Effects of the anti-inflammatory drug diclofenac and the beta-blocker metoprolol in brown trout *Salmo trutta* f. *fario* and freshwater invertebrates at different levels of biological organization

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Hier fängt die Geschichte an.

Walter Moers

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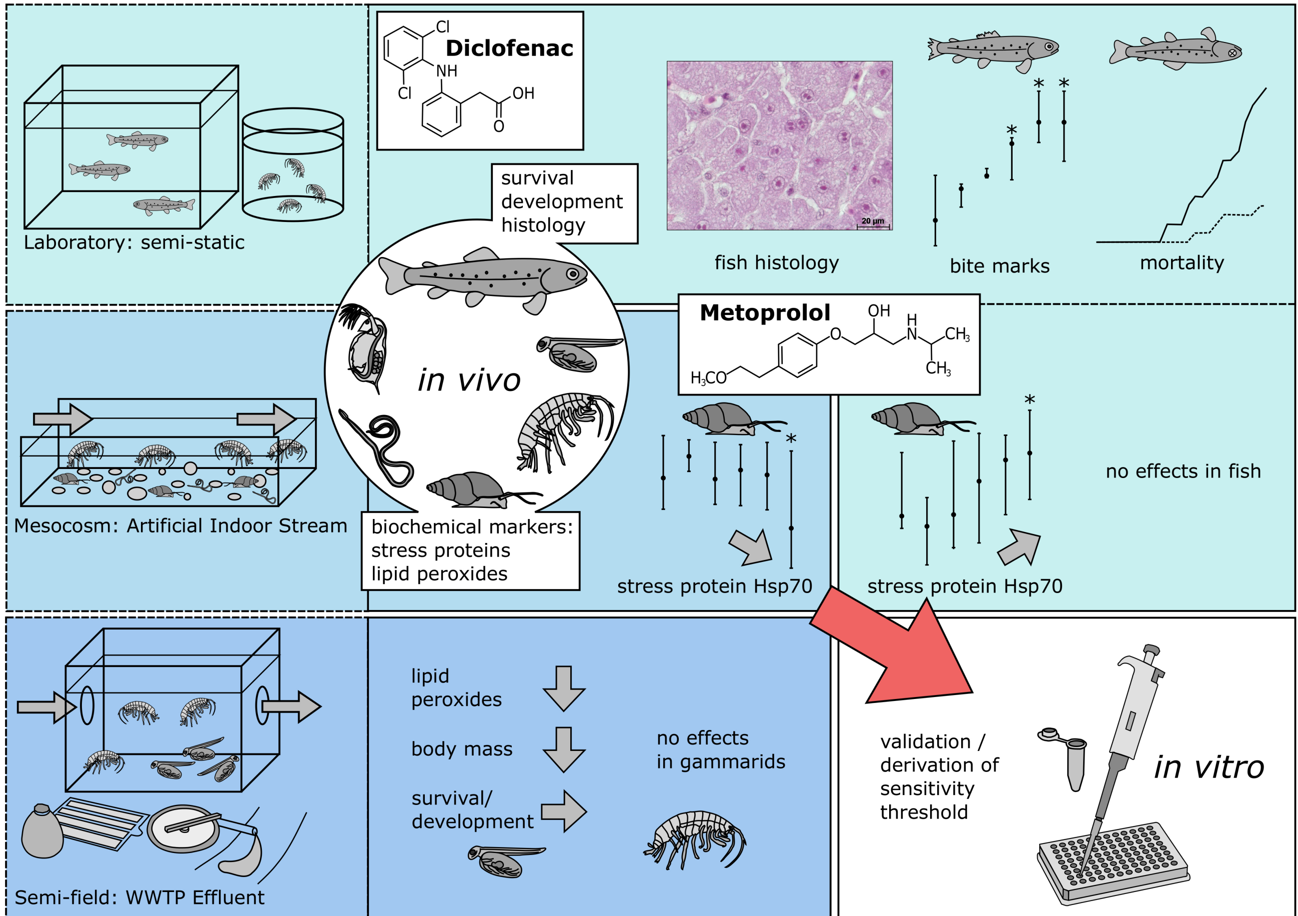
Part 1: Summary

1. Title of the thesis

Effects of the anti-inflammatory drug diclofenac and the beta-blocker metoprolol in brown trout *Salmo trutta* f. *fario* and freshwater invertebrates at different levels of biological organization

2. Graphical abstract



3. English summary

Among the wide range of chemicals released by human activities, pharmaceuticals hold a special position. They play a vital role in human and livestock health management, and their consumption increases constantly. Human pharmaceuticals reach the environment mainly through wastewater treatment plants, where they cannot be completely removed. Consequently, trace contaminations are frequently detected in aquatic ecosystems. By design, these substances are relatively stable, in the human body as well as the environment, and physiologically active at low concentrations. Furthermore, their molecular mode of action is usually highly conserved across a broad range of different taxa. This makes effects on non-target organisms likely and renders pharmaceuticals a highly relevant group of environmental chemicals, which requires further attention.

Systematic monitoring will be one fundamental cornerstone in future strategies to assess the environmental relevance of pharmaceuticals. However, the large amount of different substances can hardly be assessed by analytic-chemical means alone. In this context, biological test systems, by which effects can be monitored based on their mode of action, rather than every single substance on its own, would be a tremendous gain for further monitoring approaches.

In the project *Eff-Pharm*, the aim was to prove the feasibility of such systems by creating *in vitro* assays for pharmaceuticals. As a prerequisite for the project, and to identify pharmaceutical classes of interest, a detailed literature review provided information on the lacks of knowledge. *In vivo* studies with fish and invertebrates conducted in parallel to the biosensor development delivered manifold information necessary for the validation of the new tests. For the *in vivo* studies, we used a battery of different test organisms including the brown trout *Salmo trutta* f. *fario*, the crustaceans *Gammarus fossarum* and *Daphnia magna*, the endobenthic oligochaete *Lumbriculus variegatus* and the mudsnail *Potamopyrgus antipodarum,* which were exposed to diclofenac (non-steroidal anti-inflammatory drug) and metoprolol (beta-adrenergic receptor blocker). Gammarids, oligochaetes, and snails were exposed to metoprolol not only at laboratory conditions, but also in an artificial indoor stream system. Furthermore, brown trout and gammarids were tested with respect to their response to effluent of a wastewater treatment plant, which was known to contain both pharmaceuticals. Whereas the tests with invertebrates were conducted with adults only, effects in brown trout were analysed in different developmental stages: developing embryos starting from fertilised eggs until two months post hatch, and juveniles of six to seven months post hatch.

This thesis contains the results of the literature study, organismic and histological effects in brown trout and responses of biochemical biomarkers (changes in the levels of stress protein Hsp70 and lipid peroxides) in trout and invertebrates.

By the detailed literature review the pharmaceutical groups of non-steroidal anti-inflammatory drugs (NSAIDs) and beta-adrenergic receptor blockers (beta-blockers) were identified as most suitable for further investigations. Both are consumed in large amounts, frequently detected in surface waters, and suspected to act on aquatic organisms already in low concentrations ranges. Furthermore, both act via a specific mode of action, which can be exploited for *in vitro* assays. As active ingredient for the further studies, we chose diclofenac, the most controversially discussed NSAID with respect to its environmental effect, and metoprolol, the most consumed beta-blocker. In addition, the literature review revealed a lack of studies on regionally relevant organisms, as well as benthic invertebrates in general. Representatives of these were selected as test organisms for the further investigations.

The *in vivo* studies showed that diclofenac reduced the survival of juvenile brown trout at concentrations of 100 µg/L and led to increased signs of aggression (bite marks on fins and opercula) at 10 µg/L. Histological examinations revealed that liver, gill and kidney in the control group were already in a state of cellular reaction. However, effects in liver were more pronounced in diclofenac-

exposed animals. Brown trout embryos and sac-fry stages did not show any effects in reaction to diclofenac up to 100 µg/L. Metoprolol did not affect survival, aggression behaviour or developmental parameters in juvenile fish and fish early life stages in concentrations up to 1000 µg/L. However, it led to a conspicuous shift in the ratio of erythrocytes to leucocytes. In *P. antipodarum*, metoprolol tended to elevate the level of stress proteins in a laboratory setup, indicating proteotoxic action of the substance. In the artificial indoor stream, Hsp70 levels decreased at the highest test concentration, indicative of a breakdown of the stress protein system. Apart from this, none of the examined organisms showed clear changes of stress protein or lipid peroxide levels in reaction to any of the tested pharmaceuticals.

Brown trout embryo and sac-fry stages exposed to a wastewater treatment plant effluent containing both test chemicals in a µg/L range developed in normal time and with higher survival rate, but were lighter than lab control animals and had reduced levels of lipid peroxides. This is interpreted as the result of energy-consuming oxidative stress response.

Overall, my results demonstrate the hazard emanating from the NSAID diclofenac for fish health. At the same time, the beta-blocker metoprolol has much lower potential to evoke adverse effects in non-target organisms. Juvenile fish reacted with higher sensitivity than early life stages – which must be considered for further risk assessment approaches. Changes in the stress protein level showed to be an especially sensitive biomarker for molluscs, but not for the other tested taxa. Finally, the complex mixture of substances present in wastewater treatment plant effluents can evoke adverse effects in developing fish larvae. However, it remains to be investigated how these effects transfer to other life stages and how they will affect populations and whole ecosystems.

4. Deutsche Zusammenfassung

Unter der Vielzahl von Stoffen, die durch menschliche Aktivitäten in die Umwelt eingetragen werden, haben Arzneimittel eine besondere Stellung inne. Sie spielen eine essentielle Rolle im Gesundheitsmanagement von Menschen und Nutztieren, und ihr Verbrauch steigt stetig weiter an. Humanarzneimittel erreichen die Umwelt hauptsächlich über den Abwasserpfad, da sie in Kläranlagen nicht vollständig entfernt werden können. In der Folge werden zahlreiche Arzneimittel als Mikroverunreinigungen in Gewässern nachgewiesen. Bestimmte Eigenschaften von Arzneimitteln, die für die Behandlung von Mensch und Tier erwünscht sind, erweisen sich jedoch in der Umwelt als problematisch. Sie sind verhältnismäßig stabil, und konsequenterweise biologisch schlecht abbaubar, und bereits in geringen Konzentrationen physiologisch wirksam. Zudem ist ihr molekularer Wirkmechanismus in der Regel über eine breite Anzahl an unterschiedlichen Taxa konserviert, was Effekte auf Nichtzielorganismen wahrscheinlich macht. Arzneimittel werden deshalb als eine hochrelevante Gruppe von Mikroschadstoffen angesehen, welche dringend weiterer Aufmerksamkeit bedarf.

Ein systematisches Monitoring ist einer der Grundpfeiler weiterer Strategien, um die Umweltrelevanz von Arzneimittel abzuschätzen. Die gewaltige Menge an unterschiedlichen Substanzen kann jedoch kaum nur mit analytisch-chemischen Methoden untersucht werden. In diesem Zusammenhang wären biologische Testsysteme, durch welche Effekte von Substanzgruppen mit gleichem Wirkmechanismus gemeinsam untersucht werden, anstatt jede Substanz einzeln, ein gewaltiger Schritt in Richtung umfassender Monitoringstrategien.

Ziel des Projektes *Eff-Pharm* war es, *in vitro* Assays zum Monitoring ganzer Arzneimittelgruppen zu etablieren und ihren Einsatz für verschiedene Umwelmatrices zu validieren. Als Voraussetzung für das Projekt, und um relevante Arzneimittelgruppen zu identifizieren, lieferte eine detaillierte Literaturstudie wichtige Informationen zu derzeit bestehenden Wissenslücken. *In vivo* Untersuchungen, welche parallel zur Entwicklung der Biosensor-Zelllinien durchgeführt wurden, lieferten notwendige Informationen zur Validierung der neuen Testsysteme. Für die *in vivo* Studien wurde eine Reihe an unterschiedlichen Testorganismen eingesetzt: die Bachforelle *Salmo trutta* f. *fario*, die Crustaceen *Gammarus fossarum* und *Daphnia magna*, der endobenthische Oligochaete *Lumbriculus variegatus* und die Zwergdeckelschnecke *Potamopyrgus antipodarum*, welche gegenüber Diclofenac (nichtsteroidaler Entzündungshemmer) und Metoprolol (Betablocker) exponiert wurden. Gammariden, Oligochaeten und Schnecken wurden gegenüber Metoprolol nicht nur unter Laborbedingungen, sondern zusätzlich auch in einem Fließrinnen-Mesokosmos exponiert. Zusätzlich wurden Bachforellen und Gammariden hinsichtlich ihrer Reaktion auf den Ablauf einer kommunalen Kläranlage, welcher nachweislich beide Arzneimittel enthielt, untersucht. Während für die Untersuchungen mit Invertebraten nur adulte Tiere verwendet wurden, standen bei Bachforellen unterschiedliche Entwicklungsstadien im Fokus: Sich entwickelnde Embryonen vom befruchteten Ei bis ca. zwei Monate nach Schlupf, und juvenile Tiere im Alter von sechs bis sieben Monaten.

Die vorliegende Doktorarbeit enthält die Ergebnisse der Literaturstudie, Daten zu organismischen und histologischen Effekten bei der Bachforelle sowie zu biochemischen Biomarkern (Änderungen im Level des Stressproteins Hsp70 und von Lipidperoxiden) bei Forellen und Invertebraten.

Durch die umfassende Literaturstudie wurden die Arzneimittelgruppen der nichtsteroidalen Entzündungshemmer (NSAIDs) und beta-adrenergen Rezeptorblocker (Betablocker) als besonders geeignet für weitere Untersuchungen identifiziert. Beide Gruppen werden in großen Mengen verbraucht, entsprechend häufig in Umweltproben nachgewiesen und stehen im Verdacht bereits in geringen Konzentrationen adverse Effekte bei aquatischen Organismen hervorzurufen. Zusätzlich ist beiden Gruppen jeweils ein spezifischer Wirkmechanismus zu eigen, welcher für *in vitro* Systeme

ausgenutzt werden kann. Als stellvertretende Substanzen für unsere Studien wählten wir Diclofenac, ein NSAID, welches im Hinblick auf seine Umwelteffekte sehr kontrovers diskutiert wird, und Metoprolol, den am häufigsten konsumierten Betablocker. Die Literaturstudie ergab zudem, dass ökotoxikologische Daten zu Arzneimitteln vor allem für einheimische Arten, insbesondere benthische Invertebraten, fehlen. Deshalb wurden solche als Testorganismen für unsere weiteren Untersuchungen ausgewählt.

Die *in vivo* Untersuchungen zeigten, dass Diclofenac das Überleben von juvenilen Bachforellen in Konzentrationen von 100 µg/L und höher verringert. Darüber hinaus wiesen die Tiere ab einer Konzentration von 10 µg/L vermehrt Bissverletzungen an den Flossen und Kiemendeckeln auf, was auf verstärktes aggressives Verhalten schließen lässt. Die histologische Analyse von Leber, Kieme und Niere deuten darauf hin, dass die Tiere bereits in der Laborkontrolle in einem reaktiven physiologischen Zustand waren. Die histologischen Veränderungen in der Leber waren jedoch bei Diclofenac-exponierten Tieren noch stärker ausgeprägt. Embryonen und Dottersacklarven der Bachforelle zeigten im Gegensatz hierzu keine Reaktionen bis zur höchsten getesteten Konzentration von 100 µg/L. Der Betablocker Metoprolol hatte bis zur höchsten getesteten Konzentration von 1000 µg/L keinen Einfluss auf Überleben, Verhalten oder Entwicklung von juvenilen und embryonalen Bachforellen. Es zeigte sich jedoch eine auffällige Veränderung des Verhältnisses von Erythrozyten zu Leucozyten. Bei der Zwergdeckelschnecke *P. antipodarum* erhöhte Metoprolol die interne Konzentration des Stressproteins Hsp70, was auf einen proteotoxischen Effekt bei Mollusken schließen lässt. Unter Fließrinnen-Bedingungen waren jedoch verringerte Hsp70-Konzentrationen zu beobachten, was auf einen Zusammenbruch des Stressprotein-Systems unter diesen Bedingungen und damit auf stärkere Effekte unter naturnäheren Bedingungen schließen lässt. Abgesehen davon zeigte keine der getesteten Arten klare Veränderungen von Stressprotein- oder Lipidperoxid-Konzentrationen in Reaktion auf die getesteten Arzneimittel.

Bachforellenlarven, die gegenüber dem Kläranlagenablauf exponiert waren, entwickelten sich innerhalb der normalen Zeitspanne und zeigten eine hohe Überlebensrate. Zum Versuchsende waren die Larven jedoch leichter als die entsprechenden Kontrolltiere, und wiesen verringerte Konzentrationen von Lipidperoxiden auf. Dies kann als Resultat einer energieaufwändigen Antwort auf oxidativen Stress angesehen werden.

Insgesamt unterstreichen die ihm Rahmen meiner Dissertation erzielten Ergebnisse, dass sich das NSAID Diclofenac nachteilig auf die Fischgesundheit auswirkt. Gleichzeitig zeigte sich der Betablocker Metoprolol als deutlich weniger gefährlich für Nichtzielorganismen. Juvenile Fische reagierten deutlich sensitiver auf Arzneimittel als frühe Lebensstadien, was in der weiteren Risikobewertung berücksichtigt werden sollte. Änderungen im Stressproteinlevel erwiesen sich als sensitiver Biomarker für Mollusken, aber nicht für die anderen getesteten Organismengruppen. Es zeigte sich ebenfalls, dass die komplexe Mischung von Stoffen in Kläranlagenabläufen durchaus in der Lage ist, physiologische Effekte bei sich entwickelnden Fischlarven hervorzurufen. Es muss jedoch weiter untersucht werden, wie sich diese Effekte auf spätere Lebensstadien auswirken, und in welcher Art und Weise sich diese Auswirkungen auf Populations- oder Ökosystemebene widerspiegeln.

5. Abbreviations

AB-PAS:	Alcian blue – periodic acid Schiff staining
AIS:	artificial indoor stream
bpm:	beats per minute
C:	Celsius
cAMP:	cyclic adenosine monophosphate
CAT:	catalase
CD:	compact disc
CHPE:	cumene hydroperoxide equivalents (measurement unit of lipid peroxidation in FOX-assay)
COX:	cyclooxygenase
CYP450:	cytochrome P450
d:	day
D. magna:	*Daphnia magna* (Straus, 1820)
df	degrees of freedom
dpf:	days post fertilisation
dph:	days post hatch
dw:	dry weight
e.g.:	*exempli gratia* = for example
EU:	European Union
FSH:	follicle stimulating hormone
G. fossarum:	*Gammarus fossarum* (Koch, 1835)
GSH-Px:	glutathione peroxidase
GST:	glutathione-S-transferase
GWT-TUD:	Gesellschaft für Wissens- und Technologietransfer, Technical University of Dresden
h:	hour
HE:	Haematoxylin-eosin staining
HEPES:	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPLC-MS/MS:	high performance liquid chromatography, coupled to tandem mass spectroscopy
Hsp70:	stress proteins of the heat shock protein family with approximately 70 kilodalton
i.a.:	*inter alia* = among other things
i.e.:	*id est* = that is
ISF Langenargen:	Institut für Seenforschung, Langenargen
kDa:	kilodalton

L:	litre
L. variegatus:	*Lumbriculus variegatus* (Müller, 1774)
lh:	luteinizing hormone
LOEC:	lowest observed effect concentration
logK_{ow}:	decadic logarithm of the octanol-water partition coefficient
m:	meter
MDA:	malondialdehyde
MEC:	measured environmental concentration
µg:	microgram
mg:	milligram
mm:	millimeter
MoA:	mode of action
Ng:	nanogram
NOEC:	no observed effect concentration
NSAID:	non-steroidal anti-inflammatory drug
OECD:	Organisation for Economic Co-operation and Development
P. antipodarum:	*Potamopyrgus antipodarum* (J.E. Gray, 1843)
PEC:	Predicted environmental concentration
pH:	negative decadic logarithm of hydrogen ion activity
pKa:	decadic logarithm of the acid dissociation constant
PNEC:	predicted no-effect concentration
rcf:	relative centrifugal force
ROS:	reactive oxygen species
S. trutta:	*Salmo trutta* (Linnaeus, 1758)
SDS:	sodium dodecyl sulfate
SOD:	superoxide dismutase
T_0-control:	Control sampling of the base population at the start of the experiment
TBARS:	thiobarbituric acid reactive substances (byproduct of lipid peroxidation)
Tris:	tris(hydroxymethyl)aminomethane
WWTP:	wastewater treatment plant
WFD:	(European) Water Framework Directive

6. Introduction

Pharmaceuticals in the environment

Within the last fifty years, the problem of anthropogenic contaminations in the environment has gained increasing attention. In the beginning, the focus rested on large-scale contaminations with nutrients like nitrogen and phosphate (Carpenter *et al.* 1998), or on the devastating effects of persistent pesticides (Ratcliffe 1970). Over the years humankind started to realize that our modern human lifestyle releases a nearly unmanageable variety of substances into the environment. Large-scale agroindustry, dependent on monocultures and factory farming, would not be feasible without a multitude of pesticides, fertilizers and medicinal products. Modern manufacturing of everyday products is based on the production and processing of thousands of industry chemicals. At the same time, the diverse combustion processes, which fuel our society, release numerous organic compounds and metals into the environment. Moreover, typical everyday culture would hardly be manageable without the high abundance of pharmaceuticals, detergents, disinfectants, and cosmetics (Daughton and Ternes 1999).

The underlying pathways are diverse: several substances, like fertilizers and crop protection products, are deliberately released at one location, and reach other places via drifting or run-off (Stoate *et al.* 2001, Stoate *et al.* 2009). Substances present in exhaust gases or abrasion products are not intentionally released into the environment, but their emission and wide-range dispersal is tolerated. Apart from these diffuse entries, wastewater treatment plants are point sources responsible for a large amount of substances entering surface waters (Tixier *et al.* 2003, Triebskorn 2017). Substances of everyday use by humans are reaching wastewater through sinks, showers or toilets. Most treatment plants in Western Europe effectively remove nitrogen or phosphate, but are not designed to filter micropollutants (Luo *et al.* 2014). Consequently, many of those chemicals pass through wastewater treatment unchanged and reach surface waters. Countless studies found a wide range of trace substances in environmental compartments, like surface water, groundwater, sediments, soil and biota (e.g. Schwarzenbach *et al.* 2006).

At the same time, aquatic and terrestrial ecosystems suffer from a wide range of adverse effects. Low performance and eventual extinction of various species lead to a great loss of biodiversity (Goudie 2013). Major driving forces behind this are, certainly, habitat loss and habitat degradation, climatic changes or competition with newly introduced species. However, pollution may also play a crucial role in this detrimental development. The causal link between specific contaminations and negative environmental effects may be apparent in many cases, e.g. eutrophication of water bodies through nutrients (Smith 2003), or the drastic decline of various bird species through organochlorine compounds (Ratcliffe 1970). However, in the case of micropollutants, this causal link is hard to establish. Environmental concentrations range, like the name suggests, on a nanogram to microgram per litre scale. In many cases, it is not known whether the substances may exert any effect in such low concentrations. For many substance groups, regulating authorities impose an assessment of their eco-toxic potential, to estimate risks before a substance is introduced into the environment.

The following piece of work will be restricted to one of the previously described groups of micropollutants – pharmaceuticals. This diverse substance group is characterized by several attributes giving them potentially high environmental relevance: pharmaceuticals are designed to evoke physiological effects in the target organism – whether it is human, pet or livestock animal. The responsible biochemical pathways are usually highly conserved. Hence, it can be supposed that the effects are nearly similar in closely related species (Fent *et al.* 2006, Arnold *et al.* 2014). In contrast to crop protection products or biocides – which are poisons that aim to harm specific organisms - pharmaceuticals are commonly viewed as positive substance with “healing” properties. Still, the

evoked physiological changes and side effects are oftentimes drastic, with far-reaching effects on the whole body. For this reason, many preparations are only sold in pharmacies or available on prescription, and their package leaflets warn of the manifold side effects. Furthermore, it must not be forgotten that various active substances are explicitly designed to harm cells, proteins or genetic information - e.g. antibiotics against bacteria, virostatic agents against viruses, or cytostatic agents against the own, malignant degenerated body cells. Usually, the active ingredients are highly potent and evoke their effects already at extremely low concentrations. Experimental contamination of a lake with low ng/L-concentrations of the contraceptive 17α-ethinylestradiol, a synthetic estrogen, resulted in decreased reproduction of several fish species, and eventually large-scale alterations of the whole freshwater community (Kidd *et al.* 2014). Likewise, antipsychotics showed to alter vital behaviours, like feeding or sociality (Brodin *et al.* 2013, Brodin *et al.* 2014). Thus far, the most obvious ecologic effect on non-target organisms, directly evoked by pharmaceuticals, was the population decline of several vulture species in India and Pakistan. Birds feeding on the carcasses of diclofenac-treated cattle died from renal failure and visceral gout (Oaks *et al.* 2004) leading to the near extinction of several *Gyps* species (Prakash *et al.* 2003). These pathologies resemble common adverse side effects of the same pharmaceutical in humans (Bjorkman 1998, Kawada *et al.* 2012). However, the link between exposure and effect is usually not as clear as in this example. Apart from their high effect potential, pharmaceuticals are optimized on chemical stability, so that degradation in wastewater treatment or the environment is slow (Fent *et al.* 2006, Ebert *et al.* 2014). Overall consumption rates are constantly rising over the last years. For Germany, consumption is predicted to rise by 40 to 70 % until 2045 (Lauruschkus *et al.* 2017). One reason for this is the demographic development in the Western world. The largest amount of pharmaceuticals is consumed by elderly people. Hence, the observed general increase of consumption amounts is the logical consequence of an ageing population. Furthermore, the better availability of a constantly growing spectrum of medicinal products can also be seen as a reason. In several cases, medical indication is not necessarily given – many preparations are used as "lifestyle"-products, e.g. prophylactically in sports. Apart from usage and subsequent excretion, improper disposal is a major contributor to environmental contaminations (Ebert *et al.* 2014). In addition, in certain areas, discharges from manufacturing have led to strong pollution (Larsson 2014). Pharmaceuticals are frequently detected in marine (Arpin-Pont *et al.* 2014) and freshwater ecosystems (Ternes 1998, Santos *et al.* 2010, Aus der Beek *et al.* 2016a). Their ubiquitous occurrence in water bodies, in combination with their highly potent biological activity, slow degradation and increasing consumption explains why these substances are regarded as highly relevant pollutants.

Legal background, pharmacovigilance and need for monitoring

When politics became finally aware of the problem, they established a prospective environmental risk assessment for the approval of new pharmaceutical preparations for human (2001/83/EG) and veterinary use (2001/82/EG), transferred to national law e.g. in the German "Arzneimittelgesetz" § 22. In fact, the systematical assessment is carried out only since the implementation of the guidelines EMEA/CHMP/SWP/4447/00 (EU 2006b), CVMP/VICH/592/98-FINAL (EU 2000) and CVMP/VICH/790/03-FINAL (EU 2004), where detailed information on how to conduct the assessment is given. In addition to proving the clinical safety and efficacy, applicants have to investigate whether the compound poses a risk to the environment. Environmental risk assessment compares exposure, i.e. the calculated or measured environmental concentration, with effect concentrations obtained for organisms of different trophic levels. In cases where the predicted exposure is greater than the most sensitive effect concentration (including a safety factor addressing experimental uncertainties), an unacceptable risk is expected.

Several aspects of the current legislation are not fully satisfying: preparations already approved before the implementation of the directives, which are frequently found in relatively high concentrations in

the environment (Arnold *et al.* 2014), are exempt from environmental risk assessment. Most preparations used in everyday life, which constitute the largest amount of consumed pharmaceuticals, fall into this category of "legacy pharmaceuticals". Consequently, mainly newly developed substances feature a comprehensive environmental risk assessment. However, even if the environmental risk assessment identifies an unacceptable risk of a human pharmaceutical, the approval cannot be refused solely for environmental reasons. In this case, human health is regarded to be of paramount importance.

Besides this prospective risk assessment, there are retrospective methods to assure the safety of applied pharmaceuticals. The continuous, systematic registration and documentation of side effects in humans is called pharmacovigilance. One idea, so far not realized on a larger scale, would be to transfer this principle to the environmental sector (Wang *et al.* 2017). This eco-pharmacovigilance would collect undesired environmental effects in retrospect – and possibly lead to a re-evaluation of risk. However, this would require a large-scale, systematic monitoring of active pharmaceutical ingredients in the environment – which does not currently exist.

The extant knowledge on environmental contaminations is based on various measuring campaigns, mainly by authorities, universities and other research institutions. A comprehensive literature study concluded that the data availability is comparably good for Germany, while in many other countries only few data are available (Aus der Beek *et al.* 2016b). Additionally, it has to be born in mind that data collected in this manner does not necessarily represent the actual situation. The number of detected contaminants in a region depends largely on the number of conducted searches, and the applied techniques. For example, the high number of active pharmaceutical ingredients detected in German surface water is not necessarily a sign of an especially concerning situation, but mainly a result of the high number of studies conducted in this country. Furthermore, the detected concentrations need not be completely representative of the general situation. Analytical studies are preferably conducted at strongly contaminated sites, possibly leading to an overall bias of the data (Aus der Beek *et al.* 2016a). These points shall not trivialize the problem associated with these substances in the environment. Instead, they emphasize the need for a systematic monitoring, to allow a profound assessment of risk. However, at this moment, such a monitoring seems hardly feasible – technically as well as financially. Investigations via modern chemical-analytical methods (e.g. non-target analytics - Hernández *et al.* 2015) are complex, still difficult to interpret, and expensive, especially in the expected low concentration ranges. Moreover, most established methods can only detect the substances they are looking for. Countless other active substances, metabolites and transformation products would evade the search (Brack *et al.* 2017). Even in the hypothetic case that comprehensive analytics were possible, this could only show the presence of certain substances. The potential effect of the complex substance mixture could not be assessed. One solution to this dilemma is promised by effect-based monitoring, which was investigated in the project *Eff-Pharm*.

Scope of the project *Eff-Pharm* and the thesis

The research project "*From theory to reality – Evaluation of suitable organisms and test systems for the biomonitoring of pharmaceuticals"*, shortly titled *Eff-Pharm*, was initiated and funded by the German Environment Agency (UBA). Its basic idea was to quantify pharmaceutical ingredients via their mode of action. This mode of action is shared between all members of the same substance class, and is responsible for the actual pharmacological effect in the treated organism. Common drug targets are specific molecules, like receptors, channels or enzymes. Artificially constructed *in vitro* systems, which express the target molecule as sensor, as well as a coupled reporter system, would allow the quantification of a whole group of substances with shared mode of action at once. The feasibility of this idea was investigated in the first project part. The second part focused on the construction, establishment and validation of these systems.

Since financial resources were limited, our pioneer project could only investigate practicability for selected substance classes. Therefore, one general prerequisite for the further procedure was the identification and selection of suitable substance classes. In an extensive literature survey, we identified lacks of knowledge and compiled information on ecotoxicity of various pharmaceutical ingredients, as a basis to select relevant substances for the further project.

The effects detected in a potentially created *in vitro* system had to be related to an ecological context. Consequently, this requires a comparison of *in vitro* to *in vivo* effects. Besides the selection of suitable substance groups, one declared aim of the literature study was to assess suitable organism groups and effect endpoints for comparative *in vivo* investigations.

In the focus of the *in vivo* experiments, which were conducted in the laboratory, mesocosms and in the field (bypass of a WWTP effluent) were the substance classes of non-steroidal anti-inflammatory drugs (NSAIDs) and beta-adrenergic receptor blockers, represented by the pharmaceuticals diclofenac and metoprolol. Both are consumed in high amounts and frequently detected in the aquatic environment. Whereas numerous data on adverse effects of diclofenac in wildlife were available at the start of *Eff-Pharm* (Oaks *et al.* 2004, Schwaiger *et al.* 2004, Triebskorn *et al.* 2004, Hoeger *et al.* 2005, Mehinto *et al.* 2010), which were, however, controversially discussed with respect to their environmental relevance, little was known about the ecotoxicological effect potential of metoprolol. Hence, further effect data on both substances were urgently needed – especially on organisms of high regional environmental relevance. For this purpose, we chose brown trout and invertebrates including e.g. gammarids, as representative inhabitants of Central European stream ecosystems.

Whereas all experiments with brown trout (including embryo and sac-fry stage tests and tests with juvenile fish) were conducted by myself, the tests with invertebrates were run by the cooperation partners from Dresden, Frankfurt am Main, and Berlin-Marienfelde, who provided samples for suborganismic effect analyses to me. These focused on how, and at which concentrations, diclofenac and metoprolol affect stress protein levels and oxidative stress responses in the exposed invertebrates. In brown trout, I studied embryo development, survival, behaviour, tissue integrity and also the above mentioned stress markers.

Overarching questions of my thesis:

- Do the NSAID diclofenac or the beta-blocker metoprolol influence development or health of brown trout embryos and juveniles?
- Are there effects on the stress proteins or lipid peroxides, when fish, crustaceans, annelids or snails are exposed to diclofenac or metoprolol? Are these biochemical markers a useful addition to the traditional testing battery?
- In which concentration ranges do *in vivo* effects occur? Are there differences in sensitivity?
- Are there adverse effects, when fish or gammarids are exposed to wastewater treatment plant effluents?

It was the general aim of my thesis to provide data for effects of metoprolol and diclofenac in relevant aquatic organisms as a basis for the validation of the biosensors developed in *Eff-Pharm* with respect to necessary sensitivity and threshold values.

General remarks on the project structure and own contributions

The present thesis contains results of three separate research projects, funded by the German Environment Agency (Umweltbundesamt – UBA), entitled *"From theory to reality – Evaluation of suitable organisms and test systems for the biomonitoring of pharmaceuticals – Part I: Literature review* (FKZ 3713 634 101), - *Part II: Case studies* (FKZ 3713 63 4102) and – *Part III: Method validation* (3715 63 4120)". All three projects are based on each other and are closely intertwined. Hence, they

will be referred to as one project, shortly called *Eff-Pharm*. The following universities and research facilities conducted the work cooperatively:

- University of Tübingen: project coordination, literature research on effect data, *in vivo* studies in brown trout and biochemical analyses of fish, gammarid, oligochaete, gastropod and daphnid samples
- GWT-TUD GmbH, Dresden: literature research on effect data and *in vivo* studies in gammarids
- University of Frankfurt am Main: *in vivo* studies on sediment-dwelling oligochaetes and gastropods
- German Environment Agency, Dessau-Roßlau and Berlin-Marienfelde: funding, project supervision and *in vivo* experiments on daphnids
- DVGW Water Technology Center, Karlsruhe: chemical analyses
- Steinbeis Innovation Center Cell Culture Technology, Mannheim: literature research on *in vitro* systems, development and validation of biosensor systems

This thesis is mainly based on the tasks performed at the University of Tübingen – with a strong focus on literature review, *in vivo* studies in fish, and biochemical analyses. My own contributions to the project are summarized in Table 1. In order to provide the relevant context, the other tasks and results will be referred to - but not in close detail. The scientific background and technical realization of the *in vitro* systems was not part of this thesis. The overall results of all project parts are published in Triebskorn *et al.* (2014) and Triebskorn *et al.* (2017), and the development of the *in vitro* systems is further explained by Bernhard *et al.* (2017).

Table 1: Overview on the performed experiments and analyses. Green boxes indicate that the endpoints were evaluated as part of this thesis. Yellow boxes indicate that the experiments were performed in the overall project, but not part of this thesis. Blank boxes indicate that these experiments were not performed, or endpoints not analysed in the overall project.

		S. trutta embryo	*S. trutta* juvenile	*G. fossarum*	*D. magna*	*L. variegatus*	*P. antipodarum*
Diclofenac - single substance exposure	Organismic parameters	[green]	[green]	[yellow]	[yellow]	[yellow]	
	Hsp70		[green]	[green]	[green]	[green]	
	Lipid peroxides		[green]	[green]		[green]	
	Histology	[green]	[green]				
Metoprolol - single substance exposure	Organismic parameters	[green]	[green]	[yellow]	[yellow]	[yellow]	[yellow]
	Hsp70		[green]	[green]	[green]	[green]	[green]
	Lipid peroxides					[green]	
	Histology	[green]	[green]				
Diclofenac + Metoprolol - mixture exposure	Organismic parameters		[yellow]	[yellow]	[yellow]	[yellow]	
	Hsp70		[yellow]	[yellow]	[yellow]	[yellow]	
	Lipid peroxides		[yellow]	[yellow]		[yellow]	
	Histology		[yellow]				
Metoprolol - artificial indoor stream	Organismic parameters			[yellow]		[yellow]	[yellow]
	Hsp70			[green]			[green]
	Lipid peroxides						
	Histology						
Wastewater treatment plant effluent	Organismic parameters	[green]		[yellow]			
	Hsp70			[green]			
	Lipid peroxides	[green]		[green]			
	Histology	[green]					

7. Material and Methods

Literature study

Search details

The literature survey in 2013 compiled effect data on 90 pharmaceuticals. Relevant substances were selected according to the priority list previously published by Bergmann *et al.* (2011), based on literature data, and communications with the German Environment Agency (UBA). The UBA-selection based on measured concentrations in German waters, consumption rates, effect data from confidential regulatory studies and problematic physicochemical attributes (e.g. potential persistence, bioaccumulation). Relevant biota classes were bacteria, protists, plants/algae, molluscs, crustaceans, insects, fish, tetrapods and others (incl. communities). Since the study by Bergmann *et al.* (2011) had already evaluated literature up to 2010, we restricted our search to studies published between 2011 and 2013.

Besides studies on single substance and mixture toxicity, reviews on ecotoxicological effects of pharmaceuticals were also compiled and evaluated to obtain a better knowledge of the overall context. "Web of Knowledge" (incl. databases "Web of Science®", "BIOSIS Citation IndexSM", "BIOSIS Previews®", "MEDLINE®" and "Journal Citation Reports®") served as database for the search, with the following search terms:

- Reviews: *pharma** AND *eco**, limited by *"review"*
- Single substances: specific search entry (summarized in Table 2) for each pharmaceutical combined with the search terms: *aqua** OR *eco** OR *tox**

Substances present in our search were all either prioritized by UBA-internal data, or by the summarizing report by Bergmann *et al.* (2011). Since those prioritization schemes differed in some aspects, the respective priority classes are also described in Table 2. Judging on the information given in title and abstract, we decided whether to discard the study or evaluate it in closer detail. The sole focus rested on aquatic ecotoxicological effects. Hence, studies on effects in humans, i.e. from clinical study reports, were not relevant.

Table 2: List of pharmaceuticals with defined priority classes

P: high priority according to Bergmann et al. and UBA
Pu: high priority according to UBA
Pb: high priority according to Bergmann et al.
(P): medium priority according to Bergmann et al.
?: substances requiring further information
none: substances without priority according to Bergmann et al.

Pharmaceutical	Search entry	Priority class (UBA)	Priority class (Berg mann)	Priority class (final)	Class	CAS
"Sartanic acid"	sartane*	P		Pu	antihypertensive	
14-Hydroxyclarithromycin	hydroxyclarithromycin*	P		Pu	antibiotic	110671-78-8
17alpha-Ethinylestradiol	ethinyl* estradiol*	P	P	P	contraceptive	57-63-6
17beta-Estradiol	estradiol* AND pharma*		P	Pb	hormone	50-28-2
4-N-Methylaminoantipyrin	methylaminoantipyrin*	P		Pu	analgesic	519-98-2
6(carboxymethoxy)-4-(2-chlorophenyl)-5-(ethoxycarbonyl)-2-methylpyridine-3-carboxylic acid	amlodipin*	P		Pu	antihypertensive	
Acetyl cysteine	acetyl cystein*		?	?	mucolytic agent	616-91-1
Acetylsalicylic acid	acetylsalic*				NSAID	50-78-2
Aciclovir	aciclovir*		?	?	antiviral drug	59277-89-3

Pharmaceutical	Search entry	Priority class (UBA)	Priority class (Berg mann)	Priority class (final)	Class	CAS
Allopurinol	allopurinol*				xanthine oxidase inhibitor	315-30-0
AMDOPH	AMDOPH		(P)	(P)	analgesic	
Amlodipine	amlodipin*	P		Pu	antihypertensive	88150-42-9
Amoxicilline	amoxicillin*	P	P	P	antibiotic	26787-78-0
Atenolol/ Atenolol acid	atenolol*, tenormin*	P		Pu	betablocker	29122-68-7
Azitromycin	azitromycin*, zithromax*, azithrocin*, azin*	P		Pu	antibiotic	83905-01-5
Bezafibrate	bezafibrat*		(P)	(P)	lipid regulator	41859-67-0
Bisoprolol	bisoprolol*	P	?	Pu	betablocker	66722-44-9
Capecitabin	capectiabin*, xeloda*	P		Pu	cytostatic	15361-50-9
Carbamazepine	carbamazepin*	P	P	P	anticonvulsant	298-46-4
Cefaclor	cefaclor* OR cefachlor*		?	?	antibiotic	53994-73-3
Cefuroxime axetil	cefuroxim* axetil*		?	?	antibiotic	64544-07-6
Chloramphenicole	chloramphenicol*		P	Pb	antibiotic	56-75-7
Chlortetracycline	chlortetracyclin*		P	Pb	antibiotic	57-62-5
Ciprofloxacin	ciprofloxacin*	P	P	P	antibiotic	85721-33-1
Clarithromycine	clarithromycin*	P	P	P	antibiotic	81103-11-9
Clindamycin	clindamycin*	P	?	Pu	antibiotic	18323-44-9
Clopidogrel	clopidogrel*		?	?	antiplatelet agent	113665-84-2
Clotrimazole	clotrimazol*		(P)	(P)	antimycotic	23593-75-1
Deltamethrin	deltamethrin*, decamethrin*	P		Pu	antiparasitic	52918-63-5
Desflurane	desfluran*		?	?	anesthetic	57041-67-5
Diatrizoic acid	diatriz*, iotalam*, amidotriz*		(P)	(P)	contrast agent	737-31-5
Diazepam	diazepam*		(P)	(P)	antidepressant/ antipsychotic	439-14-5
Diclofenac	diclofenac*	P	P	P	NSAID	15307-86-5
Dienogest	dienogest*, visanne*	P		Pu	gestagan	65928-58-7
Dipyridamole	dipyridamol*		?	?	antiplatelet agent	58-32-2
Doxycycline	doxycyclin*		P	Pb	antibiotic	564-25-0
Duloxetine	duloxetin*, cymbalta*	P		Pu	antidepressant/ antipsychotic	116539-59-4
Entacapone	entacapon*		?	?	C-O-M-Inhibitor	130929-57-6
Eprosartan	eprosartan*		?	?	antihypertensive	133040-01-4
Erythromycin	erythromycin*		P	Pb	antibiotic	114-07-8
Gabapentin	gabapentin*, neurotin*		?	?	anticonvulsant	60142-96-3
Gemfibrozil	gemfibrozil*, lopid*	P		Pu	lipid regulator	25812-30-0
Glutaral	glutaral*		?	?	desinfectant	111-30-8
Hydrochlorothiazide	hydrochlorothiazid*		?	?	antihypertensive	58-93-5
Ibuprofen	ibuprofen*	P	(P)	P	NSAID	15687-27-1
Imatinib	imatinib*, gleevec*, glivec*	P		Pu	cytostatic	152459-95-5
Indometacin	indometacin*, indomethacin*		(P)	(P)	NSAID	53-86-1
Iohexol	iohexol*, omnipaq*	P	(P)	P	contrast agent	66108-95-0
Iomeprol	iomeprol*	P	(P)	P	contrast agent	78649-41-9
Iopamidol	iopamidol*		(P)	(P)	contrast agent	60166-93-0
Iopromide	iopromid*, ultravist*		(P)	(P)	contrast agent	73334-07-3
Ivermectin	ivermectin*, stromectol*	P		P	Antiparasitic	70288-86-7
Lamotrigin	lamotrigin*, lamictal*	P		Pu	anticonvulsant	84057-84-1
Levetiracetam	levetiracetam*		?	?	anticonvulsant	102767-28-2
Lincomycin	lincomycin*		(P)	(P)	antibiotic	154-21-2
Mesalazine	mesalazin*		?	?	NSAID	89-57-6
Mestranol	mestranol*		P	Pb	contraceptive	72-33-3
Metamizole	metamizol*	P		Pu	analgesic	68-89-3
Metformin	metformin*	P		Pu	antidiabetic	657-24-9
Metoprolol	metoprolol*	P		Pu	betablocker	51384-51-1
Naproxen	naproxen*	P	P	P	NSAID	22204-53-1
Norethisterone	norethisteron*		P	Pb	contraceptive	68-22-4
Opipramol	opipramol*		?	?	antidepressant/ antipsychotic	315-72-0
Oxytetracycline	oxytetracyclin*		P	Pb	antibiotic	79-57-2

Pharmaceutical	Search entry	Priority class (UBA)	Priority class (Berg mann)	Priority class (final)	Class	CAS
Pantoprazole	pantoprazol*		?	?	proton pump inhibitor	102625-70-7
Paracetamol	paracetamol*, acetaminophen*		P	Pb	analgesic	103-90-2
Piperacillin	piperacillin*		?	?	antibiotic	61477-96-1
Pregabalin	pregabalin*		?	?	anticonvulsant	148553-50-8
Primidone	primidon*		P	Pb	anticonvulsant	125-33-7
Propranolol	propanolol*, propranolol*		(P)	(P)	betablocker	525-66-6
Quetiapine	quetiapin*	P	?	Pu	antidepressant/ antipsychotic	11974-69-7
Ramipril	ramipril*		?	?	antihypertensive	87333-19-5
Roxithromycine	roxithromycin*		P	Pb	antibiotic	80214-83-1
Sevelamer	sevelamer*		?	?	phosphate binding drug	52757-95-6
Simvastatin	simvastatin*	P	?	Pu	lipid regulator	79902-63-9
Strontium ranelate	strontium ranelat*		?	?	osteoporose	135459-90-4
Sulbactam	sulbactam*		?	?	antibiotic	68373-14-8
Sulfadimethoxine	sulfadimethoxin*		P	Pb	antibiotic	122-11-2
Sulfadimidine	sulfadimidin*, sulfamethazin*		P	Pb	antibiotic	57-68-1
Sulfamethoxazole	sulfamethoxazol*	P	P	P	antibiotic	723-46-6
Tazobactam	tazobactam*		?	?	antibiotic	89786-04-9
Telmisartan	telmisartan*		?	?	antihypertensive	144701-48-4
Tetracycline	tetracyclin*		P	Pb	antibiotic	60-54-8
Tiamulin	tiamulin*	P	P	P	antibiotic	55297-95-5
Tilidine	tilidin*		?	?	analgesic	51931-66-9
Torasemide	torasemid*, torsemid*		?	?	diuretic drug	56211-40-6
Tramadol	tramadol*, ryzolt*, ultram*	P		Pu	analgesic	27203-92-5
Valproic acid	valpro*		?	?	anticonvulsant	99-66-1
Valsartan	valsartan*, angiotan*, diovan*	P	?	Pu	antihypertensive	137862-53-4
Venlafaxine	venlafaxin*		?	?	antidepressant/ antipsychotic	93413-69-5

Data evaluation and reliability analysis

All available publications were evaluated, the following parameters (Table 3) extracted and documented in the database “$OEKOTOX_{upgrade}$”:

Table 3: Information extracted from examined publications and summarized in the database “$OEKOTOX_{upgrade}$”.

Test substance	Test organism	Exposure	Effect data	Additional information
name of effective substance	test organism (species)	test concentrations in water [µg/L or µmol/L]	BCF/BMF	citation
form of the substance (salt/conjugate)	biota group	test concentrations in sediment [µg/kg or µmol/kg]	NOEC [µg/L or µmol/L]	DOI
synonyms	field/lab/mesocosm	dose [µg/kg or µmol/kg]	LOEC [µg/L or µmol/L]	full text available?
CAS number	effect endpoint	duration	EC_{10} [µg/L or µmol/L]	EndNote label
$logK_{ow}$	population relevance?	acute/sub-acute/chronic?	EC_{50} [µg/L or µmol/L]	substance in priority list?
molar mass	standard method?	analytical-chemical verification?		comments
substance class		bioaccumulation investigated?		reliability (only for selected studies)
field of application effective substance/medicinal product?				
single substance/mixture?				

In case the highest test concentration did not evoke an effect, this concentration was included as NOEC without corresponding LOEC. If already the lowest concentration evoked an effect, it was included as LOEC without corresponding NOEC. All given data on pharmaceutical ingredients was transferred to the database. As several publications investigated more than one test substance, the final database includes information on pharmaceuticals originally not defined in the priority list. For each endpoint per species and test substance given in the publications, a separate row was created. In the following, the single rows will be referred to as "database entries".

Evaluation was performed in a qualitative way, as this was more appropriate to the heterogeneous structure of the data. LOECs were compared to measured environmental concentrations (MECs), which were either available from the database given by Bergmann *et al.* (2011) or from UBA internal data. A risk quotient, calculated by dividing the maximum MEC by the minimum LOEC, served as proxy for the environmental relevance of the substance.

For substances of potentially high relevance, the reliability of the underlying publication was evaluated more closely. This was done by checking whether the studies fulfilled several reliability criteria, proposed by Wright-Walters *et al.* (2011):

"

1) A thorough description of the experimental design, including exposure regime and replication,
2) Analytical confirmation of test concentrations
3) Description of ecologically relevant endpoints and all supplemental morphological information collected
4) Use of test procedures that are based, at least generally, on internationally accepted procedures and practices. Newly developed test procedures must be able to be repeated, and meet all other required criteria
5) Clear linkage of reported findings with the exact experimental design, and
6) Sufficient reporting of results, including system performance, toxicity results, and statistical methods employed to ascertain how the data support the conclusions that are drawn

"

Criteria 1, 2, 3 and 6 were seen as most important. Studies were labelled as "reliable" when these four criteria were fulfilled or "conditionally reliable", when only three criteria were fulfilled. In cases where less than three criteria were met or apparent mistakes were identified, the study was labelled as "not reliable".

Test substances

Following the conclusions of the literature study (Chapter I), the *in vivo* experiments investigated non-steroidal anti-inflammatory drugs and beta-adrenergic receptor blockers, with diclofenac and metoprolol as representatives of their respective substance group.

Diclofenac

Diclofenac belongs to the substance group of non-steroidal anti-inflammatory drugs (NSAIDs). The mode of action of this pharmaceutical class is the inhibition of cyclooxygenase (COX) 1 and 2 (Kawada *et al.* 2012). This prevents the formation of prostaglandins - important mediators in inflammatory processes (Funk 2001). Besides this, prostaglandins are involved in various other physiological processes, like blood coagulation, vasodilation or protection of the gastric mucosa. Apart from its use as an antiphlogistic (anti-inflammatory) drug, diclofenac is commonly used as an antipyretic (anti-fever) and light analgesic (painkiller) pharmaceutical. Typical applications are creams, pills or injections to treat rheumatic symptoms, bruises and strains. The estimated global annual use was 1443 tons in

2016 (Lonappan *et al.* 2016). Various diclofenac-containing products are sold over the counter, making them a popular choice for self-medication. Particularly dermal application is regarded as problematic from an environmental point of view. Only a small fraction of active substance is absorbed, while the major part is washed off unmetabolized (Heberer and Feldmann 2005). Its occurrence in German surface waters was reported for the first time by Stumpf *et al.* (1996). Since then, it is one of the most frequently detected substances in surface waters (Aus der Beek *et al.* 2016b).

Cyclooxygenase, also called prostaglandin H synthase, is a membrane-bound haemo- and glycoprotein that consists of four domains. Highest amounts are found in the endoplasmic reticulum of prostanoid-forming cells. Its catalytic domain has hydroperoxidase, as well as cyclooxygenase activity (Vane and Botting 1998, Simmons *et al.* 2004). It metabolizes the precursor molecule arachidonic acid to the intermediate prostaglandin H2. Further metabolism depends on the respective cell type, and results in the following molecules: thromboxane A2, prostacyclin (=prostaglandin I2), prostaglandin E2, prostaglandin F2 and prostaglandin D2. Prostaglandins are responsible for the processes of inflammatory reactions, pain sensitization, fever, immunosuppression, protection of the gastrointestinal tract through mucus secretion, inhibition of vascular contraction, antagonizing platelet aggregation, vasodilation in kidney and maybe also complex integrative function in the brain and autonomous nervous system (Funk 2001). COX1 is mainly responsible for physiological endogenous processes, while the COX2 isoform is inducible by proinflammatory stimuli (Vane and Botting 1998, Simmons *et al.* 2004). NSAIDs block the COX-derived prostaglandin synthesis as a whole (Funk 2001, Kawada *et al.* 2012). Diclofenac is especially potent in inhibiting COX2, with approximately four-fold higher selectivity than for COX1 (Gan 2010). Besides the inhibition of prostaglandin and thromboxane synthesis, further mechanisms are discussed: inhibition of leukotriene production, inhibition of phospholipase A2 or action via the nitric-oxide-cGMP pathway (Gan 2010). Especially the inhibition of COX2 has recently been associated with increased cardiovascular risks in human (Cannon and Cannon 2012). Adverse side effects are often caused by reduced levels of prostaglandin E2, which is produced by both COX-isoforms (Kawada *et al.* 2012).

Diclofenac is mainly metabolized to hydroxylated and methoxylated derivatives, but also conjugated to glucuronide. A wide range of these metabolites, which still possess high similarity to the parent substance, is frequently detected in the environment (Lonappan *et al.* 2016). In the environment, direct photolysis is the main process contributing to the removal of diclofenac (Avetta *et al.* 2016). Other processes, like adsorption to particles and sediment, hydrolysis or biological degradation, are of minor importance for this substance (Buser *et al.* 1998). However, while photodegradation is of high importance in lakes, its elimination potential is negligible in rivers (Johnson *et al.* 2013). Furthermore, effective removal in conventional wastewater treatment is highly variable (Monteiro and Boxall 2010, Lonappan *et al.* 2016).

Like many other pharmaceuticals, diclofenac is an ionisable substance. It is a medium weak acid, with a pKa around four. Hence, it shifts from non-dissociated to anionic form when the pH increases. About 99.9 % of diclofenac exists as anion at physiological pH (Ferreira *et al.* 2005). This has a marked effect on its $logK_{ow}$, and consequently its bioconcentration potential (Fu *et al.* 2009, Schreiber *et al.* 2011). The K_{ow} and theoretical bioconcentration factor (BCF) are high only at low pH-ranges, which are not realistic for most aquatic ecosystems. At natural pH conditions, which are usually neutral or slightly alcalic, the K_{ow} is considerably smaller (Fu *et al.* 2009). Common models assume that uptake of pharmaceuticals into an organism is mainly based on diffusion through biomembranes and, hence, mainly depends on the lipophilicity of a substance. However, in this context it is important to notice that cellular uptake of diclofenac is not only possible through passive processes. Active transport via monocarboxylate/H^+-cotransporters, carriers originally intended for the uptake of carboxylic substrates, is highly likely (Choi *et al.* 2005).

In my experiments, diclofenac was applied as sodium salt (CAS 15307-79-6; purity: 100 %; supplier: SIGMA-ALDRICH, 3050 Spruce Street, Saint Louis, MO 63103; lot BCBN3367V). In this form, it is readily soluble in water (50 g/L) and using additional solvent was not necessary.

Metoprolol

Metoprolol is a beta-adrenergic receptor antagonist, commonly called beta-blocker. These pharmaceuticals reduce heart rate and blood pressure, and are mainly used for the treatment of hypertension, coronary heart disease and migraine. Beta-blockers act by inhibiting beta-adrenergic receptors. These receptors belong to the superfamily of G-protein-coupled receptors. Up to now, three receptor types are known: β1, mainly located in heart, kidney and adipose tissue. β2, mainly located in heart, lung, gastrointestinal tract, liver, pancreas and skeletal muscles. And β3, whose location is not as well defined (DeGeorge Jr and Koch 2007). Activation of β-adrenoceptors by the catecholamines adrenalin or noradrenalin induces a signalling pathway, which activates adenylyl cyclase and increases intracellular cAMP-levels. This induces the myocardial contractile apparatus (DeGeorge Jr and Koch 2007). Beta-blockers prevent the docking of adrenalin and noradrenalin to the receptor. Thus, they counteract the processes involved in the so-called “fight and flight” reactions, with extensive effects on an organism’s stress response (Owen *et al.* 2007). The actual physiological effects of beta-blockers strongly depend on the tissue where the receptors are present. In the heart, they influence heart rate and contractility, while they lead to vasodilation in blood vessels. In hepatic tissue, they activate glycogenolysis and gluconeogenesis. In the kidney, they promote renin release. Effects are usually small in resting organisms, but become large as soon as the sympathetic nervous system is activated (López-Sendó *et al.* 2004). In fish, the adrenergic systems is mainly responsible for modulations of cardiac output, ventilation, metabolic regulation, skeletal muscle performance and melanophore regulation (Owen *et al.* 2007).

As high blood pressure, migraine and cardiac problems are very common health impairments in the Western world, beta-blockers are an important and frequently used group of pharmaceuticals. Some beta-blockers, like propranolol, non-selectively act on beta-adrenergic receptors 1 and 2. Metoprolol, in contrast, selectively inhibits only receptor type 1. It is currently the beta-blocker with the highest prescription and consumption rate in Germany (IMS Health). Due to its mode of action, which also leads to a general “calm-down”, it is also prone to improper use to improve personal performance in exams and sports requiring concentration and accuracy (Stoschitzky 2001, Davis *et al.* 2008). Common adverse side effects in humans include bradycardia, atrioventricular block, masking of hypoglycaemia, or impotence. It is a lipophilic drug, which is rapidly absorbed and extensively metabolized in gut and liver (López-Sendó *et al.* 2004). Around 85 % is excreted as the three main metabolites (metoprolol acid, α-hydroxymetoprolol, O-desmethylmetoprolol) and only 10 % as parent substance (Rubirola *et al.* 2014). Investigations on trout liver spheroids *in vitro* came to a similar conclusion of ca. 90 % metabolite excretion, resulting from several oxidation pathways (Baron *et al.* 2017). Despite the short elimination half-life, it is frequently found in wastewater and surface waters (Godoy *et al.* 2015) – likely due to the sheer overall amount of drug consumed. Furthermore, incorrect disposal of unused metoprolol via wastewater could strongly contribute to the environmental concentrations (Brandmayr *et al.* 2015).

Like all beta-blockers, metoprolol is a weak base, with reported pK_a of 9.7, $logK_{OW}$ of 1.88/2.28 and $logD_{lipw}$ of 1.43 (Escher *et al.* 2006, Maurer *et al.* 2007). Sorption to sludge is expected to be low. Elimination of beta-blockers in wastewater treatment mainly depends on the hydraulic retention time and ranges between 25 and 75 %. Reported effluent concentrations of metoprolol range between 80 and 2000 ng/L (Maurer *et al.* 2007, Scheurer *et al.* 2010). Once the drug is excreted, it has high hydrolytic stability. Sorption potential ranges from Kd-values of 0.79 to 14.78, with decreasing potential at higher pH. It has to be considered as mobile substance, with low immobilization in

sediments. Consequently, this leads to the assumption that metoprolol and other beta-blockers may accumulate in the aquatic environment (Maszkowska *et al.* 2014a, Maszkowska *et al.* 2014b).

In my experiments, metoprolol was applied as tartrate (CAS 56392-17-7; purity: 98.7 %; supplier: SIGMA-ALDRICH, 3050 Spruce Street, Saint Louis, MO 63103; lot BCBH029V). In this form, it is readily soluble in water (4.7 g/L) and using additional solvent was not necessary.

Wastewater treatment plant Eriskirch

For the semi-field exposure to investigate a well-defined wastewater effluent, we chose the wastewater treatment plant (WWTP) Eriskirch (Gmünd 2, 88097 Eriskirch), located 500 m from the Schussen estuary at the Lake Constance. It processes wastewater of ca. 50,000 inhabitant equivalents, with a mean throughput of 11,000 m³/d, (Luddeke *et al.* 2015, Triebskorn 2017, p.41-43) in a three-staged purification (http://www.av-unteres-schussental.de/data/beschreibung.php - 04.04.2018):

- Mechanical filtration: Bar screen for the removal of coarse materials, sand trap for settleable solids and primary clarifier for the removal of primary sludge.
- Biological purification: Degradation of organic compounds in activated sludge, with upstream denitrification and downstream dosage of precipitant for nitrogen and phosphorus removal.
- Flocculation filtration: Secondary clarifier and additional treatment with precipitant for further removal of phosphorus and filterable substances.

In this manner, it is representative of the majority of medium-sized German wastewater treatment plants. The facility was also investigated in the project SchussenAktiv*plus*, led by the University of Tübingen (Triebskorn 2017). Therefore, there is much information on the facility and effluent from previous studies. This includes data on effluent concentrations of micropollutants including diclofenac and metoprolol, data on effect potentials from various laboratory *in vitro* tests, effect potentials in *P. antipodarum* and *D. rerio*, and even data from one-year old rainbow trout exposed in the effluent. Mean concentrations of diclofenac and metoprolol measured over three years were 1.3 µg/L and 1.4 µg/L, respectively.

Experiments with brown trout

Test species - Salmo trutta f. fario

Brown trout *Salmo trutta* (Linnaeus, 1758) is a prominent member of the family Salmonidae. It is native to Europe, Northern Africa and Western Asia, but has also been introduced to other parts of the world. The species shows high variability in size, growth, feeding niche and habitat use, and can adapt to a wide range of environmental conditions. Habitats range from small streams, up to large rivers, lakes, brackish and coastal waters – strongly depending on the life stage and ecotype (Klemetsen *et al.* 2003).

