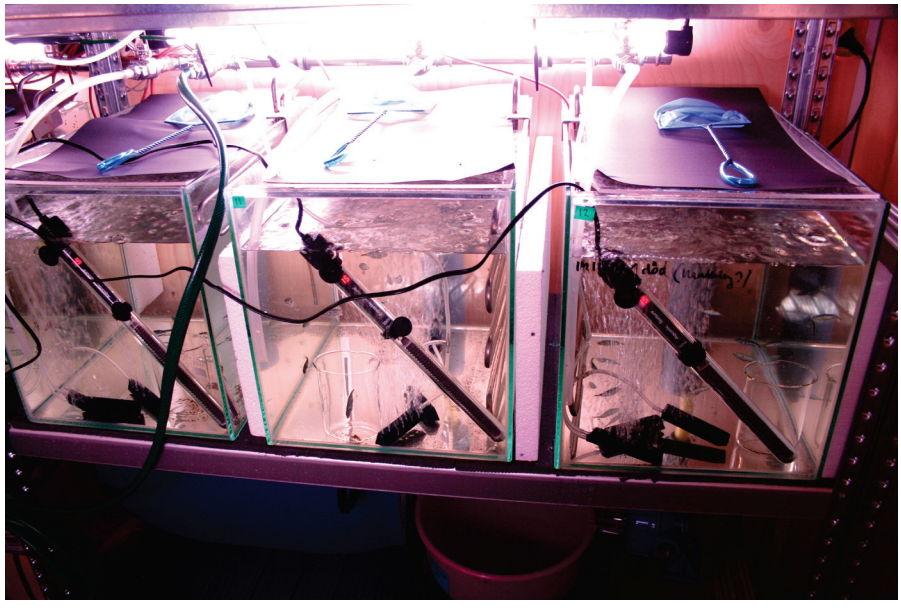

Figure 5. Aquaria setup in Paper IV.

Exposure length was around four weeks in all studies (Papers I, II and IV). The light schedule was set to 8 hours of light and 16 hours of darkness to keep the fish reproductively inactive. This is important since histological effects in both kidney and liver in reproductively active stickleback are expected. Due to spiggin production, an increase of kidney epithelial height (KEH) in tubuli can be seen in males. Hepatocellular hypertrophy, because of VTG production, can be seen in females. It may also affect social interactions in the aquaria where e.g. differences in sex ratios might induce effects within the aquaria regardless of the treatment.

3.3 Analytical chemistry

Pharmaceutical concentrations were determined in all three experiments. The chemical analysis was performed at Umeå University (UU) using tandem mass spectrometry (LC-MS/MS). In Papers I and II, this was performed to assess the actual exposure since, as described in 3.2, there are several steps in the preparation and delivery of the exposure solution that could affect the final aquarium concentration. In Paper IV, 89 different pharmaceuticals (including NSAIDs) were measured to identify the exposure from the treated

wastewater. Samples were taken several times a week from all aquaria, including the controls. Flow-proportional sampling was performed daily from the treated wastewater while grab samples were taken from the aquaria. Isotope-labelled analogues of both diclofenac and naproxen were used in studies I and II, respectively. This is important since the preparation of samples before mass spectrometry analysis includes several steps, all of which may affect the measured concentration. The isotope-labelled analogue is added at a given concentration, and its relative recovery can compensate for internal losses during the analysis and provide a more accurate quantification (Loos et al., 2016). In Paper IV, 15 different isotope-labelled analogues were used for the 89 pharmaceuticals, but for the four included NSAIDs, their corresponding labelled analogue was used. Besides analysis of aquaria water for determination of pharmaceutical exposure, concentrations in whole-fish were also measured in Papers I and II with LC-MS/MS. This facilitated the calculation of BCF.

3.4 Histology

In Papers I, II and IV, fish for histological evaluation were fixed in phosphate buffered formaldehyde (4 %) and embedded in paraffin. The kidney was sectioned *in situ*, due to the risk of damaging the organ if it were to be removed from the body cavity. In Paper I, all abdominal organs were excised, and the liver was sectioned separately. In Papers II and IV, all abdominal organs were left in situ. Even though sectioning soft organs together with the hard spine increases the risk of sectioning artefacts and scratches, it facilitates sampling standardization and reduces the total number of slides. In addition, this reduces the risk of slides being mixed up between individuals, but it also introduces a certain level of bias since all organs are available in the same section. This can be important if a certain treatment causes a specific recognizable effect in e.g., the liver. The evaluator will then consciously (or unconsciously) identify the animal as belonging to the exposed group and therefore anticipating other effects and perhaps giving them a score more often seen in exposed animals.

The kidney was sectioned longitudinally (parasagittally) at predetermined levels, and each level produced two sections with one stained with hematoxylin and eosin (H&E) and one with periodic acid-Schiff (PAS). To avoid sampling bias, the section with the largest area of the kidney was

evaluated. The liver on the chosen kidney section was simultaneously chosen except in Paper I where it was sectioned separately. A four-point scale was used in the scoring with 0 = Not present, 1 = Minimal, 2 = Mild, 3 = Moderate and 4 = Severe. The gonads were sectioned in all studies but only used for confirmation of sex. Histopathological preparation was performed at the Department of Animal Biosciences (HBIO, former Dep. of BVF) at the Swedish University of Agricultural Sciences (SLU), and the histological evaluation was done by the author and partly by another pathologist (Elisabet Ekman, Paper I) or by the author alone (Papers II and IV).

Several studies have evaluated the reliability and credibility of published histopathological data, highlighting the need for improvement (Wolf, 2021; Wolf & Maack, 2017; Wolf & Wheeler, 2018). When used in toxicological research, histopathology is typically an explorative method with the evaluator not aware of what effects are expected. Given the large number of potential effects, it is also often time consuming to evaluate histological sections. One strategy to make assessments of sections from ecotoxicological studies more standardised, directed and time efficient is to first evaluate sections from the highest concentration group (in comparison to controls). This comparison is assumed to have the highest probability of finding effects. This initial evaluation aims to refine what will be (semi)quantitatively scored or measured and is then followed by blinding and randomisation of sections from all groups. This approach was adopted in the first experiment (Paper I), in addition to evaluate previously reported effects from the literature. To reduce sampling and observation bias, standardisation at sampling and the choice of section, together with the use of masked sections are good approaches. This was done in all experimental studies (Papers I, II and IV). However, there will always be a certain level of subjectivity remaining in the histological evaluation. To strengthen the histological finding identified in Paper I, the only histological endpoint that differed significantly between diclofenac-exposed fish and controls was re-evaluated by another pathologist. The second evaluator was blinded to both treatment and the other pathologist's score. A subset of slides from Paper I were re-evaluated before the scoring of the slides in both Papers II and IV. This was done to identify a potential "diagnostic drift" between studies.

Compared to data from length measurements and qPCR analysis, it can be challenging to make histopathological analysis (semi-)quantitative as the outcome is derived from the interpretation of a complex image. However,

measurements as performed in Paper IV, can also be used. Quantification is crucial to be able to compare results between treatment groups, to perform statistical analysis and to identify effects. Qualitative (descriptive) histological analysis is not uncommon but has limited value for risk assessment.

To link histological effects with a negative outcome for the organism is far from clear-cut. Certain effects, i.e. tumour formation, extensive necrosis or malformation of structures can relatively easily be assigned as unfavourable, but affected endpoints are often much less clearly linked to impaired health. This is probably why they are not readily included in the derivation of EQS in the WFD. Still, in some contrast to gene expression analyses, they represent effects manifested as changes in the microscopic structure of organs and cells, and thus likely also in function. Taken together, compared to the apical endpoints, histology has the potential to identify sublethal effects at lower concentrations and with a greater specificity, but the biological relevance is not always evident.

Determining which organs to evaluate is an important step which obviously affects the outcome of the histological analysis. Traditionally, gills, due to their constant contact with the environment and potential contaminants, have been evaluated. However, they are prone to histological artefacts and require a high level of standardization in sampling and preparation, where euthanasia method and preservation medium can affect histological result. Gills were not analysed in any of the studies. Other commonly analysed organs are the liver and kidney, both of which were evaluated in all experimental studies (Papers I, II and IV) and in the review (Paper III). Both organs are not as easily affected by artefacts but still require sampling standardization to be able to draw firm conclusions. This is especially important in the kidney in which the relative amount of different cell types is not uniform throughout the organ. Histological investigations of gonads in ecotoxicological studies are common, mainly due to the concern about endocrine disrupting effects, especially from estrogens in wastewaters. However, the stickleback has not been identified as an especially sensitive species with regards to endocrine-related histological changes. Although cases of intersex are reported (Borg & van den Hurk, 1983; Gercken & Sordyl, 2002; Hahlbeck et al., 2004), this was also considered outside the scope of this thesis.

3.4.1 Renal histological endpoints

After the initial explorative examination where the treatment was known for the evaluator, all slides were masked and reevaluated using a specific scoring protocol. The protocol was developed beforehand and based on both previously published data from histopathological studies (Hoeger et al., 2005; Schwaiger et al., 2004) but also from the initial unmasked examination. Six different renal endpoints including presence of hematopoietic hyperplasia were evaluated and scored semiquantitatively in Papers I and II (Table 2). We hypothesized that measuring renal thickness (defined as the measurement between the ventral and dorsal border of the kidney in the section) might be a more sensitive endpoint in detecting hyperplasia, and it was therefore included in the analysis. Additional endpoints of potential importance from the review (Paper III) were added to the histological evaluation in Paper IV. This resulted in 17 different renal endpoints, some with microscopic measurements or calculations with direct correlations to the previously semiquantitatively scored endpoints (Table 2). This facilitated a more objective evaluation and with the potential of them being more sensitive endpoints for detecting differences. Additionally, these measurements and calculations produced continuous data, allowing the use of different statistical methods than the ordinal data obtained from semiquantitative scoring. Photomicrographic examples of the different scores of hematopoietic hyperplasia are shown in Figure 6 (p. 56; reproduced from Paper II) and in Paper I. Examples of nephron neogenesis are shown in Paper III and renal thickness is shown in Paper IV.

Table 2. Renal histological endpoints evaluated in the three experimental studies.

Renal histological endpoint	Paper		
	I	II	IV
Hematopoietic tissue/hematopoietic hyperplasia	X	X	X
Tubular necrosis	X	X	X
Tubular regeneration/nephron neogenesis	X	X	X
Pigmented macrophage aggregates (PMA)	X	X	X
Tubular hyaline degeneration/droplets	X	X	X
Parasites	X	X	X
Interstitial proteinaceous fluid	-	-	X
Tubular outer diameter	-	-	X
Tubular inner diameter	-	-	X
Kidney epithelial height (KEH)	-	-	X[1]
Bowman's capsule diameter	-	-	X
Glomerular diameter	-	-	X
Bowman's space	-	-	X[2]
Renal thickness at six levels	-	-	X
Renal thickness at five levels	X[3]	-	X
Dev. nephrons/basophilic clusters (DNs/BCs) per square mm	-	-	X
Glomeruli per square mm	-	-	X

[1] Calculated by subtracting the tubular inner diameter from the tubular outer diameter

[2] Calculated by subtracting the glomerular diameter from the Bowman's space diameter

[3] Evaluated and presented in Paper IV

3.4.2 Hepatic histological endpoints

The histopathological protocol for liver consisted of five endpoints in Papers I and II although hepatocellular necrosis was not reported in Paper I due to no findings (Table 3). The review (Paper III) identified four additional endpoints of interest, resulting in a total of nine hepatic endpoints evaluated in Paper IV (Table 3). The normal appearance of hepatocytes can differ a lot in fish, both between different species but also within a species, depending on factors such as nutrition, sex, age and reproductive status, which clarify the importance of relevant control fish. Photomicrographic examples of the different scores in hepatocellular vacuolation are shown in Figure 7 (p. 60; reproduced from Paper II) and images of liver from stickleback with different normal appearances can be found in Paper III together with an image on hepatocellular necrosis. Paper IV demonstrates inflammatory cell foci and pigmented macrophage aggregates.

Table 3. Hepatic histological endpoints evaluated in the three experimental studies.

Hepatic histological endpoint	Paper I	Paper II	Paper IV
Hepatocellular vacuolation	X	X	X
Pigmented macrophage aggregates (PMA)	X	X	X
Inflammatory cell foci	X	X	X
Hepatocellular necrosis	(X)[1]	X	X
Parasites	X	X	X
Fibrosis	-	-	X
Vessel alteration	-	-	X
Bile duct hyperplasia	-	-	X
Hepatocyte hyalinisation	-	-	X

[1]Hepatocellular necrosis was evaluated in Paper I but not present in any of the slides and thus not reported in the article.

3.5 Apical endpoints

Growth and condition factor was analysed in all experimental studies. Mortality and macroscopic lesions were not anticipated beforehand, and their evaluation was therefore included during the study in Paper I but also investigated in Papers II and IV. To avoid any interference with the histological analysis, the sticklebacks were held in conditions to keep them reproductively inactive. Reproduction was therefore not assessed and was also considered outside the scope of this thesis. Apical endpoints are defined as "whole-organism outcomes of exposure" with mortality and growth or reproduction as typical examples (Villeneuve & Garcia-Reyero, 2011). All those endpoints are non-specific, meaning the response is not unique to a specific contaminant and the number of substances that could e.g. cause mortality would be almost infinite, if the exposure concentration is enough. Their value for establishing cause–effect relationships in complex exposure situations, such as field studies, is therefore limited. However, there are advantages with apical endpoints such as clear relevance to ecological outcomes, and often simple assessment of effects. It is easy to measure length or weight, determine if a test object is dead or alive and to count eggs laid or fry hatched.

3.6 Gene expression analysis

Hepatic gene expression analysis was performed in experimental studies I and II to assess effects of NSAID exposure on mRNA expression level of selected target genes. The analyses were conducted at the University of Gothenburg (UGOT) using quantitative PCR (qPCR). Compared with large-scale gene expression analyses such as RNA sequencing (RNA-Seq), qPCR typically examines the expression of a limited number of target genes. In ecotoxicology, qPCR is often used to validate a subset of genes identified as differentially expressed in large-scale analyses or to confirm previously published qPCR results from exposure studies. It can also help to relate effects observed at higher biological levels, such as protein or cellular changes, to potential changes in mRNA expression on genes of interest.

Prior to running a qPCR experiment, assays need to be optimised. This can be a time-consuming and challenging process involving primer design to ensure amplification of the correct gene, testing for amplification efficiency across assays and evaluating reference genes used for normalisation. Designing primers requires DNA sequence information for the genes of interest. If the target gene is conserved between species, homologous sequences from other species may be used. An amplification efficiency of 100 % corresponds to doubling of the target amplicon during each qPCR cycle, and efficiencies of 95-105 % are generally considered acceptable. For reference genes, housekeeping genes are often selected as internal standards to normalise qPCR data and correct for variability in the amount of starting material between samples. Reference genes must be evaluated and only those showing stable expression across experimental conditions are selected.

Both target and reference genes were selected based on previously published studies (Cuklev et al., 2011; Geoghegan et al., 2008; Hogan et al., 2008; Mehinto et al., 2010; Williams et al., 2009). The primer sequences were obtained from Ensembl, NCBI GenBank or previously published studies (Geoghegan et al., 2008; Hogan et al., 2008; Williams et al., 2009).

The primary interest was the effect of NSAID exposure on genes coding for the cyclooxygenase enzymes COX-1 and COX-2. However, despite several attempts, the designed primers did not yield the expected amplicons. As qPCR was not the principal aim of the studies, no further primer optimization was performed, and these genes were therefore excluded, together with *p53* (tumor protein p53) for the same reason. The cycle threshold (C_T) for each gene of interest was normalised against the reference

gene, resulting in ΔC_T and then against the control group, producing a $\Delta\Delta C_T$. QPCR data were analysed by the method described by Livak and Schmittgen (2001) and reported as the expression fold change ($2^{-\Delta\Delta C_T}$).

3.7 Statistical analysis

Statistical analysis was performed by a statistics consultant (Papers I and II) or by the authors with support from statistical consultants (Paper IV). In experiments I, II and IV, all treatment levels were replicated three times using replicate aquaria. This results in aquaria being the experimental unit and the fish in each aquarium being technical replicates. Even though one strives to achieve as similar conditions as possible regarding to temperature, oxygen saturation, pH, light, fish density, feeding etc. in the different aquaria, there is always the possibility of specific fish interactions such as dominance, spread of pathogens, hierarchies in the aquaria which might have an effect on the outcome of the investigated endpoint. Hence, fish within one aquarium are not independent from each other. Independence between samples is one of the assumptions in the traditional analysis of variance (ANOVA) (Quinn & Keough, 2002). If an ANOVA still is performed, each fish at the same treatment level will be counted as the experimental unit. This will create pseudoreplication, which will exaggerate the statistical power and increase the risk of false positives. Hence, the possibility of assigning significant effects when there are none.

The effect from aquarium is considered as random and the treatment is considered a fixed effect. By using a linear mixed-effect model, both fixed and random effects will be accounted for in the analysis (Brown, 2021). The random effect from aquaria was included in the statistical analyses in all three experiments (Papers I, II and IV) and a mixed linear model was used for continuous variables (i.e., length, weight, condition factor, gene expression and histological measurements/calculations) followed by post-hoc testing with Dunnett's (Papers I and II) where treatment levels were compared to the zero concentration. Paper IV included only two treatment levels and post-hoc testing was therefore not needed.

Histological semiquantitative scoring produces ordinal data, which further need to be considered in the statistical analysis. A cumulative logistic response model together with post-hoc testing via a serial gatekeeping procedure (Paper I) or Dunnett-adjusted contrast (Paper II) was used. As

comparisons are done in a step-by-step fashion with a serial gatekeeping procedure, multiple comparisons are not punished in the same way as with, for example, Dunnett's test. As previously described, no post-hoc analysis were needed in Paper IV.

Sex has the potential to interfere with different response variables in different ways. However, testing for the influence of sex was done *a posteriori* in Papers I and II but included *a priori* in Paper IV. If the interaction term between treatment and sex was significant ($p < 0.05$), the response variable had to be interpreted with females and males analysed separately. This will of course reduce the power of the test since approximately half of the data will be removed from each analysis. However, if sex plays an important role, the "noise" is reduced as well, which might facilitate the detection of significant effects.

The number of fish in each experimental study was not the same. This was due to a different number of fish available at that time, and this could also affect the power, i.e., the possibility of detecting an effect.

When comparing the two pathologists histopathological scoring, Kappa statistics was used (Cohen, 1968). This tested the interobserver agreement.

3.8 Literature review

There are several types of literature reviews, including systematic reviews, mapping reviews, and rapid reviews (Grant & Booth, 2009). Different types have different working methods, provide different outcomes and are used for different purposes. The aim with the review in Paper III was to identify which histological lesions were reported in fish after exposure to NSAIDs or to treated municipal wastewater. However, predefined inclusion criteria were applied to ensure that only endpoints from studies meeting basic quality criteria were included, thereby facilitating robust conclusions. The review resembled a systematic review with a clearly defined research question, a systematic search with specified strings and dates and a search control with previously known articles. The inclusion criteria were predefined and exclusions were transparent. Included articles were summarized and a quality assessment was performed on the histological analysis. However, no preregistered protocol was used and there was only one assessor further separating it from a full systematic review. Additionally, no flow diagram (e.g. PRISMA) was included. The main reason for deviating from a

traditional systematic review was due to both time and financial constraints. An additional difference was the decision to exclude all data that reported no effects. Although there is a value in identifying conditions which resulted in no histological effects, drawing that kind of conclusions requires an extensive evaluation with the different endpoints investigated pre-specified and not only stating “no histological alterations”. The outcome of a histological evaluation also depends greatly on the assessor. This is to be compared, for example, with evaluations of mortality or parameters connected to growth, all of which can be managed without any extensive education or training. However, papers fulfilling all criteria except reporting no statistical significance were listed but not included in the review. Meta-analysis is also common in systematic reviews but the data from the included articles in the review (Paper III) were very diverse and hence, a meta-analysis would not have been meaningful. According to Grant and Booth (2009), the review would be classified as a critical review with systematic elements.

The histological endpoints from the review were assessed based on adequate quality of the histological images, which evaluated resolution and magnification The quality of the histological specimen was also evaluated based on factors such as preservation, sectioning quality and the presence of artefacts. Finally, an overall assessment was done to conclude the weight of evidence, i.e., whether the claimed diagnosis was supported by the figure or not. The last aspect comes with some subjectivity since it is based on an “expert judgement”.

Histological overlaps between fish exposed to NSAIDs and fish exposed to treated municipal wastewater or other noteworthy histological endpoints were evaluated based on reliability and relevance.

4. Results and Discussion

Results from all Papers (I–IV) are presented in full detail in the respective papers and associated supplementary material. This section presents and discusses the main results, starting with the actual measured exposure concentrations in Papers I, II and IV. Thereafter, histological effects from NSAID exposure followed by histological effects from treated municipal wastewater exposure and their identified overlap are presented. Finally, other effects of NSAID exposure are reported and discussed.

4.1 Exposure

Three-spined stickleback were exposed to diclofenac, naproxen or treated municipal wastewater. Evaluating exposure effects without determining the actual exposure concentrations adds uncertainty to the subsequent risk assessment and prevents firm conclusions. The average measured exposure concentrations in Papers I and II were 85-111 % of nominal concentration for diclofenac and 88-96 % of nominal concentration for naproxen. This can be considered a satisfactory result considering the number of factors affecting the final aquaria concentrations. Measured NSAID concentrations in other fish exposure studies are in the same range (Table 4).

Table 4. Measured NSAID exposure concentration in relation to nominal concentration.

Reference	NSAID	Measured concentration (% of nominal)
Hoeger et al. (2005)	DCF	126–230 %
Mehinto et al. (2010)	DCF	93–157 %
Bickley et al. (2017)	DCF	95–103 %
Baumann et al. (2020)	ASA	66–80 %
Birzle et al. (2023)	DCF	98–110 %

The methodology for measuring the concentrations differed between the studies which probably affected the outcome. Liquid chromatography combined with mass spectrometry, as also used in Papers I, II and IV, was the most common method. Hoeger et al. (2005), being the first published study in the review regarding NSAID exposure, used enzyme-linked immunosorbent assay (ELISA).

Internal pharmaceutical concentrations were measured in both Papers I and II. As the stickleback is a small fish, we chose to analyse whole-body concentrations of NSAIDs. The levels of the respective NSAID measured were less than the aquaria concentrations and the calculated BCF was stable over all exposure concentrations, being 0.3 for diclofenac and 0.07 for naproxen. Schwaiger et al. (2004) reported a remarkable bioconcentration factor after diclofenac exposure which varied greatly across the concentrations and the BCF ranged from 0.3 up to over 2700 depending on aquaria concentration and organ analysed. However, this is not supported in other studies with Bickley et al. (2017) reporting a stable BCF for diclofenac in fish blood plasma around 20 and Brown et al. (2007) reports a BCF of 7 and 4 for diclofenac and naproxen respectively, in fish blood plasma after laboratory exposure but a BCF of 5 and 56 after exposure to treated municipal wastewater containing diclofenac and naproxen. Fick et al. (2010a) reported a BCF in blood plasma of 2.5-29 for diclofenac and 22-28 for naproxen when fish were exposed to sewage effluent and Cuklev et al. (2011) demonstrated a relatively stable bioconcentration factor (BCF) for diclofenac of 2.5 in liver and 4 in blood plasma regardless of the exposure concentration.

In Paper IV, the determined concentrations of several NSAIDs in the municipal effluent were low but similar to reported concentrations by both UBA (2026) and by Osena et al. (2025), with the latter analysing only Swedish wastewater treatment plants (see Paper IV and Table 1 in this thesis). The sticklebacks in Paper IV were exposed to 100 % municipal effluent. However, the actual exposure concentration for wild fish in the receiving waters will be lower due to dilution and eventually further degradation. EMA uses a dilution factor of 10 when calculating the PEC in the ERA for human pharmaceuticals (EMA, 2024), but the actual dilution is variable depending on the recipient (Link et al., 2017) and as described in section 1.2.5, some streams are effluent-dominated at certain times of year. No measurements of tissue concentration could be performed for the fish in Paper IV since the samples were lost in a freezer.

