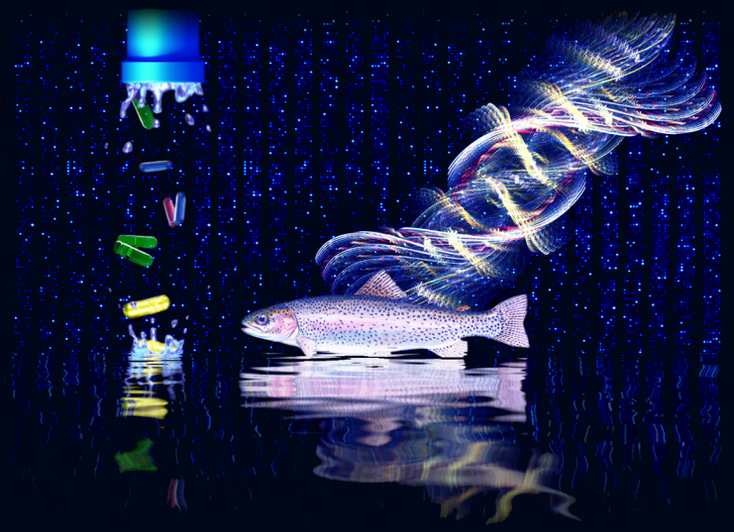
Transcriptomics and bioconcentration studies in fish to identify pharmaceuticals of environmental concern



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Till min familj

Abstract

Pharmaceuticals are frequently found in the aquatic environment. As they are most often highly biologically active, quite persistent and may accumulate in aquatic organisms, i.e. bioconcentrate, they may pose a risk to non-target organisms.

Current knowledge on environmental fate and effects of pharmaceuticals are limited, and traditional risk assessment strategies are insufficient to capture all substances posing risks for wildlife. In this thesis we explored the potential of two additional approaches to assist in the identification of substances of environmental concern. The first involved read-across between therapeutic plasma concentrations in humans and measured plasma levels of pharmaceuticals in exposed fish, in order to predict the risks for pharmacological effects in the fish. The second involved microarray analyses of gene expression to confirm pharmacological interactions, find potential biomarkers and assess the mode of action of pharmaceuticals in exposed fish.

We could show that waterborne diclofenac affects hepatic gene expression in exposed fish at water concentrations reported in treated effluents and surface waters. Pharmacological responses, resembling those found in mammals, were observed in fish at blood plasma concentrations similar to human therapeutic plasma levels, indicating a similar potency and mode of action in fish and humans. In contrast to some other reported results, the bioconcentration factor of diclofenac in fish was found to be stable across exposure concentrations.

Exposure of fish to ketoprofen at concentrations about 100 times higher than those found in treated sewage effluents resulted in plasma concentrations below 1% of human therapeutic plasma levels, suggesting low risk for effects in fish. Accordingly, no effects on hepatic gene expression could be confirmed. However, exposure of fish to complex effluents indicates a higher bioconcentration potential of NSAIDs than does exposure to single substances. Thus, laboratory experiments may underestimate risks in the environment.

Microarray analyses revealed several differentially expressed genes after exposure to conventionally treated effluents. These included estrogen-responsive genes and a biomarker for dioxin-like exposure. Further results included indications of general stress after exposure to all studied ozone treated effluents. Effluents treated with activated carbon resulted in the least responses in exposed fish.

Exposure to the glucocorticoid beclomethasone-diproprionate affected plasma glucose levels and caused oxidative stress in fish. Effects observed in fish resembled effects in humans, supporting read-across between species. Exposure to free beclomethasone did not result in any observed effects, most probably due to its inability to bioconcentrate.

Taken together, both read-across and microarray analyses have proven useful in identifying pharmaceuticals of environmental concern.

Populärvetenskaplig sammanfattning

Läkemedel är oumbärliga verktyg för att lindra, bota och förebygga sjukdomar. Dessvärre bryts många läkemedel inte ner helt i våra kroppar och aktiva substanser kan därmed transporteras vidare via reningsverk ut i våra vattendrag. Vi vet ännu ganska lite om vilka konsekvenserna av dessa utsläpp är, men det finns en uppenbar risk att flera läkemedel kan påverka djurlivet negativt, framför allt i vattenmiljön.

Läkemedel är biologiskt aktiva kemikalier. Det vill säga de är designade eller utvalda för att specifikt kunna påverka utvalda processer i våra kroppar, genom att binda till måltavlor som t.ex. receptorer eller enzymer. Detta innebär emellertid att andra djur som har dessa måltavlor också kan påverkas, om de utsätts för tillräckligt höga koncentrationer. Ett exempel på ett läkemedel som har dokumenterade effekter i miljön är det syntetiska östrogenet i p-piller, som genom att binda till östrogenreceptorn i fisk påverkar deras fortplantning redan vid väldigt låga vattenkoncentrationer. Fisk som lever i vatten där läkemedel hamnar, andas detta vatten och har därmed en risk att ta upp betydande mängder läkemedel från vattnet, och fisken har samtidigt många måltavlor som läkemedel binder till. Därför har vi valt att studera just fisk.

Även om vattenkoncentrationen av läkemedel oftast är väldigt låga, kan vissa läkemedel ändå utgöra ett problem då de ibland har förmågan att ansamlas i vattenlevande djur. Till exempel har ett syntetisk gulkroppshormon, som också används i p-piller, hittats i blodet hos fisk i koncentrationer 10 000 gånger högre än koncentration än de halter man finner i det vatten fiskarna simmat i.

I den här avhandlingen ville vi öka kunskapen kring risker med läkemedel i miljön genom att utvärdera och använda metoder som kan komplettera den traditionella miljöriskbedömningen av läkemedel. Dels utnyttjar vi befintlig kunskap om läkemedels potens och effekter i människa, dels använder vi oss av en modern, storskalig molekylärbiologisk teknik.

För att bedöma om ett läkemedel kan utgöra en risk har vi använt oss av så kallad read-across, eller extrapolering mellan arter (Studie I, II och IV). Detta innebär att vi jämför koncentrationen av ett visst läkemedel i blodet hos exponerad fisk med koncentrationer i blodet hos patienter som tar läkemedlet i fråga. Vi får på så sätt en uppfattning om den faktiska risken för att fisken ska påverkas (på något sätt) av läkemedlet som den exponeras för. Förutsatt är att den tidigare nämnda måltavlan för läkemedlet (t.ex. en receptor) även finns i fisken, men så är oftast fallet.

Om halten av läkemedel i fiskens blod tyder på en hög risk för påverkan, är det dock inte säkert att effekterna kommer att vara detsamma som de vi ser hos människor. För att få reda på mer information om hur läkemedel påverkar fisken har vi studerat genuttrycksmönstret (Studie I, II och III). Aktiviteten eller uttrycket av gener i organismer förändras hela tiden, allt eftersom miljön runt omkring oss förändras, men

det är ändå möjligt att identifiera gener vars aktivitet förändrats på grund av t.ex. läkemedelspåverkan. För att studera genuttrycksmönstret har vi använt microarray-teknik, med vilken man kan studera aktiviteten av tiotusentals olika gener samtidigt. Genom att studera så många gener samtidigt kan vi få en uppfattning om vilka biologiska processer eller system som påverkas av ett läkemedel och på så sätt få information om hur det verkar i fisken. Denna analys ger oss även möjlighet att identifiera genuttrycksförändringar som kan vara mer eller mindre specifika för exponering av en viss substans eller grupp av substanser, så kallade biomarkörer. Sådana markörer kan vara användbara för att spåra om en fisk ute i det fria har blivit exponerad för läkemedel. Dessutom kan vi med hjälp av microarray-analys få indikationer om vid vilken koncentration av läkemedlet som fisken påverkas.

In den första artikeln studerade vi diklofenak, den aktiva substansen i t.ex. Voltaren®, som tillhör gruppen icke-steroida antiinflammatoriska läkemedel, eller NSAIDs. Med hjälp av microarray lyckades vi identifiera förändringar på genuttrycket vid vattenkoncentrationer av diklofenak liknande de som har hittats i miljön. Dessutom såg vi fler och större förändringar i genuttryck ju närmare blodkoncentrationerna i fisken kom de som hittas i blodet hos människor som äter diklofenak. Resultaten tydde också på att t.ex. inflammationsprocesser påverkades i fisken, processer som man vet sedan tidigare påverkas av diklofenak i människa.

Eftersom diklofenak har pekats ut som ett läkemedel med potentiella risker för vattenmiljön var vi också intresserade av att studera risker med en annan NSAID, ketoprofen, som i vissa situationer kan utgöra ett alternativ till behandling med diklofenak, och kanske därför kunde vara säkrare ur miljösynpunkt. I denna andra studie fann vi att ketoprofen ansamlades i betydligt mindre utsträckning i fisken än diklofenak. Vid en vattenkoncentration 100 gånger högre än vad som hittats i miljön nådde koncentrationer av ketoprofen i fiskens blod bara en bråkdel av de halter man finner i blodet hos patienter som tar ketoprofen. Vi kunde heller inte påvisa några förändringar av genuttrycket i dessa fiskar. Detta experiment skulle kunna tolkas som att användning av ketoprofen inte medför någon betydande risk för effekter på fisk i våra vattendrag, i alla fall betydligt mindre risk än vad användning av diklofenak gör. Dock är bilden mer komplex när man väger in andra studier som tyder på att olika NSAIDs, särskilt ketoprofen, tenderar att ansamlas i högre utsträckning i fiskar som exponeras för renat avloppsvatten jämfört med fiskar som utsatts för ett enda läkemedel utspätt i rent vattnet. En möjlig förklaring kan ligga i att det i avloppsvatten finns andra ämnen som skulle kunna påverka upptag och/eller utsöndring av läkemedel. Detta innebär att resultat från laboratorieförsök, såsom vår studie och väldigt många andra studier, riskerar underskatta riskerna ute i miljön där många kemikalier samverkar.

Eftersom dagens reningsverk inte är designade för att ta rena bort läkemedel och andra miljögifter från avloppsvattnet, har det kommit förslag på mer avancerade reningstekniker. I den tredje studien undersökte vi olika avancerade reningsteknikers förmåga att förbättra vattenkvaliteten genom att studera genuttrycksmönstret i fisk som

exponerats för olika avloppsvatten. I fisk som exponerats för konventionellt renat avloppsvatten fanns det tydliga tecken på, bland annat, en påverkan av östrogen. Samtliga avancerade tekniker tog bort denna påverkan. Tre avloppsvatten vi studerade omfattade rening med ozon. Fisk som exponerades för dessa vatten visade tecken på stress, men vi kan inte avgöra om det var en skadlig form av stress. Den teknik som resulterade i avloppsvatten med minst påverkan på fisk var rening med aktivt kol.

I den fjärde studien studerade vi olika fysiologiska effekter hos fisk som exponerats för en glukokortikoid, beklometason. Det är ett läkemedel som används för att behandla astma. Fisken hade ökade blodsockerhalter, vilket även är en känd bieffekt hos patienter som behandlas med glukokortikoider. Dessutom visade fisken tydliga tecken på oxidativ stress, vilket kort innebär att reaktiva syreföreningar som organismen själv producerat riskerar skada celler och organ.

Sammanfattningsvis fann vi stöd för att read-across mellan människa och fisk kan bidra till att identifiera läkemedel med förhöjd miljörisk. I samtliga studier där vi använt oss av microarray analys har vi fått ytterligare information om läkemedels potens i fisk och fått en bättre uppfattning om hur läkemedel påverkar fisk och/eller identifierat möjliga biomarkörer. Dock finns det fortfarande en hel del kunskap att hämta om läkemedels effekter på miljön. De angreppssätt som presenteras i denna avhandling kan bidra till att öka vår förståelse för hur läkemedel påverkar miljön och i slutändan förhoppningsvis leda till en mer hållbar läkemedelsanvändning.

List of publications

This thesis is based on the following articles and manuscripts:

I. **Diclofenac in fish: Blood plasma levels similar to human therapeutic levels affect global hepatic gene expression**
Filip Cuklev, Erik Kristiansson, Jerker Fick, Noomi Asker, Lars Förlin, D.G. Joakim Larsson. *Environmental Toxicology and Chemistry.* 2011. Vol. 30, No. 9, pp. 2126–2134

II. **Does ketoprofen or diclofenac pose the lowest risk to fish?**
Filip Cuklev, Erik Kristiansson, Marija Cvijovic, Jerker Fick, Lars Förlin, D.G. Joakim Larsson. *Submitted*

III. **Global hepatic gene expression in fish exposed to sewage effluents: A comparison of different treatment technologies**
Filip Cuklev, Lina Gunnarsson, Marija Cvijovic, Erik Kristiansson, Carolin Rutgersson, Berndt Björlenius, D.G. Joakim Larsson. *Submitted*

IV. **Waterborne beclomethasone diproprionate affects fish while its metabolite beclomethasone is not taken up**
Bethanie Carney Almroth, Filip Cuklev, Jerker Fick, Lina Gunnarsson, Erik Kristiansson, D.G. Joakim Larsson. *In Manuscript*

Contents

Abbreviations

aRNA	amplified/antisense ribonucleic acid
API	Active pharmaceutical ingredient
BCF	Bioconcentration factor
BDP	Beclomethasone-diproprionate
BLAST	Basic local alignment search tool
BMP	Beclomethasone-17-monoproprionate
Cox	Cyclooxygenase
CR	Concentration ratio
C_t	Threshold cycle
Cyp	Cytochrome P450
DDD	Defined daily dose
DMSO	Dimethyl sulfoxid
E_1	Estrone
E_2	17-β-estradiol
E_3	Estriol
EE_2	17-α-ethinylestradiol
EMA	European medicines agency
EQS	Environmental quality standard
EST	Expressed sequence tag
FDR	False discovery rate
$F_{SS}PC$	Fish steady state plasma concentration
GC	Gas chromatography
GO	Gene ontology
H_TPC	Human therapeutic plasma concentration
LC	Liquid chromatography
LIF	Swedish pharmaceutical industry association
MS	Mass spectrometry
NOEC	No observed effect concentration
NSAID	Non-steroidal anti-inflammatory drug
PEC	Predicted environmental concentration
PNEC	Predicted no-effect concentration
qPCR	Quantitative real-time polymerase chain reaction
RTGI	Rainbow trout gene index
SSRI	Selective serotonin reuptake inhibitor
STP	Sewage treatment plant
UV	Ultra violet

When you have eliminated the impossible,
whatever remains, however improbable,
must be the truth.

Sherlock Holmes

1. Introduction

Man has always sought to prolong life and to some extent we have succeeded. Less than a century ago, the average duration of life in the western world was just above 50 years. Nowadays it is around 80 and some live to be over a hundred years old. This increase is much due to pharmaceuticals and today it is hard to imagine life without being able to take a pill to cure a headache. Unfortunately, our use of pharmaceuticals has, at least in some cases, consequences that reach beyond the intended therapeutic effects on humans. Active pharmaceutical ingredients (APIs) can also become environmental pollutants.

When a pharmaceutical is taken orally by a human, it is subjected to gastric acids and other processes threatening to modify or eliminate it, thus reducing its intended action. Pharmaceuticals are therefore of necessity designed or selected to withstand such pressures. Resistance to rapid elimination in the human body may, however, also imply resistance against degradation in sewage treatment plants (STPs) and by natural abiotic and biotic processes in surface waters. As a consequence, many APIs are quite persistent and therefore remain available in the aquatic environment for a substantial time allowing them to travel far downstream from their discharge sites. Nevertheless, their presence and availability to organisms are alone not sufficient for posing a threat. There are several reasons why APIs may pose risks to the environment. In contrast to most other pollutants, e.g. metals and plastics, pharmaceuticals are designed or selected for their biological activity, i.e. they are intended to affect biological systems in humans, and these systems may very well be present in similar forms in aquatic organisms. To perform actions on their main target in an organism, for example a receptor, and affecting other systems as little as possible, pharmaceuticals are often very potent. This results in a lower risk for non-target-related side effects in humans, but a higher potential to affect organisms in the aquatic environment as very low concentrations of high-potency substances are likely required to have an impact [1]. Taking the potency and persistence into account, APIs can indeed constitute an environmental threat, *if* the substances are taken up by organisms.

In order for a pharmaceutical, or any substance for that matter, to have the potential to be taken up from the surrounding water and accumulate in fish, or to bioconcentrate, it has to meet a number of criteria. Much like "Lipinski's rule of five" [2], used to evaluate druglikeness for orally active drugs, a substance should not be too large, not charged and not too lipophilic in order to bioconcentrate into fish. However, it should not be too hydrophilic either, and there are other properties that can influence the bioconcentration potential as well. Many pharmaceutical fulfill these criteria and some APIs (synthetic steroids) have been found to bioconcentrate over 10,000-fold into fish blood plasma, i.e. 10,000 times higher concentration in the fish compared with the surrounding water [3, 4]. Consequently, possessing all these properties make pharmaceuticals a group of high concern regarding impact in the aquatic environment.

In Sweden, the pharmaceutical industry and the Stockholm county council have developed a classification system for pharmaceuticals with regards to environmental hazard (biodegradation and bioaccumulation) and risk [5, 6]. Some county councils use this classification as one of several criteria when making their recommendations about pharmaceuticals. However, there is clearly some room for improvements in this system. This classification, as is the case with others, is generally based on standard tests and standard risk assessments, which are not always protective for the environment [7, 8] (see section 1.5).

In contrast to many other pollutants, regulations and restrictions on pharmaceuticals are very difficult to impose since the human health always is, and, at least according to my personal view, should always be priority number one. Nevertheless, precautions should be taken. The questions are for what and how. This thesis aims to be a step towards better understanding of the risks that pharmaceuticals pose to the environment and consequently to answer those questions.

1.1 Emission routes

To date, more than 160 APIs have been identified in the aquatic environment and the list is growing steadily [1, 9-15]. The concentrations are generally low with a typical detection level of ng/L up to low µg/L in treated effluents. In diluted surface waters further downstream from STPs, where the interaction between drugs and organisms

would occur, the concentrations decrease and so does the number of detected APIs. However, most data is collected in Europe, North America and limited areas of Asia and little is known regarding concentrations and occurrence in other parts of the world [1, 9-15].