Salmo trutta f. *fario* is the riverine type of the species. In contrast to the other ecotypes, the lacustrine lake trout (*Salmo trutta* f. *lacustris*) and anadromous sea trout (*Salmo trutta* f. *trutta*), they typically form resident populations in small to medium streams. The streamlined body, usually of grey to dark brown colour on the dorsal side and pale yellow to light brown colour on the ventral side, is a characteristic feature of their anatomy. Numerous red and black spots, surrounded by pale halos, mark the lateral sides. As carnivorous species, they feature a large mouth with well-developed teeth. The adipose fin, a typical feature of salmonids, is marked by a red margin (www.fishbase.se – 14.06.2018).

The ecotype prefers cold, oxygen-rich waters to forage and breed (Dußling and Berg 2001). Once per year, females spawn 500 to 1500 eggs, which are naturally deposited in sand and gravel (Klemetsen *et al.* 2003).The number of spawned eggs is mainly determined by the size of the mother, but there are also considerable differences between anadromous and resident forms of brown trout (Jonsson and Jonsson 1999). Shortly after fertilisation, eggs are highly sensitive to mechanical shocks. This sensitivity

decreases rapidly after the eggs are eyeing (Crisp 1996). As the efficiency of yolk conversion decreases with higher temperatures, eggs are larger and yolk-richer in a warmer environment, but the hatching embryos are usually smaller. After hatching, larvae remain in the gravel until the yolk sac is consumed and subsequently move to the water column to feed. Developmental time is largely dependent on ambient temperature (Killeen *et al.* 1999a, Killeen *et al.* 1999b) – but usually requires several months from fertilized egg to fully developed larvae. Spawning usually starts earlier at higher altitude and latitude, to compensate for the slower development at lower temperatures. In growing, juveniles transcend from swarming to a territorial behaviour (J. Schindler – personal communication). Young individuals become aggressive, form dominance hierarchies and compete intensively with each other (Klemetsen *et al.* 2003). All brown trout are opportunistic carnivores, but may show a certain food specialization depending on life stage and current habitat. Small individuals feed predominantly on insect larvae and zoobenthos; when growing to a certain size, they also start to become piscivorous. As cold-water organisms, their tolerate water temperature ranges between 4 and 19 °C, with optimum growth of juveniles at 13-14 °C. The annual 50-percentile oxygen concentration should be above 9 mg/L, but they can tolerate lower concentrations for a short time. Eggs and larvae have high survival, as long as oxygen concentration remains above 7 mg/L and temperature below 12.5 °C. A pH above 9.2 or below 5 is also considered harmful (Crisp 1996).

The species itself is a popular food fish, and is commonly raised in aquaculture for consumption and fishery restocking. Feral fish suffer from structural and qualitative stream degradation, as well as competition from introduced rainbow trout (*Oncorhyncus mykiss*), which has higher tolerance to a wider range of environmental conditions. However, the populations are currently considered as not endangered (Dußling and Berg 2001). In some areas, where the species was introduced, they themselves became invasive.

Fish are frequently applied in ecotoxicological research because of several reasons: they are comparably easy to keep in the lab and react sensitively to pollutants. Furthermore, their metabolic processes are largely similar to other vertebrates, including humans. The so-called read-across-hypothesis postulates that conservation of human drug targets is higher the closer a taxon is phylogenetically related to humans (Rand-Weaver *et al.* 2013). A recent meta-analysis of 194 pharmaceutical ingredients and their target proteins reported 80-90 % drug target conservation in teleosts (Verbruggen *et al.* 2018). Hence, it is not surprising that genes coding for cyclooxygenases have been found in salmonids and other teleost families (Ishikawa & Herschman 2007), though they are only partly similar to human COX (Roberts *et al.* 2000). Furthermore, the adrenergic system is well conserved in vertebrates and beta-adrenergic receptors are found in a variety of fish tissues (Massarsky *et al.* 2011). This makes it likely that the two pharmaceutical groups tested in the experiments will affect fish.

Since brown trout can only be kept in the lab at cold temperatures, spawn only once per year, and embryonic development takes a long time, they are no common testing organisms. Typical tests on fish toxicity apply model lab species, like zebrafish (*Danio rerio*), fathead minnow (*Pimephales promelas*), Japanese medaka (*Oryzias latipes*) or rainbow trout (*Oncorhynchus mykiss*), as they are much easier to handle. Although these species are of high value to derive comparable results in lab studies, they lack ecological relevance for Central Europe. For the project, it was important to work with a species of high ecological relevance for European stream ecosystems. Hence, I chose the brown trout despite its more complicated handling in the lab.

The embryonic development of brown trout is described in great detail by Killeen *et al.* (1999a), who divided the embryogenesis into 40 different steps and developed an elaborate scoring system. After fertilisation, the early embryonic development takes place: blastulation is followed by gastrulation and

subsequent differentiation of somites and development of nervous, vascular and alimentary system. Head and tail separate from the yolk sac, heart beat is visible and the embryo exhibits first spontaneous contractions. At around 30-48 d (6 °C) the eyes become pigmented (= eyed ova stage, eyed egg stage) and the yolk sac becomes vascularised, blood vessels spread through the body and body pigmentation starts. In the following, fins develop, pigmentation, muscular development and vascularisation continue and the gill filaments form. Hatching occurs usually between day 72 and 82 at 6 °C. Embryos free themselves from the surrounding chorion, but still depend on nourishment from the yolk sac (= eleutheroembryos, sac-fry, "alevins"). The final embryonic development takes place between days 82-127 (6 °C): Fins are further segmented, pigmentation continues and the dark parr marks appear on the sides. Finally, the yolk sac is fully resorbed and the fully developed larvae (= fry) enters its free-feeding stage. Temperature has a great influence on the speed of development. While the complete embryogenesis took 127 d at 6 °C, it only took 80 d at 10 °C (Killeen *et al.* 1999a).

For my experiments, I used fish obtained from a commercial fish breeder (Forellenzucht Lohmühle, Am Lohmühlebach 85, 72275 Alpirsbach-Ehlenbogen). This breeding facility is regularly controlled and rated as category I, disease-free (EU 2006a).The bred variety of brown trout is considered to be robust and close to feral forms, and also used for fishery restocking campaigns. Fish originating from this source were also applied in other field and laboratory experiments of the University of Tübingen, and performed well (Triebskorn 2017). The chosen life stages for my experiments were either eggs within 24 h of fertilisation, or juvenile fish of four to seven months post hatch.

All experiments with juvenile brown trout, and tests with larvae that continued over the time of consumption of the yolk sac, were approved by the animal welfare committee of the Regional Council of Tübingen, Germany (authorization ZP1/12).

Experimental design

The studies with brown trout consisted of seven separate experiments:

[1] May 21st 2014 – June 17th 2014: First exposure of juvenile brown trout to diclofenac (repetition necessary due to high control mortality).

[2] July 29th 2014 – August 28th/29th 2014: Exposure of juvenile brown trout to metoprolol (final sampling carried out over two days).

[3] December 1st 2014 – April 7th 2015: Exposure of brown trout embryo and sac-fry stages to diclofenac and metoprolol.

[4] December 15th 2014 – March 31st/April 14th 2015: Exposure of brown trout embryo and sac-fry stages to wastewater treatment plant effluent under semi-field conditions (control sampled at later time point, due to slower development).

[5] December 15th 2014 – May 13th 2015: Exposure of brown trout embryo and sac-fry stages to wastewater treatment plant effluent under lab conditions.

[6] July 10th 2015 – August 3rd 2015: Repeated exposure of juvenile brown trout to diclofenac.

[7] September 15th – October 13th 2015: Exposure of juvenile brown trout to mixtures of diclofenac and metoprolol (excluded from this thesis).

Juvenile brown trout – pharmaceutical exposure (Experiments 1, 2, and 6, described in chapter III)

The experiments on juvenile brown trout took place in a thermoconstant chamber (settings: 12 h/12 h light/dark; 8 °C [experiment 1 and 2], 7 °C [experiment 6]) at the University of Tübingen (Auf der Morgenstelle 28, 72076 Tübingen). In the first diclofenac exposure experiment [experiment 1], test animals aged ca. 4 month post hatch. Because these young animals performed poorly, I chose to use older individuals (ca. 6 months post hatch) for the metoprolol [experiment 2] and second diclofenac [experiment 6] exposure. All test solutions were prepared from aerated, filtered tap water (iron filter, active charcoal filter, particle filter). Five test concentrations (1st diclofenac: 0.1, 0.5, 1, 10, 100 µg/L;

2nd diclofenac: 0.1, 1, 10, 100, 200 µg/L; metoprolol: 0.1, 1, 10, 100, 1000 µg/L – all concentrations referring to the base, not the salt) and one control were tested in triplicates, arranged in a randomized block design. Each replicate was an individually aerated 25 L glass aquarium containing 13 individual fish. Overall, 18 aquaria with 234 fish were used for each experiment. All aquaria were shaded from direct light with black plastic sheets. Animals were fed daily with commercial trout feed (1.5 mm, Biomar, Brande, Denmark). Twice per week, 1/3 of the test medium was renewed, and excess food and faeces removed.

The first diclofenac experiment lasted 27 days, except the 100 µg/L treatments, which had to be terminated already after 14 days due to high mortality of the exposed fish. The metoprolol experiment was terminated on days 27 and 28 of exposure. The additional day was necessary, since the number of individual fish was too large to be sampled on one single day. In the second diclofenac experiment, sampling took place on day 24 – three days earlier than intended. This was due to the high mortality in the two highest test concentrations. The earlier sampling made sure that a sufficient number of samples for biomarker analysis was available. Fish were anesthetized and killed by an overdose of tricaine mesylate (MS-222, 1 g/L, pH 7), followed by a neck-cut. Body length and weight were measured, and the individual fish were checked for apparent peculiarities, especially bite marks as sign of aggressive behaviour. Histological samples of liver, gill, kidney and heart were fixed in glutardialdehyde. Samples of liver, head [experiment 1], and trunk kidney [experiment 6], intended for biochemical analyses, and of the remaining fish, intended for chemical analyses of the test substance, were snap frozen in liquid nitrogen and stored at -80 °C until further processing. Endpoints of interest were survival, body length and weight, marks of aggressive behaviour, levels of the stress protein Hsp70 in liver, lipid peroxides in head or trunk kidney, and histological status of liver, kidney, gill and heart (only for metoprolol).

To control for effects of the laboratory exposure itself, I conducted five samplings directly at the fish breeding facility. Samplings took place on May 9th2014, July 11th 2014, September 3rd 2014, July 7th 2015 and August 8th 2015. In this way, the samplings roughly corresponded to the start and end points of the juvenile experiments. At each time point, 20 fish were sampled in the same manner as described for the exposure experiments.

Brown trout embryo and sac-fry stages – pharmaceutical exposure (experiment 3, described in chapter III)
Testing followed the OECD guideline 212 – short term toxicity test on embryo and sac-fry stages (OECD 1998). The complete experiment took place in a thermo-constant chamber (settings: 10 h/14 h light/dark; 7 °C). Per replicate, 30 freshly fertilized eggs (within 24 h of fertilisation) were exposed in 200 mL glass petri dishes. Because early developmental stages of trout are susceptible to light (Hamdorf 1960), the eggs remained in complete darkness until they reached the eyed ova stage. Five concentrations of diclofenac (0.1, 0.5, 1, 10, 100 µg/L), five concentrations of metoprolol (0.1, 1, 10, 100, 1000 µg/L) and one water control were tested in triplicates. Test solutions were prepared from artificial water, consisting of deionized Millipore-filtered water combined with essential electrolytes (see appendix). Aeration of the single vessels was not possible, as eggs and larvae are highly sensitive to agitation (Crisp 1996). ¾ of test medium were renewed every second day.

In the course of 127 days, fertilized eggs developed to fully grown larvae. At the time, where the yolk sac was fully consumed and the larvae would transcend to a free-feeding stage, the exposure was terminated. Larvae were killed by an overdose of tricaine mesylate (MS222, 1 g/L, pH 7). Three larvae per replicate were cut into a proximal part (containing brain, gills and heart) and distal part (containing liver, kidney and alimentary system) - and subsequently fixed in glutardialdehyde solution. The remaining 18 – 27 larvae (depending on mortality rates) were snap frozen in liquid nitrogen. Although there is detailed information on the various stages of brown trout embryogenesis (Killeen *et al.* 1999a),

I only concentrated on selected developmental parameters. I focused on easily visible and assessable developmental stages: eyed ova stage, hatching and yolk-sac resorption. Other investigated parameters in the experiment were survival, heart rate seven days post hatch, body mass and, exemplarily, histology of kidney. I restricted the histological analysis to kidney, because reported reactions are supposedly strongest in this organ, and the gills were only weakly developed in the examined life stage.

Brown trout embryos and sac-fry stages – exposure in wastewater treatment plant effluent (experiments 4 and 5, described in chapter V)

The test system at the wastewater treatment plant consisted of two 250 L flow-through aquaria. A first trial of the experiment, without previous cooling of the water, was unsuccessful, as all eggs coagulated within two weeks. Therefore, effluent water after flocculation filtration was cooled down to approximately 9 °C and directed into the first aquarium for aeration. From the first aquarium, water flew into the second aquarium, where the actual exposure took place. Six stainless steel sieves were placed in the aquarium, each containing 50 freshly fertilized eggs. A control setup was not possible directly at the treatment plant. Instead, we installed a control flow-through aquarium receiving its water directly from the Lake Constance (ISF – Institut für Seenforschung; Argenweg 50/1, 88085 Langenargen). Since this water was already cool and oxygen-rich, additional cooling and aeration was not necessary. Like at the WWTP, exposure took place in six stainless steel sieves placed in a 250L aquarium, each containing 50 individual eggs. After consumption of the yolk sac, fish were fed with commercial trout feed (1.5 mm, Biomar, Brande, Denmark). Exposures were terminated three weeks after consumption of the yolk sac, which was after 106 days at the WWTP and 120 days at the control station.

In addition to the semi-field exposure, wastewater treatment plant effluent samples were tested in a laboratory experiment at the University of Tübingen (Auf der Morgenstelle 28, 72076 Tübingen). Grab samples from the effluent aquarium were transported to the laboratory and frozen at -20 °C until further use. The thermo-constant chamber was set to 7 °C; light conditions were complete darkness until the embryos reached the eyed ova stage, afterwards a 10 h/14 h light/dark cycle was applied. 30 freshly fertilized eggs were placed in 200 mL petri dishes filled with either tempered, aerated effluent or artificial water. Six replicates were set up for each treatment. Exposure lasted until the yolk sac was fully consumed – which was 149 days.

Larvae were killed by an overdose of tricaine mesylate (MS222, 1 g/L, pH 7), four individuals per sieve [experiment 4]/ three individuals per petri dish [experiment 5] fixed in cacodylate-buffered glutardialdehyde and the remaining individuals were snap frozen in liquid nitrogen. Examined endpoints were survival, time until eyed ova stage, time until hatch, heart rate seven days post hatch, body mass, lipid peroxides (full body) and kidney histology.

Additional exposure experiments in *Eff-Pharm* with invertebrates, from which samples were provided for biomarker analyses

Besides brown trout, invertebrates were examined with respect to their responses to diclofenac and metoprolol. Since the experiments were conducted by cooperation partners, who sent samples for biomarker analyses, the design of these experiments is only described briefly. Further details are given in the respective sections of the final report (Triebskorn *et al.* 2017).

Tests with Gammarus fossarum (conducted by GWT-TUD GmbH, Dresden, described in chapter IV and V)

Gammarids, like *Gammarus fossarum* (Koch, 1835), are an important part of riverine zoobenthos. Although not regularly studied in ecotoxicological tests with pharmaceuticals, they are of crucial

importance in Central European stream ecosystems. In riverine ecosystems, the genus *Gammarus* usually occurs in great abundance, which makes them the dominant macroinvertebrates in terms of biomass. They are commonly regarded as "shredders" that feed on detritus, dead plant material and biofilms ("aufwuchs"), but can also flexibly use a wide range of other food. Their feeding behaviours include carrion feeding, cannibalism and predation on other macroinvertebrates, as well as weakened vertebrates or fish eggs (MacNeil *et al.* 1997). They are abundant throughout the year, making them a highly important food source for fish, but also other vertebrates and macroinvertebrates. Therefore, gammarids are of high ecological relevance for lotic and lentic ecosystems (MacNeil *et al.* 1999).

G. fossarum applied in the studies originated from a wild population in the Tännichtgrundbach (first-order stream northwest of Dresden, Germany – which is reportedly free from domestic and industrial wastewater). They were used for single substance exposures, as well as mesocosm studies and the wastewater treatment plant exposure experiments:

Single substance exposure (described in chapter IV)

Briefly, gammarids were exposed to five concentrations of diclofenac (0.49, 1.48, 4.44, 13.33 and 40 mg/L) or metoprolol (5, 15, 45, 135, 405 mg/L) plus an untreated control over 40 days at 15 °C. Each treatment was replicated four times, with 20 individuals per replicate. Besides mortality and reproductive parameters, stress proteins and lipid peroxides were examined. In addition to the exposed animals, a small portion of the test animal stock at experimental start served as t_0-control, to investigate the effects of time and overall handling. 16-19 samples per treatment from the diclofenac exposure were available for stress protein analyses, and 13-17 samples for examinations of lipid peroxidation. From the metoprolol experiment, 30-34 samples were used to investigate stress protein levels. Lipid peroxidation was not examined for this experiment.

WWTP exposure (described in chapter V)

For the WWTP effluent exposure, gammarids were taken from the stream and subsequently transported to the WWTP Eriskirch (described above). As a t_0-control, 100 individuals were snap frozen in liquid nitrogen before the experiment began. At the WWTP, the animals were placed in 11 glass tubes with 20 gammarids and 10 preconditioned leaves of *Alnus glutinosa* each. These were exposed in the flow-through aquarium described previously for brown trout, without previous cooling of the water. A control setup was established at the Tännichtgrundbach, with steel cages, containing the glass tubes, directly exposed in the stream. Each exposure lasted for 40 days, after which 100 randomly chosen gammarids from the WWTP exposure and 70 from the stream exposure were sampled and snap frozen in liquid nitrogen. The differing sample sizes were due to higher mortality in the stream exposure. All samples stored at -20 °C until further processing and biomarker analyses.

Tests with Daphnia magna (conducted by UBA, Berlin-Marienfelde, described in chapter IV)

The water flea *Daphnia magna* (Straus, 1820) is one of the most well-known species of the crustacean order Cladocera. They inhabit small surface waters, like ponds and puddles, in high abundance. Females reach lengths up to 6 mm, males usually only around 2 mm (Bellmann 1991). *Daphnia magna* is an important part of zooplankton and a substantial food source for larger aquatic animals. As primary consumers, they feed on algae, detritus and bacteria filtered from the water. Their carapax opens to the ventral side, covering the legs, which are used for filtration. For locomotion, the species relies on its branched secondary antennae. *Daphnia magna* is one of the most commonly tested species in regulatory ecotoxicology. This is mainly due to the easy handling in the lab and fast, usually parthenogenetic reproduction. When environmental conditions are unfavourable, females produce male offspring and populations switch to sexual reproduction. After copulation, females produce ephippia, which can survive for several years. At controlled conditions, a culture can be maintained in parthenogenetic reproduction over many generations. In this case, the culture consists solely of female

animals, and offspring are largely genetically identical to their mother animal. As a side benefit, this reduces the effect of genetic confounding factors in experimental setups (Fomin *et al.* 2003). Experiments followed OECD guideline 211 (OECD 2012), and were performed with diclofenac and metoprolol over 21 days. Test concentrations were 1.9, 6.25, 12.5, 25 and 50 mg/L diclofenac and 0.1, 0.5, 2.5, 10 and 25 mg/L metoprolol. Besides survival and reproduction, which were evaluated by the project partners, I examined the levels of the stress protein Hsp70 more closely in Chapter IV.

Tests with the endobenthic oligochaete Lumbriculus variegatus (conducted by University of Frankfurt am Main, described in chapter IV)

Lumbriculus variegatus (Müller, 1774) is a sediment-dwelling oligochaete with wide distribution area. It reaches lengths of 40 to 90 mm and widths of 1.5 mm, and is coloured red or brown. It can reproduce sexually, but also asexually through splitting (morphallaxis). Usually, it populates the ground area of surface waters, where it feeds on substrates in the sediment (Grabow 2000). Hence, the examination of this organism allowed a closer look on the effects of diclofenac and metoprolol in an endobenthic organism. Through sediment dwelling and sediment-ingestion, the animal is in constant, internal and external contact with substances bound to sediment particles. The experiments in *Eff-Pharm* followed OECD guideline 225 (OECD 2007), and exposed the organism to diclofenac- and metoprolol-spiked sediment over 28 days. Apical endpoints in the original tests were only survival and reproduction. Additionally, another test was carried out per substance, with a modified range of test concentrations (0.039, 0.078, 0.156, 0.312, 0.62, 1.25, 2.5, 5 and 10 mg/kg) and the intention to provide samples for my analyses of lipid peroxides and stress proteins in Chapter IV.

Tests with the mudsnail Potamopyrgus antipodarum (conducted by University of Frankfurt am Main, described in chapter IV)

The freshwater mudsnail *Potamopyrgus antipodarum* (J.E. Gray, 1843) originates from New Zealand, but has spread to Europe in the mid-19th century. It lives in freshwater as well as brackish water, and has become one of the most abundant gastropods in European surface waters. Their cone-shaped shells are coloured red or brown, and reach a height of 4 to 6 mm (Grabow 2000). It dwells in and on sediments of stagnant and slow-flowing water, where it feeds on detritus, algae and biofilms (Fomin *et al.* 2003). Recently, it became a popular model species to assess toxicant effects in molluscs. The species has the ability to reproduce parthenogenetically, can be kept easily at lab-conditions and has a fast reproduction cycle, making it an ideal testing organism to assess reproductive toxicity. Experiments were performed according to OECD guideline 242 (OECD 2016), over 28 days with metoprolol as testing item. Nominal test concentrations were 0.1, 0.32, 1, 3.2 and 10 mg/L. In addition to an untreated negative control, a positive control with 25 ng/L 17α-ethinylestradiol and a corresponding solvent control with 10 µg/L DMSO were included in the experimental setup. Solvent was only present in the solvent and positive control, not in the negative control or the metoprolol treatments. Standard endpoints were mortality and reproduction, evaluated by the project partner, but I extended the investigation by analysing stress protein levels in Chapter IV.

P. antipodarum was only exposed to metoprolol, not diclofenac.

Mesocosms: Artificial indoor streams (AIS) (conducted by GWT-TUD GmbH, Dresden, described in chapter IV)

To take one step further towards a more realistic exposure scenario, gammarids, oligochaetes and gastropods were exposed in artificial indoor streams. These flow-through mesocosms, placed in a greenhouse at the TU Dresden, simulate the conditions of small stream ecosystems. The purpose was to examine the combined effect of metoprolol exposure and natural stressors, i.e. stream velocity.

In each AIS, the test medium had a different concentration of metoprolol (nominal: 0, 0.47, 1.9, 7.5 and 30 mg/L – measured: 0, 0.22, 0.74, 2.95, 21.95 mg/L). Water temperature was 15 °C and current velocity 0.15 m/s. Gravel and stones of various size classes provided a typical environment for the test species, but organisms were also kept in enclosed, water-permeable vessels within the stream. The system ran for 16 days before the experiment started, which subsequently lasted for 40 d. The pre-application period was necessary to establish microbial and algae growth ("aufwuchs") in the stream as food source for the exposed invertebrates. Preconditioned alder leaves (*Alnus glutinosa)* served as another food source. In each stream, 165 *G. fossarum*, 50 individuals of *P. antipodarum* and 200 *L. variegatus* were exposed without exposure vessels and therefore move freely. Furthermore, six vessels with 10 *L. variegatus* each, six vessels with 20 *P. antipodarum* each and three vessels with 20 *G. fossarum* each were exposed in each indoor stream. After 28 d, half of the exposure vessels containing *Lumbriculus* and *Potamopyrgus* were sampled (corresponding to the duration of the normal OECD tests). All other organisms were sampled when the experiment was terminated after 40 d.

Biomarker analyses

Stress proteins

Stress proteins, or "heat shock proteins" (Hsps) are a highly conserved group of proteins that are found in vertebrates as well as in invertebrates and bacteria (Sanders 1993). They are a diverse group of cytoplasmic proteins, which act as chaperones - with vital functions in the correct folding, assembly and transmembrane passage of proteins, as well as signal transduction processes (Köhler 2009). Other than their original name "heat shock proteins" would suggest, stress proteins may not only be induced by heat, but by a wide range of stressors. Furthermore, the presence of these proteins alone is no sign of stress, since many of them are present at normal conditions to maintain cellular processes. Stress protein concentrations are modulated on the one side by internal factors, e.g. during developmental and ontogenetic processes, but also by external stressors. In general, their synthesis is up-regulated as response to proteotoxicity (Sanders 1993). According to their molecular weight, Hsps are divided into four major families: Hsp90, Hsp70, Hsp60 and low molecular Hsps (Sanders 1993). The most highly conserved and largest of those families is Hsp70. Molecular similarity between Hsp70 in different phyla is very high (Sanders 1993). The production of new Hsp70 is directly dependent on the intracellular concentration of misfolded proteins. Under physiological/unstressed conditions, Hsp70 is bound to the protein Hsf (heat shock factor). Misfolded peptides competitively replace Hsf in binding to Hsp70, leading to the release of free Hsf. Trimerized Hsf binds to the *hse* (heat shock element) DNA sequence, setting further transcription of Hsp70 genes, and finally synthesis of Hsp70-protein in motion. If proteotoxic stress is increasing, so do the concentrations of Hsp70 to counteract protein damage (Morimoto 1993). Hence, stress proteins can be used as a biomarker of proteotoxic stress (Lewis *et al.* 1999).Various phyla, including fish (Iwama *et al.* 1998) and aquatic invertebrates (Sanders 1993) increase their stress protein synthesis in response to temperature changes, but also chemicals and other environmental stressors. However, very high stress intensity also damages the Hsp70-system itself, or depletes the energy resources necessary for their synthesis. Consequently, the reaction of intracellular levels of Hsp70 to increasing stress intensity follows an optimum curve. After an increase at medium stress intensities, the internal concentrations decrease again after the system is overwhelmed by high stress. Therefore, analytics of Hsp70 should preferably be used in combination with structural biomarkers (Köhler *et al.* 2001).

For the analyses presented in this thesis, the levels of the protein itself were quantified via SDS-PAGE, coupled to a quantitative immunostaining. Such immunoassays are widely applicable because of the broad crossreactivity of Hsp70 antibodies, owing to the high degree of similarity between different phyla (Sanders 1993, Lewis *et al.* 1999).

Method

Depending on the investigated organism, stress protein analyses were performed on different tissue types. For *Salmo trutta* f. *fario*, I chose to use liver samples. In the case of *Gammarus fossarum* single individuals were used *in toto*. Because single individuals were too small, pools of two individuals were used for *Daphnia magna*, three for *Lumbriculus variegatus* and four to six for *Potamopyrgus antipodarum*. Up to processing, all samples remained stored at -80 °C.

Frozen samples were mechanically homogenized on ice in extraction medium (consisting of 98 % concentrated extraction buffer (see appendix) and 2 % protease inhibitor; for *L. variegatus*, this ratio was changed to 96 % extraction buffer and 4 % protease inhibitor). For trout liver, the added volume of extraction medium was adjusted to the sample mass (dilution: 1:7). Volumes of extraction medium were 50 µL for *G. fossarum*, 60-90 µL for *P. antipodarum* (depending on the pool size), 40 µL for *L. variegatus* and 40-60 µL for *D. magna*. Homogenized samples were centrifuged at 4 °C and 20000 rcf for 10 min (Eppendorf 5424R). 5 µL of the supernatant were diluted with 245 µL of 1:10 extraction buffer for protein quantification. The remaining supernatant (maximal 60 µL) was diluted with SDS buffer (see appendix) in a proportion of two parts supernatant to one part SDS buffer, and boiled at 97 °C for 5 min. Both mixtures were stored at -20 °C until further usage.

The protein content was quantified according to Bradford (1976). The tests were performed in 96 well plates. A dilution series of bovine serum albumin (BSA) in 1:10 extraction buffer (0.4 mg/mL, 0.2 mg/mL, 0.1 mg/mL, 0.05 mg/mL, 0.025 mg/mL plus a blank) was used to gain a calibration line. Each sample was tested in triplicates. 25 µL of the samples´ supernatant mixture were mixed with 250 µL of Bradford mixture (see appendix) and the extinction at 595 nm was measured using an automated microplate reader (Bio-Tek Instruments, Winooski VT, USA).

For the electrophoresis, polyacrylamide minigels (12 % acrylamide, 0.12 % bisacrylamide) were loaded with the sample/SDS mixtures. Constant protein amounts (40 µg per sample for *S. trutta* f. *fario*, *G. fossarum*, *P. antipodarum* and *D. magna*, 60 µg per sample for *L. variegatus*) were applied to the gels, following the results of the Bradford analysis. To allow for a comparison between different gels, a standard (*S. trutta* f. *fario* full body homogenate) in duplicate was added to every gel. Gel chambers were placed in E-buffer + SDS (see appendix). Gels were run for 15 min at a voltage of 80 V, followed by 1 h at 120 V to separate the proteins. The proteins were transferred from the minigel to a nitrocellulose-membrane via semi-dry Western blot. After the blotting process, the filter was blocked in blocking solution (see appendix) for 90 minutes. Subsequently, it was rinsed in TBS for 5 min and incubated in the first antibody solution containing monoclonal α-Hsp70 antibody (mouse anti-human Hsp70, Dianova, Hamburg, Germany, dilution 1:5000 in 10 % horse serum/TBS) at room temperature overnight.

Following the first antibody incubation, the filter was rinsed in TBS for 5 min and incubated in the secondary antibody solution (goat anti-mouse IgG conjugated to peroxidase, Jackson Immunoresearch, West Grove, PA, dilution 1:1000 in 10 % horse serum/TBS) for 2 hours at room temperature. Then, the filter was rinsed in TBS for 5 min and transferred into the staining solution (see appendix) until the protein bands were visibly stained. The reaction was stopped by transferring the filter into double-distilled water.

The filters were dried for one hour and the optical volume (area of bands x average grey value after background subtraction) of each protein band was quantified using Image Studio Lite (LI-COR Biosciences, Lincoln, NE). To assure comparability, each sample was related to the Hsp70 standard.

Lipid peroxides

Oxidative stress is among the most common influences aerobic organisms have to cope with. It is induced by reactive oxygen species (ROS), like superoxide anions, hydrogen peroxide or hydroxyl radicals, which are common by-products of oxidative metabolism. Under physiological/unstressed conditions, the generation and clearance of these ROS is in a steady state. If this balance is disrupted, oxidative stress arises (Lushchak 2011). This process can, for instance, invoke chain reactions with poly-unsaturated fatty acids, generating lipid peroxides. Such alterations pose a constant threat for the functionality of biological membranes (Valavanidis *et al.* 2006). Besides internal metabolic processes, oxidative stress can be induced by external factors, like temperature, light or chemicals. Without effective countermeasures, the oxidation of proteins, lipids and nucleic acids would severely reduce an organism's viability. Usually, these countermeasures include enzymatic and non-enzymatic antioxidant defence systems, which counterbalance the effects of ROS. Non-enzymatic defence is provided e.g. by radical scavengers or oxygen quenchers. Common molecules are e.g. α-tocopherole, β-carotene, ascorbate or flavonoids. Enzymatic defence is given primarily by superoxide-dismutase (SOD), catalase (CAT) and glutathione-peroxidase (GSH-Px), supported by a range of ancillary enzymes. Consequently, the activity of these enzymes increases when an organism is exposed to oxidative stress. Additional to these defence systems, which catch free ROS and render them harmless, cells feature various other mechanisms to prevent the formation of ROS or repair inflicted damages (Sies 1993, Sies 1997).

Regardless, situations may arise where the antioxidant system is overwhelmed, leading to an imbalance of production and reduction of ROS. In these situations, products of reactive processes, like lipid peroxides or carbonylated proteins may be present in greater amounts and compromise cellular function (Valavanidis *et al.* 2006). Still, it must not be forgotten that oxidative products can also play key roles in vital cellular functions. Phagocytes release oxidative and lytic enzymes, as well as ROS as a measure of host defence against pathogens (Sies 1993, Bonga 1997). Furthermore, the production of prostaglandins is dependent on the peroxidation of arachidonic acid by cyclooxygenases, the enzymatic target of NSAIDs. It is possible that certain pharmaceuticals, like diclofenac, affect activity of antioxidative enzymes or levels of oxidative products in a negative or positive way (McRae *et al.* 2018).

There are various ways to analyse oxidative stress. For instance, one can measure the activity of anti-oxidative enzymes (Sies 1993, Sies 1997). But it is also possible to directly quantify the product of oxidative processes, like carbonylated proteins or lipid peroxides. For my studies, I chose to quantify lipid peroxides, because I wanted to focus on one of the final products of oxidative stress. While there are also other methods available, like the malondialdehyde (MDA) assay or measurement of thiobarbituric acid reactive substances (TBARS), I chose the ferrous oxidation xylenol-orange (FOX) assay, as it is a simple and reliable method (Hermes-Lima *et al.* 1995, Monserrat *et al.* 2003). The method is based on the oxidation of bivalent iron (Fe(II)) to trivalent iron (Fe(III)) via peroxides present in the sample. At acidic conditions, trivalent iron forms a complex with xylenol-orange, shifting its absorbance peak from 440 nm to 580 nm. Consequently, adding the sample to a reaction mixture of $FeSO_4$, H_2SO_4 and xylenol-orange allows quantifying the amount of lipid peroxides via spectrometric measurement.

Method

The FOX assay procedure was conducted according to a modified version of the protocol proposed by Hermes-Lima *et al.* (1995). Chosen samples were trout heads (incl. cranium, brain, gills and jaw) from the first diclofenac exposure and trout kidney from the second diclofenac exposure. Trout larvae and gammarids were used *in toto*, and for testing of *Lumbriculus variegatus,* pooling of three individuals was necessary. Samples were stored at -80 °C until further processing. The samples were weighed,

diluted in a ratio of 1:3 (trout heads), 1:7 (trout kidney), 1:2 (trout larvae) or 1:10 (*Gammarus fossarum*, *Lumbriculus variegatus*) with cooled HPLC-grade methanol and mechanically homogenized on ice. Subsequently, the homogenized samples were centrifuged at 4 °C at 15000 rcf for 5 min. The supernatants were stored at -80 °C until further usage.

The assay was performed in 96 well plates. Each well was filled with 50 µL of 0.75 mM $FeSO_4$-solution, 50 µL of 75 mM sulfuric acid, 50 µL of 0.3 mM Xylenol Orange solution, 20 µL of sample supernatant and 30 µL of double-distilled water (trout head, trout larvae, gammarids, oligochaetes) or 15 µL of supernatant and 35 µL of water (trout kidney). Each sample was tested in triplicates, and a sample blank, in which the $FeSO_4$ solution was substituted with water. All data were related to a master blank, which consisted of 200 µL of double-distilled water. The samples were incubated for 150 min (trout head, *G. fossarum*) / 135 min (trout kidney) / 300 min (trout larvae) / 48 h (*L. variegatus*) at room temperature. The absorbance at 580 nm was measured using an automated microplate reader (Bio-Tek Instruments, Winooski VT, USA). After the first measurement, 1 µL of 1 mM cumene hydroperoxide solution was added to each well. After an incubation period of 30 min (trout) / 60 min (gammarids, oligochaetes), the absorbance at 580 nm was measured again.

All measurements were automatically set in relation to the master blank value. The value obtained for the sample blank was subtracted from the sample values. Cumene hydroperoxide equivalents (CHPequiv./mg wet weight) were calculated using the following equation:

$$\frac{\text{CHPequivalents}}{\text{mg wet weight}} = \frac{\text{A580nm}}{\text{A580nmCHP}} * \text{volumeCHP} * \frac{\text{total volume}}{\text{sample volume}} * \text{dilution factor}$$

volumeCHP = 1 µL; total volume = 200 µL

Histological analyses

In addition to biochemical markers, I also studied histological alterations of liver, gills, kidney, and heart as structural biomarkers. Histological analyses allow the closer examination of organ health and integrity. As mentioned before, biochemical markers, like Hsp70, underlie an optimum response kinetic and require further information to interpret results. This information can be given by histological analyses. The severity of histological continuously increases with stress intensity. Hence, a comparison of Hsp70-levels with histopathological effects allows interpreting whether low Hsp70-levels are due to low stress intensity or to overwhelming of the defence system by high stress. Especially for fish, histopathological alterations are highly useful biomarkers because sufficient comparative data are available (e.g. Schwaiger 2001, Triebskorn *et al.* 2001, Schwaiger *et al.* 1997, Gernhöfer *et al.* 2001, Zimmerli *et al.* 2007, Wenger *et al.* 2010, Steinbach *et al.* 2014a, Wolf *et al.* 2015).

For my analyses, I examined three primary monitoring organs: gills, liver and kidney – all of high importance in the uptake, metabolism and excretion of substances. Furthermore, I investigated the heart, because of the specific action of beta-blockers on the cardiovascular system.

Gills

The gill is not only the major respiratory organ in fish, but also takes part in osmoregulation and excretion of catabolites. Teleosts have five pairs of gill arches on each body side, of which only the anterior four take part in respiration. From each gill arch protrudes a multitude of primary filaments, lined by numerous secondary lamellae on both sides. Primary filaments consist of cartilaginous support, vascular system and multi-layered epithelium. A double layer of epithelial cells builds the secondary lamellae: supporting pillar cells and pavement cells are arranged in a ladder-shaped order to form capillary channels. This filigree structure allows blood flow in close proximity to the surrounding water, which is necessary for the efficient exchange of oxygen and carbon dioxide. As the

blood flow direction is opposite to the direction of water flow in the gill, gas exchange ability is highly efficient. Further cell types frequently found in the epithelium are mucous cells, located at the edges of the primary filament, and chloride cells, on the basement of the secondary lamellae (Blüm *et al.* 1989, Takashima and Hibiya 1995).

Since the gill is in constant, close contact to the surrounding medium, it reacts fast and sensitively to environmental changes and disturbances. The first reactions to chemical or physical stressors are usually hyperthropy or hyperplasia of epithelial cells. Since the chloride cells are involved in osmoregulation and excretion, hyperplasia of these cells is a sign of osmotic or chemical stress. Other commonly found pathologies include oedema, vacuolation and inflammations as defensive response, but also necrotic changes and haemorrhage in the case of serious injuries. When the pillar cell system disintegrates, the capillary lumen expands and fills with blood cells – a symptom called aneurysm (telangiectasia). In any case, histological samples have to be interpreted with care, as many of the described symptoms can also be evoked by handling of the fish and preparation of the sample (Takashima and Hibiya 1995).

Liver

The liver is a major metabolic gland, with important function in alimentary, as well as detoxification processes. Parenchymal hepatocytes cells surround sinusoids or other blood vessels – giving the tissue its typical histological appearance. Bile canaliculi collect the bile secreted by the hepatocytes and direct it towards the bile duct. Hepatocytes contain a nucleus with a prominent nucleolus and variable amounts of heterochromatin. Furthermore, the cells usually contain large amounts of endoplasmic reticulum, due to their high activity in protein synthesis. As the liver stores energy reserves in the form of glycogen inside the hepatocytes, this energy reserve usually takes up a large portion of the cell. Other storage materials are lipids, stored in small to medium-sized droplets dispersed in the cytoplasm. A look at these storage materials allows closer insights into the nutritional status and stress of the animal, because energy has to be invested for e.g. detoxification. Glycogen can easily be stained via Periodic Acid Schiff (Blüm *et al.* 1989, Takashima and Hibiya 1995).

Common pathologies observed in hepatic tissue include hypertrophy or atrophy of hepatocytes, dilation of intercellular spaces and blood spaces due to shrinkage of the hepatocytes, loss of storage materials, and nuclear pycnosis. Another frequent observation is inflammation and increased abundance of macrophages. In the case of strong disturbances, the tissue may become necrotic and lose integrity (Takashima and Hibiya 1995).

Kidney

The major function of the kidney are excretion, osmoregulation and haematopoiesis. It is divided into head kidney, deriving from the pronephros and mainly of hematopoietic function, and the trunk kidney, deriving from the mesonephros. The functional units within the trunk kidney are nephrons, as in all vertebrates. Primary filtration takes place at the "*Corpusculum renis*", which consists of the "glomerulus", surrounded by the "Bowman's capsule". Primary urine is transported through the proximal and distal parts of the renal tubuli, where proteins and electrolytes are resorbed. The resorption of these substances is especially important in freshwater fish, which have to excrete large amounts of water entering the body via the gills. Eventually, the urine is excreted via the collecting duct and ureter. The glomerular filer consists of [1] the flat endothelial cell forming the capillaries, [2] the basal lamina underlying these cells, and [3] large amounts of podocytes, which are also connected to the basal lamina from the opposite site. The glomerular capsules consist of a fibrous outer layer of connective tissue and an inner epithelium, continuing into the renal tubular epithelium. Tubuli are formed by tubulus cells surrounding the lumen, surrounded by a layer of smooth musculature. The cells of the proximal tubulus are prismatic and characterized by a prominent "brush border" of

microvilli and high abundance of apical vacuoles. In contrast, distal tubular cells are columnar, clear and without clearly recognizable brush border. All nephron structures are embedded in interstitial hematopoietic tissue (Blüm *et al.* 1989, Takashima and Hibiya 1995).

Common pathologies may manifest in alterations of the glomerulus and surrounding structures, like hyperplasia or inflammation. Blood cells, cell debris, and foreign matter can be found in the Bowman's capsule, when the filtration structures are damaged. Typical changes in the renal tubuli are hypertrophy or atrophy of cells, or accumulations of proteinaceous matter in the tubular cells – so-called hyaline droplets. The effect is referred to as "hyaline droplet degeneration" (Schwaiger *et al.* 2004). Severe cases of this pathology may eventually lead to necrosis of tubular cells. Anomalies are often accompanied by dilated tubular lumen (Takashima and Hibiya 1995).

Heart

The heart is the central element of the teleost vascular system. From the heart, low-oxygen blood is pumped into the capillary network of the gill. After passing the gills, the oxygen-enriched blood flows directly towards the organs. After supplying the organs with oxygen, the blood returns to the heart. Here, the blood enters into the *sinus venosus* and passes through the atrium and ventricle before leaving the heart again through the *bulbus arteriosus*. All parts of the heart consist of three elements: first, an internal membrane, the endocardium, composed of endothelial cells and connective tissue. Second, an intermediate layer – made of muscle in atrium and ventricle, but connective tissue in the *sinus venosus* and *bulbus arteriosus*. Third, an external membrane, the epicardium, which consists of epithelial cells and connective tissue. The wall of the ventricle is thick and rich in muscle, while the wall of the atrium is thin and does only contain few muscle fibres. Cardiac muscle appears special in such way that its fibres consists of one cell body with only one central nucleus and the fibres bifurcate to form am complex, mesh-like structure (Blüm *et al.* 1989, Takashima and Hibiya 1995).

Pathological changes of the heart include necrosis of heart muscle cells, but also inflammations of cardiac muscle (myocarditis), endocardium or epicardium – characterized by accumulations of leucocytes (Takashima and Hibiya 1995).

Method

Histological examinations were done for samples of trout gill, kidney, liver, and – only for metoprolol-exposed animals - heart. The tissue samples were fixed in 2 % glutardialdehyde (dissolved in 0.1 M cacodylate buffer, pH 7.6) and stored at 4 °C until further processing. Since the scope of the study included only an exemplary overview on the histological effects, three samples per block and treatment were investigated, resulting in nine samples per concentration.

Prior to embedding, the tissue samples were washed three times for ten minutes in 0.1 M cacodylate buffer, followed by three times ten minute washing steps in 70 % ethanol. For gills and kidney two additional steps for decalcification in a 1:2 mixture of concentrated formic acid and 70 % ethanol was added between these steps (1. step 30 min; 2. step 24 h). Further dehydration and embedding in paraffin took place in an automated tissue infiltrator (TP 1020, Leica, Wetzlar).

Histological sections were cut with a sledge microtome (SM 2000 R, Leica, Wetzlar) set to a thickness of 3 µm. One part of the slices was stained with haematoxylin-eosin-staining (to visualize nuclei, cytoplasm, connective tissue and muscles), the other part with alcian blue-PAS-staining (to visualize mucus, glycogen and the fine structures of renal tubuli).

In a first evaluation step, slides were examined qualitatively to gain an overview and identify occurring pathologies. The second step was a semi-quantitative assessment performed in an observer-blinded way. The two slides per sample (AB-PAS/HE) were paired, the inscriptions masked, mixed and a random number was assigned to each slide pair. Each sample was classified into one of five different categories

(1: control, 2: slight reaction, 3: medium reaction, 4: strong reaction, 5: destruction) according to the criteria published by Triebskorn *et al.* 2008.

Chemical analyses (conducted by TZW)

To assure that fish were actually exposed to the indicated pharmaceutical concentrations, test media of all experiments were sampled regularly. At water sample of each single replicate and a mixture sample of all replicates belonging to the same treatment group was taken at the start of the experiment, before and after the first medium change, and at the end of the experiment. The immediately frozen samples remained stored at -20 °C until further analyses.

As resources for chemical analyses were limited, we did not analyse all samples. In all cases, the mixture samples from test start were analysed. If irregularities or strong deviations from nominal concentrations were found, the single samples were checked. Resources were allocated to the experiments, where strong effects were visible. Hence, we analysed the water samples from test end and biota samples for the second diclofenac experiment [experiment 6].

The project partner TZW carried out the actual analyses. If necessary, aqueous samples were enriched by solid-phase extraction (SPE). Diclofenac and metoprolol were analysed by high performance liquid chromatography (HPLC, 1290 series, Agilent, Waldbronn, Germany) coupled to tandem mass spectrometry (MS/MS, API 5500, AB Sciex, Foster City, USA) by multiple reaction monitoring in positive mode by electrospray ionization. For quantitative analyses, the isotopically labelled standard diclofenac-d4 was used for the correction of signal enhancement or suppression caused by matrix components.

Biota samples were freeze-dried, pooled (to achieve the required amount of sample) and homogenized. Turtle food (Engergil, JBL GmbH, Neuhofen, Germany) containing complete freeze-dried fish and crustaceans was used as co-extracted matrix. Samples were extracted in an ultrasonic bath, centrifuged and the supernatant blown down to dryness. The dry residue was reconstituted and injected into the LC-MS/MS system. A matrix matched calibration with turtle food was prepared for quantification. For the analysis of biota, samples were treated with the same analytical instrumentation as described for water samples. All samples were spiked with the isotopically labelled internal standard diclofenac-d4 for the correction of matrix effects.

The detailed procedures are described in the final *Eff-Pharm* report (Triebskorn *et al.* 2017).

Statistical evaluation

Statistical analyses were carried out using R 3.2.1 (packages: lme4, lmerTest) and SAS JMP 11. For parametric tests, data were checked for normal distribution visually via quantile-quantile-plots and histograms; homogeneity of variance was tested with either Levene's or Fligner-Killeen-Test.

For most organisms/endpoints, a lowest observed effect concentration (LOEC) and no observed effect concentration (NOEC) were derived, if possible. The LOEC is defined as the lowest tested concentration, at which a statistically significant difference to the control is visible. The NOEC is the highest tested concentration without statistically significant difference to the control.

Experiments with brown trout

Fish mortality data were analysed using COX-regression, with experimental block as nesting factor. Juvenile length and body mass were analysed with nested ANOVAs, stress protein and lipid peroxide levels with linear models, including experimental block as cofactor. In the case of lipid peroxides in [experiment 6], data had to be log10-transformed and sample mass was used as additional covariate. The occurrence of bite injuries was analysed via generalized linear mixed model (binomial distribution, aquarium identity as random factor). The general significance level was set to $\alpha=0.05$, but was adjusted

via sequential Bonferroni when multiple comparisons had to be made. In cases where no differences between groups were visible, I went without mathematical testing. Since all histological analyses were exemplary, and not carried out for all individuals, I stuck to descriptive statistics for those parameters. All results from hatchery controls were only used as qualitative references and omitted from mathematical analyses.

Experiments with invertebrates

If necessary, data were transformed to fit the assumptions of parametric testing. The respective transformations are given in the results section. For the single substance treatments, mean body mass, stress protein and lipid peroxidation levels were compared with either one-way ANOVA, when homogeneity of variance was given, or Welch-ANOVA for unequal variances. If a significant overall difference was found, Tukey-Kramer post hoc tests were used to identify differences between single treatments. In the WWTP and AIS exposure, treatments were compared using linear models. The general significance level was set to $\alpha=0.05$.

Pseudoreplication

An obvious problem with the investigated mesocosm system and the WWTP-exposure is pseudoreplication (Hurlbert 1984). The project did not offer the resources to test multiple replicates of each complex treatment. Nevertheless, we decided that these experiments under more realistic conditions were useful and should be conducted despite the experimental limitations.

For the mesocosm experiment, only five artificial indoor streams were available, which did not allow real replication of the treatments. One measure to ameliorate this problem was to expose the organisms in several separate enclosures inside each stream. This may prevent initially small-scale events from affecting the whole system, but the general pseudoreplication problem could not be solved. We chose to conduct the experiment, and interpret the results with care and based on biological plausibility. First, we had the results from the single-substance exposure in the laboratory as reference to interpret the results. Second, the testing of an ascending range of concentrations allowed identifying potential concentration-response-relationships. Especially in cases, where no clear concentration-response is visible, the results may be influenced by factors other than the treatment and have to be treated with utmost care. However, if a clear relationship is visible, this is a strong sign of biological plausibility. Third, the results obtained for different organisms and different endpoints in the same system can support each other. If several metrics point to the same direction, this is again a sign of biological plausibility.

In the semi-field exposure, I had only one aquarium at the WWTP and one at the control station. Organisms were exposed in separate compartments within the aquarium to prevent small disturbances from affecting all test organisms, but the treatment remained without real replication. Even if there had been several different aquaria at each station, pseudreplication would have persisted because the aquaria would have shared the same influent. My conclusions drawn from this experiment are therefore of qualitative, andecdotal nature. To cope with this problem, at least to a certain extent, I initiated the laboratory experiment with WWTP effluent samples. This allowed a higher degree of control over experimental conditions and better possibilities for replication, but simultaneously reduced the degree of realism. The conclusions from both experiments are restricted to the investigated WWTP in Eriskirch and cannot be generalised to other WWTPs. Interpretation in a broader context will only be meaningful in combination with results from other studies on WWTP effluent effects.

8. Results and Discussion

Chapter I: Literature study

Report: Triebskorn *et al.* 2014 - From theory to reality – Evaluation of suitable organisms and test systems for the biomonitoring of pharmaceuticals. Part I: Literature review

Aims

The first chapter is based on part 1 of *Eff-Pharm*, which consisted of a comprehensive literature review, split into two parts A and B. Part A identified suitable test substances and organisms for further *in vivo* investigations. Part B collated the basic background and possible methods for the creation of *in vitro* test systems. As the focus of the thesis rests on the *in vivo* examinations, this chapter refers to part A of the literature review only.

In a previous project funded by the UBA, Bergmann *et al.* (2011) collected monitoring and effect data on pharmaceuticals from published literature, which served as a basis for our own study. Studies on pharmaceutical effects, published between 2011 and 2013, were collected and evaluated. In combination with our own results, this evaluation had the following goals:

1) Identification of suitable substance groups for further testing.
2) Overview on the amount of published data for specific pharmaceutical and organism groups.
3) Identification of sensitive test organisms and biota groups, where information is lacking.
4) Compilation of lowest observed effect concentrations (LOECs) for priority pharmaceuticals.
5) Assessment of mixture toxicity effects.
6) Assessment of population relevance of the extracted effect endpoints.
7) Assessing reliability/credibility of studies reporting low effect concentrations.

Results

The initial screening of literature databases yielded 452 publications, which were analysed more closely. 232 were of direct relevance by providing effect data on one or several of the 90 pharmaceuticals of interest. 95 publications provided additional information on the overall topic.

In style of the original database created by Bergmann *et al.* (2011), which was named OEKOTOX, we called the newly generated database $OEKOTOX_{upgrade}$. It contained 1678 entries, extracted from the 232 relevant publications. The much higher number of entries is explained by the fact that many publications contained information on multiple pharmaceuticals, species, or effect endpoints.

The most frequently addressed drug classes were antibiotics, non-steroidal anti-inflammatory drugs (NSAIDs), anticonvulsants, antiparasitics, beta-blockers, and contraceptives. The by far most frequently investigated organism group was fish (based on the number of publications). It was followed by molluscs, plants/algae, crustaceans, and bacteria – which were all investigated in roughly the same number of studies. Only a small number of studies investigated protists, tetrapods, insects, or whole communities. Although we found studies on 144 different species/community types, only 49 species were investigated in more than one study. Only *Cyprinus carpio, Danio rerio, Oncorhynchus mykiss, Oryzias latipes, Pimephales promelas, Pseudokirchneriella subcapitata, Daphnia magna, Dreissena polymorpha,* and *Mytilus galloprovincialis* were examined in more than five different studies.

In the evaluation of relevant pharmaceutical classes, organism groups, and effect endpoints, the results have to be interpreted with care. Doubtless, the data are biased. As mentioned before, certain substance classes and organism groups are investigated more frequently than others. Therefore, there is a higher probability to find relevant effect data for these classes and groups. A first screening of the lowest LOECs extracted from the studies identified protozoans, represented by only the species *Tetrahymena pyriformis*, as most sensitive organism group. The species, investigated by the endpoint

chemotaxis behaviour, showed extremely low effect concentrations for eight substance classes. However, all data originated from a single publication, which was also judged as being not sufficiently reliable. Other sensitive organism groups were molluscs and fish. Other sensitive effect endpoints were behavioural alterations, vitellogenin induction, growth, reproduction, histopathological changes, molecular stress biomarkers, oxidative stress biomarkers, receptor binding and gene expression changes.

The actual assessment of relevance compared the obtained LOECs with measured environmental concentrations (MECs), provided by the UBA. This deviates from regular pharmaceutical risk assessment, where predicted no-effect concentrations (PNEC - calculated by dividing a NOEC by an appropriate assessment factor) are compared to predicted environmental concentrations (PEC). Consequently, the quality of the resulting MEC/LOEC quotient cannot be directly compared with a usual PEC/PNEC risk quotient. While a PEC/PNEC-quotient higher than one indicates a risk for the environment – with a high margin of safety, a MEC/LOEC-quotient higher than one indicates that environmental effects are to be expected. We considered substances with a MEC/LOEC-quotient higher than 0.1 as highly relevant, which would include a small safety factor of 10. A variety of substances showed such high quotients for several organismic groups. Among them, NSAIDs like diclofenac and ibuprofen, the analgesic paracetamol, and antibiotics like sulfamethoxazole, erythromycin, sulfadimidine and oxytetracycline were particularly noticeable. But also beta-blockers like propranolol and metoprolol, or sex hormones like 17β-estradiol and 17α-ethinylestradiol.

The assessment of reliability proved to be an essential part of the analysis. 72 publications, all reporting low effect concentrations, were examined more closely. Only nine of them were considered as reliable, while 49 were conditionally reliable and 14 were not reliable. Many studies suffered from the lack of analytic-chemical verification of test concentrations, flaws in experimental design, or improper reporting of essential study details, and lack of concentration-dependence. When excluding unreliable studies, the protozoan *Tetrahymena pyriformis* was no longer the most sensitive organism tested. Instead, the three fish species *Danio rerio*, *Oryzias latipes,* and *Oncorhynchus mykiss* remained the organisms with most sensitive reactions to pharmaceuticals. Those three were also among the most frequently investigated organisms overall. Concerning invertebrates, the mussel *Ellliptio complanata* and the mudsnail *Potamopyrgus antipodarum* reacted sensitively in the few cases where they were examined.

The original evaluation of the most relevant pharmaceuticals was strongly influenced by the reliability analysis. A large portion of LOECs close to MECs had been derived from unreliable studies. When excluding those studies, the NSAIDs diclofenac stood out by still showing high MEC/LOEC ratios in all four organismic groups. Other pharmaceuticals with high MEC/LOEC ratios in at least one or two organismic groups were sex hormones, antibiotics and beta-blockers.

Mixture toxicity was addressed in only few of the investigated studies. The obtained results are equivocal and no mode-of-action related endpoints were examined.

106 studies examined endpoints regarded as population-relevant: mainly by examining survival or mortality, growth and reproduction, but also behavioural effects and visible community changes. Especially endocrine active substances induced effects already in low concentrations.

Discussion

Overall, the literature review revealed diverse shortcomings, above all the strong heterogeneity of data. Oftentimes, only a single dataset was available for a species, rendering comparisons difficult. While several substances are intensively studied, most others are almost completely neglected. Because of this strong bias, an objective selection of test substances is hardly possible. However, in

combination with the results of the literature study on *in vitro* systems, two substance groups were promising for the further goal of developing biosensor systems. NSAIDs and beta-blockers are pharmaceutical groups acting via specific pathways. This can be exploited in the construction of *in vitro* assays. At the same time, these pharmaceutical groups are among the ones showing the most prominent effects in published studies. As representatives of the respective groups, we proposed the substances diclofenac and metoprolol. The consumption rate of diclofenac is not as high as that of other NSAIDS, like ibuprofen or acetylsalicylic acid (IMS Health). Nevertheless, reported environmental effects on vultures are drastic, and its effect on aquatic ecosystems is controversially discussed. This has led to its inclusion in the watch list of the European Water Framework directive (EU 2013). Further information on the substance was urgently needed at the time when our literature study was finished. Metoprolol is the beta-blocker with the by far highest consumption rate (IMS Health). However, relatively little is known on its effects in aquatic biota. *In vivo* examinations should help to further substantiate our knowledge on both substances.

Although the collected studies applied a wide range of different species, only a small proportion of them contributed more than a single effect value for a single substance. This renders the identification of sensitive species, based on the current literature, hardly possible. In fish, most studies applied established model species. Due to the sheer amount of studies on these species, there are several cases where they react with high sensitivity. Therefore, it was inherently more likely to find sensitive effects for these organisms. It remains unclear if other, less established species would be more sensitive and, hence, of higher relevance for ecotoxicological testing. Furthermore, studies on ecologically relevant invertebrates, like gammarids or endobenthic organisms, were scarce. Hence, it cannot be reasonably assessed how species of our regional ecosystems react to pharmaceutical contaminations.

Moreover, only few studies investigated the same endpoint in the same test organism for different pharmaceuticals – or focused on sensitivity differences of various taxa exposed to the same pharmaceuticals. The selected endpoints were highly diverse, but rarely linked to the substance mode of action, and ecological relevance remained questionable in many cases. Similar conclusions were drawn in a review by Brausch *et al.* (2012), who also stressed out the lack of chronic data on benthic invertebrates and fish, and need for mode-of action (MoA)-based endpoints.

It was especially concerning that a large proportion of the studies reporting on sensitive results was not completely reliable. Many experiments did not verify their exposure concentrations via analytical-chemical methods, but based the results solely on nominal concentrations. However, this is an essential part in sound study design in ecotoxicology – to account for dosing errors, adsorption or substance degradation. Furthermore, several publications did not report their experimental design and results in sufficient detail, which would be the fundamental pillar of transparent science. Additional problems were low sample sizes and too few tested concentrations, which give random effects much higher influence than desired. These problems with published ecotoxicological research have also been noted in other literature reviews (e.g. Harris *et al.* 2014). Recent projects like CRED (Criteria for Reporting and Evaluating ecotoxicity Data) aim to provide guidance and support for scientists and evaluators, in order to improve transparency and reliability of published data (Moermond *et al.* 2015).

As a conclusion of our literature review, we endorsed the use of non-standard species and non-standard endpoints for further analyses – as those allow investigating effects that might otherwise be overlooked. In addition, we claimed that future studies must meet a certain quality in experimental design and reporting to be of use for the scientific community.

Chapter II: Need for effect-based monitoring

Paper: Triebskorn *et al.* 2015 - Monitoring Primary Effects of Pharmaceuticals in the Aquatic Environment with Mode of Action-Specific in Vitro Biotests

Our literature research illustrated the current heterogeneity and paucity of actual knowledge on the effects of pharmaceutical effects in the environment and useful methods for their monitoring. It is a common agreement that the systematic and global monitoring of pharmaceuticals and other substance classes is a prerequisite to evaluate the risk of chemical contamination. With few exceptions, e.g. the monitoring of steroid hormones, monitoring techniques are still based on traditional analytical chemistry identifying single substances. The gained information is without doubt very useful, but the sheer complexity of environmental contaminations would overwhelm its possibilities. The number of chemical products is large and constantly increasing. Furthermore, biotic and abiotic processes generate various different metabolites and transformation products, in addition to the parent substance. Moreover, in the environment, the substances are always present in a complex mixture that might act in addition or synergism, so the focus on single substance will most likely underestimate risks.

One possible solution to integrate and, to some extent, simplify this complexity, is to monitor substances based on their mode-of-action (MoA). Many different substances act on the same molecular target, which is ultimately responsible for the evoked effects in biota. Applicability of such MoA-based techniques in monitoring has been shown (Escher *et al.* 2014), but needs to be extended. For pharmaceuticals, such techniques only exist for steroid hormones, e.g. based on the MoA via estrogen or androgen receptors. However, especially in the field of pharmaceuticals, there are much more potential applications. A variety of different substances, belonging to the same substance class, usually acts on the same molecular drug target. The basic idea for further monitoring systems is to create *in vitro* test systems, which express the drug target as a sensor plus a reporter system to recognize the sensor's state and immediately quantify the effect via fluorescence signal changes (Oldach and Zhang 2014). This would be a great improvement compared to traditional reporter gene assays, which require considerable time for the underlying signal transduction pathway, and are prone to interference by other effects. The method could elegantly bridge the current gap between analytical chemistry and *in vivo* effects. This could not only add to the battery of monitoring techniques, but be also used for the characterization of mixture effects or the examination of wastewater treatment plant efficiency.