4.2 Histopathological effects of NSAIDs

In Papers I and II, six renal and five hepatic endpoints were evaluated in H&E and PAS-stained sections (Table 2, Table 3). A total of 122 kidneys

and 73 livers were analysed from fish exposed to diclofenac while the corresponding numbers were 191 kidneys and 172 livers for fish exposed to naproxen. Two types of histopathological effects were significantly affected. In the kidney, renal hematopoietic hyperplasia was observed after both diclofenac and naproxen exposure. In the liver, a decrease in hepatocellular vacuolation was identified, after naproxen exposure (see Papers I and II for details including photomicrographs).

Seven studies evaluating effects of NSAID exposure in fish passed the inclusion criteria in the review in Paper III. Five different endpoints were reported as being affected in the liver and five types of endpoints in the kidney. The inclusion criteria were set as minimum requirements to be able to draw conclusions from reported results. Reporting only a descriptive histological analysis without quantification of data, and thus without the possibility for statistical analysis, makes it difficult to provide convincing evidence or connect cause and effect. Several of the studies in the search reported only histological indices, which is a combined score for several endpoints evaluated in the same organ. This might be a convenient approach when comparing different field stations, particularly when aiming to describe the "total load" experienced by an organism. However, the different endpoints that are added together may be completely unrelated and irrelevant findings might obscure those of importance. According to the inclusion criteria, studies only reporting histological indices were therefore excluded from the review. If histological indices are reported, the scores for the individual endpoints that constitute the index should also be reported separately.

4.2.1 Renal histopathological changes

Compared to the liver, which is a more homogeneous organ and easily accessible in most species, evaluating kidney histology can be more complex. It includes several different types of tissues including an excretory part and a hematopoietic part. There is also a normal glomerular pleomorphism which further complicates the evaluation (Wolf et al., 2015). Additionally, the kidney is located adjacent to the vertebral column, making sampling without damaging the organ difficult, especially if the fish is small.

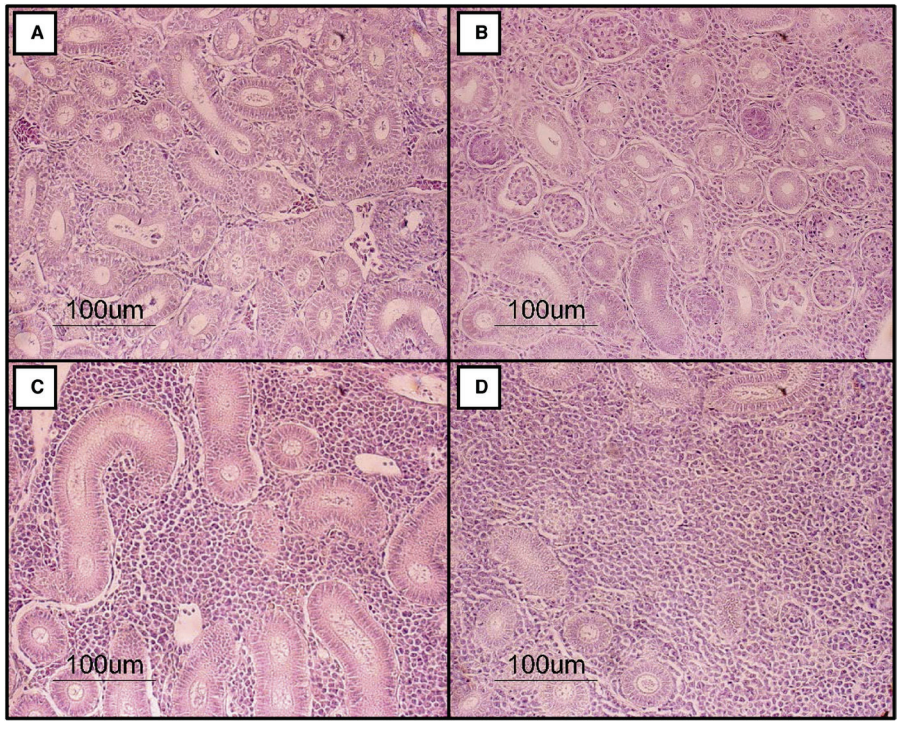

Figure 6. Scoring of renal hematopoietic hyperplasia in stickleback exposed to naproxen (magnification 200x). A) Grade 1, fish exposed to 0 µg/L; B) Grade 2, fish exposed to 18 µg/L; C) Grade 3, fish exposed to 299 µg/L; D) Grade 4, fish exposed to 1232 µg/L. Figure reproduced from Paper II.

Paper I identified a renal hematopoietic hyperplasia at the lowest tested diclofenac concentration (4.6 µg/L, measured concentration) and hence, a NOEC could not be established for that histological endpoint. It followed a clear concentration-response, and the effect was verified independently by a second pathologist who scored the finding very similarly according to interobserver statistics (see Paper I for details about interobserver variability). Paper II showed that naproxen also caused renal hematopoietic hyperplasia, but a higher concentration was required to observe a statistically significant effect (299 µg/L, measured concentration) (Figure 6). It should be noted that the number of analysed individuals was higher in the naproxen study (Paper II) compared to the diclofenac study (Paper I) due to a higher number of fish in each aquarium. This will give the statistical analysis in Paper II a higher power and hence the LOECs reported in Papers I and II are not fully comparable. Despite that, effects were first seen at a naproxen

concentration 65-times higher than for diclofenac. This difference is also in agreement with predictions from the fish plasma model as the H_TPC is considerably higher for naproxen, and the observation that diclofenac bioconcentrates more than naproxen (Papers I and II). In conclusion, this provides evidence that diclofenac is more potent than naproxen in causing hematopoietic hyperplasia.

Traditionally, renal hematopoietic hyperplasia has not been evaluated frequently in fish histopathology studies. But there are at least two studies that have reported diagnoses supportive of this after NSAID exposure. Schwaiger reported "interstitial nephritis" in rainbow trout after exposure to diclofenac. The histological images provided in the article were convincing of an effect, but no apparent inflammation was seen. In Paper I, we suggested that renal hematopoietic hyperplasia would be a more appropriate diagnosis. However, diagnosing inflammation in hematopoietic organs such as spleen and renal interstitium can be challenging due to the variable morphology together with high cellularity, and further, a diagnosis that is not especially well established. Birzle et al. (2023) reported an increase in both the relative and absolute volume of interstitial tissue (in which the hematopoietic tissue represents the major part) together with the corresponding decrease in absolute and relative nephron volume, with the most sensitive endpoint affected at 0.5 µg/L of diclofenac. This was not analysed by semiquantitative scoring but with quantitative image analysis, which has the potential to be more sensitive. However, it is not completely without bias and blinding of slides and randomization when choosing the area to analyse is equally or even more important than in semiquantitative scoring. Further, when the slides from the study by Mehinto et al. (2010) (where rainbow trout was exposed to diclofenac), were re-evaluated by a pathology working group (PWG), they concluded that almost half of the renal sections consisted mainly or exclusively of hematopoietic tissue (Wolf et al., 2014). However, without information on sampling standardisation, it is impossible to determine if hematopoietic hyperplasia was present, or if a more cranial part of the kidney, which mainly consists of hematopoietic tissue, was sampled. None of the other renal endpoints in Papers I or II were significantly affected by the NSAID exposure.

In the literature review (Paper III), kidney histology was analysed in six out of seven Papers studying NSAID exposure, and a few additional diagnoses were reported. Two studies, both receiving a weight of evidence

judged as moderate, reported an increase in developing nephrons (DNs) and basophilic clusters (BCs) (Bickley et al., 2017; Mehinto et al., 2010). Tubular regeneration, in which tubular neogenesis was included, was evaluated semiquantitatively in both Papers I and II without any significant effects identified. Neither was tubular necrosis detected, which might be an anticipated finding if an increase of DNs and BCs are detected. The diagnoses related to hematopoietic hyperplasia in Papers I and II and in Birzle et al. (2023) was scored as having a high weight of evidence, noting the potential bias when judging our own work. The rest of the diagnoses in the review, including tubular necrosis and the proposed decrease of Bowman's space were deemed as having a low weight of evidence and are evaluated more thoroughly in Paper III.

Based on several independent observations, it appears that renal hematopoietic hyperplasia is an effect linked to NSAID exposure. Hematopoietic stimulation due to NSAIDs is reported in mice (Hofer et al., 2012). In fish, hematopoietic hyperplasia can be a response to a localised or systemic inflammation (Wolf et al., 2015). It has also been described in Atlantic salmon (*Salmo salar*) with pyridoxine deficiency (Herman, 1985). The key question is however if hematopoietic hyperplasia is purely a response and an adaptation to NSAID exposure or if it has a negative effect to the organism. One thing noted but not evaluated statistically in Papers I and II is the concurrent reduction of the excretory tissue (the nephrons). In severe cases, barely any tubuli or glomeruli were seen in the whole section and effects on glomerular filtration, urinary excretion and ion balance could be expected. Mammals have a kidney reserve capacity meaning a loss of glomeruli will not affect overall kidney function until the loss drops to a certain level. Whether this overcapacity is present in fish is to the best of my knowledge not known but can be anticipated. In comparison to mammals, fish have the ability of neogenesis of nephrons, which might overcome the loss of nephrons if the stressor (i.e., the NSAID) is removed. Birzle et al. (2023) reported a significant decrease in the relative volume of nephron tissue but also the absolute volume. This confirms the loss of nephron tissue but raises the question of what happens to the disappearing nephrons? No signs of tubular necrosis were detected in any of the studies in this thesis, nor was it reported in the study by Birzle et al. (2023), neither was it convincingly identified in the articles in the review in Paper III. In Papers I and II, the kidney was sectioned longitudinally (parasagittally). As the kidney narrows

caudally, the chosen kidney section (i.e., the section with the largest kidney area) is most likely the section closest to the sagittal plane. It would therefore be possible that if the hematopoietic hyperplasia is mainly present in the centre of the kidney, theoretically, the nephron tissue could be "pushed" to the lateral borders and hence explaining the perceived lack of nephrons. However, as Birzle et al. (2023) section the kidney transversely, this contradicts this theory. In conclusion, we cannot say with certainty if hematopoietic hyperplasia (or its correlated finding decreased amount of nephron tissue) has a negative effect on the fish.

4.2.2 Hepatic histopathological changes

Paper II identified a decreased hepatocellular vacuolation but only at the highest tested naproxen concentration (1232 µg/L, measured concentration) (Figure 7). This endpoint was not significantly affected after diclofenac exposure in Paper I, but as stated previously, fewer fish were analysed in that study and hence, a decreased statistical power cannot be excluded as a potential reason for the apparent difference in observed effects. In Paper II, the fish in the two highest exposure group started to eat slower and even leaving food after about one week of exposure. The reduced food intake may very well explain the reduced hepatocellular vacuolation. In contrast to renal hematopoietic hyperplasia, hepatocellular vacuolation has a long tradition of being reported in various types of investigations and hence, this change is not specific to NSAIDs. However, as stated by Wolf et al. (2015), the reports often claim an increase in hepatocellular vacuolation and occasionally interpretate it as hepatic lipidosis, a diagnosis probably inspired by mammal or human pathology. In fish, this is not fully appropriate without concurrent evidence of adverse histological effects in the liver (Wolf et al., 2015).

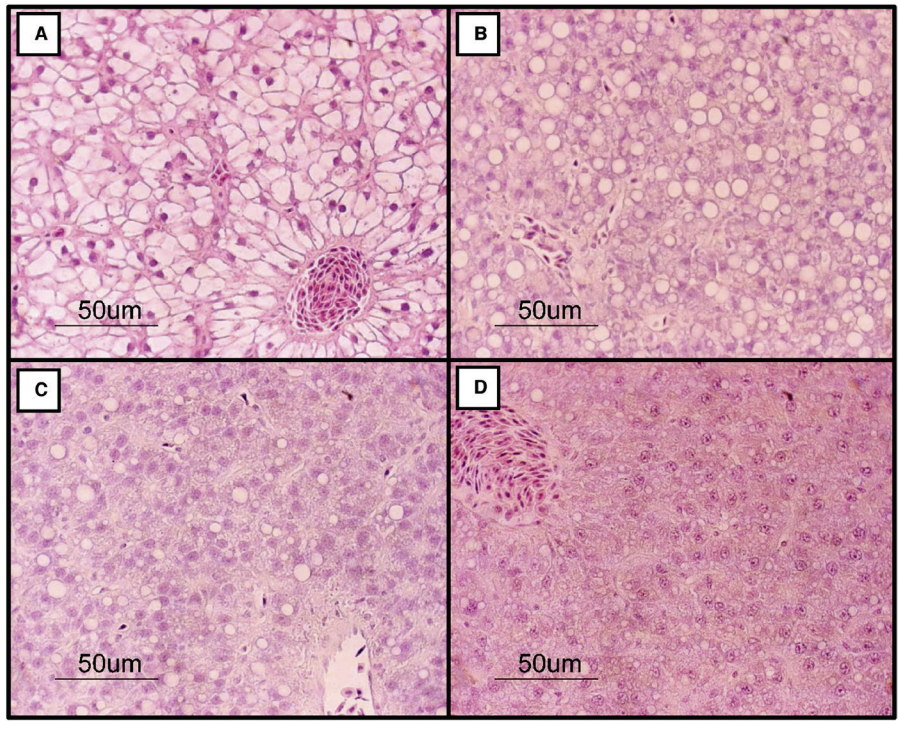

Figure 7. Scoring of hepatocellular vacuolation in stickleback exposed to naproxen (magnification 400x). A) Grade 4, fish exposed to 0 µg/L; B) Grade 3, fish exposed to 299 µg/L; C) Grade 2, fish exposed to 1232 µg/L; D) Grade 1, fish exposed to 1232 µg/L. Figure reproduced from Paper II.

Hepatocellular vacuolation can be of lipid-type or glycogen-type but special stains are needed to verify their origin. PAS together with diastase can confirm glycogen and Sudan Black/Oil Red O stains lipids (Suvarna et al., 2019) However, their appearance in H&E-stained sections can provide an indication of the type present, as demonstrated by the photomicrographs in Paper III. As vacuolation is highly variable depending on age, sex, nutritional and reproductive status, comparing individuals where such factors are reasonably similar is therefore likely crucial.

There is often (but not exclusively) a decrease of vacuolation that points to an unfavourable effect and not the opposite. In the study by Memmert et al. (2013) which exposed zebrafish to diclofenac, a change in hepatocellular vacuolation was not reported in the original study. However, when reevaluated by the PWG (Wolf et al., 2014), a decrease in hepatocellular vacuolation was identified at 1000 µg/L, which corresponds to the results in

Paper II. None of the other hepatic endpoints in Papers I or II were significantly affected by the NSAID exposure.

In the literature review (Paper III), liver histology was analysed in five out of seven papers studying NSAID exposure. Five different hepatic endpoints were identified as affected, where all except one endpoint received a moderate or high weight of evidence for the histological claim. Single-cell necrosis was reported after exposure to acetylsalicylic acid (Baumann et al., 2020) with both adequate quality of histological image and specimen together with a clear concentration-response effect and the weight of evidence was judged as high. However, the LOEC was 75 000 µg/L, which is not likely to occur in the environment. Necrosis was evaluated after both diclofenac and naproxen exposure (Papers I and II), but in close to 250 investigated sections from the same number of sticklebacks, only a single case of necrosis was detected. During the sampling of that specific fish, a nematode was found in the abdominal cavity, therefore the proposed aetiology of the necrosis was previous parasite migration (see Paper III for photomicrographs). No nematodes were found in any of the other fish during sampling. The only identified hepatic endpoint in the review with a LOEC considered possible to occur in the environment, was monocyte infiltration/accumulation (Hoeger et al., 2005). However, no histological images were presented in that paper, no concentration-response was reported and unexpectedly none of the 19 different endpoints evaluated in three different organs reported any findings in any of the control animals and the weight of evidence was judged as low.

4.3 Histopathological effects of treated municipal wastewater

The literature review (Paper III) compared histological effects in fish reported after NSAID exposure with fish exposed to treated municipal effluent. The search strings were wide with the hope of finding as many relevant papers as possible, but this also resulted in papers which were irrelevant for this review. Most of the search hits in the database search were excluded from the review. The total number of search hits together with the final number of articles included in the review should therefore not be interpreted as a large amount of relevant research data were excluded, but instead that the strings were not that restrictive. Compared to the NSAID

articles in the review, the number of fish analysed histologically in each wastewater study was not clear in 3 out of 7 papers. This data must be considered as basic information that should be included in every animal study to facilitate external quality evaluation.

None of the studies in the review on treated wastewater exposure reported a blinded histopathological evaluation, which as previously described, has the potential to induce observational bias.

To further evaluate if histological effects from NSAIDs could be identified in the aquatic environment, a study with a matched design to the experiments in Papers I and II was performed (Paper IV). Further, endpoints identified in the review (Paper III) were also evaluated and additional histological measurements and calculations were performed to increase the objectivity and possibly the sensitivity of the endpoints.

4.3.1 Renal histopathological changes

Only two papers in the review (Paper III) identified significant changes in the kidney after exposure to treated municipal wastewater, one study found no significant differences and the four remaining studies did not evaluate the kidney. An increase in the number of DNs and BCs and an increased size of renal tubuli was reported (Liney et al., 2006; Tetreault et al., 2012), both receiving a moderate weight of evidence in the review. However, there were some questions raised regarding the methodology in both claims. One example is the use of the "Field of View" (FOV) as the area in which an entity is counted, without defining the FOV. Is it the field of view seen in the microscope at a certain magnification or is it the whole section in which the organ is visible? The first definition will produce the relative occurrence of the entity and requires a randomized but still standardized sampling to avoid biases. The second definition will give the absolute (total) occurrence of the entity which is completely dependent on the section area of the kidney.

In the matched study (Paper IV), seven different renal histological endpoints were scored semiquantitatively (Table 2) but none were statistically significant compared to the controls. This included hematopoietic hyperplasia. Noting that we could not establish a NOEC for diclofenac regarding this endpoint in Paper I, the actual wastewater concentration was around 15–20 times lower compared to the LOEC. Eight different endpoints were measured or calculated, and two additional endpoints were derived from the measurements (Table 2). The statistical

analysis for renal thickness revealed a significant interaction between treatment and sex of the fish, suggesting that the treatment affects males and females differently and data must therefore be analysed separately for males and females. With the variability of sex reduced, statistical analysis showed that males exposed to treated municipal wastewater had a larger average kidney thickness compared to control fish. This was not shown in females. It was suspected that the increase could be related to fish size, given that the exposed male sticklebacks were almost significantly longer ($p=0.053$). However, the correlation between fish length and average renal thickness was not statistically significant. Birzle et al. (2023) evaluated absolute kidney volume, an endpoint directly related to renal thickness, after diclofenac exposure. Although effects on this endpoint were not statistically significant from controls, there was a strong tendency of an increased volume at 25 µg/L or higher. However, this is approximately 100 times higher than reported diclofenac levels in treated wastewater. Based on the increased renal thickness, we reevaluated sections from Paper I where sticklebacks were exposed to diclofenac. Although a tendency of increased thickness was seen with increasing diclofenac concentration, it was not statistically significant (see Paper IV for details). Since the effect was only seen in male fish, our second hypothesis was a stimulation of the androgen receptor. The gestagen levonorgestrel (LNG) used in hormonal contraceptives, have a documented androgenic effect in fish including sticklebacks (Runnalls et al., 2015; Svensson et al., 2013; Zeilinger et al., 2009). Concentrations of levonorgestrel sufficient to affect reproduction or secondary sex characters are reported in treated wastewaters (Fick et al., 2010a; Zeilinger et al., 2009). The epithelial cells in the tubuli hypertrophies after a response to androgens. As exemplified in Figure 3 in Paper III, the hypertrophy is substantial and an increase of total kidney size might be anticipated. However, we measured tubular diameter and calculated kidney epithelial height (KEH), but no significant effects were seen. The cause for increased renal thickness was not determined. No other renal endpoint was statistically significant between exposed fish and control. Notably, if comparing males to females, regardless of the treatment, there was a statistically significant change in many of the investigated renal endpoints. This speaks in favour of including sex of the fish in the statistical analysis but also, if only animals from a single sex are used, there is the possibility of effects being overlooked. However, since the

kidney is a reproductive organ in the stickleback, differences are perhaps not as evident in other species.

4.3.2 Hepatic histopathological changes

Liver histology was evaluated in five out of seven papers in the review of treated municipal effluent (Paper III), and nine different endpoints were reported. Necrosis was reported from two studies, but this finding could not be confirmed in the included photomicrographs. Both an increase and a decrease in hepatocellular vacuolation were reported. The reason for this is unclear. It might be explained by the experimental design in those studies where an increase in hepatocellular vacuolation was seen in wild-caught fish downstream from a WWTP compared to upstream (Minarik et al., 2014). The suspected nutrient-rich environment downstream could increase food availability and hence increase the hepatocellular vacuolation. The study which reported a decrease in hepatocellular vacuolation placed farmed fish in cages downstream from a WWTP and in a reference river (Giesy et al., 2003). In this scenario, the environment for the fish downstream from the WWTP could have the potential to stress the farmed fish more compared to a reference river with cleaner water. Beghin et al. (2022) reported a decrease in glycogen deposits and although no histological images demonstrating this finding were present in the article, they used quantitative image analysis on the binary image of PAS-stained sections, which likely produces reliable results. However, although statistically significant, no measures of variability were presented (neither the full data for the endpoint). All hepatic endpoints except glycogen deposits received low weight of evidence.

In the matched study (Paper IV), nine different hepatic endpoints were scored semiquantitatively (Table 3). An increase of inflammatory cell foci and pigmented macrophage aggregates (PMA) was identified in the liver from sticklebacks exposed to treated municipal effluent, but only in males. Both these endpoints were evaluated in sticklebacks exposed to diclofenac and naproxen (Papers I and II) but without significant effects. According to Ferguson (2006), an increase in PMA is reported after chronic stress, such as exposure to different pollutants. As the number of substances in a municipal effluent is substantial and not only contains pharmaceuticals, the increase of PMA not surprising. However, Beghin et al. (2022) claims a decrease in PMA after exposure to municipal effluent but as pointed out in Paper III, the evidence was not conclusive. It is unclear what caused the inflammation in

the liver. However, it was focal to multifocal and only graded as minimal to mild. As reported for the renal endpoints, an additional finding from the study in Paper IV was the apparent sex difference regarding several of the histological endpoints. This further highlighted the need to include sex as a factor in statistical analysis.

4.4 Overlapping histopathological effects

Few studies fulfilled the rather basic inclusion criteria in the review (Paper III) with only seven studies included for each exposure type. The comparison of affected endpoints between fish exposed to either NSAIDs or wastewater became difficult due to the diversity of species studied, different sizes/maturity of fish and differences in experimental designs, but primarily due to different endpoints evaluated. Additionally, the included studies had limitations, especially regarding the methodology and presentation of the histological evaluations. Three histopathological endpoints were identified as potentially overlapping between fish exposed to treated municipal wastewater and fish exposed to NSAIDs. These were hepatocellular necrosis, hepatocellular vacuolation, and DNs/BCs. None of the wastewater studies provided conclusive support for hepatocellular necrosis due to insufficient quality of the histological images or images not supporting the claim (Beghin et al., 2022; Pinto et al., 2010) and were given a low weight of evidence. However, the NSAID study demonstrating necrosis received a high weight of evidence but reported a LOEC for ASA of 75 000 µg/L (Baumann et al., 2020). Given that the median level detected in treated municipal effluent, is 0.15 µg/L (UBA, 2026), hepatocellular necrosis due to ASA is unlikely to be observed in the environment. Necrosis was not detected in the matched wastewater study with stickleback (Paper IV), thus the support for NSAIDs in treated municipal wastewater causing hepatocellular necrosis is low.