There are several sources of the APIs occurring in the aquatic environment, with STPs serving as hubs in most cases (Fig. 1) [16], where the main source is considered to be human usage. After administration, some pharmaceuticals are metabolized, while others remain intact before being excreted in urine or faeces. Topically administered substances are also washed off without any chance of being metabolized by our bodies. Consequently, a mixture of various pharmaceuticals and their metabolites enter into municipal STPs. Depending on the properties of these compounds, many are not completely removed during the sewage treatment. Unused or expired drugs which are inappropriately disposed of may also end up in the STPs, although they should be returned to pharmacies and incinerated. In Sweden, which has one of the world's most implemented return programs for unused medicines, this is considered a very small route of entrance into the aquatic environment, although in other countries and regions it may be more important [17]. Emission from the STPs can occur either via effluents or via sludge.

Although human usage of pharmaceuticals, with excreted residues accumulating at STPs, is considered the main route of emission, pharmaceutical manufacturing has recently arisen as a source of very high local emissions. Legislation on releases of APIs are generally insufficient or absent [18] and the relatively few studies on effluents and waters connected to pharmaceutical production reveal alarming results. In China, the concentration of steroidal estrogens in the effluents from an STP receiving waste water from a local contraceptives manufacturer were considerably higher than normally found in treated municipal effluents [9]. In India, the effluent from a treatment plant receiving process water from about 90 manufacturers contains extraordinary high levels of various APIs [12]. For example, the broad-spectrum antibiotic ciprofloxacin was found at concentrations up to 31 mg/L. This would correspond to 44 kg in one day, i.e. five times the entire consumed amount in Sweden every day. An example of an API with a human target is the antihistamine cetirizine, which was found at concentrations up to 10,000 times higher than is normally found in STP effluents. For 31% out of all

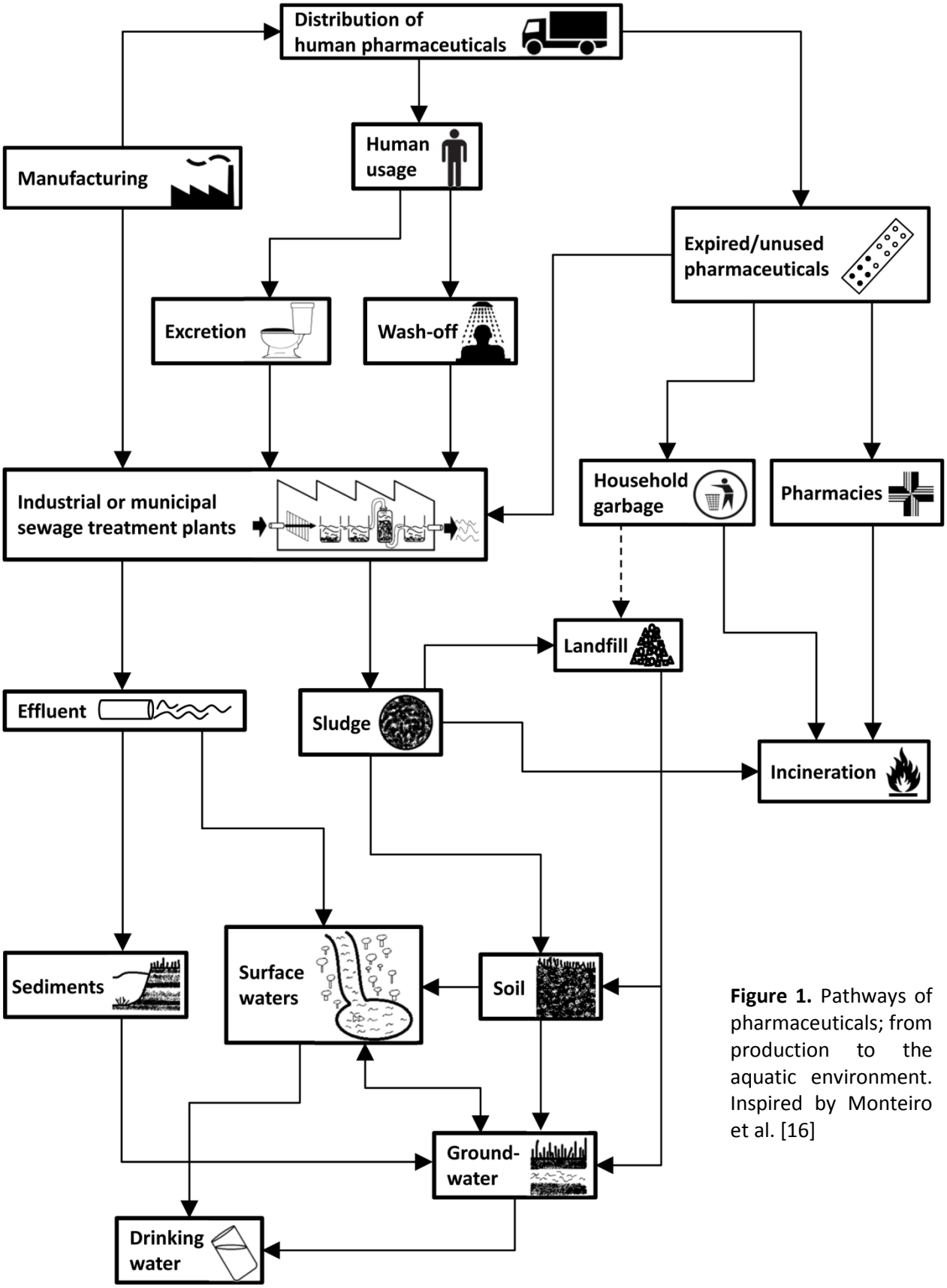

Figure 1. Pathways of pharmaceuticals; from production to the aquatic environment. Inspired by Monteiro et al. [16]

pharmaceutical products approved for the Swedish market and containing any of nine preselected substances, the API originated from manufacturers that frequently send process water to this particular Indian STP [19]. The consequences of antibiotic production release have also been observed in China where high levels of antibiotic resistance were found in bacteria strains isolated from wastewater and rivers downstream from penicillin and oxytetracycline manufacturers [13, 20]. Major releases of APIs are also documented from Western countries [21]. For example, Phillips et al. [22] found up to mg/L concentrations of certain pharmaceuticals in the effluents from two STPs in New York, USA, in comparison with 24 other STPs across the United States (including a third in New York). These two STPs received substantial flows from pharmaceutical formulation facilities, which was not the case for the other investigated STPs. An important question is how wide-spread large emissions from manufacturing sites actually are and what their impact is on the environment. However, emissions from production in particular are not further evaluated in this thesis.

1.2 Sewage treatment plants

Today's modern STPs were initially built without regards to API removal. The treatment technology is primarily designed to remove potential pathogens, to remove organic substances that may cause oxygen depletion and to reduce nutrients (phosphorus and nitrogen) that may cause over-fertilization, rather than to remove/degrade pharmaceuticals. Thus, the fate of pharmaceuticals in a conventional plant is to a large extent determined by the physical, chemical and biological properties of the substance itself and consequently the removal rate for many APIs is poor. There are three properties that determine the fate of substances in an STP system:

- Volatility
- Ability to adhere to particles
- Persistence, ability to withstand degradation

Very few pharmaceuticals are volatile and evaporation is therefore insignificant. Some adhere strongly to the sludge and end up in the sludge handling part of the plant. However, most pharmaceuticals are water soluble and will pass through the plants

intact, unless they are degraded. This incomplete removal of APIs has led to suggestions on addition of more advanced treatment steps to conventional plants. Treatment with activated carbon and ozonation are two advanced technologies proven to be particularly promising. Both have the potential to reduce the concentrations of a broad spectrum of APIs with varying properties [23-26]. Other oxidation methods, mainly based on ultra violet (UV) radiation, have also been considered [23, 27-30]. Still, the method best suited for removal of one API can differ completely from the most suitable method to remove another. In fact, for some pharmaceuticals the concentration can actually be higher in the effluent than in the influent as certain treatment steps may lead to re-generation of parent compounds from excreted metabolites, for example cleavage of glucuronide conjugates by biological treatment [31, 32].

Although chemical analyses of STP effluents have shown a general improvement in terms of substance removal, there are other aspects to consider in toxicity evaluations of effluents. Technologies based on oxidative/reductive reactions or photolytic transformation (e.g. ozonation and UV radiation) can lead to generation of transformation products that, in turn, may affect exposed organisms by unknown modes of action. Furthermore, effluent contains complex mixtures of chemicals and several substances, not only pharmaceuticals, exert their effects on organisms via similar modes of action which might lead to additive effects, whereas some substances may enhance the effects of others, i.e. synergy. Therefore, biological testing is also needed, as it provides information not possible to obtain by chemical screening alone. There are several examples where biological testing has demonstrated both increased and reduced toxicity after advanced treatments: reduced immune responses in rainbow trout after peracetic acid, UV or ozone treatment [33]; reduced induction of estrogenic biomarkers in rainbow trout after ozonation and membrane bioreactor treatment [34]; reduced induction of vitellogenin and immune gene expression in goldfish after treatment with membrane ultrafiltration followed by activated carbon filtration [24]; increased general toxicity in rainbow trout yolk sac larvae after ozonation [35]; reduced toxicity in crustaceans, bacteria and micro algae exposed to effluents treated at lower doses of ozone, but increasing toxicity with increasing ozone concentration [36-39, Hörsing et al., *manuscript*]. Nevertheless, far from all techniques have been evaluated with biological testing and studies on the mode of action of differently treated effluents in fish are

scarce. In paper III we therefore performed global hepatic gene expression analyses in fish exposed to different effluents treated with various techniques.

1.3 Effects in the environment

Very few APIs have been causally linked to adverse effects in wild organisms. The two best examples of clear links between exposure and effects are the feminization of male fish caused by exposure to estrogens, including the synthetic estrogen 17-α-ethinylestradiol (EE_2) used in many contraceptive pills, and the dramatic decline of vulture species on the Indian subcontinent caused by exposure to the non-steroidal anti-inflammatory drug (NSAID) diclofenac.

In the early 1990s, roach (*Rutilus rutilus*) with intersex characters, i.e. both male and female gonadal features in the same animal, were observed close to municipal STPs in England, and caged fish downstream from the STPs showed strong indications of exposure to estrogenic compounds [40]. Sewage effluents contain several endocrine disruptors that could theoretically be the cause: natural hormones like estrone (E_1), 17-β-estradiol (E_2) and estriol (E_3), synthetic hormones like EE_2 and industrial phenols like nonylphenol and bisphenol A. The use of nonylphenol is currently banned in Europe and detected concentrations are thus lower now than those measure in the past [41, 42]. However, several studies have together provided convincing evidence for causality between exposure to steroidal estrogens, especially EE_2, and harm to the reproduction systems in fish [4, 43-48]. These findings were the starting signal for intensified concern of pharmaceutical impact on the environment.

The case of diclofenac-poisoning of vultures describes an illustrative example of how pharmaceuticals can spread through the food chain. In Hinduism, cows are sacred and can therefore not be killed. Hence, on the Indian subcontinent they are often worked until the end of their lives and to reduce suffering they are often given diclofenac (or other NSAIDs). When they have passed away, their carcasses are disposed of naturally. In other words wildlife, including scavenging birds, is allowed to consume them. Unfortunately, vultures of the genus *Gyps*, are not able to cope with the residues of diclofenac remaining in the carcasses. Consequently, there has been an extensive decline

of >95% in vulture populations in India, Pakistan and Nepal, starting in the 1990s [49, 50]. Three *Gyps* species were even on the brink of extinction. The dead vultures showed signs of renal failure and visceral gout, which are known side effects of over-dosage of diclofenac in humans and other mammals. Accordingly, there is strong evidence, including epidemiological and experimental evidence, that diclofenac residues from dead cattle were in fact the reason for this vast vulture population decline, and in 2006, diclofenac was consequently banned for veterinary use in India, Pakistan and Nepal [49-53]. However, the recommended alternative, meloxicam, is expensive and so diclofenac is often used anyway, as are other NSAIDs like ketoprofen. Unfortunately, recent studies have shown, through experimental testing, that ketoprofen affects *Gyps* vultures in a similar manner as diclofenac and it has been suggested that ketoprofen may have contributed to the widespread vulture death despite previous indications that it was safe [51, 54].

Both EE_2 and diclofenac are examples of drugs primarily designed to interact with human drug targets, though pharmaceuticals like parasiticides and antibiotics, which target parasites and bacteria, have also been shown to affect organisms in the environment. According to field studies, the broad spectrum antiparasitic medicine ivermectin, used for veterinary purposes, affects non-target dung-feeding flies and beetles [55] and is also highly toxic to the crustacean *Daphnia magna* [56]. The previously mentioned releases of antibiotics in India and China have further raised concerns on the incidence of antibiotic resistance [12, 13, 20, 57]. Although antibiotics have the potential to affect the community structure and function of microbes such as fungi, microalgae and bacteria, the possible effects on antibiotic resistance raise particular concern because of the obvious risks for human health and the potentially global consequences [1]. However, this thesis focuses specifically on pharmaceuticals with human drug targets; hence antibiotics, parasiticides, antifungals etc. will not be further discussed.

1.4 Potential threats from pharmaceuticals in the environment

The substances mentioned in section 1.3 are pharmaceuticals for which there are relatively ample data linking to effects in the environment in one way or another.

Nevertheless, there are many pharmaceuticals that pose a potential threat and several studies have aimed to increase the knowledge of the impact on organisms by various methods. One class of pharmaceuticals that has received increased awareness and concern is progestins, i.e. synthetic forms of the female sex hormone progesterone, used in various hormonal contraceptives. They have not been reported in STP effluents very often, quite possibly because few have looked for their presence. Levonorgestrel, one of the most common progestins, used in for example emergency pills and regular contraceptive pills, has been found at approximately 1 ng/L or slightly higher on occasion [3, 58]. Concentrations of up to approximately 10 ng/L of levonorgestrel have been reported in surface and ground waters [59, 60]. However, the blood plasma concentration of levonorgestrel in fish exposed to sewage effluents can be considerably higher. Due to a considerable bioconcentration potential, concentrations up to 12 ng/ml levonorgestrel have been found, that is to say a higher plasma concentration has been measured in the exposed fish than in women taking oral contraceptives [3]. Thus, risks for effects on exposed fish are obvious (see section 1.6). Accordingly, Zeilinger et al. [61] showed that levonorgestrel concentrations of ≥0.8 ng/L inhibit reproduction in exposed fish and that higher concentrations result in masculinization of females. The effect on inhibited reproduction is in line with its intended effect on women, including feedback on the hypothalamic pituitary axis. The latter is also not surprising given that most progestin also bind to androgen receptors, although with lower affinity than to the progesterone receptor. If the findings by Fick et al. [3], Zeilinger et al. [61] and Vuillet et al. [59, 60] are representative for different species, waters and exposure situations, it is almost surprising that there are fish in certain French waters! In amphibians, levonorgestrel has been shown to impair several steps of the reproductive and developmental processes, including oocyte maturation, fertility and metamorphosis [62-64].

Among the most frequently detected APIs in both STP effluents and surface waters are the selective serotonin reuptake inhibitors (SSRIs). These antidepressants, including fluoxetine (Prozac), are generally found at low ng/L levels and on rare occasions up to µg/L levels [65-69]. The bioconcentration potential is not as high as for levonorgestrel, though fluoxetine has been found in wild fish tissue [65, 70]. Reported effects of SSRI exposure in fish include behavioral changes (aggression, appetite etc.) and reproductive alterations, though not at environmentally relevant concentrations in

most cases [66, 71]. One of the most prescribed classes of pharmaceuticals is the β-blockers. Consequently they too are frequently found in STP effluents and surface waters, generally at ng/L concentrations but occasionally up to the µg/L range [32, 72-75], though most studies show effects in fish exposed to β-blockers at relatively high concentrations (mg/L) [76].

In paper I and II, two NSAIDs are studied and in paper IV a glucocorticoid, and so a more thorough introduction to these two classes of pharmaceuticals follows.

1.4.1 Non-steroidal anti-inflammatory drugs

Non-steroidal anti-inflammatory drugs, or NSAIDs, can be found in the medicine chest of most homes in the Western world. Brand names such as Ipren, Alvedon, Treo and Voltaren are known to most Swedish consumers, and in other parts of the world Advil, Tylenol and Aspirin are just as well known. Even their active substances: ibuprofen, paracetamol, acetylsalicylic acid and diclofenac are recognized by the common man. These drugs have analgesic, antipyretic and at higher doses also anti-inflammatory effects and many of them are available over-the-counter. In paper I and II the two NSAIDs diclofenac and ketoprofen are studied and these two will therefore be in focus here, although other NSAIDs will be briefly introduced as well.

The use of NSAIDs is wide and includes short-term treatment of a variety of light to intermediate pain conditions from ordinary headache to pain reduction associated with operations. However, although they all work via the same mode of action in general, there are level differences in their action. Ibuprofen and paracetamol are often used during common cold and relatively lighter pain conditions, e.g. migraine, due to their analgesic and antipyretic effects, whereas diclofenac and ketoprofen are often the preferred alternative in association with injuries, operations and therapy for rheumatic diseases, because of the stronger anti-inflammatory and analgesic effects.

The mechanism of action of NSAIDs is not entirely known, yet the primary target is the inhibition of the cyclooxygenase enzymes Cox1 and Cox2 (also known as prostaglandin G/H synthase, or PTGS, 1 and 2) [77-79]. However, additional modes of action of individual drugs are suggested continuously [80]. The Cox enzymes convert

arachidonic acid to prostaglandin H_2, the precursor of the eicosanoid subclass prostanoids including prostaglandins, prostacyclins and thromboxanes. There is a great diversity of receptors, spread out through the human body, which means that prostanoids can have a wide variety of effects on several different physiological systems, including hyperalgesia, broncho-dilation and constriction, vasodilation and thrombosis.