Of course, it will still need a wide array of different *in vitro* systems to cover the most common pharmaceutical MoAs in environmental samples. Therefore, our first step was to exemplarily prove the feasibility of such *in vitro* systems for two highly important drug targets. First, beta-adrenoceptors, which are the drug target of antihypertensive pharmaceuticals belonging to the class of beta-blockers. Second, cyclooxygenases, an enzyme class which is targeted by the class of non-steroidal anti-inflammatory drugs (NSAIDs). Our choice of these substances was based on the fact that these two substance groups have a clearly defined MoA. At the same time, effects on their molecular drug target have wide implications on the physiology of an organism, which may lead to diverse side-effects. Our literature study listed them among the groups of highest priority, because of the high consumption and likeliness of environmental effects. When the cell culture systems are finally created, they must be optimized to not only function at sterile lab conditions, but also at environmental conditions, where various other factors might be interfering. Furthermore, they need to be validated by biomarkers and population-relevant endpoints in various biota of environmental relevance: in our case, we chose fish, crustaceans and endobenthic organisms. The results of these validation efforts are presented in the following chapters III – V:

Chapter III: Experiments with brown trout

Paper: Schwarz *et al.* 2017 - Impact of the NSAID diclofenac on survival, development, behavior and health of embryonic and juvenile stages of brown trout, *Salmo trutta* f. *fario*

Report: Triebskorn *et al.* 2017 - EFF-Pharm: Effects of pharmaceuticals (nonsteroidal anti-inflammatory drugs and beta-blockers) in fish and invertebrates and their detection by newly developed *in vitro*-bioassays – Final report → WP3A

Aims

When evaluating the available literature, it became clear that only few studies are directed to investigate effects in regionally relevant species. While it makes sense to conduct studies for direct regulatory purposes with standard model species, it is the opportunity of free academic research to deviate from the standard procedures. The goals of the studies with brown trout were:

- To investigate potential adverse effects of pharmaceuticals in a species of high ecological relevance for Central European stream ecosystems. For this, I selected brown trout, which is considered as sensitive towards environmental stressors and might be less tolerant than fish model species. By comparison to literature data, conclusions on sensitivity variations of different species can be drawn – to exemplary assess if approaches with model species are protective enough for regional biocoenosis.
- To gain further knowledge on the ecotoxic effects of diclofenac and metoprolol. Both are sold since before the European pharmaceutical legislation came into action. Hence, it is doubted that a rigorous environmental risk assessment for both substances was submitted. For diclofenac, various publically available studies hint on potential hazards, but are also controversially discussed. Metoprolol is sold and consumed in high amounts, but only few studies on its effects are available. Therefore, I aimed to further improve the knowledge basis on the environmental risk of both substances.
- Apart from the overall assessment of ecotoxicity for the species itself, I also aimed to compare different life stages. For diclofenac, studies on adult individuals are available in literature. Hence, my focus lay on juveniles (in reference to their length, also called “fingerlings”) and the earliest developmental stages from freshly fertilized egg to free-feeding larvae (fry). The testing of different life stages allows identifying vulnerable stages, which are most relevant for ecotoxicity assessment.
- Apart from standard endpoints - like development time, growth, or mortality - biomarker analyses were the prime interest of the investigations. Adding two biochemical markers - stress protein and lipid peroxide levels – allowed to identify changes on the biochemical level. Since this is no standard procedure for fish testing, these results would help to conclude whether such biochemical markers are a useful extension in risk assessment. Both biomarkers can be analysed for a broad range of taxa, including fish as well as invertebrates. Hence, I could also conduct these analyses for other test organisms and compare between taxa. Additionally, I applied histological examinations, which are a widely applied method to gain further knowledge on the health condition of the test animals. Tissue and organs react before the changes are seen on the organismic level. Hence, by histological analyses, one is able to either get more sensitive results than just looking at the organismic level, or interpret the results seen on the organismic level in a more comprehensive manner. For regulatory purposes, the method is usually considered as too expensive, since it is time consuming and requires expertise to evaluate and interpret the results.

Effects of diclofenac and metoprolol in brown trout embryo and sac-fry stages

Developing brown trout were exposed to diclofenac and metoprolol from fertilised egg until consumption of the yolk sac [experiment 3]. During the exposure, pH ranged between 6.46 and 7.3 (mean: 6.8), temperature between 6.7 and 7.6 °C (mean: 7.13 °C), and oxygen saturation between 59.6 and 81.4 % (mean: 73.4 %). Measured concentrations of diclofenac and metoprolol were, partly, below the nominal concentrations. Hence, the mean measured concentrations were used for the further comparisons (Diclofenac: 0.06, 0.51, 0.75, 7.8, 74.6 µg; Metoprolol: 0.06, 1, 9.8, 70.1, 998 µg/L).

Trout larvae developed normally within the 127 day experiment. With survival rates between 94.6 and 100 %, overall mortality was very low. Embryos of all treatments reached the eyed ova stage between 29 and 33 dpf; hatching took place between 63 and 70 dpf. At 1 µg/L diclofenac, hatching rate was slightly lower than in the control – but this effect was not seen in all other diclofenac treatments. Heart rate was on an equal level for all tested treatments and ranged between 45 and 60 bpm (mean = 51.17 bpm). Body mass was slightly lower at 7.8 µg/L diclofenac, which was mainly caused by one replicate of this treatment (mean: 78.24 mg in the replicate, compared to an overall mean of 94.1 mg; overall range between 63.9 and 123.9 mg) and non-significant (linear mixed model, df=5/11.65, F=0.7623, p=0.5944). Histological analyses of kidney revealed mild reactions in several samples of all treatments (large vacuoles in tubular cells, reactions of glomeruli, increased vesicularisation in tubuli), with no visible influence of the diclofenac treatment. The test was regarded as valid, as control survival was above 70 %, oxygen saturation above 60 % (except one replicate, where it was 59.6 %) and inter-vessel temperature differences smaller 1.5 °C.

Effects of diclofenac in juvenile brown trout

Exposure conditions during the first diclofenac exposure [experiment 1] were in an acceptable range (temperature: 7.92 ± 0.4 °C, pH: 8.49 ± 0.02, oxygen: 96.1 ± 0.65 %) and the measured concentrations of diclofenac were close to the nominal values. Hence, all evaluations of the experiment were based on nominal concentrations. Mortality in the first diclofenac exposure of juveniles was high. This was especially prominent at the highest test concentration (100 µg/L). This treatment was ended ahead of schedule, already after 14 days, due to the high mortality (>60 %) of the exposed fish. At this time point, a clear difference in comparison to the other treatment was obvious (COX-regression, df=5, χ^2=43.54, p<0.0001). However, all other treatments – including the control – experienced elevated mortality rates (≈ 40 %) during the further course of the study. Even though these did not reach the rates observed for 100 µg/L, the control mortality by far exceeded 10 %, which was the validity criterion for the test. For this reason, a repetition of the experiment was deemed necessary. Body length of fish in this first experiment varied between 2.8 and 4.4 cm (mean: 3.6 cm), body mass between 0.13 and 0.79 g (mean: 0.4 g), both measured at the end of the experiment. This was considerably larger than the fish had been at the hatchery (mean length: 3.0 cm, mean weight: 0.19 g), indicating strong growth during the exposure. There were no differences in size between the control and the treatments (length: nested ANOVA, df=5, F=1.3698, p=0.2412; weight: nested ANOVA, df=5, F=1.6892, p=0.1431). Relative stress protein levels ranged between 0.15 and 2.36 (mean: 1.38), but were not affected by any treatment (linear model, df=5/103, F=0.4174, p=0.8357). Lipid peroxide levels turned out to be highly variable, with a minimum level of 0.95 and maximum of 30.84 CHPE. A significant effect in the full model (linear model, df 6/119, F=11.1, p<0.001) was mainly driven by the covariate sample mass. The level of lipid peroxides was visibly decreased at 100 µg/L (mean: 9.7 CHPE, with two samples showing very high values, in contrast to 18.0 CHPE in the other treatments), but due to the shorter exposure time, this treatment is not directly comparable to the others. The hatchery control samples taken at a time corresponding to the end of the exposure also had low levels of lipid peroxides (mean: 8.1 CHPE)

When juvenile trout were tested in the second diclofenac experiment [experiment 6], exposure conditions were in an acceptable range (temperature: 6.91 ± 0.18 °C, pH: 8.52 ± 0.04, oxygen: 95.36 ± 1.1 %) and the real diclofenac concentrations were close to the nominal concentrations. Furthermore, tissue analyses showed diclofenac to be present in fish tissue. It was not detectable at the two lowest exposures, but at mean concentrations of 10.2, 84.5 and 169.5 µg/kg dw for the three highest treatments. Effects of diclofenac on mortality were obvious. While mortality remained within 10 % for the control, it increased in a roughly concentration-dependent manner, with highest mortality rates (>40 %) at 100 and 200 µg/L (nested COX-analysis, df=5, χ^2=13.457, p=0.0194). Step-wise comparisons revealed the two highest concentrations to be significantly different to the control, resulting in a $LOEC_{mortality}$ of 100 µg/L and $NOEC_{mortality}$ of 10 µg/L. Besides elevated mortality, animals showed bite injuries on fins and opercula, attributable to intraspecific aggression. The frequency of these injuries was low in the controls, but increased with ascending diclofenac concentration. Significance was reached at 10 µg/L (GLMM, df=5, n=161, F=5.2463, p=0.0015), resulting in a $LOEC_{injuries}$ of 10 and $NOEC_{injuries}$ of 1 µg/L. Again, standard body length (4.8 - 7.5 cm, mean: 6.02 cm), body mass (1.37 – 5.59 g, mean: 2.95 g) and stress proteins (0.87 – 2.19, mean: 1.47) were not affected by diclofenac. Lipid peroxides did show a slightly significant overall effect in the full model (linear model, df=35/123, F=1.639, p=0.02581), drawn by effects of block and sample mass, but without effect of test concentration. Overall, the variance of lipid peroxide levels was very high (12.59 – 100.7 CHPE, mean: 81.38 CHPE). Fish from the hatchery controls were of comparable size at time point 1 (start of the experiment, mean length: 6.14 g, mean weight: 3.25 g) and were considerably larger at time point 2 (end of the experiment, mean length: 7.68 cm, mean weight: 7.29 g). Stress protein levels were slightly larger in animals kept in the lab than in hatchery control animals (first hatchery control: 1.33, second hatchery control: 1.24). This effect was even more pronounced for lipid peroxides, where the levels were considerably higher in lab-exposed fish (first hatchery control: 42.96 CHPE, second hatchery control: 42.75 CHPE).

When looking at the histological samples, all examined tissues showed noticeable changes. The most prominent effects were: hypertrophy of gill epithelial cells, resulting in an overall thickened appearance of the secondary lamellae. Liver tissue was often depleted of glycogen, with dilated intercellular spaces and frequent inflammative spots. Kidneys had accumulated hyaline material in tubular cells and showed frequent aggregations of melanomacrophages. In several cases, the hematopoietic tissue showed degenerations, leading to a perforated appearance of the tissue. Many symptoms could also be seen in the control, but the frequency of strong reactions was higher in diclofenac-treated fish – especially concerning the liver. The most severe alterations were seen in the two highest treatments. These effects were seen in the qualitative, as well as the semi-quantitative assessment.

Effects of metoprolol in juvenile brown trout

Exposure conditions during the experiment [experiment 2] were in the intended range (temperature: 7.82 ± 0.18 °C, pH: 8.23 ± 0.02, oxygen: 94.9 ± 0.65 %), except for the concentrations of metoprolol – which turned out to be lower than planned. Since the concentrations deviated more than 20 % from the nominal, the evaluations were based on mean measured concentrations (0.094, 0.95, 6.9, 86, 745 µg/L).

Overall mortality in the experiment was low, with no animals at all dying in the control and only occasional mortality in the metoprolol-treated groups (nested COX-analysis; df=5, χ^2=0.0319, p=1.0). Fish showed high variation in size, with standard body length varying between 3.9 and 6.7 cm (mean = 5.2 cm) and body mass between 0.73 and 3.71 g (mean = 1.8 g). Neither of them showed any difference between the control and the metoprolol treatment groups (length: nested ANOVA, df=5, F=0.3146, p=0.9039; mass: nested ANOVA, df=5, F=0.0519, 0.9983). The animals were only slightly taller than at

the start of the experiment, where the mean fish weight was 1.52 g. A comparison with fish of the second hatchery control, where mean length was 7.33 cm and mean weight was 6.24 g, made clear that growth was much slower under the given laboratory conditions. A large percentage of 45 to 60 % of animals showed bite marks on their fins. The extent of these injuries was not as severe, as it was later observed in the second diclofenac exposure. However, the effect was present in both the treatment and the control groups (Likelihood-ratio, $n=225$, $df=10$, $\chi2=8.546$, $p=0.5757$).

The observed relative stress proteins levels ranged from 0.81 to 2.02 (mean = 1.3), with a very similar pattern for the control and each treatment group (linear model, $df=5/202$, $F=0.1355$, $p=0.984$). The hatchery control corresponding to the experimental start had a median Hsp70-level in a much higher range, but the hatchery control corresponding to the end was in a range comparable to the lab results.

Common histopathological findings in the liver were reduced glycogen content, slightly enlarged intercellular spaces, as well as occasional inflammations, large lipid vacuoles, and macrophage aggregations. In general, the tissue of fish from the hatchery control was in better condition than that of laboratory fish. Reported findings were observed in the lab control, as well as in the highest metoprolol exposure concentration, without noticeable treatment effect. The kidney of laboratory control animals showed visible reactions, like hyaline inclusions in the proximal tubuli and occasionally altered structure of the hematopoietic tissue. These reactions were even more pronounced in metoprolol-exposed fish. In addition to hyaline droplets in the tubuli, multiple samples showed reactions of the glomerular structure and necrotic changes. Yet, the effects were not completely concentration-dependent, with samples from the 6.9 µg/L treatment displaying the highest proportion of stronger reactions, and all other metoprolol treatment showing reactions of similar severity. Comparable to the effects reported for diclofenac, the gill structure appeared thickened through hyperplasia and hypertrophy of epithelial and interlamellar cells. Occasionally, other alterations, like lamellar fusion, oedema, increased abundance of mucus cells, or epithelial lifting, appeared. Again, the difference of laboratory experiment in comparison to the hatchery control was obvious, but there was no apparent treatment-related effect. The heart tissue itself was not altered, but there was a conspicuous shift in the ratio of erythrocytes to leucocytes in the blood remaining within the ventricle. Between control and the highest test concentration this was seen as trend, but not as significant difference.

Discussion

Diclofenac

Through the chemical analyses, we could show that juvenile brown trout takes up diclofenac into its tissue. However, the detected concentrations also suggest that there is no strong bioaccumulation. Due to the small size of the juvenile fish, the amount of tissue did not suffice to analyse concentrations in isolated liver or kidney samples. Instead, the tested tissue consisted mainly of muscle and spine. My results are in accordance with earlier studies (Schwaiger *et al.* 2004, Memmert *et al.* 2013), which report great variance in the bioaccumulative ability of different tissues – with usually low accumulation in muscle tissue. The low $logP_{OW}$ of diclofenac at neutral or slightly alkaline pH supports the assumption of low bioaccumulation at my experimental conditions. The tissue concentrations do not allow deriving plasma concentrations. Fish plasma models are usually based on lipophilicity (Fu *et al.* 2009, Schreiber *et al.* 2011) and would suggest a plasma accumulation factor around 2.5 at physiological fish pH. However, there may also be active processes involved in the uptake of diclofenac (Choi *et al.* 2005), further increasing plasma or tissue concentrations. Furthermore, physiological processes like enterohepatic cycling prolong the retention time in the organism (Hoeger *et al.* 2008). Trout exposed to 81 µg/L diclofenac exhibited plasma concentrations near the human therapeutic level, which suggests a plasma BCF of 4 (Cuklev *et al.* 2011). In fathead minnow, reported plasma BCFs are even higher, with the human therapeutic level being reached at water concentrations of 25 µg/L (Bickley *et*

al. 2017). I did not measure plasma concentrations in my experiments, but the cited literature suggests that concentrations of 10 to 100 µg/L diclofenac are sufficient to lead to physiologically active plasma concentrations in fish.

The lack of effects on embryonic brown trout matches with literature results. For brown trout embryos, an earlier study reported a NOEC of 500 µg/L, based on investigations of mortality and developmental parameters (LfW 2004). In most studies on other fish species, like *Danio rerio* (Hallare *et al.* 2004, van den Brandhof and Montforts 2010, Memmert *et al.* 2013), *Oncorhynchus mykiss* (Memmert *et al.* 2013) or *Cyprinus carpio* (Stepanova *et al.* 2013), only concentrations higher than 1 mg/L evoked significant effects. However, one study on *Danio rerio* showed that diclofenac and other NSAIDs increased mortality of male fish at concentrations of 1000 µg/L (Ji *et al.* 2013). Juvenile brown trout in my study reacted with much higher sensitivity than other species and also their own early life stages. Mortality increased already at concentrations in the low µg/L-range. Similar effects are reported for stickleback *Gasterosteus aculeatus* (Näslund *et al.* 2017), albeit only at slightly higher concentrations of 320 µg/L. This difference in concentration is likely due to sensitivity differences attributable to species and life stage. Furthermore, in a mesocosm experiment on diclofenac, survival of *Gasterosteus aculeatus* was reduced in two of three replicates at 4.1 µg/L (Joachim 2017). The authors concluded that the observed mortality must be due to a combination of different stressors. Apart from this, other studies did not report mortality at low diclofenac concentrations. The LC_{50} for adult carp is 71 mg/L (Islas-Flores *et al.* 2013, Saucedo-Vence *et al.* 2014), but none of the studies on trout applying a similar concentration range as in my study (Schwaiger *et al.* 2004, Hoeger *et al.* 2005, Mehinto *et al.* 2010, Memmert *et al.* 2013) reported increased mortality. Overall, this suggests that the investigated juvenile life stage of brown trout is far more susceptible to diclofenac than adult or early life stages.

Histological examinations shed further light on the physiological reasons for the increased mortality. The lab control already displayed a range of tissue reactions, which can be regarded as compensatory adaptations to the laboratory conditions. Gill and kidney are actively involved in osmoregulation and electrolyte metabolism. Hence, they may react plastically to changes in pH, salinity or temperature (Bonga 1997), which are inevitable when fish are transferred from the hatchery to the lab. Reactions in the liver were, to a certain extent, already present in fish from the hatchery. This and the high weight gain of hatchery fish in the same time are indications of the high energy demand and metabolic activity of the tested juvenile life stage. During the exposure, the fish received a diet that sufficed for maintenance, but did not allow the strong growth targeted in commercial trout farming. This means that the test fish had potentially less energy reserves to deal with stressful situations than fish fed ad libitum. Additionally, the lab exposure was semi-static with regular water exchanges. This is a less natural and more stressful condition, in contrast to the large flow-through system at the hatchery. However, the lab situation alone cannot be the reason for the observed mortality rates at high diclofenac concentrations. Mortality in lab controls was low, compared to the other treatments. Results suggest an adverse effect of the pharmaceutical treatment, because severity of histological reactions, especially in the liver, tended to be higher in diclofenac-exposed individuals. A variety of previous studies found evidence for histological effects of diclofenac in fish. 18 month old *Salmo trutta* exposed to 1.15 µg/L diclofenac suffered from increased monocyte infiltration in liver, telangiectasis in gill, and hyaline droplets and mild tubular necrosis in kidney (Hoeger *et al.* 2005). In rainbow trout *Oncorhynchus mykiss*, histological examinations by Schwaiger *et al.* (2004) reported pillar cell necrosis in gills and tubular hyaline droplet degeneration and interstitial nephritis in kidney, but no effects in liver at 5 µg/L. Ultrastructural examinations of the same animals (Triebskorn *et al.* 2004) confirmed the pathologies in gills and kidneys. Furthermore, they found effects in liver tissue, like the collapse of cellular compartmentation, glycogen depletion, and macrophage infiltration. In a later study, which applied the same species, Mehinto *et al.* (2010) documented symptoms like renal tubular cell necrosis

and hyperplasia of intestinal villi at 1 µg/L diclofenac. Birzle (2015) did the most thorough examination of histological alterations in rainbow trout: through morphometric measurements of histological sections he showed that diclofenac, i.a. increased the amount of hyaline droplets in kidney tubuli at 25 µg/L and evoked thickening of secondary gill lamellae at 5 µg/L. In a rainbow trout ELS test, Memmert *et al.* 2013 did not find any histological effects up to 320 µg/L diclofenac, but slight effects on gills at 1000 µg/L. Bickley *et al.* (2017) examined kidney histology in fathead minnow *Pimephales promelas*, and found increased cellularity and inflammations in fish exposed to 25 µg/L. In three-spined stickleback *Gasterosteus aculeatus*, examined kidneys featured renal hematopoietic hyperplasia at 5 µg/L. In contrast to the findings in rainbow trout (Schwaiger *et al.* 2004, Birzle 2015), fathead minnow (Bickley *et al.* 2017) or stickleback (Näslund *et al.* 2017), there was no specific pathology associated with my diclofenac exposure. These studies showed clear pathological reactions of fish kidney but less conclusive results on liver – which is the opposite of my findings. The mentioned differences in histological reactions are likely due to species and life-stage. Overall, my data suggest that a combination of basal level of stress, sensitive life-stage and diclofenac as toxic agent led to the observed pathologies and high mortality rates in µg/L concentrations.

Another unexpected finding was the apparent effect of diclofenac on behaviour, significant at concentrations higher than 1 µg/L. The true extent of this reaction was likely masked by the high mortality in most diclofenac treatments. Most recovered carcasses showed bite marks, but I could not clearly determine whether they were inflicted *ante* or *post mortem*. Hence, the actual frequency of bite marks may have been even higher than shown in the results. Fin erosion is among the frequently observed phenomena in commercial salmonid farming. The parameter varies in relation to factors like stocking density (Jones *et al.* 2011), feeding conditions (Noble *et al.* 2007, Cañon Jones *et al.* 2010), or water parameters (Bosakowski and Wagner 1994). Aggression behaviour in reaction to analgesic exposure has not yet been reported. However, prostaglandins play an important role in many cellular processes. It has been shown that environmentally relevant concentrations of diclofenac drastically decrease dopamine levels in the brain of the African catfish *Rhamdia quelen* (Guiloski *et al.* 2017). This influence on the levels of neurotransmitters could manifest in complex behavioural changes. Other behavioural reactions, like loss of balance, respiratory stress or erratic swimming, were reported for *Rhamdia quelen* at much higher concentrations of diclofenac (Ajima *et al.* 2015). Nassef *et al.* (2010) reported that diclofenac affected feeding but not swimming behaviour, and concluded that behaviour is a more sensitive endpoint than mortality. Unfortunately, this study only examined one concentration of 1 mg/L. Moreover, several studies suggest that NSAIDs influence the levels of sex steroids. In the tiger fish *Hoplias malabaricus*, diclofenac reduced plasma testosterone (Guiloski *et al.* 2015) and in the African clawed frog *Xenopus laevis*, it increased aromatase activity and altered male calling behaviour (Efosa *et al.* 2017). Sex hormones play a major role in mating and territoriality behaviour – which could also explain the observed effects. In my case, I did not actually observe the aggression behaviour, and could only indirectly judge by the frequency of bite marks. It may be that diclofenac does not directly increase aggressiveness, but instead reduces defensive behaviour. Through its pain-relieving ability or the general weakening of the body condition, diclofenac may dullen the senses of victim fish, making them more prone to conspecific attacks. The suffered injuries may also be linked to the increased mortality rates. Potentially lethal infections are more likely to occur when animals are injured. On the other hand, the increased aggression behaviour could also be triggered by density changes through mortality. Young brown trout from hatcheries transcend from swarming to territorial behaviour, when density is low (J. Schindler – personal communication). If mortality reduces the density below a threshold level, this may lead to an aggression response. However, bite mark frequency was low in the two lowest test concentrations, despite the relatively high mortality in these treatments. Another explanation comes from the findings of Birzle (2015), who showed that diclofenac leads to perforations of the cornea in rainbow trout. Trout rely heavily on visual cues for orientation and foraging (Klemetsen

et al. 2003). If a similar effect is evoked in brown trout, the behavioural effects can be interpreted as panic reactions due to visual disturbance. There were no apparent macroscopic effects on the brown trout eyes in my study, but due to strong pigmentation, this effect is more difficult to quantify in brown trout compared to rainbow trout. Finally, I must acknowledge that my experimental setup was not designed to answer questions on behavioural effects in full detail. These effects were not anticipated, and therefore not included in the planning. Further studies could focus on behavioural endpoints by monitoring activity and inter-individual behaviours to put my findings into perspective.

Diclofenac had no effect on Hsp70 and lipid peroxidation in brown trout juveniles. There are few studies on the effects of NSAIDs on stress protein induction. Hallare *et al.* (2004) could not find any effect of diclofenac on Hsp70 in *Danio rerio* up to 2 mg/L. However, ibuprofen led to increased Hsp70-levels in rainbow trout fry (Gravel and Vijayan 2007). The same study found no such effect for salicylic acid, another NSAID. Several studies investigated oxidative stress biomarkers in response to diclofenac exposure, with inconsistent results. Oxidative damage is the major reason suspected to be responsible for the strong adverse effects of acidic NSAIDs on gastric mucosa in mammals (Sánchez *et al.* 2002). Diclofenac increased hydroperoxide content and lipid peroxidation in carp at 7.1 mg/L (Saucedo-Vence *et al.* 2014) and induced reactive oxygen species in rainbow trout cell assays with an EC_{50} of 44.5 µg/L (Fernandez *et al.* 2013). Oxidative stress induced by NSAIDs is likely mediated through superoxide anions generated during metabolism by CYP450. This is, for example, seen in the livers of inanga *Galaxias maculatus* exposed to diclofenac (McRae *et al.* 2018), where lipid peroxidation and CAT activity were both increased. In contrast, renal lipid peroxidation of the same animals was reduced. In hepatocytes of *Oreochromis niloticus*, the expression of GST-genes was up-regulated in a concentration dependent manner (Gröner *et al.* 2015). *Danio rerio* had decreased levels of lipid peroxides at 20 µg/L diclofenac, but none of the other parameters of the oxidative stress response were affected up to 60 mg/L (Praskova *et al.* 2014). Mortality rates of *Cyprinus carpio* were first affected at concentrations of 3 mg/L, but oxidative stress biomarkers, e.g. TBARS, were significantly lower already at 30 µg/L (Stepanova *et al.* 2013). Trophic exposure of tiger fish *Hoplias malabaricus* to 2 µg/kg diclofenac increased SOD and CAT activity, as well as the hydroperoxide level, but reduced GST activity (Guiloski *et al.* 2015). In the catfish *Rhamdia quelen*, various oxidative stress biomarkers, like lipid peroxidation, CAT-activity or SOD-activity, were reduced at 0.2 µg/L (Guiloski *et al.* 2017). However, the activity of GST was increased, which can be interpreted as a protective effect – a possible explanation why no protein carbonylation or DNA damage was seen in this study. For the same species, Ghelfi *et al.* (2016) reported that diclofenac increased the activity of SOD in kidney at 0.2 µg/L, but had no effect on lipid peroxides – which would fit this pattern. In stickleback *Gasterosteus aculeatus* exposed to diclofenac in a mesocosm, antioxidative defence was increased at 4.1 µg/L. Moreover, the authors reported immune destabilisation due to leucoyte oxidative stress (Joachim 2017). The oxidative stress response is a complex process, which may be an explanation for the divergent findings. If diclofenac induces protection mechanism against ROS, like increased activity of superoxide dismutase, glutathione-S-transferase, or catalase, this may prevent the peroxidation of lipids, proteins or DNA. Furthermore, cyclooxygenases themselves possess peroxidase activity (Simmons *et al.* 2004). Their inhibition through NSAIDs could inhibit lipid peroxidation, thereby counteracting or masking other effects.

Metoprolol

Our study could not identify an influence of metoprolol on hatching or survival of brown trout. A recent study by Gröner *et al.* (2017b) exposed Nile tilapia *Oreochromis niloticus* to metoprolol from fertilized egg until 80 dph came to a similar conclusion. However, Gröner *et al.* (2017b) showed growth (measured by wet weight, length and condition index) to be reduced in a concentration-dependent manner, reaching significance at 11 µg/L. The experiment, in view of the much faster development of

tilapia compared to trout, spanned over a much broader range of life stages. The growth effect was not yet visible after only 8 dhp, which suggests that it is mainly relevant in fry and juveniles, not in larval stages. My embryo and sac-fry exposure did not continue into these later stages - explaining the lack of a growth effect. In tilapia, there were no effects on mRNA expression of biotransformation enzymes (Gröner *et al.* 2017b), but decreased expression of lh (luteinizing hormone) and FSH (follicle-stimulating hormone) mRNA, and increased vitellogenin mRNA hint on a potential endocrine activity of the substance. Histological effects in the gill were only vague, and seen as first defensive reactions towards adverse conditions in the surrounding medium. In comparison, effect concentrations as low as 1 µg/L were found for cytological and histological changes in adult rainbow trout *Oncorhynchus mykiss* (Triebskorn *et al.* 2007). In the liver, these effects (reduction of glycogen stores, membrane material within cells, changes of the endoplasmic reticulum, loss of compartmentation) were most pronounced and concentration-dependent. In kidney, thickening of basal membrane, altered endocytic channels and increased amounts of macrophages were only observed at 1 and 500 µg/L, but not in the other test concentrations. In gills, the major effects were epithelial lifting, hypertrophy of mucus and chloride cells, macrophage infiltration, and dilation of the endoplasmic reticulum. These effects are, in great parts, different to the observations in my experiment. However, it is important to bear in mind the substantial differences in the experimental design: first, the applied species was different, although rainbow trout and brown trout are closely related. Second, the animals in my experiments were of a much younger life stage. Third, many of the effects described by Triebskorn *et al.* (2007) were on an ultrastructural level, which views the cellular structures in greater detail and, thus, has higher sensitivity. Fourth, reactions to metoprolol in the liver of brown trout may be concealed by the already sub-optimal state of the control animals. Steinbach *et al.* (2014b) observed histological effect on the vascular system, visible by liver congestion and changes of the pericardium and myocardium of juvenile rainbow trout exposed to atenolol. Furthermore, they identified beta-blocker effects on several blood parameters, like lactate and haemoglobin content, glucose concentration, or haematocrit. Although I could not observe a negative effect on liver or heart tissue integrity, my results also pointed towards alterations of fish blood parameters. The observed difference in the ratio of blood cells is only a first indication. It was an incidental finding during the examination of the heart – and we had originally not intended to examine the blood. Further studies, with blood smears taken exactly for this purpose, will be necessary to make a broader statement concerning this endpoint.

Stress proteins were not affected by any treatment at all. Given the large sample size, it is reasonable to assume that metoprolol does not exert proteotoxic effects in trout.

Considering the overall results, with medium-intensity stress evoked by the lab exposure itself, the results are not that surprising. The stress response in fish is largely mediated by catecholamines. Via beta-adrenergic receptors, these stress hormones lead to mobilization of energy reserves in liver and increased ventilation and heart rate, which also manifests in tissue alterations – primarily in the gill (Bonga 1997). Metoprolol blocks the respective receptors and may prevent this stress response. This could actually counteract effects like the ones observed in my experiments. This could lead to a situation, where the negative implications of increased detoxification activity are balanced by beneficial effects through catecholamine receptor blocking.

Sensitivity of different life stages to diclofenac and metoprolol

In my experiments, I exposed two different life stages of the same species and the same brood stock to similar concentrations of diclofenac and metoprolol. For metoprolol, both life stages did not show strong effects. In the experiments with diclofenac, juvenile fish showed severe effects, while early life stages did not react at all. The pH of both exposures differed: embryos developed in artificial water with neutral pH, whereas juveniles were exposed to slightly alkaline filtered tap water. This can have

great influence on the toxicity of ionisable pharmaceuticals (Boström and Berglund 2015). For alkaline substances like metoprolol, toxicity should increase with pH, while for acidic substances, like diclofenac, toxicity should be lower at higher pH. However, the juvenile trout reacted sensitively to diclofenac, despite the high pH. Consequently, I conclude that brown trout embryos and eleutheroembryos are less sensitive towards diclofenac than older life stages. This could be due to their independence from external feeding, but also differences in metabolism. In cell culture experiments, van Leeuwen *et al.* (2011) suggested that the actual toxic effect of diclofenac is due to intermediate products of the CYP450-metabolism. CYP450 or other enzymes can differ in their life-stage-specific expression (Andersson and Förlin 1992), which influences the reactions to toxicants. The low susceptibility of early life stages was also seen in other studies on brown trout (LfW 2004) and rainbow trout (Memmert *et al.* 2013). All studies reporting sensitive reactions of fish exposed to diclofenac (Schwaiger *et al.* 2004, Triebskorn *et al.* 2004, Birzle 2015, Näslund *et al.* 2017) used older individuals. When ecotoxicity data are only obtained for early life stages, this might underestimate the risk. Future studies must be aware of this fact and should apply the most susceptible age class for a protective risk assessment.

Chapter IV: Biomarker effects in invertebrates

Paper: Jungmann *et al.* 2017 - Health effects of metoprolol in epibenthic and endobenthic invertebrates – A basis to validate future *in vitro* biotests for effect-based biomonitoring

Report: Triebskorn *et al.* 2017 - EFF-Pharm: Effects of pharmaceuticals (nonsteroidal anti-inflammatory drugs and beta-blockers) in fish and invertebrates and their detection by newly developed in vitro-bioassays – Final report → WP3A, WP4, WP6

Aims

For the assessment of pharmaceutical ecotoxicity to invertebrates, the water flea *Daphnia magna* is usually the most common investigated organism. In our studies, we ventured beyond this. Not only by additionally testing pharmaceutical effects in gammarids, gastropods, and sediment-dwelling oligochaetes, but also by investigating additional endpoints of toxicity. Invertebrate studies focus mainly only on organismic parameters, like survival, reproduction, or growth. I expanded this range of endpoints with biochemical analyses of stress proteins and lipid peroxides.

Biomarker effects of diclofenac in gammarids, daphnids and oligochaetes

In general, analyses of stress proteins in gammarids turned out to be more difficult than in fish. A considerable portion of samples showed no or only a very weak signal after immunostaining. Relative Hsp70-levels varied between 0 and 1.03, with a mean of 0.19. Exposure to diclofenac did not affect the stress protein levels of gammarids (ANOVA, df=5/97, F=0.7660, p=0.5765; data transformed by cubic root). Lipid peroxide levels ranged between 5.18 and 57.16 CHPE, with a mean of 18.23 CHPE – which was close to the mean of 17.94 CHPE for the t_0 control. There was a slight, non-significant tendency for the degree of lipid peroxidation to decrease with increasing diclofenac concentration (Welch-ANOVA, df=5, F=2.0571, p=0.0898). In contrast, body mass seemed to slightly increase with exposure concentration. Interestingly, the animals of the t_0-control were heavier than the control animals sampled at the end of the experiment (mean t0: 16.96 mg; mean experiment: 13.63 mg).

Hsp70-levels in *D. magna* varied between 0.27 and 1.64 (mean: 1.0) and showed no difference between the treatments (linear model, df=5/15, F=0.3356, p=0.8835). Sample size was small, since pools of two individuals were necessary to achieve the required protein amount, and was even smaller for the two highest diclofenac treatments, which experienced high mortality.

The analysis of stress proteins in the oligochaetes proved to be a great technical challenge for the applied method. Several adjustments of the standard procedure were necessary to obtain quantifiable

amounts of Hsp70 (pooling of samples, loading with higher protein mass – all changes are already mentioned in the Material and Methods section). Finally, the immunostaining yielded weak, barely measurable signals. However, the necessity to pool the samples, both for the analysis of stress proteins and lipid peroxides, resulted in a very low number of replicates for each treatment. Except for a single sample of the highest treatment (rel. Hsp70-level: 0.8651), all stress protein levels were in a comparable, low range (0.0015-0.4740, mean: 0.1546). The observed variability appeared higher at diclofenac-treated groups, with several visible outliers, but there was no overall treatment-related difference (ANOVA, df=5/24, F=0.5211, p=0.7579, data transformed by cubic root). Lipid peroxide levels varied between 13.59 and 36.68 CHPE, with a mean of 20.98 CHPE, without apparent differences between treatments (ANOVA, df=5/24, F=0.4661, p=0.7975, data transformed by square-root).

Biomarker effects of metoprolol in gammarids, daphnids, gastropods and oligochaetes

Due to 100 % mortality in the 135 and 405 mg/L metoprolol treatments, stress proteins could only be examined for the three lowest test concentrations in the gammarid experiment. For the 45 mg/L treatment, sample size was reduced due to the already high mortality in this group. Relative Hsp70-levels of gammarids exposed to metoprolol varied between 0.018 and 1.23, with a mean of 0.47. There were no differences between the treatments and the control (ANOVA, df=3/98, F=0.1333, p=0.9400; data transformed by square-root), but the levels were overall higher than in the corresponding t_0-control (mean: 0.29).

When exposed in the artificial indoor streams, the stress protein levels were visibly reduced (0.003-1.14, mean: 0.23), compared to the respective t_0 control (mean: 0.42). However, none of the AIS-treatments differed from the AIS-control (linear model, df=5/67, F=1.835, p=0.1178). Individuals exposed to the highest treatment (21.95 mg/L) were smaller than control animals (linear model, df=5/84, F=3.168, p=0.0114), while there was no difference to the other treatments.

Stress protein levels of metoprolol-treated *D. magna* ranged between 0.29 and 1.29 (mean: 0.75). Like for diclofenac, sample size in the higher treatments was greatly reduced due to high mortality. The treatments with sufficient sample size did not show an apparent difference to the control group.

P. antipodarum experienced an obvious, concentration-dependent increase of relative Hsp70-level with rising metoprolol-concentration in the single-substance laboratory experiment. In the highest test concentrations of 10 mg/L, Hsp70-levels were significantly higher than in the negative control (linear model, df=5/30, F=3.22, p=0.01915). This difference was even more pronounced in contrast to the solvent control. However, since the only other treatment receiving solvent was the positive control, a direct comparison between solvent control and metoprolol treatments is not meaningful. Overall, levels varied between 0.04 and 1.0. In contrast to the batch experiment, Hsp70-levels were not affected in the AIS-setup after 28 d (linear model, df=5/36, F=1.819, p=0.1339). After 40 days, the highest concentration (21.95 mg/L) led to reduced Hsp70-levels (linear model, df=5/36, F=2.906, p=0.02645).

In oligochaetes exposed to metoprolol, stress protein levels showed high variability (0.0002-0.3685, mean: 0.1161), compared to constantly low levels in the control (0.0151-0.1078, mean: 0.0481). However, only the lowest treatment concentration differed visibly from the control. The higher-concentrations treatments differed only slightly from the control, and the observed difference in means was non-significant (ANOVA, df=5/20, F=2.2054, p=0.0942; data transformed by cubic root). Lipid peroxide levels tended to be higher at metoprolol-treatments (Kendall's Tau, n=30, τ=0.2847, p=0.0371), but there was no clearly observable difference of any treatment compared to the control. Lipid peroxide levels varied between 16.89 and 25.47 CHPE, with a mean of 20.74 CHPE. In the AIS setup, the oligochaetes escaped from their enclosures and investigation of biochemical markers was not possible.

Discussion

Overall, I could not observe any response in stress protein levels of *G. fossarum* exposed to either diclofenac or metoprolol. Even in treatment groups that already experienced high mortality, Hsp70-levels did not differ from the control. This suggests that both pharmaceuticals do not exhibit proteotoxic effects. However, it is also possible that the Hsp70-system was already overwhelmed in these organisms and therefore did not show any further changes. In this case, the applied concentrations were either generally too high to see the reaction phase of the stress protein response, or the test concentration spacing was an unfortunate choice. The observed difference in stress protein levels between lab-kept animals and the t_0-control from the field shows that, in general, changes can be evoked. The difference could, on the one side, be due to stress of the animals by handling and experimental procedure. On the other side, it may be also attributable to the different age and reproductive state. The tendency of decreased levels of lipid peroxides with increasing diclofenac-concentrations could hint at an ameliorative effect of the pharmaceutical, like it is observed for fish (McRae *et al.* 2018). Via its mode of action, diclofenac inhibits oxygenases, which have peroxidation ability. Due to the high variation observed in the data, the trend is vague and non-significant. The lower proportion of adult individuals and egg-bearing females in diclofenac-exposed experimental groups explains the observed tendency of higher body mass in gammarids in those groups. With less energy invested in reproduction, the individuals can remain heavier. The lower weight of lab-exposed individuals overall could also be due to this energy conservation, but also to the potentially stressful lab conditions. Furthermore, the different age structure could also confound the results obtained in my biomarker analyses. In summary, there was no difference in stress protein levels evoked by the tested concentrations of diclofenac and metoprolol, even in concentrations leading to drastic mortality and reduction of reproductive parameters. Lipid peroxides were, if anything, reduced by diclofenac – showing no indication for oxidative stress exhibited by the substance, but rather an ameliorating effect. Yet, I am restricted to a conclusion concerning lipid peroxides, since no further tests concerning other oxidative parameters (e.g. catalase or superoxide-dismutase activity) were within the scope of this study. In the amphipod *Hyalella azteca*, effluents from a NSAID manufacturing plant led to increased activities of antioxidative enzymes and also increased levels of hydroperoxides, lipid peroxides and carbonylated proteins (Novoa-Luna *et al.* 2016). The effluent concentrations of four measured NSAIDs, diclofenac, ibuprofen, naproxen and paracetamol, ranged between 1 and 3 mg/L each. This complex mixture is hardly comparable to the single substance exposure in our case.

In an earlier study on the effects of diclofenac in *D. magna*, the lowest concentration to evoke changes of the Hsp70-levels was 30 mg/L (Haap *et al.* 2008). At these concentrations, the mortality in our experiment was so high that I did not have enough samples to make a reliable statement on the stress protein levels. At lower test concentrations, where the sample size was not reduced in such a drastic way, both diclofenac and metoprolol did not evoke differences in stress protein levels, compared to the untreated control.

Unfortunately, several factors complicate the interpretation of the results obtained for *L. variegatus*. The applied technique for stress protein quantification did not work as well on this organism as it did on the others. Overall, blotted protein bands were only weakly stained, resulting in low measured signal, which gives noise much greater influence. For the FOX-assay, a high dilution and long incubation times were necessary – still resulting only in low measured extinction values. All this introduces further inaccuracies into the final calculation of the CHP-equivalents. However, the biggest problem is the very limited number of replicates. Instead of originally planned fifteen individuals, I ended up with results on five pooled samples. Although the observed results did to some extent hint on potential effects of the tested pharmaceuticals, the database is not clear enough to make a sound statement.

The only tested invertebrate with a visible reaction of the Hsp70-system was *P. antipodarum*. Stress protein levels increased with ascending metoprolol concentration and were significantly higher in the highest tested concentration compared to the negative control. Because the variation in the negative control was high, these differences were not as clear for the lower metoprolol concentrations. A comparison to the solvent control is difficult, because none of the metoprolol treatments used a solvent, too. The solvent control served only as a reference for the positive control with ethinyl estradiol, which is required by the underlying guideline 242 (OECD 2016). However, in general the results from the solvent control support the conclusion drawn from the comparisons to the negative control. Metoprolol is likely to exert proteotoxic action in this species. In the highest test concentration, which resulted in drastic reduction of the reproductive output, the Hsp70-system was still in reaction and had not reached a state of degradation. In tendency, the biomarker already reacted in a concentration range that did not affect the standard parameter reproduction. Therefore, future approaches could benefit from the inclusion of stress protein analyses in molluscs - increasing sensitivity by detecting physiological changes before the alterations manifest in reduced reproduction. The method worked well for this organism, but again, individuals had to be grouped into larger pools. This reduced the sample size, and resulted in only six pools per treatment. Further investigations with higher sample size would be helpful before drawing definitive conclusions. Contardo-Jara *et al.* (2010) found an increase of *hsp70*-mRNA in the zebra mussel *Dreissena polymorpha* at concentrations as low at 0.534 µg/L metoprolol, which is close to the concentrations where Hsp70-levels tended to increase in our study. It may be that the stress protein system of molluscs reacts to metoprolol in a more sensitive manner than in other invertebrate taxa. Further studies with other species of gastropods or bivalves could help shedding light on this issue. The results of the AIS-experiment stand in contrast to the batch experiments, because the stress protein level was decreased by metoprolol in the mesocosms. However, the concentration evoking a decrease of Hsp70 in the AIS was considerably higher than the ones tested in the batch experiment. This high concentration, combined with physically stressful conditions in the flow-through, may be responsible for a beginning breakdown of the Hsp70-system. While the animals were still coping with the combined stress of stream and metoprolol after 28 days, the system might be overwhelmed after a longer time – resulting in strongly reduced levels of Hsp70 at the highest test concentration.

Chapter V: Laboratory and semi-field studies with effluents

Report: Triebskorn *et al.* 2017 - EFF-Pharm: Effects of pharmaceuticals (nonsteroidal anti-inflammatory drugs and beta-blockers) in fish and invertebrates and their detection by newly developed in vitro-bioassays – Final report → WP5

Aims

One intended field of application for the developed *in vitro* biotests is the quality assessment of wastewater and/or wastewater treatment. In this context, it is important to know whether the mixture of substances present in treated wastewater is able to evoke effects in biota. Hence, I wanted to gain own experimental experience with respect to the question whether wastewater treatment plant effluents exert an impact on developing brown trout embryos and gammarids. In separate experiments, I exposed brown trout from fertilized egg to free-feeding larvae to the effluent of a conventional three-stage treatment plant: once in a semi-field situation directly at the plant, and once in a laboratory setting with water samples taken from the effluent. The evaluation of organismic parameters was complemented by analysing lipid peroxidation and assessing histological integrity of larval organs. Gammarids were only exposed in a semi-field experiment, which was complemented by biochemical analyses of lipid peroxides and stress proteins.

Organismic and biomarker effects in embryonic brown trout

In the WWTP Eriskirch, mean temperature was 8.87 °C, conductivity 1261.4 µS/cm and oxygen saturation 76 %. These conditions differed strongly from the field control side at the ISF Langenargen (temperature: 7.42 °C, conductivity: 317.4 µS/cm, O_2-saturation: 97 %). Chemical analyses of the water showed concentrations of 1.44 µg/L diclofenac and 1.39 µg/L metoprolol in the WWTP effluent. Previous analyses of the effluent already showed that the conventional treatment effectively reduces turbidity, but does not remove micropollutants like 1-H-benzotriazole, carbamazepine, diclofenac or sulfamethoxazole to a sufficient proportion (<50 % elimination). Another peculiarity were increased levels of perfluorooctanesulfonic acid (PFOS) in this particular WWTP (Triebskorn 2017, p166-170). The control water from the Lake Constance was not contaminated with measurable concentrations of both diclofenac and metoprolol. At both sites, the embryos reached the eyed ova stage after 31 dph. They finished hatching on day 50 at the WWTP and on day 64 at the control site. At the WWTP, embryonic development was finished 79 dph and the experiment ended after 106 days. Development took longer at the control site, where yolk sacs were consumed 99 dph and the experiment was terminated after 120 days. Unfortunately, the larvae at the WWTP escaped from one of the sieves, leaving only five of six sieves for sampling at the end of the experiment. Mortality was higher in the control (20 %) than in the WWTP effluent exposure (9 %), but a large part of the control mortality occurred after swim-up of the larvae. The number of malformed larvae was negligible in relation to the overall number (3 of 434 individuals at the final sampling). Heart rate was slightly higher at the WWTP than at the control site (76 vs. 74 bpm) but overall variation was high (60-99 bpm). Larvae from the control site were much larger (235.3 vs. 106.1 mg) than those from the WWTP and had slightly lower levels of lipid peroxides (4.34 vs. 5.0 CHPE).

The water parameters of the lab experiment were in the intended range. Temperature was between 6.1 and 6.7 °C (mean: 6.36 °C), pH between 7.6 and 8.2 (mean: 7.8) and oxygen saturation between 92.5 and 123 % (mean: 102 %). The conductivity of the WWTP effluent (mean: 1288.5 µS/cm) was considerably higher than that of artificial water (mean: 755.5 µS/cm). At 35 dph, the vast majority of embryos of both treatments reached the eyed ova stage. Last stragglers had reached this stage at 43 dph. First embryos hatched at 71 dph and all embryos had hatched at 84 dph, with one replicate of the control not starting until 81 dph. For the biggest part of this phase, embryos exposed to WWTP effluent seemed to develop slightly faster, but the control embryos joined up fast during the last days. Heart rate varied between 42 and 54 bpm (mean: 45.1 bpm). Embryogenesis was finished after 149 days of exposure. Mortality rates were variable (0-55 %), but on average not different between the two treatments. Observed mortality happened mainly after the larvae had hatched. Like in the field experiment, malformation rates were negligible. Control larvae were considerably heavier than the ones exposed to WWTP effluent (control: 73-147 mg, mean: 115.5 mg; effluent: 48-130 mg, mean: 147 mg; linear model, df=1/138, F=17.42, p<0.0001). A similar effect was seen for lipid peroxides (linear model, df=1/138, F=42.714, p=0.0018), which were also lower in the effluent-exposed larvae (control: 0.07-8.80 CHPE, mean: 4.67 CHPE; effluent: 1.79-7.41 CHPE, mean: 3.79 CHPE). Overall, the kidney of larvae were in a good histological state. There were no histological differences between the control and the effluent-exposed larvae. Most individuals were in a control state, few showed mild to moderate reactions. Observed peculiarities of renal histology were vacuolization in tubular cells - likely an age-related effect that was also observed in the single-substance exposure of trout larvae - and slight alterations of the hematopoietic tissue (large vacuoles in hematopoietic cells). In two cases, necrosis of single cells was observed.

Due to the large variations in overall exposure conditions, the direct comparison between both field sites is not meaningful. The obtained values should rather be taken as overall qualitative reference for the lab experiment. The most important finding in the field experiment was that embryos/larvae

exposed to WWTP effluent developed normally and did not exhibit high mortality. Therefore, I can conclude that the effluent of the WWTP Eriskirch does not have drastic effects on this particular life stage of brown trout. However, this finding is exemplary and cannot be generalized to other WWTPs, which may deal with a completely different mixture of chemicals and other purification rates.

The high heartbeat rate in the field exposures compared to the lab data can be attributed to the higher temperature in in the field. Likewise, the faster hatching in the field is also due to temperature effects. This may also be seen in the differences in hatching time between the single substance exposure experiments [experiment 3] and the WWTP effluent exposure experiment in the lab [experiment 5]. While hatching in the single substance exposure was finished after 70 days, it took 84 days for the WWTP effluent exposure. These experiments took place in separate thermo-constant chambers – whose mean temperatures differed by approximately 1 °C. This further illustrates the strong effect of even slight temperature differences on trout development. However, compared to the data published by Killeen *et al.* 1999a, the development of the embryos in my laboratory experiments seems slow. In the published study, eyed ova stage is reached after 30 days, hatching is finished after 82 days and the embryo is fully developed after 127 days at 6 °C. Hence, embryos in my experiments developed slower, even though they were exposed at a higher temperature. In the single-substance exposure experiment, embryonic development was also slower than suggested by literature data. A likely explanation is plasticity in this parameter, for example depending on the genetic origin. Female brown trout show great variability in egg number and egg size depending on genetic and environmental parameters (Jonsson and Jonsson 1999). Hence, there may also be variability in developmental times. All eggs/embryos used for my experiment came from the same stock of animals and all my obtained results are consistent within themselves. In the previous experiments with effluent samples in the project SchussenAktiv*plus*, effluent after flocculation filtration had no significantly negative effects on hatching or survival of zebrafish embryos (Triebskorn 2017, p.228-229), which matches my results. However, various *in vitro* assays pointed at adverse effect potentials in the water: those included increased mutagenicity, dioxin-like activity, estrogenic, anti-estrogenic and androgenic activities (Triebskorn 2017, p.202-226).

The most interesting effect of the effluent exposure experiment in the lab was the reduced body mass compared to the control. In contrast to the field experiment, the larvae did not receive any feed, since the exposure was terminated before the yolk sac was fully consumed. Therefore, I can assume that all exposed larvae were supported with the same amount of resources from beginning to end. This suggests that larvae from effluents could not allocate as much resources into growth as their conspecifics in the control did. Most likely, they had to invest more into detoxification of the chemical cocktail they received through the exposure medium. This could be pharmaceuticals like diclofenac and metoprolol, but also other organic substances (biocides, detergents…) or salts – which is reflected in the much higher conductivity of the effluent.

Regarding the results on lipid peroxides, there are two possible explanations for the reduced level in the effluent-exposed larvae. On the one side, certain substances in wastewater (e.g. pharmaceuticals on a variety of synthetic anti-oxidants (Sies 1993)) may exhibit an anti-oxidative effect, reducing oxidative stress. Even with other substances present, which might increase oxidative stress, the net effect still could be positive. On the other side, the vast diversity of different trace substances in the effluent could induce the overall oxidative stress response of the organism, e.g. by protective enzymes. In contrast, organisms raised in pristine water containing no more than the essential electrolytes, have less reason for this induction. Such effects were discussed for NSAIDs in Chapter III. Increased levels of antioxidant enzymes would reduce the effects of oxidative stress (Martinez-Rodriguez *et al.* 2018), like the level of lipid peroxides, but also come at an increased resource cost. In view of the reduced body mass in effluent exposed larvae in the lab, this hypothesis is plausible. One year-old rainbow trout

exposed in effluent of the same WWTP displayed increased levels of the detoxifying enzyme Cyp1A1, and showed histological reactions in the liver tissue (Triebskorn 2017, p.235-238). This can be interpreted as result of higher metabolic activity, and would fit well to my results – though the investigated life stage was different. Future studies could investigate effects on oxidative stress more closely by having a look on the activity of antioxidative enzymes like catalase, ascorbate-peroxidase or superoxide dismutase.

Biomarker effects in gammarids

Environmental parameters also differed in this experiment. In the WWTP effluent, pH was 6.1, conductivity 1053 µS/cm, temperature 14.35 °C and oxygen saturation 93 %. At the first sampling in the stream (t_0), pH was 6.7, conductivity 731 µS/cm, temperature 12.0 °C and oxygen saturation 86 %. At the second sampling in the field (t_{40}), corresponding to the end of the exposure, pH was 7.6, conductivity 732 µS/cm, temperature 13.2 °C and oxygen saturation 92 %.

Lipid peroxides were of a comparable level in effluent-exposed gammarids and the t_{40} control (mean: 57.0 CHPE). Values obtained for the control at the start of the experiment (t_0) were higher (mean: 68.4 CHPE), but did not differ significantly (linear model, df=2/91, F=2.651, p=0.07604, log-transformed data). Body mass showed great differences between all treatment groups (linear model, df=2/91, F=25.39, p<0.0001, fourth-root transformed). Control individuals sampled after 40 days (8.6-22.5 mg, mean: 14.77 mg) were heavier than at the beginning (5.6-20.5 mg, mean: 11.28 mg), and individuals exposed in the effluent (10.5-21.9 mg, mean: 17.35 mg) had an even bigger body mass than t_{40} and t_0. Hsp70-levels in t_0 control animals (mean: 0.36) were lower than those of the t_{40} control, which resulted in an overall significant difference (linear model, df=2/98, F=3.845, p=0.02469, square-root transformed). There were no differences in Hsp70-level between the t_{40} control and the effluent-exposed animals (0-1.02, mean: 0.42).

Based on our results it seems likely that the chemicals present in the wastewater effluent exhibit no oxidative or proteotoxic effects on gammarids. On the other hand, it would also be possible that the oxidative and anti-oxidative substances balance each other so that no net effect remains to be seen. Moreover, it may also be that the induction of cellular defence mechanisms prevents damage. Since the Hsp70-system of gammarids did not react in the single substance exposure at concentrations exceeding the wastewater effluent concentrations by orders of magnitude, my results are not surprising. One year-old rainbow trout exposed to the effluent had no increased levels of Hsp70 (Triebskorn 2017, p.235-238). In a field study on *G. fossarum* at wastewater-exposed streams, Hsp70 expression depended on season, but the reactions to effluent exposure were inconclusive. However, the stress protein Hsp90, which is closely associated with steroid hormone interactions, was decreased downstream the WWTP discharge (Schirling *et al.* 2005). In a supposedly polluted stream, which had previously suffered from high crayfish mortality, Hsp70-levels in gammarids and trout were distinctly elevated (Triebskorn *et al.* 2002). Gammarids from the Schussen river, in which the WWTP Eriskrich discharges its effluent, showed higher Hsp70-levels at sites downstream of WWTPs (Triebskorn 2017, p.323-324). The differences in body weight between all samplings are most likely due to age, temperature and nutritional conditions. All animals derived from the same stock population, but the effluent and t_{40} animals had 40 days more to feed than the t_0 control animals. Moreover, the effluent seems to provide a warm environment with good nutritional conditions (high bacterial biomass/aufwuchs) for gammarids – reflected in the higher body mass. This also seems to counterbalance potential chemical stress on the first glance. However, females exposed to the effluent also showed reduced fecundity, which fits to adverse effects on *P. antipodarum* reproduction in earlier studies (Triebskorn 2017, p.231-233). Hence, the long-term population effects of effluent exposure should be more drastic. Comparable effects were found in *Gammarus pulex* exposed to estrogen-containing wastewaters (Schneider *et al.* 2015): effluent-exposed cohorts were bigger, but also had

altered sex ratio and fecundity. In another case, fecundity and fertility of *G. fossarum* were reduced, but enzymatic parameters like glutathione-S-transferase activity were unaltered by WWTP effluents (Wigh *et al.* 2017). Besides several sensitive insect orders, *Gammarus pulex* and *Gammarus fossarum* are among the most sensitive invertebrates in river ecosystems, and wastewater discharge is one important cause for the degradation of these systems (Berger *et al.* 2016).

9. Summarizing assessment of data obtained in *Eff-Pharm*

In the project *Eff-Pharm*, we investigated the impact of the pharmaceuticals diclofenac and metoprolol in a wide variety of different organisms, quantified via several endpoints. Table 4 summarizes the obtained effect concentrations of all experiments. In the following, I will provide a summarizing assessment of these combined results.

Table 4: Summary of effect concentrations obtained in Eff-Pharm. Results in grey were not part of this thesis. The most prominent effects identified in this thesis are highlighted in red.

Test organism	Endpoint	Diclofenac	Metoprolol
Salmo trutta (embryo)	Survival Growth Development Histological state	NOEC ≥ 74.6 µg/L	NOEC ≥ 998 µg/L
Salmo trutta (juvenile)	Survival	NOEC = 10 µg/L - decrease	NOEC ≥ 745 µg/L
	Bite marks	NOEC = 1 µg/L - increase	
	Stress proteins	NOEC ≥ 200 µg/L	NOEC ≥ 745 µg/L
	Lipid peroxides		Not tested
	Histological state	Reactions in all treatments	Kidney reacting at 0.094 µg/L
Gammarus fossarum (batch)	Mortality	NOEC = 8000 µg/L - increase	NOEC = 15000 µg/L - increase
	Reproduction	NOEC = 790 µg/L - decrease	NOEC = 5000 µg/L - decrease
	Stress proteins	No effect up to 24,100 µg/L	NOEC ≥45,000 µg/L
	Lipid peroxides		Not tested
Gammarus fossarum (AIS)	Mortality	Not tested	NOEC ≥ 21,950 µg/L
	Reproduction		NOEC = 740 µg/L - decrease
	Stress proteins		NOEC ≥ 21,950 µg/L
	Lipid peroxides		NOEC ≥ 21,950 µg/L
	Body mass		NOEC = 2950 µg/L - decrease
Daphnia magna	Immobility	EC_{50} = 25,200 µg/L - increase	EC_{50} = 2000 µg/L - increase
	Reproduction	EC_{50} = 15,000 µg/L - decrease	EC_{50} = 2900 µg/L - decrease
	Stress proteins	NOEC ≥ 53,700 µg/L	NOEC ≥ 3200 µg/L
Lumbriculus variegatus	Reproduction	NOEC = 80 mg/kg dw	NOEC = 160 mg/kg dw
	Stress proteins	Increasing variance	Tendentially increasing
	Lipid peroxides	NOEC ≥ 10 mg/kg dw	Increase
Potamopyrgus antipodarum (batch)	Reproduction	Not tested	NOEC = 3200 µg/L - decrease
	Stress proteins		NOEC = 3200 µg/L - increase
Potamopyrgus antipodarum (AIS)	Reproduction	Not tested	NOEC < 220 µg/L – decrease
	Stress proteins		NOEC = 2950 µg/L - decrease

Effects of diclofenac

Diclofenac had negative effects on gammarid survival at concentrations in the low mg/L range. For daphnids, effect concentrations were even higher. The endobenthic oligochaete *Lumbriculus variegatus* was first affected at concentrations of 160 mg/kg diclofenac. In a study by Nieto *et al.* (2017) another sediment invertebrate, the non-biting midge *Chironomus riparius*, showed reduced emergence at 34 mg/kg diclofenac. In comparison to the tested invertebrates, brown trout was a much more sensitive test organism, with negative effects on behaviour and mortality at low to medium µg/L-concentrations. One possible explanation for these differences is that human cyclooxygenases (prostaglandin synthase) share more structural similarities with those of teleosts than to those of invertebrates (Gunnarsson *et al.* 2008, Rand-Weaver *et al.* 2013, Verbruggen *et al.* 2018, http://ecodrug.org/ - 18.06.2018). In almost all studies that reported effects of diclofenac in low concentrations, fish were the examined organism (Schwaiger *et al.* 2004, Birzle 2015, Bickley *et al.* 2017, Näslund *et al.* 2017). An exception is the sea urchin *Paracentrotus lividus*, which showed to be an invertebrate with high sensitivity to diclofenac (Ribeiro *et al.* 2015). Sea urchins are deuterostomians, and therefore phylogenetically more closely related to humans than protostomian invertebrates. Further conclusions on the general sensitivity of echinoderms are difficult, because they are rarely applied in ecotoxicological research.