A decrease in hepatocellular vacuolation was reported in Giesy et al. (2003) after exposure to treated wastewater. However, an apparent correlation between weight and vacuolation score raises questions about differences in fish size between sampling sites, which can itself affect hepatic vacuolation. The included histological image was not of adequate quality for external evaluation. Beghin et al. (2022) claimed to have scored vacuolation, but no data are presented. They investigated glycogen deposits in an adequate way, but no histological images are included to support their

claims. It should be added that two studies in the review claims the opposite, an increase in hepatocellular vacuolation or a related diagnosis ((Minarik et al., 2014; Scott et al., 2018). No statistically significant differences in vacuolation were detected in the matched stickleback study with wastewater (Paper IV). We reported a decrease in hepatocellular vacuolation after naproxen exposure, but only at the highest tested concentration (1232 µg/L, measured concentration) (Paper II). Memmert et al. (2013) did not report any histological changes in the liver after diclofenac exposure and were therefore not included in the review. A re-evaluation of the slides by the PWG described earlier, detected a decreased vacuolation with a LOEC of 1000 µg/L diclofenac (Wolf et al., 2014). In conclusion, there is some evidence for treated municipal effluent causing a decrease in hepatocellular vacuolation. The evidence of NSAID causing this is clearer but effects only occur at or around 1 mg/L which is not likely to be encountered in the environment, perhaps except downstream from discharge points from production sites for NSAIDs.

The only overlapping endpoint in the kidney after NSAID or treated wastewater exposure was an increase in DNs and BCs (Bickley et al., 2017; Liney et al., 2006; Mehinto et al., 2010). The overall quality of evidence for this histological claim was moderate for all studies, and the finding was reported at considerably lower NSAID concentrations compared to both necrosis and the decrease in hepatocellular vacuolation. However, the reliability of the studies varied, with remaining uncertainties with regards to the standardization of analyses. Additionally, if the increase of DNs and BCs is a consequence of renal damage, one would expect some evidence of degeneration or necrosis of tubular cells, none of which were found or verified. We evaluated tubular regeneration semiquantitatively in all experimental studies, and no statistically significant results were found after diclofenac, naproxen or wastewater exposure (Papers I, II and IV). In the matched wastewater study (Paper IV), we also included the calculation of DN/BC density (i.e. number of DNs/BCs per mm^2) but found no significant differences compared to controls. This endpoint is not completely dismissed but the evidence is inconclusive.

As mentioned earlier, hematopoietic hyperplasia is not frequently reported. None of the wastewater studies in the review reported to have evaluated this endpoint. Three studies in the NSAID review reported this or corresponding diagnoses, where two originated from the author of this thesis.

All papers reported significant increases in the amount of interstitial tissue or decreases of nephron tissue. The lowest significant concentration was 0.5 µg/L diclofenac (Birzle et al., 2023). In Paper I, significant effects on renal hematopoietic hyperplasia were seen at the lowest tested concentration (4.6 µg/L diclofenac) and in Paper II at 299 µg/L naproxen. In the matched study with wastewater (Paper IV), renal hematopoietic hyperplasia was not significantly affected between treatment groups. However, the average renal thickness was increased in fish exposed to treated municipal effluent. This led us to reanalyse sections from male sticklebacks in Paper I by measuring renal thickness. Even though a trend was seen, this was not statistically significant and a correlation between renal thickness and renal hematopoietic hyperplasia could not be confirmed (Paper IV).

Although an almost identical design and methodology were adopted in Paper IV, the histological effects detected did not mimic those identified in NSAID-exposed fish. The overall conclusion was that no clear histopathological effects connected to NSAID exposure are seen after exposure to treated municipal effluent. This is, however, not the same as claiming no effects of NSAIDs in the environment as effects in other organs, in other species or at sites with higher exposure concentrations cannot be excluded.

4.5 Apical effects after NSAID exposure

4.5.1 Mortality

In both studies I and II, mortalities in the highest tested concentrations (271 µg/L and 1232 µg/L, measured concentration of diclofenac and naproxen, respectively) warranted euthanasia and sampling of fish one week earlier for those aquaria. This was not anticipated and the reason for the mortality was unclear. It should be noted that the study from Memmert et al. (2013), used the exact same nominal concentration (320 µg/L) among others, when exposing zebrafish to diclofenac, and their NOEC for survival was 320 µg/L (nominal concentration, 336 µg/L measured concentration). That study was performed on eggs from laboratory-bred zebrafish, while the sticklebacks were wild-caught and exposed when around 10 months old, although that does not in itself explain the differences. Joachim et al. (2021) performed an extensive mesocosm study with sticklebacks which to date is

the driver for the EQS for diclofenac. However, they experienced high mortalities overall, especially in other species in the mesocosm. The LOEC regarding mortality of sticklebacks were 3.82 µg/L. Schwarz et al. (2017) reported significant mortalities at 100 µg/L of diclofenac in brown trout (*Salmo trutta*) after 25 days of exposure. In the study in Paper IV, one fish died in a control aquarium on the first day of the study. The dead fish was found a few hours after transfer to the aquaria and was therefore most likely related to handling trauma. No further mortalities occurred during that study.

4.5.2 Jaw Lesions

The fish in the highest exposure groups of both diclofenac and naproxen were observed to have lower jaw lesions (see Figure 3 in Paper II), and the prevalence showed a concentration-response (Papers I and II). Mandibular effects have also been identified by Yokota et al. (2018) in Japanese medaka (*Oryzias latipes*) exposed to diclofenac and the proposed aetiology was disruption of osteoclast function. Stancova et al. (2014) have also reported lesions in the lower jaw in tench (*Tinca tinca*). However, they exposed the fish to a mixture of pharmaceuticals, including but not limited to NSAIDs, therefore a complete causality could not be determined. The LOEC for jaw lesions in the diclofenac and naproxen study were 271 and 299 µg/L, respectively. No jaw lesions were detected in the study in Paper IV. The appearance of jaw lesions might be one explanation behind the reduced food intake (and subsequent decreased hepatocellular vacuolation) observed in the highest exposure groups.

4.5.3 Growth

Both diclofenac and naproxen caused significant reductions in the fish condition factor but only at the highest tested concentration in the respective studies (271 µg/L and 1232 µg/L, measured concentrations of diclofenac and naproxen, respectively). However, since there was a significant increase in jaw lesions at the highest concentrations in those studies, this is not surprising since mandibular lesions is likely to affected food intake. The fish in those aquaria appeared to eat slower and even leave food after around one to one and a half weeks of exposure. No effects on condition factor were seen in the study in Paper IV.

4.6 Hepatic Gene Expression

Complement component 7 (*c7*), a gene coding for proteins involved in the innate immune system, showed a consistent response to NSAID exposure in experimental studies I and II as well as in Cuklev et al. (2011). In studies I and II, a concentration-response relationship was observed, with similar expression fold changes for both NSAIDs, although effects occurred at lower concentrations for diclofenac, adding evidence for diclofenac being the more potent NSAID. No statistically significant effects were detected for the genes targeted by the primers in Mehinto et al. (2010) (i.e. *cox1* and *cox2*, encoding cytochrome c oxidase and not cyclooxygenase). In the naproxen study, *cyp1a*, *gr* and *sod-1*, genes coding for proteins involved in detoxification and oxidative stress were significantly affected at the highest or second highest concentration. No gene expression analyses were performed in Paper IV but given the LOEC for *c7* for both diclofenac and naproxen, a detectable effect would not be expected.

Other studies have also investigated hepatic gene expression. One study that stands out is Hong et al. (2007), which reported an increased expression of *vtg*, *cyp1a* and *p53* in Japanese medaka exposed to diclofenac at concentrations as low as 1 µg/L. However, this conclusion was based on a single sample consisting of three pooled fish per treatment, providing very limited data. Bickley et al. (2017) investigated gene expression in the anterior kidney of fathead minnow (*Pimephales promelas*) exposed diclofenac and reported effects on genes involved in immune function and inflammation.

Bereketoglu et al. (2020) exposed zebrafish larvae to eight different NSAIDs, all at 100 µg/L, and analysed hepatic expression for several genes including those encoding for cyclooxygenases and vitellogenin. Although six of the genes showed significant results for only one to three of the substances, seven of the genes were consistently downregulated by all NSAIDs at the same exposure concentration. Relative expression was approximately 0.5 for all genes, indicating a strong and similar effect size across all tested substances, which is surprising. The authors also reported altered sex ratios after NSAID exposure, with a higher proportion of males. In Paper II, hepatic *vtg* expression was analysed, but no significant effects were detected at any concentration. In Bereketoglu et al. (2020), exposure concentrations were not analytically confirmed and some ambiguities regarding replication were noted. Although the reported effects are clear,

replication of the experiment, preferably in an additional lab, would be valuable for confirmation.

5. Conclusions and implications

5.1 Main conclusions

This thesis has conclusively identified that renal hematopoietic hyperplasia occurs in three-spined stickleback after waterborne exposure to the NSAIDs diclofenac and naproxen. The NSAID exposure also caused jaw lesions, a reduced condition factor and mortality in a concentration-response manner. Diclofenac exposure generally caused effects at lower concentrations compared to naproxen. The most sensitive endpoint was renal hematopoietic hyperplasia, but effects were only evaluated down to 4.6 µg/L, a concentration around 15–20 times higher than those typically measured in treated municipal effluents.

Further, after a standardised review, this thesis identified a few potential overlaps regarding histological effects in the kidney and liver of fish after exposure to NSAIDs or treated municipal wastewater. Large differences in design and methodology together with some ambiguities made comparisons of studies challenging. Both reliability and relevance were questioned in several of the studies. Taken together, the evidence for NSAID-induced histological effects in fish exposed to municipal effluent is low.

To facilitate comparisons, a study with treated municipal wastewater exposure was matched to previous studies with stickleback exposed to NSAIDs. Previously identified histological effects after NSAID exposure were not confirmed in wastewater-exposed fish, nor were the apical endpoints including jaw lesions, decreased condition factor and mortality.

In conclusion, different NSAIDs induce histological effects in fish, but there is yet only strong evidence for such effects at concentrations more rarely encountered in the environment. Additionally, such effects have not been observed in the environment. Therefore, there is currently no clear evidence of NSAIDs causing histological effects in fish in the wild and discussion should be limited to *risks* for effects. It should be added that only the kidney and liver were evaluated in this thesis and conclusions are therefore restricted to these organs.

This thesis also highlights the importance of well-designed studies with transparency in methodology and data presentation and a stringent interpretation of data. Failing to do so can lead to waste of resources, unnecessary use of research animals, wrong and ineffective efforts and

measures, or even lack of relevant measures. This will also lead to decreased trust in research and science, an issue that is more important than ever.

5.2 Implications for risk management

This thesis has demonstrated that NSAIDs have different potencies in causing effects upon exposure. Diclofenac was proven to affect fish at lower concentration compared to naproxen. If appropriate from a clinical point of view, substituting diclofenac with naproxen will lead to a reduction in environmental concentrations of diclofenac. That in turn will reduce the risk to aquatic organisms.

Ibuprofen, another possible alternative to diclofenac, has also been under evaluation for setting an EQS in the WFD with a proposed annual average quality standard (AA-QS) for surface water of 0.14 µg/L (SCHEER, 2022b). This is not so different from the proposed AA-QS for diclofenac of 0.04 µg/L (SCHEER, 2022a). However, based on available data, risks are considerably lower for ibuprofen as well. The lowest concentration of ibuprofen demonstrated to have an effect in a study used for deriving the proposed EQS was 265.4 µg/L (Constantine et al., 2020). In contrast, the lowest comparable concentration of diclofenac was 3.82 µg/L, a difference of almost 70 times indicating that diclofenac is considerably more toxic to aquatic biota. The still rather similar EQS are mainly a consequence of different assessment factors (safety factors). In addition, concentrations of ibuprofen in treated wastewaters in Sweden are typically about 20 times lower than those of diclofenac, despite more than twice as high sales of ibuprofen based on DDD (Table 5). Hence, risk quotients based on lowest observed effect levels and exposure concentrations in treated municipal wastewater are approximately 1400 [1] times higher for diclofenac – despite lower use. This supports that substitution of diclofenac with ibuprofen also reduces overall environmental risks.

[1] Calculated by (265.4/0.014)/(3.82/0.279)

Table 5. Comparisons of average yearly sales with wastewater median concentrations and removal rates in Sweden for diclofenac and ibuprofen. Abbreviations: DCF=diclofenac; IBU=ibuprofen; M=Millions

	Yearly sales[1] (2004-2021) (kg/year)	**DDD[2] (g)**	**DDD[3] sold (per year)**	**Wastewater Osena[4] (2004-2021)**		
				Untreated (µg/L)	Treated (µg/L)	Removal
DCF	4200	0.1	42 M	0.333	0.279	16 %
IBU	106200	1.2	88.5 M	2.621	0.014	99 %

[1]E-hälsomyndigheten (2026)

[2]WHO Collaborating Centre for Drug Statistics Methodology (2026)

[3]The value was calculated as DDD divided by yearly sales

[4]Osena et al. (2025)

Improving wastewater treatment will also reduce concentrations in effluents and subsequently the risk of negative effects from NSAIDs in the environment. It is unclear if NSAIDs cause harm to aquatic organisms downstream from WWTPs. Therefore, in relation to NSAIDs specifically, the benefit of upgrading all large European WWTPs with advanced pharmaceutical treatment as stipulated under the revised Urban Wastewater Treatment Directive (UWWTD) (European Commission, 2024) is about decreasing risks, not about reducing known effects. Having said that, advanced treatment will reduce risks with many trace pollutants, known and unknown. On a global scale, poorly treated wastewater or wastewater not treated at all probably poses a greater risk to both aquatic and other species, including humans. Hence, installing basic treatment should always be the top priority.

In risk assessment, one needs to account for differences between species in terms of sensitivity. Also, a certain margin is needed. The proposed EQS for diclofenac in surface water is still awaiting approval, but based on the data from this thesis, an AA-QS of 0.04 µg/L will most likely be protective for aquatic species. In Sweden, based on the data from Osena et al. (2025) and including a dilution factor in receiving surface waters, there is probably a low risk of exceeding the AA-QS. However, as reported by Svenskt Vatten (2020), rivers and streams with higher concentrations do occur and might warrant further investigations.

5.3 Implications for future research

Whether NSAIDs have effects in the aquatic environment is still unknown, hence more independent work on effect concentrations as well as observational field studies is warranted. Few studies have investigated effects of diclofenac or other NSAIDs on fish in a comprehensive way. Based on this thesis I conclude that renal hematopoietic hyperplasia should be included as a relevant and potentially sensitive endpoint, both in studies with fish exposed to NSAIDs and in fish exposed to wastewaters. As it seems to be a relatively specific endpoint, it could contribute to linking cause and effect in complex exposure situations. Additional organs would be valuable to study, not least the gills, as they are in constant contact with the water. In humans, gastrointestinal side effects are a well-known consequence of NSAID use (Cryer & Kimmey, 1998), indicating that the gastrointestinal tract may represent a potential target organ in fish as well. As demonstrated by the vulture population crash, future studies should also address differences in sensitivity between species.

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Popular science summary

Medicines are invaluable for preventing, alleviating and curing many diseases. After use, they often reach the environment in different ways and some of them may also cause unwanted effects. Typically, a portion of the drug is metabolised in the body while the rest is excreted unchanged in urine or faeces, eventually reaching wastewater treatment plants. However, most treatment plants are not designed to remove pharmaceuticals, and many substances therefore end up in lakes and rivers.

Pharmaceuticals are designed to affect biological processes in the human body. These biological systems are also present in other species, particularly other vertebrates such as fish. Because many pharmaceuticals are effective at very low concentrations, sometimes even small emissions may cause biological effects, although the concentrations required to affect organisms in the environment are often unclear. Emissions from pharmaceutical manufacturing can sometimes lead to very high environmental concentrations. In addition, there are special risks linked to discharges of antibiotics and the development of antibiotic resistance, which in the long term threatens our ability to prevent and treat various bacterial infections.

Studies have shown that several pharmaceuticals such as NSAIDs and steroid hormones can cause effects in the environment. NSAIDs are a group of medicines including diclofenac, naproxen and ibuprofen, and are used for treating pain, fever and inflammation. Several vulture species in India and Pakistan came close to extinction after feeding on the carcasses of cattle that had been treated with diclofenac. The drug damaged their kidneys and rapidly caused renal failure and death.

Laboratory studies in fish exposed to low concentrations of diclofenac have also reported changes in different organs, including the kidneys. This led to diclofenac being placed on an EU watch list, to monitor its occurrence and concentrations in European waters. Because of ambiguities in earlier studies reporting effects of diclofenac, we chose to investigate how and at what concentrations NSAIDs affect fish. We exposed three-spined sticklebacks in aquaria containing either diclofenac or naproxen. After one month of exposure, we observed clear changes in the kidney when examined under the microscope. The effects were seen at lower concentrations of diclofenac compared to naproxen, although the changes were mainly

observed at concentrations higher than those usually measured in the aquatic environment.

We also performed a literature review to evaluate which organ changes have been reported when fish are exposed to either different NSAIDs or to treated municipal wastewater, which often contains several NSAIDs. The review showed no clear evidence of organ changes common to both types of exposure, at least not at concentrations normally found in the aquatic environment. However, comparing results between different studies can be difficult when the experimental design differs. Therefore, we replicated our previous laboratory studies with sticklebacks and NSAIDs but instead exposed the fish in aquaria receiving treated municipal wastewater. We then investigated whether similar effects occurred. As in the literature review, we concluded that the organ changes observed after exposure to diclofenac or naproxen could not be verified when fish were exposed to treated wastewater. This was probably because the concentrations of NSAIDs in the wastewater were too low.

In conclusion, there is currently no strong evidence that NSAIDs cause effects in wild fish. However, since this thesis only investigated changes in kidney and liver, effects in other organs or in other species cannot be excluded. Diclofenac caused effects at lower concentrations than naproxen. Another NSAID, ibuprofen, is efficiently removed during wastewater treatment, and studies show that much higher concentrations are required to cause effects in the environment compared with diclofenac. One way to reduce the risk of environmental effects is therefore to choose naproxen or ibuprofen instead of diclofenac when possible and medically appropriate.

Populärvetenskaplig sammanfattning

Läkemedel är oumbärliga för att förhindra, lindra och bota många sjukdomar. När vi använder läkemedel kan de dock komma ut i miljön på olika sätt och ge oönskade effekter. Vanligen bryts en del av läkemedlet ner i kroppen, medan resten utsöndras med urin eller avföring, och når därefter reningsverken. Dagens reningsverk är inte byggda för att rena bort läkemedel och därför hamnar många ämnen i våra sjöar och vattendrag.

Läkemedel är utvecklade för att påverka biologiska processer i kroppen. Dessa biologiska system kan även finnas hos andra arter, särskilt ryggradsdjur såsom fiskar. Eftersom många läkemedel är effektiva vid mycket låga koncentrationer kan även små utsläpp vara tillräckliga för att orsaka biologiska effekter, även om det ofta är oklart vilka koncentrationer som krävs för att ge effekter i miljön. Utsläpp från tillverkningen av läkemedel kan ibland resultera i mycket höga koncentrationer i miljön. När det gäller utsläpp av antibiotika finns det speciella risker kopplade till utveckling av antibiotikaresistens, vilket i förlängningen äventyrar våra möjligheter att förebygga och behandla olika bakteriella infektioner.

Flera läkemedel, såsom NSAID och hormonella preparat, har visat sig ha miljöeffekter. NSAID är en grupp av läkemedel mot smärta, feber och inflammation där bland annat diklofenak, naproxen och ibuprofen ingår. I Indien och Pakistan blev flera arter av gamar nästintill utrotade när de åt av kadaver från nötkreatur som behandlats med diklofenak. Läkemedlet var skadligt för deras njurar och orsakade snabbt njursvikt och död.

I laboratoriestudier med fiskar som fått simma i vatten med diklofenak har man också rapporterat förändringar i olika organ, inklusive njuren, redan vid låga koncentrationer. Detta ledde till att diklofenak placerades på en övervakningslista inom EU för att undersöka dess förekomst och koncentrationer i europeiska vattendrag. Eftersom resultaten från tidigare studier varit oklara valde vi att undersöka hur och vid vilka koncentrationer NSAID påverkar fisk. Vi exponerade storspigg i akvarier som innehöll antingen diklofenak eller naproxen. Efter en månads exponering kunde tydliga förändringar i njurarna ses vid mikroskopisk undersökning. Effekterna sågs vid lägre koncentrationer av diklofenak än av naproxen. Förändringarna sågs dock främst vid koncentrationer som är högre än som vanligen uppmäts i vattenmiljön.

Vi genomförde även en litteraturstudie för att undersöka vilka organförändringar som rapporterats när fiskar exponeras antingen för olika NSAID eller för renat avloppsvatten, som ofta innehåller flera NSAID. Översikten visade inga tydliga belägg för organförändringar som är gemensamma för båda typerna av exponering, åtminstone inte vid koncentrationer som normalt förekommer i miljön. Att jämföra resultat mellan olika studier kan dock vara svårt när försöksuppläggen skiljer sig åt. Vi valde därför att upprepa upplägget för våra tidigare studier med spigg och NSAID, men exponerade i stället fiskarna för renat kommunalt avloppsvatten. Därefter undersökte vi om liknande effekter uppstod. I likhet med litteraturstudien var slutsatsen att de organförändringar som uppstått efter exponering för diklofenak eller naproxen inte kunde verifieras när spiggar exponerades för renat kommunalt avloppsvatten. Detta berodde antagligen på att koncentrationerna av NSAID i avloppsvattnet var för låga.

Sammanfattningsvis finns det i dagsläget inga starka belägg för att NSAID orsakar effekter hos vilda fiskar. Eftersom vi bara undersökt njure och lever kan man inte utesluta effekter i andra organ eller i andra arter. Effekterna av diklofenak sågs vid lägre koncentrationer än för naproxen. Ibuprofen, ett annat NSAID, avlägsnas effektivt i reningsverken och enligt litteraturen krävs det dessutom betydligt högre koncentrationer av ibuprofen än för diklofenak för att orsaka effekter i miljön. Ett sätt att minska risken för miljöeffekter är därför att välja naproxen eller ibuprofen i stället för diklofenak när det är möjligt och medicinskt lämpligt.

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Diclofenac affects kidney histology in the three-spined stickleback (*Gasterosteus aculeatus*) at low µg/L concentrations

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ABSTRACT

Diclofenac, a commonly used non-steroidal anti-inflammatory drug, is considered for regulation under the European water framework directive. This is because effects on fish have been reported at concentrations around those regularly found in treated sewage effluents (~1 µg/L). However, a recent publication reports no effects on fish at 320 µg/L. In this study, three-spined sticklebacks (*Gasterosteus aculeatus*) were exposed to 0, 4.6, 22, 82 and 271 µg/L diclofenac in flow-through systems for 28 days using triplicate aquaria per concentration. At the highest concentration, significant mortalities were observed already after 21 days (no mortalities found up to 22 µg/L). Histological analysis revealed a significant increase in the proportion of renal hematopoietic tissue (renal hematopoietic hyperplasia) after 28 days at the lowest concentration and at all higher concentrations, following a clear dose-response pattern. Skin ulcerations of the jaw were noted by macroscopic observations, primarily at the two highest concentrations. No histological changes were observed in the liver. There was an increase in the relative hepatic mRNA levels of *c7* (complement component 7), a gene involved in the innate immune system, at 22 µg/L and at all higher concentrations, again following a clear dose-response. The bioconcentration factor was stable across concentrations, but lower than reported for rainbow trout, suggesting lower internal exposure to the drug in the stickleback. In conclusion, this study demonstrates that diclofenac causes histological changes in the three-spined stickleback at low µg/L concentrations, which cause concern for fish populations exposed to treated sewage effluents.