In humans, several different side effects have been observed. Those occurring most commonly are gastrointestinal bleeding, renal and cardiovascular problems and, when administered topically, skin irritations [8]. It has been suggested that side effects caused by NSAIDs originate in the inhibition of Cox1, while the anti-inflammatory actions are a result of Cox2 inhibition [81]. Traditional NSAIDs affect Cox1 and Cox2 with relative equipotency, though the Cox2 selective coxibs, e.g. rofecoxib (Vioxx), were developed and made available on the market in 1999 [82]. Although both Cox1 and Cox2 exert the same converting action, there are differences. Cox1 is responsible for the baseline levels of prostaglandins, whereas Cox2 produces prostaglandins through stimulation by e.g. proinflammatory cytokines [79, 83, 84]. In theory, selectivity for Cox2 would allow coxibs to reduce inflammation and hyperalgesia while minimizing adverse side effects. However, the results have not been as expected. In spite of the Cox2 selectivity, several side effects including renal failure and cardiovascular effects could still be observed. Many of these side effects are probably due to an increased synthesis of thromboxanes. Consequently most coxibs have been withdrawn from the market, including the infamous Vioxx (rofecoxib). Non-selective NSAIDs usually tend to preferentially affect one of Cox1 and Cox2 slightly more than the other, rather than acting equally. Diclofenac binds preferentially to Cox2 and ketoprofen to Cox1 (Fig. 2) [85]. This may explain some differences in characteristics and effects between different “non-selective” NSAIDs.

Non-steroidal anti-inflammatory drugs come in several formulations. Pills and tablets taken orally have been the general form of administration, though injections and suppositories are also widely used [8]. In the past decade, the use of topically administered gels has increased more and more [86]. As mentioned in section 1.1, one important point of origin for drug emission into the environment is the human body and this is mainly through excretion. When discussing NSAIDs, one must take gels into account since topical administration results in residues that are washed off straight down the drain. When administered orally, the major part of the drugs are excreted as

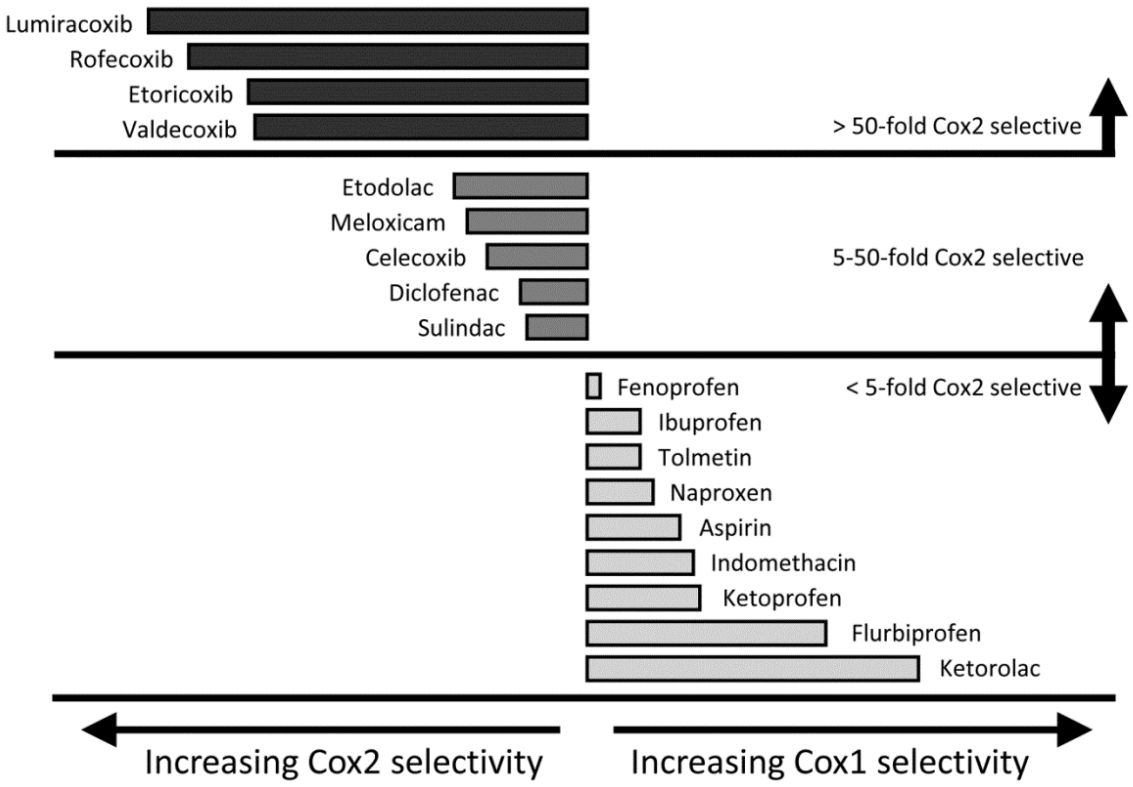

Figure 2. Graphical overview of the Cox-selectivity of different NSAIDs [85].

metabolites, e.g. glucuronides [8], but the washed-off gel residues contain the parent compound. Furthermore, as this formulation requires an additional barrier to be crossed, i.e. the skin, before reaching its target within the body, the total amount of substance used in a single treatment may be higher than when it is taken as a pill. Notably, gels are not taken into account in calculations of defined daily dose (DDD) and sales per active substance [86].

Perhaps the most drastic effects caused by pharmaceuticals in the environment (thus far) are the previously mentioned reports on *Gyps* vultures on the Indian subcontinent [49, 50]. The initial reports were on how some vulture species that had fed on diclofenac-treated livestock developed renal failure. This subsequently led to visceral gout and death, to such a degree that some species were pushed to the edge of extinction. Consequently, in 2006 the use of diclofenac for veterinary purposes was banned in India, Nepal and Pakistan [52]. However, after a few years it turned out that another NSAID, ketoprofen, may have contributed to this dramatic decline in vulture populations, although the evidence and environmental causality are not as conclusive as for diclofenac [51, 54]. Ketoprofen-related mortality has in addition been reported in

male eider ducks given the drug intentionally [87]. The symptoms were identical to those found in the *Gyps* vultures exposed to diclofenac, i.e. renal failure and visceral gout.

For diclofenac and ketoprofen, the removal rate in STPs varies in most cases from low to moderate (5-70%) [31, 88-90], though occasionally higher removal rates are found for ketoprofen [31]. Consequently, they are found in STP effluents very frequently. Detected concentrations obviously vary as well, though measured levels are often approximately 1 μg/L or just below for both compounds [3, 31, 88-91]. In some contrast, the removal efficiency for ibuprofen is very high (>90%-100%) [26, 31, 32, 88]. Nevertheless, ibuprofen have also be found at approximately 1 μg/L [88], though the influent concentrations are generally much higher than for diclofenac and ketoprofen [26, 88] due to considerably higher usage. In surface waters the concentrations are lower, though both ketoprofen and diclofenac can still be found at concentrations of up to 100 ng/L and occasionally higher [32, 88, 92].

Both cyclooxygenase enzymes (Cox1 and 2) have been characterized in a number of teleosts [66] and effects on several endpoints have been documented following experimental exposure to NSAIDs. Cytological and histological studies in fish exposed to diclofenac have reported effects including glycogen depletion of hepatocytes in the liver, hyaline droplet degeneration in the kidney and pillar cell necrosis in the gills [93-96]. Some effects were observed at water concentrations as low as to 1 μg/L. Nevertheless, the mode of action of diclofenac in fish is unknown and gene expression data subsequent to exposure is lacking. We therefore performed global hepatic gene expression analysis in rainbow trout exposed to diclofenac to increase the knowledge of the actions of diclofenac in fish (Paper I). We also performed bioconcentration analyses, as there have been some uncertainties about the bioconcentration potential of diclofenac [3, 91, 94].

Based on documented effects on birds, and reported sublethal effects in fish in laboratory exposures at around 1 μg/L, diclofenac was, in 2012, included in the substance priority list within the EU Water Framework Directive (together with EE2 and E2) [97]. This means that EU Member States will have to ensure that set limit values, Environmental Quality Standards (EQS), are met by 2021. The EQS for diclofenac in inland surface waters is set to 0.1 μg/L. Importantly, measures to reach the EQS should not jeopardize human health by inferring with the possibilities to prescribe diclofenac or

restrict its availability for non-prescription use. Improved removal during sewage treatment is hence a reasonable mitigation alternative, though most likely very expensive. Another option is substitution of API in clinical situations where there are alternative substances with a similar mode of action and potency as diclofenac, but with less potential of posing a risk in the environment. Ibuprofen was under consideration for inclusion in the priority list, but was excluded at the end of the process. De Lange et al. [98] reported effects on the activity of the crustacean *Gammarus pulex* at a very low exposure concentration (10 ng/L). However, the reliability of the study by De Lange et al. is questionable due to the lack of a dose-response relationship (no effects at higher concentrations of ibuprofen), reproducibility and understanding of the mechanism behind the effects [99]. Effects on fish include an increasing change in reproductive patterns of Japanese medaka (*Oryzias latipes*) with increasing exposure concentration of ibuprofen, though only significantly at a water concentration of 100 µg/L [100]; reduced concentrations of prostaglandin E2 in gills upon exposure to ibuprofen at 50 and 100 µg/L [101]; disturbance in the osmoregulatory, metabolic and cortisol responses in rainbow trout at the relatively high concentration of 1 mg/L ibuprofen or salicylate [102]. Studies on the effects of ketoprofen exposure in fish are however very scarce. Thus, in paper II we aimed to analyze the bioconcentration potential and pharmacological responses of ketoprofen, in a manner similar to that used to address effects of diclofenac in paper I, to assess whether ketoprofen could pose as a better alternative with regards to effects on fish.

1.4.2 Glucocorticosteroids

The potential of several steroids to have an impact on aquatic organisms has already been demonstrated (see section 1.3 and opening paragraph in section 1.4). However, most focus has been on sex steroids, e.g. synthetic hormones used in contraceptive pills, and effects directly connected to reproductive processes. In paper IV, we aimed to evaluate another group of steroids, glucocorticosteroids, or glucocorticoids in short. They are widely used in treatment of a large variety of human diseases as they are important for many systems in vertebrate physiology, though their role in the immune response has proven most useful. Medical indications caused by an overactive

immune system, such as allergies, asthma and autoimmune diseases, are among the main treatment areas.

Glucocorticoids act by binding to the ubiquitous glucocorticoid receptor, which in turn initiates gene transcription via glucocorticoid response elements [103]. One of the resulting gene transcriptions is the induction of genes involved in gluconeogenesis [104], from which the name glucocorticoids derive. Their anti-inflammatory effects are mediated by inhibition of the transcription factors, e.g. activating protein-1 and nuclear factor κB, which leads to a decreased expression of genes involved in inflammatory responses [105]. Due to their anti-asthmatic properties, glucocorticoids are often administered via inhalers for delivery to lung tissue where they have local effects. They are mainly excreted via faeces as metabolites [8]. However, like NSAIDs, several substances are also administered topically, thus they may also enter the sewage systems as parent compounds. There are also some concerns regarding inappropriate disposal of inhaler devices, which, at least in some regions, may also add to the amount of unmetabolized compound reaching the environment. Accordingly several different glucocorticoids, natural and synthetic, have been measured in sewage effluents and surface waters at low ng/L concentrations [106, 107] and in addition, they have the potential to bioconcentrate [108].

In rainbow trout, two glucocorticoid receptors have been identified (GR1 and GR2) [109]. Since conserved drug targets strongly increase the probability for pharmacological interactions to occur at low doses of APIs such as those found in the aquatic environment [110], conditions are favorable for physiological effects as a consequence of glucocorticoid exposure in the field. The internal corticoid system of teleost fish differs, however, from mammals in that fish lack mineralcorticoids, thus the principle glucocorticoid cortisol fills both mineral- and glucocorticoid functions [111]. Aside from their important roles in metabolism and immune function, glucocorticoids are also involved in osmoregulation, which is of additional importance in anadromous species like rainbow trout as they migrate between fresh and salt water, i.e. smoltification [112]. Furthermore, they are important in the larval metamorphosis in fish [112] and a known side effect in humans is the growth inhibition and pubertal delay [8, 113]. Taking all these aspects into account suggests multiple types of ecotoxicological effects by glucocorticoids in the aquatic environment. Accordingly, reported effects of

exposure to the synthetic glucocorticoid dexamethasone include changes in reproduction, growth, and development, though at relatively high doses (500 μg/L) [114-116].

In paper IV we aimed to investigate the potential of the synthetic glucocorticoid beclomethasone and its prodrug beclomethasone-diproprionate (BDP) to affect fish. As BDP is mainly used in treatment of asthmatic disorders it is primarily administered as an inhalant, although it is also available in gel-form [8]. Soon after administration, BDP is metabolized to beclomethasone-17-monoproprionate (BMP), beclomethasone-21-monoproprionate (inactive) and free beclomethasone in humans via esterases present in numerous tissues of the body [117]. Beclomethasone-17-monoproprionate is considered the active metabolite with an affinity of approximately 18 times higher than that of free beclomethasone in humans [118]. A dose of 0.8 mg/day of the prodrug BDP yields an H_TPC of 0.33 ng/ml BMP [113, 119]. However, although beclomethasone and BDP are considered to be inactive metabolites compared with BMP, their binding affinities are similar to that of dexamethasone. In fish, this affinity relationship has been reported to be similar to that in humans [108].

Although administered for local effects, dose-related systemic effects have been established in humans upon BDP inhalation, including growth rate reduction, adrenal suppression and adverse effects on skin, bone and eyes [8, 113]. Published data on effects of beclomethasone on fish are few, though recently Kugathas et al. [108] demonstrated effects of waterborne BDP, at nominal concentrations of 1 μg/L, on plasma glucose levels and white blood cell counts in fish. Although neither effluents nor surface waters concentrations of any of the beclomethasone formulations are known at present, it can be assumed that most of the consumed prodrug BDP has been metabolized into the less lipophilic forms BMP, beclomethasone and additional metabolites before reaching the environment. Unused doses and residues from topical administration may however enter the environment in prodrug form either via sewage or landfills [120]. In paper IV we have therefore investigated the potential of both the prodrug BDP and its metabolite free beclomethasone to bioconcentrate and affect physiological parameters in exposed fish.

1.5 Traditional risk assessment

In 2006 the European Medicines Agency (EMA) established guidelines (OECD; http://www.oecd.org) for risk assessments of pharmaceuticals, which are required for the approval of a new product [7]. These are in principal based on the ratio between a predicted exposure concentration (PEC) and a predicted no-effect concentration (PNEC). The PEC for an API is calculated using information on predicted usage and assuming a reasonable worst case scenarios regarding emission, i.e. no metabolism takes place, everything that is consumed is diluted in 200 liters of water (estimated usage per capita and day), that no API is removed during the sewage treatment process and that the final effluent is diluted 1/10 in the receiving aquatic environment. The PEC calculation does not, however, take into account the fact that some streams may be effluent dominated and that consumption could be higher in certain regions compared to others. Thus the PEC value may in some cases underestimate the worst-case scenario. The PNEC is based on the lowest available experimental no observed effect concentration (NOEC) which is obtained through a set of recommended standard toxicity tests (http://www.oecd.org) [7]: growth inhibition test on algae (OECD 201), reproduction test on *Daphnia* (OECD 211) and early-life stage test on fish (OECD 210). However, the effects of APIs in the environment are not standard and cannot always be evaluated by classical toxicity parameters, e.g. survival (LC_{50}) and hatching success. For example, chronic exposure to SSRIs may theoretically lead to decreased fish populations, but through effects on the behavior (e.g. less aggressiveness leading to less mating or feeding, or increased risk for predation) rather than direct lethality. As a matter of fact, there is an ongoing discussion on which effects are relevant to include in formal risk assessments and which will ultimately protect populations in the field. Reproduction tests have high relevance for the protection of populations. However, reproduction data from, for example, *Daphnia* may not be protective for other species, as the number and similarity of conserved human drug targets in crustaceans is much lower than in for example fish [110]. Accordingly, the standard tests on *Daphnia* did not capture the high risk of EE_2 since one of the drug targets lacking in this species is the estrogen receptor. The NOEC of EE_2 from the standard early-life stage test in fish was certainly lower, though non-standard tests have showed induced intersex in fish at concentrations of EE_2 an additionally hundred times lower [121, 122]. Nevertheless, the standard tests in EU are still better than those

implemented in the USA where no tests on fish are mandatory and the environmental risk assessment may be based on acute responses (lethality) alone [123].

Regardless of the outcome of the environmental risk assessment for human drugs by EMA and FDA, the aim is not to affect whether a product is approved for usage in humans. Additionally, in EU risk assessment requirements apply to new drugs; no risk assessment is required for products approved before 2006. As mentioned in the opening paragraph, these standard tests form the foundation on which recommendations and information for physicians in Sweden are based, i.e. the product-based classification coordinated by the Swedish Pharmaceutical Industry Association (LIF) at http://www.fass.se and the API-based classification by the Stockholm county council at http://www.janusinfo.se. However, these are voluntary systems for classification and are not tied up by EMA legislations [5, 6, 8]. Thus, a few modifications are implied, including use of actual sales figures (if available) for the total volume of the API in PEC calculations and excretion form and biodegradability are considered. Additionally, this is applied on all products on the Swedish market, not only new substances.

Nevertheless, in order to be able to conduct an environmentally fair risk assessment of both new APIs and products already out on the market, more tests and, above all, tests aiming to study the proper endpoints are needed. In this thesis, examples of other strategies to identify potential risks are presented and applied.