Recent studies also pointed out that molluscs may be sensitive towards NSAIDs: Ericson *et al.* (2010) examined the marine blue mussel *Mytilus edulis* and reported on a lower scope for growth at 100 µg/L. However, the effect was not concentration-dependent and strong effects, like lower byssus strength and lower number of byssus threads, were first evoked at concentrations of 10 mg/L. Schmidt *et al.* (2011) showed that diclofenac leads to DNA-damage and induces oxidative stress in *Mytilus spec.* at concentrations as low as 1 µg/L. Bonnefille *et al.* (2018) found effects on tyrosine and tryptophan metabolism of *Mytilus galloprovincialis* at 122 µg/L diclofenac, which could influence osmoregulation or reproduction. They also pointed out that other modes of action beyond COX-inhibition, i.e. effects on monoamine oxidase, may be primarily responsible for the effects observed in molluscs. In the mesocosm experiments described by Joachim (2017) and James-Casas and Andres (2017), which investigated a broad range of different taxa in the same system, suborganismic effects in molluscs were even more sensitive endpoints than those of fish. Unfortunately, molluscs are not a regular test species in the environmental risk assessment of pharmaceuticals. Due to their sensitivity, it is advisable that further studies are conducted, especially on freshwater-dwelling species, to enlarge the database for this taxon.

A mechanism which is not tackled in my own studies, is the suspected action of NSAIDs on the vertebrate reproductive system. *In vitro* studies pointed out that diclofenac has the potential to interfere with steroid metabolism and inhibits the metabolism of important sex hormones like 17α-hydroxyprogesterone or androstendione (Fernandes *et al.* 2011). In hepatocytes of *Oreochromis niloticus* it up-regulated the expression of genes for the production of vitellogenin (Gröner *et al.* 2015). The same result was seen in *Oryzias latipes* already at concentrations of 1 µg/L (Hong *et al.* 2007). In the frog *Xenopus laevis*, diclofenac altered sex hormone levels, induced the synthesis of vitellogenin, and altered male calling behaviour (Efosa *et al.* 2017). Chae *et al.* (2015) reported teratogenic effects on the protein level for the same species. Various reproductive parameters, like hatchability and fecundity, were decreased in medaka in a two-generation experiment, albeit only at a concentration of 10 mg/L (Lee *et al.* 2011). In a medaka ovulation assay, Yokota *et al.* (2016) demonstrated an *in vitro* antiovulatory effect of diclofenac at 30 mg/L. Moreover, the effect was clearly shown to be a cyclooxygenase-mediated effects, because it was negated by co-exposure to prostaglandin E2. *In vivo*, the effect was much more pronounced: fecundity was reduced at 0.1 mg/L, fertility at 0.05 mg/L, and

the gonadosomatic index already at 0.0125 mg/L. The higher sensitivity in this case is likely due to the longer exposure time in the *in vivo* experiment.

Diclofenac is among the most controversially discussed pharmaceuticals in respect to its environmental risk. After its detrimental effects on Asian vulture populations was uncovered (Oaks *et al.* 2004), various studies examined its effects in other taxa and ecosystems. In fish, multiple studies found histological alterations at low µg/L concentrations (Schwaiger *et al.* 2004, Triebskorn *et al.* 2004, Hoeger *et al.* 2005, Mehinto *et al.* 2010), while others did not find these effects at all, or only at much higher concentrations (Memmert *et al.* 2013, Stepanova *et al.* 2013, Praskova *et al.* 2014). A study re-evaluating several of the published findings questioned the reliability of the low-concentration effects (Wolf *et al.* 2014). It is true that histological analyses require the assessor to have profound knowledge and are, to a certain degree, prone to subjective interpretation. However, the growing body of evidence compiled during the last years supports the hypothesis that low-concentrations of diclofenac can actually affect the integrity of several fish organs (Bickley *et al.* 2017, Gröner *et al.* 2017a, Näslund *et al.* 2017). For those studies, histological assessment was done in a blinded fashion, increasing objectivity of the results. Birzle (2015) even conducted elaborate biometric measurements of histological sections to avoid observer bias – and received highly convincing results. My results add right into this body of evidence. While my histological results were not as clear as seen in some other studies, the effects on the organismic level are of high relevance.

The most sensitive organism to diclofenac was the brown trout *Salmo trutta* f. *fario*, with a $\text{LOEC}_{\text{bite marks}}$ of 10 µg/L.

Effects of metoprolol

Metoprolol did not evoke substantial effects in brown trout embryos and juveniles up to 998 or 745 µg/L, respectively. The observed qualitative histological reactions did not transfer to observable health impairments. Acute tests in fish already showed that fish mortality is not affected by metoprolol until concentrations reach higher mg/L-ranges (Huggett *et al.* 2002, van den Brandhof and Montforts 2010, Moermond and Smit 2016). The crustaceans *G. fossarum* and *D. magna* showed first reactions in the high µg/L to low mg/L-range. Likewise, the gastropod *P. antipodarum* displayed effects in a comparable concentration range. Effect in *L. variegatus* are based on sediment concentrations, and are therefore not directly comparable to water concentrations. For gammarids, as well as gastropods, sensitivity to metoprolol increased when the organisms dwelled in an artificial stream situation (Buchberger *et al.* 2018). Sensitivity comparisons between the tested vertebrate and invertebrate species are difficult. The highest tested concentrations in the fish experiment approximately correspond to the lowest in the invertebrate experiments. I do not know how the fish would have reacted, if they were exposed to slightly higher concentrations. Sun *et al.* (2014) reported that zebrafish embryos exposed to 16 mg/L metoprolol suffered reduced hatching and increased mortality. Furthermore, heart rate was reduced at 1 mg/L, albeit not in a completely concentration-dependent manner. Due to restrictions of animal testing, elaborate pre-testing and range-finding experiments were not possible for fish in my experiments. Therefore, the concentrations based on literature data for adult rainbow trout (Triebskorn *et al.* 2007). In retrospect, these concentrations were too low to derive effect concentrations. However, they traversed an environmentally relevant range, as well as considerably higher concentrations, without finding worrying effects. In a comparative study published by Moermond and Smit (2016), which was based on regulatory and literature data, crustaceans were identified as the most sensitive taxon in terms of traditional endpoints, with a lowest NOEC of 3.1 mg/L. The observed effect was reduced reproduction in a 9 d test with *Daphnia magna* (Dzialowski *et al.* 2006). A study over six generations of *D. magna* by Dietrich *et al.* (2010) yielded a very low LOEC of 1.2 µg/L, but cannot be regarded as fully reliable : the authors tested only a single concentration, and the results were not consistent with those of a mixture treatment in the same experiment. So far, only

results for acute tests are available for crustacean species other than *D. magna* (Huggett *et al.* 2002, Fraysse and Garric 2005, Nalecz-Jawecki and Persoone 2006). Chronic effect data on algae, also reviewed by Moermond and Smit (2016), ranged from 6.14 to 24.3 mg/L. The review based its assessment of fish toxicity on a reported NOEC on fish growth and development of 12.6 mg/L for *Danio rerio* (van den Brandhof and Montforts 2010). However, the more recent results by Gröner *et al.* (2017b), which reported at NOEC for fish growth of 1.1 µg/L, should also be taken into account.

In view of the obtained biomarker results, the most striking finding is the increase of stress protein levels in *P. antipodarum* exposed to metoprolol. The decrease of Hsp70, observed in snails exposed to the highest concentration in the AIS, does not necessarily contradict this finding. Due to technical difficulties in the test setup, there was a concentration gap between 2.95 and 21.95 mg/L. I can only speculate on potential effects, if further intermediate concentrations would have been tested. In the 40d-sampling, reproduction decreased to near zero at the highest test concentration, but was not affected at lower concentrations. It is likely that the organisms exposed to 21.95 suffered drastic physiological stress, which also led to overwhelming of the Hsp70-system (Köhler *et al.* 2001). In my studies, I did not examine the effects of metoprolol on lipid peroxidation in the single substance experiments with trout, gammarids, and gastropods. Lipid peroxide levels of gammarids exposed to metoprolol in the AIS did not react to any of the exposure concentrations. However, in *L. variegatus* the levels of lipid peroxides tended to be higher at rising test concentrations. A recent study showed that metoprolol can lead to oxidative stress in carp *Cyprinus carpio*, visible by increased levels of hydroperoxides, lipid peroxides and carbonylated protein, as well as increased activity of superoxide-dismutase and catalase, in concentrations as low as 10 ng/L (Martinez-Rodriguez *et al.* 2018). The underlying assumption is that the metabolism of metoprolol produces and releases free radicals. A similar effect may be true for the tested oligochaetes. Because the trend was weak and the results are only based on one indicator of oxidative stress, I view my results as a first indication of oxidative effects of metoprolol in invertebrates, rather than definitive evidence.

In comparison to reported environmental concentrations, our own observed lowest effect concentrations do not seem worrisome. WWTP effluent concentrations range up to 5 µg/L (Maurer *et al.* 2007, Scheurer *et al.* 2010, Meyer *et al.* 2016), which is high, compared to other pharmaceuticals, but still considerably lower than our effect values. Moermond and Smit (2016) derived an EQS of 62 µg/L from the above cited studies for metoprolol ecotoxicity. This is considerably higher than surface water and even WWTP effluent concentrations. An environmental risk assessment of atenolol, another beta-blocker, also identified a low risk quotient for the substance (Küster *et al.* 2010). In contrast, ERAs by Godoy *et al.* (2015) concluded that that atenolol, metoprolol and propranolol can pose a risk, but also referred to the paucity of relevant effect data. Within the substance class of beta-blockers, propranolol seems to be the one with the greatest adverse effect potential. It affects sea urchin development at concentrations higher 5 µg/L (Ribeiro *et al.* 2015), has a LC50 of 130 µg/L for *Danio rerio* larvae (Sun *et al.* 2014), decreases growth of medaka at 500 µg/L (Huggett *et al.* 2002) and was generally identified as one of the pharmaceuticals with the highest risk ratios (Fent *et al.* 2006, Donnachie *et al.* 2016). Another potential problem arises from suspected endocrine effects of beta-blockers (Massarsky *et al.* 2011), which are supported by the recent findings of tilapia mRNA expression (Gröner *et al.* 2015, Gröner *et al.* 2017b) and effects of propranolol on medaka sex steroid levels (Huggett *et al.* 2002). None of the conducted studies so far, including ours, can answer the question, whether beta-blockers have adverse long-term effects on fish populations through interference with endocrine pathways. For this purpose, elaborate fully-life-cycle or multi-generation tests will be necessary. One must not forget that the cited evaluations base on single substances, whereas a mixture of all different beta-blockers is present in the environment. Combination toxicity

must be assessed for these substances with shared mode of action to achieve a more realistic risk assessment.

The most sensitive organism to metoprolol in a laboratory setting was the water flea *Daphnia magna*, with a LC_{50} of 2 mg/L. The most sensitive organism in the AIS was the mudsnail *Potamopyrgus antipodarum*, with a $LOEC_{reproduction}$ of 0.22 mg/L.

Stress proteins and lipid peroxides as additional endpoints

In the majority of cases, the levels of stress proteins and lipid peroxides were not significantly affected by the treatment at all. Nevertheless, differences attributable to other factors were common. Gammarids showed distinct differences in Hsp70 according to their age, when animals sampled at the end of the experiments were compared to the t0-control. Likewise, trout exposed in the lab differed in comparison to their conspecifics at the hatchery. In addition to literature data, this demonstrates that the Hsp70-system can react plastically to environmental conditions (Schirling *et al.* 2005). Field data, which showed that fish exposed to differentially polluted streams differ in their stress protein levels, support this assumption (Triebskorn *et al.* 2002, Wilhelm *et al.* 2017). Besides pollution, other environmental (Iwama *et al.* 1998) and seasonal effects are of high importance (Köhler *et al.* 2001). My results were always standardized to the total amount of protein, and effects were always quantified in relation to the respective control. Hence, the effect of such confounding factors should be low in our laboratory experiments, but is nicely demonstrated by the differences to hatchery or t_0 controls. In our case, hints on induced changes by pharmaceuticals were only seen for metoprolol in snails. In the past, the gastropod stress protein system has already proved to be highly inducible by environmental stressors, like in the terrestrial snail *Xeropicta derbentina* exposed to heat (Dieterich *et al.* 2015). This suggests that quantification of Hsp70 is an especially useful additional biomarker for gastropods. For the other tested taxa, there is no sensitivity benefit in comparison to the traditional endpoints. However, this conclusion is restricted to the two tested pharmaceuticals and cannot be generalized for other stressors. Especially for brown trout and gammarids, the method itself worked fairly well and the sample sizes were sufficient to allow a confident interpretation of the results.

Lipid peroxidation was a useful addition to the testing battery for trout embryos exposed to effluent samples. In adult individuals, the results were highly variable but did not show an overall tendency, despite the high sample size. In this context, future studies will fare better when examining a wider range of oxidative stress biomarkers. Not only the final, damaged molecular components, like lipid peroxides and carbonylated proteins, but also defensive systems like catalase, glutathione-S-transferase, or superoxide-dismutase activity. This will allow putting results of one endpoint into a broader physiological context, but is of course only possible when there is sufficient sample mass for all assays.

A major problem encountered in the biochemical analyses of most tested invertebrates was the low sample size. The numbers derived from the official OECD guidelines and originally sufficed for the determination of apical endpoints. However, the need for pooling two, three, or even six individuals to achieve the necessary weight, drastically reduced the sample size. Therefore, statistical security of the results is low, especially in combination with high variation. For small invertebrates (below 10 mg) the test setups according to standard OECD guidelines do not yield enough samples for a thorough analysis of the applied biochemical markers. These tests would need to apply a higher number of replicates, which is difficult to implement and even more labour-intensive. The Hsp70 quantification was easy to implement for *S. trutta* and *P. antipodarum*, challenging for *D. magna* and *G. fossarum*, and very problematic for *L. variegatus*. Protein content was low, and there was hardly any signal after protein band staining, even when applying a higher-than-usual protein amount. These major problems could be due to a large proportion of the animals consisting of digestive tract and its content, which

decreases the volume of actual annelid sample and introduces further confounding factors. It may also be that *Lumbriculus* has in general a very low base level of Hsp70. Overall, I cannot recommend this technique for further investigations on this particular organism.

Stress proteins are endorsed as additional biomarkers for experiments with molluscs, but not for aquatic oligochaetes.

Environmental relevance

Reported environmental concentrations of diclofenac and metoprolol are diverse, and differ considerably depending on country and water body. For metoprolol, Ternes (1998) reported median concentrations of 0.045 µg/L, but the much newer review of Hughes *et al.* (2013) concluded on a median of 0.1045 µg/L. In strongly contaminated rivers, maximum metoprolol concentrations can reach up to 0.52 µg/L (Meyer *et al.* 2016) or even 8.04 µg/L (Hughes *et al.* 2013). However, even these extreme concentrations are much lower than the lowest NOECs resulting from our studies. Hence, an environmental risk of metoprolol as single substance is unlikely. Aus der Beek *et al.* (2016b) summarized global concentrations of diclofenac and reported a mean concentration of 0.16 µg/L for German surface waters. Correspondingly, Letzel *et al.* (2009) calculated a worst-case PEC of 0.14 µg/L. Other reported PECs vary from low to medium ng/L-ranges, rarely above 0.1 µg/L (Letzel *et al.* 2009, Johnson *et al.* 2013). The obtained NOEC of 1 µg/L for brown trout behaviour was in the range of wastewater treatment plant effluent concentrations (Ternes 1998, Letzel *et al.* 2009) and only one order of magnitude higher than some reported MECs for surface water. This is an alarming result, especially considering that the effects were on an organismic level, with high relevance for the overall population. Moreover, the endpoints behaviour, mortality, and histological integrity also reacted at lower concentrations, albeit not to an extent to reach statistical significance. Transfer of data from pure laboratory setups to the environment is always associated with uncertainties. It is likely that the artificial fishkeeping conditions acted as additional stressor. But, as shown in the flow channel experiments for metoprolol, environmental conditions may also act as additional stressors and increase sensitivity. In the wild, fish encounter a range of challenging situations, like fluctuations in water parameters, limited food availability, competition or exposure to adverse chemical stressors. In such stressful conditions, toxicants like diclofenac could act as the final trigger leading to large-scale detrimental effects. To date, I am not aware of any studies linking fish population declines to NSAIDs. In fact, such studies are hardly possible and not realistic, because natural streams always contain complex mixtures of chemicals. Our own studies on WWTP effluents demonstrated the high plasticity of biological endpoints, and their variability in reaction to environmental parameters. There were no obvious adverse effects on brown trout and gammarids, but the field experiments lacked controls that allowed sufficient comparability. When additional environmental effects were accounted for in the lab experiment, the effluent alone had a considerable influence on brown trout larval growth, even though it did not yet transfer to effects on survival. Considering that the applied early life stage is less sensitive than juvenile fish, the effluent may lead to stronger effects in older individuals. Other studies have shown that reduced pollution through WWTP upgrade can in fact improve the health of fish in the receiving water (Maier *et al.* 2015, Wilhelm *et al.* 2017). Although fish were the most sensitive organism in our study, we must not neglect effects on invertebrates. Sensitivities may greatly differ for other substances. A wide range of insects, crustaceans, and other invertebrates showed to be highly sensitive to changes in river systems through wastewater treatment plant discharges (Berger *et al.* 2016). Undoubtedly, effects of chemicals are only one of many stressors: degenerated habitat structure, competition by invasive species, or climate change are also of major importance in freshwater ecosystems. Nevertheless, as there is reason to believe that pharmaceuticals influence the health and survival of freshwater biota, we must take action to ameliorate these effects.

The veterinary use of diclofenac, with specific reference to effects in necrophagous birds, was assessed by the European Medicines Agency (EMEA/V/A/107 - EMA 2014). In view of the high risk for vultures, its use is largely restricted in European countries. In many European states, including Germany, it is not allowed to be used for this purpose at all. In contrast to the acknowledgement of terrestrial environmental risk, the situation is not as clear for the aquatic environment. At the time when the project *Eff-Pharm* was initiated, diclofenac was added to the watch list for potential priority substances of the European framework directive (EU 2013, EU 2015). The aim was to generate further monitoring data and gain information on potential environmental implications (Schröder *et al.* 2016). A tremendous amount of studies found evidence for adverse effects of the pharmaceutical at low µg/L-concentrations, with our own studies adding to this body of evidence. James-Casas and Andres (2017) summarized effect concentrations from literature and a mesocosm study conducted at the same institution (Joachim 2017), and compared them to measured environmental concentrations: they concluded that, if only standard endpoints on the organismic level are considered (PNEC = 1.11 µg/L), calculated risk quotients are acceptable in all but a worst-case urban scenario. However, if suborganismic effects are included (PNEC = 0.025 µg/L), there is a high risk for both rural and urban scenarios. In a preliminary report of the joint research council (Loos *et al.* 2017), it is concluded that the data situation for diclofenac is by now sufficient. Hence, it can be removed from the watch list. The substance's risk quotient is worrisome – it remains to be seen which further actions will be implemented in European legislation to cope with this problem.

10. Conclusions

In vivo studies on NSAIDs, beta-blockers and wastewater treatment plant effluents

Our studies revealed that diclofenac, but not metoprolol, poses a high risk to the aquatic organisms by adversely affecting juvenile fish in concentrations near those found in the environment. Mortality increased at 100 µg/L, histological state of major metabolic organs deteriorated, and fish showed conspicuous behavioural alterations already at 10 µg/L. Earlier life stages of the same species proved to be more resilient to the pharmaceutical exposure. In contrast to fish, freshwater invertebrates reacted with much lower sensitivity to diclofenac. In general, these invertebrates reacted slightly more sensitive to metoprolol, albeit in concentration ranges considerably higher than those reported for European surface waters. Stress proteins and lipid peroxides as biomarkers of effect did not, if at all, react more sensitively than traditional endpoints for the majority of investigated biota. One exception were gastropods, where the Hsp70-system reacted sensitively to metoprolol exposure. In this particular case, the addition of this biomarker might be a useful addition to the testing battery – but should be examined in greater detail. However, the suitability of Hsp70 and lipid peroxides as biomarkers may be different when substances with other modes of action are investigated. Our studies on trout larvae and gammarids revealed reactions when the organisms were exposed to the complex mixture of substances present in wastewater treatment plant effluents. While the results were inconclusive, and likely influenced by other environmental factors, for gammarids, the body mass of trout larvae was considerably reduced by the effluent water. At the same time, analyses of lipid peroxides proved to be a useful additional biomarker in this case, but should be supplemented with other biochemical markers of oxidative stress to facilitate interpretation of the results.

In vitro systems as potential monitoring tools

Besides the wide range of generated *in vivo* effect data, the project *Eff-Pharm* also managed to create the desired *in vitro* systems for quantification of beta-blockers and cyclooxygenase-inhibitors (Bernhard *et al.* 2017). The systems achieve the final result within few minutes, largely limiting the confounding effect of cytotoxicity in the sample. Detection limits were 2 µg/L for beta-blockers (measured in metoprolol-equivalents) and 0.5 µg/L for NSAIDs (measured in diclofenac-equivalents). Therefore, the sensitivity is below the most sensitive effects found in our own studies, and in the range

of the most sensitive effects overall. Further testing and modifications are undertaken to adapt the procedure for application in the field. Moreover, the basic principle can be adapted to a variety of other pharmaceutical groups, to generate a larger battery of useful *in vitro* systems.

These systems will eventually allow quantification of pharmaceutical mixtures and other substances sharing the same mode of action in environmental samples. This will be a highly useful additional technique for the monitoring of surface waters. One of the major challenges in the implementation of the WFD is the vast number of chemicals and mixtures, with insufficient analytical procedures available. Here, effect-based tools promise to be a way for solution-oriented monitoring (Brack *et al.* 2017). Furthermore, comparisons of influent and effluent effects could pose an efficient tool for the investigation of wastewater treatment plant purification efficiency. Another potential use is the assessment of metabolites and transformation product, in respect to their remaining effects according to the parent mode of action. They may also guide further, systematic assessments of mixture toxicity *in vivo*: a combination of *in vitro* analyses and *in vivo* ecotoxicity tests of pharmaceutical mixtures could give us further insights into the mechanisms of mixture toxicity. It remains to be seen how these techniques can be implemented into a broader, regulatory context.

11. Bibliography

Ajima, M. N. O., Ogo, O. A., Audu, B. S. and Ugwoegbu, K. C. (2015). "Chronic diclofenac (DCF) exposure alters both enzymatic and haematological profile of African catfish, *Clarias gariepinus*." *Drug and chemical toxicology* **38**(4): 383-390.

Andersson, T. and Förlin, L. (1992). "Regulation of the cytochrome P450 enzyme system in fish." *Aquatic Toxicology* **24**(1): 1-19.

Arnold, K. E., Brown, A. R., Ankley, G. T. and Sumpter, J. P. (2014). "Medicating the environment: assessing risks of pharmaceuticals to wildlife and ecosystems." *Philosophical Transactions of the Royal Society B-Biological Sciences* **369**(1656): 11.

Arpin-Pont, L., Bueno, M. J. M., Gomez, E. and Fenet, H. (2014). "Occurrence of PPCPs in the marine environment: a review." *Environmental Science and Pollution Research* **23**(6): 4978-4991.

Aus der Beek, T. A., Weber, F. A. and Bergmann, A. (2016). "Pharmaceuticals in the environment: Global occurrence and potential cooperative action under the Strategic Approach to International Chemicals Management (SAICM)". FKZ 371265408. Umweltbundesamt. *UBA Texte*. **67/2016**.

Aus der Beek, T. A., Weber, F. A., Bergmann, A., Hickmann, S., Ebert, I., Hein, A. and Kuster, A. (2016). "Pharmaceuticals in the environment - global occurrences and perspectives." *Environmental Toxicology and Chemistry* **35**(4): 823-835.

Avetta, P., Fabbri, D., Minella, M., Brigante, M., Maurino, V., Minero, C., Pazzi, M. and Vione, D. (2016). "Assessing the phototransformation of diclofenac, clofibric acid and naproxen in surface waters: Model predictions and comparison with field data." *Water Research* **105**: 383-394.

Baron, M. G., Mintram, K. S., Owen, S. F., Hetheridge, M. J., Moody, A. J., Purcell, W. M., Jackson, S. K. and Jha, A. N. (2017). "Pharmaceutical metabolism in fish: using a 3-D hepatic *in vitro* model to assess clearance." *Plos One* **12**(1): 13.

Bellmann, H. (1991). "Spinnen, Krebse, Tausendfüßer - Die farbigen Naturführer". München, *Mosaik Verlag*.

Berger, E., Haase, P., Oetken, M. and Sundermann, A. (2016). "Field data reveal low critical chemical concentrations for river benthic invertebrates." *Science of the Total Environment* **544**: 864-873.

Bergmann, A., Fohrmann, R. and Weber, F. A. (2011). "Zusammenstellung von Monitoringdaten zu Umweltkonzentrationen von Arzneimitteln". German Environment Agency. *UBA Texte*. **66**.

Bernhard, K., Stahl, C., Martens, R., Köhler, H.-R., Triebskorn, R., Scheurer, M. and Frey, M. (2017). "Two novel real time cell-based assays quantify beta-blocker and NSAID specific effects in effluents of municipal wastewater treatment plants." *Water Research* **115**: 74-83.

Bickley, L. K., van Aerle, R., Brown, A. R., Hargreaves, A., Huby, R., Cammack, V., Jackson, R., Santos, E. M. and Tyler, C. R. (2017). "Bioavailability and kidney responses to diclofenac in the fathead minnow (*Pimephales promelas*)." *Environmental Science & Technology* **51**(3): 1764-1774.

Birzle, C. F. (2015). "Etablierung und Validierung quantitativ-morphologischer Parameter bei Regenbogenforellen im Rahmen ökotoxikologischer Fragestellungen". PhD, *University of Munich*.

Bjorkman, D. (1998). "Nonsteroidal anti-inflammatory drug-associated toxicity of the liver, lower gastrointestinal tract, and esophagus." *The American Journal of Medicine* **105**(5, Supplement 1): 17S-21S.

Blüm, V., Casado, J., Lehmann, J. and Mehring, E. (1989). "Farbatlas der Histologie der Regenbogenforelle". Berlin, *Springer Verlag*.

Bonga, S. E. W. (1997). "The stress response in fish." *Physiological Reviews* **77**(3): 591-625.

Bonnefille, B., Gomez, E., Alali, M., Rosain, D., Fenet, H. and Courant, F. (2018). "Metabolomics assessment of the effects of diclofenac exposure on *Mytilus galloprovincialis*: Potential effects on osmoregulation and reproduction." *Science of The Total Environment* **613-614**(Supplement C): 611-618.

Bosakowski, T. and Wagner, E. J. (1994). "A survey of trout fin erosion, water quality, and rearing conditions at state fish hatcheries in Utah." *Journal of the World Aquaculture Society* **25**(2): 308-316.

Boström, M. L. and Berglund, O. (2015). "Influence of pH-dependent aquatic toxicity of ionizable pharmaceuticals on risk assessments over environmental pH ranges." *Water Research* **72**: 154-161.

Brack, W., Dulio, V., Ågerstrand, M., Allan, I., Altenburger, R., Brinkmann, M., Bunke, D., Burgess, R. M., Cousins, I., Escher, B. I., Hernández, F. J., Hewitt, L. M., Hilscherová, K., Hollender, J., Hollert, H., Kase, R., Klauer, B., Lindim, C., Herráez, D. L., Miège, C., Munthe, J., O'Toole, S., Posthuma, L., Rüdel, H., Schäfer, R. B., Sengl, M., Smedes, F., van de Meent, D., van den Brink, P. J., van Gils, J., van Wezel, A. P., Vethaak, A. D., Vermeirssen, E., von der Ohe, P. C. and Vrana, B. (2017). "Towards the review of the European Union Water Framework Directive: recommendations for more efficient assessment and management of chemical contamination in European surface water resources." *Science of The Total Environment* **576**: 720-737.
Bradford, M. M. (1976). "Rapid and sensitive method for quantitation of microgram quantities of protein utilizing principle of protein-dye binding." *Analytical Biochemistry* **72**(1-2): 248-254.
Brandmayr, C., Kerber, H., Winker, M. and Schramm, E. (2015). "Impact assessment of emission management strategies of the pharmaceuticals Metformin and Metoprolol to the aquatic environment using Bayesian networks." *Science of The Total Environment* **532**: 605-616.
Brausch, J. M., Connors, K. A., Brooks, B. W. and Rand, G. M. (2012). "Human pharmaceuticals in the aquatic environment: a review of recent toxicological studies and considerations for toxicity testing." *Reviews of Environmental Contamination and Toxicology* **218**: 1-99.
Brodin, T., Fick, J., Jonsson, M. and Klaminder, J. (2013). "Dilute concentrations of a psychiatric drug alter behavior of fish from natural populations." *Science* **339**(6121): 814-815.
Brodin, T., Piovano, S., Fick, J., Klaminder, J., Heynen, M. and Jonsson, M. (2014). "Ecological effects of pharmaceuticals in aquatic systems-impacts through behavioural alterations." *Philosophical Transactions of the Royal Society B-Biological Sciences* **369**(1656): 10.
Buchberger, A.-K., Brettschneider, D., Berg, K., Jungmann, D., Oehlmann, J., Scheurer, M. and Oetken, M. (2018). "Effects of metoprolol on aquatic invertebrates in artificial indoor streams." *Journal of Environmental Science and Health, Part A* **53**(8): 1-12.
Buser, H.-R., Poiger, T. and Müller, M. D. (1998). "Occurrence and fate of the pharmaceutical drug diclofenac in surface waters: rapid photodegradation in a lake." *Environmental Science & Technology* **32**(22): 3449-3456.
Cannon, C. P. and Cannon, P. J. (2012). "COX-2 inhibitors and cardiovascular risk." *Science* **336**(6087): 1386-1387.
Cañon Jones, H. A., Hansen, L. A., Noble, C., Damsgård, B., Broom, D. M. and Pearce, G. P. (2010). "Social network analysis of behavioural interactions influencing fin damage development in Atlantic salmon (*Salmo salar*) during feed-restriction." *Applied Animal Behaviour Science* **127**(3–4): 139-151.
Carpenter, S. R., Caraco, N. F., Correll, D. L., Howarth, R. W., Sharpley, A. N. and Smith, V. H. (1998). "Nonpoint pollution of surface waters with phosphorus and nitrogen." *Ecological Applications* **8**(3): 559-568.
Chae, J.-P., Park, M. S., Hwang, Y.-S., Min, B.-H., Kim, S.-H., Lee, H.-S. and Park, M.-J. (2015). "Evaluation of developmental toxicity and teratogenicity of diclofenac using *Xenopus* embryos." *Chemosphere* **120**: 52-58.
Choi, J. S., Jin, M. J. and Han, H. K. (2005). "Role of monocarboxylic acid transporters in the cellular uptake of NSAIDs." *Journal of Pharmacy and Pharmacology* **57**(9): 1185-1189.
Contardo-Jara, V., Pflugmacher, S., Nützmann, G., Kloas, W. and Wiegand, C. (2010). "The β-receptor blocker metoprolol alters detoxification processes in the non-target organism *Dreissena polymorpha*." *Environmental Pollution* **158**(6): 2059-2066.
Crisp, D. T. (1996). "Environmental requirements of common riverine European salmonid fish species in fresh water with particular reference to physical and chemical aspects." *Hydrobiologia* **323**(3): 201-221.
Cuklev, F., Kristiansson, E., Fick, J., Asker, N., Förlin, L. and Larsson, D. G. J. (2011). "Diclofenac in fish: blood plasma levels similar to human therapeutic levels affect global hepatic gene expression." *Environmental Toxicology and Chemistry* **30**(9): 2126-2134.
Daughton, C. G. and Ternes, T. A. (1999). "Pharmaceuticals and personal care products in the environment: agents of subtle change?" *Environmental Health Perspectives* **107**: 907-938.

DeGeorge Jr, B. R. and Koch, W. J. (2007). "Beta blocker specificity: A building block toward personalized medicine." *Journal of Clinical Investigation* **117**(1): 86-89.
Dieterich, A., Troschinski, S., Schwarz, S., Di Lellis, M. A., Henneberg, A., Fischbach, U., Ludwig, M., Gärtner, U., Triebskorn, R. and Köhler, H.-R. (2015). "Hsp70 and lipid peroxide levels following heat stress in *Xeropicta derbentina* (Krynicki 1836) (Gastropoda, Pulmonata) with regard to different colour morphs." *Cell Stress and Chaperones* **20**(1): 159-168.
Dietrich, S., Ploessl, F., Bracher, F. and Laforsch, C. (2010). "Single and combined toxicity of pharmaceuticals at environmentally relevant concentrations in *Daphnia magna* – A multigenerational study." *Chemosphere* **79**(1): 60-66.
Donnachie, R. L., Johnson, A. C. and Sumpter, J. P. (2016). "A rational approach to selecting and ranking some pharmaceuticals of concern for the aquatic environment and their relative importance compared with other chemicals." *Environmental Toxicology and Chemistry* **35**(4): 1021-1027.
Dußling, U. and Berg, R. (2001). "Fische in Baden-Württemberg". Stuttgart, Germany, *Ministerium für Ernährung und Ländlichen Raum Baden-Württemberg*.
Dzialowski, E. M., Turner, P. K. and Brooks, B. W. (2006). "Physiological and reproductive effects of beta adrenergic receptor antagonists in *Daphnia magna*." *Archives of Environmental Contamination and Toxicology* **50**(4): 503-510.
Ebert, I., Amato, R., Hein, A. and Konradi, S. (2014). "Arzneimittel in der Umwelt - vermeiden, reduzieren, überwachen". Umweltbundesamt.
Efosa, N. J., Kleiner, W., Kloas, W. and Hoffmann, F. (2017). "Diclofenac can exhibit estrogenic modes of action in male *Xenopus laevis*, and affects the hypothalamus-pituitary-gonad axis and mating vocalizations." *Chemosphere* **173**: 69-77.
EMA (2014). "CVMP assessment report under Article 30(3) of Regulation (EC) No 726/2004 - On the risk to vultures and other necrophagous bird populations in the European Union in connection with the use of veterinary medicinal products containing the substance diclofenac". EMEA/V/A/107. European Medicines Agency.
Ericson, H., Thorsén, G. and Kumblad, L. (2010). "Physiological effects of diclofenac, ibuprofen and propranolol on Baltic Sea blue mussels." *Aquatic Toxicology* **99**(2): 223-231.
Escher, B. I., Allinson, M., Altenburger, R., Bain, P. A., Balaguer, P., Busch, W., Crago, J., Denslow, N. D., Dopp, E., Hilscherova, K., Humpage, A. R., Kumar, A., Grimaldi, M., Jayasinghe, B. S., Jarosova, B., Jia, A., Makarov, S., Maruya, K. A., Medvedev, A., Mehinto, A. C., Mendez, J. E., Poulsen, A., Prochazka, E., Richard, J., Schifferli, A., Schlenk, D., Scholz, S., Shiraish, F., Snyder, S., Su, G. Y., Tang, J. Y. M., van der Burg, B., van der Linden, S. C., Werner, I., Westerheide, S. D., Wong, C. K. C., Yang, M., Yeung, B. H. Y., Zhang, X. W. and Leusch, F. D. L. (2014). "Benchmarking organic micropollutants in wastewater, recycled water and drinking water with *in vitro* bioassays." *Environmental Science & Technology* **48**(3): 1940-1956.
Escher, B. I., Bramaz, N., Richter, M. and Lienert, J. (2006). "Comparative ecotoxicological hazard assessment of beta-blockers and their human metabolites using a mode-of-action-based test battery and a QSAR approach." *Environmental Science & Technology* **40**(23): 7402-7408.
EU (2000). "CVMP/VICH/592/98-FINAL - guideline on environmental impact assessment (EIAS) for veterinary medicinal products - phase I". T. E. A. f. t. E. o. M. Products. London.
EU (2004). "CVMP/VICH/790/03-FINAL - guideline on environmental impact assessment for veterinary medicinal products phase II". E. M. Agency. London.
EU (2006). "Council Directive 2006/88/EC on animal health requirements for aquaculture animals and products thereof, and on the prevention and control of certain diseases in aquatic animals". *Official Journal of the European Union*. **49**: 14-56.
EU (2006). "EMEA/CHMP/SWP/4447/00 corr 2 - guideline on the environmental risk assessment of medicinal products for human use". E. M. Agency. London.
EU (2012). "COM(2011) 876 final 2011/0429 (COD) proposal for a Directive of the European Parliament and of the Council amending Directives 2000/60/EC and 2008/105/EC as regards priority substances in the field of water policy".

EU (2013). "Directive 2013/39/EU of the European Parliament and of the Council of 12 August 2013 amending Directives 2000/60/EC and 2008/105/EC as regards priority substances in the field of water policy". *Official Journal of the European Union*. **L226**: 1-17.
EU (2015). "Commission implementing Decision (EU) 2015/495 of 20 March 2015 establishing a watch list of substances for Union-wide monitoring in the field of water policy pursuant to Directive 2008/105/EC of the European Parliament and of the Council". *Official Journal of the European Union*. **L78**: 40 - 42.
Fent, K., Weston, A. A. and Caminada, D. (2006). "Ecotoxicology of human pharmaceuticals." *Aquatic Toxicology* **76**(2): 122-159.
Fernandes, D., Schnell, S. and Porte, C. (2011). "Can pharmaceuticals interfere with the synthesis of active androgens in male fish? An *in vitro* study." *Marine Pollution Bulletin* **62**(10): 2250-2253.
Fernandez, C., Carbonell, G. and Babin, M. (2013). "Effects of individual and a mixture of pharmaceuticals and personal-care products on cytotoxicity, EROD activity and ROS production in a rainbow trout gonadal cell line (RTG-2)." *Journal of Applied Toxicology* **33**(11): 1203-1212.
Ferreira, H., Lúcio, M., Lima, J. L. F. C., Matos, C. and Reis, S. (2005). "Effects of diclofenac on EPC liposome membrane properties." *Analytical and Bioanalytical Chemistry* **382**(5): 1256-1264.
Fomin, A., Oehlmann, J. and Markert, B. (2003). "Praktikum zur Ökotoxikologie - Grundlagen und Anwendungen biologischer Testverfahren". Landsberg, *ecomed verlagsgesellschaft AG & Co. KG*.
Fraysse, B. and Garric, J. (2005). "Prediction and experimental validation of acute toxicity of β-blockers in *Ceriodaphnia dubia*." *Environmental Toxicology and Chemistry* **24**(10): 2470-2476.
Fu, W., Franco, A. and Trapp, S. (2009). "Methods for estimating the bioconcentration factor of ionizable organic chemicals." *Environmental Toxicology and Chemistry* **28**(7): 1372-1379.
Funk, C. D. (2001). "Prostaglandins and leukotrienes: advances in eicosanoid biology." *Science* **294**(5548): 1871-1875.
Gan, T. J. (2010). "Diclofenac: an update on its mechanism of action and safety profile." *Current Medical Research and Opinion* **26**(7): 1715-1731.
Gernhöfer, M., Pawert, M., Schramm, M., Müller, E. and Triebskorn, R. (2001). "Ultrastructural biomarkers as tools to characterize the health status of fish in contaminated streams." *Journal of Aquatic Ecosystem Stress and Recovery* **8**(3): 241-260.
Ghelfi, A., Ribas, J. L. C., Guiloski, I. C., Bettim, F. L., Piancini, L. D. S., Cestari, M. M., Pereira, A. J., Sassaki, G. L. and Silva de Assis, H. C. (2016). "Evaluation of biochemical, genetic and hematological biomarkers in a commercial catfish *Rhamdia quelen* exposed to diclofenac." *Bulletin of environmental contamination and toxicology* **96**(1): 49-54.
Godoy, A. A., Kummrow, P. and Pamplin, P. A. Z. (2015). "Occurrence, ecotoxicological effects and risk assessment of antihypertensive pharmaceutical residues in the aquatic environment - A review." *Chemosphere* **138**: 281-291.
Gonzalez-Rey, M. and Bebianno, M. J. (2014). "Effects of non-steroidal anti-inflammatory drug (NSAID) diclofenac exposure in mussel *Mytilus galloprovincialis*." *Aquatic Toxicology* **148**: 221-230.
Goudie, A. S. (2013). "The human impact on the natural environment : past, present, and future". Chichester, *WILEY Blackwell*.
Grabow, K. (2000). "Farbatlas Süßwasserfauna Wirbellose". Stuttgart, *Eugen Ulmer GmbH & Co.*
Gravel, A. and Vijayan, M. M. (2007). "Non-steroidal anti-inflammatory drugs disrupt the heat shock response in rainbow trout." *Aquatic Toxicology* **81**(2): 197-206.
Gröner, F., Hohne, C., Kleiner, W. and Kloas, W. (2017). "Chronic diclofenac exposure affects gill integrity and pituitary gene expression and displays estrogenic activity in nile tilapia (*Oreochromis niloticus*)." *Chemosphere* **166**: 473-481.
Gröner, F., Hohne, C., Kleiner, W. and Kloas, W. (2017). "Chronic exposure to the beta-blocker metoprolol reduces growth and alters gene expression of gonadotropins and vitellogenin in Nile tilapia (*Oreochromis niloticus*)." *Ecotoxicology and Environmental Safety* **141**: 271-279.
Gröner, F., Ziková, A. and Kloas, W. (2015). "Effects of the pharmaceuticals diclofenac and metoprolol on gene expression levels of enzymes of biotransformation, excretion pathways and estrogenicity in

primary hepatocytes of Nile tilapia (*Oreochromis niloticus*)." *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology* **167**: 51-57.
Guiloski, I. C., Ribas, J. L. C., Pereira, L. d. S., Neves, A. P. P. and Silva de Assis, H. C. (2015). "Effects of trophic exposure to dexamethasone and diclofenac in freshwater fish." *Ecotoxicology and Environmental Safety* **114**: 204-211.
Guiloski, I. C., Stein Piancini, L. D., Dagostim, A. C., de Morais Calado, S. L., Fávaro, L. F., Boschen, S. L., Cestari, M. M., da Cunha, C. and Silva de Assis, H. C. (2017). "Effects of environmentally relevant concentrations of the anti-inflammatory drug diclofenac in freshwater fish *Rhamdia quelen*." *Ecotoxicology and Environmental Safety* **139**: 291-300.
Gunnarsson, L., Jauhiainen, A., Kristiansson, E., Nerman, O. and Larsson, D. G. J. (2008). "Evolutionary conservation of human drug targets in organisms used for environmental risk assessments." *Environmental Science & Technology* **42**(15): 5807-5813.
Haap, T., Triebskorn, R. and Köhler, H. R. (2008). "Acute effects of diclofenac and DMSO to *Daphnia magna*: immobilisation and hsp70-induction." *Chemosphere* **73**(3): 353-359.
Hallare, A. V., Köhler, H. R. and Triebskorn, R. (2004). "Development toxicity and stress protein responses in zebrafish embryos after exposure to diclofenac and its solvents, DMSO." *Chemosphere* **56**(7): 659-666.
Hamdorf, K. (1960). "Die Beeinflussung der Embryonal- und Larvalentwicklung der Regenbogenforelle (*Salmo irideus* Gibb.) durch Strahlung im sichtbaren Bereich." *Zeitschrift für vergleichende Physiologie* **42**(6): 525-565.
Harris, C. A., Scott, A. P., Johnson, A. C., Panter, G. H., Sheahan, D., Roberts, M. and Sumpter, J. P. (2014). "Principles of sound ecotoxicology." *Environmental Science & Technology* **48**(6): 3100-3111.
Heberer, T. and Feldmann, D. (2005). "Contribution of effluents from hospitals and private households to the total loads of diclofenac and carbamazepine in municipal sewage effluents—modeling versus measurements." *Journal of Hazardous Materials* **122**(3): 211-218.
Hermes-Lima, M., Willmore, W. G. and Storey, K. B. (1995). "Quantification of lipid-peroxidation in tissue-extracts based on Fe(III)xylenol orange complex formation." *Free Radical Biology and Medicine* **19**(3): 271-280.
Hickey, E. J., Raje, R. R., Reid, V. E., Gross, S. M. and Ray, S. D. (2001). "Diclofenac induced in vivo nephrotoxicity may involve oxidative stress-mediated massive genomic DNA fragmentation and apoptotic cell death." *Free Radical Biology and Medicine* **31**(2): 139-152.
Hoeger, B., Dietrich, D. R., Schmid, D., Hartmann, A. and Hitzfeld, B. (2008). "Distribution of intraperitoneally injected diclofenac in brown trout (*Salmo trutta* f. *fario*)." *Ecotoxicology and Environmental Safety* **71**(2): 412-418.
Hoeger, B., Köllner, B., Dietrich, D. R. and Hitzfeld, B. (2005). "Water-borne diclofenac affects kidney and gill integrity and selected immune parameters in brown trout (*Salmo trutta* f. *fario*)." *Aquatic Toxicology* **75**(1): 53-64.
Hong, H. N., Kim, H. N., Park, K. S., Lee, S. K. and Gu, M. B. (2007). "Analysis of the effects diclofenac has on Japanese medaka (*Oryzias latipes*) using real-time PCR." *Chemosphere* **67**(11): 2115-2121.
Huggett, D. B., Brooks, B. W., Peterson, B., Foran, C. M. and Schlenk, D. (2002). "Toxicity of select beta adrenergic receptor-blocking pharmaceuticals (B-blockers) on aquatic organisms." *Archives of Environmental Contamination and Toxicology* **43**(2): 229-235.
Hughes, S. R., Kay, P. and Brown, L. E. (2013). "Global synthesis and critical evaluation of pharmaceutical data sets collected from river systems." *Environmental Science & Technology* **47**(2): 661-677.
Hurlbert, S. H. (1984). "Pseudoreplication and the design of ecological field experiments." *Ecological Monographs* **54**(2): 187-211.
Islas-Flores, H., Gomez-Olivan, L. M., Galar-Martinez, M., Colin-Cruz, A., Neri-Cruz, N. and Garcia-Medina, S. (2013). "Diclofenac-induced oxidative stress in brain, liver, gill and blood of common carp (*Cyprinus carpio*)." *Ecotoxicology and Environmental Safety* **92**: 32-38.
Iwama, G. K., Thomas, P. T., Forsyth, R. B. and Vijayan, M. M. (1998). "Heat shock protein expression in fish." *Reviews in Fish Biology and Fisheries* **8**(1): 35-56.

James-Casas, A. and Andres, S. (2017). "Report on the aquatic environmental risk assessment for diclofenac". Development of robust tools for the assessment of pharmaceuticals in aquatic media (DOREMIPHARM). Verneuil-En-Halatte, Oise, France. *INERIS*. DRC-16-129627-12069A. 55.
Ji, K., Liu, X., Lee, S., Kang, S., Kho, Y., Giesy, J. P. and Choi, K. (2013). "Effects of non-steroidal anti-inflammatory drugs on hormones and genes of the hypothalamic-pituitary-gonad axis, and reproduction of zebrafish." *Journal of Hazardous Materials* **254-255**: 242-251.
Joachim, S. (2017). "Bottom-up and top-down effects in a diclofenac contaminated model ecosystem - Task 2: Mesocosms study". Development of robust tools for the assessment of pharmaceuticals in aquatic media (DOREMIPHARM). Verneuil-En-Halatte, Oise, France. *INERIS*. DRC-16-129627-11897A. 87.
Johnson, A. C., Dumont, E., Williams, R. J., Oldenkamp, R., Cisowska, I. and Sumpter, J. P. (2013). "Do concentrations of ethinylestradiol, estradiol, and diclofenac in European rivers exceed proposed EU environmental quality standards?" *Environmental Science & Technology* **47**(21): 12297-12304.
Jones, H. A. C., Noble, C., Damsgard, B. and Pearce, G. P. (2011). "Social network analysis of the behavioural interactions that influence the development of fin damage in Atlantic salmon parr (*Salmo salar*) held at different stocking densities." *Applied Animal Behaviour Science* **133**(1-2): 117-126.
Jonsson, N. and Jonsson, B. (1999). "Trade-off between egg mass and egg number in brown trout." *Journal of Fish Biology* **55**(4): 767-783.
Kawada, N., Moriyama, T., Kitamura, H., Yamamoto, R., Furumatsu, Y., Matsui, I., Takabatake, Y., Nagasawa, Y., Imai, E., Wilcox, C. S., Rakugi, H. and Isaka, Y. (2012). "Towards developing new strategies to reduce the adverse side-effects of nonsteroidal anti-inflammatory drugs." *Clinical and Experimental Nephrology* **16**(1): 25-29.
Kidd, K. A., Paterson, M. J., Rennie, M. D., Podemski, C. L., Findlay, D. L., Blanchfield, P. J. and Liber, K. (2014). "Direct and indirect responses of a freshwater food web to a potent synthetic oestrogen." *Philosophical Transactions of the Royal Society B: Biological Sciences* **369**(1656).
Killeen, J., McLay, H. A. and Johnston, I. A. (1999). "Development in *Salmo trutta* at different temperatures, with a quantitative scoring method for intraspecific comparisons." *Journal of Fish Biology* **55**(2): 382-404.
Killeen, J. R., McLay, H. A. and Johnston, I. A. (1999). "Temperature and neuromuscular development in embryos of the trout (*Salmo trutta* L.)." *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology* **122**(1): 53-64.
Klemetsen, A., Amundsen, P. A., Dempson, J. B., Jonsson, B., Jonsson, N., O'Connell, M. F. and Mortensen, E. (2003). "Atlantic salmon *Salmo salar* L., brown trout *Salmo trutta* L. and Arctic charr *Salvelinus alpinus* (L.): a review of aspects of their life histories." *Ecology of Freshwater Fish* **12**(1): 1-59.
Köhler, H.-R. (2009). "Die Rolle von Stressproteinen bei der Anpassung an Umweltbedingungen: Ökophysiologische, ökotoxikologische und evolutionsbiologische Implikationen." *Umweltwissenschaften und Schadstoff-Forschung* **21**: 150-159.
Köhler, H. R., Bartussek, C., Eckwert, H., Farian, K., Gränzer, S., Knigge, T. and Kunz, N. (2001). "The hepatic stress protein (hsp70) response to interacting abiotic parameters in fish exposed to various levels of pollution." *Journal of Aquatic Ecosystem Stress and Recovery* **8**(3-4): 261-279.
Küster, A., Alder, A. C., Escher, B. I., Duis, K., Fenner, K., Garric, J., Hutchinson, T. H., Lapen, D. R., Péry, A., Römbke, J., Snape, J., Ternes, T., Topp, E., Wehrhan, A. and Knacker, T. (2010). "Environmental risk assessment of human pharmaceuticals in the European Union: A case study with the β-blocker atenolol." *Integrated Environmental Assessment and Management* **6**(S1): 514-523.
Larsson, D. G. J. (2014). "Pollution from drug manufacturing: review and perspectives." *Philosophical Transactions of the Royal Society B: Biological Sciences* **369**(1656).
Lauruschkus, F., Wiedmer, S., Buhnar, K. and Aettner, J. (2017). "Arzneimittelverbrauch im Spannungsfeld des demografischen Wandels". Civity Management Consultants GmbH & Co. KG.
Lee, J., Ji, K., Kho, Y. L., Kim, P. and Choi, K. (2011). "Chronic exposure to diclofenac on two freshwater cladocerans and Japanese medaka." *Ecotoxicology and Environmental Safety* **74**(5): 1216-1225.

Letzel, M., Metzner, G. and Letzel, T. (2009). "Exposure assessment of the pharmaceutical diclofenac based on long-term measurements of the aquatic input." *Environment International* **35**(2): 363-368.
Lewis, S., Handy, R. D., Cordi, B., Billinghurst, Z. and Depledge, M. H. (1999). "Stress proteins (HSP's): methods of detection and their use as an environmental biomarker." *Ecotoxicology* **8**(5): 351-368.
LfW (2004). "Ökotoxikologische Auswirkungen von Arzneimitteln. Langzeitwirkungen bei Fischen.". *Abschlussbericht des Bayerischen Landesamtes für Wasserwirtschaft zum Forschungs- und Entwicklungsvorhaben 2001–2003.*
Lonappan, L., Brar, S. K., Das, R. K., Verma, M. and Surampalli, R. Y. (2016). "Diclofenac and its transformation products: environmental occurrence and toxicity - a review." *Environment International* **96**: 127-138.
Loos, R., Gawlik, B. M., Locoro, G., Rimaviciute, E., Contini, S. and Bidoglio, G. (2009). "EU-wide survey of polar organic persistent pollutants in European river waters." *Environmental Pollution* **157**(2): 561-568.
Loos, R., Marinov, D., Sanseverino, I., Napierska, D. and Lettieri, T. (2017). "Draft: Review of the 1st Watch List under the Water Framework Directive and recommendations for the 2nd Watch List". European Commission. *JRC Technical reports*.
López-Sendó, J., Swedberg, K., McMurray, J., Tamargo, J., Maggioni, A. P., Dargie, H., Tendera, M., Waagstein, F., Kjekshus, J., Lechat, P., Pedersen, C. T., Priori, S. G., Alonso García, M. A., Blanc, J.-J., Budaj, A., Cowie, M., Dean, V., Deckers, J., Fernandez Burgos, E., Lekakis, J., Lindahl, B., Mazzotta, G., McGregor, K., Morais, J., Oto, A., Smiseth, O. A., Alonso García, M. A., Ardissino, D., Avendano, C., Lundqvist, C. B., ment, D. C., Drexler, H., Ferrari, R., Fox, K. A., Julian, D., Kearney, P., Klein, W., ber, L. K., Mancia, G., Nieminen, M., Ruzyllo, W., Simoons, M., Thygesen, K., Tognoni, G., Tritto, I. and Wallentin, L. (2004). "Expert consensus document on β-adrenergic receptor blockers The Task Force on Beta-Blockers of the European Society of Cardiology." *European Heart Journal* **25**(15): 1341-1362.
Luddeke, F., Hess, S., Gallert, C., Winter, J., Gude, H. and Loffler, H. (2015). "Removal of total and antibiotic resistant bacteria in advanced wastewater treatment by ozonation in combination with different filtering techniques." *Water Research* **69**: 243-251.
Luo, Y. L., Guo, W. S., Ngo, H. H., Nghiem, L. D., Hai, F. I., Zhang, J., Liang, S. and Wang, X. C. C. (2014). "A review on the occurrence of micropollutants in the aquatic environment and their fate and removal during wastewater treatment." *Science of the Total Environment* **473**: 619-641.
Lushchak, V. I. (2011). "Environmentally induced oxidative stress in aquatic animals." *Aquatic Toxicology* **101**(1): 13-30.
MacNeil, C., Dick, J. T. and Elwood, R. W. (1997). "The trophic ecology of freshwater *Gammarus* spp. (Crustacea: Amphipoda): Problems and perspectives concerning the functional feeding group concept." *Biological Reviews of the Cambridge Philosophical Society* **72**(3): 349-364.
MacNeil, C., Dick, J. T. A. and Elwood, R. W. (1999). "The dynamics of predation on *Gammarus* spp. (Crustacea: Amphipoda)." *Biological Reviews* **74**(4): 375-395.
Maier, D., Blaha, L., Giesy, J. P., Henneberg, A., Kohler, H. R., Kuch, B., Osterauer, R., Peschke, K., Richter, D., Scheurer, M. and Triebskorn, R. (2015). "Biological plausibility as a tool to associate analytical data for micropollutants and effect potentials in wastewater, surface water, and sediments with effects in fishes." *Water Research* **72**: 127-144.
Martinez-Rodriguez, H., Donkor, K., Brewer, S., Galar-Martinez, M., SanJuan-Reyes, N., Islas-Flores, H., Sanchez-Aceves, L., Elizalde-Velazquez, A. and Gomez-Olivan, L. M. (2018). "Metoprolol induces oxidative damage in common carp (*Cyprinus carpio*)." *Aquatic Toxicology* **197**: 122-135.
Massarsky, A., Trudeau, V. L. and Moon, T. W. (2011). "β-blockers as endocrine disruptors: the potential effects of human β-blockers on aquatic organisms." *Journal of Experimental Zoology Part A: Ecological Genetics and Physiology* **315A**(5): 251-265.
Maszkowska, J., Stolte, S., Kumirska, J., Lukaszewicz, P., Mioduszewska, K., Puckowski, A., Caban, M., Wagil, M., Stepnowski, P. and Bialk-Bielinska, A. (2014). "Beta-blockers in the environment: Part II. Ecotoxicity study." *Science of the Total Environment* **493**: 1122-1126.