1. Introduction

Pharmaceuticals are used for curing, treating, diagnosing or preventing disease in both human and animals. In the last decades, the awareness of the risk pharmaceutical residues in the environment may pose has increased. Since pharmaceuticals are designed to affect biological systems, they have the potential to affect non-target organisms when present in the environment (Gunnarsson et al., 2008). They are often stable molecules which are biologically active at low concentrations. Moreover, sewage treatment plants are normally not developed to remove these substances. There is an increasing concern about their environmental impact and various drugs have received much attention for their presence and anticipated effects in the environment (Schwaiger et al., 2004; Kidd et al., 2007; Brodin et al., 2013). The most striking example is diclofenac which is responsible for the dramatic decline and almost extinction of several vulture populations in Pakistan over the past years. The birds were exposed by eating from the carcasses of previously diclofenac-treated livestock, which resulted in kidney failure and death (Oaks et al., 2004; Prakash et al., 2012).

Diclofenac is a common non-steroidal anti-inflammatory drug (NSAID) widely used in both human and veterinary medicine to reduce inflammation and pain. The common mode of action of all NSAIDs is the inhibition of cyclooxygenase (COX) enzymes which results in reduced prostanoid synthesis. There are many different biochemical processes that are affected by prostanoids, not only those associated with inflammation but also physiological functions such as gastric mucus secretion and hemodynamics. This is the reason why NSAIDs can give rise to adverse effects on e.g. the gastrointestinal tract and the kidney, where the latter is partially due to reduced renal blood flow which causes ischemia and subsequent necrosis.

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There is also a concern that diclofenac affects fish; In Europe, diclofenac concentrations of around 1 µg/L are frequently found in treated sewage effluents (Brown et al., 2007; Fick et al., 2010; UBA, 2017, Meyer et al., 2016). Several authors have reported that diclofenac can bioconcentrate in fish, even though reported bioconcentration factors differ greatly between the studies (Schwaiger et al., 2004; Brown et al., 2007; Fick et al., 2010; Cuklev et al., 2011). For instance, Schwaiger et al. (2004) reports a highly concentration-dependent bioconcentration factor of 12–2732 between liver in rainbow trout and water, while Cuklev et al. (2011) reports a stable bioconcentration of 2.5 in the same organ of the same species. In addition, cyclooxygenases are conserved between humans and fish (Gunnarsson et al., 2008), providing an opportunity for high-affinity interaction with the intended targets of NSAIDs. Accordingly, Cuklev et al. (2011) identified effects on mRNA expression for genes involved in the immune system in rainbow trout exposed to diclofenac. Several laboratory studies in salmonid fish exposed to low µg/L concentrations of diclofenac have reported a range of histological and cytological changes in gill, liver, kidney and intestine (Schwaiger et al., 2004; Triebskorn et al., 2004; Hoeger et al., 2005; Mehinto et al., 2010). Mehinto et al. (2010) also reports changes in the expression of several genes including cyclooxygenase. This has led to a widespread concern for detrimental effects of diclofenac in European waterways. As a result, diclofenac has recently been included in the watch list of priority substances within the European Water Framework Directive, as one of the first pharmaceuticals (EU, 2013). If Environmental Quality Standards for diclofenac come into place, then major investments may be required in wastewater infrastructure. A more recent laboratory study conducted by Novartis, the company that developed diclofenac, is, however, strongly contradictory to the studies previously mentioned. The study included both rainbow trout and zebrafish exposed to diclofenac, and has histology as one major endpoint. The authors propose a NOEC at 320 µg/L which is about 100 times higher than the highest concentrations found in European aquatic environments (Memmert et al., 2013). Therefore additional, independent data, including effects on other fish species, would be valuable to make informed decisions.

A suitable fish model in this context is the three-spined stickleback (*Gasterosteus aculeatus*), a commonly used fish in ecotoxicological research due to its small size and adaptability to laboratory conditions. The stickleback kidney plays an important role in reproduction, as the male kidney produces a glue ("spiggin") used for building a nest where the female lays her eggs (Jakobsson et al., 1999). Changes in kidney function could thus very well lead to reproductive disturbances in this species. An additional advantage in using the stickleback is its completely sequenced genome which facilitates gene expression analysis.

The principal aim of this study was to investigate if diclofenac affects kidney and liver histology in the three-spined stickleback at µg/L concentrations. We also aimed to assess the bioconcentration of diclofenac and study if diclofenac alters the hepatic mRNA levels of several genes in this species.

2. Material and methods

2.1. Animals

Juvenile (~4–5 months old) three-spined sticklebacks with no external macroscopic signs of disease were caught from wild populations by ring nets in Öresund on the Swedish southwest coast in October 2012. They were transported in well aerated tubs to the aquatic facility at the department of Biomedicine and Veterinary Public Health, SLU, Uppsala. Upon arrival, the fish were acclimatized to laboratory conditions by gradually adding carbon-filtered tap water and increasing the temperature slowly during several days. A few days after arrival to the facility, all fish were treated with a diluted formaldehyde bath (2.5 mL 37% formaldehyde to 10 L of water) to remove any external parasites. The fish were then kept in 100 L aerated and filtered glass aquaria with continuous flow-through of new carbon-filtered tap water at a temperature of ~17–18 °C. The fish were fed frozen blood worms once or twice daily and the photoperiod was set to 8 h light and 16 h darkness to keep them in a reproductive inactive condition. The fish were adapted to these conditions until the start of the experiment in March 2013 (~5 months). The experiment was approved in advance by the Uppsala Ethical Committee on Animal Research (C198/12).

2.2. Test chemical

Diclofenac sodium salt (CAS: 15307-79-6, purity ≥98%) was purchased from Sigma-Aldrich (Steinheim, Germany).

2.3. Experimental design

Before the start of the exposure, 180 fish were randomly distributed in fifteen 54 l well aerated aquaria (n = 12/aquarium) with continuous flow-through of carbon filtrated tap water. The water was pumped into the aquaria by a multi-channel peristaltic pump (Ismatec®) with PharMed Ismaprene tubing in the peristaltic pump and silicone tubing as extensions. The water renewal rate was approximately 100% per day. The tanks, placed in mixed order, were provided with glass jars made non-transparent by sand-blasting to provide cover/enrichment for the fish and airstones. The fish were acclimatized to the experimental aquaria for ten days.

Diclofenac sodium salt was weighed and mixed with MilliQ water on a magnetic stirrer for a minimum of 15 min in darkness for the making of a "superstock" solution with a diclofenac concentration of 640 mg/L (only taking into account the diclofenac, not the salt). No solvents were used. From the superstock, different volumes together with MilliQ water were taken to produce the stock solutions of 0, 2, 8, 32 and 128 mg/L. Every aquarium had its own stock solution bottle. The stock solutions were delivered into all aquaria by a multi-channel peristaltic pump with PharMed Ismaprene tubing and polytetrafluoroethylene (PTFE) tubing as extensions. The nominal concentrations in the aquaria were 0, 5, 20, 80 and 320 µg/L and each concentration was tested in triplicate aquaria (producing four different exposure concentration groups and one control group, each containing three aquaria). The stock solutions were renewed once a week.

At the onset of the experiment (day 0), different volumes of the superstock solution were added to each aquarium to immediately reach the desired exposure concentration. The fish were fed frozen blood worms one to two times daily. Cleaning was done every third day by siphoning fecal matter and mechanically cleaning the outflow net. The temperature was checked daily (range 15.7–17.6 °C) and the pH varied between 6.7 and 7.0. The photoperiod was set to 8/16 h (L/D). Aquaria water was sampled from each aquaria two times a week and frozen at −20 °C until analysis of diclofenac concentration. The fish were checked every day and moribund fish were removed and euthanized by decapitation followed by immediate destruction of the brain, in accordance with the approved ethical permit.

2.4. Sampling

The fish were sampled on day 28 except in the 320 µg/L group which was sampled on day 21 due to high mortalities in all three replicates. The fish were euthanized as described in Section 2.3, blotted dry and their length and weight were determined to the nearest mm and mg respectively. Apparent macroscopic lesions (including jaw lesions and parasite infections) were noted for each fish. The abdomen was cut open from lateral to the anus to the septum transversum and the sex of the fish was determined through macroscopical examination of the gonads in all fish. The liver (without gallbladder) from at least two females and two males from each aquarium were dissected, weighed, put in cryotubes and immediately frozen in liquid nitrogen and thereafter put in −80 °C for gene expression analysis. The remaining body of

the fish was fixed in phosphate buffered formaldehyde (4%) for histological analysis. Two to three fish from each aquarium were packed in tin foil and frozen at −20 °C until analysis for whole-body diclofenac concentration. The remaining fish were fixed in phosphate buffered formaldehyde (4%) for histological analysis.

2.5. Chemical analysis

Diclofenac concentrations in water samples were determined by chemical analysis using an in-line SPE column coupled to liquid chromatography-tandem mass spectrometry. In short, a triple stage quadrupole MS/MS TSQ Quantum Ultra EMR (Thermo Fisher Scientific, San Jose, CA, USA) coupled with an Accela and a Surveyor LC pump (Thermo Fisher Scientific, San Jose, CA, USA) and a PAL HTC autosampler (CTC Analytics AG, Zwingen, Switzerland) were used as analytical system. Full details about the chromatography and mass spectrometric settings have been published previously (Grabic et al., 2012). Whole-body samples were pre-treated as follows: internal standard was added (50 ng of D3-diclofenac), and the sample extracted sequentially with 1.5 mL acetonitrile twice. Samples were homogenized for 4 min at 42,000 oscillations per minute, using a Mini Beadbeater (Biospec. Bartlesville, USA) with zirconium beads and then centrifuged at 14,000 rpm for 10 min. Both supernatants were combined, evaporated to 20 µL and reconstituted in 100 mL methanol.

2.6. Bioconcentration factor

The average bioconcentration factor was calculated for each aquarium by dividing the concentration of diclofenac in each fish with the average concentration of diclofenac in the water throughout the experiment.

2.7. Condition factor

Fulton's condition factor was calculated for each fish by multiplying the total weight in grams by 100 and dividing by cube of the total length in centimeters.

2.8. Histological analysis

The fixated fish were dissected by removing the abdominal organs except for the kidney. Tissue from liver was put in plastic cassettes. The fish were trimmed by cutting off the head just caudally to the gills and cutting off the tail caudally to the anus and the remaining part (the mid part containing the whole kidney) of the fish was put on the lateral side in plastic cassettes. The samples were processed routinely and embedded in paraffin blocks. Tissue from liver were sectioned at three different levels and stained with Hematoxylin-Eosin (HE). Since the kidney was sectioned in situ, approximately 400 µm was cut away by parasagittal sectioning to remove the tissue lateral to the kidney. Then two consecutive, 4 µm thick, parasagittal sections were taken every 200 µm at three levels. One of the consecutive sections was stained with Hematoxylin-Eosin (HE) and the other with Periodic acid-Schiff (PAS).

The stickleback kidney is composed by an anterior part with primarily hematopoietic tissue and a posterior (renal) part dominated by nephrons. In this study, only the renal part of the kidney (here defined as the tissue caudal to the narrowest part in the ventrodorsal plane of the kidney) was assessed. Kidney sections from six to nine fish from each aquarium were examined by light microscopy. The different number of fish was due to mortalities in some aquaria. In an attempt to standardize the section level examined in the kidney, the section which showed the largest size of the kidney was examined. If more than one section had the approximate same size of the kidney, the section which had the largest size of the corpuscle of Stannius was chosen.

As recommended by the Best Practice Guidelines concerning toxicologic histopathology (Crissman et al., 2004), the initial review of a subset of kidney and liver sections from fish from the control aquaria and the 320 µg/L aquaria were performed where the veterinary pathologist (JN) was aware of the treatment group. This was done to recognize treatment-related histopathological lesions and to get an estimation of their range. Thereafter, all liver and kidney slides were coded and followed by a masked evaluation and scored semiquantitatively. This method, postexamination masking, is common in histopathologic examinations (Gibson-Corley et al., 2013). In the kidney, the amount of hematopoietic tissue was evaluated and the presence of tubular necrosis, tubular regeneration, pigmented macrophage aggregates (PMA), tubular hyaline degeneration/droplets and parasites were examined and scored. In the liver, hepatocyte vacuolation, inflammatory cell foci, pigmented macrophage aggregates (PMA) and the presences of parasites were scored. Lesions were chosen based on the initial review together with previous literature (Schwaiger et al., 2004; Hoeger et al., 2005; Mehinto et al., 2010; Memmert et al., 2013) and a 4-point scoring system was used with 0 = Not present, 1 = Minimal, 2 = Mild, 3 = Moderate and 4 = Severe. Lesions with statistically significant differences between the control group and the exposure concentration groups were reexamined blindly by a second veterinary pathologist (EE) to validate the finding.

2.9. Quantitative real-time PCR

Liver samples from two males and two females from each aquarium were analyzed. Relative hepatic mRNA levels from *ptgs1A* (*prostaglandin-endoperoxide synthase 1A*), *ptgs1B* (*prostaglandin-endoperoxide synthase 1B*), *ptgs2B* (*prostaglandin-endoperoxide synthase 2B*), *cox1* (*cytochrome c oxidase subunit I*), *cox2* (*cytochrome c oxidase subunit II*), *p53* (*tumor protein p53*), *cyp1A* (*cytochrome P450 1A*) and *c7* (*complement component 7*) were investigated and their sequences were obtained from Ensembl and NCBI GenBank. The genes were selected from published diclofenac exposure studies in fish (Mehinto et al., 2010; Cuklev et al., 2011). *β-Actin*, *rpl8* and *β-tubulin* were chosen as potential reference genes. Primer sequences were obtained from previous published studies (Hogan et al., 2008; Geoghegan et al., 2008; Williams et al., 2009) or designed using Beacon Designer™ (Palo Alto, CA) and synthesized by Eurofins MWG Operon (Ebersberg, Germany). Total RNA was isolated using the RNeasy Plus Mini Kit (Qiagen). RNA purity and quantity were analyzed using a NanoDrop ND 1000. Total RNA was reverse transcribed to cDNA using the iScript cDNA Synthesis kit following the manufacturer's protocol. Quantitative PCR was done in triplicate and performed on an ABI 7900HT. Non template controls (NTC) and no reverse transcriptase controls (NoRT) from pooled samples were used to check for primer dimer formation or DNA contamination. The primers for *ptgs1A*, *ptgs1B*, *ptgs2B* and *p53* were not producing amplicons/correct amplicons, and since effects on gene expression was not considered the most prioritized aim of the study, no further attempts were made to optimize primers for these genes. The concentration and annealing temperature for each primer pair were optimized, resulting in efficiencies between 93.4% and 103.2% for all of the used primer pairs. Hereafter, the median cycle threshold (C_T) of the triplicate was used. None of the potential reference genes were significantly regulated between the groups but *β-actin* showed the least variability ($p = 0.72$) and were chosen as the reference gene. The C_T value of the gene of interest was normalized by subtracting the corresponding C_T value of the reference gene in each fish, producing ΔC_T. The ΔC_T for each fish in the exposure concentration groups was normalized to the average ΔC_T in the control group, producing $\Delta\Delta C_T$ for each fish and reported as the expression fold change ($2^{-\Delta\Delta C_T}$). For full details, see supplementary data.

2.10. Statistical analysis

The statistical analyses has taken into account both variation between fish within aquaria as well as variation between aquaria within

treatments. All calculations were performed in SAS (SAS version 9.3, SAS Institute Inc., Cary, N.C., USA).

The SAS procedure "MIXED" was used for continuous dependent variables (length, weight, condition factor and relative hepatic mRNA levels (ΔC_T)). The nominal aquaria diclofenac concentration was regarded as a fixed factor and aquaria was regarded as a random factor. Multiple comparisons were performed by the Dunnett method comparing the zero concentration with the other concentrations. The p-values ($\alpha = 0.05$) and confidence intervals were adjusted according to Dunnett.

Ordinal variables (histological lesions) were analyzed using a cumulative logistic response model ("GLIMMIX" procedure in SAS) with the grade of histological lesion as a dependent variable. The nominal aquaria diclofenac concentration was regarded as a fixed factor and aquaria as a random factor with $\alpha = 0.05$. For subsequent multiple comparisons, a serial gatekeeping procedure adapted for dose-response relationships was used (Dmitrienko et al., 2009). First, the highest exposure concentration group (320 µg/L, nominal) was compared against the control group. If $p > 0.05$, then no further comparisons were made, whereas if $p < 0.05$ the second highest exposure concentration group was compared against the control group, and so on. To evaluate the agreement between the different pathologists regarding the scoring of renal hematopoietic tissue, a linear weighted Cohen's kappa was calculated (Cohen, 1968).

Mortality data as well as the presence/absence of jaw lesions were analyzed using logistic regression ("LOGISTIC" procedure in SAS) with mortality/jaw lesion as the dependent variable and nominal aquaria diclofenac concentration and aquaria as independent variables. Quasiseparation was detected as a consequence of the lack of mortalities in most aquaria, and therefore Firth's bias correction was used (Heinze, 2006). The α-level was set to 0.05.

3. Results

3.1. Chemical analysis

A total of 129 water samples were analyzed. Diclofenac was not detected in any of the samples from the control aquaria. In the exposure aquaria, the average measured water concentration of diclofenac per each exposure concentration group was 85–111% of the nominal concentration (Table 1). Thirty-eight fish were analyzed for diclofenac residues (two samples lost). Diclofenac was not detected in any of the fish from the control aquaria (Table 1). The bioconcentration factor was relatively stable (average 0.3) across all exposure concentrations (Fig. 1; See supplementary data for full details).

3.2. Mortality

After approximately one and a half week of exposure, the fish in the highest exposure concentration group appeared to be eating slower compared to the other groups and a week later, this group also started leaving food. After an additional week, the fish in the next highest exposure concentration group started leaving food. One fish in the highest exposure concentration group (320 µg/L, nominal) and one fish in the second highest exposure concentration group (80 µg/L, nominal) were found moribund and were euthanized (hereafter counted as mortality). The mortality in the highest exposure concentration group (320 µg/L, nominal) was significantly increased compared to the control group ($p < 0.0001$) after 20 days of exposure (Fig. 2). For animal welfare reasons, the remaining fish in these three aquaria were therefore sampled the following day. In the aquaria exposed to a nominal concentration of 80 µg/L, there were mortalities in the two replicate aquaria with the highest measured diclofenac concentration. There were no mortalities in any of the other aquaria throughout the experiment. For full details, see supplementary data.

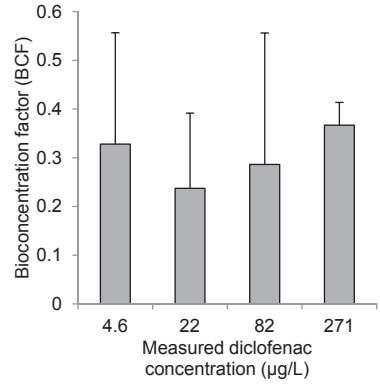

Fig. 1. Bioconcentration factors (BCFs; whole fish to water) in stickleback exposed to diclofenac. Grey bars show the average BCF of three aquaria replicates +S.D.

3.3. Jaw lesions

During the last weeks of the experiment, skin ulcerations on the jaw (predominately the lower jaw) were seen in some of the fish that were removed due to mortality. At the sampling on day 21 and day 28, additional fish with jaw lesions were noted. Table 2 shows a summary of the number of fish with skin ulcerations of the jaw per treatment group. The overall frequency of this finding was 15% (27/180). However, the lesion was not seen in any of the control aquaria. The overall statistical test, which was based on both aquaria and individual replication (see Section 2.10), was not significant ($p = 0.122$). This could be interpreted that one should not proceed with individual comparisons. Still, if this is done, including control for multiple comparisons using firth correction, the highest exposure concentration group fall out as significantly different from the controls ($p = 0.0225$, one-tailed test).

3.4. Length, weight and condition factor

Six fish (two fish from the 0 µg/L group (not in the same aquaria), one in 4.6, 22, 82 and 271 µg/L each) was infected with *Schistocephalus*

Table 1
Mean measured diclofenac concentrations in water (µg/L) and fish (ng/g).

	Diclofenac concentration				
Nominal aquaria concentration (µg/L)	0	5	20	80	320
Measured concentration in water (µg/L; mean ± S.D.) (n)	< LOQ[a] (27)	4.6 ± 1.9 (27)	22 ± 7 (27)	82 ± 22 (27)	271 ± 59 (21)
Measured concentration in fish (ng/g; mean ± S.D.) (n)	< LOQ[b] (9)	1.4 ± 0.9 (8)	5.8 ± 4.9 (9)	25 ± 23 (6)	113 ± 55 (6)

n = number of samples.
[a] LOQ = Limit of quantification 5 ng/L.
[b] LOQ = Limit of quantification 0.1 ng/g.

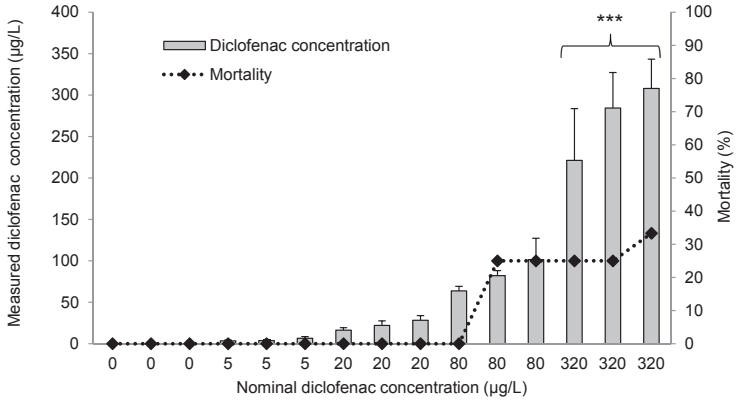

Fig. 2. Measured diclofenac concentration and mortality of exposed stickleback. Each bar represents an aquarium replicate + S.D. ***p < 0.0001 for mortality.

Table 2
Frequencies of skin ulcerations on the jaw in stickleback exposed to diclofenac.

Treatment group (µg/L)	Fish with jaw lesions
0	0/36 (0%)
4.6	1/36 (3%)
22	5/36 (14%)
82	11/36 (31%)
271	10/36 (28%)

Table 3
Average condition factor in stickleback exposed to diclofenac.

Treatment group (µg/L)	Condition factor (g/cm^3 ± S.D.)
0	0.73 ± 0.09
4.6	0.68 ± 0.08
22	0.68 ± 0.09
82	0.71 ± 0.09
271	0.66 ± 0.09**

** p = 0.0054.

solidus, a very common tapeworm in wild-caught three-spined stickleback. These fish were not included in weight or condition factor analyses because of the relatively large contribution from the parasite on those parameters. There was no significant difference in the length or weight of the fish between the different treatment groups. The condition factor was significantly different between treatment groups (p = 0.034), with 271 µg/L tending to be lower compared to the control group according to Dunnett's post hoc test (p = 0.0054) (Table 3). This is in line with the lower food intake observed in this group. However, it should also be noted that these fish were sampled one week earlier than the rest.

3.5. Histology

Hematopoietic cells can always be found in the renal part of the normal stickleback kidney and hence, no fish were given the grade 0. In renal hematopoietic tissue hyperplasia, an increased amount of hematopoietic cells is observed in the renal part of the kidney, interspersed between the tubules and glomeruli. The grading of renal hematopoietic tissue (interpreted as the proportion of hematopoietic tissue), by pathologist JN is illustrated in Fig. 3, showing fish classified as grade 1–3. For details on the different features seen, see Fig. 4. The grades of renal hematopoietic tissue were significantly different between the treatment groups (p = 0.032) and followed a clear concentration-response, with higher grades and consequently a larger proportion of hematopoietic tissue at higher exposure concentrations (Fig. 5). Post-hoc analysis revealed a significant increase at all exposure concentration groups (p = 0.044, 0.010, 0.0018 and 0.0040 at concentrations 4.6, 22, 82 and 271 µg/L, respectively; one-tailed test based on previous literature (Schwaiger et al., 2004)) compared to the control group. A second independent grading of renal hematopoietic tissue by pathologist EE was also significantly different between the treatment groups (p = 0.0023), following a similar concentration-response and post-hoc analysis revealed significant differences to the control group at all exposure concentration groups (p = 0.027, 0.0018, 0.00030 and 0.00020 at concentrations 4.6, 22, 82 and 271 µg/L respectively; one-tailed test). The inter-rater agreement between the two pathologists was 0.66. Such strength of agreement is considered "substantial" according to Landis and Koch (1977). In no case did the pathologists score differently with more than one grade. There was no significant differences between treatment groups for any of the remaining renal lesions (pigmented macrophage aggregates p = 0.80; tubular regeneration p = 0.80 and hyaline degeneration/droplets p = 0.54). Only 9 of the examined fish had parasites (mainly protozoa) in their kidneys and these fish were in the control group and the 22 µg/L group. No fish showed signs of tubular necrosis. Similarly, no differences were found for any of the endpoints in the liver (hepatic vacuolation p = 0.72; inflammatory cell foci p = 0.74; pigmented macrophage aggregates p = 0.73 and parasites p = 0.77). For full details, see supplementary data.