1.6 Combining bioconcentration and read-across

The concentrations of APIs found in surface waters are generally low and several magnitudes below the human therapeutic plasma concentrations (H_TPC; also referred to as C_{max}), i.e. the concentrations found in the blood plasma of human patients being treated with the drug. Considering these parameters alone, the probability for a pharmacological interaction leading to adverse effects to occur in the environment is relatively small. However, a direct extrapolation from water concentration to the levels of APIs encountered by the drug targets in a water-living organism is neither fair nor correct. In fact, the concentration of an API in, for example fish blood plasma, may very well be extensively higher than in the surrounding water, due to bioconcentration.

1.6.1 Bioconcentration – accumulation of waterborne substances in organisms

Simply put, bioconcentration is a process whereby a waterborne substance is taken up by an aquatic organism to the extent that the concentration in the organism has stabilized (steady state) at a higher level than that of the surrounding water. The rate at which a substance is able to bioconcentrate into a specific tissue (e.g. blood plasma) is often presented as a bioconcentration factor (BCF), i.e. the ratio between water concentration and the tissue. For example, a BCF of 50 into fish blood plasma means that the concentration of the substance in the plasma is 50 times higher than the surrounding water.

Factors influencing uptake and bioconcentration potential are similar to the previously mentioned criteria used to evaluate druglikeness for orally active drugs, i.e. "Lipinski's rule of five" [2]. Although there has been some controversy regarding the applicability of the rule to the aquatic environment, it is at least a start. The substance should neither be too large nor charged to bioconcentrate. On the other hand, Lipinski further states that the substance should not be too lipophilic, though according to our model the BCF increases with an increasing lipophilicity (see below). However, the availability would most likely decrease, since very lipophilic substances tend to adhere to particles and are removed in STPs. Nevertheless, although the availability increases with increasing hydrophilicity, the bioconcentration potential decrease since very hydrophilic chemicals are not partitioned in the lipids and lipid membranes of organisms, in contrast to lipophilic chemicals.

Empirical data on the BCF of aquatic organisms is lacking for the overwhelming majority of pharmaceuticals, thus theoretical values obtained by predictive models are often used instead. In the model proposed by Fitzsimmons et al. [124], the only predictors used are the lipophilicity of the molecule, i.e. the octanol-water coefficient (log K_{OW}), and the water concentration. For moderately lipophilic, nonpolar contaminants, this provides a rather good estimate of BCF and there are studies showing that log K_{OW} is in fact a decent predictor of the BCF for many pharmaceuticals [3, 91]. Naturally, there are exceptions as other elements may influence the uptake of substances as well, e.g. pH and endogenous carriers.

To date there are very few studies reporting concentrations of pharmaceuticals in fish exposed to effluents and surface waters. Nevertheless, over twenty APIs have been found in various tissues of fish. These APIs include pharmaceuticals from several classes, including steroids, NSAIDs and SSRIs [3, 4, 65, 69, 91]. The bioconcentration potential for different pharmaceuticals can differ extensively. Some APIs have a BCF over 10,000, e.g. the progestin levonorgestrel to blood plasma [3] and EE_2 to bile [4], whereas some do not bioconcentrate at all.

1.6.2 Read-across using the fish plasma model

In combination with knowledge of the water concentration of a certain API, information on the BCF to blood plasma, estimated or experimentally obtained, provides a more relevant measure of the actual exposure, i.e. the internal dose to which the organism is exposed. Since we already have substantial knowledge of the potency of pharmaceuticals in humans, including H_TPC, this could further be used to assess the likelihood for a pharmacological interaction or effect to occur in exposed aquatic organisms, i.e. read-across. In 2003, Hugget et al. [125] presented a simplistic model on how to apply this strategy: "the fish plasma model". It is based on the ratio (concentration ratio; CR [3]) between measured H_TPC and measured or predicted fish steady state plasma concentration ($F_{SS}PC$; Equation 1) and the lower the CR, the greater the potential for a pharmacological response in fish. However, this risk identification strategy may only be performed if the drug target of the API is conserved in the investigated species (see section 1.8).

$$CR = \frac{H_TPC}{F_{SS}PC}$$

Equation 1. The formula used in "the fish plasma model". CR = concentration ratio, H_TPC = human therapeutic plasma concentration, $F_{SS}PC$ = fish steady state plasma concentration.

The "fish plasma model" approach can be very powerful and provides the possibility to screen a large set of pharmaceuticals relatively quickly. If there are no measured values of $F_{SS}PC$, BCF to blood plasma or concentrations in the aquatic environment available, one may use predictions obtained by methods mentioned above.

However, the time saved by using predictions may result in loss of power as predictions incorporate an additional source of error. In paper I, II and IV we have used an approach based on "the fish plasma model" with measured plasma concentrations in exposed fish and calculated BCFs to blood plasma for the two NSAIDs diclofenac and ketoprofen, as well as two forms of the glucocorticoid beclomethasone, its pro-drug form and a metabolite. The results were used in a comparison with H_TPC for the respective drug in relation to observed effects or responses.

1.7 Biomarkers

Simply put, a biomarker is an indicator for a certain biological state. Within ecotoxicology, biomarkers may be divided into three classes: biomarkers of susceptibility, exposure and effect [126]. Susceptibility biomarkers could be, for example, genetic differences that can explain or predict individual or species variability in the response to a given toxicant. An exposure biomarker is mainly used to determine whether an organism has been exposed to a given chemical or group of chemicals but offers limited possibilities to assess the risks for adverse effects. Biomarkers of effects, on the other hand, are different types of documented effects linked to more or less specific toxicants, e.g. feminization of male fish upon estrogenic exposure. However, the distinctions between the types of biomarkers are not always strict.

For example, one of the most commonly used biomarkers of exposure in ecotoxicology is the induction of vitellogenin (*vtg*) in male and juvenile fish as a result of exposure to estrogenic compounds. The gene(s) for vitellogenin encodes for a precursor to egg yolk proteins and is produced in the liver of sexually maturing females, hence the gene is normally not expressed (or expressed at very low levels) in males or juvenile fish. Vitellogenin as a biomarker was a major factor in the discovery of EE_2 as a main contributor to the feminization of fish downstream STPs [4, 40, 43, 44, 47, 48].

The optimal biomarker is sensitive enough to be detected at a desired threshold and correlates well with the magnitude of the exposure. It should also be specific for certain individual or group of substances/effects and sufficiently robust for usage in different exposure scenarios and by different measuring techniques. Unfortunately, one

biomarker rarely fulfills all these criteria, though several biomarkers and different types of biomarkers can be used in combination, particularly on a molecular level, to become more informative.

Molecular responses in an organism are often fast and short exposure times may be sufficient to trigger a detectable response. Thus, exploratory molecular analyses may serve both to increase our understanding of the mode of action (including toxicity) of pharmaceuticals in aquatic organisms and to provide biomarkers, following proper evaluation. One of the aims in paper I and II was to use an exploratory technique, i.e. microarray, to discover new potential biomarkers. In paper III we have used the same technique in search of already established biomarkers to gain information on the exposure and possible ensuing effects.

1.8 Genomics

There is no universally accepted definition of genomics, though in this thesis the term applies to studies of the genome or gene-products on a large scale. The whole concept of genomics is based on the central dogma of molecular biology: DNA can be copied to DNA (DNA replication), DNA information can be copied into mRNA (transcription) and mRNA can then serve as a template for the synthesis of amino acids that are assembled into proteins (translation; Fig. 3). Most things that occur in an organism are related to an effect of a protein and therefore to the previous steps: DNA transcription to mRNA and mRNA translation to proteins. Thus, the study of mRNA, or transcriptomics, can reveal possible effects at the protein level and thus physiological processes.

1.8.1 DNA - genomic information to predict susceptibility

"*As a general rule, extrapolations across species require knowledge of species-specific physiology"* [99]. This could in short be interpreted within the framework of this thesis as follows: pharmaceutical effects in humans can only be extrapolated to other organisms if the species in question possess the specific drug target. Pharmaceuticals are designed to exert their intended clinical effects through relatively specific, high-

affinity interactions with target proteins, e.g. receptors, while affecting other systems as little as possible. Since APIs are generally present at very low concentrations in aquatic environments, such high-affinity interactions with proteins are likely the most relevant in wildlife. Many human proteins are conserved in wild organisms, thus a pharmaceutical may interact with a similar protein in exposed wildlife species [110]. Although pharmacological interactions are possible if the drug target protein is not present, there is an increased risk for effects at the low concentrations of pharmaceutical residues found in the environment if the target is conserved. Gunnarsson et al. [110] showed that fish and frogs have a corresponding target protein for >80% of 1,318 investigated human drug targets, whereas the water flea *Daphnia pulex* only shared 61% and green algae 35%. For example, the presence of estrogen receptors in fish indicates their susceptibility to estrogen exposure, whereas a lack of the receptors, as in algae and water fleas (*Daphnia*), indicates a relative insensitivity. Accordingly, there are documented strong effects of EE_2 and other estrogens at low concentrations in fish but not in water fleas or algae. Furthermore, this highlights the flaws in traditional standard tests for assessing effects of pharmaceuticals (see section 1.5).

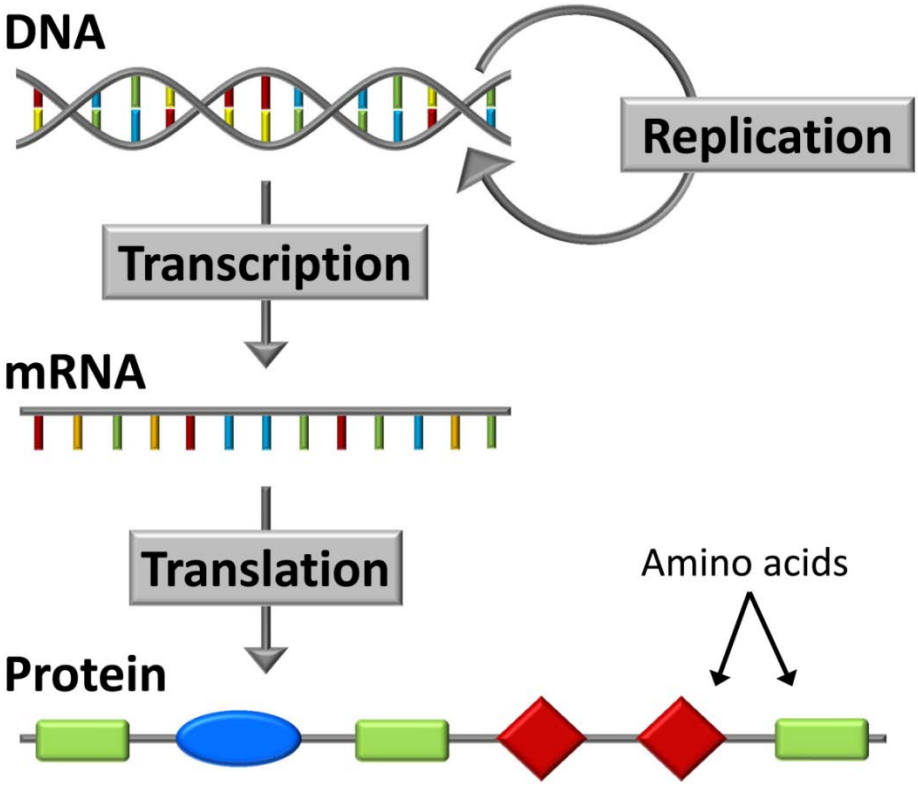

Figure 3. The central dogma of molecular biology.

1.8.2 mRNA – applying microarrays to ecotoxicology

As previously stated, several drug targets are well conserved in fish and by using information on the well-known modes of action in mammalians one has the possibility to create hypotheses of potential molecular responses in fish, i.e. which gene-products to study. Nevertheless, even if a drug target is evolutionarily well conserved, the stimulation of the target might lead to different physiological events in different organisms. Thus, studying a broader set of responses in the tested species rather than one or a few responses hypothesized from known responses in humans would provide additional and valuable information. Microarrays (Fig. 4) provide an efficient tool for studying thousands of potential gene responses simultaneously by analyses of the abundance of thousands of expressed, specific mRNA sequences (transcripts).

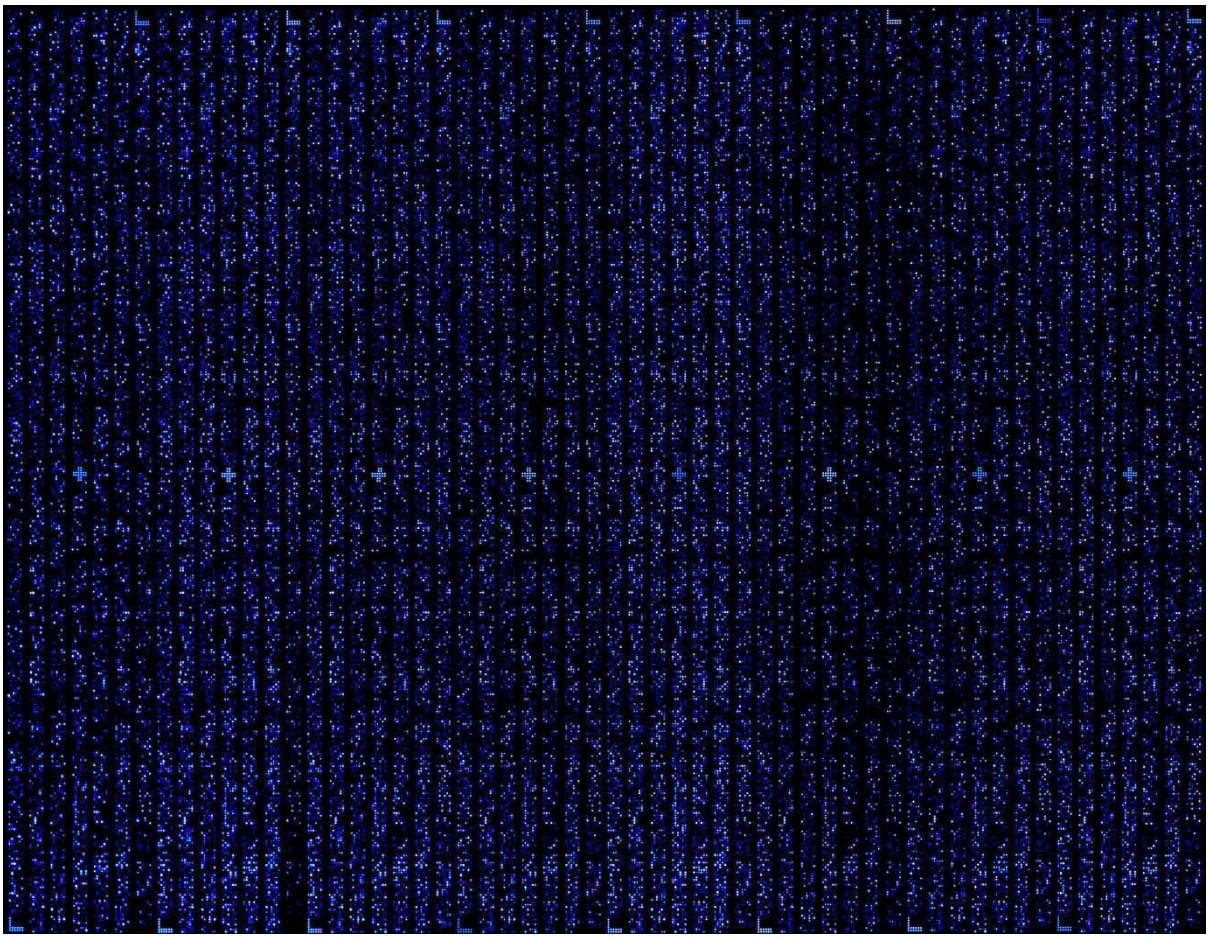

Figure 4. A microarray chip from paper I, showing the gene expression in the liver from eight individual rainbow trout. The light intensity of each spot reflects the expression of one specific mRNA transcript.

Microarray technology has in the past decade been successfully applied to several different areas within biology, ranging from cancer diagnostics and cell-signaling in yeast to ecotoxicological research. Several different types of microarrays exist, though they are all based on the basic principle of measuring mRNA abundance corresponding to individual genes. There are also several commercial microarray platforms available with probes selected to cover genes of general interest or sometimes the entire transcriptome, i.e. the set of all RNA molecules, of a species. The benefits from using these commercial arrays include their readymade protocols and support as well as their generally high quality. However, although market availability continues to increase, there are few commercial arrays designed for environmentally relevant species. For use in ecotoxicological research, non-commercial microarrays have been developed mainly by academia, such as those for water flea (*Daphnia magna*) [127], rainbow trout (*Oncorhynchus mykiss*) [128, 129] and eelpout (*Zoarces viviparous*) [130]. In paper I, II and III, updated versions of the array initially developed by Gunnarsson et al. [129] are used.

The main purposes of a microarray analysis within ecotoxicology are fourfold:

- Providing information on the mode of action of a substance
- Assisting in the discovery of new potential biomarkers
- Revealing information about the potency of a substance
- Aiding in the identification of substances within a mixture

In this thesis, all these purposes have been applied. In paper I and II, the aims were connected to the first three purposes, i.e. mode of action information, biomarker discovery and potency information. In paper III, the fourth purpose, identification in a mixture, was applied by comparing the genes differentially expressed following exposure to STP effluents with differentially expressed genes known to respond to exposure to individual drugs (e.g. from paper I and II). Furthermore, the mode of action and the potency of differently treated effluents were studied and effects on already established biomarkers (e.g. *vtg*) were assessed. Accordingly, microarrays provide a powerful and multifaceted tool within ecotoxicology.

2. Aims of thesis

There are several ways to address the growing concern of environmental effects of pharmaceuticals. In this thesis we have explored the use of bioconcentration and transcriptomics as complements to traditional risk assessment strategies, with a potential to provide additional possibilities to identify pharmaceuticals of environmental concern.