Maszkowska, J., Stolte, S., Kumirska, J., Łukaszewicz, P., Mioduszewska, K., Puckowski, A., Caban, M., Wagil, M., Stepnowski, P. and Białk-Bielińska, A. (2014). "Beta-blockers in the environment: Part I. Mobility and hydrolysis study." *Science of The Total Environment* **493**: 1112-1121.
Maurer, M., Escher, B. I., Richle, P., Schaffner, C. and Alder, A. C. (2007). "Elimination of β-blockers in sewage treatment plants." *Water Research* **41**(7): 1614-1622.
McRae, N. K., Glover, C. N., Burket, S. R., Brooks, B. W. and Gaw, S. (2018). "Acute exposure to an environmentally relevant concentration of diclofenac elicits oxidative stress in the culturally important galaxiid fish *Galaxias maculatus*." *Environmental Toxicology and Chemistry* **37**(1): 224-235.
Mehinto, A. C., Hill, E. M. and Tyler, C. R. (2010). "Uptake and biological effects of environmentally relevant concentrations of the nonsteroidal anti-inflammatory pharmaceutical diclofenac in rainbow trout (*Oncorhynchus mykiss*)." *Environmental Science & Technology* **44**(6): 2176-2182.
Memmert, U., Peither, A., Burri, R., Weber, K., Schmidt, T., Sumpter, J. P. and Hartmann, A. (2013). "Diclofenac: new data on chronic toxicity and bioconcentration in fish." *Environmental Toxicology and Chemistry* **32**(2): 442-452.
Meyer, W., Reich, M., Beier, S., Behrendt, J., Gulyas, H. and Otterpohl, R. (2016). "Measured and predicted environmental concentrations of carbamazepine, diclofenac, and metoprolol in small and medium rivers in northern Germany." *Environmental Monitoring and Assessment* **188**(8): 16.
Moermond, C. T. A., Kase, R., Korkaric, M. and Ågerstrand, M. (2015). "CRED: Criteria for reporting and evaluating ecotoxicity data." *Environmental Toxicology and Chemistry*: n/a-n/a.
Moermond, C. T. A. and Smit, C. E. (2016). "Derivation of water quality standars for carbamazepine, metoprolol, and metformin and comparison with monitoring data." *Environmental Toxicology and Chemistry* **35**(4): 882-888.
Monserrat, J. M., Geracitano, L. A., Pinho, G. L. L., Vinagre, T. M., Faleiros, M., Alciati, J. C. and Bianchini, A. (2003). "Determination of lipid peroxides in invertebrates tissues using the Fe(III) xylenol orange complex formation." *Archives of Environmental Contamination and Toxicology* **45**(2): 177-183.
Monteiro, S. C. and Boxall, A. B. A. (2010). "Occurrence and fate of human pharmaceuticals in the environment." *Reviews of Environmental Contamination and Toxicology* **202**: 53-154.
Morimoto, R. I. (1993). "Cells in stress: Transcriptional activation of heat shock genes." *Science* **259**(5100): 1409-1410.
Nalecz-Jawecki, G. and Persoone, G. (2006). "Toxicity of selected pharmaceuticals to the anostracan crustacean *Thamnocephalus platyurus* - Comparison of sublethal and lethal effect levels with the 1h Rapidtoxkit and the 24h Thamnotoxkit microbiotests." *Environmental Science and Pollution Research* **13**(1): 22-27.
Näslund, J., Fick, J., Asker, N., Ekman, E., Larsson, D. G. J. and Norrgren, L. (2017). "Diclofenac affects kidney histology in the three-spined stickleback (*Gasterosteus aculeatus*) at low µg/L concentrations." *Aquatic Toxicology* **189**: 87-96.
Nassef, M., Matsumoto, S., Seki, M., Khalil, F., Kang, I. J., Shimasaki, Y., Oshima, Y. and Honjo, T. (2010). "Acute effects of triclosan, diclofenac and carbamazepine on feeding performance of Japanese medaka fish (*Oryzias latipes*)." *Chemosphere* **80**(9): 1095-1100.
Nieto, E., Corada-Fernández, C., Hampel, M., Lara-Martín, P. A., Sánchez-Argüello, P. and Blasco, J. (2017). "Effects of exposure to pharmaceuticals (diclofenac and carbamazepine) spiked sediments in the midge, *Chironomus riparius* (Diptera, Chironomidae)." *Science of The Total Environment* **609**: 715-723.
Noble, C., Mizusawa, K., Suzuki, K. and Tabata, M. (2007). "The effect of differing self-feeding regimes on the growth, behaviour and fin damage of rainbow trout held in groups." *Aquaculture* **264**(1-4): 214-222.
Novoa-Luna, K. A., Romero-Romero, R., Natividad-Rangel, R., Galar-Martinez, M., SanJuan-Reyes, N., Garcia-Medina, S., Martinez-Vieyra, C., Neri-Cruz, N. and Gomez-Olivan, L. M. (2016). "Oxidative stress induced in *Hyalella azteca* by an effluent from a NSAID-manufacturing plant in Mexico." *Ecotoxicology* **25**(7): 1288-1304.
Nunes, B., Campos, J. C., Gomes, R., Braga, M. R., Ramos, A. S., Antunes, S. C. and Correia, A. T. (2015). "Ecotoxicological effects of salicylic acid in the freshwater fish *Salmo trutta fario*: antioxidant

mechanisms and histological alterations." *Environmental Science and Pollution Research* **22**(1): 667-678.
Oaks, J. L., Gilbert, M., Virani, M. Z., Watson, R. T., Meteyer, C. U., Rideout, B. A., Shivaprasad, H. L., Ahmed, S., Chaudhry, M. J. I., Arshad, M., Mahmood, S., Ali, A. and Khan, A. A. (2004). "Diclofenac residues as the cause of vulture population decline in Pakistan." *Nature* **427**(6975): 630-633.
OECD (1998). "Test No. 212: Fish, Short-term toxicity test on embryo and sac-fry stages", *OECD Publishing*.
OECD (2007). "Test No. 225: Sediment-Water *Lumbriculus* Toxicity Test Using Spiked Sediment", *OECD Publishing*.
OECD (2012). "Test No. 211: *Daphnia magna* Reproduction Test", *OECD Publishing*.
OECD (2016). "Test No. 242: *Potamopyrgus antipodarum* Reproduction Test", *OECD Publishing*.
Oldach, L. and Zhang, J. (2014). "Genetically encoded fluorescent biosensors for live-cell visualization of protein phosphorylation." *Chemistry & Biology* **21**(2): 186-197.
Owen, S. F., Giltrow, E., Huggett, D. B., Hutchinson, T. H., Saye, J., Winter, M. J. and Sumpter, J. P. (2007). "Comparative physiology, pharmacology and toxicology of β-blockers: Mammals versus fish." *Aquatic Toxicology* **82**(3): 145-162.
Prakash, V., Pain, D. J., Cunningham, A. A., Donald, P. F., Prakash, N., Verma, A., Gargi, R., Sivakumar, S. and Rahmani, A. R. (2003). "Catastrophic collapse of Indian white-backed *Gyps bengalensis* and long-billed *Gyps indicus* vulture populations." *Biological Conservation* **109**(3): 381-390.
Praskova, E., Plhalova, L., Chromcova, L., Stepanova, S., Bedanova, I., Blahova, J., Hostovsky, M., Skoric, M., Marsalek, P., Voslarova, E. and Svobodova, Z. (2014). "Effects of subchronic exposure of diclofenac on growth, histopathological changes, and oxidative stress in zebrafish (*Danio rerio*)." *The Scientific World Journal* **2014**: 645737.
Rand-Weaver, M., Margiotta-Casaluci, L., Patel, A., Panter, G. H., Owen, S. F. and Sumpter, J. P. (2013). "The read-across hypothesis and environmental risk assessment of pharmaceuticals." *Environmental Science & Technology* **47**(20): 11384-11395.
Ratcliffe, D. A. (1970). "Changes attributable to pesticides in egg breakage frequency and eggshell thickness in some british birds." *Journal of Applied Ecology* **7**(1): 67-+.
Ribeiro, S., Torres, T., Martins, R. and Santos, M. M. (2015). "Toxicity screening of diclofenac, propranolol, sertraline and simvastatin using *Danio rerio* and *Paracentrotus lividus* embryo bioassays." *Ecotoxicology and Environmental Safety* **114**: 67-74.
Rubirola, A., Llorca, M., Rodriguez-Mozaz, S., Casas, N., Rodriguez-Roda, I., Barcelo, D. and Buttiglieri, G. (2014). "Characterization of metoprolol biodegradation and its transformation products generated in activated sludge batch experiments and in full scale WWTPs." *Water Research* **63**: 21-32.
Sánchez, S., Martín, M. J., Ortiz, P., Motilva, V. and Lastra, C. A. D. L. (2002). "Effects of dipyrone on inflammatory infiltration and oxidative metabolism in gastric mucosa: Comparison with acetaminophen and diclofenac." *Digestive Diseases and Sciences* **47**(6): 1389-1398.
Sanders, B. M. (1993). "Stress proteins in aquatic organisms: An environmental perspective." *Critical Reviews in Toxicology* **23**(1): 49-75.
Santos, L., Araujo, A. N., Fachini, A., Pena, A., Delerue-Matos, C. and Montenegro, M. (2010). "Ecotoxicological aspects related to the presence of pharmaceuticals in the aquatic environment." *Journal of Hazardous Materials* **175**(1-3): 45-95.
Saucedo-Vence, K., Dublán-García, O., López-Martínez, L. X., Morachis-Valdes, G., Galar-Martínez, M., Islas-Flores, H. and Gómez-Oliván, L. M. (2014). "Short and long-term exposure to diclofenac alter oxidative stress status in common carp *Cyprinus carpio*." *Ecotoxicology* **24**(3): 527-539.
Scheurer, M., Ramil, M., Metcalfe, C. D., Groh, S. and Ternes, T. A. (2010). "The challenge of analyzing beta-blocker drugs in sludge and wastewater." *Analytical and Bioanalytical Chemistry* **396**(2): 845-856.
Schirling, M., Jungmann, D., Ladewig, V., Nagel, R., Triebskorn, R. and Kohler, H. R. (2005). "Endocrine effects in *Gammarus fossarum* (Amphipoda): Influence of wastewater effluents, temporal variability, and spatial aspects on natural populations." *Archives of Environmental Contamination and Toxicology* **49**(1): 53-61.

Schmidt, W., O’Rourke, K., Hernan, R. and Quinn, B. (2011). "Effects of the pharmaceuticals gemfibrozil and diclofenac on the marine mussel (*Mytilus spp.*) and their comparison with standardized toxicity tests." *Marine Pollution Bulletin* **62**(7): 1389-1395.

Schneider, I., Oehlmann, J. and Oetken, M. (2015). "Impact of an estrogenic sewage treatment plant effluent on life-history traits of the freshwater amphipod *Gammarus pulex*." *Journal of Environmental Science and Health Part a-Toxic/Hazardous Substances & Environmental Engineering* **50**(3): 272-281.

Schreiber, R., Gundel, U., Franz, S., Küster, A., Rechenberg, B. and Altenburger, R. (2011). "Using the fish plasma model for comparative hazard identification for pharmaceuticals in the environment by extrapolation from human therapeutic data." *Regulatory Toxicology and Pharmacology* **61**(3): 261-275.

Schröder, P., Helmreich, B., Skrbic, B., Carballa, M., Papa, M., Pastore, C., Emre, Z., Oehmen, A., Langenhoff, A., Molinos, M., Dvarioniene, J., Huber, C., Tsagarakis, K., Martinez-Lopez, E., Pagano, S., Vogelsang, C. and Mascolo, G. (2016). "Status of hormones and painkillers in wastewater effluents across several European states-considerations for the EU watch list concerning estradiols and diclofenac." *Environmental Science and Pollution Research* **23**(13): 12835-12866.

Schwaiger, J. (2001). "Histopathological alterations and parasite infection in fish: indicators of multiple stress factors." *Journal of Aquatic Ecosystem Stress and Recovery* **8**(3): 231-240.

Schwaiger, J., Ferling, H., Mallow, U., Wintermayr, H. and Negele, R. D. (2004). "Toxic effects of the non-steroidal anti-inflammatory drug diclofenac Part 1: histopathological alterations and bioaccumulation in rainbow trout." *Aquatic Toxicology* **68**(2): 141-150.

Schwaiger, J., Wanke, R., Adam, S., Pawert, M., Hönnen, W. and Triebskorn, R. (1997). "The use of histopathological indicators to evaluate contaminant-related stress in fish." *Journal of Aquatic Ecosystem Stress and Recovery* **6**(1): 75-86.

Schwarzenbach, R. P., Escher, B. I., Fenner, K., Hofstetter, T. B., Johnson, C. A., von Gunten, U. and Wehrli, B. (2006). "The challenge of micropollutants in aquatic systems." *Science* **313**(5790): 1072-1077.

Sies, H. (1993). "Strategies of antioxidant defense." *European Journal of Biochemistry* **215**(2): 213-219.

Sies, H. (1997). "Oxidative stress: oxidants and antioxidants." *Experimental Physiology* **82**(2): 291-295.

Simmons, D. L., Botting, R. M. and Hla, T. (2004). "Cyclooxygenase isozymes: the biology of prostaglandin synthesis and inhibition." *Pharmacological Reviews* **56**(3): 387-437.

Smith, V. H. (2003). "Eutrophication of freshwater and coastal marine ecosystems - A global problem." *Environmental Science and Pollution Research* **10**(2): 126-139.

Sørensen, J. G., Kristensen, T. N. and Loeschcke, V. (2003). "The evolutionary and ecological role of heat shock proteins." *Ecology Letters* **6**(11): 1025-1037.

Steinbach, C., Burkina, V., Fedorova, G., Grabicova, K., Stara, A., Velisek, J., Zlabek, V., Schmidt-Posthaus, H., Grabic, R. and Kocour Kroupova, H. (2014). "The sub-lethal effects and tissue concentration of the human pharmaceutical atenolol in rainbow trout (*Oncorhynchus mykiss*)." *Science of the Total Environment* **497-498**: 209-218.

Steinbach, C., Burkina, V., Fedorova, G., Grabicova, K., Stara, A., Velisek, J., Zlabek, V., Schmidt-Posthaus, H., Grabic, R. and Kroupova, H. K. (2014). "The sub-lethal effects and tissue concentration of the human pharmaceutical atenolol in rainbow trout (*Oncorhynchus mykiss*)." *Science of the Total Environment* **497**: 209-218.

Stepanova, S., Praskova, E., Chromcova, L., Plhalova, L., Prokes, M., Blahova, J. and Svobodova, Z. (2013). "The effects of diclofenac on early life stages of common carp (*Cyprinus carpio*)." *Environmental Toxicology and Pharmacology* **35**(3): 454-460.

Stoate, C., Báldi, A., Beja, P., Boatman, N. D., Herzon, I., van Doorn, A., de Snoo, G. R., Rakosy, L. and Ramwell, C. (2009). "Ecological impacts of early 21st century agricultural change in Europe - A review." *Journal of Environmental Management* **91**(1): 22-46.

Stoate, C., Boatman, N. D., Borralho, R. J., Carvalho, C. R., De Snoo, G. R. and Eden, P. (2001). "Ecological impacts of arable intensification in Europe." *Journal of Environmental Management* **63**(4): 337-365.

Stumpf, M., Ternes, T. A., Haberer, K., Seel, P. and Baumann, W. (1996). "Nachweis von Arzneimittelrückständen in Kläranlagen und Fließgewässern." *Vom Wasser* **86**: 291-303.

Sun, L. W., Xin, L. H., Peng, Z. H., Jin, R., Jin, Y. X., Qian, H. F. and Fu, Z. W. (2014). "Toxicity and enantiospecific differences of two beta-blockers, propranolol and metoprolol, in the embryos and larvae of zebrafish (*Danio rerio*)." *Environmental Toxicology* **29**(12): 1367-1378.

Takashima, F. and Hibiya, T. (1995). "An atlas of fish histology: normal and pathological features". Tokyo, *Kodansha ltd. - Gustav Fischer Verlag*.

Ternes, T. A. (1998). "Occurrence of drugs in German sewage treatment plants and rivers." *Water Research* **32**(11): 3245-3260.

Tixier, C., Singer, H. P., Oellers, S. and Muller, S. R. (2003). "Occurrence and fate of carbamazepine, clofibric acid, diclofenac, ibuprofen, ketoprofen, and naproxen in surface waters." *Environmental Science & Technology* **37**(6): 1061-1068.

Triebskorn, R. (2017). "Weitergehende Abwasserreinigung: ein wirksames und bezahlbares Instrument zur Verminderung von Spurenstoffen und Keimen im Wasserkreislauf: gemeinsamer Schlussbericht der vom Bundesministerium für Bildung und Forschung sowie vom Ministerium für Umwelt, Klima und Energiewirtschaft Baden-Württemberg geförderten Projekte SchussenAktiv, SchussenAktiv*plus* und SchussenAktiv*plus+*", *Universitätsbibliothek Tübingen*.

Triebskorn, R., Adam, S., Casper, H., Honnen, W., Pawert, M., Schramm, M., Schwaiger, J. and Köhler, H.-R. (2002). "Biomarkers as diagnostic tools for evaluating effects of unknown past water quality conditions on stream organisms." *Ecotoxicology* **11**(6): 451-465.

Triebskorn, R., Berg, K., Ebert, I., Frey, M., Jungmann, D., Oehlmann, J., Oetken, M., Sacher, F., Scheurer, M., Schmieg, H., Schwarz, S. and Köhler, H. R. (2015). "Monitoring primary effects of pharmaceuticals in the aquatic environment with mode of action-specific *in vitro* biotests." *Environmental Science & Technology* **49**(5): 2594-2595.

Triebskorn, R., Böhmer, J., Braunbeck, T., Honnen, W., Köhler, H.-R., Lehmann, R., Oberemm, A., Schwaiger, J., Segner, H., Schüürmann, G. and Traunspurger, W. (2001). "The project VALIMAR (VALIdation of bioMARkers for the assessment of small stream pollution): objectives, experimental design, summary of results, and recommendations for the application of biomarkers in risk assessment." *Journal of Aquatic Ecosystem Stress and Recovery* **8**(3): 161-178.

Triebskorn, R., Casper, H., Heyd, A., Eikemper, R., Köhler, H. R. and Schwaiger, J. (2004). "Toxic effects of the non-steroidal anti-inflammatory drug diclofenac Part II. Cytological effects in liver, kidney, gills and intestine of rainbow trout (*Oncorhynchus mykiss*)." *Aquatic Toxicology* **68**(2): 151-166.

Triebskorn, R., Casper, H., Scheil, V. and Schwaiger, J. (2007). "Ultrastructural effects of pharmaceuticals (carbamazepine, clofibric acid, metoprolol, diclofenac) in rainbow trout (*Oncorhynchus mykiss*) and common carp (*Cyprinus carpio*)." *Analytical and Bioanalytical Chemistry* **387**(4): 1405-1416.

Triebskorn, R., Schwarz, S., Köhler, H.-R., Berg, K., Jungmann, D., Frey, M., Oehlmann, J. and Oetken, M. (2014). "From theory to reality – Evaluation of suitable organisms and test systems for the biomonitoring of pharmaceuticals - Part I: Literature review". *UBA Texte*. **64/2014**.

Triebskorn, R., Schwarz, S., Schmieg, H., Köhler, H.-R., Jungmann, D., Berg, K., Buchberger, A.-K., Frey, M., Scheurer, M., Sacher, F., Oetken, M. and Oehlmann, J. (2017). "EFF-Pharm: Effects of pharmaceuticals (nonsteroidal anti-inflammatory drugs and beta-blockers) in fish and invertebrates and their detection by newly developed *in vitro*-bioassays". FKZ 3713 63 4102/3715 63 4120. Umweltbundesamt. *UBA Texte*. **44/2017**.

Triebskorn, R., Telcean, I., Casper, H., Farkas, A., Sandu, C., Stan, G., Colărescu, O., Dori, T. and Köhler, H.-R. (2008). "Monitoring pollution in River Mureş, Romania, part II: Metal accumulation and histopathology in fish." *Environmental Monitoring and Assessment* **141**(1): 177-188.

Valavanidis, A., Vlahogianni, T., Dassenakis, M. and Scoullos, M. (2006). "Molecular biomarkers of oxidative stress in aquatic organisms in relation to toxic environmental pollutants." *Ecotoxicology and Environmental Safety* **64**(2): 178-189.

van den Brandhof, E.-J. and Montforts, M. (2010). "Fish embryo toxicity of carbamazepine, diclofenac and metoprolol." *Ecotoxicology and Environmental Safety* **73**(8): 1862-1866.

van Leeuwen, J. S., Vredenburg, G., Dragovic, S., Tjong, T. F. J., Vos, J. C. and Vermeulen, N. P. E. (2011). "Metabolism related toxicity of diclofenac in yeast as model system." *Toxicology Letters* **200**(3): 162-168.

Vane, J. R. and Botting, R. M. (1998). "Anti-inflammatory drugs and their mechanism of action." *Inflammation Research* **47**(2): 78-87.

Verbruggen, B., Gunnarsson, L., Kristiansson, E., Österlund, T., Owen, S. F., Snape, J. R. and Tyler, C. R. (2018). "ECOdrug: a database connecting drugs and conservation of their targets across species." *Nucleic Acids Research* **46**(D1): D930-D936.

Wang, J., He, B., Yan, D. and Hu, X. (2017). "Implementing ecopharmacovigilance (EPV) from a pharmacy perspective: A focus on non-steroidal anti-inflammatory drugs." *Science of The Total Environment* **603-604**(Supplement C): 772-784.

Wenger, M., Ondračková, M., Machala, M., Neča, J., Hyršl, P., Šimková, A., Jurajda, P., Von Der Ohe, P. and Segner, H. (2010). "Assessing relationships between chemical exposure, parasite infection, fish health, and fish ecological status: A case study using chub (*Leuciscus cephalus*) in the Bílina River, Czech Republic." *Environmental Toxicology and Chemistry* **29**(2): 453-466.

Wigh, A., Geffard, O., Abbaci, K., Francois, A., Noury, P., Berge, A., Vulliet, E., Domenjoud, B., Gonzalez-Ospina, A., Bony, S. and Devaux, A. (2017). "*Gammarus fossarum* as a sensitive tool to reveal residual toxicity of treated wastewater effluents." *Science of the Total Environment* **584**: 1012-1021.

Wilhelm, S., Henneberg, A., Kohler, H. R., Rault, M., Richter, D., Scheurer, M., Suchail, S. and Triebskorn, R. (2017). "Does wastewater treatment plant upgrading with activated carbon result in an improvement of fish health?" *Aquatic Toxicology* **192**: 184-197.

Wolf, J. C., Baumgartner, W. A., Blazer, V. S., Camus, A. C., Engelhardt, J. A., Fournie, J. W., Frasca, S., Jr., Groman, D. B., Kent, M. L., Khoo, L. H., Law, J. M., Lombardini, E. D., Ruehl-Fehlert, C., Segner, H. E., Smith, S. A., Spitsbergen, J. M., Weber, K. and Wolfe, M. J. (2015). "Nonlesions, misdiagnoses, missed diagnoses, and other interpretive challenges in fish histopathology studies: A guide for investigators, authors, reviewers, and readers." *Toxicologic Pathology* **43**(3): 297-325.

Wolf, J. C., Ruehl-Fehlert, C., Segner, H. E., Weber, K. and Hardisty, J. F. (2014). "Pathology working group review of histopathologic specimens from three laboratory studies of diclofenac in trout." *Aquatic Toxicology* **146**: 127-136.

Wright-Walters, M., Volz, C., Talbott, E. and Davis, D. (2011). "An updated weight of evidence approach to the aquatic hazard assessment of Bisphenol A and the derivation a new predicted no effect concentration (Pnec) using a non-parametric methodology." *Science of The Total Environment* **409**(4): 676-685.

Yokota, H., Eguchi, S., Hasegawa, S., Okada, K., Yamamoto, F., Sunagawa, A., Tanaka, M., Yamamoto, R. and Nakano, E. (2016). "Assessment of in vitro antiovulatory activities of nonsteroidal anti-inflammatory drugs and comparison with *in vivo* reproductive toxicities of medaka (*Oryzias latipes*)." *Environmental Toxicology* **31**(12): 1710-1719.

Zimmerli, S., Bernet, D., Burkhardt-Holm, P., Schmidt-Posthaus, H., Vonlanthen, P., Wahli, T. and Segner, H. (2007). "Assessment of fish health status in four Swiss rivers showing a decline of brown trout catches." *Aquatic Sciences* **69**(1): 11-25.

Part 2: Own contribution to the submitted publications and manuscripts

Publication I: From theory to reality – Evaluation of suitable organisms and test systems for the biomonitoring of pharmaceuticals. Part I: Literature review (2014)

Rita Triebskorn, Simon Schwarz, Heinz-R. Köhler, Kristin Berg, Dirk Jungmann, Manfred Frey, Jörg Oehlmann, Matthias Oetken

UBA Texte 64/2014, Dessau-Rosslau - Report on behalf of the Federal Environment Agency (Germany), used for Chapter I

- 100 % of literature analyses on vertebrate, plant, bacteria and protist studies
- 60 % of data compilation and evaluation
- 25 % of final written report

The project partner GWT-TUD (K. Berg, D. Jungmann) reviewed and compiled literature on invertebrates and assessed reliability of the studies reporting sensitive effect concentrations. M. Frey reviewed the entire literature on *in vitro* monitoring systems. R. Triebskorn coordinated the project and the writing of the final report.

The figures and tables concerning the evaluation of effect data were created by me, together with K. Berg and R. Triebskorn.

Publication II: Monitoring Primary Effects of Pharmaceuticals in the Aquatic Environment with Mode of Action-Specific in Vitro Biotests (2015)

Rita Triebskorn, Kristin Berg, Ina Ebert, Manfred Frey, Dirk Jungmann, Jörg Oehlmann, Matthias Oetken, Frank Sacher, Marco Scheurer, Hannah Schmieg, Simon Schwarz, Heinz-R. Köhler

Environmental Science & Technology 49, 2594-2595 – used for Chapter II

- 10 % of manuscript revision

R. Triebskorn wrote the manuscript, based on the collective conclusions of all authors, drawn from the previous literature review.

The summarizing figure in the publication was created by R. Triebskorn.

Publication III: Impact of the NSAID diclofenac on survival, development, behavior and health of embryonic and juvenile stages of brown trout, Salmo trutta f. fario (2017)

Simon Schwarz, Hannah Schmieg, Marco Scheurer, Heinz-R. Köhler, Rita Triebskorn

Science of the Total Environment 607-608, 1026-1036 – used for Chapter III

- 90 % of planning and experimental design
- 80 % of experiment conduction
- 80 % of biochemical and histological analyses
- 100 % of statistical evaluation
- 80 % of final manuscript

H. Schmieg helped with the conduction of the exposure experiment and the evaluation of biochemical biomarkers. M. Scheurer verified diclofenac concentrations in water and fish samples via chemical

analyses. R. Triebskorn supervised the overall project and helped in the finalisation of the manuscript, together with H.-R. Köhler.

All figures and tables in the publication were created by me.

The content of this publication partially overlaps with the content of publication IV.

Publication IV: EFF-Pharm: Effects of pharmaceuticals (nonsteroidal anti-inflammatory drugs and beta-blockers) in fish and invertebrates and their detection by newly developed in vitro-bioassays – Final report (2017)

Rita Triebskorn, Simon Schwarz, Hannah Schmieg, Heinz-R. Köhler, Dirk Jungmann, Kristin Berg, Anna Buchberger, Manfred Frey, Marco Scheurer, Frank Sacher, Matthias Oetken, Jörg Oehlmann

UBA Texte 44/2017, Dessau-Rosslau – Report on behalf of the German Environment Agency, used for Chapters III, IV, V

- 90 % of planning and experimental design of fish experiments
- 80 % of conduction of fish experiments
- 80 % of biochemical and histological analyses of fish
- 80 % of lipid peroxide and stress protein analyses of invertebrates
- 90 % of statistical evaluation on fish experiments and biomarker responses in invertebrates
- 80 % final written report on fish experiments
- 90 % final written report on biomarker responses in invertebrates

Together with me, H. Schmieg performed the laboratory exposure of juvenile trout to metoprolol and evaluated biochemical and histological biomarkers as part of her master thesis. All exposure experiments on invertebrates were conducted by GWT-TUD (K. Berg, A. Buchberger, D. Jungmann), University of Frankfurt (M. Oetken, J. Oehlmann) or the UBA. TZW (M. Scheurer, F. Sacher) were responsible for analytic-chemical verification of exposure concentrations. M. Frey developed the *in vitro* biotests. All authors wrote their respective section of the final report. R. Triebskorn and H.-R. Köhler coordinated the project and the writing of the final report.

I created only the figures and tables concerning effects in brown trout, together with H. Schmieg, and the figures and tables concerning stress protein and lipid peroxide evaluations in invertebrates. The other figures and tables were created by the authors of the respective sections: summary (R. Triebskorn, H.-R. Köhler), tests with gammarids (D. Jungmann, K. Berg, A. Buchberger), tests with gastropods and oligochaetes (M. Oetken, J. Oehlmann), chemical analyses (M. Scheurer, F. Sacher), *in vitro* test systems (M. Frey).

The content of this publication partially overlaps with the content of publications III and V.

Publication V: Health effects of metoprolol in epibenthic and endobenthic invertebrates – A basis to validate future in vitro biotests for effect-based biomonitoring (2017)
Dirk Jungmann, Kristin Berg, Andreas Dieterich, Martin Frank, Tonya Gräf, Marco Scheurer, Simon Schwarz, Carmen Siewert, Matthias Oetken

Journal of Environmental Science and Health, Part A, 52/3, 189-200 – used for Chapter IV

- 90 % of biomarker (stress proteins and lipid peroxides) analyses
- 100 % of biomarker (stress proteins and lipid peroxides) evaluation
- 10 % of final written manuscript

D. Jungmann and M. Oetken coordinated the writing of the manuscript. The exposure experiments were performed by GWT-TUD (D. Jungmann, K. Berg) and University of Frankfurt (M. Oetken, M. Frank, T. Gräf). A. Dieterich and C. Siewert assisted in the processing of biochemical samples.

I only created the tables and figures concerning stress proteins and lipid peroxides in gammarids and oligochaetes. The other figures and tables were created by the respective co-authors: gammarids (D. Jungmann, K. Berg), oligochaetes (M. Frank, T. Gräf, M. Oetken).

The content of this publication partially overlaps with the content publication IV.

Part 3: Study reports

Publication I: From theory to reality – Evaluation of suitable organisms and test systems for the biomonitoring of pharmaceuticals - Part I: Literature review

Rita Triebskorn[1,2], Simon Schwarz[1], Heinz-R. Köhler[1], Kristin Berg[3], Dirk Jungmann[3], Manfred Frey[4], Jörg Oehlmann[5], Matthias Oetken[5]

[1] Animal Physiological Ecology, University of Tübingen;

[2] Steinbeis Transfer Center for Ecotoxicology and Ecophysiology, Rottenburg

[3] GWT, Technical University Dresden

[4] Steinbeis Transfer Center for Applied Biological Chemistry, Mannheim

[5] Aquatic Ecotoxicology, University of Frankfurt

Study report on behalf of the Federal Environment Agency (Germany) published in UBA TEXTE 64/2014

Environmental Research of the Federal Ministry for the Environment, Nature Conservation, Building and Nuclear Safety, Project No. (FKZ) 3713 634 101 Report No. (UBA-FB) 001941/E

The report is shortened to exclude the part on *in vitro* systems, which was not part of this thesis.

Abstract

In order to identify sensitive organisms, biological endpoints *in vivo* and suitable *in vitro* test systems for the biomonitoring of pharmaceuticals, a two-part literature search was conducted.

In the first part, the database OEKOTOX established by Bergmann et al. (2011) [1] was upgraded with effect data published between 2011 and 2013 for 90 pharmaceuticals of high priority. From all available data the lowest effect concentrations were identified and the most sensitive organisms and effect endpoints were determined. In addition, effect data were assessed with respect to their relation to measured environmental concentrations (MECs) in German surface waters by calculations of risk quotients $MEC_{max}/LOEC_{min}$. Publications providing the lowest effect data were evaluated with respect to their reliability according to the criteria of Wright-Walters et al. (2011) [2]. Out of 72 publications investigated for their reliability, 9 were "reliable", 49 "conditionally reliable" and 14 "not reliable" (of which 4 were, in part, conditionally reliable).

Prior to the reliability check, the analgesics paracetamol, diclofenac and ibuprofen, the ß-blocker propranolol, the antibiotics sulfamethoxazole and erythromycin and the lipid regulator gemfibrozil were identified as environmentally most relevant pharmaceuticals. The relevance was defined by $MEC_{max}/LOEC_{min}$-values >0.1 for a minimum of 3 different biota classes. After assessment of publication reliability, however, only for diclofenac $MEC_{max}/LOEC_{min}$-values >0.1 were found in more than 3 biota classes. As further important pharmaceuticals propranolol, sulfamethoxazole, bezafibrate, 17α-ethinlyestradiol, 17β-estradiol and oxytetracycline were identified with $MEC_{max}/LOEC_{min}$-values >0.1 for 2 biota classes.

Prior to the reliability assessment, the ciliate *Tetrahymena pyriformis*, zebrafish (*Danio rerio*), rainbow trout (*Oncorhynchus mykiss*), medaka (*Oryzias latipes*), the mussel *Elliptio complanata*, and the mudsnail *Potamopyrgus antipodarum* were identified as most sensitive organisms for pharmaceuticals. The most sensitive effect endpoints were chemotaxis, behavior, vitellogenin synthesis, growth rate, reproduction, histopathological alterations, molecular stress biomarkers, oxidative stress markers, receptor binding, and gene expression. All extremely low LOECs (in the range of pg/L) for chemotaxis alterations in *Tetrahymena pyriformis*, however, derive from only a single publication by Lang & Köhidai (2012) [3], the reliability of which, however, could not be proven.

In the second part, a literature search was conducted with the aim to evaluate the state of knowledge in the field of *in vitro* testing of pharmaceutical effects, to discover advantages and disadvantages of biochemical and cell-based assays and to suggest possibilities for the realisation of cell-based tools for a mode of action-based biomonitoring of pharmaceuticals. Promising approaches for ß-adrenoreceptor blockers and cyclooxygenase-inhibitors (analgesics) are presented.

As a future perspective for a biomonitoring of pharmaceuticals, the development of mode of action-based *in vitro* test systems for ß-blockers and NSAIDs are recommended. Their suitability to reflect *in vivo* responses of organisms which have been identified to be sensitive in part 1 of this study, or which are relevant for German aquatic ecosystems should be assessed, and they should be validated by *in vivo* studies to be conducted in parallel to their establishment.

Kurzbeschreibung

Mit dem Ziel, sensitive Organismen und organismische Endpunkte sowie geeignete *in vitro*-Testsysteme für ein Biomonitoring von Arzneimitteln zu identifizieren, wurde eine zweiteilige Literaturstudie durchgeführt.

Im ersten Teil wurde die von Bergmann et al. (2011) [1] erstellte Datenbank OEKOTOX um Wirkdaten für 90 Arzneimittel von hoher Priorität bis 2013 erweitert. Von allen verfügbaren Daten wurden die niedrigsten Effektwerte identifiziert und die sensitivsten Organismen bzw. Effektendpunkte bestimmt. Darüber hinaus wurden die Effektkonzentrationen zu gemessenen Umweltkonzentrationen in Deutschen Oberflächengewässern (MECs) in Beziehung gestellt und Risikoquotienten $MEC_{max}/LOEC_{min}$ berechnet. Diejenigen Publikationen, in denen die empfindlichsten Endpunkte bzw. Organismen enthalten waren, wurden auf der Basis der von Wright-Walters et al. (2011) [2] genannten Kriterien auf Reliabilität geprüft. Von 72 auf Reliabilität überprüften Publikationen wurden 9 als „reliabel“, 49 als „bedingt reliabel“ und 14 als „nicht reliabel“ (von denen 4 in Teilen bedingt reliabel waren) klassifiziert.

Vor der Reliabilitätsprüfung wurden in der vorliegenden Literaturstudie als Pharmazeutika mit höchster Umweltrelevanz die Schmerzmittel Paracetamol, Diclofenac und Ibuprofen, der ß-Blocker Propranolol, die Antibiotika Sulfamethoxazol und Erythromycin und der Lipidsenker Gemfibrozil identifiziert. Die Umweltrelevanz wurde auf der Basis eines Risikoquotienten ($MEC_{max}/LOEC_{min)}$ >0.1 für mindestens 3 Organismengruppen) definiert. Nach erfolgter Reliabilitätsprüfung zeigten sich $MEC_{max}/LOEC_{min)}$-Werte >0.1 bei mehr als 3 Organismengruppen jedoch nur noch für Diclofenac. Als weitere wichtige Pharmazeutika wurden Propranolol, Sulfamethoxazol, Bezafibrat, 17α-Ethinlyestradiol, 17β-Estradiol und Oxytetrazyclin mit $MEC_{max}/LOEC_{min)}$-Werten >0.1 bei 2 Organismengruppen identifiziert.

Als sensitivste Organismen für Arzneimittel hatten sich vor der Reliabilitätsprüfung der Ciliat *Tetrahymena pyriformis*, der Zebrabärbling (*Danio rerio*), die Regenbogenforelle (*Oncorhynchus mykiss*), Medaka (*Oryzias latipes*), die Muschel *Elliptio complanata* und die Zwergdeckelschnecke *Potamopyrgus antipodarum* erwiesen. Die sensitivsten Effektendpunkte waren Chemotaxis, Verhalten, Vitellogeninsynthese, Wachstum, Reproduktion, histopathologische Veränderungen, molekulare und oxidative Stressmarker, Genexpression und Rezeptorbindung. Alle extrem niedrigen LOECs (im Bereich von pg/L) für die Beeinflussung der Chemotaxis bei *Tetrahymena pyriformis* stammen allerdings von einer einzigen Publikation von Lang & Köhidai (2012) [3], die sich in der durchgeführten Reliabilitätsprüfung als „nicht reliabel“ erwies, so dass der Zebrabärbling als bedeutendster Testorganismus und das Verhalten als sensitivster Wirkendpunkt nachrückt.

Im zweiten Teil der Studie wurde eine Literaturrecherche durchgeführt, die zum Ziel hatte, den Wissensstand im Bereich von *in vitro*-Verfahren zum Nachweis von Arzneimittel-Effekten zu

beleuchten, Vor- und Nachteile von biochemischen und zellbasierten Assays herauszuarbeiten und Möglichkeiten für die Realisierung von *in vitro* Testsystemen für ein Wirkmechanismus-basiertes Biomonitoring von Arzneimitteln vorzuschlagen. Am Ende dieses Teils werden vielversprechende Wege zur Etablierung solcher Testsysteme für den Nachweis der Wirkungen von ß-Blockern und Cyclooxygenase-Hemmern (Schmerzmitteln) dargestellt.

Für ein künftiges Biomonitoring von Arzneimitteln wird empfohlen, Wirkstoffklassen-spezifische *in vitro*-Testsysteme für ß-Blocker und Schmerzmittel zu entwickeln. Durch parallel durchzuführende *in vivo* Untersuchungen soll überprüft werden, inwieweit diese Testsysteme geeignet sind, *in vivo*-Reaktionen von Organismen abzubilden, die in Teil 1 der vorliegenden Studie als sensitiv für Arzneimittel identifiziert wurden, oder die als relevant für deutsche Fließgewässer bekannt sind.

List of Abbreviations - Part 1

CAS: Chemical Abstracts Service
DOI: Digital Object Identifier
LOEC: Lowest observed effect concentration
$LOEC_{min}$: Minimum of lowest observed effect concentrations reported
$LogK_{OW}$: Octanol/water partitioning coefficient (logarithmic form)
MEC: Measured environmental concentration
MEC_{max}: Maximum of measured environmental concentrations reported
MOA: Mode of action
NOEC: No observed effect concentration
NSAID: Non-steroidal anti-inflammatory drug
PBT: persistent, bioaccumulating, and toxic
PEC: Predicted environmental concentration
PNEC: Predicted no effect concentration
TER: Toxicity exposure ratio
UBA: Umweltbundesamt der Bundesrepublik Deutschland

1. Introduction

Chemical analysis has regularly revealed the presence of human and veterinary pharmaceuticals in wastewater and surface water in nanogram to microgram per liter concentrations (e.g. Fent et al., 2006 [4], Brauch, 2011 [5]). Decisions on the environmental relevance of these substances usually rely on data recorded for current and future consumption rates, environmental concentrations, environmental fate and pathways (persistence), and ecotoxicological effects recorded in laboratory studies. The problem with this approach is the fact that ecotoxicological routine testing mainly focuses on acute or chronic unspecific toxicity, whereas pharmaceuticals, according to their envisaged specific action in man (human medicine) or companion animals (veterinary drugs) more likely exert specific effects based on their mode of action (MOA) (Brausch et al. 2012 [6]). Information on the effects of active pharmaceutical ingredient classes, either acting on targets or exerting unwanted adverse side effects are, therefore, mandatory prerequisites for an effect-directed monitoring of pharmaceuticals in aquatic ecosystems.

One focus of the present literature review study thus was on the question which organisms sensitively respond to pharmaceuticals and which sublethal parameters can be used as warning sentinels (biomarkers) to monitor action of pharmaceuticals in a sensitive and specific way.

In a comprehensive report of Bergmann et al. (2011) [1] a prioritization of human and veterinary pharmaceuticals was undertaken on the basis of data on the presence of compounds in the aquatic environment, their ecotoxicological effects, and their consumption rates up to the year 2011. This report lists analytical data for 274 ingredients and ecotoxicological effect data for 251 ingredients, all of them incorporated in both the MEC (Measured Environmental Concentrations) and ÖKOTOX (effect

data) databases. Bergmann et al. (2011) [1] have outlined 24 substances with high priority for environmental monitoring programs. However, the databases also provide evidence that 31 pharmaceuticals with partially high and steadily increasing consumption rates lacked ecotoxicological data until 2009 resulting in a very high uncertainty regarding the assessment of their environmental relevance. Supplementary to the substances regarded as relevant for further research by Bergmann et al. (2011) [1], the German Federal Ministry for the Environment, Nature Conservation and Nuclear Safety has identified further substances as to be of high priority for an evaluation with respect to possible environmental effects. In the present study, we therefore focused on a set of 90 substances, prioritized by Bergmann et al. (2011) [1] and UBA, for which literature was analyzed.

Bergmann et al. (2011) [1] concluded from their research that already the number of substances with high priority (24) would exceed the capacity of routine chemical monitoring. They therefore suggested having leading substances defined for particular ingredient classes that should be included in monitoring programs. The apparent but crucial drawback of such an approach, however, lies in the risk of overlooking effective concentrations of non-leading substances. In contrast to it, the use of tests that could visualize specific molecular interactions of chemicals exhibiting the same mode of action may enable a monitoring of an entire class of compounds. Particularly in view of the vast number of pharmaceuticals with increasing consumption rates but lacking ecotoxicological effect data, a mode of-action-specific *in vitro* assay is a matter of paramount interest; also because new and future compounds that act in the same way on the same molecular target can easily be traced, once such an assay has been established.

In vitro systems using highly sensitive fluorescence detection technologies are already used by the pharmaceutical industry to identify compound classes as promising candidates in the development of new pharmaceuticals (Eggeling et al., 2003) [7]. A biomonitoring on the basis of such specific mechanisms of action could be possible for e.g. non-steroidal anti-inflammatory drugs (NSAIDs) or ß-blockers. For these, the inhibition of the cyclooxygenase and the blocking of β-receptors could be used as mode of actions to be targeted. In contrast to Escher et al. (2005) [8] who use "*in vitro* Assessment of Modes of Toxic Action" in that sense that specific mode of actions defined by the test system itself are investigated (e.g. photosynthesis inhibition of pharmaceuticals by a photosynthesis inhibition assay), we thus favor an approach to use the specific target molecules for pharmaceutical classes (responsible for the pharmacological effect of the respective substance class) as monitoring tools. In the second part of this study, we therefore evaluated different *in vitro* test systems with respect to their suitability as future test systems in routine monitoring of pharmaceutical classes.

In summary, this study reviews (1) the current state of knowledge in effect-directed research and analyzes present data with focus on suitable compounds, organisms, and endpoints which may be combined in a monitoring approach, and (2) in addition, the state of knowledge for *in vitro* testing of pharmaceuticals as a prerequisite for the development of mode of-action-based monitoring tools.

2. Materials and Methods

2.1 Literature search and data collection

The literature search is divided into two parts.

In the first part, effect data for 90 pharmaceuticals defined as relevant according to either Bergmann et al. (2011) [1] or UBA were collected for several biota classes (bacteria, protists, plants/algae, mollusks, crustaceans, insects, fish, tetrapods, others incl. communities). The list of pharmaceuticals and the priority classes are shown in table 1. The prioritization of UBA is based on MECs obtained from the German counties, consumption rates (tendencies between 2002 and 2012), effect values of UBA-internal studies or literature, suspicion to be a PBT compound, degradability and metabolism. The

prioritization of Bergmann et al. (2011) [1] is only based on literature data which resulted in partial differences between the two lists of priority substances available.

The search was restricted to literature published between 2011 and 2013, since all earlier published data have been analyzed by Bergmann et al. (2011) [1]. For the search, Web of Knowledge (including the databases “Web of Science®”, “BIOSIS Citation Index℠”, “BIOSIS Previews®”, “MEDLINE®” and “Journal Citation Reports®”) were used. The following search items were defined:

For Reviews: pharma* AND eco*, limited by “review”

For single substances: the specific search entry for each pharmaceutical (see table 1) combined with the search terms: aqua* OR eco* OR tox*

The following research areas were seen as irrelevant and therefore excluded from the literature search:

Anesthesiology, Anthropology, Biomedical, Social Sciences, Biophysics, Business Economics, Communication, Computer Science, Criminology Penology, Critical Care Medicine, Cultural Studies, Demography, Dentist, Oral Surgery Medicine, Dermatology, Education, Educational Research, Electrochemistry, Emergency Medicine, Energy Fuels, Engineering, Ethnic Studies, Food Science Technology, General Internal Medicine, Genetics Heredity, Geography, Geology, Geriatrics, Gerontology, Government, Law, Health Care Sciences, Services, History, Imaging Science, Photographic Technology, Infectious Diseases, Information Science, Library Science, Instruments Instrumentation, Integrative Complementary Medicine, International Relations, Legal Medicine, Materials Science, Mathematical Computational Biology, Mathematics, Medical Informatics, Medical Laboratory Technology, Meteorology Atmospheric Sciences, Nursing, Nutrition Dietetics, Obstetrics, Gynecology, Oncology, Ophthalmology, Orthopedics, Otorhinolaryngology, Parasitology, Pediatrics, Pharmacology, Pharmacy, Physics, Polymer Science, Psychiatry, Psychology, Radiology, Nuclear Medicine, Medical Imaging, Rehabilitation, Research Experimental Medicine, Respiratory System, Social Issues, Sociology, Sport Sciences, Substance Abuse, Surgery, Transplantation, Tropical Medicine, Urology, Nephrology, Virology

Table 1: List of pharmaceuticals with defined priority classes

P: *high priority according to Bergmann et al. and UBA*
Pu: *high priority according to UBA*
Pb: *high priority according to Bergmann et al.*
(P): *medium priority according to Bergmann et al.*
?: *substances requiring further information*
none: *substances without priority according to Bergmann et al.*

Pharmaceutical	Search entry	Priority class (UBA)	Priority class (Berg mann)	Priority class (final)	Class	CAS
"Sartanic acid"	sartane*	P		Pu	antihypertensive	
14-Hydroxyclarithromycin	hydroxyclarithromycin*	P		Pu	antibiotic	110671-78-8
17alpha-Ethinylestradiol	ethinyl* estradiol*	P	P	P	contraceptive	57-63-6
17beta-Estradiol	estradiol* AND pharma*		P	Pb	hormone	50-28-2
4-N-Methylaminoantipyrin	methylaminoantipyrin*	P		Pu	analgesic	519-98-2
6(carboxymethoxy)-4-(2-chlorophenyl)-5-(ethoxycarbonyl)-2-methylpyridine-3-carboxylic acid	amlodipin*	P		Pu	antihypertensive	
Acetyl cysteine	acetyl cystein*		?	?	mucolytic agent	616-91-1
Acetylsalicylic acid	acetylsalic*				NSAID	50-78-2
Aciclovir	aciclovir*		?	?	antiviral drug	59277-89-3
Allopurinol	allopurinol*				xanthine oxidase inhibitor	315-30-0

Pharmaceutical	Search entry	Priority class (UBA)	Priority class (Berg mann)	Priority class (final)	Class	CAS
AMDOPH	AMDOPH		(P)	(P)	analgesic	
Amlodipine	amlodipin*	P		Pu	antihypertensive	88150-42-9
Amoxicilline	amoxicillin*	P	P	P	antibiotic	26787-78-0
Atenolol/ Atenolol acid	atenolol*, tenormin*	P		Pu	betablocker	29122-68-7
Azitromycin	azitromycin*, zithromax*, azithrocin*, azin*	P		Pu	antibiotic	83905-01-5
Bezafibrate	bezafibrat*		(P)	(P)	lipid regulator	41859-67-0
Bisoprolol	bisoprolol*	P	?	Pu	betablocker	66722-44-9
Capecitabin	capectiabin*, xeloda*	P		Pu	cytostatic	15361-50-9
Carbamazepine	carbamazepin*	P	P	P	anticonvulsant	298-46-4
Cefaclor	cefaclor* OR cefachlor*		?	?	antibiotic	53994-73-3
Cefuroxime axetil	cefuroxim* axetil*		?	?	antibiotic	64544-07-6
Chloramphenicole	chloramphenicol*		P	Pb	antibiotic	56-75-7
Chlortetracycline	chlortetracyclin*		P	Pb	antibiotic	57-62-5
Ciprofloxacin	ciprofloxacin*	P	P	P	antibiotic	85721-33-1
Clarithromycine	clarithromycin*	P	P	P	antibiotic	81103-11-9
Clindamycin	clindamycin*	P	?	Pu	antibiotic	18323-44-9
Clopidogrel	clopidogrel*		?	?	antiplatelet agent	113665-84-2
Clotrimazole	clotrimazol*		(P)	(P)	antimycotic	23593-75-1
Deltamethrin	deltamethrin*, decamethrin*	P		Pu	antiparasitic	52918-63-5
Desflurane	desfluran*		?	?	anesthetic	57041-67-5
Diatrizoic acid	diatriz*, iotalam*, amidotriz*		(P)	(P)	contrast agent	737-31-5
Diazepam	diazepam*		(P)	(P)	antidepressant/ antipsychotic	439-14-5
Diclofenac	diclofenac*	P	P	P	NSAID	15307-86-5
Dienogest	dienogest*, visanne*	P		Pu	gestagan	65928-58-7
Dipyridamole	dipyridamol*		?	?	antiplatelet agent	58-32-2
Doxycycline	doxycyclin*		P	Pb	antibiotic	564-25-0
Duloxetine	duloxetin*, cymbalta*	P		Pu	antidepressant/ antipsychotic	116539-59-4
Entacapone	entacapon*		?	?	C-O-M-Inhibitor	130929-57-6
Eprosartan	eprosartan*		?	?	antihypertensive	133040-01-4
Erythromycin	erythromycin*		P	Pb	antibiotic	114-07-8
Gabapentin	gabapentin*, neurotin*		?	?	anticonvulsant	60142-96-3
Gemfibrozil	gemfibrozil*, lopid*	P		Pu	lipid regulator	25812-30-0
Glutaral	glutaral*		?	?	desinfectant	111-30-8
Hydrochlorothiazide	hydrochlorothiazid*		?	?	antihypertensive	58-93-5
Ibuprofen	ibuprofen*	P	(P)	P	NSAID	15687-27-1
Imatinib	imatinib*, gleevec*, glivec*	P		Pu	cytostatic	152459-95-5
Indometacin	indometacin*, indomethacin*		(P)	(P)	NSAID	53-86-1
Iohexol	iohexol*, omnipaq*	P	(P)	P	contrast agent	66108-95-0
Iomeprol	iomeprol*	P	(P)	P	contrast agent	78649-41-9
Iopamidol	iopamidol*		(P)	(P)	contrast agent	60166-93-0
Iopromide	iopromid*, ultravist*		(P)	(P)	contrast agent	73334-07-3
Ivermectin	ivermectin*, stromectol*	P		P	Antiparasitic	70288-86-7
Lamotrigin	lamotrigin*, lamictal*	P		Pu	anticonvulsant	84057-84-1
Levetiracetam	levetiracetam*		?	?	anticonvulsant	102767-28-2
Lincomycin	lincomycin*		(P)	(P)	antibiotic	154-21-2
Mesalazine	mesalazin*		?	?	NSAID	89-57-6
Mestranol	mestranol*		P	Pb	contraceptive	72-33-3
Metamizole	metamizol*	P		Pu	analgesic	68-89-3
Metformin	metformin*	P		Pu	antidiabetic	657-24-9
Metoprolol	metoprolol*	P		Pu	betablocker	51384-51-1
Naproxen	naproxen*	P	P	P	NSAID	22204-53-1
Norethisterone	norethisteron*		P	Pb	contraceptive	68-22-4
Opipramol	opipramol*		?	?	antidepressant/ antipsychotic	315-72-0
Oxytetracycline	oxytetracyclin*		P	Pb	antibiotic	79-57-2
Pantoprazole	pantoprazol*		?	?	proton pump inhibitor	102625-70-7

Pharmaceutical	Search entry	Priority class (UBA)	Priority class (Berg mann)	Priority class (final)	Class	CAS
Paracetamol	paracetamol*, acetaminophen*		P	Pb	analgesic	103-90-2
Piperacillin	piperacillin*		?	?	antibiotic	61477-96-1
Pregabalin	pregabalin*		?	?	anticonvulsant	148553-50-8
Primidone	primidon*		P	Pb	anticonvulsant	125-33-7
Propranolol	propanolol*, propranolol*		(P)	(P)	betablocker	525-66-6
Quetiapine	quetiapin*	P	?	Pu	antidepressant/ antipsychotic	11974-69-7
Ramipril	ramipril*		?	?	antihypertensive	87333-19-5
Roxithromycine	roxithromycin*		P	Pb	antibiotic	80214-83-1
Sevelamer	sevelamer*		?	?	phosphate binding drug	52757-95-6
Simvastatin	simvastatin*	P	?	Pu	lipid regulator	79902-63-9
Strontium ranelate	strontium ranelat*		?	?	osteoporose	135459-90-4
Sulbactam	sulbactam*		?	?	antibiotic	68373-14-8
Sulfadimethoxine	sulfadimethoxin*		P	Pb	antibiotic	122-11-2
Sulfadimidine	sulfadimidin*, sulfamethazin*		P	Pb	antibiotic	57-68-1
Sulfamethoxazole	sulfamethoxazol*	P	P	P	antibiotic	723-46-6
Tazobactam	tazobactam*		?	?	antibiotic	89786-04-9
Telmisartan	telmisartan*		?	?	antihypertensive	144701-48-4
Tetracycline	tetracyclin*		P	Pb	antibiotic	60-54-8
Tiamulin	tiamulin*	P	P	P	antibiotic	55297-95-5
Tilidine	tilidin*		?	?	analgesic	51931-66-9
Torasemide	torasemid*, torsemid*		?	?	diuretic drug	56211-40-6
Tramadol	tramadol*, ryzolt*, ultram*	P		Pu	analgesic	27203-92-5
Valproic acid	valpro*		?	?	anticonvulsant	99-66-1
Valsartan	valsartan*, angiotan*, diovan*	P	?	Pu	antihypertensive	137862-53-4
Venlafaxine	venlafaxin*		?	?	antidepressant/ antipsychotic	93413-69-5

2.2. Created library and data files

2.2.1 Endnote library

All references analyzed were included into an Endnote library ("EndNote Library – Pharmaceuticals – Literature study part 1 / 2 /3") in the format ".CIW".

The three partners involved in the literature search used the following labels for their citations:

BER 1-x: GWT Dresden (R-: Review, A-: Additional Information, I-: Irrelevant)

SCH 1-x: University Tübingen (R-: Review, A-: Additional Information, I-: Irrelevant)

FRE 1-x: STZ Frey

The library contains the following folders:

Part 1:

Tuebingen - relevant studies: Studies on vertebrates, plants, protozoans and bacteria, which were directly included into the database.

Tuebingen - additional information: Studies, mainly reviews, on vertebrates, plants, protozoans and bacteria, which were used as help for the general interpretation of the data, but not directly included into the database.

Tuebingen - irrelevant studies: Studies, which were analyzed but were not included into the database.

Tuebingen- evaluation not possible: Studies, for which an evaluation was not possible because the full text was not available and the abstract did not contain sufficient information.

Dresden - relevant studies: Studies on invertebrates, which were directly included into the database.

Dresden - additional information: Studies, mainly reviews, on invertebrates, which were used as help for the general interpretation of the data, but not directly included into the database.

Dresden - irrelevant studies: Studies, which were analyzed but were not included into the database.

Part 2:

Mannheim - *in vitro* techniques: Studies on *in vitro* techniques, which were used for the second part of the literature study.

Part 3:

Additional literature used for this report

2.2.2 OEKOTOX$_{upgrade}$

In part 1 of the study, all publications available for the 90 substances were analyzed and evaluated with respect to the following criteria:

Name of effective substance, investigated form of the substance (salt/conjugate), synonyms, CAS number, $LogK_{ow}$, molar mass, substance class, field of application, effective substance or medical product, single substance or mixture, test organisms (species), biota group, field/lab/mesocosm test, effect endpoint, population relevance yes/no, standard method yes/no, test concentration in water (µg/L, mol/L), test concentration in sediment (if necessary), applied dose (if necessary), duration of test, acute/chronic/sub-acute, chemical analyses present, accumulation data present, bioaccumulation factor, NOEC (µg/L, µmol/L), LOEC (µg/L, µmol/L), EC_{10} (µg/L, µmol/L), EC_{50} (µg/L, µmol/L), citation, DOI, full text available, Endnote label, chemical present in priority list yes/no, comments, reliability analysis (only for selected publications).

In case no effect was observed in the study, the highest concentration tested (without effect) has been included as „NOEC" without corresponding LOEC. Furthermore, numerous studies reported effects already at the lowest tested concentration. These values are recorded as LOECs without a corresponding NOEC. It should be kept in mind that there is a possibility that lower values than the reported may also cause an effect.

Besides data for the priority substances defined in table 1, data for several other pharmaceuticals is also reported in the database. These effects were reported in the analyzed publications besides the ones for the target substances, and therefore included. However, the data sets for these substances are not complete, since they are mere "byproducts" of the original search.

All data were included into the database OEKOTOX$_{upgrade}$

A separate row was created for every chemical, species and endpoint used in each study. Each single row was defined as a "database entry". Because many publications report on multiple chemicals, organisms or endpoints, the total number of database entries is much higher than the number of publications.

2.2.3. "Evaluation database" for assessment of data

With the aim to evaluate the collected data with respect to (1) their suitability for the study, (2) data number per pharmaceutical and organism group, (3) most sensitive organisms, (4) lowest effect

concentrations, (5) mixture toxicity, and (6) population relevance, the "evaluation database" has been created.

In data sheet (4) the lowest and second lowest effect data and measured environmental concentrations (MECs) obtained either from the MEC database of Bergmann et al. (2011) [1] or UBA were included with the aim to assess the environmental relevance of the lowest effect data. However, not for all 90 substances both values MECs and LOECs were available. As a result, only for 32 substances a risk quotient ($MEC_{max}/LOEC_{min}$) could be calculated as a proxy for their environmental relevance. These substances were: paracetamol, tramadol, amoxicilline, chloramphenicole, chlortetracycline, ciprofloxacin, doxycycline, erythromycin, lincomycin, oxytetracycline, sulfadimethoxine, sulfadimidine, sulfamethoxazole, carbamazepine, diazepam, clotrimazole, ivermectin, atenolol/ atenolol acid, metoprolol, propranolol, 17alpha-ethinylestradiol, 17beta-estradiol, norethisterone, diatrizoic acid, bezafibrate, gemfibrozil, acetyl cysteine, acetylsalicylic acid, diclofenac, ibuprofen, indometacin, naproxen. For tetracycline, quetiapine, venlafaxine and deltamethrin no MECs were available.

With the aim to compare mixture toxicity data with LOECs for isolated substances, the "mixture toxicity" datasheet was created. The data sheet "population relevance" has been used as a basis to quantify available data with relevance for the population level.

3. Results

3.1. Effects of pharmaceuticals in vivo

3.1.1 Number of publications and database entries

Altogether, 452 publications were analyzed for 90 pharmaceuticals. 325 papers contained data for vertebrates, plants/algae, protozoa, or bacteria (analyzed by the University of Tübingen), 179 papers contained data for invertebrates (analyzed by the GWT, TU Dresden) , and 50 of them contained data for invertebrates and either vertebrates, plants, protozoa or bacteria (analyzed by both, University of Tübingen and GWT TU Dresden). 232 of these publications were of relevance for the database, 95 provided additional information, 134 were not relevant, and for 6 publications the evaluation was not possible, since the full PDF was either not available or did not contain enough information to analyze the study.

All in all, 1678 entries were included in the database $OEKOTOX_{upgrade}$ (1434 for vertebrates, plants, protozoa and bacteria and 244 for invertebrates). Because many studies investigated multiple chemicals and endpoints, the number of database entries is much higher than the number of studies.

Figs. 1 and 2 show that most of the studies were conducted with antibiotics, followed by NSAIDs, anticonvulsants, antiparasitics, ß-blockers and contraceptives, and that the number of publications per pharmaceutical class is reflected by the number of database entries.

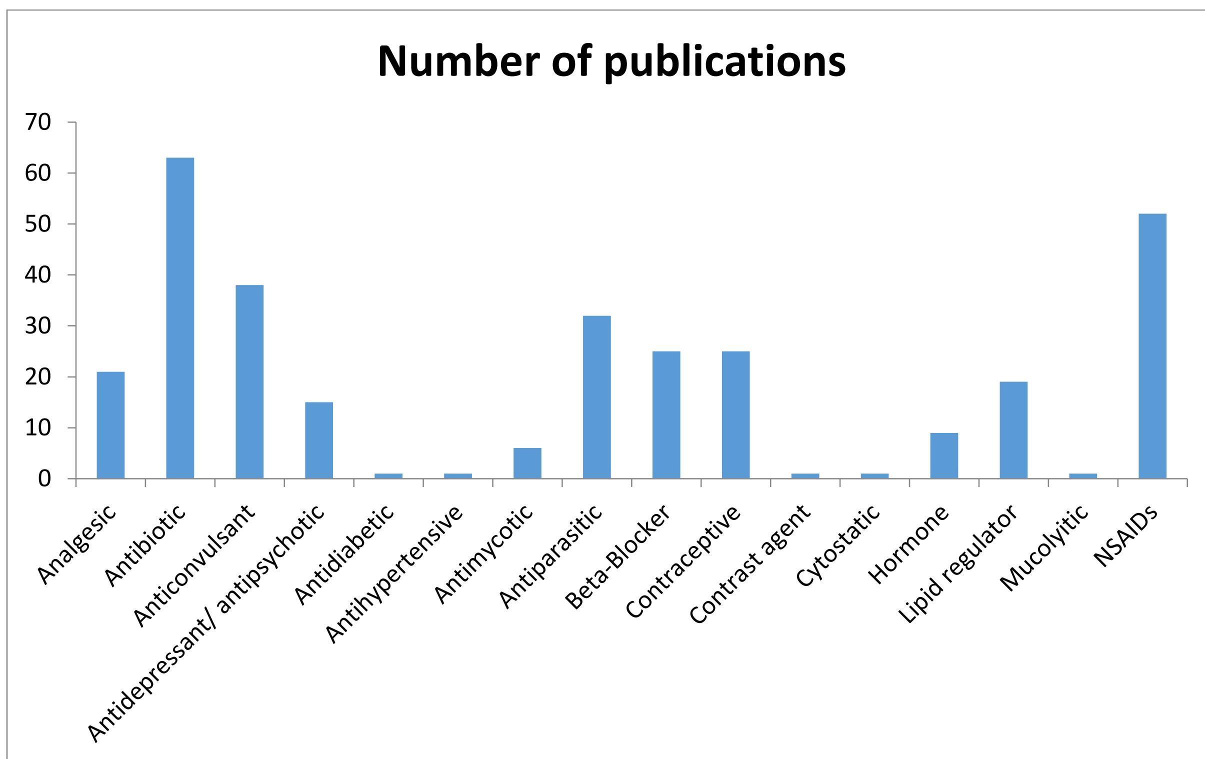


Figure 1: Number of publications per substance class

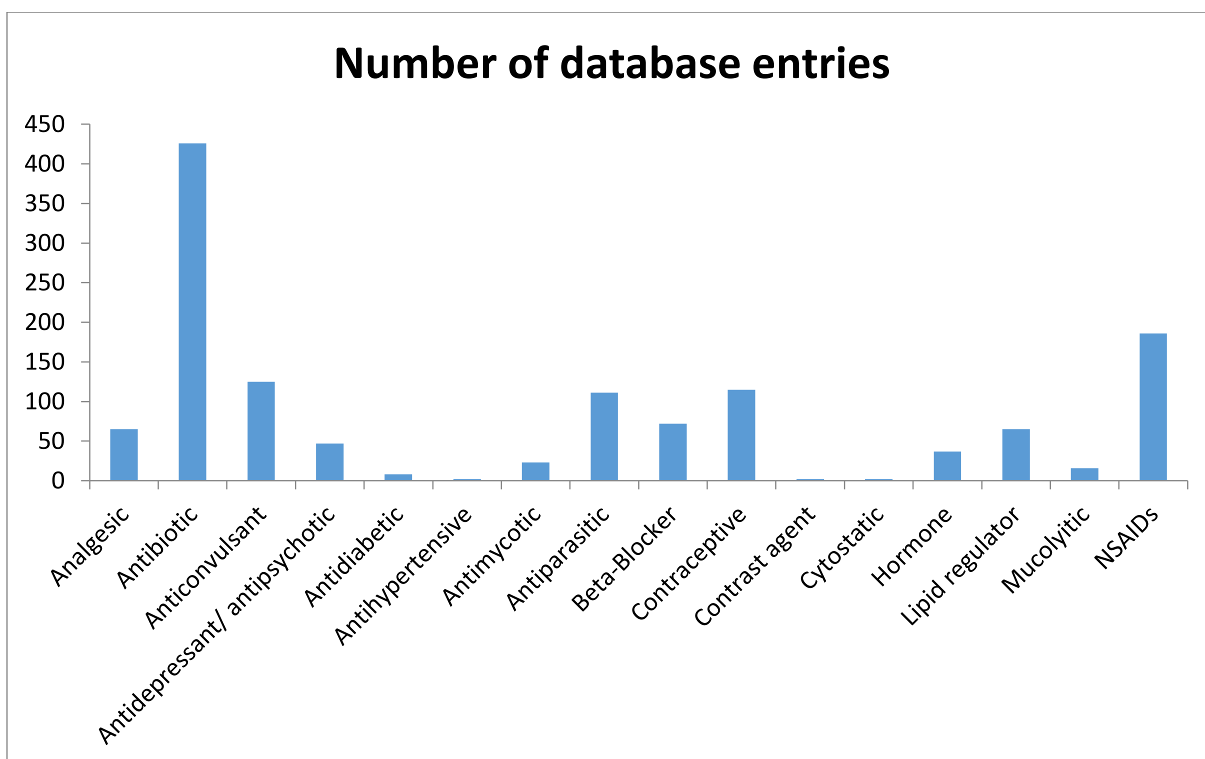


Figure 2: Number of database entries per substance class

3.1.2 Number of publications per organism group

Most publications on effects of pharmaceuticals in biota have been found for fish, followed by mollusks, plants/algae, crustaceans, and bacteria (Fig. 3). In general, this distribution pattern for the publication number is reflected by the number of database entries per organism group (Fig. 4), however, for plants/algae more data were available per publication than for mollusks, and for bacteria more database entries have been conducted than for crustaceans.