3.6. QPCR

The relative hepatic mRNA levels (ΔC_T) of gene *c7* were significantly different between the treatment groups (p < 0.0001). The post-hoc test revealed a decreased ΔC_T (resulting in an increased $2^{-\Delta\Delta C}T$, Fig. 6) in all but the lowest (4.6 µg/L) exposure concentration group compared to the control group (p = 0.027, 0.0088 and < 0.0001 at concentrations 22, 82 and 271 µg/L respectively, two-tailed test) and followed a concentration-response pattern (Fig. 6). *Cox1*, *cox2* and *cyp1A* were not significantly regulated in the exposure concentration groups compared to the control group. For full details, see supplementary data.

4. Discussion

This study shows that diclofenac exposure affects kidney histology in the three-spined stickleback. Effects on renal hematopoietic tissue were found down to the lowest concentration tested, i.e. 4.6 µg/L. This confirms the earlier findings in rainbow trout and brown trout (Schwaiger

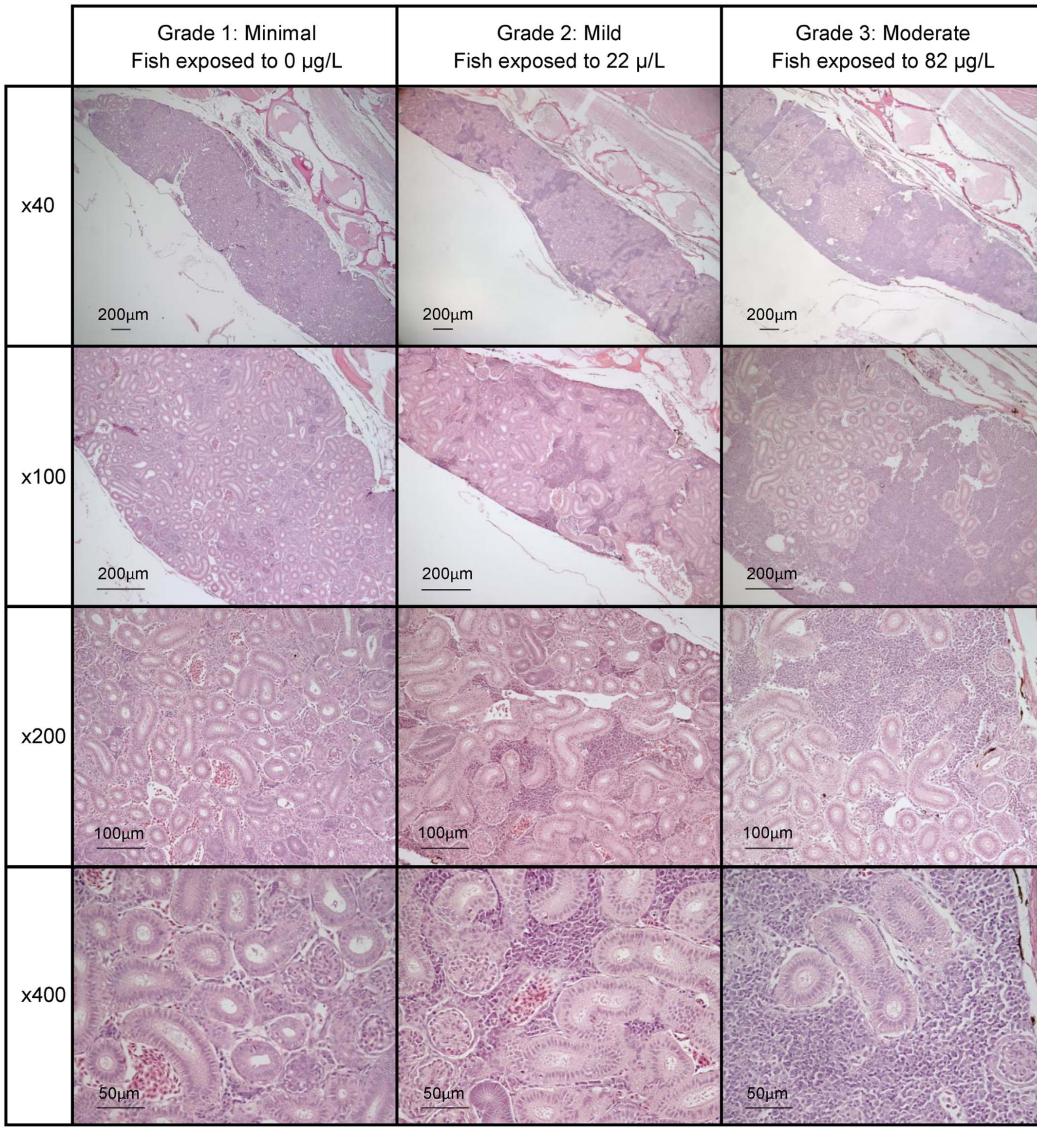

Fig. 3. Grading of renal hematopoietic tissue in stickleback exposed to diclofenac, examples from three individual fish. Both pathologists (JN and EE) gave these fish the same grading. The numbers in the left column refers to the microscope magnification.

et al., 2004; Hoeger et al., 2005; Mehinto et al., 2010) but differs from a more recent study on rainbow trout and zebrafish (Memmert et al., 2013). Whereas the latter study reported a NOEC for the most sensitive sublethal endpoint at 320 µg/L, we observed significant mortalities at 271 µg/L. Although we did not detect any histological changes in the liver, hepatic mRNA levels were affected at 22 µg/L. Diclofenac showed a stable but somewhat lower bioconcentration factor compared to results in previous studies (Cuklev et al., 2012; Memmert et al., 2013; Bickley et al., 2017), which suggests that stickleback might be slightly less sensitive to diclofenac than other investigated fish species given the same water concentration. Taken together, there is a risk that fish will be affected by diclofenac in effluent-dominated waters as concentrations in treated sewage effluents may reach up to low µg/L levels (Brown et al., 2007; Fick et al., 2010; UBA, 2017; Meyer et al., 2016).

An increased proportion of renal hematopoietic tissue was observed at all exposure concentrations. The effect followed a classical concentration-response pattern which is one of several important criteria for evaluating cause and effect relationships (Fox, 1991). Furthermore, the effect was

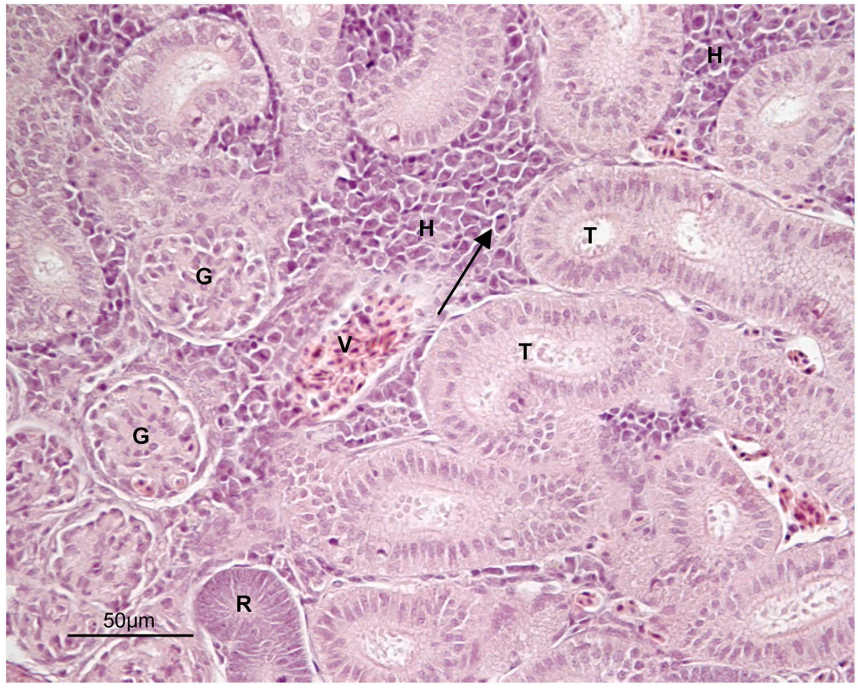

Fig. 4. Detailed image from Fig. 3 (kidney from stickleback exposed to 22 µg/L diclofenac). Glomeruli (G), tubules (T), regenerating tubule (R), hematopoietic tissue (H) and a blood vessel (V) are shown. The arrow shows a mitosis.

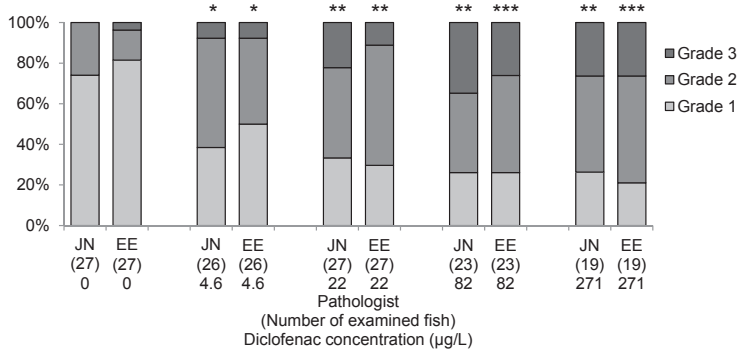

Fig. 5. Comparison of renal hematopoietic tissue scoring in stickleback exposed to diclofenac by study pathologists Johanna Näslund (JN) and Elisabet Ekman (EE). Each bar represents the proportion of different scores in three replicate aquaria. Levels of significance compared to the control group were *$0.05 \geq p > 0.01$; **$0.01 \geq p > 0.001$; ***$p \leq 0.001$.

observed independently by two veterinary pathologists with special training in fish histopathology. The scoring was done using coded slides, and there was substantial agreement between the pathologists rating. Independent observations of similar effects by different people, in different labs and in different species increase the degree of evidence (Fox, 1991). Renal hematopoietic hyperplasia has been reported previously in fish exposed to diclofenac, but then referred to as "interstitial nephritis" (Schwaiger et al., 2004). There is no apparent signs of inflammation in neither our samples, nor those presented in the study by Schwaiger et al. (2004). The observed effect is rather an increased proportion of hematopoietic tissue in the kidney. Hence we think the term "renal hematopoietic hyperplasia" would be more appropriate.

We applied an experimental design with independent aquaria replication of the different exposure concentrations, hence not basing conclusions on pseudoreplication within aquaria. The level of significance is an additional criterion for linking cause and effect (Fox, 1991). For the lowest concentration tested, the p-value for increase in renal hematopoietic tissue was modest but significant. As we a priori, based on previous literature (Schwaiger et al., 2004), had a hypothesis that diclofenac would cause this effect, we argue that our application of a one-sided statistical test is valid. For all other tested concentrations, the p-value was stronger. We therefore conclude that diclofenac has the ability to cause renal hematopoietic hyperplasia in fish at low micrograms per liter.

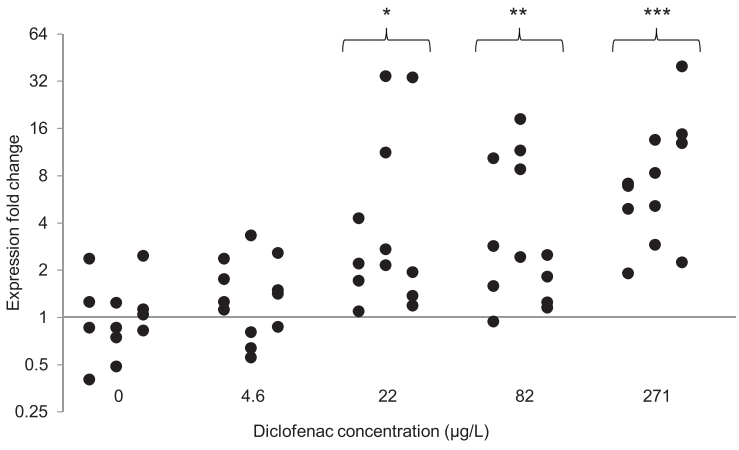

Fig. 6. Expression fold change ($2^{-\Delta\Delta C_T}$) of hepatic *c7* mRNA in the different aquaria. One dot represents one fish. The fish in the same aquarium are depicted on top of each other and hence the replicates within each treatment group are shown. The statistical analysis is based on ΔC_T values. Levels of significance compared to the control group were $^*0.05 \geq p > 0.01$; $^{**}0.01 \geq p > 0.001$; $^{***}p \leq 0.001$.

In light of this, it is intriguing why Memmert et al. (2013) did not report any effects on any endpoint at concentrations up to 320 µg/L. It is hardly a species effect as several previously published studies used rainbow trout and found effects at considerably lower concentrations (Schwaiger et al., 2004; Cuklev et al., 2011). In the present study, we terminated the highest exposure concentration group (271 µg/L) one week earlier than planned due to high mortalities, and these fish also showed a significant decrease in condition factor. Noteworthy, there were no mortalities at all in any of the aquaria up to 22 µg/L. Memmert et al. (2013) studied very young fish, whereas both we and Schwaiger et al. (2004) investigated the effects on older fish. It is possible that renal hematopoietic hyperplasia is more easily induced in older fish.

The study by Wolf et al. (2014) describes a re-evaluation by a pathology working group of three histopathological studies, all assessing effects of diclofenac on trout (Hoeger et al., 2005; Mehinto et al., 2010; Memmert et al., 2013), whereas material from the study by Schwaiger et al. (2004) was not available. An overall conclusion was that the low NOECs for histopathological changes reported by Hoeger et al. (2005) and Mehinto et al. (2010) could not be confirmed. In agreement, we could not see any signs of tubular necrosis or significantly increased occurrence of tubular regeneration in any of the exposure concentration groups. With regards to the study by Memmert et al. (2013) the pathology working group identified significantly decreased hepatic glycogen at 1000 µg/L, an alteration that was not originally noted by the evaluator in the study. We did not see any sign of decreased liver glycogen, although the highest concentration in our study was only 271 µg/L and the exposure time was shorter. A very recent study showed a significant positive correlation between the diclofenac exposure concentration and the number of developing nephrons in fathead minnow (Bickley et al., 2017). As only a correlation analysis was made, a LOEC or NOEC was not established. The same study also reports inflammation of glomeruli and tubules in the kidney at the highest exposure concentration (25 µg/L), but no quantitative assessment was presented. Based on the available material, it is a bit difficult to judge if the hypercellularity is caused by inflammation or variations in the thickness of sections (see Fig. S1 in Supporting information in Bickley et al. (2017)). We found no apparent signs of inflammation in the stickleback kidneys. Neither Schwaiger et al. (2004), Hoeger et al. (2005), Mehinto et al. (2010) nor Memmert et al. (2013) states that their histological evaluations were done blindly and in none of the studies were sections assessed by more than one evaluator. As histopathology involves subjective judgements and requires a trained eye, blind scoring of the key findings by more than one experienced histopathologist is advisable (Wolf et al., 2015). The lack of this might explain some of the discrepancies between previous studies.

The bioconcentration of diclofenac in fish have been evaluated in several studies (Schwaiger et al., 2004; Mehinto et al., 2010; Kallio et al., 2010; Lahti et al., 2011; Cuklev et al., 2011; Bickley et al., 2017). Schwaiger et al. (2004) show a very irregular bioconcentration factor with a decrease of the bioconcentration factor with increasing exposure concentration, and the bioconcentration factor varying from almost zero up to 2732 in various organs. Cuklev et al. (2011) demonstrated a stable bioconcentration factor of 2.5 in the liver and 4.0 in the blood plasma, independently of exposure concentration. This is in agreement with the present study although the bioconcentration factor in our study is lower. It should be noted that the bioconcentration factor in our study refers to diclofenac concentrations in whole fish. Memmert et al. (2013) similarly demonstrated a bioconcentration factor between whole rainbow trout and water, but higher (3–5) than found in the stickleback. This could be a species-effect.

Cuklev et al. (2011) showed effects of diclofenac on the hepatic mRNA levels of different genes including the *c7* (*complement component 7*) gene. In line with these findings, increased hepatic mRNA levels of *c7* by diclofenac were found in the stickleback in this study. The changes followed a clear concentration-response, with significant effects at 22 µg/L and all higher concentrations. This gene is involved in the innate immune system and together with other complement components the c7 protein form a membrane attack complex initiating lysis of foreign cells (Delves and Roitt, 2011). An effect on the immune system could potentially offer an explanation on why there was a tendency for a concentration-dependent occurrence of jaw lesions in the fish. The three-spined sticklebacks used in this study were originally wild-caught and may carry pathogens. An altered immune system could pave the way for infections. Although speculative, the hematopoietic hyperplasia seen in the kidneys could be an indirect effect of diclofenac, via impairing the immune system, rendering the fish more susceptible to infections. If that were the case, it would still be an effect initiated by diclofenac exposure, and still highly relevant, as fish in the wild have a far from sterile environment, and a well-functioning immune system is crucial for their well-being and survival. On the other hand, in mice, NSAIDs are well known to stimulate hematopoiesis directly (Hofer et al., 2012), hence providing an explanation to the effects found in fish that does not need to involve co-exposure to an infectious agent. The other genes evaluated in this study, i.e. *cox1*, *cox2* and *cyp1A*, were not significantly affected by diclofenac. These genes were chosen from the study by Mehinto et al. (2010), in which *cox1* and *cox2* were significantly downregulated in the liver at 1 µg/L and *cyp1a1*

upregulated in the liver at 1 µg/L. Although the authors claim they have investigated the genes *cyclooxygenase 1* and *2*, the primers specified in the paper (Supporting information, Table S1) do not support this. The primers fit the genes *cytochrome oxidase subunit I* and *II* which can have the same annotation as *cyclooxygenase 1* and *2* and this can perhaps explain the mix-up. Bickley et al. (2017) showed effects of 25 µg/L diclofenac on the expression of several genes in the anterior kidney, including genes involved in inflammation and the immune system. Hong et al. (2007) reported effects of diclofenac on gene expression in medaka but results are ambiguously reported and lack replication.

Studies of the effects of diclofenac on fish are accumulating, and critical evaluations of these are needed to get a better understanding of the risks. Guiloski et al. (2017) reports that diclofenac at 0.2 µg/L affects several enzymes in the liver (SOD, hydroperoxides, GSH and GST) and in the testis (SOD, GST hydroperoxides and GPx). Some of the effects did not follow a concentration-response, and for all enzymes in the testis except SOD, the effect was only seen at the lowest tested concentration, 0.2 µg/L. The lack of aquarium replication also makes the interpretation difficult. Prokkola et al. (2015) reported effects of diclofenac on hepatic gene expression in sticklebacks both in the absence and presence of hypoxia. On the other hand, Lubiana et al. (2016) found no effects of diclofenac alone on gene expression in the gills in the same species. However, some support for effects on gene expression by co-exposure with hypoxia was found. Both studies used only one exposure concentration (1 µg/L, nominal), and a concentration-response study would be valuable to better assess the causality. In another recent study, Nile tilapia was exposed to diclofenac (Groner et al., 2017). They report that diclofenac causes histological changes in the gills at 0.1 µg/L. In their supplementary material they show examples of the gill histopathological lesions claiming chloride cell hypertrophy but judging by the picture, the chloride cells looks more like mucus cells. Their experimental design included four aquaria replicates per treatment group, however in the statistical analysis all individual fish were considered as independent biological replicates. Praskova et al. (2014) report no histopathological effects on zebrafish exposed to up to 60 mg/L diclofenac, but provide no information at all on how the analyses was done. It is possible that some of the discrepancies between the studies can be due to differences between fish species, age and exposure conditions, including temperature. However, some of the contradicting results in the literature are likely a reflection of limitations in study design and reporting standards.

In the present study, histopathological effects on kidneys i.e. hyperplasia of hematopoietic tissue were seen down to the lowest tested concentration (4.6 µg/L) and a no-effect concentration could not be determined. Similar effects have been found previously in rainbow trout (Schwaiger et al., 2004). The studies by Schwaiger et al. (2004) and Triebskorn et al. (2004) report LOECs on kidney histology (5 µg/L and 1 µg/L, respectively) based on "mean assessment values" which is a measure including several lesions. Mehinto et al. (2010) and Hoeger et al. (2005) report LOECs for individual kidney lesions down to 5 µg/L and 50 µg/L, respectively. What these changes means in terms of adverse effects is not known. The increase in renal hematopoietic tissue, the increased hepatic *c7* mRNA, the jaw lesions and the primary mode of action of diclofenac in mammals together point in the direction of effects on the immune system of fish. Standard toxicity tests are not designed to evaluate such disturbances, although in the real environment, a well functional defense against infectious agents would be critical. Given that the stickleback appear to bioconcentrate diclofenac to a lower degree than rainbow trout, it is quite possible that the stickleback is not the most sensitive fish species. Additionally, diclofenac is not the only NSAID present in treated sewage and surface waters. Considering their overlapping mechanisms of action, additive effects are expected. Based on the above, the proposed environmental quality standard of 100 ng/L for diclofenac in inland surface waters (EU, 2012) therefore seems reasonable if a precautionary principle is to be applied.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.aquatox.2017.05.017.

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

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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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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.

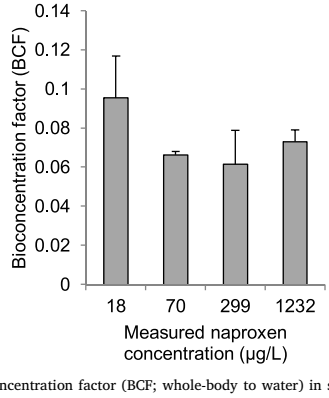

Fig. 1. Bioconcentration factor (BCF; whole-body to water) in stickleback exposed to naproxen. Grey bars show the average BCF of three aquarium replicates + S.D.

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

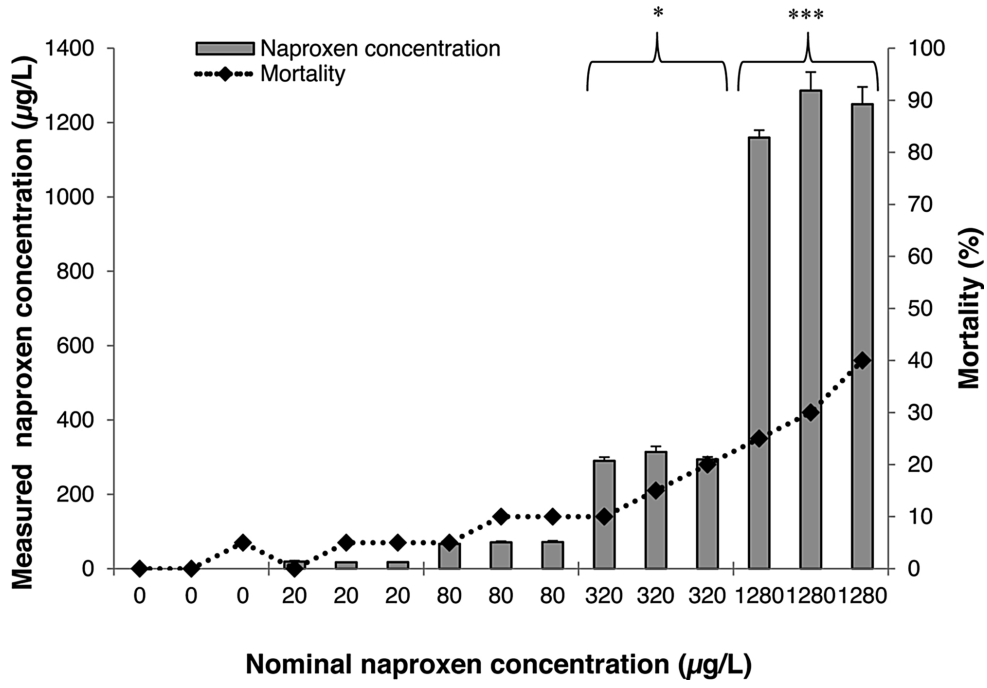

Fig. 2. Measured naproxen concentration and mortality of exposed stickleback. Each grey bar represents the average naproxen concentration in one aquarium replicate + S.D. Asterisks indicate significant differences in mortality to the control group, * $p = 0.04$ (d. 27) and *** $p < 0.001$ (d. 21).