The major aims of this thesis were:

- To investigate if read-across between therapeutic plasma concentrations in humans and measured plasma levels of pharmaceuticals in exposed fish can be used to predict the likelihood for pharmacological effects in the fish

- To assess the suitability of microarray analyses to confirm pharmacological interactions in fish exposed to pharmaceuticals

- To better understand the mode of action of pharmaceuticals in fish and find potential biomarkers through transcriptome analyses

- To evaluate the ability of differently treated sewage effluents to affect fish, through analyses of global hepatic gene expression

3. Methodological considerations

The workflow in the papers in this thesis generally started with the exposure of fish to a single pharmaceutical substance dissolved in water, or exposure to complex effluents. This has been followed by gene expression analyses in paper I, II and III, whereas bioconcentration studies and accompanying chemical analyses were performed in paper I, II and IV. In paper IV analyses of effects on the physiology of the fish were performed. In this chapter the different types of experiments and analyses will be presented.

3.1. Fish exposures

In this thesis, all exposure experiments were performed using the salmonid rainbow trout (*Oncorhynchus mykiss*) obtained from local fish farms. The rainbow trout is highly suitable for the combination of different analyses performed here. Its physiology is relatively well-known compared with most other fish species. Although its genome is not yet fully described, as for e.g. zebrafish (*Danio rerio*), information on gene sequences are sufficient for performing microarray studies. The Institute for Genomic Research Rainbow Trout Gene Index (RTGI) database (http://compbio.dfci.harvard.edu/tgi/) has an extensive library of expressed sequence tags (EST) available for this species [131] and in contrast to zebrafish, rainbow trout are sufficiently large for collecting the amount of blood plasma required for subsequent chemical analyses. Rainbow trout thrive well in laboratory conditions and have a high tolerance to stressors occurring in different exposure situations. Although not a native species of the Swedish coastal or freshwater nature, they tolerate conditions found in Swedish waters, thus there is the possibility of performing field studies (e.g. caged downstream STPs). In order to be able to evaluate if effluents caused estrogenic effects in fish, juvenile trout were used in the studies in this thesis, since maturing female fish naturally express *vtg* and other biomarkers for estrogenic exposure.

Microarray experiments are relatively costly and there is consequently often a limitation on the number of biological replicates applied in such studies. Therefore, it is of great importance to reduce the biological (and technical) variation. Due to this, all exposure experiments in this thesis were performed under controlled laboratory

conditions. Furthermore, to reduce variation between individuals, the fish were not fed during the experiments, as dominant fish in the aquaria often feed considerably more than subordinate fish. Rainbow trout cope well without food for two weeks, which is the applied exposure length in all studies here, and lipid content differs very little from fed fish [132]. Applying an exposure length of two weeks is a compromise as there are advantages as well as disadvantages with any exposure length selected. Firstly, a relatively long exposure allows chemicals to bioconcentrate, though we cannot be entirely sure whether they have reached $F_{SS}PC$ with the experimental setup used in this thesis. Responses are expected to be more stable after a longer period, whereas acute responses are often more variable. On the other hand, initial effects may be stronger and not observed after a longer exposure due to compensation. Other advantages with a two week exposure compared with longer exposure periods are: the possibility to let the fish starve while still avoiding strong effects of food deprivation; the time period is short enough to avoid unexpected incidents (e.g. water pump failure), as failure tend to increase with experimental time length; longer exposures require more of the substance to be studied; possibility to perform online effluent exposures which can be used for comparisons to field exposures. Within the field of ecotoxicology the exposure length of two weeks is at least considered semi-chronic, if not chronic.

The exposures to single substances in paper I, II and IV, were performed at several water concentrations to investigate the dose-response relationship. In paper I and II the lowest concentrations of pharmaceutical used corresponded to levels measured in effluents. The higher concentrations were used to guide the identification of gene responses in the lower concentrations, as genes with small changes in regulation may be difficult to identify when thousands of genes are analyzed in parallel. However, this strategy, previously applied by Gunnarsson et al. [128] to identify gene responses at low exposure concentrations to estrogen, makes the assumption that genes that are differentially expressed at a low exposure are also differentially expressed at a higher exposure. Additionally, if a response is observed after exposure at several concentrations of a substance, the probability of the response being false-positive decreases, especially if there is a dose-response pattern [99]. The chosen organ for the gene expression analyses was the liver as it is the major detoxification organ and is affected by many pharmaceuticals. All fish experiments were approved by the local animal committee in Gothenburg (permission no. 36-2007 and 216-2010).

3.2 Bioconcentration

3.2.1 Customized bioconcentration studies versus OECD 305

In all the exposure experiments in this thesis, a continuous flow-through setup was used. This is more preferable than a semi-static setup for several reasons: it allows a higher load density of fish and a continuous flow of the substance; it results in a higher water quality and a closer resemblance to the environment; it subjects the fish to less handling stress as the water is not changed manually. According to the OECD 305 guidelines – "Bioconcentration: Flow-through fish test" (http://www.oecd.org) [133], there are a number of criteria that should be fulfilled in a bioconcentration study. However, because the studies in this thesis also included other analyses (e.g. gene expression), a number of these criteria were not met. Firstly, no depuration period was included in any of the bioconcentration studies, because although a continuous flow-through setup was used, there were still loading density limitations (approximately n=10 depending on fish size) and thus all fish were subjected to the subsequent analyses. However, the guidelines do state that a depuration period is always necessary, *unless* the uptake of the substance is low, e.g. a BCF less than 10. As previously mentioned the fish were not fed to reduce variation in the subsequent analyses. Thus, the exposure time was shortened to 14 days instead of the requested 28 days for reasons stated in the previous section (3.1). The purpose of having a 28-day exposure is primarily to ensure that steady state has been reached, which is most likely acquired for at least diclofenac and ketoprofen within only a few days [91].

The guidelines further state that the concentration of the substance should be sufficiently low to be dissolved in water and the use of solvents is not recommended, though acceptable if necessary. In paper IV, we aimed to reach fish plasma concentrations of the substance corresponding to H_TPC and/or set the highest water concentrations to levels previously shown to have effects on exposed fish. However, for this to be met, exposure concentrations needed to be at such high levels that a solvent was required. In addition, although not among the recommended in the guidelines, dimethyl sulfoxide (DMSO) was the solvent of choice for a number of reasons. For example, the substances could be dissolved using DMSO at concentrations where no effects of fish have been documented [134, 135]. Control fish were exposed to DMSO at

the highest concentration used to dissolve the substances. The concentrations of the substances should also be below the chronic effect levels. This could not be met since a major aim of the studies was to assess possible effects and relate them to the plasma concentration.

Measurements of biological responses and internal exposures on individuals rather than on pooled samples are usually preferred as this increases the possibility to establish cause and effect. However, in paper I, plasma samples were pooled in pairs, due to the small amount of blood that could be extracted from each fish (average fish weight of approximately 40 g). The method for the chemical measurements of diclofenac was not yet sufficiently refined for detection at such low concentrations in such small plasma volumes.

In all bioconcentration studies in this thesis, aquaria duplicates were used for each concentration. Each aquarium contained at least eight fish which gives $n_1+n_2 \geq 16$. However, in bioconcentration studies, individual fish are sometimes argued to be pseudo replicate and that the number of true replicates is the number of aquaria of each exposure concentration, i.e. n=2 here. The practice used in this thesis is, however, very common. This may possibly be due to the major infrastructural challenges required to apply comprehensive replications in each experiment with, for example eight aquaria per group times four treatments, i.e. 32 aquaria, each supplied with individual flow-through and dosing systems. In fact, in many well-cited fish studies performed by other labs on pharmaceuticals and fish, only one aquarium [94, 136-140], or sometimes two replicate aquaria [141, 142], have been used per concentration.

3.2.2 Chemical analyses

In this thesis, chemical analyses were performed on liver (Paper I), blood plasma and water (Paper I, II and IV) to assess the actual, or internal, exposure. In this section, the workflow is briefly presented [143, 144]. In all three studies, surrogate standards were used and added to all samples (labeled naproxen (methyl-^{13}C; methyl-D_3) in paper I and II and D_6-amitryptiline in paper IV). All water samples were filtered through a membrane filter before extraction, hence no particles were included in the water samples. Plasma samples were diluted with aqueous formic acid before filtration. The

next step was the extraction of the substance, which was performed by first applying the samples to solid-phase extraction columns and then eluting the compound of interest with methanol. Solid-phase extraction was not performed on the liver samples and they were measured differently. They were only homogenized, extracted with acetonitrile and then filtered using a membrane filter.

In paper I, gas chromatography (GC) was used as separation method for the plasma and water samples, whereas liquid chromatography (LC) was used for the liver samples as they required additional selectivity. In paper II and IV, the same LC system was used for all samples. Both chromatography systems are based on transportation of the samples in a mobile phase which is then forced through a solid phase. Due to the differences in distribution of the analytes between the two phases, they will be separated from each other via travel speed through the solid phase.

In GC the sample is vaporized and swept by a stream of carrier gas (the mobile phase) through a heated column containing an involatile liquid, i.e. the solid phase. The limitation of GC is the requirement of the substances to be easily vaporized and thermally stable. However, the advantage using GC is that the outcome is both qualitative (identification of individual components) and quantitative (concentrations of individual compounds).

Although GC has been used extensively, the technique has been overtaken by LC, which is the most widely used analytical separation method nowadays. As the name suggests, the mobile phase is liquid and the technique does not require volatile or thermally stable compounds. Another major benefit to using LC is the possibility to analyze water soluble substances. However, although the sensitivity of an LC is high, it does not reach the sensitivity level of a GC.

There are several different types of detectors for both techniques. However, mass spectrometry (MS) is ideal as it provides both quantitative and qualitative information. It consists of three major parts: the ion source, the mass analyzer and the detector. Since the mass spectrometer uses electric and magnetic fields to move and manipulate the analytes, it is a requirement that the analytes are ionized. In addition, it requires that the ions are in gas phase. Different ion sources are used in GC and LC but both generate ions in gas phase. In the analysis using GC, the principle behind the ion source is that the

sample is bombarded with high energy electrons which removes an electron from the analyte molecule on impact. In the analysis using LC, electrospray ionization is used which is based on a nebulizer which atomizes the mobile phase to a vapor. At the tip of the nebulizer is a high voltage, which produces charged droplets. Evaporation of the droplets will make the charge density on the surface too large and the droplets explode. When the droplets eventually become small enough, they transfer the charges on the surface to the organic molecules. The ions are transported from the ion source into the vacuum of the mass analyzer where they are separated according to their mass to charge ratio (m/z). There are several types of mass analyzers as well, though here quadrupoles were used. In a quadrupole, a range of m/z ratio can be applied and consequently only compounds of interest reach the detector where the ions are transformed into a usable signal. To increase the selectivity and sensitivity, mass analyzers can be used in series (tandem MS), for example the triple-stage quadrupoles coupled with the LC used in this thesis, i.e. LC-MS/MS. The result from the GC/LC-MS analysis is an output showing peaks of different heights, which indicate the concentration, separated by molecular weight (Fig. 5).

To ensure high quality results from the analyses, several precautions have been taken. Standards and blanks were run at several time-points and recoveries of solid-phase extractions were measured (spiking of non-exposed samples).

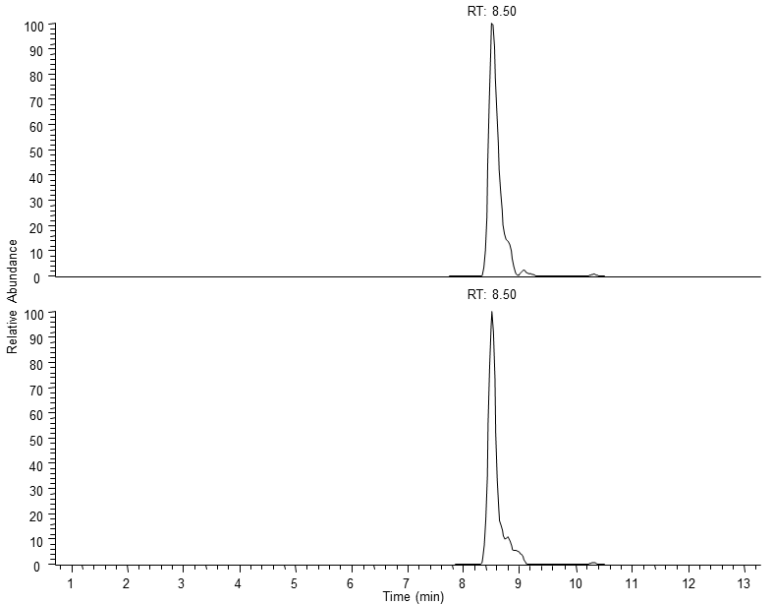

Figure 5. A chromatogram showing the results from the LC-MS/MS analysis of diclofenac in paper I. The upper peak represents the quantification ion (294 → 250) and the lower peak represents the quality ion (296 → 252).

3.3 Microarray

3.3.1 From design to raw data

There are several different types of microarray platforms. In this thesis, we have used the Geniom/RT-analyzer platform, which is an oligonucleotide array provided by febit (Heidelberg, Germany). Due to the limited availability of commercial array designs for environmentally relevant species, a custom design was required. At the time, few companies besides febit offered this possibility. Unfortunately, the initial probe design strategy and hybridization and washing protocols suggested by febit resulted in low correlation between array and qPCR data. Hence, we evaluated our own probe design and modification to the experimental process. The result was an array:qPCR correlation matching the quality of the best commercial arrays [145].

OligoArray 2.1 was used to design 50-mer probes [130, 146] using transcripts from the RTGI database, which contained roughly 80,000 transcripts [131]. As the Geniom platform allows 15,000 probes, not all putative rainbow trout genes could be included, thus well-annotated genes were primarily selected. These genes included homologs to drug targets predicted by Gunnarsson et al. [110], genes associated with pharmacological processes described in the Pharmacogenetics and Pharmacogenomics Knowledge database (PharmGKB; http://www.pharmgkb.org/index.jsp), genes described in Comparative Toxicogenomics Database [147], homologs to all cytochrome P450 genes annotated in zebrafish etc. To predict the rainbow trout homologs, the Washington University Basic Local Alignment Search Tool 2.2.6 (BLAST; http://blast.wustl.edu) [148] was used in tblastx mode. The remaining space on the array was assigned to randomly selected rainbow trout ESTs. To annotate the microarray, the transcripts associated with the probes were compared with UniProtKB/Swiss-Prot [149] and Ensembl [150].

Although all array platforms are based on the same general principles, there are a few differences between them. In this thesis, we have used an oligonucleotide array and following is a simplified description of the experimental progress. The tissue of choice is homogenized and total RNA is extracted. The mRNA is subsequently converted into biotinylated (fluorescence-labeled) amplified RNA (or antisense RNA; aRNA) and hybridized to the chip. When a specific transcript binds to its matching probe on the

chip, a fluorescent signal can be induced and visualized by a camera (Fig. 4). The signal increases depending on the amount of binding, i.e. a measurement of the mRNA abundance, and the intensity is extracted using image analysis softwares, which in our case was the Geniom Wizard (no longer available due to reconstructions of febit).

3.3.2 Data analysis

The output data from a microarray analysis must be further processed prior to statistical analysis and biological interpretations, i.e. background correction and normalization. To remove artifacts originated from the synthesis/hybridization process and noise, background correction is performed, usually through subtraction of the background from the intensity in the probe spots [130]. Subsequently, the array is normalized to compensate for overall signal differences due to unequal concentrations of the added aRNA, variations in hybridization efficiency etc., to enable comparisons between different samples/arrays. There are several methods for normalization. The one used here was the quantile-quantile algorithm [151], which is a method used to make the distribution of probe intensities similar for every sample.

Standard statistical methods, such as t-tests, are usually not sufficient for analyzing microarray data. Since such a high number of genes is present on an array, treating each gene independently disregards information as many properties may be shared among genes, e.g. their within-group variability. Therefore, implementing additional criteria is crucial. Here, moderated/Bayesian t-test was used which, rather than repeatedly estimates the within-group variability for each gene, pools the information from many similar genes. This type of test additionally includes a fold-change criterion [152]. However, the result may still include false-positives, because of the high number of genes analyzed. Thus an adjusted p-value should be provided. In paper I, II and III, the p-value was adjusted by calculation of the Benjamini–Hochbergs false discovery rate (FDR) [153], though referred to as adjusted p-value in paper II and III. By setting a threshold at an FDR of, for example, 0.3, the list of potentially differentially expressed genes is estimated to contain 30% false-positives.

3.3.3 Gene ontology analysis

In order to identify the type of biological response or the mode of action after an exposure, several strategies can be applied. One might simply study the genes connected to the known drug target in humans, though a large amount of information may be lost and the purpose of using a microarray with thousands of genes is somehow lost. Fortunately, there are tools to assist in the analysis process. In paper I and III, analyses of enriched gene ontology (GO) terms were performed. The GO project is a major collaborative initiative which aims to systemically assign genes and gene products descriptions within three different ontologies: biological process, molecular function and cellular component (http://www.geneontology.org) [154]. For example, the differentially expressed gene in paper I, complement component C7, is assigned to biological processes connected to the innate immune response, e.g. complement activation – alternative pathway (GO:0006957). By studying a set of plausibly differentially expressed genes on an array, e.g. FDR<0.2, one may identify several genes belonging to the same processes. One way to achieve this is by using GOrilla (http://cbl-gorilla.cs.technion.ac.il/) [155], which was done in paper I and III, where enriched processes within a data set are searched for, i.e. processes that include several genes in the selected data set. Although a threshold of FDR<0.2 would include false-positives, this type of analysis allows more false-positives as these are less likely to have the same annotation as the truly differentially expressed genes [145].

Nevertheless, it should be noted that this type of analysis is just a tool to generate biological hypotheses and to aid in the identification of differentially expressed genes of interest. In other words, just because the process for dibenzo-p-dioxin metabolism is enriched does not necessary mean that the fish have been exposed to polychlorinated dibenzo-p-dioxins (PCDDs). In addition, few rainbow trout proteins have been assigned to GO terms and thus GO analyses must be performed on orthologous proteins in other species. The analyses performed in GOrilla depend highly on the choice of reference species as the GO term assignments are directly linked to available studies in the literature. For example, in the human database the GO term process complement activation (GO:0006956) has 192 products, whereas the same process in the zebrafish database only has 5 products. Furthermore, one of the major problems with GO term analysis using reference species is that the genes are assigned to GO terms according to

their function etc. in the selected reference species. Whether or not the gene has the same function in rainbow trout remains uncertain in many cases. Taking all these aspects together, GO term analysis may indeed be a powerful tool, though it highlights the issue that conclusions concerning affected physiological processes are highly influenced by the available knowledge of protein functionality in the species.