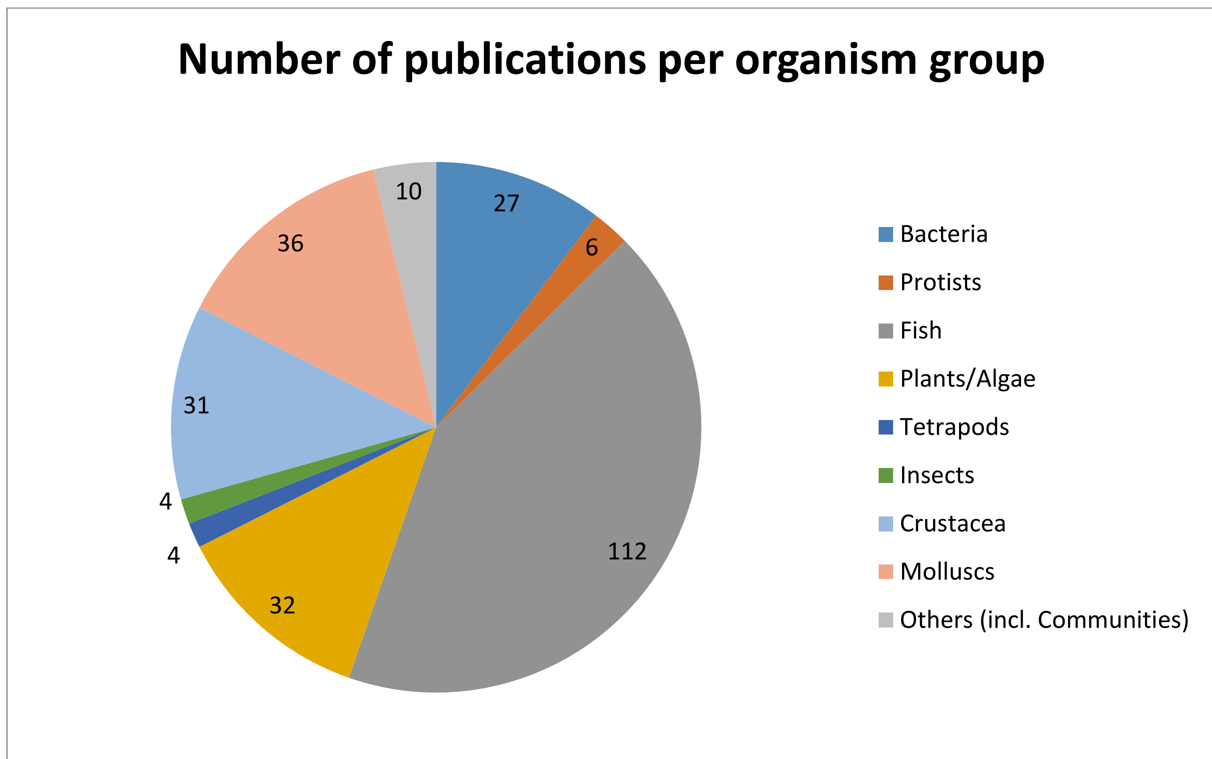


Figure 3: Number of studies per biota group

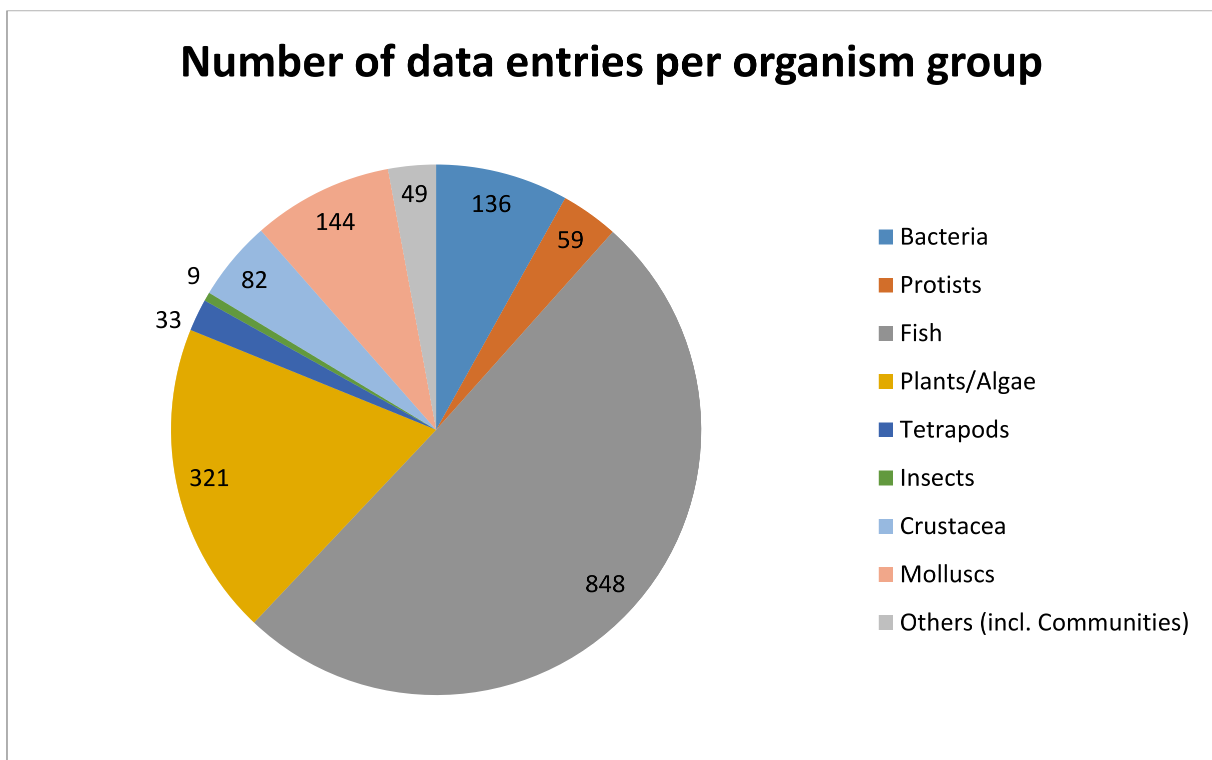


Figure 4: Number of database entries per biota group

3.1.3. Evaluation of data

3.1.3.1 Most sensitive biota classes for pharmaceuticals

In a first step, the lowest and second lowest effect data were analyzed with respect to their relation to distinct organism classes (Fig. 5).

It became evident that most of the lowest plus second lowest effect values were recorded for protozoans (represented by only a single test species: *Tetrahymena pyriformis*). Mollusks were shown to be very sensitive for anticonvulsants, antipsychotics, lipid regulators, analgesics (other than NSAIDs), hormones and contraceptives. The LOECs were in the range of ng/L – lower µg/L values.

Lowest or second lowest effect values for fish (several species) were determined for hormones, mycolytica, anticonvulsants, antiparasitics and lipid regulators. Also for them, the LOECs are in the range of ng/L - lower µg/L values.

Bacteria and plants were shown to be highly sensitive to antibiotics, crustaceans were very sensitive to antiparasitics.

In a second step, data were analyzed in a more detailed way by determining the number of pharmaceuticals for which defined LOECs (< 0.1, 1 or 10µg/L) were reported for the investigated species in order to identify the most sensitive species for pharmaceuticals.

Figure 6 shows that the most sensitive species with LOECs < 0.1 for 2-8 pharmaceuticals were the ciliate *Tetrahymena pyriformis*, zebrafish (*Danio rerio*), rainbow trout (*Oncorhynchus mykiss*), medaka (*Oryzias latipes*), the mussel *Elliptio complanata* and the mudsnail *Potamopyrgus antipodarum*.

All extremely low LOECs (in the range of pg/L) for *Tetrahymena pyriformis* derive from only a single publication by Lang & Köhidai (2012) [3], the reliability of which, however, could not be proven (chapter 3.1.5). Additionally, the conclusion on the most sensitive species is biased by the fact that these species are also among the most frequently used test organisms.

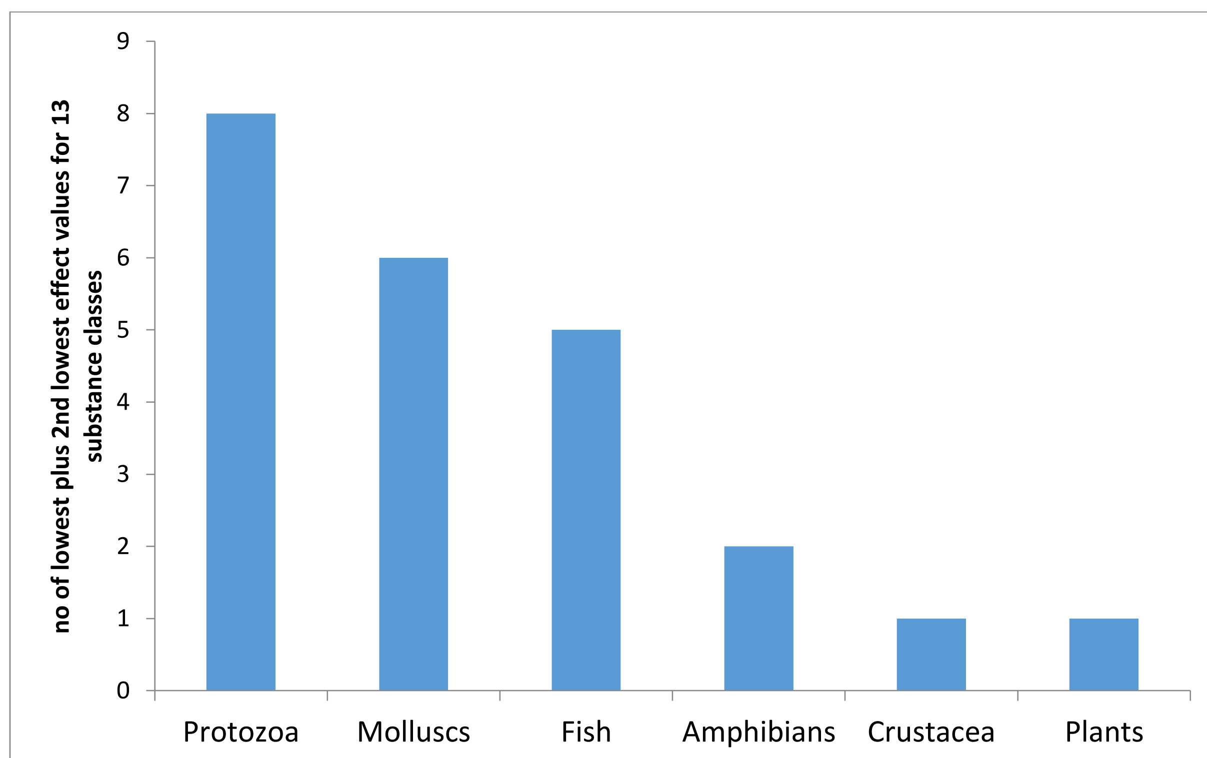


Figure 5: Number of lowest plus second lowest effect values for 13 pharmaceutical classes

Figure 6: Number of pharmaceuticals with defined LOECs for investigated species

3.1.3.2 Most sensitive effect endpoints per substance classes

In order to identify most sensitive effect endpoints for pharmaceuticals, data were analyzed with respect to the number of pharmaceuticals with defined LOECs (< 0.1, 1 or 10 µg/L) for the investigated effect endpoints. Figure 7 makes evident that the most sensitive endpoints with LOECs < 0.1 for 3-8 pharmaceuticals were chemotaxis, behavior, vitellogenin induction, growth rate, reproduction, histopathological alterations, molecular stress biomarkers, oxidative stress markers, receptor binding, and gene expression. All extremely low LOECs (in the range of pg/L) for chemotaxis derive from the above mentioned study of Lang & Köhidai (2012) [3] who used *Tetrahymena pyriformis* as test organism. As mentioned above, the reliability of this study, however, could not be proven (shown in chapter 3.1.5).

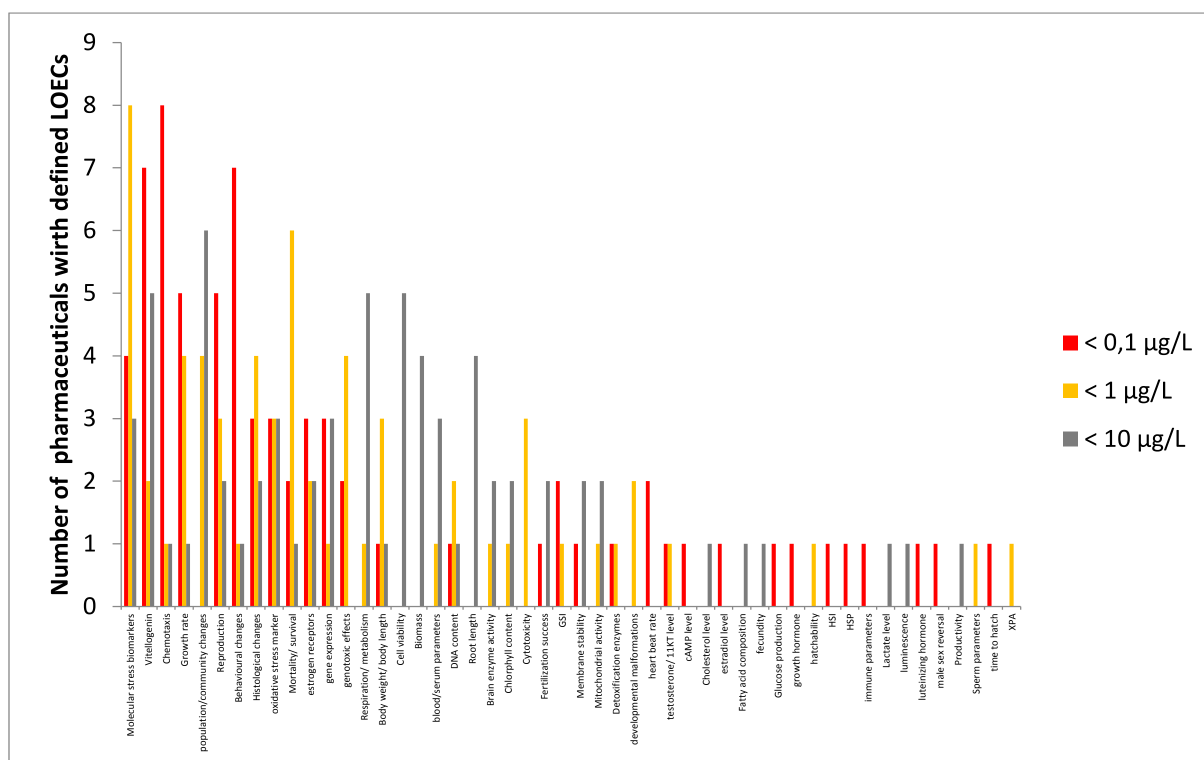


Figure 7: Number of pharmaceuticals with defined LOECs for investigated effect endpoints

3.1.3.3 Identification of pharmaceuticals of highest relevance

To identify the risk exerted by chemicals for the aquatic environment, different types of risk quotients are calculated in ecological risk assessment (PEC/PNEC ratios for industrial chemicals and pharmaceuticals, TER for pesticides). The PEC/PNEC ratio is defined by (1) the PEC, which represents the concentration of a chemical supposed to occur in the environment (predicted environmental concentration), and (2) the PNEC (predicted no effect concentration) which is calculated on the basis of the lowest observed effect concentration in any group of biota (LOEC), divided by a safety factor which itself depends on the size and quality of the data for different trophic levels. A possible risk for the environment is indicated by a risk quotient larger than 1.

In the present study, the calculation of the risk quotient slightly differs from this routine procedure: For those pharmaceuticals, for which both Measured Environmental Concentrations (MECs) (either from Bergmann et al., 2011 [1] or UBA; summarized in data sheet ""MECs & LOECs")") and LOEC values were available, MEC/LOEC quotients were calculated as a proxy for the environmental relevance of LOECs by using the highest available MEC (MEC_{max}) and the lowest LOEC ($LOEC_{min}$). In contrast to the established prospective risk assessment procedure, we used LOECs (lowest observed effect concentrations) instead of NOECs. This can be justified by the necessity to identify threshold values for effects as a prerequisite for pharmaceutical monitoring. A further reason is that our literature survey aims at identifying pharmaceuticals of environmental concern retrospectively, and thus had to take into consideration concentrations measured in the environment (MECs). Finally, only for 37% of the LOECs corresponding NOECs were available. If NOECs were used, the database would have become too small for the envisaged analyses.

For the calculations of MEC_{max}/$LOEC_{min}$ values also data from Bergmann et al. (2011) [1] were analysed.

Figure 8 makes evident that most pharmaceuticals with LOECs leading to risk quotients above 0.1 were antibiotics and NSAIDs followed by β-blockers and lipid regulators, analgesics different from NSAIDs and contraceptives/hormones.

When analyzing data with focus on $MEC_{max}/LOEC_{min}$ values for the respective biota classes (Fig. 9) it became evident that most pharmaceuticals, for which risk quotients >0.1 were calculated were investigated in plants/algae and invertebrates closely followed by vertebrates. For bacteria, only few risk quotient > 0.1 were found.

For the following substances, the risk quotient values were >1 (>0.1):

Vertebrates: >1: atenolol, carbamazepine, diclofenac, 17α-ethinylestradiol, 17β-estradiol, gemfibrozil, ibuprofen, metoprolol, paracetamol, propranolol (>0.1: bezafibrate).

Invertebrates: >1: bezafibrate, carbamazepine, diclofenac, erythromycin, 17α-ethinylestradiol, ibuprofen, ivermectin, oxytetracycline, paracetamol, propranolol, sulfamethoxazole (>0.1: ciprofloxacin, gemfibrozil, 17β-estradiol)

Plant/Protozoa: >1: acetylsalicylic acid, diatrizoic acid, diclofenac, erythromycin, lincomycin, metoprolol, naproxen, oxytetracycline, paracetamol, propranolol, sulfadimidine, sulfamethoxazole (>0.1: clotrimazole, ibuprofen)

Bacteria: >1: paracetamol, erythromycin, sulfadimidine, sulfamethoxazole (>0.1: diclofenac, gemfibrozil).

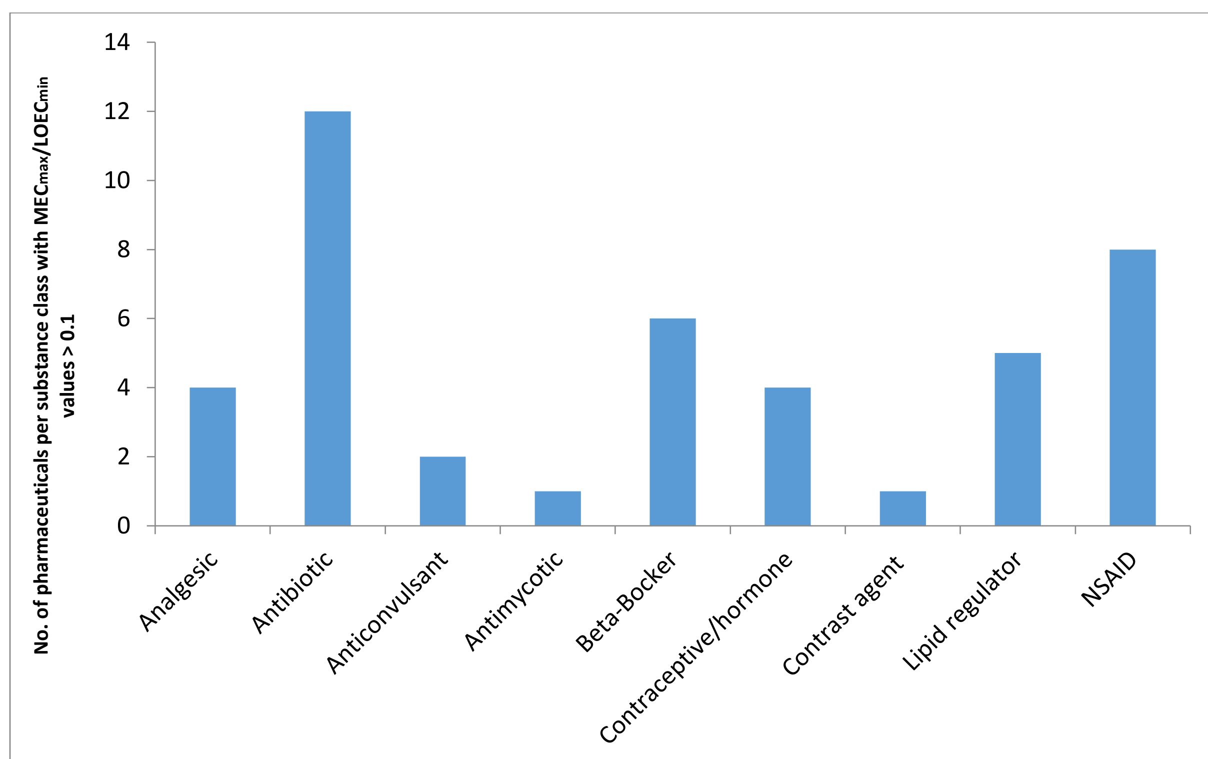


Figure 8: Number of pharmaceuticals per pharmaceutical class with $MEC_{max}/LOEC_{min}$ values >0.1

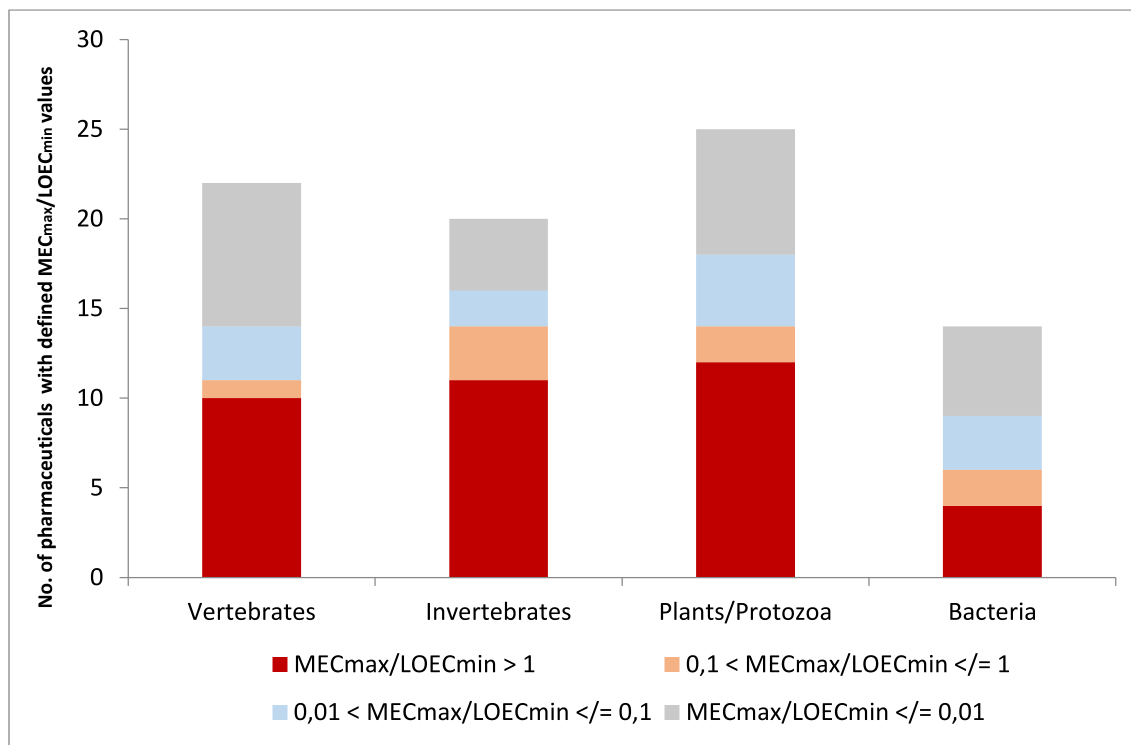


Figure 9: Number of pharmaceuticals with defined $MEC_{max}/LOEC_{min}$ values per organism group

Table 2 makes evident that, according to the calculated risk quotients, the pharmaceuticals of highest relevance (with $MEC_{max}/LOEC_{min}>0.1$) in at least three different biota classes were:

- Paracetamol (analgesic, acetaminophen),
- Diclofenac, ibuprofen (analgesic, NSAIDs - non-steroidal anti-inflammatory drugs)
- Propranolol (ß-blocker)
- Sulfamethoxazole, Erythromycin (antibiotics)
- Gemfibrozil (lipid regulator)

Table 2: Priority pharmaceuticals for which $MEC_{max}/LOEC_{min}$ values were >0.1 in 1, 2, 3 or 4 biota groups before reliability evaluation, including data from $OEKOTOX_{upgrade}$ and Bergmann et al. (2011) [1]

$MEC_{max}/LOEC_{min}$ >0.1 in 4 organismic groups	**$MEC_{max}/LOEC_{min}$ >0.1 in 3 organismic groups**	**$MEC_{max}/LOEC_{min}$ >0.1 in 2 organismic groups**	**$MEC_{max}/LOEC_{min}$ >0.1 in 1 organismic groups**
Paracetamol	Ibuprofen	Bezafibrate	Acetylsalicylic acid
Diclofenac	Propranolol	Carbamazepine	Atenolol
	Sulfamethoxazole	17β-estradiol	Ciprofloxacin
	Gemfibrozil	17α-ethinylestradiol	Clotrimazole
	Erythromycin	Metoprolol	Diatrizoic acid
		Oxytetracycline	Lincomycin
		Sulfadimidine	Naproxen
			Ivermectin

3.1.3.4 Data for mixtures

In general, only for a few cases, a direct comparison between LOECs for an isolated substance and its toxicity in a pharmaceutical mixture could be realized, since data differed with respect to species,

effect endpoints or test designs. However, for a few substances, such a comparison was possible (summarized in Table 3)

The table makes evident that, dependent on - at least - the chemical tested and the effect endpoint under investigation, the toxicity of a single pharmaceutical could be lower, higher or equal to its toxicity when applied in a mixture with other compounds.

However, since no mode of action-based effect endpoints were investigated in any of these studies, the specific contribution of the respective substance to the toxicity of the chemical mixture cannot be quantified.

Table 3: Comparison of pharmaceutical effect concentrations applied either isolated or mixture of pharmaceuticals

	Species	Effect	LOEC single	Effect conc. Mixture	Reference
Paracetamol	*Danio rerio*	Reproduction output	10	0.5	Galus et al. 2013a [9, 10]
	Danio rerio	Mortality	0.5	10	Galus et al. 2013a [9, 10]
Propranolol	*Mytilus galloprovincialis*	cAMP-level, PKA activity	0.0003	NOEC 0.0003	Franzelitti et al. 2011, 2013 [11, 12]
Diclofenac	*Dreissena polymorpha*	Molecular stress level	0.3	0.1	Parolini et al. 2013 [13]

3.1.3.5. Data with population relevance

All data collected in the present study were investigated with respect to their population relevance. We defined data as being relevant for the population level as containing information on effects related to (1) community changes, (2) reproduction, fecundity, fertility, embryo development, sex ratio, intersex, imposex (summarized as “reproduction”), (3) behavior including mating behavior, (4) growth, and (5) survival / mortality.

Altogether, 106 studies and 561 database entries were found to be related to population-relevant endpoints. Figure 10 shows that most database entries contained information of influences of pharmaceuticals on survival and mortality, growth and reproduction. Studies related to behavioral and community changes were in the minority. Hormone-like acting substances did induce population-relevant effects already in relatively low concentrations.

Number of database entries with population-relevant effect-endpoints

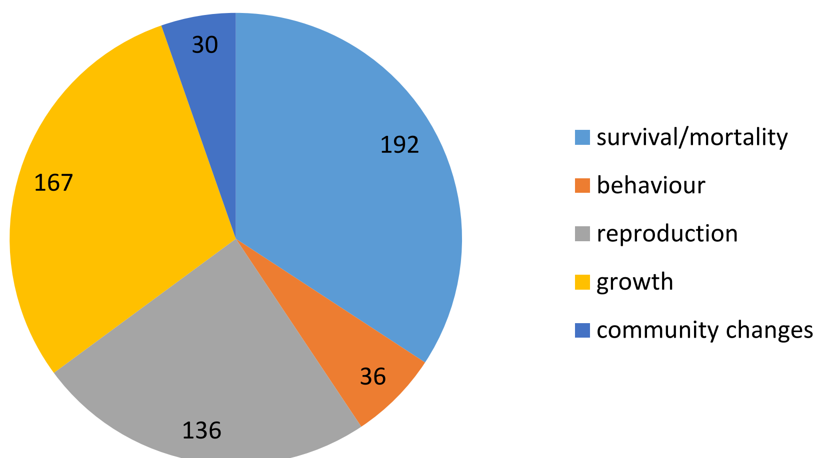


Figure 10: Number of database entries with population-relevant endpoints

3.1.4 Reliability of publications

For those studies which contained data for the most relevant pharmaceuticals (lowest LOECs) and for the most sensitive endpoints and organisms, the reliability was investigated according to the criteria of Wright-Walters et al. (2011) [2].

These are the following:

"

1) A thorough description of the experimental design, including exposure regime and replication,
2) Analytical confirmation of test concentrations
3) Description of ecologically relevant endpoints and all supplemental morphological information collected
4) Use of test procedures that are based, at least generally, on internationally accepted procedures and practices. Newly developed test procedures must be able to be repeated, and meet all other required criteria
5) Clear linkage of reported findings with the exact experimental design, and
6) Sufficient reporting of results, including system performance, toxicity results, and statistical methods employed to ascertain how the data support the conclusions that are drawn

"

If criteria 1, 2, 3 and 6 were met the study was designated as "reliable". If only 3 of these criteria were met, it was designated as "conditionally reliable", If less than 3 criteria were met or mistakes became obvious, the study was designated to be "not reliable".

Consideration was also given to whether the studies were conducted according to GLP and whether both NOEC and LOEC values were provided; however, these criteria did not influence the decision about reliability.

Taking all together, 72 publications have been checked for reliability. Nine of them were reliable, 49 conditionally reliable and 14 not reliable (of which 4 were, in part, conditionally reliable). In Table 4, the results of the reliability assessment are summarized. For studies which were not reliable, further information is given in Table 5. Except for the study of Fairchild et al. (2011) [14], none of the investigations were conducted under GLP.

Table 4: Publications checked for reliability, reliability criteria met, and decision on reliability

Reference	Reliability criteria met	Reliabilty
Antunes et al., 2013 [15]	1,3,6	conditionally reliable
Backhaus et al., 2011 [16]	3, (6)	not reliable
Bajet, 2012[17]	1,3,6	conditionally reliable
Benstead, 2011 [18]	1,2,3	conditionally reliable
Boltes et al., 2012 [19]	1,3,6	conditionally reliable
Boonstra et al., 2011 [20]	1,2,3	conditionally reliable
Chandra et al., 2012 [21]	1,3,6	conditionally reliable
Chen et al., 2012 [22]	1,3, 6	conditionally reliable
Claessens et al., 2013 [23]	1,3,6	conditionally reliable
Contardo-Jara, 2011 [24]	1,2,3	conditionally reliable
Doyle et al., 2013 [25]	1,3,6	conditionally reliable
Fairchild et al., 2011 [14]	1,2,3,6	reliable
Feito et al., 2012 [26]	1,3,(6)	conditionally reliable (mitochondrial activity, DNA content), not reliable (lipid peroxidation, chlorophyll content)
Feito et al., 2013 [27]	1,3, (6)	conditionally reliable (DNA-content), not reliable (mitochondrial activity)
Finn et al., 2012 [28]	2,3,6	conditionally reliable
Fong & Hoy, 2012 [29]	1,3,6	conditionally reliable
Franzellitti et al., 2011 [11]	1,3,6	conditionally reliable
Franzellitti et al., 2013 [12]	1,3,6	conditionally reliable
Gagné et al., 2012 [30]	2,3,6	conditionally reliable
Galus et al., 2013 [10]	(1),2,3,(6)	not reliable
Gonzalez-Rey, 2011 [31]	1,3,6	conditionally reliable
Gust et al., 2012 [32]	1,3,6	conditionally reliable
Gust et al., 2013 [33]	1,3,6	conditionally reliable
Hallgren et al., 2011 [34]	(1),3,6	conditionally reliable
Hallgren et al., 2012 [35]	1,2,3,6	reliable
Hillis et al., 2011 [36]	1,3,6	conditionally reliable
Hoffmann & Kloas, 2012 [37]	1,3,6	conditionally reliable
Huynh Thi et al., 2012 [38]	1,2,3,6	reliable
Ings et al., 2012 [39]	1,3,6	conditionally reliable
Johns et al. 2011 [40]	1,3,6	conditionally reliable
Kaptaner et al., 2011 [41]	1,3,6	conditionally reliable
Lang & Kohidai, 2012 [3]	1,3,(6)	not reliable
Lange et al., 2012 [42]	2,3,6	conditionally reliable
Lawrence et al., 2012 [43]	1,3,6	conditionally reliable
Lei et al., 2013 [44]	1,3,6	conditionally reliable
Liu et al., 2011 [45]	1,3,6	conditionally reliable
Liu et al., 2011 [46]	1,2,3,6	reliable
Madureira et al., 2011 [47]	1,3,6	conditionally reliable

Meina et al., 2013 [48]	1,3,6	conditionally reliable
Notch &Mayer, 2013 [49]	3,(6)	not reliable
Oliveira et al., 2012 [50]	1,3,6	conditionally reliable
Ozdemir et al., 2011 [51]	(1),3,6	conditionally reliable
Parolini et al., 2011 [52]	3,6	not reliable
Parolini et al., 2011 [53]	1,3,6	conditionally reliable
Parolini et al., 2013 [13]	3,6	not reliable
Ragugnetti et al., 2011 [54]	1,3,6	conditionally reliable
Reyhanian et al., 2011 [55]	1,3,6	conditionally reliable
Ribeiro et al., 2012 [56]	1,3,6	conditionally reliable
Rocco et al., 2012 [57]	(3), 6	not reliable
Rocco et al., 2012 [58]	(3), (6)	not reliable
Saravanan et al., 2011 [59]	1,3,6	conditionally reliable
Sarria et al., 2011 [60]	1,3,(6)	conditionally reliable,
Shen et al., 2012 [61]	1,2,3,6	conditionally reliable
Shi et al., 2012 [62]	1,3,(6)	conditionally reliable (chronic test), not reliable (acute test)
Silva et al., 2012 [63]	1,2,3,6	reliable
Skolness et al., 2012 [64]	1,2,3,(6)	reliable
Sponchiado et al. 2011 [65]	3,6	not reliable
Stange et al., 2012 [66]	1,3,6	conditionally reliable
Stange et al., 2012 [67]	1,3,(6)	cond. reliable (reproduction), not reliable (gene expression)
Thomas et al., 2012 [68]	1,3,6	conditionally reliable
Toumi et al., 2013 [69]	1,2,3,6	reliable
van Leeuwen et al., 2012 [70]	(1),3,6	conditionally reliable
Veach et al., 2012 [71]	1,3,6	conditionally reliable
Wang & Gunsch, 2012 [72]	1,2,3,6	reliable
Wang et al., 2011 [73]	1,3,6	conditionally reliable
Wu et al., 2012 [74]	1,3,6	conditionally reliable
Yan et al., 2013 [75]	1,2,3,6	reliable
Yergeau et al., 2012 [76]	(1), 6	not reliable
Yonar et al., 2011 [77]	1,3,6	conditionally reliable
Zhang & Gong, 2013 [78]	1,3,6	conditionally reliable
Zhang et al., 2012 [79]	1,3,6	conditionally reliable
Zhang et al, 2012 [80]	1,3,6	conditionally reliable

Table 5: Further information on pharmaceuticals tested, test organisms, effect endpoints and reasons for lacking reliability for not reliable studies

Reference	Pharmaceutical tested	Test organism	Effect endpoint	Reason for lacking reliability
Backhaus et al 2011 [16]	Fluoxetine, Propranolol, Clotrimazole	Periphyton community	Inhibition of total pigment content (biomass)	Lacking replicates for some test substances, but not specified (N= 1 to N=5). Results from other studies were involved for calculations of some concentration-response-curves without representing them.
Feito et al 2012 [26]	Diclofenac	*Danio rerio, Polystichum subspicatum*	Lipid peroxidation, Chlorophyll autofluorescence	Partly not reliable for lipid peroxidation in zebrafish and Chlorophyll content in Polystichum due to lacking concentration-effect relationships. .
Feito et al 2013 [27]	Venlafaxine	*Polystichum spicatum*	Mitochondrial activity	Partly not reliable for mitochondrial activity due to lacking concentration-effect relationships.
Galus et al 2013 [10]	Paracetamol, Venlafaxine, Carbamazepine; Gemfibrozil	*Danio rerio*	Reproductive output, Embryonic mortality, Developmental malformations; Histopathological changes, Plasma estradiol level, Blood 11-Ketotestosterone level	Experimental mistake obvious with high concentrations of pharmaceuticals in control treatments, for Gemfibrozil higher than the treatment “low”. This information can be obtained from the “Supplementary data”, and it is mentioned in one sentence of the discussion. The effective concentrations highly differ from the nominal concentrations for which results are presented. For acetaminophen, e.g., the effective concentrations were only about 10% of the nominal concentrations.
Láng & Köhidai 2012 [3]	Acetylsalicylic acid, Diclofenac, Fenoprofen, Ibuprofen, Naproxen, Paracetamol, Erythromycin, Lincomycin, Sulfamethoxazole, Trimethoprim, Metoprolol, Propranolol, Timolol, Diatrizoic acid	*Tetrahymena pyriformis*	Growth rate, Chemotactic behaviour	Calculation of EC50 values for growth inhibition unclear; no concentration-effect relationships for chemotaxis; authors recommend themselves to use the test system not as a quantitative, but a qualitative assay to prove for environmental effects of chemicals.
Notch & Mayer 2013 [49]	17alpha-ethinylestradiol	*Danio rerio*	Embryonic vitellogenin mRNA, embryonic Cyp1a mRNA, embryonic XPC mRNA (genome repair pathway), embryonic XPA mRNA (genome repair pathway)	Lacking replicates and insufficient description of exposition conditions. Effects for XPC/XPA-mRNA und CYP1a-mRNA which only occurred after 24h or 48 h. The authors wanted to show that some effects disappear after longer exposure times. Effects for vtg-mRNA remained stable for the entire exposure time.
Parolini et al 2011 a [52]	Ibuprofen	*Dreissena polymorpha*	Molecular stress biomarkers	Lacking replicates. Chemical analysis only in stock solution.
Parolini et al 2013 [13]	Ibuprofen, Diclofenac, Paracetamol	*Dreissena polymorpha*	Molecular stress biomarkers	Lacking replicates. Chemical analysis only in stock solution.
Rocco et al 2012 a [57]	Gemfibrozil	*Danio rerio*	Comet assay, Diffusion assay, RAPD-PCR	Insufficient description of experimental design (replica, organisms, test concentrations); results from controls not presented.
Rocco et al 2012 b [58]	Lincomycin, Erythromycin	*Danio rerio*	Micronucleus test, Comet assay	Insufficient description of experimental design (replica, organisms, test concentrations); no significant effects.
Shi et al 2012 [62]	Clotrimazole	*Xenopus laevis*	Embryo mortality, Embryo body length	lacking concentration-effect relationships in acute test

Sponchiado et al 2011 [65]	17beta-estradiol	Oreochromis niloticus	Micronucleus test, Nucleus abnormalities, Comet assay	Lacking replicates. Exposure from 5 to 35 days, but no renewal of test substances.
Stange et al 2012b [67]	17alpha-ethinylestradiol	*Potamopyrgus antipodarum*	Gene expression (estrogen receptor)	Effects on gene expression of estrogen receptor only occurred after 7 days, but not after 28 days, lacking time-effect relationship.
Yergeau et al 2012[76]	Erythromycin, Sulfamethoxazole, Sulfadimidine, Gemfibrozil, Erythromycin	Bacterial community	Microbial community composition (DNA level)	Insufficient description of experimental design (substances, solvents for stock solution, organisms). Exposure time 8 weeks without renewal

3.1.5 Consequences of reliability assessment for the described results

Our literature study revealed *Tetrahymena pyriformis* as the most sensitive test organism for pharmaceuticals prior to the reliability check of the publications. This assessment, however, is based on the fact, that for this protozoan extremely low LOECs are reported for a total of 8 substances by Láng & Köhidai (2012) [3] who used "chemotaxis" as an effect endpoint. Since the reliability assessment could not prove the reliability of this publication, three fish species (zebrafish, rainbow trout and medaka) can be identified as most sensitive organisms for pharmaceuticals followed by bivalves and snails. The publications which provide the lowest LOECs for these species did successfully pass the reliability assessment. Table 6, however, also makes evident, that for the three fish species the database was much larger (in total 489 database entries) than for the mentioned molluskan species (only 8 database entries). Also for crustaceans which represent ecologically important species of aquatic ecosystems only few data are available compared to fish.

Table 6: Assessment of data for most sensitive species with respect to reliability of publications

	Number of publications	Number of database entries	Number of database entries with LOEC ≤0.1	Number of reliable database entries with LOEC ≤0.1	Lowest LOEC [µg/L]	Lowest reliable LOEC [µg/L]
Tetrahymena pyriformis	1	28	13	--	0,0000151 (Láng & Köhidai 2012) [3]	--
Zebrafish (*Danio rerio*)	36	333	36	21	0,0104 (Lange et al 2012) [42]	0,0104 (Lange et al 2012) [42]
Rainbow trout (*Oncorhynchus mykiss*)	18	92	5	5	0,00266 (Ings et al 2012) [39]	0,00266 (Ings et al 2012) [39]
Medaka (*Oryzias latipes*)	8	64	12	12	0,001 (Lei et al 2013) [44]	0,001 (Lei et al 2013) [44]
mussel (*Elliptio complanata*)	2	4	3	3	0,04 (Gust et al, 2012) [32]	0,04 (Gust et al, 2012) [32]
mudsnail (*Potamopyrgus antipodarum*)	2	4	4	3	0,05 (Stange et al, 2012a) [66]	0,05 (Stange et al, 2012) [66]
Daphnia magna	12	42	2	2	0,011 (Toumi et al, 2013) [69]	0,011 (Toumi et al, 2013) [69]
Gammarus spec	6	11	0	0	1 (Boonstra et al, 2011) [20]	1 (Boonstra et al, 2011) [20]

With respect to the determination of the most important pharmaceuticals for environmental effects the reliability assessment had a more important influence on the final result. In chapter 3.1.4.3 all data available from the OEKOTOX and the $\mathrm{OEKOTOX}_{\mathrm{upgrade}}$ databases were assessed with the result summarized in table 2. Since the publications of the OEKOTOX database analyzed by Bergmann et al. (2011) [1], however, were not at our disposal for reliability checks, we could only compare the results

for the most important pharmaceuticals based on data of the $OEKOTOX_{upgrade}$ database prior (table 7) and after reliability assessment (table 8). The results of the analyses based on data from $OEKOTOX_{upgrade}$ only before reliability evaluation did not differ much from those when the OEKOTOX data were included. . This is due to the fact that the original OEKOTOX database mainly reports on EC_{50} values and includes only a low number of LOECs. Only a slight difference becomes obvious for Metoprolol with $MEC_{max}/LOEC_{min}$>0.1 in only 1 organismic group instead of 2. After assessment of publication reliability, however, only for diclofenac $MEC_{max}/LOEC_{min}$-values >0.1 were found in >3 biota classes. As further important pharmaceuticals propranolol, sulfamethoxazole, bezafibrate, 17α-ethinlyestradiol, 17β-estradiol and oxytetracycline were identified with $MEC_{max}/LOEC_{min}$-values >0.1 for 2 biota classes.

Table 7: Priority pharmaceuticals before reliability evaluation, only based on data from $OEKOTOX_{upgrade}$

$MEC_{max}/LOEC_{min}$ >0.1 in 4 organismic groups	**$MEC_{max}/LOEC_{min}$ >0.1 in 3 organismic groups**	**$MEC_{max}/LOEC_{min}$ >0.1 in 2 organismic groups**	**$MEC_{max}/LOEC_{min}$ >0.1 in 1 organismic groups**
Paracetamol	Ibuprofen	Bezafibrate	Acetylsalicylic acid
Diclofenac	Propranolol	Carbamazepine	Atenolol
	Sulfamethoxazole	17β-estradiol	Ciprofloxacin
	Gemfibrozil	17α-ethinylestradiol	Clotrimazole
	Erythromycin	Oxytetracycline	Diatrizoic acid
		Sulfadimidine	Lincomycin
			Naproxen
			Metoprolol

Table 8: Priority pharmaceuticals after reliability evaluation, only based on data from $OEKOTOX_{upgrade}$

$MEC_{max}/LOEC_{min}$ >0.1 in 4 organismic groups	**$MEC_{max}/LOEC_{min}$ >0.1 in 3 organismic groups**	**$MEC_{max}/LOEC_{min}$ >0.1 in 2 organismic groups**	**$MEC_{max}/LOEC_{min}$ >0.1 in 1 organismic groups**
Diclofenac		Propranolol	Ibuprofen
		Sulfamethoxazole	Paracetamol
		Bezafibrate	Carbamazepine
		17β-estradiol	Erythromycin
		17α-ethinylestradiol	Gemfibrozil
		Oxytetracycline	Sulfadimidine
			Atenolol
			Ciprofloxacin

3.1.6 Summary of part 1

The literature survey conducted in the present project reviewed publications on pharmaceutical effects in the environment from 2011- July 2013, and thus completed the database OEKOTOX established by Bergmann et al. (2011) [1] with the database $OEKOTOX_{upgrade}$. An Endnote library was created which contains all publications analyzed. In addition, a data evaluation sheet was created as a base for the identification of the most sensitive organisms, the most sensitive effect endpoints, and the ecologically most relevant pharmaceuticals.

For data published between 2011 and 2013, the following results were found:

- Most studies were conducted with antibiotics, followed by NSAIDs, anticonvulsants, antiparasitics, ß-blockers and contraceptives.
- Most of the research data are related to pharmaceutical effects in fish, followed by effects in mollusks, plants/algae, crustaceans, and bacteria.
- After the reliability evaluation three fish species (zebrafish, rainbow trout and medaka) were identified as most sensitive organisms for pharmaceuticals followed by bivalves and snails.
- Sensitive effect endpoints were behavior, vitellogenin induction, growth rate, reproduction, histopathological alterations, molecular stress biomarkers, oxidative stress markers, receptor binding, and gene expression

In order to evaluate the lowest and the second lowest effect values from both the OEKOTOX and the $OEKOTOX_{upgrade}$ database with respect to their ecological relevance, risk quotients were calculated as MEC_{max}/LOECmin. Risk quotients >0.1 were defined as to be of ecological relevance. The analyses provided the following results:

- Most pharmaceuticals for which risk quotients >0.1 were calculated were investigated in plants/algae and invertebrates closely followed by vertebrates. For bacteria, only few risk quotient >0.1 were found.
- Antibiotics, analgesics (NSAIDs), and ß-Blockers were the pharmaceutical classes for which data with the highest ecological relevance were found.
- After reliability assessment diclofenac was identified as the pharmaceutical of highest relevance (with $MEC_{max}/LOEC_{min}$ >0.1 in > 3 biota classes). For propranolol, sulfamethoxazole, bezafibrate, 17α-ethinlyestradiol, 17β-estradiol and oxytetracycline $MEC_{max}/LOEC_{min}$-values >0.1 were calculated for 2 biota classes.

Only in a few cases, the toxicity of an isolated substance could be compared to its toxicity in a pharmaceutical mixture. It could be shown that, at least dependent on the chemical tested and the effect endpoint under investigation, the toxicity of a single pharmaceutical can either be lower, higher, or equal to its toxicity when applied in a mixture together with other compounds. Since no mode of action-based effect endpoints were investigated it was impossible to quantify the specific contribution of the respective substance to the toxicity of the chemical mixture under investigation.

106 studies and 561 database entries were found to be related to population-relevant endpoints with most data on survival/mortality followed by growth and reproduction. Studies related to community changes and behavioral endpoints were in the minority. Direct population relevance, for example induced changes in the composition of bacterial, protozoan or algal communities could only be shown in few studies.

From part 1 of this literature review the following shortcomings could be identified:

- More studies which fulfill the reliability criteria are necessary, especially more chemical analyses should be integrated
- Only few data for invertebrates are available
- Data for ecologically relevant crustaceans are lacking
- Data on sediment toxicity are lacking
- More population-relevant community data are necessary
- In order to be able to identify the contribution of isolated pharmaceuticals to chemical mixtures, mode of action-based effect endpoints have to be investigated.

4. Conclusions

As a basis for the development of an effect-based strategy to biomonitor pharmaceuticals, a literature review has been conducted which revealed the necessity to develop mode of action-based biotests for routine monitoring of distinct pharmaceutical classes. In this context, a strategy to monitor entire classes of pharmaceuticals with the same mode of action is given preference because a monitoring programme comprising different biotests for each single substance would cause unrealistically high costs, and a monitoring programme which focusses on just a few lead substances would drastically underestimate the risk exerted by a plethora of pharmaceuticals excluded from analysis. Consequently, priority should be given to develop effect-oriented *in vitro* tests for pharmaceutical classes and, within those, to analgesics, ß-blockers, and antibiotics. Such *in vitro* assays need to be evaluated with in-vivo test systems in parallel. In view to incorporate living organisms here, the literature review revealed ciliate, fish, and mollusk species to exhibit particular sensitivity to pharmaceuticals. Most sensitive endpoints were behavior (chemotaxis), vitellogenin synthesis, growth, reproduction, histological responses, biochemical stress markers, changes in gene expression profiles, receptor binding, and, with reservation, the heart rate. Numerous pharmaceuticals have also been tested for their impact on parameters that directly influence population development: reproduction, fecundity, ontogeny, mating patterns. Here, most significance has been assigned to pharmaceuticals with endocrine action. In contrast to single substances, effects of mixtures of pharmaceuticals can hardly be assessed reliably, since data are not consistent. Consequently, mode of action-based biotests are necessary to decipher the contribution of single substances to the toxicity exerted by a mixture of pharmaceuticals. Endpoints which may be candidates to track mode of action-specific effects of pharmaceuticals are receptor affinity, vitellogenin induction (for estrogenic hormones), specific induction of gene expression, specific repression of enzymes (e.g. COX) or the formation of biochemical secondary products (e.g. lipid peroxides).

Overall, the constructed database represents an extensive compilation of recently generated data on pharmaceutical effects, which will help researchers orienting in this quickly growing field. Besides the identification of certain promising test species and effect endpoints, there is growing evidence that several pharmaceuticals bear the potential to exhibit effects at environmentally relevant concentrations. They may therefore pose serious risks towards aquatic ecosystems and further studies are urgently needed.

The evaluation also showed that researchers need to put a higher effort into improving the reliability of their reported data; this includes a conclusive experimental design as well as a comprehensibly documentation and interpretation of the results.

5. Identification of shortcomings

The literature review generally revealed a strong heterogeneity of data, frequently resulting in just a single dataset for a species. Furthermore, in fish, most studies have been conducted with model species or with species of only local relevance. Consequently, single studies providing numerous data for a large number of chemicals which have been conducted with a single test organism in a single test run will bias the empirical evidence, particularly if the reliability of this test or study is in question.

In view to the relevance of data for Germany, a major shortcoming is the scarcity of data on ecologically relevant invertebrates (gammarids or biota of the sediment). Thus, the sensitivity of key species of home waters cannot be reliably assessed. Generally, data on sediment toxicity are limited in number. Only few publications report on studies that have used the same endpoint in the same test organism for different pharmaceuticals, and mode of action-specific endpoints are rarely used both in studies on single substances and mixtures. Quite often, chemical analytics supplementing biological tests is missing, and only about 70% of publication reviewed met the reliability criteria of Wright-Walters et

al. (2011) to a sufficient extent. In general, a mode of action-based *in vitro* test for non-hormonal pharmaceuticals does not exist.

6. Future perspectives in the biomonitoring of pharmaceuticals

- We suggest to coin biotests that are based on mode of action-specific mechanisms and thus are specific for pharmaceutical classes and can be implemented in monitoring programs. Advantages of such biotests would be
  - the integration of overall chemicals belonging to an mode of action-specific class of pharmaceuticals, irrespective of their accessibility by chemical analytics which can be limited by constraints posed by methodological detection limits, laboratory capacities, or budgetary limits,
  - a pre-adaptation for the monitoring of future pharmaceuticals that exhibit the same mode of action as those the test has been developed for,
  - and the integration of combinatory effects of mixtures of pharmaceuticals.

- Suitable prototypes implementing this idea would be *in vitro* tests for analgesics, like NSAIDs, and *in vitro* tests for ß-blockers.
- The development of these *in vitro* biotests must go in line with *in vivo* experiments on ecologically relevant species which represent water and sediment biota in order to validate the sensitivity of the novel *in vitro* tests and to “ecologically calibrate” their signals. In these *in vivo* studies, identical endpoints shall be investigated in the laboratory and in field-relevant exposure systems, both for single pharmaceuticals and their mixtures. This strategy will provide necessary information regarding
  - the relevance of *in vitro* test signals for the situation *in vivo*,
  - the necessity to artificially concentrate water samples,
  - the relevance of laboratory studies for the field situation,
  - the significance of mixture toxicity, and
  - differences in the toxicity of pharmaceuticals to water- and sediment-living biota.
- These suggestions are completely in line with the postulations of Ankley et al. (2007) who emphasize the impotance of mode of action-based studies for pharmaceutical monitoring, and of Brausch et al. (2012) [6] who stress the necessity of

  “...(a) chronic toxicity data for individual pharmaceuticals to benthic invertebrates, including bivalves, and fish is lacking;
  (b) Effects of pharmaceuticals on threatened or endangered species, which warrant protection at the individual level of biological organization;
  (c) MOA-based studies, in which biochemical and histological alterations are investigated or studies in which genetic alterations are monitored in response to long-term pharmaceutical exposure;
  (d) Techniques capable of detecting sensitive endpoints in aquatic organisms, such as *in vitro* and computational toxicology, for prioritizing chemicals and pathways for future studies;
  (e) Data on complex mixtures of pharmaceuticals that found in WWTP effluents”

7 References - Part 1:

[1] **Bergmann, A., Fohrmann, R., Weber, F.A.** (2011): Zusammenstellung von Monitoringdaten zu Umweltkonzentrationen von Arzneimitteln. *UBA Texte 66*, 2011.
[2] **Wright-Walters, M., Volz, C., Talbott, E., Davis, D.** (2011): An updated weight of evidence approach to the aquatic hazard assessment of Bisphenol A and the derivation a new predicted no effect concentration (Pnec) using a non-parametric methodology. *Sci. Tot. Env.* 409: 676–685.
[3] **Lang, J., Kohidai, L.** (2012): Effects of the aquatic contaminant human pharmaceuticals and their mixtures on the proliferation and migratory responses of the bioindicator freshwater ciliate *Tetrahymena*. *Chemosphere* 89, 592-601.
[4] **Fent, K. Weston; A.A., Caminada, D.** (2006): Ecotoxicology of human pharmaceuticals. *Aquat Toxicol.* 76(2):122-59.

[5] **Brauch, H.-J.** (2011): Organische Spurenstoffe in Gewässern. Vorkommen und Bewertung. *Gwf-Wasser/Abwasser*, 12, 1206-1211

[6] **Brausch, J.M., Connors, K.A., Brooks, B.W., Rand, G.M.** (2012): Human Pharmaceuticals in the aquatic environment: A review of recent toxicological studies and considerations for toxicity testing. in: **Whitacre, D.M.** (Ed.), *Reviews of Environmental Contamination and Toxicology*, Vol 218. Springer, New York, pp. 1-99.

[7] **Eggeling, C., Brand, L., Ullmann, D., Jäger, S.** (2003): Highly sensitive fluorescence detection technology currently available for HTS. *Drug Discov Today*. 8(14): 632-41.

[8] **Escher BI, Bramaz N, Eggen RIL, Richter M** (2005): *In vitro* assessment of modes of toxic action of pharmaceuticals in aquatic life. *Environ Sci Technol* 39:3090–3100

[9] **Galus, M., Jeyaranjaan, J., Smith, E., Li, H., Metcalfe, C., Wilson, J.Y.** (2013a): Chronic effects of exposure to a pharmaceutical mixture and municipal wastewater in zebrafish. *Aquatic toxicology* (Amsterdam, Netherlands) 132-133, 212-222.

[10] **Galus, M., Kirischian, N., Higgins, S., Purdy, J., Chow, J., Rangaranjan, S., Li, H.X., Metcalfe, C., Wilson, J.Y.** (2013b): Chronic, low concentration exposure to pharmaceuticals impacts multiple organ systems in zebrafish. *Aquat. Toxicol.* 132, 200-211.

[11] **Franzellitti, S., Buratti, S., Valbonesi, P., Capuzzo, A., Fabbri, E.** (2011): The beta-blocker propranolol affects cAMP-dependent signalling and induces the stress response in Mediterranean mussels, *Mytilus galloprovincialis*. *Aquat. Toxicol.* 101, 299-308.

[12] **Franzellitti, S., Buratti, S., Valbonesi, P., Fabbri, E.** (2013): The mode of action (MOA) approach reveals interactive effects of environmental pharmaceuticals on *Mytilus galloprovincialis*. *Aquat. Toxicol.* 140, 249-256.

[13] **Parolini, M., Pedriali, A., Binelli, A.** (2013): Application of a Biomarker Response Index for Ranking the Toxicity of Five Pharmaceutical and Personal Care Products (PPCPs) to the Bivalve *Dreissena polymorpha*. *Arch. Environ. Contam. Toxicol.* 64, 439-447.

[14] **Fairchild, W., Doe, K., Jackman, P., Akaishi, F., Arsenault, J., Aube, J., Losier, M., Cook, A.** (2011): Acute and chronic toxicity of two formulations of the pyrethroid pesticide deltamethrin to amphipods, sand shrimp and lobster larvae (PO). *Canadian Technical Report of Fisheries and Aquatic Sciences* 2949, 64-65.

[15] **Antunes, S.C., Freitas, R., Figueira, E., Goncalves, F., Nunes, B.** (2013): Biochemical effects of acetaminophen in aquatic species: edible clams Venerupis decussata and Venerupis philippinarum. *Environ. Sci. Pollut. Res.* 20, 6658-6666.

[16] **Backhaus, T., Porsbring, T., Arrhenius, A., Brosche, S., Johansson, P., Blanck, H.** (2011): Single-substance and mixture toxicity of five pharmaceuticals and personal care products to marine periphyton communities. *Environ. Toxicol. Chem.* 30, 2030-2040.

[17] **Bajet, C.M., Kumar, A., Calingacion, M.N., Narvacan, T.C.** (2012): Toxicological assessment of pesticides used in the Pagsanjan-Lumban catchment to selected non-target aquatic organisms in Laguna Lake, Philippines. *Agric. Water Manage.* 106, 42-49.

[18] **Benstead, R.S., Baynes, A., Casey, D., Routledge, E.J., Jobling, S.** (2011): 17 beta-Oestradiol may prolong reproduction in seasonally breeding freshwater gastropod mollusks. *Aquat. Toxicol.* 101, 326-334.

[19] **Boltes, K., Rosal, R., Garcia-Calvo, E.** (2012): Toxicity of mixtures of perfluorooctane sulphonic acid with chlorinated chemicals and lipid regulators. *Chemosphere* 86, 24-29.

[20] **Boonstra, H., Reichman, E.P., van den Brink, P.J.** (2011): Effects of the veterinary pharmaceutical ivermectin in indoor aquatic microcosms. *Arch. Environ. Contam. Toxicol.* 60, 77-89.

[21] **Chandra, K., Bosker, T., Hogan, N., Lister, A., MacLatchy, D., Currie, S.** (2012): Sustained high temperature increases the vitellogenin response to 17 alpha-ethynylestradiol in mummichog (*Fundulus heteroclitus*). *Aquat. Toxicol.* 118, 130-140.