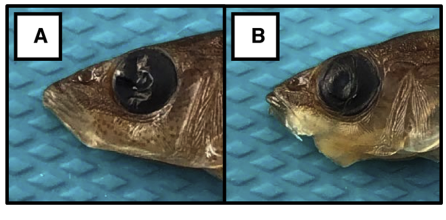

Fig. 3. Formalin-fixated stickleback. **A:** Fish from control group with no signs of jaw lesions. **B:** Fish exposed to naproxen (1232 µg/L) with lesions on the lower jaw.

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

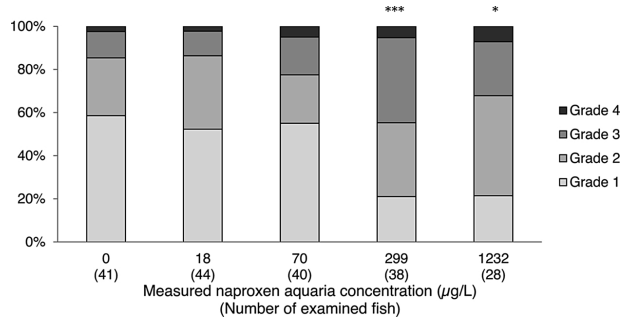

Fig. 4. Grading of renal hematopoietic hyperplasia in stickleback exposed to naproxen, where grade 4 is the most severe hyperplasia. Asterisks indicate significant differences to the control group,* p = 0.011; *** p = 0.001.

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

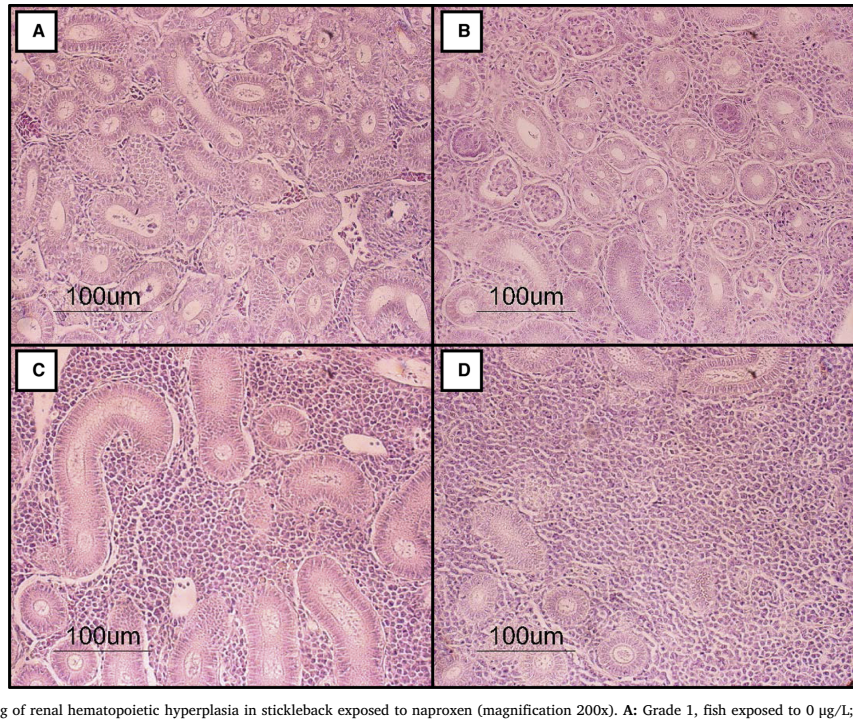

Fig. 5. Grading of renal hematopoietic hyperplasia in stickleback exposed to naproxen (magnification 200x). **A:** Grade 1, fish exposed to 0 µg/L; **B:** Grade 2, fish exposed to 18 µg/L; **C:** Grade 3, fish exposed to 299 µg/L; **D:** Grade 4, fish exposed to 1232 µg/L.

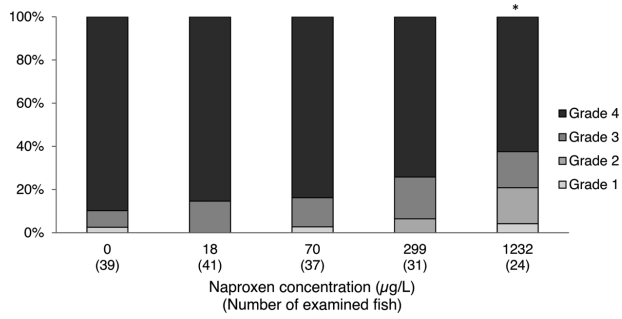

Fig. 6. Grading of hepatocellular vacuolation in stickleback exposed to naproxen where grade 4 is the most extensive vacuolation and in this study considered "normal". Asterisks indicate significant differences to the control group, * $p = 0.014$.

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

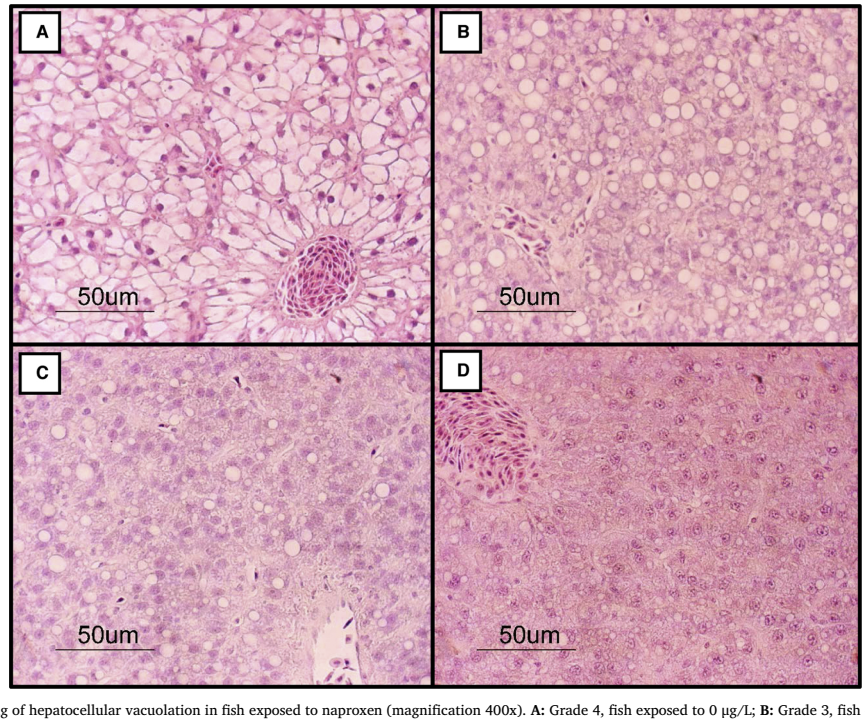

Fig. 7. Grading of hepatocellular vacuolation in fish exposed to naproxen (magnification 400x). **A:** Grade 4, fish exposed to 0 μg/L; **B:** Grade 3, fish exposed to 299 μg/L; **C:** Grade 2, fish exposed to 1232 μg/L; **D:** Grade 1, fish exposed to 1232 μg/L.

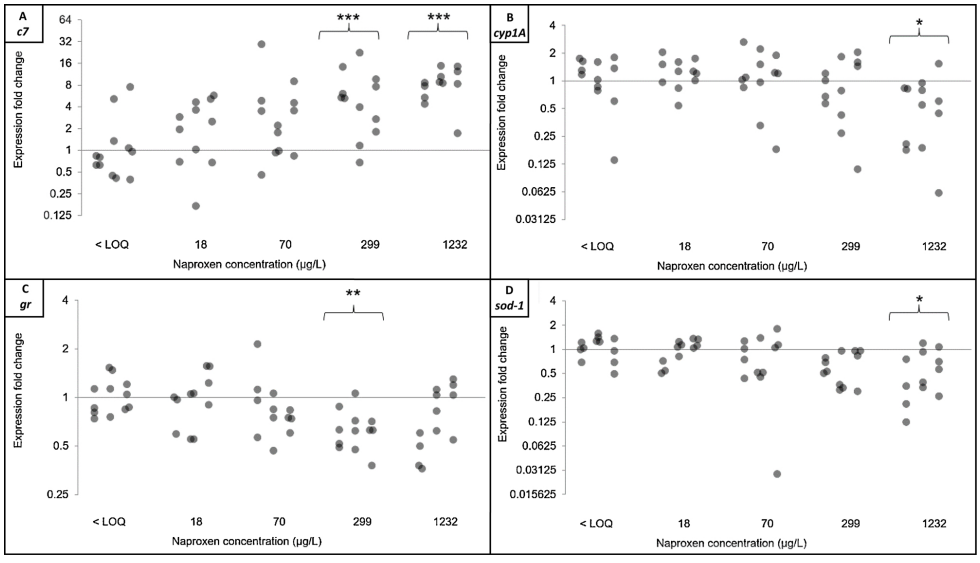

Fig. 8. Expression fold change ($2^{-\Delta\Delta C_T}$) of hepatic *c7*, *cyp1A*, *gr* and *sod-1* mRNA in the different aquaria in relation to the control group. One dot represent one fish and the fish in the same aquarium are depicted on top of each other. Thus, the three aquarium replicates in the 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 (*Onchorhyndus 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_2PC (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 (1997b)	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)
$BCF_{Whole-Body}$ (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 (*cyp7*)	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 *cyp1A1* (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.

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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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III

Review Article

No Clear Evidence of Histopathological Effects Linked to NSAIDs in the Kidney or Liver of Fish Exposed to Treated Municipal Wastewaters

Toxicologic Pathology
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Abstract

Non-steroidal anti-inflammatory drugs (NSAIDs) can cause histopathological changes in the kidney and liver of fish. Still, it is unclear whether exposure to treated municipal wastewater that contains NSAID residues causes similar effects. We therefore conducted a comprehensive, critical review on claimed histopathological changes in fish exposed either to NSAIDs or to treated municipal wastewater in the laboratory or downstream from treatment plants. A detailed scrutinization questioned the basis for several findings. Hepatocellular necrosis, hepatocellular vacuolation, and an increase of developing nephrons/basophilic clusters (DNs/BCs) were overlapping findings, but the lowest observed effect concentrations (LOEC) for the hepatic endpoints were well above concentrations frequently encountered in treated effluents. An increase of DNs/BCs were reported at lower NSAID concentrations, but with some concerns regarding reliability. Hence, there is no clear documentation that histopathological effects caused by NSAIDs are present in fish exposed to municipal effluents. Study design, including the species studied, exposure regimes, endpoints analyzed, and applied methodology varied widely between studies, all of which could make overlapping effects difficult to detect. In addition, limitations in both experimental design and reporting standards in fish histopathology studies prevent any firm conclusions. More comparable study designs in future studies would facilitate comparisons.

Keywords

fish, wastewater treatment plant, sewage, histopathology, NSAID, diclofenac

Introduction

Over the past decades, there has been a growing concern about pharmaceuticals in the environment. The most remarkable example is the near extinction of several vulture species in India and Pakistan caused by exposure to the non-steroidal anti-inflammatory drug (NSAID) diclofenac. When vultures were scavenging on carcasses of livestock previously treated with diclofenac, their kidney function rapidly deteriorated, leading to severe visceral gout, and ultimately death.[28,30] The resulting lack of vultures also led to vast amounts of animal carcasses not being eaten, thereby becoming a public health risk as vectors for infectious diseases.[31] To protect the vultures, diclofenac was banned for veterinary use in India and Pakistan in 2006, however, diclofenac could still be detected in carcasses of ungulates years after the ban, which points to an illegal use.[9]

As of today, diclofenac is still a very commonly used drug in humans to treat inflammation, pain, and fever. A well-recognized side effect from NSAIDs, including diclofenac, is altered blood flow to the kidneys, which in certain patients can lead to renal failure.[33] This indicates that kidney impairment due to diclofenac may be found in a wide range of vertebrates. Indeed, laboratory studies in fish exposed to diclofenac reports histopathological effects, including various changes in the kidneys, at low µg/L.[5,27] A recent mesocosm study also report increased mortality in three-spined sticklebacks at 3.82 µg/L.[18] In Europe, levels of diclofenac around or exceeding 1 µg/L can often be found in effluents from municipal wastewater treatment plants (WWTPs),[6,13,24,38] raising concern about possible effects in the aquatic environment as well. Such observations led to diclofenac being added to the watch list of priority substances within the Water Framework Directive in the European Union[11] with the objective of improving knowledge on environmental exposure levels. In 2020, an expert group was assigned by the department Directorate-General Environment (DG ENV) at the European Commission (EC) to propose a draft for an Environmental Quality Standard for diclofenac derived from the available literature. During this exercise, the reliability of some of the diclofenac studies was questioned.[10] The draft

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was then evaluated by the Scientific Committee on Health, Environmental, and Emerging Risks (SCHEER) on behalf of DG ENV. An Annual Average Quality Standard (AA-QS) of 40 ng/L in fresh water and 4 ng/L in sea water was suggested by the expert group and supported by SCHEER, but no decision has yet been made by the European Council or the European Parliament.[34]

Studies reporting effects in fish at low or even sub µg/L of NSAIDs together with observed or predicted concentrations in waterways suggest that effects of diclofenac are possible, and even plausible. Still, there seems to be a lack of studies directly addressing if effects typically caused by diclofenac exposure, can be found in fish exposed to treated municipal wastewater. Such municipal wastewater contains mixtures of chemicals, including several NSAIDs. Given the similar mode of action of different NSAIDs, one can expect that they would act additively in fish, although direct empirical evidence appears to be scarce. There are also data suggesting that bioconcentration of NSAIDs may be higher in fish exposed to complex wastewaters than single compounds,[8] potentially leading to underestimation of risks if only effect data from single-compound exposures are used.

Sublethal effects in fish, such as histopathological alterations, can add evidence of causality but the specificity and sensitivity of such effects are important to consider. Here we aimed to conclude if specific histopathological alterations in fish identified after exposure to NSAIDs also are reported in fish exposed to treated municipal wastewaters. This was addressed by a comprehensive review where histopathological effects in fish after NSAID or wastewater exposure were compared and evaluated based on both relevance and reliability. The chosen approach is most similar to a critical review, as defined by Grant and Booth,[15] but has some of the characteristics of a systematic review, such as a clearly defined research question, a standardized and fully transparent search and clear inclusion/exclusion criteria. As histopathology and especially fish histopathology is a complex scientific field where misdiagnoses and misinterpretations are common,[40] a secondary aim was to evaluate the technical (section preparation and imaging) and diagnostic quality for each reported histopathological effect.

Materials and Methods

Rationale for Scope

The current review focuses on kidney and liver only, two commonly analyzed organs where histopathological effects of NSAIDs at low concentrations have been reported.[17,27] Gills are also frequently analyzed with reported effects after NSAID exposure.[5,23,35] However, from our own experience and also stressed by Wolf et al,[40] out of the commonly investigated organs in fish histopathology, gills are the most technically challenging during preparation and they are also prone to artifacts to a higher degree than liver and kidney. For this reason, we did not evaluate reported effects on gills here and limit our conclusions to liver and kidney.

There is a vast literature on effects of gonad histology linked to exposure to treated municipal wastewater. Although gonads in general are relatively easy to prepare for histological analyses and are more resilient to artifacts than gills, some of the observed effects on gonads are linked to endocrine disrupting chemicals in the wastewater.[2,19] Also, we are not aware of any extensive support for specific histopathological effects on fish gonads by NSAIDs at low exposure concentrations. This motivated our choice to not include gonad histology in the present review.

Database Search on Treated Municipal Wastewater Exposure

The search was performed by JN using the database Web of Science™.[7] A research question was formulated: *What histopathological findings are reported in the kidney or liver of fish exposed to treated municipal wastewaters?* The search settings were "Database: Web of Science Core Collection" and "Editions: All" and the publication date was set to January 1, 1970, to March 31, 2025. Full search strings can be found in the supplementary material (Table S1). To ensure an accurate search, previously downloaded articles regarding treated municipal wastewater exposure and histology in fish were sought for among the final hits, and 11/12 were found which was considered acceptable. The remaining article not found, was added to the final search hits (supplementary material, Table S3).

Database Search on NSAID Exposure

The search for NSAID articles was also performed by JN using the database Web of Science™,[7] with the corresponding research question: *What histopathological findings are reported in the kidney or liver of fish exposed to NSAIDs?* The search strings were similar to the wastewater search but replacing the first string with appropriate terms for NSAIDs as well as approximately fifty different NSAIDs (supplementary material, Table S2, Figure S1). This string was divided into three sub-strings due to space limitations in the search engine and for clarification. The same settings as for the wastewater search were used. Previously downloaded relevant articles were sought for among the final hits and 12/13 were found. The missing article was added to the final search hits (supplementary material, Table S4).

Inclusion Criteria and Screening Workflow

Only original articles in English were included in the screening. Prior to the screening of the search hits, a set of inclusion criteria was formulated. Only articles quantitatively or semiquantitatively analyzing liver or kidney histopathology in fish exposed to municipal WWTP effluent or exposed to a single NSAID or a mixture of NSAIDs and compared with a suitable control were included in the review (Table 1).

Table 1. Article inclusion criteria.

	Inclusion criteria municipal wastewater review	Inclusion criteria NSAID review
Species	Fish	Fish
Exposure	Treated municipal wastewater with at least secondary (biological or chemical) treatment, but not chlorination of effluent	Waterborne NSAID exposure, including mixtures of NSAIDs but not mixtures with other pharmaceutical classes
Control	A relevant control group for comparison (upstream of the WWTP, reference area, tap/ground water, etc)	A relevant control group where the only difference between treatment and control is the NSAID exposure
Endpoint	Quantitative or semiquantitative histopathological data reported from liver and/or kidney for both exposed and control fish, excluding studies only reporting composite organ indexes	
Statistical support for claims of histopathological effects	Stated significance and/or reported p-value of claims showing significance ($P < .05$) *(Non-significant findings in studies otherwise fulfilling all of the above criteria are compiled in the supplementary material, Tables S9-10)*	

Effluents from non-municipal sources were considered irrelevant since they would not normally contain NSAIDs. Untreated or only primary treated wastewaters could certainly contain elevated NSAID levels but were also considered out of scope here. Chlorinated effluents were excluded due to the special nature of such effluents, including risks for generating toxic disinfection byproducts and/or residual chlorine. Furthermore, NSAID exposure had to be waterborne to facilitate a comparison between the exposure levels with levels found in wastewaters or surface waters. Only articles reporting statistically significant differences in histological effects between exposed fish and fish used as controls were included in the detailed evaluation. This was mainly because of the many additional challenges in interpreting reported negative results in non-standardized tests. These also include unspecific claims, such as "no histological alterations," leaving the reader unaware of which alterations that actually were scored. For completeness, non-significant findings in studies otherwise fulfilling all of the above criteria were compiled in the supplementary material, Tables S9 and S10. Another cause for exclusion was when the histological diagnoses were added together, generating an organ index without reporting individual effects. This is problematic for several reasons, including that it does now allow exact identification of what was affected, which makes comparisons between studies practically impossible. While detailed information on both methods used for obtaining measurements and on approaches to minimizing bias (eg, randomized sampling and blinded evaluation), are important for judging validity of quantitative or semiquantitative data, it was not used as formal inclusion criteria.

The screening was performed by JN and started with the article title and if it was clear that the article was out of scope, it was excluded with no further evaluation. Otherwise, the abstract was evaluated and articles not providing evidence for exclusion continued to full-text evaluation. After full-text evaluation, if an article was to be excluded, at least one of the reasons for exclusion was recorded. DGJL performed an additional evaluation in case of any ambiguities with regards to inclusion/exclusion. Articles that remained after the full-text evaluation were included in the review. See supplementary material (Tables S3-S8) for full details, including exclusion reasons after the full-text evaluation.

Review of Included Articles

General information regarding study design and histological analysis was compiled for each article in tables. A general comment was also added when relevant, which illustrated identified ambiguities within each paper. All claimed histological diagnoses in liver and kidney were also compiled in tables.

A comparison of histological findings in fish exposed to wastewaters or NSAIDs was made to identify, and highlight overlaps and potential lack thereof. Findings with representative photomicrographs included, were evaluated by a fish pathologist (JN) based on criteria modified from Wolf and Maack,[41] created for scoring credibility of histopathology data. "Figure image quality" was defined as an adequate technical quality including image resolution and magnification of the provided figure, while "histologic specimen quality" was defined as adequate preservation and processing of the tissue. Both criteria were adopted but instead of a numeric combined score as in Wolf and Maack,[41] only yes/no or N/A (= not assessable) was used. If the image quality of the histological figure is not adequate, the quality of the histological specimen is often impossible to assess and thus the need for N/A. Diagnostic accuracy was discussed on a case per case basis as we found it too complex to present in a tabular way.

We then assigned a "weight of evidence" scored as low, moderate, or high, to each claim (each histological endpoint in each article). This was based on an overall assessment of experimental design including clarity of methodology, relevance, and number of control groups, coherence in results between multiple control groups, potential confounders between groups (eg, sex, maturation, and size differences), observed clear dose-responses, the level of statistical replication, in particular with regards to aquaria replication, appropriate statistical handling

of data, the level of statistical significance, and if the diagnosis was supported or not in provided histological images. Although there will inevitably remain some level of subjectivity in the determination of "weight of evidence," to achieve the score "high," the article must provide histological figures of adequate technical and histological quality. Given the low number of articles in the review with even fewer including photomicrographs, and the evaluator's (JN) familiarity with the articles in the NSAID review, blinding the photomicrographs before the evaluation would not have increased the objectivity and hence, the assessment was performed with the evaluator aware of the origin of the histological images. Finally, each overlapping diagnosis was then evaluated, including a discussion of relevance and reliability.

Results and Discussion

Database Search on Treated Municipal Wastewater and NSAID Exposure

A total of 866 articles were generated in the Web of Science search for histological effects from municipal effluent exposure. Adding the previously downloaded article not found in the search resulted in 867 articles. Since the search was wide to retrieve as many relevant articles as possible, many were clearly out of scope. After the title and abstract screening, only 68 articles continued to full-text evaluation where only seven articles passed the criteria to be included in the review. The majority of the 68 articles were excluded due to factors, such as effluent arising from non-municipal sources (eg, metallurgical effluent) or the wastewater was untreated, only primary treated or chlorinated. There were also papers where the control was not suitable, that is, other pollution sources than treated municipal wastewater differed between exposure and control sites, or in some cases, controls were missing and hence, made it impossible to assign any histological outcomes to the wastewater exposure. For additional information, see supplementary material (Tables S5 and S7). No study passed all inclusion criteria while simultaneously claiming no statistical significance for any of the histological effect investigated.

As for the NSAID exposure articles, 177 articles were generated in the Web of Science search producing a total of 178 when the previously downloaded article not found in the search was added. Thirty-six articles were still included after the title and abstract screening but only seven of them remained after full-text evaluation. Reasons for exclusion were similar to the evaluation on municipal effluent, where many of the search hits were addressing a completely different subject. Regarding the articles that did expose fish to NSAIDs, the majority of them lacked a quantitative or semiquantitative histological analysis, which is necessary to statistically evaluate the results. Some articles were also excluded because only organ indexes were reported. For additional information, see supplementary material (Tables S6 and S8). One article fulfilled all criteria except claiming any statistical significance for histological endpoints evaluated in liver and kidney.[23]

The applied inclusion criteria largely represent a set of minimum requirements to facilitate an evaluation of claimed findings. The low number of studies fulfilling these criteria could reflect either the lack of studies in this area, or a sign of suboptimal experimental design in existing studies. The latter is problematic in many ways, ranging from the unnecessary use of research animals to the risk of misdirecting policies and decisions based on non-robust data.