3.4 Quantitative PCR

Microarrays do provide an extensive amount of information. However, other methods are generally needed to quantify the actual differences in mRNA abundance of genes that are identified as differentially expressed by microarrays. Due to artifacts (e.g. cross hybridization) and the relatively small dynamic range, which is an issue if exact quantification of mRNA is important, the microarray data and hypotheses need validation. Quantitative real-time polymerase chain reaction (qPCR) suites this purpose as it is one of the most sensitive tools for measuring mRNA abundance. Nevertheless, it does require optimization. Design of specific primers is crucial though may be a challenge for rainbow trout, partly due to the uncertainties in nucleotide sequences available (only EST library). There are other issues that may influence the outcome data as well, e.g. primer-dimers and contamination by genomic DNA. In this thesis, a dissociation stage was added at the end of the amplification cycles to evaluate the specificity of the amplification. Additionally, for every sample conducted in the qPCR analyses, no reverse transcriptase (NoRT) samples were run to ensure that no genomic DNA was present at levels that could interfere.

To adjust for differences between samples not caused by the exposure, for example dilution and pipetting errors, the qPCR data need to be normalized, just like the microarray. Therefore, the usage of good reference (housekeeping) genes is also crucial when conducting a qPCR analysis. Optimally a reference gene should be expressed at levels similar to the genes of interest, should vary little between individuals and should not be affected by the treatment. There are a number of genes that are very commonly used as reference genes, including β-actin (*actb*) and ubiquitin (*ubq*). It is desirable to use two or even more reference genes and use the average expression for normalization, though one gene is often considered sufficient. In paper II, both *actb* and *ubq* were used,

whereas only *ubq* was used in paper III due to material limitations. In paper I, however, both *actb* and *ubq* tended to be affected by the exposure and thus two other genes, calnexin and thiopurine S-methyltransferase, were used and both proved to be suitable [156]. The normalization was subsequently performed by subtracting the relative threshold cycle (C_t) values of the reference gene (or average if two were used) for each sample from the C_t values for each gene and sample. The resulting value, ΔC_t, was then used in the subsequent statistical analyses.

4. Results and Discussion

Many pharmaceuticals are found in the aquatic environment and effects on wildlife species are a growing concern. In 1998, Beland wrote: "Searching for definite proofs in a traditional sense is illusory and should not bog us down into inaction when the survival of an important element of our environment is at stake" [157]. It may be a bit harsh to relate this citation to the traditional risk assessment of pharmaceuticals in the environment, though today's traditional test strategies are from many perspectives insufficient to capture the potential risks of some substances (see section 1.5). Therefore, there is an increasing interest in other complementary methods that may take the understanding of the impact of pharmaceuticals in aquatic organisms to the next level.

4.1. Bioconcentration

If an API is to have an effect on an aquatic organism, it has to be taken up and the more that is taken up, the greater is the likelihood for a pharmacological interaction to result in a meaningful effect. This is, in its simplest meaning, the background for studying bioconcentration potential in order to identify pharmaceuticals that may be of concern in the aquatic environment. In paper I, II and IV we have exposed fish to single substances and measured their plasma concentrations to calculate BCFs and to read-across from H_TPC and thereby estimate the probability for a pharmacological interaction to occur.

4.1.1. Bioconcentration of NSAIDs (Paper I and II)

Non-steroidal anti-inflammatory drugs are a group of pharmaceuticals of high concern when it comes to environmental impact, much due to their very frequent occurrence in STP effluents and surface waters at relatively high concentrations (up to µg/L). Diclofenac has particularly been highlighted and is one of the most well studied drugs regarding potentiality to affect aquatic organisms. In paper I, the BCF of diclofenac to blood plasma was found to be approximately 4, which is rather similar to most

previously reported BCFs to blood plasma ranging from 2.5 to 29 with a median of 5 (Fig. 6a) [3, 91, 138]. The determined BCF to liver was in the same range as the BCF to plasma, at approximately 2.5. Importantly, both BCFs were stable throughout the exposure concentrations, which is a prerequisite for applying the predictive bioconcentration models proposed by Fitzsimmons et al. [124] and Hugget et al. [125]. In contrast to our findings, Schwaiger et al. [94] reported that the BCF of diclofenac from spiked aquaria water to rainbow trout liver varied from 2,732 at a water concentration of 1 µg/L, to only 12 for a water concentration of 500 µg/L, i.e. roughly a 200-fold drop. This pattern was also observed for ketoprofen, though to a lesser extent, in paper II where the BCF to blood plasma dropped 6-fold over a 100-fold water concentration increase, from 0.042 to 0.007 (Fig. 6b). Decreasing BCFs with increasing water concentrations are not commonly reported, though they have been observed for other chemicals in other species (*Oryzias latipes*, *Perna viridis* and *Dreissena polymorpha*) [158-160]. Such trends could be explained by for example a saturation of binding sites for the bioconcentrating chemical in the organism or by insufficient energy required to bind the chemical at higher concentrations [160]. However, none of these explanations seem adequate for the observations in the study by Schwaiger et al. [94] since the highest reported BCF of >2000 is several magnitudes higher than in paper I. Differences in BCF magnitudes could possibly be explained due to feeding differences. In the paper I, the fish were not fed, in contrast to the study by Schwaiger et al. However, after a 28-d starvation period, the lipid content in the liver of juvenile rainbow trout differs very little from that of fed fish, although there is a twofold decrease in muscle lipids that could possibly favor a shift of lipophilic contaminants from the muscle to the liver [132]. Nevertheless, it seems highly unlikely that this could be a major part of the explanation behind the approximately 1,000-fold differences in liver BCF between the studies. It should also be mentioned that in contrast to the other studies on diclofenac, including paper I and II, DMSO (0.12‰) was used as solvent by Schwaiger et al. [94], though the impact of DMSO (or other solvents for that matter) on BCF remains. To sum up, we cannot explain why the results of Schwaiger et al. differ from those in paper I. However, given the quality assurance of the analytical method in paper I, as well as the consistency over a series of water concentrations and the coherence between the liver BCF and the plasma BCFs in the same study and other studies [3, 91, 138], it appears more reasonable that the results in paper I reflect the actual bioconcentrating behavior of

diclofenac. In addition, although the predicted BCF of diclofenac (93) [3] is higher compared with the BCFs in paper I, it is much closer to the BCFs of diclofenac in paper I than the highest reported BCF by Schwaiger et al [94].

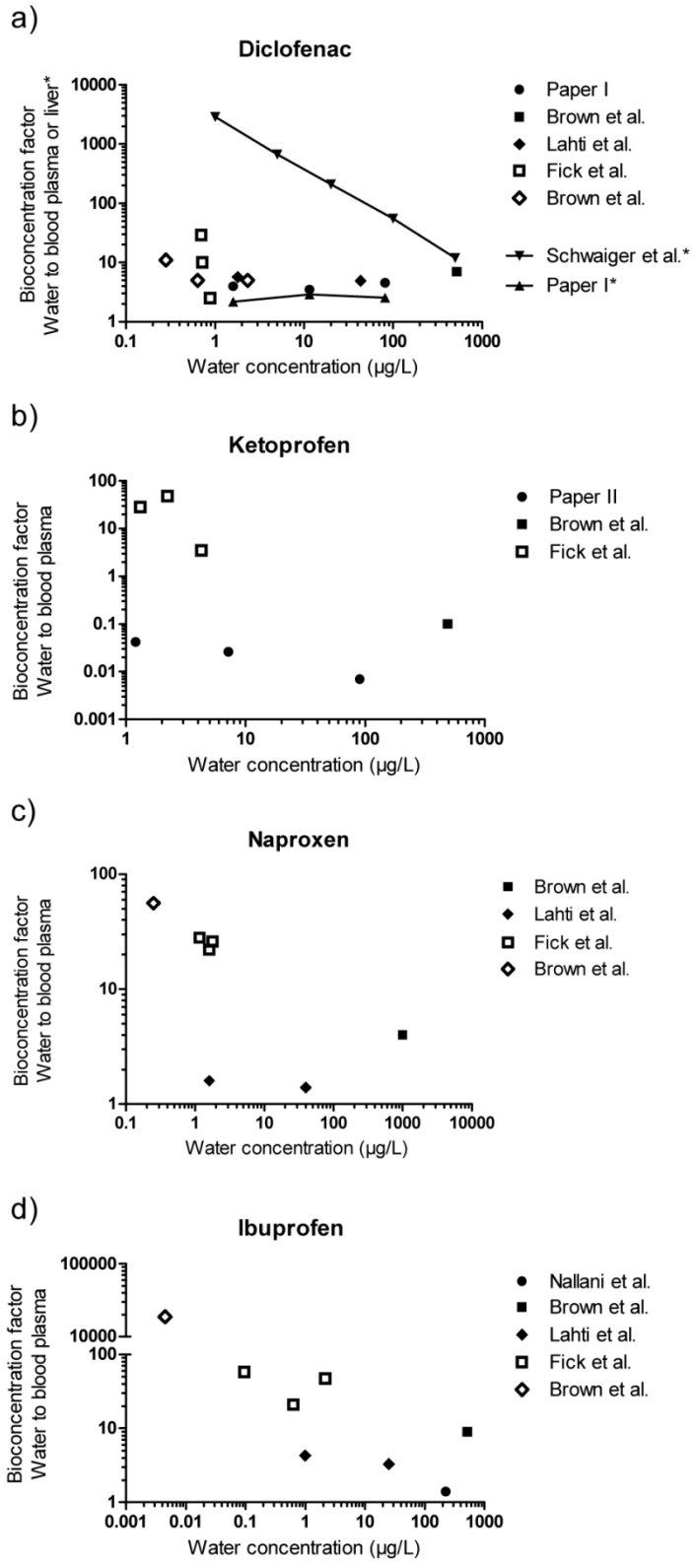

Figure 6. A comparison of bioconcentration factors of four NSAIDs to blood plasma and liver* in fish exposed to single substances or a mixture of a few substances in pure water under controlled lab conditions (closed symbols) versus fish exposed to undiluted sewage effluents (open symbols). Data was collected from the paper I and II as well as from previously published studies Fick et al. [3], Brown et al. [91], Schwaiger et al. [94], Lahti et al. [138] and Nallani et al. [164]. In the study by Brown et al. [91] only nominal concentrations were used for the lab exposure.

In the fish exposed to the lowest concentration of diclofenac in paper I, the plasma concentrations were approximately 6 ng/ml, which corresponds to less than 1.5% of the diclofenac H_TPC of ≥420 ng/ml [8, 161, 162]. If the read-across strategy is applied to these results, diclofenac would have moderate, if any, effects on fish at concentrations found in effluents. Accordingly, few effects were seen on the global hepatic gene expression at the lowest exposure concentration, though increasingly distinguishable when plasma levels in the fish approached H_TPC ($F_{SS}PC$ of approximately 88% of H_TPC at the highest exposure concentration; see section 4.2.1).

To address whether ketoprofen, a drug with similar mode of action and clinical applications, would be a better alternative to diclofenac with regards to effects in fish, the same strategies and experimental setup as for diclofenac in paper I was applied in paper II. Similarly to diclofenac, reported BCFs of ketoprofen to fish blood plasma varies as well, from 0.1 to 48 [3, 91]. The results from the ketoprofen study in paper II showed that waterborne ketoprofen bioconcentrates considerably less than does diclofenac under controlled laboratory conditions. In fact, ketoprofen did not bioconcentrate at all, since measured plasma concentrations were lower than the surrounding waters. At the highest exposure concentration of ketoprofen, i.e. roughly 100 times higher than levels found in undiluted sewage effluents, the plasma levels reached less than 1% of H_TPC (>1000ng/ml) [163]. Thus, the probability of pharmacological interactions rendering physiological effects is very small according to “the fish plasma model” [125]. Accordingly, no effects on the global hepatic gene expression could be confirmed (see section 4.2.1), which is in contrast to diclofenac at corresponding water concentrations. These results support our hypothesis that the use of ketoprofen rather than diclofenac may pose lower risks for exposed fish.

However, the BCF for ketoprofen to blood plasma of <0.05 found in paper II differs considerably from studies where rainbow trout were exposed to undiluted sewage effluents (BCF=3.5-48) [3, 91], but is more similar to the BCF of 0.1 previously reported for rainbow trout exposed to nominal concentrations of ketoprofen together with four other pharmaceuticals in pure water under controlled lab conditions [91]. In fact, higher BCFs in fish exposed to undiluted effluents compared to single substances in pure water is a collective trend for several NSAIDs as revealed by a meta-analysis on naproxen and ibuprofen (Fig. 6) [3, 91, 138, 164]. Even diclofenac shows a similar

tendency, though not as clearly [3, 91, 138] (Paper I). This suggests that other constituents of treated sewage effluents may influence the uptake, distribution, metabolism or excretion of NSAIDs and perhaps other pharmaceuticals to an extensive degree. If true, the concentration of other substances is just as, or even more, important than the studied substance and it furthermore raises the question on how relevant the BCFs for some APIs generated under controlled lab conditions with single substances in pure water are for reflecting risks in the field environment. In traditional risk assessment, this type of substance behavior is not taken into account, thus substances considered environmentally safe and non-bioconcentrating may in fact pose serious threats [7, 123]. Nevertheless, neither undiluted effluents, nor exposure to a single drug in pure water reflect the exposure situation for wild fish and it remains to be seen which scenario provides the best approximation.

If the highest reported BCF of ketoprofen to blood plasma of 48 [3] is applied in a scenario where fish are exposed to sewage waste water in an effluent-dominated stream with a surface water concentration of 1 µg/L ketoprofen, this would give a predicted plasma concentration of only 5% of H_TPC in the exposed fish [163]. Thus, the probability for pharmacological interactions to lead to (adverse) effects is still quite small [125]. However, because diclofenac has been reported to cause effects on gene expression at plasma concentrations that are considerably lower than the corresponding H_TPC [141] (Paper I), we cannot yet reject the possibility of effects in fish exposed to ketoprofen at concentrations found in the aquatic environment.

4.1.2. Uptake of the glucocorticoid beclomethasone-diproprionate and its metabolite beclomethasone (Paper IV)

Beclomethasone is administered as a prodrug, beclomethasone-diproprionate (BDP), which is metabolized into its more active forms beclomethasone-17-monoproprionate (BMP) and free beclomethasone as well as other inactive forms and conjugates. When investigating the bioconcentration potential of this drug and applying read-across, it is therefore not as straightforward as it is for e.g. diclofenac and ketoprofen.

Exposure to BPD resulted in all three active forms of the drug in the blood plasma, though not at quantifiable levels for free beclomethasone, whereas no uptake could be confirmed upon exposure to free beclomethasone. Furthermore, both BMP and beclomethasone were found in the water where fish were exposed to BDP, indicating metabolism and excretion by the fish. Free beclomethasone was detected at much lower levels than BMP, suggesting that the primary excretion product from fish may be BMP and not free beclomethasone. While it cannot be excluded that BDP may transforms to BMP and free beclomethasone in the aquarium water, BDP has previously been reported to be relatively stable in aqueous culture medium at 37°C [165]. Based on the concentrations of all three forms of beclomethasone measured in the water and in the plasma of the fish, the calculated BCFs were 3.34 (low dose) and 0.75 (high dose).

In humans, BDP plasma levels are reduced by >99% 30 minutes after a single intravenous dose and consequently, BMP levels rapidly reach more than ten times the concurrent BDP concentration [117]. This indicates a very rapid metabolism of BDP and if applicable in fish as well, the uptake in the fish is most likely very rapid too, since we find a high BDP:BMP ratio in the blood plasma of the fish exposed to high dose BDP. In the fish plasma, each metabolite is at equilibrium with the surrounding water, in contrast to humans (Fig. 7). This means that a high metabolic conversion of BDP to BMP may not be reflected in a very high concentration of plasma BMP since BMP (and free beclomethasone) is expected to be lost not only through further metabolism as in humans, but also to the surrounding water through the gills. However, a high BDP:BMP ratio in the fish plasma may also be the result of a slow metabolism of BDP. Nonetheless, because both BMP and free beclomethasone were found in both the blood plasma and in the water, metabolism of BDP is evident. The plasma concentration of BMP was quite similar over the two exposure concentrations of BDP even though the plasma concentration of BDP increased. This may be explained by a limited metabolic capacity or differential distribution between organs within the fish.