[22] **Chen, X., Zhou, Y., Yang, D., Zhao, H., Wang, L., Yuan, X.** (2012): CYP4 mRNA expression in marine polychaete Perinereis aibuhitensis in response to petroleum hydrocarbon and deltamethrin. *Marine Pollution Bulletin* 64, 1782-1788.

[23] **Claessens, M., Vanhaecke, L., Wille, K., Janssen, C.R.** (2013): Emerging contaminants in Belgian marine waters: Single toxicant and mixture risks of pharmaceuticals. *Marine pollution bulletin* 71, 41-50.

[24] **Contardo-Jara, V., Lorenz, C., Pflugmacher, S., Nuetzmann, G., Kloas, W., Wiegand, C.** (2011): Exposure to human pharmaceuticals Carbamazepine, Ibuprofen and Bezafibrate causes molecular effects in *Dreissena polymorpha*. *Aquat. Toxicol.* 105, 428-437.

[25] **Doyle, M.A., Bosker, T., Martyniuk, C.J., Maclatchy, D.L., Munkittrick, K.R.** (2013): The effects of 17-alpha-ethinylestradiol (EE2) on molecular signalling cascades in mummichog (*Fundulus heteroclitus*). *Aquatic toxicology* (Amsterdam, Netherlands) 134-135, 34-46.

[26] **Feito, R., Valcarcel, Y., Catala, M.** (2012): Biomarker assessment of toxicity with miniaturised bioassays: diclofenac as a case study. *Ecotoxicology* 21, 289-296.

[27] **Feito, R., Valcarcel, Y., Catala, M.** (2013): Preliminary data suggest that venlafaxine environmental concentrations could be toxic to plants. *Chemosphere* 90, 2065-2069.

[28] **Finn, J., Hui, M., Li, V., Lorenzi, V., de la Paz, N., Cheng, S.H., Lai-Chan, L., Schlenk, D.** (2012): Effects of propranolol on heart rate and development in Japanese medaka (*Oryzias latipes*) and zebrafish (*Danio rerio*). *Aquat. Toxicol.* 122, 214-221.

[29] **Fong, P.P., Hoy, C.M.** (2012): Antidepressants (venlafaxine and citalopram) cause foot detachment from the substrate in freshwater snails at environmentally relevant concentrations. *Marine and Freshwater Behaviour and Physiology* 45, 145-153.

[30] **Gagne, F., Andre, C., Fortier, M., Fournier, M.** (2012): Immunotoxic potential of aeration lagoon effluents for the treatment of domestic and hospital wastewaters in the freshwater mussel *Elliptio complanata*. *J. Environ. Sci.* 24, 781-789.

[31] **Gonzalez-Rey, M., Bebianno, M.J.** (2011): Non-steroidal anti-inflammatory drug (NSAID) ibuprofen distresses antioxidant defense system in mussel *Mytilus galloprovincialis* gills. *Aquat. Toxicol.* 105, 264-269.

[32] **Gust, M., Gelinas, M., Fortier, M., Fournier, M., Gagne, F.** (2012): *In vitro* immunotoxicity of environmentally representative antibiotics to the freshwater mussel *Elliptio complanata*. *Environ. Pollut.* 169, 50-58.

[33] **Gust, M., Fortier, M., Garric, J., Fournier, M., Gagne, F.** (2013): Effects of short-term exposure to environmentally relevant concentrations of different pharmaceutical mixtures on the immune response of the pond snail *Lymnaea stagnalis*. *Sci. Total Environ.* 445, 210-218.

[34] **Hallgren, S., Volkova, K., Reyhanian, N., Olsen, K.H., Hallstrom, I.P.** (2011): Anxiogenic behaviour induced by 17 alpha-ethynylestradiol in male guppies (*Poecilia reticulata*). *Fish Physiol. Biochem*. 37, 911-918.

[35] **Hallgren, P., Sorita, Z., Berglund, O., Persson, A.** (2012): Effects of 17 alpha-ethinylestradiol on individual life-history parameters and estimated population growth rates of the freshwater gastropods *Radix balthica* and *Bithynia tentaculata*. *Ecotoxicology* 21, 803-810.
[36] **Hillis, D.G., Fletcher, J., Solomon, K.R., Sibley, P.K.** (2011): Effects of ten antibiotics on seed germination and root elongation in three plant species. *Arch. Environ. Contam. Toxicol.* 60, 220-232.
[37] **Hoffmann, F., Kloas, W.** (2012): Estrogens Can Disrupt Amphibian Mating Behavior. *PloS one* 7.
[38] **Huynh Thi, T., Silvestre, F., De Meulder, B., Thome, J.-P., Nguyen Thanh, P., Kestemont, P.** (2012): Combined effects of deltamethrin, temperature and salinity on oxidative stress biomarkers and acetylcholinesterase activity in the black tiger shrimp (*Penaeus monodon*). *Chemosphere* 86, 83-91.
[39] **Ings, J.S., George, N., Peter, M.C.S., Servos, M.R., Vijayan, M.M.** (2012): Venlafaxine and atenolol disrupt epinephrine-stimulated glucose production in rainbow trout hepatocytes. *Aquat. Toxicol.* 106, 48-55.
[40] **Johns, S.M., Denslow, N.D., Kane, M.D., Watanabe, K.H., Orlando, E.F., Sepulveda, M.S.** (2011): Effects of estrogens and antiestrogens on gene expression of fathead minnow (*Pimephales promelas*) early life stages. *Environ. Toxicol.* 26, 195-206.
[41] **Kaptaner, B., Unal, G.** (2011): Effects of 17 alpha-ethynylestradiol and nonylphenol on liver and gonadal apoptosis and histopathology in *Chalcalburnus tarichi*. *Environ. Toxicol.* 26, 610-622.
[42] **Lange, A., Katsu, Y., Miyagawa, S., Ogino, Y., Urushitani, H., Kobayashi, T., Hirai, T., Shears, J.A., Nagae, M., Yamamoto, J., Ohnishi, Y., Oka, T., Tatarazako, N., Ohta, Y., Tyler, C.R., Iguchi, T.** (2012): Comparative responsiveness to natural and synthetic estrogens of fish species commonly used in the laboratory and field monitoring. *Aquat. Toxicol.* 109, 250-258.
[43] **Lawrence, J.R., Zhu, B., Swerhone, G.D.W., Roy, J., Tumber, V., Waiser, M.J., Topp, E., Korber, D.R.** (2012): Molecular and microscopic assessment of the effects of caffeine, acetaminophen, diclofenac, and their mixtures on river biofilm communities. *Environ. Toxicol. Chem.* 31, 508-517.
[44] **Lei, B., Kang, J., Yu, Y., Zha, J., Li, W., Wang, Z.** (2013): Beta-estradiol 17-valerate affects embryonic development and sexual differentiation in Japanese medaka (*Oryzias latipes*). *Aquatic toxicology* (Amsterdam, Netherlands) 134-135, 128-134.
[45] **Liu, B.Y., Liu, W.Q., Nie, X.P., Guan, C., Yang, Y.F., Wang, Z.H., Liao, W.** (2011): Growth response and toxic effects of three antibiotics on Selenastrum capricornutum evaluated by photosynthetic rate and chlorophyll biosynthesis. *J. Environ. Sci.* 23, 1558-1563.
[46] **Liu, B.Y., Nie, X.P., Liu, W.Q., Snoeijs, P., Guan, C., Tsui, M.T.K.** (2011): Toxic effects of erythromycin, ciprofloxacin and sulfamethoxazole on photosynthetic apparatus in *Selenastrum capricornutum*. *Ecotoxicology and Environmental Safety* 74, 1027-1035.
[47] **Madureira, T.V., Cruzeiro, C., Rocha, M.J., Rocha, E.** (2011): The toxicity potential of pharmaceuticals found in the Douro River estuary (Portugal)-Experimental assessment using a zebrafish embryo test. *Environmental Toxicology and Pharmacology* 32, 212-217.
[48] **Meina, E.G., Lister, A., Bosker, T., Servos, M., Munkittrick, K., MacLatchy, D.** (2013): Effects of 17 alpha-ethinylestradiol (EE2) on reproductive endocrine status in mummichog (*Fundulus heteroclitus*) under differing salinity and temperature conditions. *Aquat. Toxicol.* 134, 92-103.
[49] **Notch, E.G., Mayer, G.D.** (2013): Impact of environmental estrogens on nucleotide excision repair gene expression in embryonic zebrafish. Comparative biochemistry and physiology. *Toxicology & pharmacology: CBP* 157, 361-365.
[50] **Oliveira, C., Almeida, J., Guilhermino, L., Soares, A.M.V.M., Gravato, C.** (2012): Acute effects of deltamethrin on swimming velocity and biomarkers of the common prawn *Palaemon serratus*. *Aquat. Toxicol.* 124, 209-216.
[51] **Ozdemir, A., Duran, M., Sen, A.** (2011): Potential use of the oligochaete *Limnodrilus profundicola* V., as a bioindicator of contaminant exposure. *Environ. Toxicol.* 26, 37-44.
[52] **Parolini, M., Binelli, A., Provini, A.** (2011a): Chronic effects induced by ibuprofen on the freshwater bivalve *Dreissena polymorpha*. *Ecotoxicology and Environmental Safety* 74, 1586-1594.
[53**] Parolini, M., Quinn, B., Binelli, A., Provini, A.** (2011b): Cytotoxicity assessment of four pharmaceutical compounds on the zebra mussel (*Dreissena polymorpha*) haemocytes, gill and digestive gland primary cell cultures. *Chemosphere* 84, 91-100.
[54**] Ragugnetti, M., Adams, M.L., Guimaraes, A.T.B., Sponchiado, G., de Vasconcelos, E.C., de Oliveira, C.M.R.** (2011): Ibuprofen genotoxicity in aquatic environment: An experimental model using *Oreochromis niloticus. Water Air Soil Pollut.* 218, 361-364.
[55] **Reyhanian, N., Volkova, K., Hallgren, S., Bollner, T., Olsson, P.E., Olsen, H., Hallstrom, I.P.** (2011): 17 alpha-Ethinyl estradiol affects anxiety and shoaling behavior in adult male zebra fish (*Danio rerio*). *Aquat. Toxicol.* 105, 41-48.
[56] **Ribeiro, C., Urbatzka, R., Castro, L.F.C., Carrola, J., Fontainhas-Fernandes, A., Monteiro, R.A.F., Rocha, E., Rocha, M.J.** (2012): *In vitro* exposure of Nile tilapia (*Oreochromis niloticus*) testis to estrogenic endocrine disrupting chemicals: mRNA expression of genes encoding steroidogenic enzymes. *Toxicol. Mech. Methods* 22, 47-53.
[57] **Rocco, L., Frenzilli, G., Zito, G., Archimandritis, A., Peluso, C., Stingo, V.** (2012a): Genotoxic effects in fish induced by pharmacological agents present in the sewage of some Italian water-treatment plants. *Environ. Toxicol.* 27, 18-25.
[58] **Rocco, L., Peluso, C., Stingo, V.** (2012b): Micronucleus test and comet assay for the evaluation of zebrafish genomic damage induced by erythromycin and lincomycin. *Environ. Toxicol.* 27, 598-604.
[59] **Saravanan, M., Karthika, S., Malarvizhi, A., Ramesh, M.** (2011): Ecotoxicological impacts of clofibric acid and diclofenac in common carp (*Cyprinus carpio*) fingerlings: Hematological, biochemical, ionoregulatory and enzymological responses. *J. Hazard. Mater.* 195, 188-194.
[60] **Sarria, M.P., Santos, M.M., Reis-Henriques, M.A., Vieira, N.M., Monteiro, N.M.** (2011): The unpredictable effects of mixtures of androgenic and estrogenic chemicals on fish early life. *Environ. Int.* 37, 418-424.
[61**] Shen, M.-F., Kumar, A., Ding, S.-Y., Grocke, S.** (2012): Comparative study on the toxicity of pyrethroids, alpha-cypermethrin and deltamethrin to *Ceriodaphnia dubia*. **Ecotoxicology and Environmental Safety** 78, 9-13.
[62] **Shi, H.H., Sun, Z., Liu, Z., Xue, Y.G.** (2012): Effects of clotrimazole and amiodarone on early development of amphibian (*Xenopus tropicalis*). *Toxicol Environ. Chem.* 94, 128-135.

[63] **Silva, P., Rocha, M.J., Cruzeiro, C., Malhao, F., Reis, B., Urbatzka, R., Monteiro, R.A.F., Rocha, E.** (2012): Testing the effects of ethinylestradiol and of an environmentally relevant mixture of xenoestrogens as found in the Douro River (Portugal) on the maturation of fish gonads-A stereological study using the zebrafish (*Danio rerio*) as model. *Aquat. Toxicol.* 124, 1-10.
[64] **Skolness, S.Y., Durhan, E.J., Jensen, K.M., Kahl, M.D., Makynen, E.A., Villeneuve, D.L., Ankley, G.T.** (2012): Effects of gemfibrozil on lipid metabolism, steroidogenesis, and reproduction in the fathead minnow (*Pimephales promelas*). *Environ. Toxicol. Chem.* 31, 2615-2624.
[65] **Sponchiado, G., Reynaldo, E., de Andrade, A.C.B., de Vasconcelos, E.C., Adam, M.L., de Oliveira, C.M.R.** (2011): Genotoxic effects in erythrocytes of *Oreochromis niloticus* exposed to nanograms-per-liter concentration of 17 beta-estradiol (E-2): An assessment using micronucleus test and comet assay. *Water Air Soil Pollut.* 218, 353-360.
[66**] Stange, D., Oehlmann, J.** (2012a): Identification of oestrogen-responsive transcripts in *Potamopyrgus antipodarum*. *Journal of Molluscan Studies* 78, 337-342.
[67] **Stange, D., Sieratowicz, A., Horres, R., Oehlmann, J.** (2012b): Freshwater mudsnail (*Potamopyrgus antipodarum*) estrogen receptor: Identification and expression analysis under exposure to (xeno-)hormones. *Ecotoxicology and Environmental Safety* 75, 94-101.

[68] **Thomas, M.A., Joshi, P.P., Klaper, R.D.** (2012): Gene-class analysis of expression patterns induced by psychoactive pharmaceutical exposure in fathead minnow (*Pimephales promelas*) indicates induction of neuronal systems. *Comp. Biochem. Physiol. C-Toxicol. Pharmacol.* 155, 109-120.
[69] **Toumi, H., Boumaiza, M., Millet, M., Radetski, C.M., Felten, V., Fouque, C., Ferard, J.F.** (2013): Effects of deltamethrin (pyrethroid insecticide) on growth, reproduction, embryonic development and sex differentiation in two strains of *Daphnia magna* (Crustacea, Cladocera). *Sci. Total Environ.* 458, 47-53.
[70] **van Leeuwen, J.S., Unlu, B., Vermeulen, N.P.E., Vos, J.C.** (2012): Differential involvement of mitochondrial dysfunction, cytochrome P450 activity, and active transport in the toxicity of structurally related NSAIDs. *Toxicol. Vitro* 26, 197-205.
[71] **Veach, A., Bernot, M.J., Mitchell, J.K.** (2012): The influence of six pharmaceuticals on freshwater sediment microbial growth incubated at different temperatures and UV exposures. *Biodegradation* 23, 497-507.
[72] **Wang, S.Y., Gunsch, C.K.** (2011): Effects of selected pharmaceutically active compounds on the ammonia oxidizing bacterium *Nitrosomonas europaea*. *Chemosphere* 82, 565-572.
[73] **Wang, H.P., Wang, J.J., Wu, T.T., Qin, F., Hu, X.Q., Wang, L.H., Wang, Z.Z.** (2011): Molecular characterization of estrogen receptor genes in *Gobiocypris rarus* and their expression upon endocrine disrupting chemicals exposure in juveniles. *Aquat. Toxicol.* 101, 276-287.
[74] **Wu, N., Wei, H., Shen, H., Wu, T.T., Guo, M.** (2012): Acute toxic effects of deltamethrinon red swampcrayfish, *Procambarus clarkia* (decapoda, cambaridae). *Crustaceana* 85, 993-1005.
[75] **Yan, Z.H., Lu, G.H., Yang, X.F.** (2013): Single and combined effects of estrone and 17 beta-estradiol on male goldfish. *Biomed. Environ. Sci.* 26, 176-184.
[76] **Yergeau, E., Sanschagrin, S., Waiser, M.J., Lawrence, J.R., Greer, C.W.** (2012): Sub-inhibitory concentrations of different pharmaceutical products affect the meta-transcriptome of river biofilm communities cultivated in rotating annular reactors. *Environ. Microbiol. Rep.* 4, 350-359.
[77] **Yonar, S.M., Sakin, F., Yonar, M.E., Ispir, U., Kirici, M.** (2011): Oxidative stress biomarkers of exposure to deltamethrin in rainbow trout fry (*Oncorhynchus mykiss*). *Fresenius Environ. Bull.* 20, 1931-1935.
[78] **Zhang, X.Y., Gong, Z.Y.** (2013): Fluorescent transgenic zebrafish Tg(nkx2.2a:mEGFP) provides a highly sensitive monitoring tool for neurotoxins. *PloS one* 8.
[79] **Zhang, Y.Y., Wang, H.P., Qin, F., Liu, S.Z., Wu, T.T., Li, M., Xu, P., Zhang, X., Wang, X.Q., Hu, G.J., Yuan, C., Wang, Z.Z.** (2012c): Molecular characterization of estrogen receptor genes in loach Paramisgurnus dabryanus and their expression upon 17 alpha-ethinylestradiol exposure in juveniles. *Gen. Comp. Endocrinol.* 178, 194-205.
[80] **Zhang, W., Zhang, M., Lin, K.F., Sun, W.F., Xiong, B., Guo, M.J., Cui, X.H., Fu, R.B.** (2012a): Eco-toxicological effect of carbamazepine on *Scenedesmus obliquus* and *Chlorella pyrenoidosa*. *Environmental Toxicology and Pharmacology* 33, 344-352.

Publication II: Monitoring Primary Effects of Pharmaceuticals in the Aquatic Environment with Mode of Action-Specific in Vitro Biotests

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The continuously growing and aging world population and the intensified livestock farming worldwide are expected to raise the global consumption and the number of human and veterinary pharmaceuticals on the market in the future. As a result, pharmaceuticals that are incompletely removed by wastewater treatment will occur in higher concentrations and in immense multiplicity in the water cycle. Except for synthetic steroid hormones, environmental monitoring of pharmaceuticals still remains to be based upon analytical chemistry of single substances which is incapable to encompass entities of pharmaceutical compounds with, for example, the same mode of action (MOA) at once, and thus will soon be overstrained by the sheer number of compounds, metabolites, and new product developments. For all the major classes of pharmaceuticals, we will therefore need MOA-based cell culture assays that report on the immediate interaction of compounds with their specific molecular target and, thus, on possible effects on organisms in the environment.

The topic’s relevance is without doubt. Globally, pollution by pharmaceuticals has been identified as a matter of concern by environmental policy. Recently, a nomination dossier was submitted to the United Nations that proposed ‘environmentally persistent pharmaceutical pollutants’ as an emerging policy issue under the framework of the ‘Strategic Approach to International Chemicals Management’. Last year, representatives of 24 countries and representatives of industry and academia agreed on the necessity to establish systematic and global monitoring programs on pharmaceuticals as a basis for a constant evaluation of potential risks to man and the environment.[1] Moreover, in the last amendment of the European Water Framework Directive, the European Commission was mandated to develop a strategy to reduce possible environmental impacts of pharmaceuticals. As a consequence, diclofenac, 17β-estradiol and 17α-ethinylestradiol are discussed to be placed on a watch list for which EU-wide monitoring data must be gathered. According to the EU, new pharmaceuticals have to undergo an environmental risk assessment (ERA) before marketing approval. For several pharmaceuticals, potential risks have been already identified and, in contrast to human medication, marketing authorization can be denied or restricted for veterinary medicinal products (VMP). A major drawback of the current approach for pharmaceutical ERA, however, is the lack of suitable data on exposure and ecotoxicological effects for the vast majority of the “old” active ingredients in pharmaceuticals which were already on the market before the requirement for an ERA was introduced into legislation. In addition, long-term effects resulting from chronic exposure of nontarget organisms and the potential of mixture effects as well as interactions with biotic and abiotic confounding factors are far from being well understood.

As an advanced tool for an ERA, different strategies of pharmaceutical prioritization have been proposed among which the most promising ones incorporate ecotoxicity data that are plausibly linked to specific modes of action (MOA). Furthermore, for monitoring purposes, MOA-based tools have been identified (e.g., in the U.S. Tox21 and ToxCast programmes) as auspicious instruments that can provide information on potential pharmaceutical class-related effects in environmental samples. A detailed study giving numerous examples of application of MOA-based techniques to environmental samples has been published recently.[2] Nevertheless, all these approaches have focused on nonpharmaceutical compounds with the exception of synthetic steroid hormones.

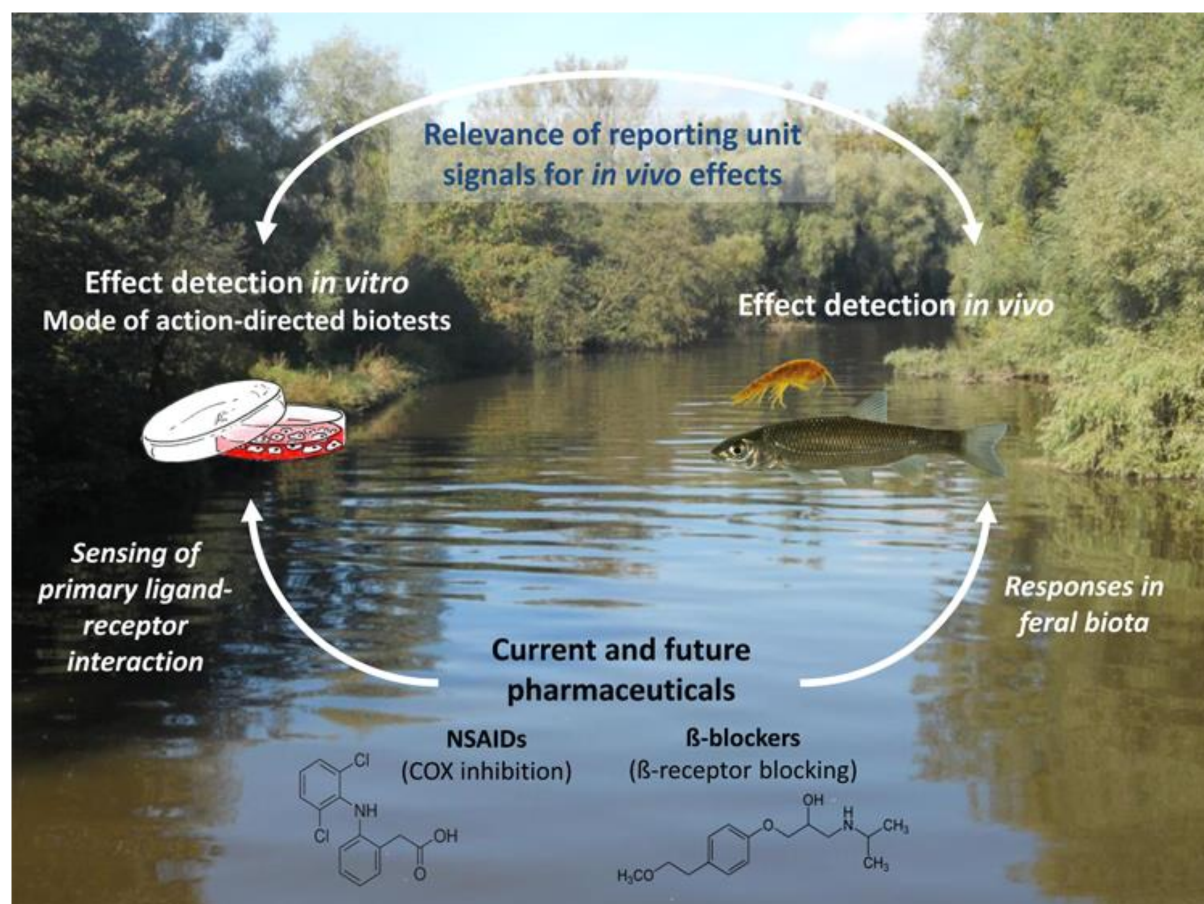

Figure 1: Pharmaceuticals may, phenomenologically, exert effects in nontarget biota and pose environmental risks. The vast number of these compounds and their metabolites makes it impossible to routinely screen their abundance by analytical chemistry. MOA specific life cell biosensing could trace primary action of pharmaceutical classes and attribute in vivo effects to them.

However, MOA-based techniques are within reach also for other classes of pharmaceuticals. Theoretical approaches to predict interactions of human pharmaceuticals with receptors of wildlife species have already been developed.[3] Even though a high diversity of biota with different physiology is potentially affected in the environment, the biochemical function of receptors remains rather phylogenetically conserved within a clade, at least among vertebrates. *In vitro* assays based on highly conserved signal transduction pathways including ligand-receptor interactions are, therefore, supposed to be relevant for a high number of vertebrate species, including fish. Although the use of such MOA-based *in vitro* assays for environmental monitoring is already common practice for nonpharmaceutical compounds or sex steroids, for other classes of pharmaceuticals such *in vitro* tests have not left a mark in ecotoxicology yet. This is the more surprising because target-based assays focusing on receptor-ligand binding, coupled to the generation of measurable signals have been, historically, the mainstay of substance screening in pharmacological research and development.

In this context, the most recent developments make use of the enormous potential of genetically encoded fluorescent sensors expressed by recombinant cell lines comprising a sensing unit that recognizes its interaction with a chemical and a reporting unit which indicates the sensing unit's state and, accordingly, leads to immediate fluorescence signal changes[4] - in contrast to reporter gene assays which generate a signal at the downstream end of a long signal transduction pathway allowing side effects by interfering substances at every step along this cascade. Adapting such methods to construct cell lines that generate immediate fluorescent signals following a pharmaceutical-target interaction should therefore substantially advance MOA-directed analysis of the primary impact of pharmaceuticals in environmental samples. Own current research revealed a fluorescence resonance energy transfer (FRET)-based cell line created to monitor β-adrenoreceptor binding to provide an optical signal for β-blockers in the concentrations range of the lowest observed effect concentration (LOECs) reported for the most sensitive biota (10 to 100 nmol/L).[5]

Of course, the transfer of these cell culture technologies from the "clean" conditions of active component screening to the "dirty" composition of environmental samples will likely pose challenges for an appropriate processing of these samples. Furthermore, receptor activation as a primary effect of environmental compounds needs to be validated by biomarkers and population-relevant endpoints in environmentally relevant biota including fish, crustaceans, and sediment-dwelling organisms for the same environmental situation − a labor-intensive but achievable task (Figure 1). Consequently, novel MOA-directed *in vitro* assays on primary pharmaceutical action will serve as both compound class-selective and effect-oriented tools, which bridge the still existing gap between the analytical chemistry of pharmaceuticals in waters and sediments and their *in vivo* effects in an elegant way. They need to be urgently implemented in environmental monitoring programmes and the routine assessment of environmental quality.

Notes

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Acknowledgements

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References

(1) Workshop Documentation: Pharmaceuticals in the Environment - Global occurrence, effects, and options for action. April 8th and 9th 2014, International Environment House II, Geneva, Switzerland; German Federal Environment Agency (UBA): Dessau-Roßlau, Germany, 2014; http://www.pharmaceuticals-in-the-environment.org/files/en/bereich_2/application/pdf/workshop_documentation_global-relevance-ofpharmaceuticals-in-the-environment.pdf.

(2) Escher, B. I.; Allinson, M.; Altenburger, R.; Bain, P. A.; Balaguer, P.; Busch, W.; Crago, J.; Denslow, N. D.; Dopp, E.; Hilscherova, K.; Humpage, A. R.; Kumar, A.; Grimaldi, M.; Jayasinghe, B. S.; Jarosova, B.; Jia, A.; Makarov, S.; Maruya, K. A.; Medvedev, A.; Mehinto, A. C.; Mendez, J. E.; Poulsen, A.; Prochazka, E.; Richard, J.; Schifferli, A.; Schlenk, D.; Scholz, S.; Shiraish, F.; Snyder, S.; Su, G. Y.; Tang, J. Y. M.; van der Burg, B.; van der Linden, S. C.; Werner, I.; Westerheide, S. D.; Wong, C. K. C.; Yang, M.; Yeung, B. H. Y.; Zhang, X. W.; Leusch, F. D. L. Benchmarking organic micropollutants in wastewater, recycled water and drinking water with *in vitro* bioassays. Environ. Sci. Technol. 2014, 48 (3), 1940−1957 DOI: 10.1021/es403899t.

(3) Walker, S. D.; McEldowney, S. Molecular docking: a potential tool to aid ecotoxicity testing in environmental risk assessment of pharmaceuticals. Chemosphere 2013, 93 (10), 2568−2577 DOI: 10.1016/j.chemosphere.2013.09.074.

(4) Oldach, L.; Zhang, J. Genetically encoded fluorescent biosensors for live-cell visualization of protein phosphorylation. Chem. Biol. 2014, 21 (2), 186−197 DOI: 10.1016/j.chembiol.2013.12.012.

(5) Triebskorn, R.; Schwarz, S.; Köhler, H.-R.; Berg, K.; Jungmann, D.; Frey, M.; Oehlmann, J.; Oetken, M. From Theory to Reality - Evaluation of Suitable Organisms and Test Systems for the Biomonitoring of Pharmaceuticals - part I: Literature Review; Texte 64/2014: Dessau- Roßlau, Germany, 2014; www.umweltbundesamt.de/publikationen/from-theory-to-reality-evaluation-of-suitable.

Publication III: Impact of the NSAID diclofenac on survival, development, behaviour and health of embryonic and juvenile stages of brown trout, *Salmo trutta* f. *fario*

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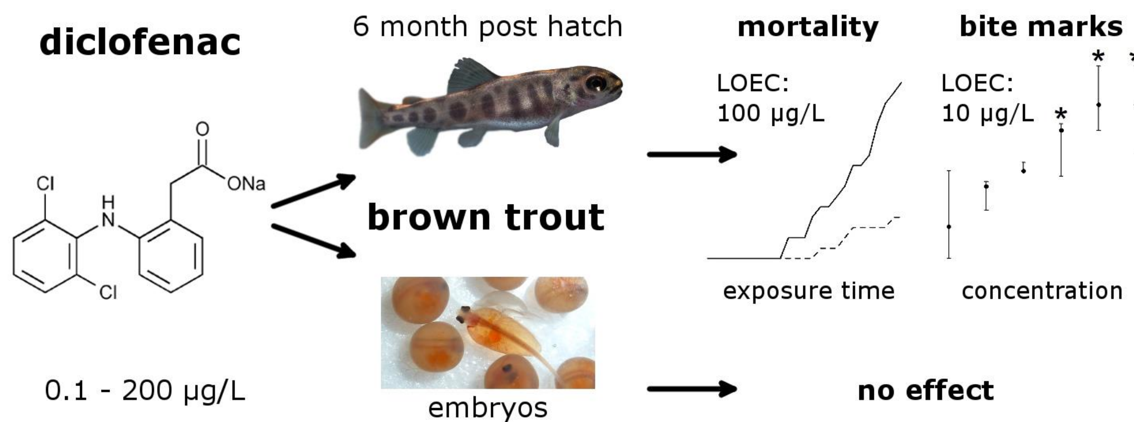
Graphical abstract



Abstract

The NSAID diclofenac is controversially discussed with respect to its environmental relevance. Since further information is need to assess whether diclofenac should be included as substance of priority in the EU water framework directive, we investigated the impact of this analgesic on the embryonic development of brown trout (*Salmo trutta* f. *fario*) from fertilized egg until the end of sac-fry stage and studied effects in juvenile fish six months post hatch.

Embryos were exposed to five test concentrations (0.1, 0.5, 1, 10, 100 µg/L) over 127 days at 7 °C. None of the treatments affected mortality, hatching, development or heart rate. Six months old juveniles exposed to five concentrations (0.1, 1, 10, 100, 200 µg/L) over 25 days at 7 °C, however, showed increased mortality, reaching significance at 100 µg/L. Furthermore, a significantly higher proportion of juvenile animals bore injuries at concentrations higher 10 µg/L. Neither the levels of the stress protein Hsp70, nor the amount of lipid peroxides was affected by any of the treatments. Histological analyses of gill, liver and kidney revealed visible tissue reactions in fish from all experimental groups. Histological responses in livers of diclofenac-exposed fish outstripped the status of laboratory control fish, particularly when exposed to the two highest concentrations. Chemical analyses of fish muscle tissue revealed concentration-dependent uptake of DCF into the animal, but no relevant bioconcentration.

Our study supports earlier findings indicating a lower sensitivity of trout early life stages compared to older individuals, suggesting that studies for risk assessment of diclofenac should predominantly focus on later life stages. Furthermore, fish mortality was found to increase with rising diclofenac concentrations, and the lowest observed effect concentration of 10 µg/L on the organismic level emphasises the classification of diclofenac as a micropollutant that requires close attention.

Keywords
Mortality - lipid peroxides – stress proteins – histology – embryo test

1. Introduction

As a result of the rising production of chemicals and their manifold usage in everyday life, the complexity of the micropollutant mixture in the water cycle is continuously growing (Schwarzenbach *et al.* 2006 Luo *et al.* 2014). Yet, much uncertainty remains concerning their risk for humans and wildlife. During the last decade, pharmaceuticals were targeted as environmentally relevant emerging pollutants (Triebskorn *et al.* 2014), which is not astonishing since they possess the innate and by design desired ability to elicit biologically relevant effects already at low concentrations. As such, effects on aquatic biota are within the realms of possibility, especially when considering that organisms are exposed throughout their entire life span (Fent *et al.* 2006).

Within the wide range of pharmaceutically active compounds, the non-steroidal anti-inflammatory drug (NSAID) diclofenac is among the most frequently used, studied – and with respect to its environmental relevance - controversially discussed (Triebskorn *et al.* 2014, Lonappan *et al.* 2016). The therapeutic action of DCF is the inhibition of cyclooxygenase I and II, leading to reduced production of prostaglandins (Gan 2010). Prostaglandins are involved in a variety of physiological processes, including inflammation, platelet aggregation, vasodilation, renin release and presumably complex integrative functions in brain and autonomic nervous systems (Funk 2001, Simmons *et al.* 2004). The well-known side effects of this drug in humans, however, seem to be resembled by pathologies in other, even wildlife vertebrates. Since the discovery of diclofenac's detrimental effects on vulture populations by eliciting acute renal failure and visceral gout (Oaks *et al.* 2004), a wide spectrum of studies focused on examining its risk for aquatic ecosystems.

As a commonly used over-the-counter analgesic, its concentration in sewage treatment plant effluents can range up to several micrograms per litre (Meyer *et al.* 2016). As a direct consequence, measureable amounts of diclofenac are present in most European streams (Loos *et al.* 2009, Aus der Beek *et al.* 2016b) and are occasionally found even in ground water (Monteiro and Boxall 2010). However, the reported surface water concentrations show great variation: while most reported medians and means are in the low ng/L-range between 5 and 32 ng/L, there remain various events where it was detected in concentrations above 100 ng/L (Ternes 1998, Loos *et al.* 2009, Aus der Beek *et al.* 2016b). Letzel *et al.* (2009), who based their study on measured data rather than available consumption data, calculated a worst case predicted environmental concentration (PEC) of 140 ng/L for German rivers.

Although the detected concentrations are varying, the almost ubiquitous presence of diclofenac in surface waters is undoubted. Far more discordance exists on effect concentrations in non-target organisms. While several studies found lowest observed effect concentrations (LOECs) in close range to the PEC (Schwaiger *et al.* 2004, Triebskorn *et al.* 2004, Hoeger *et al.* 2005, Triebskorn *et al.* 2007, Mehinto *et al.* 2010, Ribeiro *et al.* 2015, Näslund *et al.* 2017), others feature effect values that exceed the environmental concentrations by several orders of magnitude (Lee *et al.* 2011, Memmert *et al.* 2013). Currently, diclofenac has been included into the watch list of chemicals that are in discussion as priority substances in the European Water Framework Directive (EU 2006a, EU 2012, EU 2013). Therefore, further information regarding effects of diclofenac on aquatic organisms is crucially needed.

The aim of our study was to further substantiate the knowledge on this controversially discussed pharmaceutical by testing two supposedly sensitive life stages of brown trout. Tests on embryos and sac-fry stages are common for other fish species like zebrafish or medaka, but are rarely applied for cold-water fish. The main reasons for this may be the long embryonic development period, which exceeds several months, and the fact that eggs are only available for a short time frame once per year. However, as brown trout are a species of high local ecological relevance for Central European stream

ecosystems (Dußling and Berg 2001), their examination provides crucial information for the evaluation of potential adverse effects related to chemical exposure. Juveniles of about 6 months post hatch pose another sensitive life stage, since these animals are still in growth and lack the constitution and energy reserves of fully grown adults.

The major aim of the test with embryos and sac-fry stages was to examine developmental parameters like mortality, hatching rate and time, malformations and heart rate, accompanied by histological examinations. In the experiment with juveniles, the focus originally was put on sublethal parameters. Several NSAIDs are suspected to alter the level of oxidative stress, but there are reports on decreases as well as increases (Hickey *et al.* 2001, Fernandez *et al.* 2013, Gonzalez-Rey and Bebianno 2014, Nunes *et al.* 2015). Consequently, we wanted to have a closer look at one of these parameters – lipid peroxides. Lipid peroxide formation is among the final results of imbalances in the dynamic steady state of reactive oxygen species, and increased levels lead to compromised cellular function (Lushchak 2011). Moreover, the proteotoxic action of a substance can be assessed via measurements of the stress protein Hsp70. The Hsp70 system is induced by intracellular malfolded and degraded proteins, and assists in the restoration of damaged proteins. Therefore, increased levels of Hsp70 are a general sign of proteotoxic stress (Sørensen *et al.* 2003). Besides these biochemical analyses, histopathological examination was a further part of the study. Tissue integrity is a parameter that can react sensitively and integrate over a broad range of adverse effects – making it a suitable tool for the assessment of general health condition (Triebskorn *et al.* 2007). Furthermore, biometric measurements, mortality, as well as morphological and behavioural peculiarities of the test animals were recorded in the experiment.

Our objective was to identify whether diclofenac has the potential to adversely affect the development of brown trout; and whether the health of juveniles is compromised after sub-chronic exposure. To achieve this, we applied a battery of different tests to different life stages of the same fish species, complemented by chemical analyses, to shed further light on the risk emanating from the NSAID diclofenac for aquatic wildlife.

2. Materials and Methods

2.1 Test organism

Brown trout (*Salmo trutta* f. *fario*) is a salmonid teleost native to Central European countries. As a typical inhabitant of the upper river regions, it prefers cool, oxygen-rich waters (Dußling and Berg 2001). Embryonic development from fertilised egg to fully developed larvae requires several months, strongly depending on the ambient temperature (Killeen *et al.* 1999a).

Fertilised eggs and juvenile animals were obtained from a commercial fish breeder (Forellenzucht Lohmühle, D- 72275 Alpirsbach-Ehlenbogen). The breeding facility is subject to regular controls and rated as category I, disease-free (EU 2006a, Council Directive 2006/88/EC) . Since the breeder supplies animals for fishery restocking campaigns in German streams, the chosen variety is considered robust and close to feral forms. Eggs were exposed directly after purchase. Juvenile animals were acclimatized to lab conditions in two 250 L aquaria (filtered, aerated tap water – iron filter, particle filter, activated charcoal filter; constant particle filtration and aeration during exposure) for one week prior to the experiments.

2.2 Test substance

In all experiments, diclofenac was applied as sodium salt (CAS 15307-79-6, Sigma-Aldrich, 89555 Steinheim, Germany). The addition of organic solvents was not necessary. In the following, all given concentrations refer to the amount of pure diclofenac, not the salt.

2.3 Exposure of trout embryos and sac-fry stages

The experiment followed the OECD guideline 212 (OECD 1998): in the semi-static test setup freshly fertilized eggs of brown trout (*Salmo trutta* f. *fario*) were exposed to diclofenac in concentrations of 0, 0.1, 0.5, 1, 10, and 100 µg/L at 7 °C. Until the eyed ova stage, the embryos developed in darkness - subsequently the conditions changed to a 10 h/14 h light/dark cycle. Treatments were tested in triplicates, where each replicate consisted of a glass Petri dish containing 200 mL test medium and 30 individual eggs. Every second day, in each Petri dish 150 mL of test solution were replaced with fresh solution prepared from aerated artificial water (294 mg/L $CaCl_2$ x 2 H_2O, 123.25 mg/L $MgSO_4$ x 7 H_2O, 64.75 mg/L $NaHCO_3$, 5.75 mg/L KCl). Water conditions (pH, oxygen content, temperature) were in a tolerable range at the end of the experiment (mean pH = 6.8 ± 0.21, mean oxygen = 8.4 ± 0.68 mg/L, mean temperature = 7.2 ± 0.19 °C). Oxygen saturation was above the threshold level of 60 % in all vessels except for one, which had a final oxygen saturation of 59.6 % (block 1 – 0.1 µg/L diclofenac). The exposure lasted for 127 days and was terminated before the fry had fully consumed their yolk-sac. Recorded parameters were the time until eyed ova stage, time to hatch, heart rate one week post hatch and survival (excluding unfertilized eggs). At the end of the experiment, all larvae were anesthetised and killed by an overdose of MS-222 (tricaine methanesulfonate, 1 g/L, buffered with $NaHCO_3$). Three larvae were fixed in a solution of 2 % glutardialdehyde diluted in 0.1 M cacodylate buffer (pH 7.6) for further histological examination of kidney and liver. The remaining larvae (between 20 and 26 individuals per treatment) were used to determine body mass.

2.4 Exposure of juvenile trout

Juvenile brown trout (approx. 6 months post hatch) were exposed for 25 days in a semi-static three-block design. Each block consisted of six 25 L aquaria (0, 0.1, 1, 10, 100, 200 µg/L diclofenac) containing 13 individual fish each. This led to a total of 234 fish exposed in 18 different aquaria. Ambient conditions of the thermoconstant chamber were set to 7 °C and a 12 h/12 h light/dark cycle, with the aquaria being shaded from direct light. Twice a week, in each aquarium one third of the test medium was exchanged with fresh diclofenac solution (prepared from filtered, aerated tap water – iron filter, particle filter, activated charcoal filter). Additionally, excess food and faeces were removed. Water conditions (pH, oxygen content, temperature, conductivity) were checked regularly and remained within the acceptable range throughout the experiment (mean pH = 8.5 ± 0.04, mean oxygen = 10.97 ± 0.15 mg/L, mean temperature = 6.9 ± 0.18 °C, mean conductivity = 446 ± 5.5 µS/cm). Fish were fed twice a day with commercial trout feed (1.5 mm, Biomar, Brande, Denmark), and mortality was recorded. At the end of the exposure period, fish were anesthetised and killed by an overdose of MS-222 (1 g/L, buffered with $NaHCO_3$), followed by a neck-cut. Two researchers recorded body lengths, body mass, as well as any apparent morphological abnormalities. This included the assessment of mutually inflicted injuries (bite marks on fins, snout and opercula) by scoring whether they were absent or present. Histological samples of gill, liver, and trunk kidney were fixed in a solution of 2 % glutardialdehyde diluted in 0.1 M cacodylate buffer (pH 7.6). Samples of liver (for stress protein analysis), trunk kidney (for lipid peroxide analysis), and all remaining fish tissue (for chemical analysis of diclofenac concentration in tissues) were frozen in liquid nitrogen and stored at - 80 °C until further usage.

In addition to the lab exposure, 20 animals were sampled directly at the fish hatchery at time points comparable to start and end of the experiment. Dissection and preparation of samples followed the same routine as described above. These “hatchery controls” were necessary to assure ourselves that the animal stock designated for our experiment was free of injuries, parasites and pathogens, and served as reference to check for potential effects of the lab exposure itself.

2.5 Chemical analyses

The real concentrations of diclofenac in the test aquaria as well as concentrations in exposed fish were determined by chemical analyses. Water samples were taken at the start and the end of the experiment, as well as before and after the first water exchange – and frozen at -20 °C until processing. Analyses were performed on mixture samples of the three replicate aquaria belonging to the same treatment. If necessary, aqueous samples were enriched by solid-phase extraction (SPE). A 50 mL sample was adjusted to pH 3 with hydrochloric acid and the analyte was pre-concentrated with a polymeric sorbent material (Strata-X, 200 mg, Phenomenex, Aschaffenburg, Germany). Subsequently, the sorbent was dried under a gentle stream of nitrogen and eluted with 8 mL methanol and 2 mL acetonitrile. Diclofenac was analyzed by high performance liquid chromatography (HPLC, 1290 series, Agilent, Waldbronn, Germany) coupled to tandem mass spectrometry (MS/MS, API 5500, AB Sciex, Foster City, USA) by multiple reaction monitoring in positive mode by electrospray ionization. The optimized interface and MS/MS parameters are displayed in Table 1 in the supplementary information.

For quantitative analyses, the isotopically labelled standard diclofenac-d_4 was used for the correction of signal enhancement or suppression caused by matrix components.

For the extraction and analysis of diclofenac in fish, several parameters for optimum extraction like organic solvents, extraction pH and injection volume were tested to assess the effect of co-extracted matrix. For this purpose, turtle food (Engergil, JBL GmbH, Neuhofen, Germany) containing complete freeze-dried fish and crustaceans was used as matrix. The matrix was ground in a blender to obtain a fine powder for extraction experiments. 0.5 g of freeze-dried matrix was extracted with 3 mL of the respective organic solvent for 15 min in an ultrasonic bath. After extraction, the vessel was centrifuged for 15 min at about 3,000 g and the supernatant was transferred into a glass vial. Extraction and subsequent centrifugation were repeated with the same amount of extraction solvent and the two supernatants were merged and blown down to dryness under a gentle stream of nitrogen. The dry residue was reconstituted with 200 µL of methanol and 0.8 mL of ultra-pure water. After micro-centrifugation an aliquot was injected into the LC-MS/MS system. A matrix matched calibration with turtle food was prepared for quantification.

For the analysis of biota, samples were treated with the same analytical instrumentation as described for water samples, but different LC buffers and another LC gradient program were used due to co-eluting matrix components. Eluents were (A) ultrapure water + 5 mM ammonium formate + 0.05 % formic acid and (B) methanol / acetonitrile (⅓ / ⅔, v/v) + 5 mM ammonium formate. Chromatographic separation started with 10 % B, which was increased to 15 % within 2 min, further increased to 20 % within 6 min, to 50 % within 4 min, to 60 % within 8 min and to 100 % within 2 min. Then eluent composition was held at 100 % B for 6 min and decreased within one minute to the initial conditions (total gradient time 29 min). Between two injections the column was re-equilibrated for 6 min. After exposure time, fish were freeze dried, pooled and homogenized. By that means, two pool samples for every tests concentration including the control could be provided for analysis (total number of twelve). The extraction efficiency was ensured by supplementing one of the control samples from the test series with juvenile trout with 20 µg/kg diclofenac. All samples were spiked with the isotopically labelled internal standard diclofenac-d_4 for the correction of matrix effects.

2.6 Stress protein analysis

The stress protein Hsp70 was quantified in liver of the exposed fish as described by Dieterich *et al.* (2015). After homogenisation and protein quantification (Bradford 1976), sample protein (standardized to 40 µg total protein) was separated via SDS-PAGE and blotted to a nitrocellulose membrane. Protein bands were immune-stained with a monoclonal α-Hsp70 IgG (Dianova, Hamburg, Germany) and a secondary peroxidase-coupled α-IgG (Jackson Immunoresearch, West Grove, PA) and

the optical volume was finally quantified and related to an internal Hsp70 standard. Details on the procedure are given in the supplementary information.

2.7 Determination of lipid peroxides

The FOX (ferrous oxidation xylenol orange) assay was conducted according to a modified version of the protocols proposed by Hermes-Lima *et al.* (1995) and Monserrat *et al.* (2003). Frozen kidney samples were diluted 1:7 in HPLC-grade methanol, homogenized and centrifuged at 15,000 rcf for 5 min. The assay was performed in 96 well plates. Each well was filled with 50 µL of 0.75 mM $FeSO_4$-solution, 50 µL of 75 mM sulfuric acid, 50 µL of 0.3 mM xylenol orange solution, 15 µL of sample supernatant and 35 µL of bidestilled water. Each sample was tested in triplicate, and a sample blank, in which the $FeSO_4$ solution was substituted with water, was added. All data were related to a master blank, which consisted of bidestilled water. The samples were incubated for 135 min at room temperature. The absorbance at 570 nm was measured using an automated microplate reader (Bio-Tek Instruments, Winooski VT, USA). After the first measurement, 1 µL of 1 mM cumene hydroperoxide solution was added to each well. After another incubation period of 30 min, the absorbance at 570 nm was measured again. Cumene hydroperoxide equivalents (CHPequiv./mg wet weight) were calculated using the following equation:

$$\frac{A570nm}{A570nmCHP} * volumeCHP * \frac{total\ volume}{sample\ volume} * dilution\ factor = \frac{A570nm}{A570nmCHP} * 1 * \frac{200}{15} * 7$$

2.8 Histological analyses

Three samples per aquarium were further processed for the exemplary histological evaluation. Prior to embedding, the fixed tissue samples were washed three times for ten minutes in 0.1 M cacodylate buffer (pH 7.6), followed by three 15 min washing steps in 70 % ethanol. For gills and kidney, an additional step for decalcification in a 1:2 mixture of concentrated formic acid and 70 % ethanol was added between these steps. Dehydration and paraffin embedding took place in an automated tissue infiltrator (TP 1020, Leica, Wetzlar). Histological sections were cut with a sledge microtome (SM 2000 R, Leica, Wetzlar) set to a thickness of 3 µm. One part of the slices was stained with hematoxilin-eosin (to visualize nuclei, cytoplasm, connective tissue and muscles), the other part with alcian blue-PAS (to visualize mucus and glycogen). In a first evaluation step, slides were examined qualitatively to gain an overview and identify occurring pathologies. The second step was a semi-quantitative assessment performed in an observer-blinded way. The two slides per sample (AB-PAS/HE) were paired, the inscriptions masked, mixed and a random number was assigned to the slide pair. Each sample was classified into one of five different categories (1: control, 2: slight reaction, 3: medium reaction, 4: strong reaction, 5: destruction) according to the criteria published by Triebskorn *et al.* (2008). The classification of each number to the corresponding treatment was done after the assessment of all slides had been finished.

2.9 Statistical analysis

Statistical evaluation was done using R 3.2.1 (packages: lme4, lmerTest) and SAS JMP 11. Mortality data were analysed using COX-regression, using experimental block as nesting factor. Embryonic body mass was analysed by linear mixed model with Petri dish identity as random factor. Juvenile body length and body mass were analysed via nested ANOVA, data on stress proteins and lipid peroxidation using a linear model, including test block as cofactor. For lipid peroxides, data had to be log-transformed and sample mass was used as additional covariate. Occurrence of injuries was compared using a generalized linear mixed model (binomial distribution, aquarium identity as random factor). Base significance level was set to α=0.05; in cases of multiple comparisons α was adjusted via sequential Bonferroni. If visible differences between groups were absent, we refrained from applying statistical tests. Due to the exemplary nature of the histological analyses we did not perform a

mathematical test on these data and thus restricting ourselves to descriptive statistics. Hatchery controls were only used as qualitative reference and not included in the mathematical analyses.

2.10 Animal welfare

The conducted experiment on juvenile brown trout was approved by the animal welfare committee of the Regional Council of Tübingen, Germany (authorisation ZP1/12).

2.11 Credibility of data

For an overview on the fulfilment of the criteria proposed by Moermond *et al.* (2015), see supplementary information.

3. Results

3.1 Chemical analyses

In the exposure experiment with larvae, the real water concentrations of diclofenac were slightly lower than the nominal concentrations (Table 1), while there were only minimal differences in the experiments with juveniles (Table 2). The limit of quantification (LoQ) for the analyses of water samples was 10 ng/L.

When analysing biota samples, the extraction pH had a distinct impact on the extracted matrix components. Methanol extraction resulted in the most colored extract and produced a very fine precipitant, which may result in problems in subsequent clean-up steps. Acetonitrile and ethyl acetate extraction were additionally performed with the addition of 2 % formic acid or 5 % NH_4OH for acidic and alkaline extraction, respectively. Best results were obtained for neutral extraction with acetonitrile (supplementary information – Figure 1). The LoQ of the analytical method for biota samples was set to 5 µg/kg. At this concentration a signal-to-noise ratio of higher than 10 was achieved for diclofenac in the respective test matrix. Based on the LoQ, minimum bio-concentration factors (BCF) between 50 L/kg and 0.025 L/kg were calculated for the different test concentrations (0.1 µg/L to 200 µg/L).

In the control samples and the two lowest test concentrations (0.1 µg/L and 1µg/L) no diclofenac was detected in the fish in concentrations above the LoQ. In samples deriving from fish tanks with higher test concentrations of diclofenac, concentrations in the fish samples positively correlated with the test concentrations in the water (supplementary information – Figure 2). In the spiked control sample, the expected concentration was found proving the applicability of the method also for trout samples.

3.2 Exposure of trout embryos and sac-fry stages

Eye pigmentation started 30 days post fertilisation (dpf) and was finished 33 dpf; hatching began 64 dpf and was finished 70 dpf. Both parameters did not differ between any of the tested treatments. The frequency of malformations was negligibly low. Furthermore, diclofenac affected neither embryo nor larvae survival and had no influence on heart beat rate (Table 1). The mean body mass was slightly lower at 10 µg/L, but these differences were due to a single replicate and no significant differences were found (linear mixed model, df=5/11.65, F=0.7623, p=0.5944). Histological examination revealed neither strong reactions in any of the examined larvae, nor any further effect of the exposure to diclofenac.

Validity of the test was given, with control survival above 70 %, oxygen saturation above 60 % (sole exception: 59.6 % in block 1 – 0.1 µg/L diclofenac) and temperature differences smaller than 1.5 °C between test vessels.

Table 1: Summary on brown trout embryos and sac-fry stages exposed to diclofenac (DCF) for 127 days. Times until eyed ova stage and hatch are given in days post fertilisation [dpf]. Survival rates were calculated excluding unfertilised eggs. The heart rate is given in beats per minute [bpm]; for both heart rate and body mass both the arithmetic mean and the standard deviation (SD) are given.

Treatment (nominal concentration)	Real water concentration [µg/L]	Mean time until eyed ova stage [dpf]	Mean time until hatch [dpf]	Overall survival rate [%]	Mean ± SD heart beat rate [bpm]	Mean ± SD body mass [mg]
0 µg/L DCF	< LoQ	30.46	65.08	97.57	50.8 ± 2.5	95.06 ± 10.93
0.1 µg/L DCF	0.06	30.62	65.33	96.38	52.4 ± 2.9	94.81 ± 10.39
0.5 µg/L DCF	0.51	30.61	65.70	98.77	50.9 ± 2.8	94.11 ± 11.44
1 µg/L DCF	0.75	30.66	65.83	97.28	50.8 ± 2.5	94.58 ± 11.06
10 µg/L DCF	7.8	30.52	65.05	95.06	51.0 ± 2.2	89.02 ± 13.87
100 µg/L DCF	74.6	30.68	65.90	100.00	50.7 ± 2.4	95.05 ± 11.61

3.3 Exposure of juvenile trout

The biometric measurements did not show any differences between the treatments (body mass: nested ANOVA, df=5, n=231, F=1.1950, 0.3127; body length: nested ANOVA, df=5, n=232, F=1.4084, p=0.2224). Fish sampled at the hatchery resembled the lab-exposed animals in body size and weight at time point 1 (beginning of the experiment), but had grown considerably larger at time point 2 (end of the experiment).

Juvenile fish showed a concentration-dependent increase in mortality (nested COX-analysis, df=5, n=234, $\chi 2$=13.457, p=0.0194) (Figure 1). Mortality rate exceeded 40 % in the two highest tested concentrations on day 25, while not exceeding 10 % in the control group. Step-wise comparison revealed significantly increased mortality at 200 and 100 µg/L, and a trend towards elevated mortality rates in the other test concentrations.

Furthermore, the animals showed conspicuous bite marks on fins and opercula as signs of intraspecific aggression (Figure 2). We observed that in some aquaria, there was a higher tendency for bites on fins, while in other aquaria the animals bore a higher frequency of body injuries (snout and opercula). Evaluation was based on the prevalence of overall injuries, irrespective of harmed area and severity. While the frequency of these mutually inflicted injuries was low in the control group, it increased with diclofenac concentrations, with significant differences at concentrations of 10 µg/L and higher (GLMM, df=5, n=161, F=5.2463, p(10 µg/L)=0.0015).

Biochemical analyses did not reveal any influence of diclofenac on the level of the stress protein Hsp70 (linear model, df=5/151, F=1.046, p=0.3928), but revealed a slight increase of the Hsp70 level through the lab exposure itself. Lipid peroxidation was considerably higher in the lab exposure, compared to the samples from the hatchery. Results showed high variability, and were influenced by block and also the covariate sample mass, but not by diclofenac (linear model, df=35,123, F=1.639, p=0.02581). All presented biometric, biochemical and histological results are based on data from fish surviving until the termination of the exposure experiment. Data are summarized in Table 2; detailed results of statistical analyses are given in the supplementary information.

Histopathological evaluation of juvenile fish revealed hypertrophy of gill pavement cells in all treatments, leading to an overall thickening of secondary lamellae. Livers, in general, exhibited low amounts of stored glycogen, dilated intercellular spaces, and frequent inflammative areas. Further effects were seen in kidney: most samples showed accumulations of hyaline material in tubular cells

and, frequently, high abundances of melanomacrophages. Moreover, several samples featured degenerative changes in the hematopoietic tissue leading to a "perforated" appearance. Individuals sampled directly at the hatchery (only evaluated for time point 1, since these animals were of comparable size to those in the experiment) showed a low frequency of histological alterations in kidney and gill – and slight reactions of the hepatic tissue (reduced glycogen, inflammations and aggregations of macrophages). Although most of the symptoms were also present in laboratory control animals, the frequency of strong reactions was higher in diclofenac-treated fish, especially in the liver. Most severe pathologies were observed in the two highest diclofenac treatments. A selection of observed pathologies is illustrated in Figure 3.

These findings are reflected by the blinded semi-quantitative assessment. Regarding the gills, hatchery controls, kept in ponds, were predominantly in the control state, while lab controls, kept in aquaria, were in a state of slight to medium reaction. Diclofenac-exposed animals showed a slightly increased proportion of stronger reactions. While the kidneys of hatchery animals were in very good condition, most animals from the lab experiment had to be classified at least into the state of medium reaction. The two highest diclofenac concentrations also led, in few cases, to symptoms of severe destruction of renal tissue. The histology of the liver was already in a slight to medium reaction state in most samples from the hatchery. Lab-exposed animals often exhibited symptoms of medium to strong cellular reaction, but the proportion of strong reactions was considerably higher in fish exposed to diclofenac, particularly at 100 and 200 µg/L. Data are displayed in Figure 4. A detailed overview on the occurrence of the most frequent symptoms is given in the supplementary information.

Table 2: Summary on juvenile brown trout exposed to diclofenac (DCF) over 25 days. LoQ was 0.01 µg/L for water and 5 µg/kg for biota samples. Injuries were only taken into account for animals surviving until day 25, and are displayed in total number of animals bearing injuries divided by the total number of surviving individuals. Mass, length, Hsp70 level and lipid peroxidation are displayed as arithmetic means and standard deviations (SD). Standard length refers to the length from snout to the base of the tail fin. Lipid peroxidation is given as cumene hydroperoxide equivalents per mg wet weight [$CHP_{equiv.}$/mg ww]. While the treatments did neither affect the biometric nor the biochemical measurements, increasing diclofenac concentrations elevated mortality rates and led to higher proportions of mutually inflicted injuries. Asterisks (*) depict significant differences at the adjusted α vs. the lab control; hatchery controls 1 and 2 were excluded from statistical comparisons.