Review of Treated Municipal Wastewater and NSAID Studies

Seven articles were included in the municipal wastewater exposure review and seven in the NSAID exposure review. The experimental setup on wastewaters differed widely between studies. None of them studied the same fish species and the studies were conducted on three different continents. Most studies investigated free-living wild fish in rivers downstream from WWTPs, but fish were also held in cages in rivers downstream from WWTPs or exposed to treated municipal wastewater in aquaria. The controls consisted mainly of river water upstream from the WWTPs, but also from nearby lakes or ponds or tap water. The exposure duration lasted from three weeks to up to close to a year. Exposure duration was obviously undetermined for all studies that analyzed free-living wild fish. Choosing to study free-living wild fish when it comes to evaluating effects of WWTP effluent exposure can come with some limitations as many uncontrolled variables may affect the outcome. Water temperature, food availability, fish density, or other properties may differ between sites, while not being attributed to the wastewater exposure as such. Hence, although field studies in some sense reflect actual exposure scenario better than laboratory studies, it can be difficult to link effects observed in fish to the wastewater exposure specifically. It is also impossible to know the exact level or timing of exposure since the fish in most cases can migrate closer to or further from where the effluent enters the study area. The characteristics of the control conditions are equally important. Comparing caged fish in a river downstream from a WWTP with fish in a tank receiving tap water can have its disadvantages since tap water and river water (without the wastewater) may also differ in their effects on fish. Exposing control fish in cages upstream from the WWTP would be more suitable. More details on the respective study design can be found in Table 2.

All studies included in the NSAID review were conducted in Europe. The most investigated drug was diclofenac (5/7 studies). All included studies measured the actual exposure levels, which increases their overall reliability, and many argue this should be a requirement in these types of studies.[16] The exposure concentrations were in the µg or sub-µg/L range, except in one study where only mg/L-concentrations were evaluated. The latter is not considered relevant for any exposure scenario, with the possible exception of direct discharges from manufacturing.[20] Salmonid species were used in three out of seven studies and all studies lasted for 21 to 28 days. Studies on pharmacokinetics of NSAIDs in fish are sparse but suggest this is probably

Table 2. Summary of study design in the wastewater articles.

Paper no. Reference	Country of origin	Species, age at study start, sex	Study design	Treatment		Exposure time	Replication and number of fish in study	Number of fish analyzed histologically
				Exposure	Control			
1 Gisey et al[14]	United States of America	Goldfish *(Carassius auratus)*, adults, ♂♀	Field study (caged fish in river)	Downstream from WWTP outfall (4 sites)	Reference river (1 site) and pond (1 site)	42-43 days	Replication at one site (pond). Claimed 10♂ and 10♀ per site.	"From each fish" but unclear number (see comment)
Comment: *The authors claimed that the study started with 10♂ and 10♀ per site but this did not match the numbers in the appendix. Based on the appendix, one can assume that 6 to 13 fish per sex and site were analyzed histologically except at the pond where 18 to 22 fish per sex were analyzed (due to cage replication). The two reference sites differed widely from each other on different biological endpoints (length, weight, etc).*								
2 Liney et al[21]	United Kingdom	Roach *(Rutilus rutilus)*, fertilized eggs, ♂♀	Laboratory study (mesocosm-aquaria)	WWTP effluent (20%, 40%, and 80%) diluted with tap water	Dechlorinated tap water	300 days	Not specified, (60 fish per treatment were used for different analyses)	30 fish per treatment (4 treatments)
Comment: *The authors reported no sex-related differences for any of the outcomes, but it is unclear how undifferentiated fish were dealt with.*								
3 Pinto et al[29]	Portugal	Gudgeon (*Gobio gobio*) and Mullet *(Mugil cephalus)*, –, –	Field study (wild fish)	Downstream from WWTP outfall (one site)	Upstream from WWTP outfall (one site)	N/A	No, 22-23 gudgeons per site, 7 mullets per site	22-23 gudgeons per site & 7 mullets per site (2 sites)
Comment: *The authors did not report the sex of the fish or took it into consideration.*								
4 Tetreault et al[37]	Canada	Fathead minnow *(Pimephales promelas)*, –, ♂♀	Field study (wild fish)	Downstream from WWTP outfall (one site)	Upstream from WWTP outfall (one site)	N/A	Yes (sampling on two consecutive years), aimed sampling of 20♀ and 20♂ (per site?)	Not specified
Comment: *The authors reported sampling of 20♀ and 20♂ (per site?) in 2006 and 20♀ and 20♂ (per site?) "when possible" in 2007. This did not match the numbers in Table 2 in their article. One could assume that 11 to 22 fish per sex, site, and year were analyzed histologically as they were sampled for other purposes. The authors stated that fish were sampled for kidney histology in 2006 but presented data from 2007 as well.*								
5 Minarik et al[25] *(field study)*	United States of America	Common carp *(Cyprinus carpio)*, adults, ♂♀	Field study (wild fish)	WWTP effluent channel (one site) + downstream from WWTP (one site)	Upstream from WWTP (one site) and reference lake (one site)	N/A	No, 12-21♂ and 19-49♀ per site	12-49 fish per sex and site (4 sites)
Comment: *Only the field study was included in this review since no statistically significant histological endpoints were reported from the laboratory exposure.*								
6 Scott et al[36]	Australia	Mosquitofish *(Gambusia holbrooki)*, –,♂♀	Field study (wild fish)	Downstream WWTP outfall (one site)	Reference area (two sites)	N/A	No, 10-29♂ and 14-19♀ per site	2-11 fish per sex and site (3 sites)
Comment: *Only the site downstream from WWTP and the reference sites were included in this review. Only two fish sampled for histology at one site.*								
7 Beghin et al[3]	Belgium	Rainbow trout *(Oncorhynchus mykiss)*, 9 months, ♂♀	Field study (caged fish in river)	Downstream from WWTP outfall (one site)	Upstream from WWTP outfall (one site)	21 days	Yes, 3 cages per site, 14 fish in each cage (42 fish per site)	Presumably 12-16 female fish per site (2 sites, see comment)
Comment: *The initial sampling of fish before exposure (T0) was deemed irrelevant for this review. Only females were analyzed due to too few males. It was somewhat unclear how many fish were analyzed histologically. There were no significant differences between the responses recorded between the three replicate cages at each site (based on few analyzed fish per cage), hence each fish was considered the experimental unit, potentially inflating statistical power. A nested statistical approach would have been more appropriate.*								

Abbreviations: -, Information missing; N/A, Not applicable since wild free-living fish were sampled.

sufficient to allow reasonably steady states of internal exposure concentrations to be achieved.[6] On the contrary, one cannot exclude that certain histological changes could take longer to manifest, particularly at lower concentrations. Additional details on the respective study designs are presented in Table 3.

Regarding the histological analysis in both reviews, most of the articles (11/14) did not report if the scoring was done with the evaluator unaware of the treatment (Tables 4 and 5). Due to the subjective nature of histological scoring where a semiquantitative approach is common, it is important to minimize observer bias by masking the slides before the final analysis. Blinded scoring was, however, not used as an inclusion criterion since it was suspected to exclude too many papers, which unfortunately could be confirmed afterwards with only 3 of 14 claiming a blinded evaluation. Some of the articles reported which histological diagnoses they would analyze, and this varied between one diagnosis to more than ten different diagnoses. Others applied a more general but unspecific approach and stated they would analyze "histological alterations." This could be problematic since lesion severity, and the experience level of the examiner will affect the likelihood of a finding to be detected. In addition, it is not always the case of identifying an entity with increased magnitude but also detecting what is missing. Therefore, a histological investigation with "no findings" is not always equal to no effect. As clearly demonstrated in the review by Wolf,[39] regarding morphologic findings involving fish exposures to diclofenac, out of 12 papers with a total of 62 claimed histological findings (electron microscopy analysis excluded), less than 15% were judged as credible or highly credible. Approximately, 65% of the findings were judged as having no or dubious credibility. Although that study was funded by a company that markets products with diclofenac and by a single author, we judge the reliability of the credibility assessment as high. This is not only due to Wolf's recognized experience in fish histopathology, but also from our own previous study independently raising very similar critique about the histological assessment in some of the reviewed papers.[27] We strongly recommend describing which diagnoses have been evaluated (eg, necrosis, vacuolation, and inflammation) and not only "histological alterations." This will make the reader aware if a specific finding has been evaluated or not. Such standardization will also decrease subjectivity since the same diagnoses are evaluated in all slides.

Some of the histological findings reported from the articles were consistent within and between the exposure types but contradictory diagnoses were also identified. In 2/14 articles, no histological figures were provided. Even though it is not mandatory to include histological figures in papers analyzing histology, we strongly encourage it. This will facilitate the possibility for the reader to not only evaluate the histological quality of the samples but also allows for an external evaluation of the diagnosis. While single images are not sufficient to reliably confirm a diagnosis, they play an important role in supporting (or contradicting) claimed diagnoses. Since article space is limited, there is always the possibility to include large amounts of data including histological figures as supplementary material. The reported histopathological diagnoses will be reviewed further in the next section.

Evaluation of Reported Histological Changes

All diagnoses from Tables 4 and 5 are divided by organ and exposure type, with similar diagnoses grouped together (Table 6). The majority of the included histological images were of both adequate image and histological specimen quality. However, the overall weight of evidence for respective histological claim was considered low in 15/33 cases (Table 6). Three histological diagnoses showed some overlap between wastewater and NSAID-exposed fish but there were also diagnoses with contradictory results. Hepatocellular necrosis, hepatocellular vacuolation and developing nephrons/basophilic clusters (DNs/BCs) were reported in both exposure types. The overlaps, however, should be viewed considering the limitations pointed out in Tables 4 and 5 where both reliability and relevance are questionable in several of the studies. This is further evaluated below the respective diagnosis. To illustrate overlapping histological findings as well as renal hematopoietic hyperplasia, we include photomicrographs from our own collections of slides from three-spined stickleback. Reports of no significant effects are listed in Tables S9 and S10.

Hepatocellular Necrosis

Hepatocellular necrosis (Figure 1A and B) in fish can be caused by a wide range of etiologies, both infectious and non-infectious but it is also frequently overdiagnosed in the scientific literature.[40] Pinto et al[29] and Beghin et al[3] both reported hepatocellular necrosis to be increased after exposure to treated municipal wastewater and Baumann et al[1] after NSAID exposure (Table 6). None of the two wastewater studies provide histological images that conclusively support their claims. In the study of Beghin et al,[3] the liver is stained with an untraditional stain, nuclear fast red and picro-indigo-carmine, why we prefer to be a bit cautious in our interpretation. There are some signs that could point to cellular alterations, such as focal loss of cell nuclei and occasional more dark-stained hepatocytes. However, in our view, they are not typical necrotic cells and artifacts cannot be excluded. The image intended to illustrate necrosis in the study by Pinto et al[29] was unfortunately not of sufficient quality to support the proposed finding. In addition, Pinto et al[29] did not take the sex of the wild-caught fish into consideration. The hepatosomatic index was also significantly larger in downstream fish, possibly related to different sex ratios and/or exposure to estrogenic substances. This bears relevance as hepatocellular necrosis of reproductively active female fish has been described previously by Wolf and Wheeler.[43] This is only one of the reasons why sex of the fish should be accounted for in histopathological investigations. Beghin et al[3] analyzed females only but applied a statistical approach with exaggerated power. Baumann et al[1] provided adequate images of high quality supporting the

Table 3. Summary of study design in the NSAID articles.

Paper no. Reference	Country	Species, age at study start, sex	Study design	Treatment: Exposure (nominal concentrations)	Treatment: Control	Exposure time	Replication and number of fish in study	Number of fish analyzed histologically
8 Hoeger et al[17]	Germany	Brown trout (*Salmo trutta)*, 18 months, –	Laboratory study (fish in aquaria)	Diclofenac (0.5, 5, and 50 µg/L)	Lake water	21 days	No replicate aquaria, 36 fish per treatment	6 fish per tissue and treatment (4 treatments)
9 Mehinto et al[22]	United Kingdom	Rainbow trout (*Onchorhynchus mykiss)*, juveniles, ♀	Laboratory study (fish in aquaria)	Diclofenac (0.5, 1, 5, and 25 µg/L)	"Water control"	21 days	2 replicate aquaria, 15 fish in each (= 30 fish per treatment)	10 fish per tissue and treatment (5 treatments)
10 Näslund et al[27]	Sweden	Three-spined stickleback *(Gasterosteus aculeatus)*, 4-5 months, ♂♀	Laboratory study (fish in aquaria)	Diclofenac (5, 20, 80, and 320 µg/L)	Tap water	28 days (320 µg/L-21 days)	3 replicate aquaria, 12 fish in each (= 36 fish per treatment)	19-27 fish (kidney)/12-17 fish (liver) per treatment (5 treatments)
Comment: *Paper 10 originates from the authors of the present review.*								
11 Bickley et al[4]	United Kingdom	Fathead minnow *(Pimephales promelas)*, 11 months, ♂	Laboratory study (fish in aquaria)	Diclofenac (0.2, 1, 5, and 25 µg/L)	Dechlorinated, filtered and UV-treated mains water	21 days	2 replicate aquaria, 24 fish in each (= 48 fish per treatment)	16 fish per treatment (5 treatments)
12 Baumann et al[1]	Germany	Zebrafish (*Danio rerio)*, 8 months, ♂♀	Laboratory study (fish in aquaria)	Acetyl salicylic acid (10, 50, 75, and 100 mg/L)	Not stated	21 days	3 replicate aquaria, 20 fish in each (= 60 fish per treatment)	15♂ and 15♀ per treatment (5 treatments)
13 Näslund et al[26]	Sweden	Three-spined stickleback *(Gasterosteus aculeatus)*, 8-9 months, ♂♀	Laboratory study (fish in aquaria)	Naproxen (20, 80, 320, and 1280 µg/L)	Tap water	27 days (1280 µg/L-21 days)	3 replicate aquaria, 20 fish in each (= 60 fish per treatment)	28-44 fish (kidney)/24-41 fish (liver) per treatment (5 treatments)
Comment: *Paper 13 originates from the authors of the present review.*								
14 Birzle et al[5]	Germany	Rainbow trout (*Onchorhynchus mykiss)*, 1.5 years, ♂♀	Laboratory study (fish in aquaria)	Diclofenac (0.1, 0.5, 1, 5, 25, and 100 µg/L)	Spring water	28 days	2 aquaria per treatment with 11♂ in one aquarium and 11♀ in the other (=22 fish per treatment)	10♂ and 10♀ per treatment (7 treatments)

Abbreviation: -, information missing.

Table 4. Summary of histological analysis in the wastewater articles.

Paper no. Reference	Organ: Histological endpoints examined	Reported statistically significant histological endpoints (LOEC) The arrows indicate an increase (↑) or a decrease (↓) of the diagnosis	Claimed blinded histological scoring
1 **Gisey et al[14]**	**Liver:** Hepatocellular vacuolation	**Liver:** ↓ Hepatocellular vacuolation	No
	Kidney: Not analyzed	**Kidney:** Not analyzed	
Comment: *Only one (of four) exposure site was statistically different to only one (of two) of the reference sites. There was an apparent relation between weight and vacuolation score, especially in males (compare Figures 2 and 12 in Gisey et al[14]), suggesting that any effect on vacuolation could be a consequence of growth/size that differed widely between sites.*			
2 **Liney et al[21]**	**Liver:** Not analyzed	**Liver:** Not analyzed	NO
	Kidney: "Abnormalities," tubule diameter, number of glomeruli/ field of view (FOV), number of DNs/FOV, number of BCs/FOV	**Kidney:** ↑Tubule diameter ↑Number of DNs/FOV ↑Number of BCs/FOV	
Comment: *Potential influence by differences in fish size and maturational stage after the long exposure (300 days) on measured parameters were not addressed. Field of view (FOV) was not specified/defined, making it somewhat unclear if the analyzed kidney area was identical between all fish.*			
3 **Pinto et al[29]**	**Liver:** Necrosis, hydropic vacuolation, lipid vacuolation, vacuolation foci, unspecific granuloma, lymphocytic foci, disaggregated cells foci, macrophage aggregate, perivascular necrosis, vacuolation, parasitic granuloma	**Liver:** ↑Hepatocellular necrosis (in gudgeon)	NO
	Kidney: Not analyzed	**Kidney:** Not analyzed	
Comment: *Hepatocellular necrosis was not assessable due to too low resolution in the figure. Two different P values ($P < .001$ and $P < .05$) were reported for the endpoint necrosis.*			
4 **Tetreault et al[37]**	**Liver:** Not analyzed	**Liver:** Not analyzed	No
	Kidney: Accumulation of inflammatory cells by measuring proximal tubule area and Bowman's capsule area	**Kidney:** ↑ Proximal tubule area (♂♀ 2006 and ♂ 2007) ↑Proximal tubule outer area (♂♀ 2006 and ♂ 2007) (↑Bowman's capsule area (♀ 2007) $P \leq .063$) ↑Bowman's space area (♀ 2007)	
Comment: *Information was missing regarding methodology (staining, selection/measuring of tubuli/Bowman's capsule). While effect patterns on tubuli were consistent across years and sexes, this was not the case for effect patterns on glomeruli and Bowman's capsule. The authors unconventionally claimed $P \leq .063$ as significant, despite a stated α-value of $< .05$. In the discussion section, a reduction of Bowman's space was claimed but the figure showed a statistically significant increase, and only in one year and only in females.*			
5 **Minarik et al[25]**	**Liver:** Vacuolation, "presence/absence of eosinic staining/ proteinaceous fluid"	**Liver:** ↑ Hepatocellular vacuolation	No
	Kidney: Not analyzed	**Kidney:** Not analyzed	
Comment: *No histological figures were included. Histological scores (ordinal variables) were incorrectly treated as continuous variables in the statistical analysis. Data was not reported on differences in sizes between sites of wild-caught fish, potentially influencing hepatocellular vacuolation.*			

(continued)

Table 4. (continued)

Paper no. Reference	Organ: Histological endpoints examined	Reported statistically significant histological endpoints (LOEC) The arrows indicate an increase (↑) or a decrease (↓) of the diagnosis	Claimed blinded histological scoring
6 **Scott et al**[36]	**Liver:** Fat storage ("fatty liver"), granulomas, liquification/ hemorrhage, degenerative fatty necrosis	**Liver:** ↑ Fat storage	No
	Kidney: Liquification/hemorrhage, inclusions	**Kidney:** No statistically significant differences	
Comment: *The authors used quantitative image analysis. The claim that vacuoles in the liver were fat was based on HE-stain, which is inconclusive. Fat storage was only significantly increased when compared with one of the two reference sites. No results for degenerative fatty necrosis or granulomas were reported. The assignment of potential significant differences was unclear.*			
7 **Beghin et al**[3]	**Liver:** Melanomacrophage centers (MMC), fibrosis, vessel alteration, hepatocyte vacuolation, altered/lytic or necrotic hepatocytes, glycogen content	**Liver:** ↑ Cellular death ↓Glycogen deposit ↓ MMC ↑ Fibrosis ↑ Vessel alteration	No
	Kidney: Not analyzed	**Kidney:** Not analyzed	
Comment: *The authors used an untraditional staining for the liver (nuclear fast red [NR] and picro-indigo-carmine [PIC]) The claimed endpoints were not adequately supported in figures: Cells identified as necrotic were not typical; For glycogen deposits, alternative suitable staining (PAS) was used and assessed through quantitative image analysis, but no figures were shown. Also, no data on variability (eg, standard deviations) were reported for this endpoint (Table 6 in Beghin et al*[3]*). For MMC, it is unclear if all claimed MMCs are correctly identified (Figure 3D). Neither fibrosis nor vessel alterations were apparent from the figures. While hepatocyte vacuolation was investigated according to the methods section, no results were presented on this endpoint. Histological scores (ordinal variables) were incorrectly treated as continuous variables in the statistical analysis (not applying to glycogen deposits).*		

Table 5. Summary of the histological analysis in the NSAID articles.

Paper no. (NSAID) Reference	Organ: Histological endpoints examined	Reported statistically significant histological endpoints (LOEC) The arrows indicate an increase (↑) or a decrease (↓) of the diagnosis	Claimed blinded histological scoring
8 **(DCF)** **Hoeger et al**[17]	**Liver:** Histopathological alterations **Head and trunk kidney:** Histopathological alterations	**Liver:** ↑ Monocyte infiltration/accumulation (5 µg/L) **Trunk kidney:** ↑ Interstitial proteinaceous fluid (50 µg/L) ↑ Tubular necrosis (50 µg/L)	No
Comment: *No histological figures were included. There were no reported alterations in any control fish (questionable). There was no dose-response pattern in monocyte infiltration/accumulation. A reevaluation by a PWG found no exposure-related findings in the liver or kidney, or no tubular necrosis in the examined sections.*[42] *Monocyte infiltration was graded as "dubious credibility" (score 2 of 5) and interstitial proteinaceous fluid was graded as "no credibility" (score 1 of 5) by Wolf.*[39]			
9 **(DCF)** **Mehinto et al**[22]	**Liver:** Histological alterations **Kidney:** Histological alterations	**Liver:** No visible lesions **Kidney:** ↑ Number of DNs (5 µg/L) ↓ Bowman's space (5 µg/L) ↑ Tubular necrosis (25 µg/L)	No
Comment: *Tubular necrosis was not supported in the figure. A reevaluation by a PWG found no exposure-related findings in liver or kidney.*[42] *Increased DNs was graded as "dubious credibility" (score 2 of 5), decreased Bowman's space and tubular necrosis was graded as "no credibility" (score 1 out of 5) by Wolf.*[39]			
10 **(DCF)** **Näslund et al**[27]	**Liver:** Hepatocellular vacuolation, inflammatory cell foci, PMA, parasites **Kidney:** Hematopoietic tissue, tubular necrosis, tubular regeneration, PMA, tubular hyaline degeneration/ droplets, parasites	**Liver:** No statistically significant differences **Kidney:** ↑ Hematopoietic hyperplasia (4.6 µg/L)	Yes
Comment: *Paper 10 originates from the authors of the present review. The sex of the fish was not taken into consideration in the histological evaluation. Increased hematopoietic tissue was rated as "credible" (score 4 of 5) by Wolf.*[39]			
11 **(DCF)** **Bickley et al**[4]	**Liver:** Not analyzed **Kidney:** Number of glomeruli per mm^2, number of BCs and DNs per mm^2	**Liver:** Not analyzed **Kidney:** ↑ Number of BCs and DNs per mm^2 (N/A)	Yes
Comment: *Only correlations were performed, hence no LOECs or NOECs were derived. The provided images with different magnifications complicate the comparisons. Increased DNs was graded "equivocal credibility" (score 3 of 5) by Wolf.*[39]			

(continued)

Table 5. (continued)

Paper no. (NSAID) Reference	Organ: Histological endpoints examined	Reported statistically significant histological endpoints (LOEC) The arrows indicate an increase (↑) or a decrease (↓) of the diagnosis	Claimed blinded histological scoring
12 **(ASA)** **Baumann et al**[1]	**Liver:** Hepatocyte necrosis, hepatocellular vacuolation, cystic degeneration, hyalinized hepatocytes, inflammatory cells	**Liver:** ↑ Single-cell necrosis (75 mg/L) ↑ Bile duct hyperplasia (75 mg/L) ↑ Hepatocyte hyalinization (75 mg/L)	No
	Kidney: Not analyzed	**Kidney:** Not analyzed	
Comment: *Note the high effect-concentrations.*			
13 **(NPX)** **Näslund et al**[26]	**Liver:** Hepatocellular vacuolation, inflammatory cell foci, PMA, hepatocellular necrosis, parasites	**Liver:** ↓ Hepatocellular vacuolation (1232 µg/L)	Yes
	Kidney: Hematopoietic hyperplasia, tubular necrosis, tubular regeneration, PMA, tubular hyaline degeneration/ droplets, parasites	**Kidney:** ↑ Hematopoietic hyperplasia (299 µg/L)	
Comment: *Paper 13 originates from the authors of the present review. The sex of the fish was not taken into consideration in the histological evaluation.*			
14 **(DCF)** **Birzle et al**[5]	**Liver:** Not analyzed	**Liver:** Not analyzed	No
	Kidney: Relative and absolute interstitial volume, relative and absolute nephron volume, absolute kidney volume, kidney-to-body weight ratio	**Kidney:** ↑ Relative and absolute interstitial volume (25 µg/L) ↓ Relative nephron volume (25 µg/L) ↓ Absolute nephron volume (0.5 µg/L)	
Comment: *The authors used quantitative stereology. Increased hematopoietic tissue (largely equivalent to interstitial volume) and decreased nephron volume were rated as "credible" (score 4 of 5) based on the material included in the PhD-thesis underlying this publication.*[39]			

Table 6. Overview of the claimed significant histological changes from studies of fish exposed to treated municipal wastewater or NSAIDs alone.