In addition to its intended local effects, BMP can have adverse systemic side effects at H_TPC (0.33 ng/ml) via hypothalamic-pituitary-adrenal suppression [113, 119, 166]. Therefore the H_TPC values are relevant for read-across in paper IV, at least from this perspective. The concentrations of BMP measured in the current study are close to the H_TPC and effects on the transcriptional activity on the glucocorticoid receptor 2 in

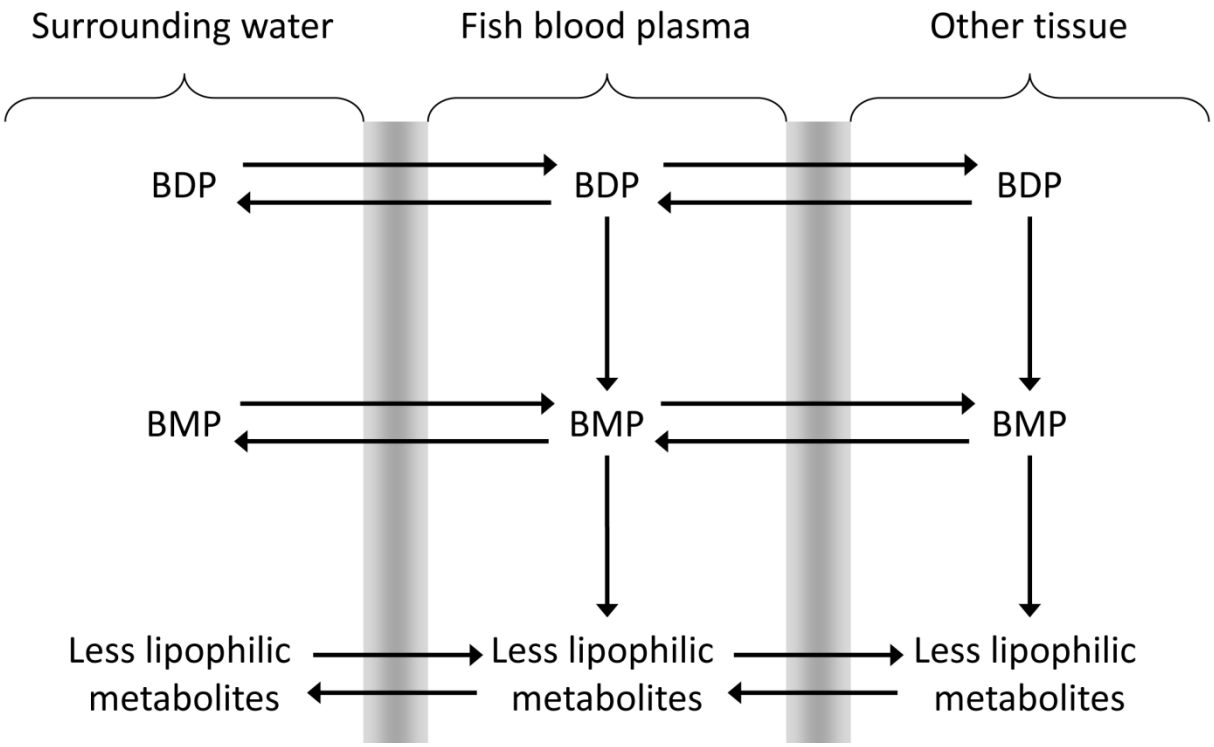

Figure 7. Equilibrium of beclomethasone-diproprionate (BDP), beclomethasone-17-monoproprionate (BMP) and less lipohilic metabolites in fish.

fish have previously been reported near these concentrations *in vitro*, though not *in vivo* [108]. However, since BDP, BMP and free beclomethasone all bind the receptor, though with different affinities [118, 165], it is somewhat difficult to apply read-across from plasma concentrations in humans to predict potential effects in model organisms. Although BMP was found at rather similar plasma concentrations in both groups of BDP exposure, the physiological effects studied here were only significant at the highest exposure concentration of BDP. Thus, we cannot exclude a contribution from other active forms of beclomethasone than BMP. In fact, the relative transcriptional activities of BDP, BMP and free beclomethasone in fish are not known.

4.1.3. The applicability of bioconcentration studies and the read-across strategy

The results from paper I on diclofenac support the assumptions made by Hugget et al. [125] in "the fish plasma model" and this study is, to the best of my knowledge, the first where the relation between blood plasma levels of fish exposed to a pharmaceutical in the water, and responses in the fish, are specifically addressed and documented. At a

plasma concentration well below the H_TPC in exposed fish, observed responses were moderate, but became more distinct closer to the H_TPC. Interestingly, the observed responses showed apparent similarities with effects observed in humans (see section 4.2.1). In paper II, plasma concentrations of ketoprofen in the exposed fish were orders of magnitudes below the H_TPC, and accordingly no effects could be detected. Unfortunately, none of the exposure concentrations led to sufficiently high plasma concentrations of ketoprofen for us to be able to confirm a pharmacological interaction. Thus the possibilities for applying read-across according to "the fish plasma model" could only be tested with regards to the fact that effects were absent when the plasma concentration of the drug was low, but not in the sense that effects were seen at or close to the H_TPC. In paper IV applicability of read-across according to "the fish plasma model" was not as clear-cut, possibly due to the complicated nature of pharmaceuticals with multiple active forms binding with different affinity (see section 4.1.2) [108, 118].

Although the read-across concept is a powerful strategy from many perspectives, particularly in identifying pharmaceuticals posing environmental risks, one should be careful and not draw the conclusion that plasma levels far below the H_TPC will not result in any effects. On a further note, as the studied organ in paper I and II is the liver, extrapolating from responses in human liver to fish by using the read-across strategy may not be entirely correct. At similar plasma concentrations in fish and human, e.g. at H_TPC, the human liver would often be subjected to higher concentrations than the fish liver if the drug is administered orally. This is because in humans, the exposure would occur through the intestine which carries high concentrations via the portal vein directly to the liver and then out in the bloodstream, while fish take up the drug straight into the bloodstream via the gills. As the liver is the main location of metabolism for many drugs, the concentration of the substance is often much lower when the blood leaves the liver and enters into the circulatory system, where H_TPC is measured. In this event, a modification of "the fish plasma model" is therefore desirable, as a higher concentration is needed in the fish plasma in comparison with humans in order to have the same exposure in the liver. However, if forms of administration where the substance does not undergo metabolism in the liver before reaching the bloodstream are used (e.g. intravenous or topical with a systemic target), "the fish plasma model" is applicable as the concentration reaching the liver would be similar for fish and humans at similar plasma concentrations. It may also be used without modification if the studied organ is

not the liver, as the concentration present in the plasma is also the concentration to which the studied organ is exposed. On the other hand, if the pharmaceutical is locally administered, as beclomethasone-diproprionate, or BDP, in paper IV, a straightforward use of "the fish plasma model" is also questionable as the concentration in the target organ would most likely be higher than in the plasma. Though for BDP, systemic effects have been reported at H_TPC in humans [104], thus read-across is still applicable.

Bioconcentration studies can be used for other applications as well, in addition to the read-across combination. For example, when conducting a screening of concentrations of substances in the aquatic environment, measurements in biota (e.g. in fish) may at times be a better strategy than measurements in water. To put this in context, the discussion here is exemplified by the EU Water Framework directives [97], where concentration limit values, or Environmental Quality Standards (EQS; see section 1.4.1), for some chemicals are set for biota as well as for water. For very hydrophobic substances the biota EQS should primarily be applied as these substances accumulate in biota and are hardly detectable in water even when using the most advanced analytical techniques. Unfortunately, this does not apply to all chemicals, since EQS have only been set for surface waters for the recently included APIs, EE_2, E_2 and diclofenac. There is, however, a number of advantages with measuring these compounds, and other chemicals, in biota, e.g. fish. Firstly, as previously described, concentrations of substances in the surrounding waters are not the only factors of importance when predicting risks to organisms, as the rate of uptake and bioconcentration etc. must be taken into account as well. Although it is possible to extrapolate BCFs from other studies and apply them to measured water concentrations, it is, according to the meta-analysis in paper II, not that simple since BCFs may vary between different exposure conditions. However, uncertainties associated with extrapolations are avoidable by instead measuring substances in organisms exposed to the water of interest, thereby providing information on the actual (internal) exposure. Secondly, water concentrations may vary considerably over time in the field. A water sample collected at one time-point may differ completely from that of another time-point, especially in effluent-dominated streams, and it may be a matter of only a few hours, e.g. morning and evening [167]. The concentration in a fish, on the other hand, is not likely to be reflective of short term variations and, for substances not likely to reach equilibrium very quickly, biota samples will provide a better cumulative view of the general situation. There are exceptions,

however, where $F_{SS}PC$ is reached in a very short time, which most likely is the case for BDP as observed in paper IV. Thirdly, considering the relatively low EQS for EE_2 (0.035 ng/L) and the fact that steroids and other APIs are commonly present at very low concentrations associated with risks, the limitations in today's analytical techniques may in many cases lead to no detection in surface waters. However, due to the high bioconcentration potential of many steroids (and some other APIs), such high sensitivity in analytical techniques is often not expected to be required to detect the substance in biota. Fourthly, measuring APIs in biota also provides the possibility of read-across as much is known regarding effects versus internal exposure concentrations (dose) in humans, though the previously mentioned advantages apply for any chemical. Measuring in biota does nevertheless have downsides as well, including analytical challenges regarding both sensitivity and selectivity due to higher noise in biota samples. Collecting biota samples is also highly time-consuming and requires the use and sampling of animals. Predicting internal exposure from measured water concentrations by applying theoretical models for bioconcentration is more in agreement with the three Rs of experimental animal work; refine, reduce and replace. However, as shown in this thesis, there are still major knowledge gaps regarding which factors are important for bioconcentration of pharmaceuticals. Therefore, more empirical data, involving animal studies, are required in order to develop and validate bioconcentration models that can be accurately applied in the field for such a diverse group of chemicals as pharmaceuticals.

4.2. Gene expression

In order to evaluate the potential risk of APIs in the aquatic environment, it is an advantage to have information about the expected mode of action in exposed species, as this may provide insights as to possible adverse outcomes. Although the human drug targets are well conserved for the investigated drugs and the group of species studied in this thesis [110], pharmacological interactions may not lead to gene-response cascades identical to those observed in humans. The broad analytical approach provided by microarray analyses is therefore well suited for the purpose of identifying modes of action in fish exposed to various APIs. Furthermore, microarrays provide a possibility to

search for several biomarker responses in organisms exposed to a mixture; they provide the possibility to identify new potential biomarkers in organisms exposed to individual drugs or type of drugs, and they can provide an indication of the potency of a given drug/exposure. The technique was successfully used in paper I, II and III from all three different perspectives.

4.2.1. Gene expression of the NSAIDs diclofenac and ketoprofen (Paper I and II)

In paper I and II we used microarrays and qPCR to study the hepatic gene expression in fish exposed to the two NSAIDs diclofenac and ketoprofen. For diclofenac in paper I, all exposure concentrations led to differentially expressed genes. At the lowest exposure concentration of 1 µg/L, which corresponds to concentrations frequently detected in effluents [3, 31, 88-91] and occasionally in surface waters [32], the response was relatively moderate. Although 11 transcripts had a p-value <0.001, the FDR was above 0.78 indicating a high rate of false-positives [153]. Nevertheless, one of these 11 transcripts was differentially expressed in the higher doses as well. This consistency between responses at different doses increases the likelihood for being a true-positive [99]. In general, both the p-values and the corresponding FDRs decreased with an increasing exposure concentration and at the highest concentration, 70 transcripts had a p-value <0.001 (FDR>0.005) indicating an increasing response in a dose-response manner. As the likelihood for a response to be true increases additionally if it follows a dose-response trend [99], we performed a robust linear regression analysis as well. This resulted in 623 transcripts with an FDR below 0.3 and these were subsequently used for the GO-term enrichment analysis using GOrilla [155]. Although allowing a 30% chance of a transcript being a false-positive may seem quite high, GO-term enrichment analysis can accommodate a larger number of false-positives since falsely differentially expressed genes are less prone to be involved in the enriched pathways [145]. According to the GOrilla analyses against the human database, most enriched GO-term processes were connected to immune response and inflammation (Table 1), e.g. complement activation (GO:0006956). Hence, there is a strong indication of a similar mode of action of diclofenac in rainbow trout and humans as diclofenac and other NSAIDs exert their anti-inflammatory and analgesic effects mainly through inhibition of Cox, which takes part in several biological processes, including the

inflammatory response. Several of the genes included in these areas were complement components (e.g. *c7* and *c6*), which are part of the innate immune response, more specifically the complement activation (GO:0006956). Accordingly, complement components were induced in diclofenac treated mouse liver as well as other genes connected to the above mentioned processes [168].

Notably, when analyzing the same dataset, but against the zebrafish database using zebrafish gene annotations, the outcome differed completely. Most GO-term processes connected to metabolism rather than immune response and inflammation. This highlights the issue on choosing the correct reference species, as described in section 3.3.3. All processes, including immune response and inflammation, are well described and studied in humans, thus there are more genes assigned to GO-terms in these areas in comparison with zebrafish where the actual functions of different fish proteins have been investigated considerably less.

To confirm the results from the microarray analyses, qPCR was performed on a set of genes selected from different aspects, e.g. included among the top regulated transcripts or enriched GO-term processes. Although the Geniom platform had been used before and shown good correlation with the qPCR analyses in the hands of our group [129, 130], probes, annotations etc. differed from the design in paper I. Therefore, the qPCR was performed on the same individuals present on the microarray, i.e. a technical validation, and according to these results, the performance of the microarray was highly satisfactory. There was a high correlation between the microarray and qPCR data, indicating high quality of the microarray, i.e. low cross-hybridization etc.

In addition to providing information on the mode of action of diclofenac in fish, the gene expression results in paper I furthermore support read-across according to "the fish plasma model" [125] with clearer responses closer to H_TPC. In paper II, where fish were exposed to ketoprofen in an experimental set-up similar to that in paper I, the plasma concentration only reached a few parts per thousands of the H_TPC at the highest exposure concentration (100 µg/L). Therefore, the expected response would be low [125] and microarray analyses were thus only conducted on the fish exposed to the highest concentration of ketoprofen. Accordingly, the microarray analyses revealed very limited responses, if any, compared with the non-exposed fish: only 58 transcripts had a p-value <0.05 and the FDR was >0.99 for all (no transcripts had p<0.001, for comparison

with paper I). Although the microarray design had been further developed with new probes in comparison with paper I, we felt confident about the quality and performance due to the results from the previous studies using Geniom/RT analyzer (Paper I) [129, 130, 169]. Thus, the validation using qPCR could be performed on other individuals than the ones included in the microarray analyses, i.e. a biological validation. The genes selected for the qPCR analysis included transcripts with a relatively low p-value on the microarray. However, because the FDR was >0.99, most differentially expressed genes were most likely false-negatives. Accordingly, none of the genes were significantly differentially expressed when measured by the qPCR. As both plasma concentrations and gene expression were measured on an individual level, in contrast to paper I where two plasma samples were pooled to generate replicates, the results from the qPCR could be directly related to individual plasma concentrations. Nevertheless, controlling for individual differences in internal exposure did not reveal any significant effects. Consequently, we could not confirm any response in the fish exposed to ketoprofen at a concentration of 100 µg/L, and could therefore not assess the mode of action and compare it with diclofenac.

4.2.2. Evaluation of sewage treatment technologies using microarrays (Paper III)

To identify effects caused by substances frequently encountered in STP effluents, several research groups have used microarrays on individual drugs, metals etc. [127, 129, 170-172], including the studies in paper I and II. Using this approach, modes of action and biomarkers of exposure for these individual substances, or groups acting via the same targets (e.g. NSAIDs), have been identified or suggested. A combination of microarray studies on fish exposed both to single substances as well as to complex effluents, provides a possibility to identify which specific groups of compounds in the effluent indeed affect exposed organisms [173, 174]. This subsequent analysis strategy was applied in paper III. By using known biomarkers of exposure and mode of actions of different substances in fish, the effects of differently treated effluents on fish were studied, and consequently different treatment techniques could be evaluated with regards to these endpoints. The exposure was performed at Henriksdal STP, Stockholm, Sweden, using a large-scale pilot plant with parallel treatment lines. The different sewage treatment techniques included in this study were: conventional treatment with

activated sludge and sand filter alone or in combination with granular activated carbon, ozone 5 mg/L, ozone 15 mg/L, ozone 5 mg/L + a moving bed biofilm reactor or irradiation by UV radiation + hydrogen peroxide. A control group representing "clean" water was also included; this consisted of fish exposed to tap water treated by activated carbon and mixed with an addition of 2% conventionally treated effluent (without the added effluent the water is too clean for the fish). The fish exposed to any of the generated effluents were compared to the control group in order to assess which effluent had the least effect in fish.

The microarray analyses revealed differentially expressed transcripts, with a significance level at FDR<0.2 (referred to as "adjusted p-value" in the manuscript) [153], in the groups of fish exposed to conventionally treated effluents as well as the groups with the additional advanced treatment steps ozone 15 mg/L, ozone 5 mg/L + a moving bed biofilm reactor and irradiation by UV radiation + hydrogen peroxide. With a less stringent cut off ($p<0.01$ without multiple adjustment), all treatment groups had significantly regulated genes. This cut off ($p<0.01$) was subsequently used for the GO-term enrichment analysis. Overall, the fish exposed to conventionally treated effluents as well as the groups with the additional advanced treatment steps ozone 5 mg/L + a moving bed biofilm reactor and irradiation by UV radiation + hydrogen peroxide had far more putatively differentially expressed transcripts compared to the other groups.

To evaluate the modes of action affected in the exposed fish, GO-term enrichment analysis was performed using GOrilla (see section 3.3.3) [155]. In the fish exposed to the conventionally treated effluents, most enriched GO-term processes were connected to metabolism, including the most enriched GO-term process: xenobiotic metabolic process (GO:0006805). The most recurrent group of genes was the cytochrome P450s, or Cyps. The detoxification enzyme Cyp1a was represented in most of the enriched GO-term processes and the transcript was induced in several of the treatment groups (Fig. 8a). The induction of the Cyp1a gene (*cyp1a*) is often mediated by xenobiotics binding to the cytosolic aryl hydrocarbon receptor, though other induction mechanisms are possible. Examples of groups of chemicals that induce *cyp1a* via the aryl hydrocarbon receptor are polycyclic aromatic hydrocarbons (PAHs), polychlorinated dibenzo-p-dioxins (PCDDs) and polychlorinated biphenyls (PCBs) [175], though we cannot say which chemical(s) induced the *cyp1a* expression in this particular study.

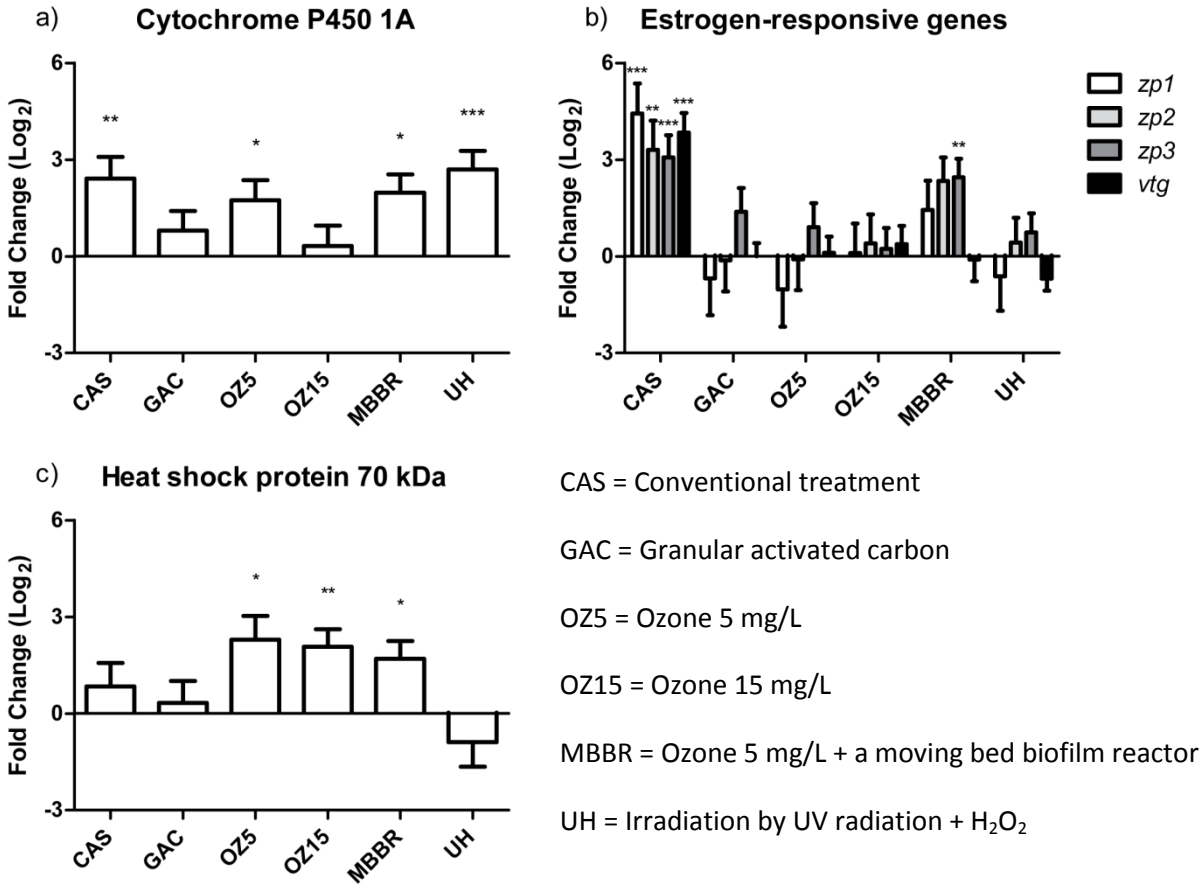

Figure 8. Hepatic gene expression changes on selected genes, measured by qPCR, in rainbow trout exposed to differently treated effluents for two weeks. Values on the y axis are fold change (log2) compared to control fish (exposed to granular activated carbon-treated tap water with an addition of 2% conventionally treated effluent). Statistical analysis was performed using one-way ANOVA and Dunnett's test of multiple comparisons. Levels of significance were *0.01<p<0.05; **0.001<p<0.01; ***p<0.001.

As described in section 1.3, STP effluents are major point sources for release of estrogenic substances and exposures to effluents from different STPs have been associated with reproductive effects in fish [4, 43-47]. In paper III, the microarray analyses revealed the induction of two estrogen-responsive genes, zona pellucida 1 and 2 (*zp1* and *zp2*), in the fish exposed to the conventionally treated effluents. Additionally, there was an induction of two other estrogen-responsive genes, *zp3* and *vtg*, as measured by the qPCR (Fig. 8b). Alongside of paper III, chemical analyses of APIs were performed on the different effluents [26], though EE_2 could not be detected (detection limit was 0.1 ng/L). Nevertheless, APIs with a high BCF and potency, such as synthetic steroid hormones, can potentially affect organisms even at exposure concentrations below the detection limits. The induction of the estrogen-responsive genes was not present in any of the other exposure groups, with the exception of the group of fish

exposed to the effluents treated with ozone 5 mg/L + a moving bed biofilm reactor. In this group, *zp3* was significantly induced, whereas *zp1* and *zp2* showed tendencies to be induced, though not significantly (Fig. 8b).

According to the chemical analyses, the addition of ozone treatment improved the effluent quality to a higher extent with regards to reduced API-concentrations, as only 5-15% of the total API concentration remained compared with the conventionally treated effluents [26]. The number of differentially expressed genes was also substantially lower according to the microarray analyses. However, the fish exposed to any of the ozone-treated effluents (including ozone 5 mg/L + a moving bed biofilm reactor) showed an induction of one of the most common biomarkers for stress in general, heat shock protein 70 kDa (*hsp70*; Fig. 8c) [176]. Ozone-produced oxidants have previously been reported to induce the expression of *hsp70* in gills and liver of exposed fish [177], though in the same study additional biomarkers, indicating oxidative stress, were induced. This was not the case in paper III, thus suggesting that rather than oxidative stress caused by ozone produced oxidants (e.g. free radicals) as in the study by Reiser et al. [177], *hsp70* was induced due to other stressors e.g. metabolites of chemicals in the effluent formed during the ozone treatment process. As stated in section 1.2, oxidative/reductive technologies, such as ozone treatment, can create harmful degradation products. Ozone-treated effluents in particular have accordingly been shown to have unwanted effects in previous studies, including changes in the metabolome in exposed rainbow trout [178] and an increased general toxicity in rainbow trout yolk sac larvae [35]. In exposed crustaceans, bacteria and micro algae, the outcome of ozone-treatment seems to be dose-dependent, since low doses of ozone are associated with a reduced toxicity and high doses with an increased toxicity [36-39, Hörsing et al. *manuscript*]. Recently, Bundschuh et al. [179] performed a meta-analysis where they found that biological tests often leads to an identification of increased toxicity by ozonation, although the isolated use of more narrow endpoints (e.g. specific biomarkers) often indicate a decreased toxicity.

To remove the new products formed by oxidative/reductive technologies, several different additional methods have been suggested, e.g. sandfilter [35]. In paper III, the addition of a moving bed biofilm reactor post ozone 5 mg/L treatment was for this purpose, i.e. an attempt to remove possible transformation products. However, the

outcome of this additional treatment was poor based on the microarray analyses, which revealed a relatively high number of differentially expressed transcripts, including the previously mentioned induction of the estrogen-responsive gene *zp3*. Interestingly, the effluents treated with ozone alone did not cause this high number of significantly affected genes in the exposed fish, thus subsequently suggesting that a moving bed biofilm reactor creates new compounds and/or renders conjugated compounds bioavailable.

The addition of irradiation by UV radiation + hydrogen peroxide treatment was not effective in improving effluent quality, as assessed by both the gene expression analyses and API concentrations [26]. In addition to the relatively modest reduction of 60% of the total API concentration, compared with the conventionally treated effluents, this treatment technology is, like ozonation, also based on oxidative/reductive reactions and thus the problem with harmful transformation products regards to these effluents as well. Accordingly, the microarray analyses revealed a high number of differentially expressed genes in the exposed fish. This, in combination with several of the enriched GO-term processes that were connected to apoptosis and cell death, suggests that the fish exposed to the effluents treated with irradiation by UV radiation + hydrogen peroxide suffered from more stress than the fish exposed to most of the other effluents. In addition, several of the enriched GO-term processes were connected to metabolism and were similar to several of those enriched in most of the other groups, although the genes contributing to the enrichment differed substantially compared with the other groups, suggesting different modes of action. However, the most significantly differentially expressed transcript in the fish exposed to the effluents treated with irradiation by UV radiation + hydrogen peroxide, was carbonyl reductase (*cbr*) which can, like several Cyps, be induced by polycyclic aromatic hydrocarbons, such as β-naphthoflavone, via the aryl hydrocarbon receptor [180]. Because *cbr* was induced in the fish exposed to the conventionally treated effluents as well, it is possible that the removal of e.g. polycyclic aromatic hydrocarbons by irradiation by UV radiation + hydrogen peroxide is incomplete. This is additionally supported by the highly significant induction of *cyp1a*.

The advanced technology that generated the effluents causing the least response in fish was granular activated carbon treatment. None of the genes analyzed by qPCR

were significantly differentially expressed in this group (Fig. 8) and the chemical analyses revealed >95% removal of the total API concentration compared with the conventionally treated effluents. Furthermore, all transcripts on the array had an FDR>0.79, indicating a high level of false-positives.

4.2.3. The applicability of microarray analyses

Although a gene is differentially expressed, it does not necessarily mean that there will be physiological or adverse effects. Therein lies the main criticism on the use of microarrays in ecotoxicology [181]. However, obtaining hard evidence on physiological effects is not the purpose of studying gene expression as it rather represents the earliest response. In ecotoxicology, there are at least three main uses of microarrays: information on the mode of action, potency indications and finally searching for new biomarkers or studying already established biomarkers. In this thesis, all three uses have been applied.

In paper I, the results from the GO-term enrichment analysis indicated a similar mode of action of diclofenac in fish as in humans. Furthermore, responses in gene expression were identified at concentrations of diclofenac found in effluents and on occasions in surface waters [3, 31, 32, 88-91], and although the FDR was high, the top ranked genes are potential biomarkers of diclofenac exposure. However, more studies are needed to evaluate whether they fulfill the biomarker criteria (see section 1.7).

In paper II, a notion on the potency of ketoprofen was provided for exposed fish, under the given conditions. The microarray analyses revealed a very limited response in the gene expression and given the large number of endpoints studied in a microarray experiment, some genes may be falsely identified as differentially expressed. Indeed, our estimates show a very high FDR for all transcripts, thus suggesting that those plausibly differentially expressed may certainly, to a large extent, be false positives. Accordingly, neither of the genes analyzed by qPCR was differentially expressed. This highlights the importance of confirming microarray results by qPCR due to the high risk of assigning false-positives, i.e. type I errors, in studies where many endpoints are studied in parallel. Still, we cannot exclude that the expression of a few isolated genes is indeed slightly affected by the treatment.

In paper III, microarray analyses were successfully used to search for previously established biomarkers in complex mixtures, i.e. effluents, which provided an indication of the potential of additional treatments to remove certain substances. For example, several estrogen-responsive genes were induced by conventionally treated effluents, though not induced in fish exposed to most of the additionally treated effluents. Furthermore, GO-term enrichment analysis revealed several GO-term processes that provided additional information on the nature of the differently treated effluents. On a further notice, because the threshold set for the transcripts included in the GOrilla analysis in paper I (FDR<0.3) would not include transcripts from all groups in paper III, a less strict threshold ($p<0.01$) was set instead. This threshold would indeed include a larger number of false-positives, yet as stated in sections 3.3.3 and 4.2.1, GO-term enrichment analysis can accommodate a larger number of false-positives since falsely differentially expressed genes are less prone to be involved in the enriched processes [145]. On the other hand, due to the outcome of the GOrilla analyses in paper III, this statement may be questioned as there were several enriched GO-term processes in the groups where the effluents had been additionally treated with granular activated carbon or ozone 5 mg/L, suggesting very limited improvements compared with conventional treatment. However, although the same p-value cut off for transcript selection was used for all groups, the FDR was much higher in both these additionally treated groups in comparison to the fish exposed to the conventionally treated effluents. Therefore, it is likely that several of the transcripts included in the putatively enriched GO-term processes are not differentially expressed, i.e. false-positives, which was confirmed for some genes by the qPCR analysis. Nevertheless, what supports the possibility that some of the enriched GO-term processes in the fish exposed to activated carbon or ozone 5 mg/L treated effluents are in fact enriched as a consequence of the exposure, is the fact that they, in contrast to e.g. the fish exposed to ozone 15 mg/L treated effluents, are similar to those enriched in the conventionally treated group, where the FDRs are much lower. Regardless, GO-term enrichment analysis should be used merely for formation of biological hypotheses and not as evidence of certain exposures or effects.

4.3. Physiological effects

Microarrays may indeed be powerful from many perspectives, yet they only provide a partial view of the effects and not evidence as to whether an exposure would lead to adverse effects [181]. Studies on physiological changes, on the other hand, are closer on that matter. In all of the studies in this thesis, the liver somatic index was calculated and compared between the different exposures within each study. In paper III, the heart somatic index was also studied. In neither paper I, II or paper IV, were there any differences between the control fish and the exposed fish. In paper III, however, there was an increase in both liver and heart size in the fish exposed to the conventionally treated effluents compared to the control group. These increases could not be seen in any of the other groups, though this increase in liver size was only significantly prevented by granular activated carbon, ozone 5 mg/L and ozone 15 mg/L treatment. As mentioned in section 4.2.2, there was an increased expression of *vtg*, and induction of hepatic vitellogenin synthesis can lead to an increased liver size. Nevertheless, liver enlargements may be a consequence of exposure to a large variety of xenobiotics and it is therefore difficult to point out which chemical(s) caused the observed liver size increase in the fish exposed to the conventionally treated effluents. In a previous study at the same STP, the liver size was also increased in fish exposed to conventionally treated effluents, containing estrogens [34]. The increase in heart size is, however, not likely caused by estrogens. It has been suggested that exposure to the β-adrenoreceptor antagonist, or β-blocker, propranolol may increase heart size in fish [76, Gunnarsson et al., unpublished], though it is unclear whether β-blockers were the cause in this case.

4.3.1. Effects upon glucocorticoid exposure

In paper IV, the exposure response was assessed through studies on physiological effects, though we plan to perform microarray analyses here as well. No effects were observed upon the exposures to free beclomethasone or low dose BDP and the discussion hereinafter will therefore be related merely to the high BDP exposure (648 ng/L in aquarium water).

Several of the studied physiological endpoints in paper IV were significantly affected in the fish exposed to high dose BDP. Plasma glucose levels increased in rainbow trout, as has been seen in humans [104]. BDP binds to and activates the glucocorticoid-receptor which in turn may bind to the glucocorticoid response element resulting in transcription of genes involved in gluconeogenesis [104]. In rainbow trout and in fathead minnow, increase in plasma glucose levels have previously been reported upon exposure to cortisol (intraperitoneal implants) [182] and waterborne BDP [108], respectively. However, plasma glucose levels have been used as indicators of general environmental stress in fish and are thus not specific for glucocorticoid exposure [183]. Exposure to high concentrations of BDP also resulted in changes in glutathione levels and an increased catalase activity. These changes indicate oxidative stress, which can result in damage to cellular molecules and eventually, cell death. Glutathione is an important molecular antioxidant that functions as a reducing agent in antioxidant enzyme reactions, as a scavenger of reactive oxygen species and as a conjugation molecule important in excretion of xenobiotics. Catalase can protect against oxidative damage resulting from hydrogen peroxide, which is produced during normal cellular metabolism or as a result of chemical actions of xenobiotics, by catalyzing its decomposition to water and oxygen [184]. Interestingly, similar effects have also been shown in humans where levels of glutathione decreased while catalase activity increased in erythrocytes upon BDP treatment [185].

5. Conclusions

In this thesis, transcriptomics and bioconcentration studies have been used to identify pharmaceuticals of environmental concern. Transcriptomics have also been used to evaluate responses in fish exposed to differently treated sewage effluents. Below are the main conclusions drawn from each paper followed by considerations on the main aims of this thesis.

Paper I - Diclofenac

- Diclofenac affected hepatic gene expression in exposed fish at water concentrations reported in treated effluents and surface waters.
- Pharmacological responses were observed in fish at blood plasma concentrations similar to human therapeutic plasma levels, indicating a similar potency in fish and humans, thus supporting read-across between species.
- Responses identified in fish resembled those found in mammals, further supporting read-across.
- Potential biomarkers for diclofenac or NSAID exposure were identified.
- A stable bioconcentration factor for diclofenac across exposure concentrations was demonstrated.

Paper II – Ketoprofen

- Exposure of fish to ketoprofen at concentrations about 100 times higher than those found in treated sewage effluents resulted in plasma concentrations below 1% of human therapeutic plasma levels, suggesting low risk for effects in fish.
- At these exposure concentrations, no effects on gene transcription were found, in agreement with the proposed read-across strategy.
- Exposure of fish to effluents indicates a higher bioconcentration potential than exposure to single NSAIDs, thus laboratory experiments may underestimate risks in the environment.

Paper III – Sewage treatment technologies

- Microarray analyses revealed several differentially expressed genes after exposure to conventionally treated effluents, including estrogen-responsive genes and a biomarker for dioxin-like exposure (*cyp1a*).
- Most advanced treatments resulted in effluents with no or minor estrogenic responses in exposed fish.
- Treatment with activated carbon or a high dose of ozone resulted in effluents where no dioxin-like response was observed in exposed fish.
- Activated carbon treatment generated the effluent that lead to the least responses on exposed fish.
- Exposure to all ozone-treated effluents caused induction of *hsp70*, a biomarker indicating a general stress response in fish.

Paper IV – Beclomethasone

- Exposure to the glucocorticoid beclomethasone-diproprionate affected plasma glucose levels and caused oxidative stress in fish.
- Effects observed in fish resembled effects in human, supporting read-across between species.
- No effects were observed in fish exposed to free beclomethasone, most likely because it did not bioconcentrate.

Thesis conclusions

The combination of bioconcentration studies and read-across between species proved to be a strategy with high potential for identifying pharmaceuticals of environmental concern. Microarrays and gene expression were successfully used to provide 1) potential biomarkers for diclofenac/NSAID exposure, 2) information on the mode of action of diclofenac in fish, 3) an assessment of the potency of NSAIDs in fish and 4) identification of sewage treatment techniques resulting in less risk for effluent-exposed fish. Studies on gene expression and modes of action in non-target organism have thus proven useful to complement traditional environmental risk assessment strategies for pharmaceuticals.

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