Treated municipal wastewater				NSAID			
Liver Histological diagnosis (paper no.)	Adequate quality of histological image	Adequate quality of histological specimen	Weight of evidence for histological claim	Liver Histological diagnosis (LOEC [paper no.])	Adequate quality of histological image	Adequate quality of histological specimen	Weight of evidence for histological claim
↑ Hepatocellular necrosis (3)	***No***	***N/A***	***Low***	***↑ Single-cell necrosis (ASA 75 mg/L [12])***	***Yes***	***Yes***	***High***
↑ Cellular death (7)	***Yes***	***Yes***	***Low***				
↓ Hepatocellular vacuolation (1)	***No***	***N/A***	***Low***	***↓ Hepatocellular vacuolation (NPX 1232 µg/L [13])***	***Yes***	***Yes***	***High***[a]
↓Glycogen deposit (7)	—	—	***Moderate***				
↑ Hepatocellular vacuolation (5)	—	—	Low				
↑Fat storage (6)	Yes	Yes	Low				
↑ Fibrosis (7)	Yes	Yes	Low	↑ Monocyte infiltration/accumulation (DCF 5 µg/L [8])	—	—	Low
↓ MMC (7)	Yes	Yes	Low	↑ Hepatocyte hyalinization (ASA 75 mg/L [12])	—	—	Moderate
↑ Vessel alteration (7)	Yes	Yes	Low	↑ Bile duct hyperplasia (ASA 75 mg/L [12])	—	—	Moderate
Kidney Histological diagnosis (paper no.)				Kidney Histological diagnosis (LOEC [paper no.])			
↑Number of DNs/FOV (2)	***Yes***	***Yes***	***Moderate***	***↑ Number of DNs (DCF 5 µg/L [9])***	***Yes***	***Yes***	***Moderate***
↑Number of BCs/FOV (2)	***Yes***	***Yes***	***Moderate***	***↑ Number of BCs or DNs /mm² (DCF N/A [11])***	***Yes***	***Yes***	***Moderate***
↑Bowman's space area (4)	Yes	Yes	Low	↓ Bowman's space (DCF 5 µg/L [9])	Yes	Yes	Low
(↑Bowman's capsule area (4))	Yes	Yes	Low				
↑Tubule diameter (2)	Yes	Yes	Moderate	↑ Hematopoietic hyperplasia (DCF 4.6 µg/L [10])	Yes	Yes	High[a]
↑ Proximal tubule area (4)	Yes	Yes	Moderate	↑ Hematopoietic hyperplasia (NPX 299 µg/L [13])	Yes	Yes	High[a]
↑ Proximal tubule outer area (4)	Yes	Yes	Moderate	↑ Relative interstitial volume (DCF 25 µg/L [14])	Yes	Yes	High
				↑ Absolute interstitial volume (DCF 25 µg/L [14])	Yes	Yes	High
				↓ Relative nephron volume (DCF 25 µg/L [14])	Yes	Yes	High
				↓ Absolute nephron volume (DCF 0.5 µg/L [14])	Yes	Yes	High
				↑ Tubular necrosis (DCF 50 µg/L [8])	—	—	Low
				↑ Tubular necrosis (DCF 25 µg/L [9])	Yes	Yes	Low
				↑Interstitial proteinaceous fluid (DCF 50 µg/L [8])	—	—	Low

Related diagnoses are grouped. Text in combined boldface and italics further indicate similar effects reported both in fish exposed to wastewater and to NSAIDs.
[a]This study originates from the authors of the present review.
Abbreviations: ASA, acetyl salicylic acid; DCF, diclofenac; NPX, naproxen; N/A, not assessable due to inadequate quality of histological figure; -, no histological image representing endpoint included in article.

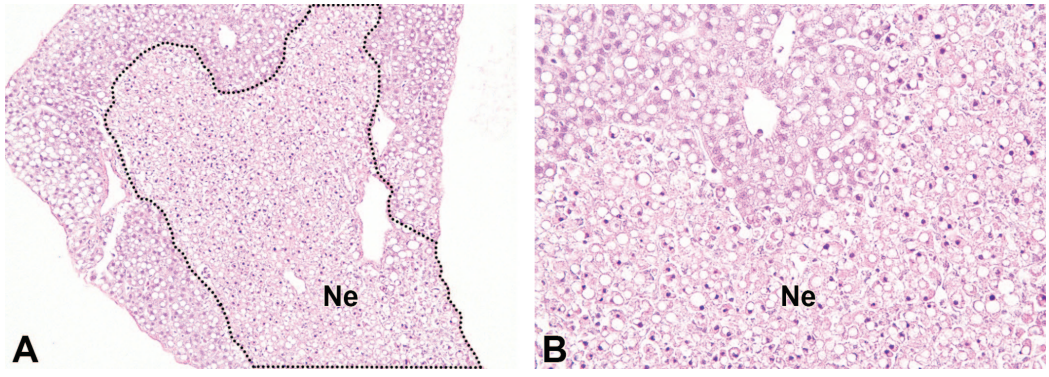

Figure 1. Hepatocellular necrosis in the three-spined stickleback (*Gasterosteus aculeatus*). (A) Centrally, there is a large necrotic area (Ne, dashed line) with disintegrated cells with pale eosinophilic cytoplasm, karyolysis, and nuclear pyknosis. There is also a generalized hepatocellular vacuolation of lipid type (clear round cytoplasmatic vacuoles) across the entire section. A previous parasite migration was the suspected etiology of the necrosis. H&E, original objective 20X. (B) Higher magnification of the necrotic area (Ne) from the liver section in A. H&E, original objective 40X.

diagnosis and supported even further by an evident dose-response. However, the LOEC/NOEC (lowest observed effect concentration/no observed effect concentration) were 75,000/50,000 µg/L (acetyl salicylic acid), which is well above concentrations found in treated municipal wastewaters. In addition, in our own studies, only one case of liver necrosis was detected in close to 250 sticklebacks exposed to two different NSAIDs. The sole case of necrosis detected was most likely caused by a previous parasite migration.[26,27] Taken together, the support that treated municipal wastewater can cause hepatocellular necrosis and that this finding is caused by NSAIDs is low.

Hepatocellular Vacuolation

Hepatocellular vacuolation is a commonly scored endpoint in fish histology but evaluation demands caution due to high variability of the "normal" level. Differences in the level of vacuolation are, for example, due to species, sex, and nutritional and reproductive status. The appearance of the vacuoles can provide a hint on their origin (glycogen or lipid), but special stains are required for correct identification. Figure 2A to D demonstrates different levels and types of hepatocellular vacuolation in the three-spined stickleback. Two wastewater studies and one NSAID study identified a decrease in hepatocellular vacuolation or a related diagnosis (glycogen storage) (Table 6). Giesy et al[14] studied caged fish upstream and downstream from WWTPs and reported a statistically significant decrease only at one out of four exposure sites and only when compared with one of the two reference sites. However, the observed difference was similar for both females and males, but there was an apparent relation between weight and vacuolation score, which suggest that vacuolation could be a consequence of growth/size which differed widely between sites. In addition, the images provided were not of sufficient quality to support the diagnosis. Beghin et al[3] studied caged fish upstream and downstream from one WWTP. They claimed they evaluated and scored hepatocyte vacuolization, but no data or conclusions were presented. They reported decreased glycogen, but no supportive images were included. However, they used periodic acid Schiff (PAS) staining to identify glycogen and quantitative image analysis to evaluate the amount in the liver, which is considered a good approach. While claiming a significant difference between the two sites, results were presented without providing any measure of variance.

Näslund et al[26] (own study) identified a decrease in hepatocellular vacuolation after exposure to naproxen. The study design included three replicate aquaria with both females and males, but the sex of the fish was not accounted for in the statistical comparisons. However, this has been investigated in retrospect, and including sex in the statistical analyses did not affect the previously reported conclusions (unpublished data). Critically, the LOEC of naproxen was 1232 µg/L, which is much higher than levels reported in treated municipal wastewater.

In addition, even though Memmert et al[23] did not report any significant histological changes in trout liver after chronic exposure to diclofenac and hence, are not included in this review, a pathology working group (PWG) reanalyzed the histological slides and identified decreased glycogen as a novel finding.[42] No histological images were included in the original article, or in the PWG article. The LOEC was 1000 µg/L, which is in line with the findings for naproxen by Näslund et al.[26] In conclusion, there is some evidence that wastewater exposure leads to decreased hepatocellular vacuolization/glycogen storage, noting that both Minarik et al[25] and Scott et al[36] report the opposite. The latter studies investigated wild fish which complicates comparisons. Decreased hepatocellular vacuolation after NSAID exposure is a reliable finding, but it has only been demonstrated at around 1 mg/L or higher. Hence, the relative

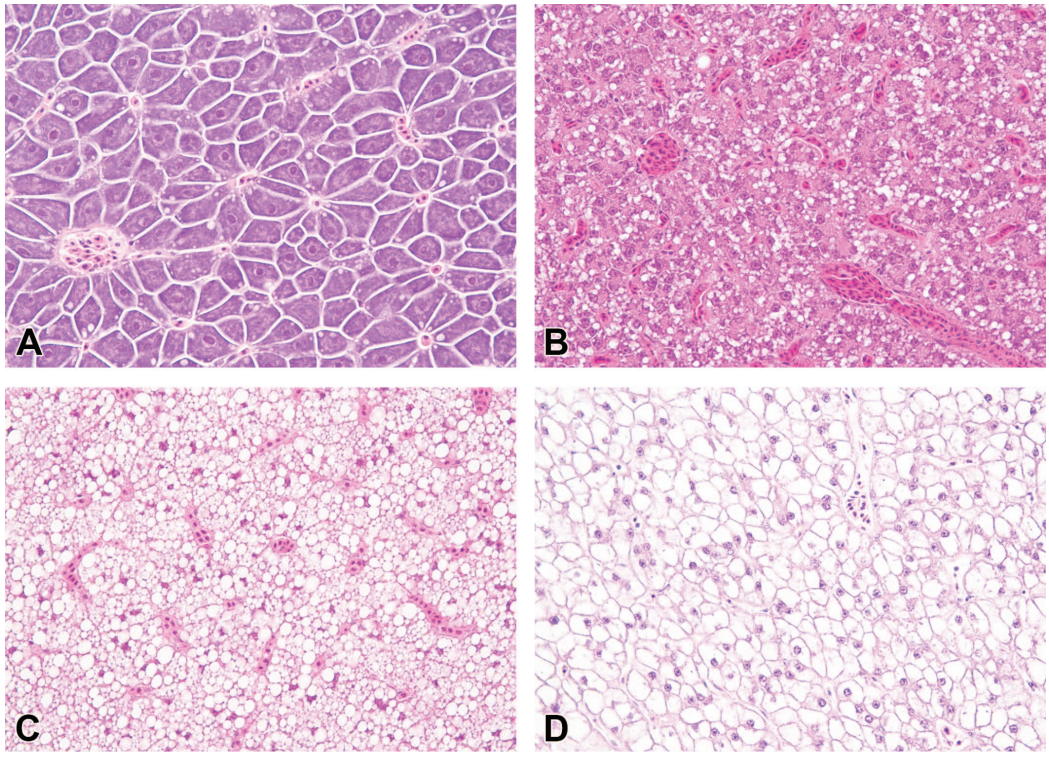

Figure 2. Liver from three-spined stickleback (*Gasterosteus aculeatus*) with different degrees of vacuolation. (A) Reproductively active female with minimal vacuolation, and hypertrophied, basophilic hepatocytes. (B) Reproductively active male with a higher degree of hepatocellular vacuolation and a more eosinophilic cytoplasm compared to the female in A. (C) Reproductively inactive male with extensive hepatocellular vacuolation with clear round vacuoles in the cytoplasm suggestive of a lipid origin. The nucleus is commonly located more peripherally, but this is not always easily discerned. (D) Reproductively inactive male with extensive hepatocellular vacuolation with more irregular shapes of the vacuoles and occasionally a more centrally located nucleus suggestive of glycogen type. H&E, original objective 40X (A-D).

insensitivity makes it implausible that NSAIDs in treated municipal wastewaters could cause such effects. As mentioned earlier, several factors such as species, sex, and reproductive and nutritional status can all affect hepatocellular vacuolation. A preferred strategy is therefore to account for such factors either experimentally or in the statistical analysis.

Developing Nephrons/Basophilic Clusters

Renal tubuli can regenerate after injury but the fish kidney has also the ability to develop new nephrons. DNs arise from BCs and the presence of both BCs and DNs in histological sections (Figure 3A and B), can be a normal finding, especially in growing animals.[12] However, Reimschuessel et al[32] demonstrated an increase of DNs/BCs after renal injury. Increasing numbers of DNs/BCs were reported in roach exposed for 300 days to treated wastewater[21] and in rainbow trout[2] and fathead minnow[4] exposed to diclofenac. The image quality and the quality of the histological specimens were considered adequate for all three articles and DNs/BCs could be identified in the provided figures.

In Figure 4 in the article by Liney et al[21], the kidney from exposed fish is approximately twice as thick compared to the depicted control. Accordingly, the author mentioned significant differences in fish size between treatments. Hence, differential growth over the 300 days exposure could very well have led to secondary effects, including differences in the maturational stage of the kidneys. Furthermore, the field of view (FOV) was not defined, which raises the question whether the absolute number (eg, per cross-section, which in turn is inherently related to fish size) or the relative number (eg, per area unit) were reported.

Mehinto et al[22] reported a clear concentration-response, with significant increased number of DNs at 5 and 25 µg/L, but not

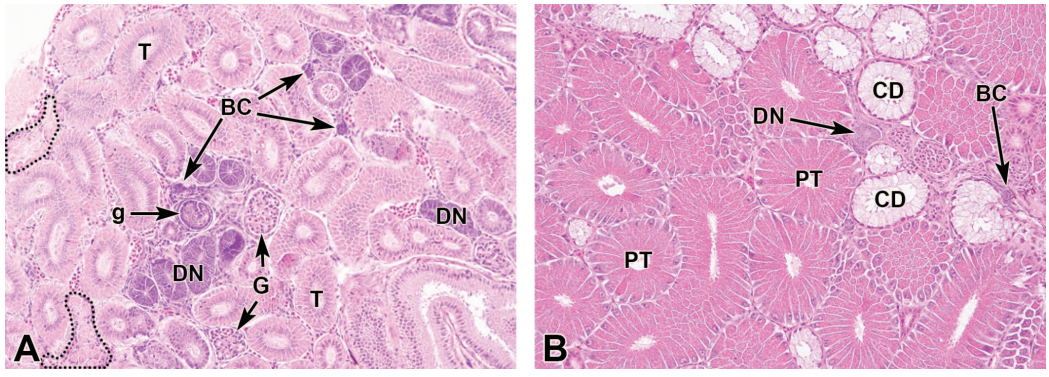

Figure 3. Kidney with nephron neogenesis in the three-spined stickleback (*Gasterosteus aculeatus*). The renal tubules may appear very different in reproductively active male sticklebacks compared to females or to reproductively inactive male sticklebacks. (A) Kidney from a reproductively active female stickleback, basophilic clusters (BCs) and developing nephrons (DNs) are easily recognized in H&E. A newly formed glomerulus (g) is also present and can be compared with older glomeruli (G). Numerous tubuli (T) and two pigmented macrophage aggregates (dashed line) are also visible. (B) Kidney from a reproductively active male stickleback. The proximal tubuli (PTs) are hypertrophied and more eosinophilic due to spiggin accumulation. Basophilic clusters (BCs) and developing nephrons (DNs) are also visible and are of comparable sizes as in A, hence demonstrating the massive increase in tubuli size. The collecting ducts (CDs) are clear as they contain large amounts of acidic mucus. H&E, original objective 20X (A-B).

at lower concentrations. Histological slides from a subset of fish (5) from the control group and the groups exposed to 0.5 and 1 µg/L were reevaluated blindly by the PWG mentioned earlier.[42] No significant differences were observed, which is in agreement with the original study at these concentrations. Histological sections from the 5 and 25 µg/L groups were additionally reevaluated by a single reviewing pathologist who reported no increase of DNs at any concentration but without providing a fully comparable quantitative comparison across all groups.[42] Notably, it was pointed out that slides from 40% of the fish in the study did not cover any or enough urinary kidney tissue for diagnostic purposes. This raises the question of what part of the kidney that was studied in the original study.

Bickley et al[4] reported the relative amount of DNs and BCs to the cross-sectional area. However, they sectioned the kidney transversely, and at several levels along the longitudinal axis, but it is unclear which levels they have compared.

In conclusion, an increase of DNs and BCs were reported at considerably lower NSAID concentrations than the histopathological changes in the liver, but the reliability of the finding in both Mehinto et al[22] and Bickley et al[4] is not without doubt. In addition, if the increase of DNs or BCs is due to toxic insult to the kidney, one might expect other histological signs of this, such as vacuolation or necrosis of tubuli cells. Mehinto et al[22] do indeed claim increased tubular necrosis, but this finding is neither supported by the histological images provided in the paper, nor was it detected by the PWG reanalysis mentioned earlier. The reliability of the findings by Liney et al[21] is also uncertain. Although not completely dismissed, the evidence for NSAID residues causing an increased number of DNs or BCs in fish exposed to treated municipal wastewater is weak.

Other Diagnoses

Among all histopathological diagnoses identified in this review, one diagnosis particularly worth mentioning is renal hematopoietic hyperplasia (Figure 4A and B). As stated by Wolf et al[40] some diagnoses, including renal hematopoietic hyperplasia, have not been traditionally evaluated in the past and hence there is a risk of them being underreported. We have earlier demonstrated an increase in renal hematopoietic hyperplasia, both after exposure to diclofenac and naproxen with an LOEC for diclofenac at 4.6 µg/L and for naproxen at 299 µg/L.[26,27] The paper by Birzle et al[5] also supports this where both the absolute and relative volume of interstitial tissue, which mainly consists of hematopoietic tissue, were increased and the corresponding finding of an absolute and relative volume of nephron tissue was decreased. The LOEC for the most sensitive endpoint was 0.5 µg/L of diclofenac. This was analyzed by quantitative image analysis, a method that may be highly sensitive and have potential to overcome some sources of subjective errors. As stated by Wolf et al,[42] 40% of the kidney sections examined in Mehinto et al[22] consisted mainly or solely of hematopoietic kidney where few or no urinary elements were found. If this was due to differences in sampling levels along the longitudinal axis of the kidney, or an actual increase in hematopoietic tissue with concurrent decrease of urinary tissue remains unknown. Unfortunately, none of the papers in the municipal wastewater review reported an evaluation of this specific lesion and there is therefore a risk that this might be overlooked.

Conclusion

This study investigated whether there is support that fish exposed to treated municipal wastewater develop similar histological

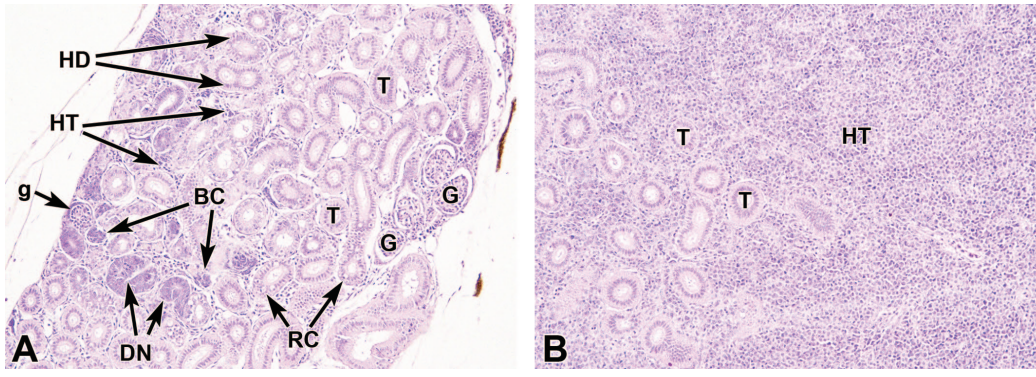

Figure 4. Kidney from three-spined stickleback (*Gasterosteus aculeatus*) with different degrees of hematopoietic hyperplasia. Hyperplasia of hematopoietic tissue can occur as a general response to different stimuli, both infectious and non-infectious. (A) Renal hematopoietic hyperplasia scored as minimal (normal level in the three-spined stickleback) with barely visible hematopoietic tissue (HT). Tubuli (T), glomeruli (G), developing nephrons (with several mitoses) (DNs), basophilic clusters (BCs), a newly formed glomerulus (g), hyaline droplets (HDs), and rodlet cells (RCs) are also seen. (B) Renal hematopoietic hyperplasia scored as severe. There is a marked proliferation of hematopoietic tissue (HT) and very few tubuli (T) can be found. Due to the hyperplasia, the kidney is much larger than in the fish illustrated in A and therefore extends beyond the entire field of view despite the same magnification. Parasagittal section of kidney in situ, H&E, original objective 20X (A-B).

alterations in liver and kidney as those identified after NSAID exposure. Comprehensive literature reviews of relevant articles fulfilling basic criteria including relevant controls and statistical comparisons only resulted in seven studies for each exposure type. Most of the included studies still had significant limitations, such as unclear methodology, lack of histological figures, histological figures with insufficient quality or not supporting the diagnoses, and use of only unrealistically high exposure concentrations, to name a few. Based on this critical review, we find no clear evidence in the literature that histological alterations in fish exposed to treated municipal wastewaters are caused by residues of NSAIDs. However, limitations in both experimental design and reporting standards of fish histopathology studies prevent any firm conclusions. Well-designed studies evaluating effects from wastewater or NSAID exposure, in which as many factors as possible, with regards to experimental design and analysis, have been kept constant, would certainly facilitate interpretations. In addition, given the largely unlimited possibilities to provide supplementary data with today's publications, including histological images of adequate quality, representing both treated and control animals, with both high and low magnification, and with clear notations should not be an issue and are strongly encouraged. The current lack of evidence for effects of NSAID residues on liver and kidney histology in fish does not rule out histopathological effects in other organs, including gills. It also does not preclude other types of effects in fish or in other taxa.

Author Contributions

Johanna Näslund contributed to conceptualization, methodology, formal analysis, investigation, writing—original draft, writing—review and editing, visualization, and project administration.

Leif Norrgren contributed to conceptualization, resources, writing—review and editing, and supervision.

D. G. Joakim Larsson contributed to conceptualization, methodology, investigation, writing—original draft, writing—review and editing, resources, and supervision.

Declaration of Conflicting Interests

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Ethical Considerations

This article does not contain any studies with human or animal participants.

Consent to Participate

Not applicable.

Consent for Publication

Not applicable.

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Supplemental Material

Supplemental material for this article is available online.

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Pharmaceuticals are widely detected in aquatic environments, raising concerns about effects on wildlife. This thesis investigates whether common anti-inflammatory drugs (NSAIDs), such as diclofenac and naproxen, cause tissue damage in fish. Studies in three-spined sticklebacks revealed kidney effects, but mainly at concentrations higher than those typically found in the environment. Diclofenac was more potent than naproxen. No overlapping effects were found between exposure to NSAIDs and treated municipal wastewater. The results suggest that the choice of NSAID influences environmental risk.

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