Treatment (nominal concentration)	Real water concentration [µg/L]	Biota concentration [µg/kg dw]	Occurrence of injuries / number of survivors	Mean ± SD body mass [g]	Mean ± SD standard length [cm]	Mean ± SD Hsp70 level [rel. grey value]	Mean ± SD lipid peroxides [$CHP_{equiv.}$/mg ww]
Hatchery 1	-	-	0/20	3.25 ± 1.04	6.15 ± 0.54	1.34 ± 0.22	42.95 ± 20.71
Hatchery 2	-	-	0/20	7.29 ± 1.75	7.68 ± 0.56	1.24 ± 0.26	42.75 ± 23.41
0 µg/L DCF	< LoQ	< LoQ	7/35	3.18 ± 0.67	6.19 ± 0.46	1.52 ± 0.27	74.33 ± 26.83
0.1 µg/L DCF	0.095	< LoQ	9/26	2.90 ± 0.65	5.96 ± 0.44	1.47 ± 0.27	85.18 ± 20.69
1 µg/L DCF	0.975	< LoQ	14/30	2.81 ± 0.52	6.00 ± 0.38	1.46 ± 0.25	84.08 ± 23.47
10 µg/L DCF	9.6	10.2	16/26*	2.82 ± 0.79	5.96 ± 0.47	1.52 ± 0.25	83.62 ± 21.32
100 µg/L DCF	98.5	84.5	17/22*	3.03 ± 0.72	6.00 ± 0.49	1.37 ± 0.30	79.15 ± 26.84
200 µg/L DCF	200	169.5	16/22*	2.84 ± 0.74	5.93 ± 0.51	1.45 ± 0.27	82.15 ± 18.28

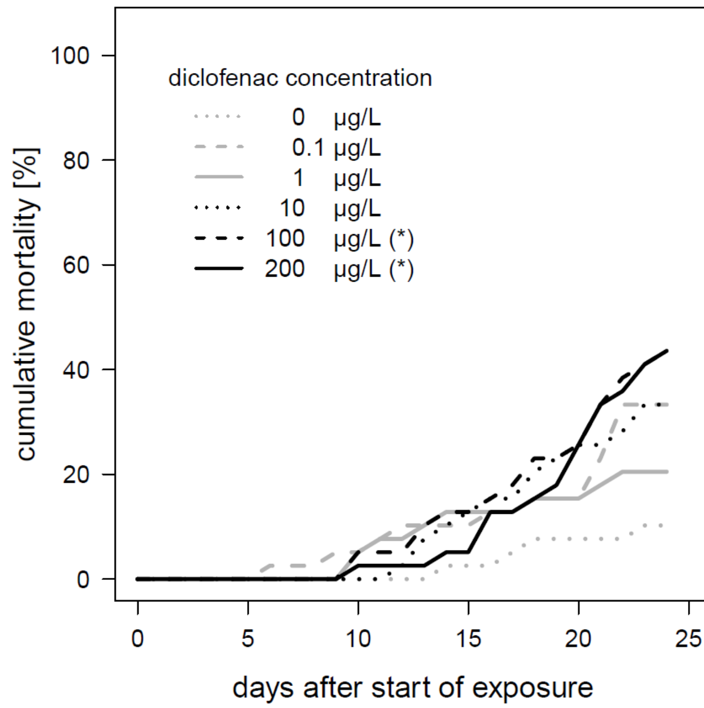


Figure 1: Mean cumulative mortality of juvenile brown trout exposed to diclofenac vs. exposure time. Diclofenac led to a concentration-dependent increase of mortality, reaching significance at 100 µg/L and 200 µg/L (asterisks).

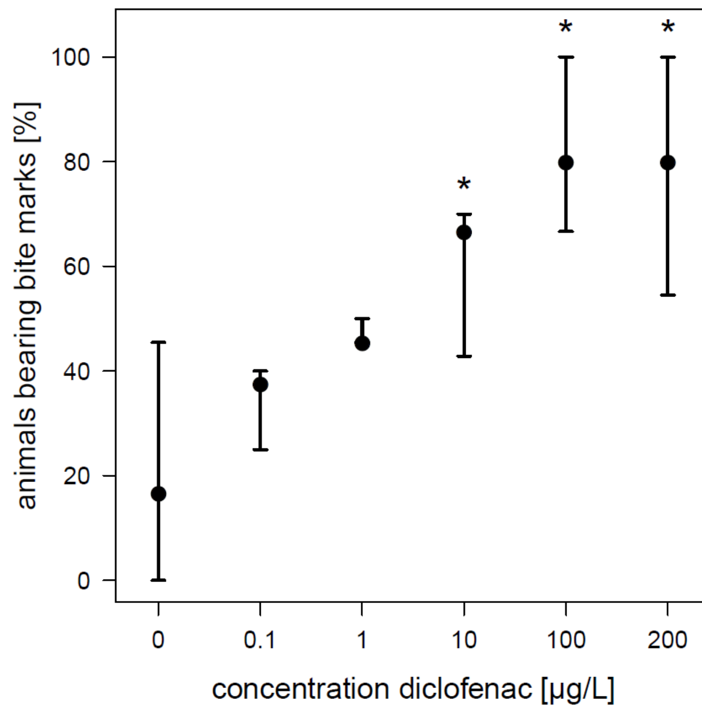


Figure 2: Percentage of brown trout showing bite marks on fins and body after 25 days exposure to diclofenac. Filled circles indicate the mean, whiskers the minimum and maximum of the tree replicates. Diclofenac increased the frequency of mutually inflicted injuries from concentrations higher than 10 µg/L (asterisks).

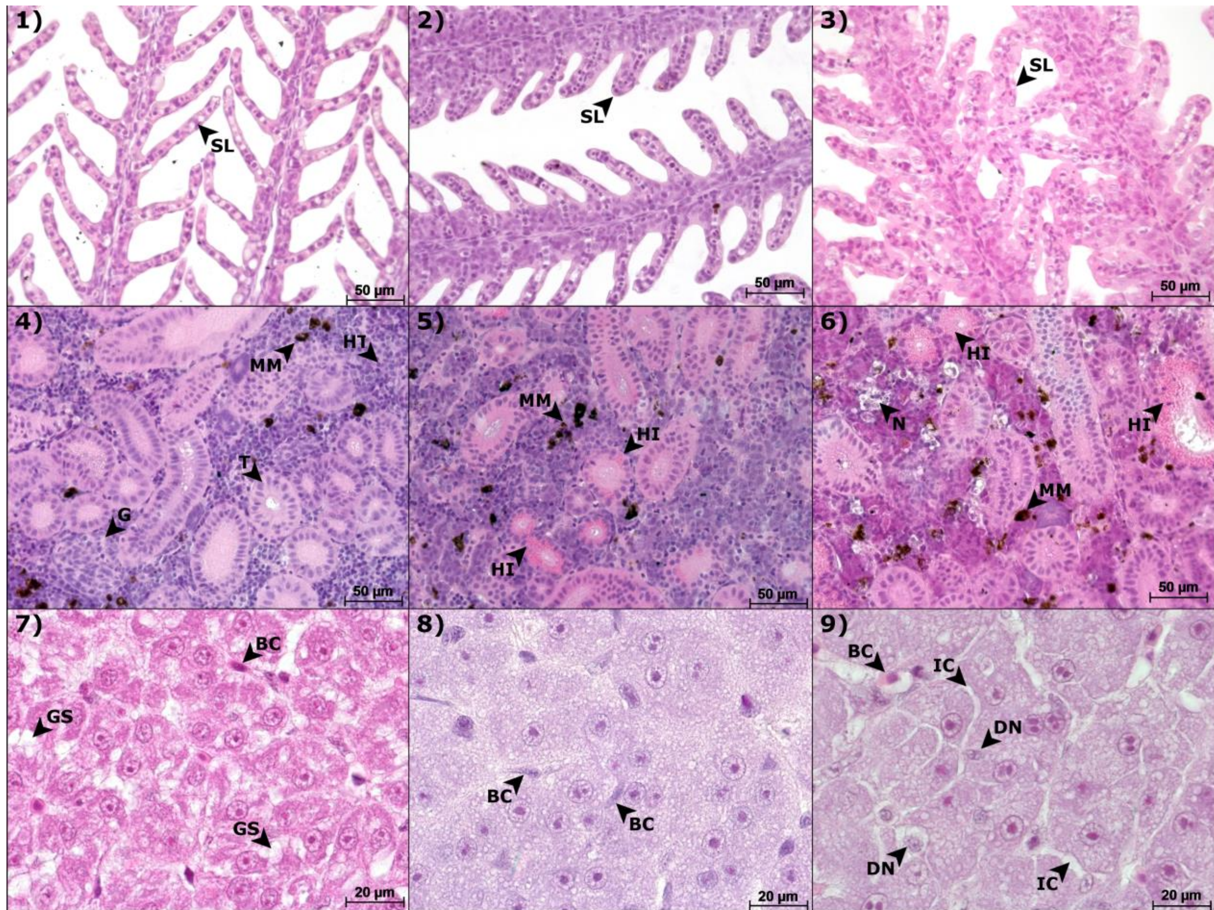


Figure 3: Overview of selected histological findings (haematoxylin-eosin-stained sections) in brown trout juveniles: 1) Gill of a hatchery control individual, displaying secondary lamellae (SL) in the control state. 2) Gill of a lab control individual, featuring slight hypertrophy of gill epithelial cells, leading to thickened appearance of secondary lamellae (SL). 3) Gill of an individual exposed to 100 µg/L diclofenac, with thickened secondary lamellae (SL) resulting from strong hypertrophy of epithelial cells and ionocytes. 4) Kidney of a hatchery control individual, displaying the normal state of glomeruli (G), tubuli (T) and hematopoietic tissue (HT), as well as few melanomacrophages (MM). 5) Kidney of an individual exposed to 0.1 µg/L diclofenac, characterised by hyaline inclusions (HI) in tubular cells. 6) Kidney of individual exposed to 100 µg/L diclofenac, featuring hyaline inclusions (HI) in tubular cells, increased abundance of melanomacrophages (MM) and necrotic areas (N) leading to "perforated" appearance of hematopoietic tissue. 7) Liver of a hatchery control individual, characterised by hepatocytes with large light areas containing glycogen (GL); blood cells (BC) are visible between hepatocytes. 8) Liver of an individual exposed to 10 µg/L diclofenac: the highly vesiculated hepatocytes lack of glycogen. 9) Liver of an individual exposed to 100 µg/L diclofenac, characterised irregularly shaped and vesiculated hepatocytes with lack of glycogen storage and degenerating nuclei (DN); enlarged intercellular spaces (IC) are visible.

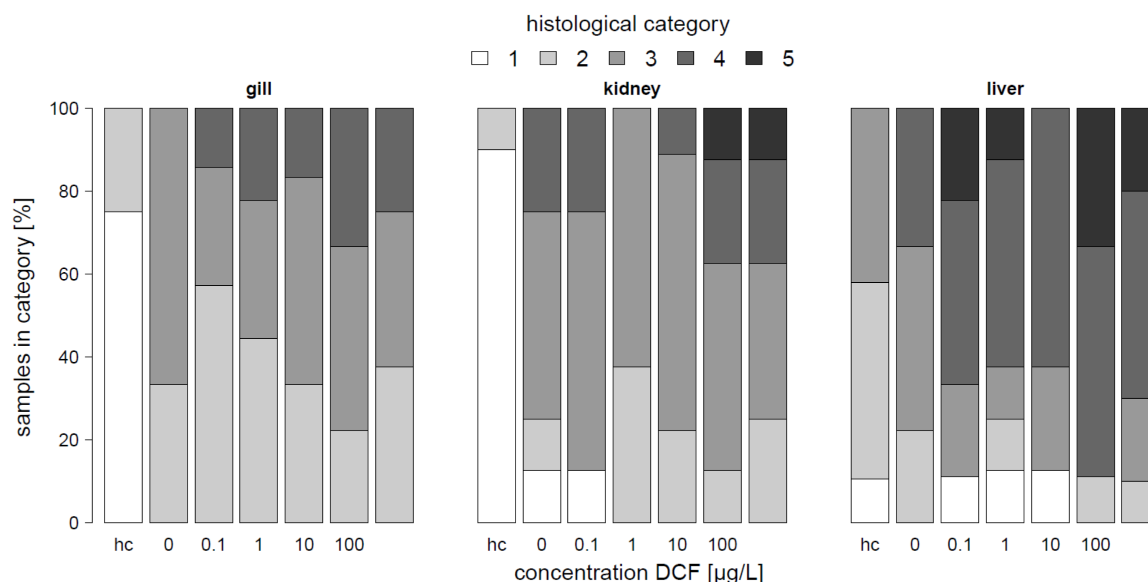


Figure 4: Semi-quantitative evaluation of histological sections of gill (left), kidney (middle) and liver (right). Hatchery controls (hc) are fish from the same stock, but sampled directly at the hatchery. Explanation is given in the text. Particularly for liver, there is a trend for increasing severity of pathological alterations at diclofenac exposure.

4. Discussion

Our results show that diclofenac can affect juvenile brown trout on the organismic level, even at concentrations as low as 10 µg/L. In contrast, no effects could be observed when examining early life stages of the same species.

4.1 Internal diclofenac concentrations

Chemical analyses of fish tissue confirmed diclofenac to be taken up by the animals, but did not indicate strong bioaccumulation. The samples used in our study consisted mainly of muscle tissue and spine. Due to the small size of the juvenile fish we were not able to provide enough material for further chemical analyses of liver or kidney after taking samples for biochemistry and histology. Our findings are in accordance with earlier studies (Schwaiger *et al.* 2004, Memmert *et al.* 2013), which reported high variability in bioaccumulation depending on the tissue type - with low bioaccumulative potential of muscle. This is supported by the low $\log P_{O/W}$ of diclofenac at neutral or, in our case, slightly alkaline conditions (ca. 0.7 at a pH of 8.5) suggesting low potential for bioaccumulation. While our analyses are useful in confirming that concentration-dependent uptake has taken place, they do not allow direct inference on fish plasma concentrations. A simple fish plasma model based on lipophilicity (Fu *et al.* 2009, Schreiber *et al.* 2011) would suggest a bioaccumulation factor of 2.5 at pH 7.6. However, lipophilicity may not be the only driving force behind uptake of NSAIDs because other, active mechanisms could contribute to this process (Choi *et al.* 2005). Cuklev *et al.* (2011) have measured plasma concentrations close to the human therapeutic level in trout exposed to 81 µg/L diclofenac, suggesting a BCF of 4. In a recent study by Bickley *et al.* (2017) on fathead minnow, accumulation in plasma was even higher, reaching the human therapeutic level at water concentrations of 25 µg/L. Effects in concentration ranges of 10 to 100 µg/L, as found in our study, may therefore be due to sufficiently large plasma concentrations to trigger a biological response.

4.2 Embryo test

The exposure of embryonic brown trout to diclofenac did not show any evidence for embryotoxicity of this pharmaceutical up to concentrations of 100 µg/L. These results match with a previous study on brown trout embryos, which reports a NOEC of 500 µg/L for mortality, hatching, development and teratogenicity (LfW 2004). Similar results were found for embryonic and larval stages of *Danio rerio*

(Hallare *et al.* 2004, van den Brandhof and Montforts 2010, Memmert *et al.* 2013), *Oncorhyncus mykiss* (Memmert *et al.* 2013) or *Cyprinus carpio* (Stepanova *et al.* 2013), where significant effects were found only at concentrations higher than 1 mg/L.

4.3 Mortality

On the contrary, juvenile brown trout responded in a far more sensitive way to diclofenac exposure than larvae. The increase in mortality, already occurring at diclofenac concentrations in the low µg/L-range, is alarming. Aside the moderate mortality rate already at the lowest test concentration, the effect was concentration dependent. This result matches a recent study by Näslund *et al.* (2017), which reported concentration-dependent increases in mortality of sticklebacks exposed to diclofenac, reaching significance at 320 µg/L. Assuming slight sensitivity differences attributable to species and life stage, the effect concentrations are in a similar range. Apart from this, diclofenac exposure-related mortality has never been reported in such low concentrations ranges before. Acute tests on adult fish reported an EC_{50} of 71 mg/L for carp (Islas-Flores *et al.* 2013, Saucedo-Vence *et al.* 2014), and none of the studies on trout that have examined a concentration range similar to our study (Schwaiger *et al.* 2004, Hoeger *et al.* 2005, Mehinto *et al.* 2010, Memmert *et al.* 2013) has reported mortality increases worth mentioning. The present data suggest that the investigated life stage of brown trout (juveniles at an age of approx. 6 months post hatch) is far more susceptible to diclofenac than both the adult and early life stages.

4.4 Behavioural effects

Similarly surprising is the apparent effect of diclofenac on the inter-individual relationship in exposed fish. The proportion of individuals bearing marks of aggressive behaviour largely increased with increasing diclofenac concentrations, with a LOEC of 10 µg/L. The real frequency of fin bites was presumably even higher, since most carcasses recovered during the experiment bore bite marks. However, we could not clearly determine whether these damages were inflicted *post mortem* and have, consequently, excluded them from the analyses. The symptomatology of fin erosion is a frequently observed phenomenon in salmonids raised in commercial fish farming. Studies found the aggression behaviour to be influenced by stocking density (Jones *et al.* 2011), feeding conditions (Noble *et al.* 2007, Cañon Jones *et al.* 2010) and water parameters (Bosakowski and Wagner 1994). However, an effect of analgesics on the aggression behaviour has, to our knowledge, not been reported so far. Ajima *et al.* (2015) have reported behavioural changes of African catfish *Rhamdia quelen* at 25 mg/L diclofenac. Those included respiratory distress, loss of balance, and erratic swimming, but no signs of aggression. Besides a possible direct increase of aggressiveness, also a reduced ability or disposition for defensive actions might be the reason for this effect. As a pain reliever, diclofenac might dullen the senses of affected individuals, making them vulnerable to conspecific attacks. Furthermore, the generally weakened condition of the animals, suggested by the high mortality, may have a similar effect on the defensive abilities of the fish. Näslund *et al.* (2017) described sticklebacks leaving food and bearing higher proportions of skin ulcerations when exposed to diclofenac – which can be seen as signs of weakened body condition. Overall, a link between suffered injuries and mortality cannot be ruled out. Open wounds may act as entrance for various pathogens, rendering the animals prone to, potentially lethal, infections. On the other hand, diclofenac-induced mortality may have caused increasing aggression behaviour through fish density alterations. Young brown trout transcend from swarming to territorial behaviour when density is low, so high mortality rates could reduce the density below this threshold level. The main reason against this hypothesis is that no increased proportion of injuries was observed in the two lowest concentrations, despite higher mortality rates than in the control. One further possible explanation for the observed increased aggressiveness in juvenile fish derives from the study of Birzle (2015), who identified that diclofenac leads to perforations of the cornea in rainbow trout. Assuming a similar effect

for brown trout juveniles, the behavioural abnormalities could be attributed to panic reactions due to impaired eyesight. Macroscopic examination of the trout eye at our sampling did not reveal any abnormalities, but since the eyes of brown trout appear darker than those of rainbow trout, alterations *per se* cannot be recognized as easily. Since the behavioural effect was not anticipated at the beginning of our experiment, the set-up was not designed to answer these questions exhaustively. Further studies focussing on behavioural endpoints, e.g. via monitoring of activity and inter-individual behaviours, should help to put our findings into perspective.

4.5 Histological effects

Histological evaluation gives additional insights into the causes of the observed fatalities: the exposure in the laboratory itself elicited a physiological response in the animals. Tissue reactions can be seen as adaptations to altered water conditions (e.g. pH and salinity) - particularly in gill and kidney, organs that are directly involved in osmoregulation and electrolyte metabolism. Furthermore, livers showed symptoms of cellular reaction already in the individuals that have been sampled at the hatchery - a strong indication of the high energy demand and metabolic activity of the tested life stage. The high weight gain in fish sampled at the hatchery after the experiment supports this assumption. Commercial fish farming targets fast weight gain, while our feeding regime in the experiment aimed at maintenance. Therefore, the lab animals may have had less energy reserves to cope with stress than fish fed *ad libitum*. It must also be stressed out that the test could not be performed under flow-through conditions, but in a semi-static set-up with regular water exchanges. These circumstances may have transferred the animals into an artificial situation they were not accustomed to. However, the lab situation alone cannot be seen as the sole reason for the observed fatalities, since lab controls showed a comparably low mortality rate of 10 %. The prevalence of strong reactions and destructions in tissue was by trend higher at diclofenac exposure, especially regarding the liver, pointing at an explicit negative effect of the pharmaceutical. We cannot attribute a specific pathology to the treatment, as it has been described in other studies for older individuals of rainbow trout (Schwaiger *et al.* 2004, Birzle 2015), fathead minnow (Bickley *et al.* 2017) or stickleback (Näslund *et al.* 2017). In these published studies, the kidney is the major affected organ, showing the clearest pathological symptoms. Our results are, in this respect, less conclusive. The already high prevalence of reactions in control animals complicates the assessment of further deteriorations. The liver was the organ showing the clearest effects, in contrast to other studies where the liver histology was inconspicuous (Schwaiger *et al.* 2004, Näslund *et al.* 2017). However, ultrastructural examinations of rainbow trout livers revealed several comparable symptoms, like glycogen reduction and macrophage infiltration (Triebskorn *et al.* 2004). Differences in the severity of reactions between studies are in this case most likely attributable to species and life stage. Overall, no observed reaction type was exclusive to the diclofenac treatment, but we can see a qualitative difference in severity. Considering the high mortality in the diclofenac treatment, we also assume that animals suffering from the most severe pathologies, resulting in complete organ failure, did not survive until the time point of histological sampling. Based on the present data it is most likely that the basal level of stress, acting on a particularly susceptible life stage, led to highly sensitive reactions towards diclofenac and, consequently, resulted in fatal effects already at concentrations in the µg/L-range. We have to stress out that the purpose of the, exemplary, histological analyses in our study merely was the assessment of general health condition in view to assist in the interpretation of the other results. A more comprehensive description of histological alterations by diclofenac is given by Birzle (2015), who applied biometric measurements on histological samples, or the recent study by Näslund *et al.* (2017).

4.6 Biochemical responses

The lack of induction of the stress protein Hsp70 level is in accordance with studies on *Danio rerio* that did not reveal any effect of diclofenac on Hsp70 in concentrations up to 2 mg/L (Hallare *et al.* 2004).

Similar findings were obtained for the freshwater invertebrate *Daphnia magna*, for which a LOEC of 30 mg/L has been reported (Haap *et al.* 2008). Gravel and Vijayan (2007), however, reported elevated Hsp70-levels in trout exposed to ibuprofen, another NSAID, but not salicylate, and complex interactions with heat shock-stimulated responses. Based on the results of our study, we draw the conclusion that the proteotoxic potential of diclofenac is negligible in trout and, probably, also in other teleost species.

The amount of lipid peroxides was not affected by any of the treatments. Yet, the FOX-assay solely quantifies lipid peroxides but not the overall oxidative stress response of an exposed organism. The high complexity of the oxidative stress response system, however, in this context may be the reason for divergent findings that are present in the literature. A study on carp (Saucedo-Vence *et al.* 2014) found a significant increase of hydroperoxide content and lipid peroxidation at a concentration of 7.1 mg/L diclofenac. However, the authors were not able to establish a concentration-response relationship as they had tested a single concentration only. Fernandez *et al.* (2013) have found a concentration-dependent induction of reactive oxygen species (ROS) by diclofenac in rainbow trout cell assays with an EC_{50} of 44.5 µg/L. On the contrary, Praskova *et al.* (2014) have reported a decrease of lipid peroxidation in *Danio rerio* at 20 µg/L diclofenac, without other parameters of the oxidative stress response being affected at concentrations up to 60 mg/L. A recent study by Ghelfi *et al.* (2016) found an increase in the activity of the enzyme superoxide dismutase in catfish kidney at 0.2 µg/L, but no influence on lipid peroxidation. If protection mechanisms against ROS, like superoxide dismutase, are induced by diclofenac, the final result – peroxidation of lipids, proteins, or DNA – may be prevented. Additionally, it has to be born in mind that the catalytic domains of cyclooxigenases possess peroxidase activity (Simmons *et al.* 2004). As a direct physiological consequence, COX-inhibitors may inhibit peroxidation of lipids. This could counterbalance the increase of lipid peroxides by cellular stress and, thereby, mask the actual effects.

4.7 Comparisons of embryonic stages and juveniles

In our study, two different life stages of the same species, originating from the same life stock, were exposed to similar concentrations of diclofenac. Yet, the responses were entirely different. Here, one may raise the difference in the pH between both studies. While the embryos developed in artificial water with neutral pH, juveniles were exposed in filtered tap water, which is typically alkaline in the test region. Earlier studies showed that the pH of the exposure medium can have a great influence on the toxicity of ionisable pharmaceuticals (Boström and Berglund 2015). However, for acidic substances like diclofenac one would assume a lower toxicity at higher pH, contrary to the findings in our experiment. Thus, our results lead to the conclusion that brown trout embryos and eleutheroembryos are more resilient towards diclofenac toxicity than older life stages. Reasons may be the independence from external feeding, but also differences in metabolic pathways. Studies on cell cultures have pointed out that the toxic action of diclofenac might be due to intermediate products of the CYP450-metabolism (van Leeuwen *et al.* 2011). Life-stage dependent differences in the expression of CYP450 (Andersson and Förlin 1992) or other enzymes therefore may influence the reaction towards toxicants. The low susceptibility of brown trout embryos to diclofenac, compared to older life stages, has been previously reported (LfW 2004). Memmert *et al.* (2013) exposed rainbow trout from early embryonic up to early juvenile stages, without finding effects until concentrations of 1 mg/L diclofenac. Studies, which actually reported effects of diclofenac in a low µg/L-range, were all conducted on older individuals (Schwaiger *et al.* 2004, Birzle 2015, Näslund *et al.* 2017). Solely relying on data obtained from early life stage tests would probably underestimate the risk. Consequently, future studies, particularly those with a focus on risk assessment, should address the differences in sensitivity in different life-stages of trout and other fish species to determine the most susceptible and, therefore, most relevant age class.

4.8 Environmental relevance

Reported environmental concentrations of diclofenac are diverse and can reach the high ng/L-range. Measured environmental concentrations (MECs) differ considerably depending on the country and, frequently, data availability is lower than desired. In an extensive summary, Aus der Beek *et al.* (2016b) have reported a mean concentration of 0.16 µg/L for surface waters in Germany, for which the largest set of environmental data is available. These results fit well to the study of Letzel *et al.* (2009), from whose study a worst-case PEC of 0.14 µg/L has derived. Other PECs range in the low to medium ng/L-range (Letzel *et al.* 2009, Johnson *et al.* 2013), rarely exceeding 0.1 µg/L. Our lowest tested diclofenac concentration of 0.1 µg/L is, therefore, in a range that is not the normal case but can be found in surface waters. The second-lowest concentration of 1 µg/L is still in the range of wastewater treatment plant effluent concentrations (Ternes 1998, Letzel *et al.* 2009). Higher concentrations, which elicited significant effects in our study, have not been measured in the environment so far. Nevertheless, our results are alarming since the safety margin between worst-case environmental concentration and experimental lowest effect concentration is small. Especially since the affected parameters mortality and behaviour are on an organismic level, which is of high relevance for the whole population and would warrant the application of further safety factors. Furthermore, survival rates, inviolacy, and the histological integrity of liver were, by trend albeit not significantly, affected at even lower concentrations. Even though deduction of environmental risks from laboratory experiments is always associated with uncertainty, the intensity of basic but moderate stress factors elicited by, e.g. the fishkeeping conditions in aquaria can be regarded comparable to other confounding stress conditions that frequently occur in the environment. Feral fish encounter various events, like seasonal or irregular changes in water parameters, periods of limited food availability, intraspecific competition or co-exposure to other chemicals (e.g. nitrite or ammonia). In all these cases, further chemical stressors like diclofenac may act as a final trigger leading to detrimental effects on the individual and population level. To date, we are not aware of any study clearly linking fish population declines to contaminations with NSAIDs. Nevertheless, in view of other effects reported for diclofenac in the low µg/L-range in freshwater (Schwaiger *et al.* 2004, Triebskorn *et al.* 2004, Birzle 2015, Näslund *et al.* 2017) as well as in marine organisms (Ribeiro *et al.* 2015), the currently proposed environmental quality standards of 0.1 µg/L and 0.01 µg/L, respectively, (EU 2012, EU 2013, Schröder *et al.* 2016) seem very reasonable.

4.9 Perspectives

Targeting diclofenac alone would be a grave underestimation of the general situation in the environment. The wide abundance of a range of other NSAIDs and further micropollutants is current reality in our aquatic ecosystems. Reliable effect-based tools would be a great gain for further evaluation of environmental risks (Triebskorn *et al.* 2015, Brack *et al.* 2017). In a recent study, Bernhard *et al.* (2017) have reported on novel mode-of-action based bioassays for the quantification of cyclooxygenase-inhibitors, like diclofenac, in aqueous samples. Assuming that the observed effects of diclofenac in aquatic species depend on the pharmaceutical's physiological mode-of-action, future studies will relate the collective inhibitory action of NSAID mixtures to the effects observed in exposed biota. This change from substance-based to mode-of-action based evaluation will be a necessary step towards a more holistic assessment of the risk posed by pharmaceuticals in our environment.

5. Conclusions

Our results clearly indicate that embryos and larvae of brown trout are more resilient against the NSAID diclofenac than six months-old juveniles, which exhibited drastic effects. Environmentally relevant endpoints like mortality and behaviour were significantly affected at concentrations 10-100 times above reported effluent and surface-water concentrations. Since the range between lowest effect and highest surface water concentration are within only two orders of magnitude, the results are alarming. Particularly because other NSAIDs that are also present in surface waters likely will exert

concomitant effects via the same mode-of-action pathway. Other studies also reported on adverse effects of diclofenac, on the histological as well as organismic level, in a comparably low concentration range. Taking this into account and applying a precautionary principle, it seems appropriate to monitor diclofenac closely and aim for compliance to the proposed environmental quality standard.

6. Acknowledgements

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7. Conflict of interest

The authors declare that there is no conflict of interests.

8. References

Ajima, M. N. O., Ogo, O. A., Audu, B. S. and Ugwoegbu, K. C. (2015). "Chronic diclofenac (DCF) exposure alters both enzymatic and haematological profile of African catfish, *Clarias gariepinus*." *Drug and chemical toxicology* **38**(4): 383-390.

Andersson, T. and Förlin, L. (1992). "Regulation of the cytochrome P450 enzyme system in fish." *Aquatic Toxicology* **24**(1): 1-19.

Arnold, K. E., Brown, A. R., Ankley, G. T. and Sumpter, J. P. (2014). "Medicating the environment: assessing risks of pharmaceuticals to wildlife and ecosystems." *Philosophical Transactions of the Royal Society B-Biological Sciences* **369**(1656): 11.

Arpin-Pont, L., Bueno, M. J. M., Gomez, E. and Fenet, H. (2014). "Occurrence of PPCPs in the marine environment: a review." *Environmental Science and Pollution Research* **23**(6): 4978-4991.

Aus der Beek, T. A., Weber, F. A. and Bergmann, A. (2016a). "Pharmaceuticals in the environment: Global occurrence and potential cooperative action under the Strategic Approach to International Chemicals Management (SAICM)". FKZ 371265408. Umweltbundesamt. *UBA Texte*. **67/2016**.

Aus der Beek, T. A., Weber, F. A., Bergmann, A., Hickmann, S., Ebert, I., Hein, A. and Kuster, A. (2016b). "Pharmaceuticals in the environment - global occurrences and perspectives." *Environmental Toxicology and Chemistry* **35**(4): 823-835.

Avetta, P., Fabbri, D., Minella, M., Brigante, M., Maurino, V., Minero, C., Pazzi, M. and Vione, D. (2016). "Assessing the phototransformation of diclofenac, clofibric acid and naproxen in surface waters: Model predictions and comparison with field data." *Water Research* **105**: 383-394.

Baron, M. G., Mintram, K. S., Owen, S. F., Hetheridge, M. J., Moody, A. J., Purcell, W. M., Jackson, S. K. and Jha, A. N. (2017). "Pharmaceutical metabolism in fish: using a 3-D hepatic *in vitro* model to assess clearance." *Plos One* **12**(1): 13.

Bellmann, H. (1991). "Spinnen, Krebse, Tausendfüßer - Die farbigen Naturführer". München, *Mosaik Verlag*.

Berger, E., Haase, P., Oetken, M. and Sundermann, A. (2016). "Field data reveal low critical chemical concentrations for river benthic invertebrates." *Science of the Total Environment* **544**: 864-873.

Bergmann, A., Fohrmann, R. and Weber, F. A. (2011). "Zusammenstellung von Monitoringdaten zu Umweltkonzentrationen von Arzneimitteln". German Environment Agency. *UBA Texte*. **66**.

Bernhard, K., Stahl, C., Martens, R., Köhler, H.-R., Triebskorn, R., Scheurer, M. and Frey, M. (2017). "Two novel real time cell-based assays quantify beta-blocker and NSAID specific effects in effluents of municipal wastewater treatment plants." *Water Research* **115**: 74-83.

Bickley, L. K., van Aerle, R., Brown, A. R., Hargreaves, A., Huby, R., Cammack, V., Jackson, R., Santos, E. M. and Tyler, C. R. (2017). "Bioavailability and kidney responses to diclofenac in the fathead minnow (*Pimephales promelas*)." *Environmental Science & Technology* **51**(3): 1764-1774.

Birzle, C. F. (2015). "Etablierung und Validierung quantitativ-morphologischer Parameter bei Regenbogenforellen im Rahmen ökotoxikologischer Fragestellungen". PhD, *University of Munich*.

Bjorkman, D. (1998). "Nonsteroidal anti-inflammatory drug-associated toxicity of the liver, lower gastrointestinal tract, and esophagus." *The American Journal of Medicine* **105**(5, Supplement 1): 17S-21S.

Blüm, V., Casado, J., Lehmann, J. and Mehring, E. (1989). "Farbatlas der Histologie der Regenbogenforelle". Berlin, *Springer Verlag*.

Bonga, S. E. W. (1997). "The stress response in fish." *Physiological Reviews* **77**(3): 591-625.

Bonnefille, B., Gomez, E., Alali, M., Rosain, D., Fenet, H. and Courant, F. (2018). "Metabolomics assessment of the effects of diclofenac exposure on *Mytilus galloprovincialis*: Potential effects on osmoregulation and reproduction." *Science of The Total Environment* **613-614**(Supplement C): 611-618.

Bosakowski, T. and Wagner, E. J. (1994). "A survey of trout fin erosion, water quality, and rearing conditions at state fish hatcheries in Utah." *Journal of the World Aquaculture Society* **25**(2): 308-316.

Boström, M. L. and Berglund, O. (2015). "Influence of pH-dependent aquatic toxicity of ionizable pharmaceuticals on risk assessments over environmental pH ranges." *Water Research* **72**: 154-161.

Brack, W., Dulio, V., Ågerstrand, M., Allan, I., Altenburger, R., Brinkmann, M., Bunke, D., Burgess, R. M., Cousins, I., Escher, B. I., Hernández, F. J., Hewitt, L. M., Hilscherová, K., Hollender, J., Hollert, H., Kase, R., Klauer, B., Lindim, C., Herráez, D. L., Miège, C., Munthe, J., O'Toole, S., Posthuma, L., Rüdel, H., Schäfer, R. B., Sengl, M., Smedes, F., van de Meent, D., van den Brink, P. J., van Gils, J., van Wezel, A. P., Vethaak, A. D., Vermeirssen, E., von der Ohe, P. C. and Vrana, B. (2017). "Towards the review of the European Union Water Framework Directive: recommendations for more efficient assessment and management of chemical contamination in European surface water resources." *Science of The Total Environment* **576**: 720-737.

Bradford, M. M. (1976). "Rapid and sensitive method for quantitation of microgram quantities of protein utilizing principle of protein-dye binding." *Analytical Biochemistry* **72**(1-2): 248-254.

Brandmayr, C., Kerber, H., Winker, M. and Schramm, E. (2015). "Impact assessment of emission management strategies of the pharmaceuticals Metformin and Metoprolol to the aquatic environment using Bayesian networks." *Science of The Total Environment* **532**: 605-616.

Brausch, J. M., Connors, K. A., Brooks, B. W. and Rand, G. M. (2012). "Human pharmaceuticals in the aquatic environment: a review of recent toxicological studies and considerations for toxicity testing." *Reviews of Environmental Contamination and Toxicology* **218**: 1-99.

Brodin, T., Fick, J., Jonsson, M. and Klaminder, J. (2013). "Dilute concentrations of a psychiatric drug alter behavior of fish from natural populations." *Science* **339**(6121): 814-815.

Brodin, T., Piovano, S., Fick, J., Klaminder, J., Heynen, M. and Jonsson, M. (2014). "Ecological effects of pharmaceuticals in aquatic systems-impacts through behavioural alterations." *Philosophical Transactions of the Royal Society B-Biological Sciences* **369**(1656): 10.

Buchberger, A.-K., Brettschneider, D., Berg, K., Jungmann, D., Oehlmann, J., Scheurer, M. and Oetken, M. (2018). "Effects of metoprolol on aquatic invertebrates in artificial indoor streams." *Journal of Environmental Science and Health, Part A* **53**(8): 1-12.

Buser, H.-R., Poiger, T. and Müller, M. D. (1998). "Occurrence and fate of the pharmaceutical drug diclofenac in surface waters: rapid photodegradation in a lake." *Environmental Science & Technology* **32**(22): 3449-3456.

Cannon, C. P. and Cannon, P. J. (2012). "COX-2 inhibitors and cardiovascular risk." *Science* **336**(6087): 1386-1387.

Cañon Jones, H. A., Hansen, L. A., Noble, C., Damsgård, B., Broom, D. M. and Pearce, G. P. (2010). "Social network analysis of behavioural interactions influencing fin damage development in Atlantic salmon (*Salmo salar*) during feed-restriction." *Applied Animal Behaviour Science* **127**(3–4): 139-151.

Carpenter, S. R., Caraco, N. F., Correll, D. L., Howarth, R. W., Sharpley, A. N. and Smith, V. H. (1998). "Nonpoint pollution of surface waters with phosphorus and nitrogen." *Ecological Applications* **8**(3): 559-568.

Chae, J.-P., Park, M. S., Hwang, Y.-S., Min, B.-H., Kim, S.-H., Lee, H.-S. and Park, M.-J. (2015). "Evaluation of developmental toxicity and teratogenicity of diclofenac using *Xenopus* embryos." *Chemosphere* **120**: 52-58.

Choi, J. S., Jin, M. J. and Han, H. K. (2005). "Role of monocarboxylic acid transporters in the cellular uptake of NSAIDs." *Journal of Pharmacy and Pharmacology* **57**(9): 1185-1189.

Contardo-Jara, V., Pflugmacher, S., Nützmann, G., Kloas, W. and Wiegand, C. (2010). "The β-receptor blocker metoprolol alters detoxification processes in the non-target organism *Dreissena polymorpha*." *Environmental Pollution* **158**(6): 2059-2066.

Crisp, D. T. (1996). "Environmental requirements of common riverine European salmonid fish species in fresh water with particular reference to physical and chemical aspects." *Hydrobiologia* **323**(3): 201-221.

Cuklev, F., Kristiansson, E., Fick, J., Asker, N., Förlin, L. and Larsson, D. G. J. (2011). "Diclofenac in fish: blood plasma levels similar to human therapeutic levels affect global hepatic gene expression." *Environmental Toxicology and Chemistry* **30**(9): 2126-2134.

Daughton, C. G. and Ternes, T. A. (1999). "Pharmaceuticals and personal care products in the environment: agents of subtle change?" *Environmental Health Perspectives* **107**: 907-938.

Davis, E., Loiacono, R. and Summers, R. J. (2008). "The rush to adrenaline: Drugs in sport acting on the β-adrenergic system." *British Journal of Pharmacology* **154**(3): 584-597.

DeGeorge Jr, B. R. and Koch, W. J. (2007). "Beta blocker specificity: A building block toward personalized medicine." *Journal of Clinical Investigation* **117**(1): 86-89.

Dieterich, A., Troschinski, S., Schwarz, S., Di Lellis, M. A., Henneberg, A., Fischbach, U., Ludwig, M., Gärtner, U., Triebskorn, R. and Köhler, H.-R. (2015). "Hsp70 and lipid peroxide levels following heat stress in *Xeropicta derbentina* (Krynicki 1836) (Gastropoda, Pulmonata) with regard to different colour morphs." *Cell Stress and Chaperones* **20**(1): 159-168.

Dietrich, S., Ploessl, F., Bracher, F. and Laforsch, C. (2010). "Single and combined toxicity of pharmaceuticals at environmentally relevant concentrations in *Daphnia magna* – A multigenerational study." *Chemosphere* **79**(1): 60-66.

Donnachie, R. L., Johnson, A. C. and Sumpter, J. P. (2016). "A rational approach to selecting and ranking some pharmaceuticals of concern for the aquatic environment and their relative importance compared with other chemicals." *Environmental Toxicology and Chemistry* **35**(4): 1021-1027.

Dußling, U. and Berg, R. (2001). "Fische in Baden-Württemberg". Stuttgart, Germany, *Ministerium für Ernährung und Ländlichen Raum Baden-Württemberg*.

Dzialowski, E. M., Turner, P. K. and Brooks, B. W. (2006). "Physiological and reproductive effects of beta adrenergic receptor antagonists in *Daphnia magna*." *Archives of Environmental Contamination and Toxicology* **50**(4): 503-510.

Ebert, I., Amato, R., Hein, A. and Konradi, S. (2014). "Arzneimittel in der Umwelt - vermeiden, reduzieren, überwachen". Umweltbundesamt.

Efosa, N. J., Kleiner, W., Kloas, W. and Hoffmann, F. (2017). "Diclofenac can exhibit estrogenic modes of action in male *Xenopus laevis*, and affects the hypothalamus-pituitary-gonad axis and mating vocalizations." *Chemosphere* **173**: 69-77.

EMA (2014). "CVMP assessment report under Article 30(3) of Regulation (EC) No 726/2004 - On the risk to vultures and other necrophagous bird populations in the European Union in connection with the use of veterinary medicinal products containing the substance diclofenac". EMEA/V/A/107. European Medicines Agency.

Ericson, H., Thorsén, G. and Kumblad, L. (2010). "Physiological effects of diclofenac, ibuprofen and propranolol on Baltic Sea blue mussels." *Aquatic Toxicology* **99**(2): 223-231.

Escher, B. I., Allinson, M., Altenburger, R., Bain, P. A., Balaguer, P., Busch, W., Crago, J., Denslow, N. D., Dopp, E., Hilscherova, K., Humpage, A. R., Kumar, A., Grimaldi, M., Jayasinghe, B. S., Jarosova, B., Jia, A., Makarov, S., Maruya, K. A., Medvedev, A., Mehinto, A. C., Mendez, J. E., Poulsen, A., Prochazka, E., Richard, J., Schifferli, A., Schlenk, D., Scholz, S., Shiraish, F., Snyder, S., Su, G. Y., Tang, J. Y. M., van der Burg, B., van der Linden, S. C., Werner, I., Westerheide, S. D., Wong, C. K. C., Yang, M., Yeung, B. H. Y., Zhang, X. W. and Leusch, F. D. L. (2014). "Benchmarking organic micropollutants in wastewater, recycled water and drinking water with *in vitro* bioassays." *Environmental Science & Technology* **48**(3): 1940-1956.

Escher, B. I., Bramaz, N., Richter, M. and Lienert, J. (2006). "Comparative ecotoxicological hazard assessment of beta-blockers and their human metabolites using a mode-of-action-based test battery and a QSAR approach." *Environmental Science & Technology* **40**(23): 7402-7408.
EU (2000). "CVMP/VICH/592/98-FINAL - guideline on environmental impact assessment (EIAS) for veterinary medicinal products - phase I". T. E. A. f. t. E. o. M. Products. London.
EU (2004). "CVMP/VICH/790/03-FINAL - guideline on environmental impact assessment for veterinary medicinal products phase II". E. M. Agency. London.
EU (2006a). "Council Directive 2006/88/EC on animal health requirements for aquaculture animals and products thereof, and on the prevention and control of certain diseases in aquatic animals". *Official Journal of the European Union*. **49**: 14-56.
EU (2006b). "EMEA/CHMP/SWP/4447/00 corr 2 - guideline on the environmental risk assessment of medicinal products for human use". E. M. Agency. London.
EU (2012). "COM(2011) 876 final 2011/0429 (COD) proposal for a Directive of the European Parliament and of the Council amending Directives 2000/60/EC and 2008/105/EC as regards priority substances in the field of water policy".
EU (2013). "Directive 2013/39/EU of the European Parliament and of the Council of 12 August 2013 amending Directives 2000/60/EC and 2008/105/EC as regards priority substances in the field of water policy". *Official Journal of the European Union*. **L226**: 1-17.
EU (2015). "Commission implementing Decision (EU) 2015/495 of 20 March 2015 establishing a watch list of substances for Union-wide monitoring in the field of water policy pursuant to Directive 2008/105/EC of the European Parliament and of the Council". *Official Journal of the European Union*. **L78**: 40 - 42.
Fent, K., Weston, A. A. and Caminada, D. (2006). "Ecotoxicology of human pharmaceuticals." *Aquatic Toxicology* **76**(2): 122-159.
Fernandes, D., Schnell, S. and Porte, C. (2011). "Can pharmaceuticals interfere with the synthesis of active androgens in male fish? An *in vitro* study." *Marine Pollution Bulletin* **62**(10): 2250-2253.
Fernandez, C., Carbonell, G. and Babin, M. (2013). "Effects of individual and a mixture of pharmaceuticals and personal-care products on cytotoxicity, EROD activity and ROS production in a rainbow trout gonadal cell line (RTG-2)." *Journal of Applied Toxicology* **33**(11): 1203-1212.
Ferreira, H., Lúcio, M., Lima, J. L. F. C., Matos, C. and Reis, S. (2005). "Effects of diclofenac on EPC liposome membrane properties." *Analytical and Bioanalytical Chemistry* **382**(5): 1256-1264.
Fomin, A., Oehlmann, J. and Markert, B. (2003). "Praktikum zur Ökotoxikologie - Grundlagen und Anwendungen biologischer Testverfahren". Landsberg, *ecomed verlagsgesellschaft AG & Co. KG*.
Fraysse, B. and Garric, J. (2005). "Prediction and experimental validation of acute toxicity of β-blockers in *Ceriodaphnia dubia*." *Environmental Toxicology and Chemistry* **24**(10): 2470-2476.
Fu, W., Franco, A. and Trapp, S. (2009). "Methods for estimating the bioconcentration factor of ionizable organic chemicals." *Environmental Toxicology and Chemistry* **28**(7): 1372-1379.
Funk, C. D. (2001). "Prostaglandins and leukotrienes: advances in eicosanoid biology." *Science* **294**(5548): 1871-1875.
Gan, T. J. (2010). "Diclofenac: an update on its mechanism of action and safety profile." *Current Medical Research and Opinion* **26**(7): 1715-1731.
Gernhöfer, M., Pawert, M., Schramm, M., Müller, E. and Triebskorn, R. (2001). "Ultrastructural biomarkers as tools to characterize the health status of fish in contaminated streams." *Journal of Aquatic Ecosystem Stress and Recovery* **8**(3): 241-260.
Ghelfi, A., Ribas, J. L. C., Guiloski, I. C., Bettim, F. L., Piancini, L. D. S., Cestari, M. M., Pereira, A. J., Sassaki, G. L. and Silva de Assis, H. C. (2016). "Evaluation of biochemical, genetic and hematological biomarkers in a commercial catfish *Rhamdia quelen* exposed to diclofenac." *Bulletin of environmental contamination and toxicology* **96**(1): 49-54.
Godoy, A. A., Kummrow, P. and Pamplin, P. A. Z. (2015). "Occurrence, ecotoxicological effects and risk assessment of antihypertensive pharmaceutical residues in the aquatic environment - A review." *Chemosphere* **138**: 281-291.
Gonzalez-Rey, M. and Bebianno, M. J. (2014). "Effects of non-steroidal anti-inflammatory drug (NSAID) diclofenac exposure in mussel *Mytilus galloprovincialis*." *Aquatic Toxicology* **148**: 221-230.
Goudie, A. S. (2013). "The human impact on the natural environment : past, present, and future". Chichester, *WILEY Blackwell*.
Grabow, K. (2000). "Farbatlas Süßwasserfauna Wirbellose". Stuttgart, *Eugen Ulmer GmbH & Co.*
Gravel, A. and Vijayan, M. M. (2007). "Non-steroidal anti-inflammatory drugs disrupt the heat shock response in rainbow trout." *Aquatic Toxicology* **81**(2): 197-206.
Gröner, F., Hohne, C., Kleiner, W. and Kloas, W. (2017a). "Chronic diclofenac exposure affects gill integrity and pituitary gene expression and displays estrogenic activity in nile tilapia (*Oreochromis niloticus*)." *Chemosphere* **166**: 473-481.
Gröner, F., Hohne, C., Kleiner, W. and Kloas, W. (2017b). "Chronic exposure to the beta-blocker metoprolol reduces growth and alters gene expression of gonadotropins and vitellogenin in Nile tilapia (*Oreochromis niloticus*)." *Ecotoxicology and Environmental Safety* **141**: 271-279.
Gröner, F., Ziková, A. and Kloas, W. (2015). "Effects of the pharmaceuticals diclofenac and metoprolol on gene expression levels of enzymes of biotransformation, excretion pathways and estrogenicity in primary hepatocytes of Nile tilapia (*Oreochromis niloticus*)." *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology* **167**: 51-57.
Guiloski, I. C., Ribas, J. L. C., Pereira, L. d. S., Neves, A. P. P. and Silva de Assis, H. C. (2015). "Effects of trophic exposure to dexamethasone and diclofenac in freshwater fish." *Ecotoxicology and Environmental Safety* **114**: 204-211.
Guiloski, I. C., Stein Piancini, L. D., Dagostim, A. C., de Morais Calado, S. L., Fávaro, L. F., Boschen, S. L., Cestari, M. M., da Cunha, C. and Silva de Assis, H. C. (2017). "Effects of environmentally relevant concentrations of the anti-inflammatory drug diclofenac in freshwater fish *Rhamdia quelen*." *Ecotoxicology and Environmental Safety* **139**: 291-300.
Gunnarsson, L., Jauhiainen, A., Kristiansson, E., Nerman, O. and Larsson, D. G. J. (2008). "Evolutionary conservation of human drug targets in organisms used for environmental risk assessments." *Environmental Science & Technology* **42**(15): 5807-5813.
Haap, T., Triebskorn, R. and Köhler, H. R. (2008). "Acute effects of diclofenac and DMSO to *Daphnia magna*: immobilisation and hsp70-induction." *Chemosphere* **73**(3): 353-359.
Hallare, A. V., Köhler, H. R. and Triebskorn, R. (2004). "Development toxicity and stress protein responses in zebrafish embryos after exposure to diclofenac and its solvents, DMSO." *Chemosphere* **56**(7): 659-666.

Hamdorf, K. (1960). "Die Beeinflussung der Embryonal- und Larvalentwicklung der Regenbogenforelle (*Salmo irideus* Gibb.) durch Strahlung im sichtbaren Bereich." *Zeitschrift für vergleichende Physiologie* **42**(6): 525-565.

Harris, C. A., Scott, A. P., Johnson, A. C., Panter, G. H., Sheahan, D., Roberts, M. and Sumpter, J. P. (2014). "Principles of sound ecotoxicology." *Environmental Science & Technology* **48**(6): 3100-3111.

Heberer, T. and Feldmann, D. (2005). "Contribution of effluents from hospitals and private households to the total loads of diclofenac and carbamazepine in municipal sewage effluents—modeling versus measurements." *Journal of Hazardous Materials* **122**(3): 211-218.

Hermes-Lima, M., Willmore, W. G. and Storey, K. B. (1995). "Quantification of lipid-peroxidation in tissue-extracts based on Fe(III)xylenol orange complex formation." *Free Radical Biology and Medicine* **19**(3): 271-280.

Hernández, F., Ibáñez, M., Portolés, T., Cervera, M. I., Sancho, J. V. and López, F. J. (2015). "Advancing towards universal screening for organic pollutants in waters." *Journal of Hazardous Materials* **282**: 86-95.

Hickey, E. J., Raje, R. R., Reid, V. E., Gross, S. M. and Ray, S. D. (2001). "Diclofenac induced in vivo nephrotoxicity may involve oxidative stress-mediated massive genomic DNA fragmentation and apoptotic cell death." *Free Radical Biology and Medicine* **31**(2): 139-152.

Hoeger, B., Dietrich, D. R., Schmid, D., Hartmann, A. and Hitzfeld, B. (2008). "Distribution of intraperitoneally injected diclofenac in brown trout (*Salmo trutta* f. *fario*)." *Ecotoxicology and Environmental Safety* **71**(2): 412-418.

Hoeger, B., Köllner, B., Dietrich, D. R. and Hitzfeld, B. (2005). "Water-borne diclofenac affects kidney and gill integrity and selected immune parameters in brown trout (*Salmo trutta* f. *fario*)." *Aquatic Toxicology* **75**(1): 53-64.

Hong, H. N., Kim, H. N., Park, K. S., Lee, S. K. and Gu, M. B. (2007). "Analysis of the effects diclofenac has on Japanese medaka (*Oryzias latipes*) using real-time PCR." *Chemosphere* **67**(11): 2115-2121.

Huggett, D. B., Brooks, B. W., Peterson, B., Foran, C. M. and Schlenk, D. (2002). "Toxicity of select beta adrenergic receptor-blocking pharmaceuticals (B-blockers) on aquatic organisms." *Archives of Environmental Contamination and Toxicology* **43**(2): 229-235.

Hughes, S. R., Kay, P. and Brown, L. E. (2013). "Global synthesis and critical evaluation of pharmaceutical data sets collected from river systems." *Environmental Science & Technology* **47**(2): 661-677.

Hurlbert, S. H. (1984). "Pseudoreplication and the design of ecological field experiments." *Ecological Monographs* **54**(2): 187-211.

Islas-Flores, H., Gomez-Olivan, L. M., Galar-Martinez, M., Colin-Cruz, A., Neri-Cruz, N. and Garcia-Medina, S. (2013). "Diclofenac-induced oxidative stress in brain, liver, gill and blood of common carp (*Cyprinus carpio*)." *Ecotoxicology and Environmental Safety* **92**: 32-38.

Iwama, G. K., Thomas, P. T., Forsyth, R. B. and Vijayan, M. M. (1998). "Heat shock protein expression in fish." *Reviews in Fish Biology and Fisheries* **8**(1): 35-56.

James-Casas, A. and Andres, S. (2017). "Report on the aquatic environmental risk assessment for diclofenac". Development of robust tools for the assessment of pharmaceuticals in aquatic media (DOREMIPHARM). Verneuil-En-Halatte, Oise, France. *INERIS*. DRC-16-129627-12069A. 55.

Ji, K., Liu, X., Lee, S., Kang, S., Kho, Y., Giesy, J. P. and Choi, K. (2013). "Effects of non-steroidal anti-inflammatory drugs on hormones and genes of the hypothalamic-pituitary-gonad axis, and reproduction of zebrafish." *Journal of Hazardous Materials* **254-255**: 242-251.

Joachim, S. (2017). "Bottom-up and top-down effects in a diclofenac contaminated model ecosystem - Task 2: Mesocosms study". Development of robust tools for the assessment of pharmaceuticals in aquatic media (DOREMIPHARM). Verneuil-En-Halatte, Oise, France. *INERIS*. DRC-16-129627-11897A. 87.

Johnson, A. C., Dumont, E., Williams, R. J., Oldenkamp, R., Cisowska, I. and Sumpter, J. P. (2013). "Do concentrations of ethinylestradiol, estradiol, and diclofenac in European rivers exceed proposed EU environmental quality standards?" *Environmental Science & Technology* **47**(21): 12297-12304.

Jones, H. A. C., Noble, C., Damsgard, B. and Pearce, G. P. (2011). "Social network analysis of the behavioural interactions that influence the development of fin damage in Atlantic salmon parr (*Salmo salar*) held at different stocking densities." *Applied Animal Behaviour Science* **133**(1-2): 117-126.

Jonsson, N. and Jonsson, B. (1999). "Trade-off between egg mass and egg number in brown trout." *Journal of Fish Biology* **55**(4): 767-783.

Kawada, N., Moriyama, T., Kitamura, H., Yamamoto, R., Furumatsu, Y., Matsui, I., Takabatake, Y., Nagasawa, Y., Imai, E., Wilcox, C. S., Rakugi, H. and Isaka, Y. (2012). "Towards developing new strategies to reduce the adverse side-effects of nonsteroidal anti-inflammatory drugs." *Clinical and Experimental Nephrology* **16**(1): 25-29.

Kidd, K. A., Paterson, M. J., Rennie, M. D., Podemski, C. L., Findlay, D. L., Blanchfield, P. J. and Liber, K. (2014). "Direct and indirect responses of a freshwater food web to a potent synthetic oestrogen." *Philosophical Transactions of the Royal Society B: Biological Sciences* **369**(1656).

Killeen, J., McLay, H. A. and Johnston, I. A. (1999a). "Development in *Salmo trutta* at different temperatures, with a quantitative scoring method for intraspecific comparisons." *Journal of Fish Biology* **55**(2): 382-404.

Killeen, J. R., McLay, H. A. and Johnston, I. A. (1999b). "Temperature and neuromuscular development in embryos of the trout (*Salmo trutta* L.)." *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology* **122**(1): 53-64.

Klemetsen, A., Amundsen, P. A., Dempson, J. B., Jonsson, B., Jonsson, N., O'Connell, M. F. and Mortensen, E. (2003). "Atlantic salmon *Salmo salar* L., brown trout *Salmo trutta* L. and Arctic charr *Salvelinus alpinus* (L.): a review of aspects of their life histories." *Ecology of Freshwater Fish* **12**(1): 1-59.

Köhler, H.-R. (2009). "Die Rolle von Stressproteinen bei der Anpassung an Umweltbedingungen: Ökophysiologische, ökotoxikologische und evolutionsbiologische Implikationen." *Umweltwissenschaften und Schadstoff-Forschung* **21**: 150-159.

Köhler, H. R., Bartussek, C., Eckwert, H., Farian, K., Gränzer, S., Knigge, T. and Kunz, N. (2001). "The hepatic stress protein (hsp70) response to interacting abiotic parameters in fish exposed to various levels of pollution." *Journal of Aquatic Ecosystem Stress and Recovery* **8**(3-4): 261-279.

Küster, A., Alder, A. C., Escher, B. I., Duis, K., Fenner, K., Garric, J., Hutchinson, T. H., Lapen, D. R., Péry, A., Römbke, J., Snape, J., Ternes, T., Topp, E., Wehrhan, A. and Knacker, T. (2010). "Environmental risk assessment of human pharmaceuticals in the European Union: A case study with the β-blocker atenolol." *Integrated Environmental Assessment and Management* **6**(S1): 514-523.

Larsson, D. G. J. (2014). "Pollution from drug manufacturing: review and perspectives." *Philosophical Transactions of the Royal Society B: Biological Sciences* **369**(1656).

Lauruschkus, F., Wiedmer, S., Buhnar, K. and Aettner, J. (2017). "Arzneimittelverbrauch im Spannungsfeld des demografischen Wandels". Civity Management Consultants GmbH & Co. KG.

Lee, J., Ji, K., Kho, Y. L., Kim, P. and Choi, K. (2011). "Chronic exposure to diclofenac on two freshwater cladocerans and Japanese medaka." *Ecotoxicology and Environmental Safety* **74**(5): 1216-1225.

Letzel, M., Metzner, G. and Letzel, T. (2009). "Exposure assessment of the pharmaceutical diclofenac based on long-term measurements of the aquatic input." *Environment International* **35**(2): 363-368.

Lewis, S., Handy, R. D., Cordi, B., Billinghurst, Z. and Depledge, M. H. (1999). "Stress proteins (HSP's): methods of detection and their use as an environmental biomarker." *Ecotoxicology* **8**(5): 351-368.

LfW (2004). "Ökotoxikologische Auswirkungen von Arzneimitteln. Langzeitwirkungen bei Fischen.". *Abschlussbericht des Bayerischen Landesamtes für Wasserwirtschaft zum Forschungs- und Entwicklungsvorhaben 2001–2003.*

Lonappan, L., Brar, S. K., Das, R. K., Verma, M. and Surampalli, R. Y. (2016). "Diclofenac and its transformation products: environmental occurrence and toxicity - a review." *Environment International* **96**: 127-138.

Loos, R., Gawlik, B. M., Locoro, G., Rimaviciute, E., Contini, S. and Bidoglio, G. (2009). "EU-wide survey of polar organic persistent pollutants in European river waters." *Environmental Pollution* **157**(2): 561-568.

Loos, R., Marinov, D., Sanseverino, I., Napierska, D. and Lettieri, T. (2017). "Draft: Review of the 1st Watch List under the Water Framework Directive and recommendations for the 2nd Watch List". European Commission. *JRC Technical reports*.

López-Sendó, J., Swedberg, K., McMurray, J., Tamargo, J., Maggioni, A. P., Dargie, H., Tendera, M., Waagstein, F., Kjekshus, J., Lechat, P., Pedersen, C. T., Priori, S. G., Alonso García, M. A., Blanc, J.-J., Budaj, A., Cowie, M., Dean, V., Deckers, J., Fernandez Burgos, E., Lekakis, J., Lindahl, B., Mazzotta, G., McGregor, K., Morais, J., Oto, A., Smiseth, O. A., Alonso García, M. A., Ardissino, D., Avendano, C., Lundqvist, C. B., ment, D. C., Drexler, H., Ferrari, R., Fox, K. A., Julian, D., Kearney, P., Klein, W., ber, L. K., Mancia, G., Nieminen, M., Ruzyllo, W., Simoons, M., Thygesen, K., Tognoni, G., Tritto, I. and Wallentin, L. (2004). "Expert consensus document on β-adrenergic receptor blockers The Task Force on Beta-Blockers of the European Society of Cardiology." *European Heart Journal* **25**(15): 1341-1362.

Luddeke, F., Hess, S., Gallert, C., Winter, J., Gude, H. and Loffler, H. (2015). "Removal of total and antibiotic resistant bacteria in advanced wastewater treatment by ozonation in combination with different filtering techniques." *Water Research* **69**: 243-251.

Luo, Y. L., Guo, W. S., Ngo, H. H., Nghiem, L. D., Hai, F. I., Zhang, J., Liang, S. and Wang, X. C. C. (2014). "A review on the occurrence of micropollutants in the aquatic environment and their fate and removal during wastewater treatment." *Science of the Total Environment* **473**: 619-641.

Lushchak, V. I. (2011). "Environmentally induced oxidative stress in aquatic animals." *Aquatic Toxicology* **101**(1): 13-30.

MacNeil, C., Dick, J. T. and Elwood, R. W. (1997). "The trophic ecology of freshwater *Gammarus* spp. (Crustacea: Amphipoda): Problems and perspectives concerning the functional feeding group concept." *Biological Reviews of the Cambridge Philosophical Society* **72**(3): 349-364.

MacNeil, C., Dick, J. T. A. and Elwood, R. W. (1999). "The dynamics of predation on *Gammarus* spp. (Crustacea: Amphipoda)." *Biological Reviews* **74**(4): 375-395.

Maier, D., Blaha, L., Giesy, J. P., Henneberg, A., Kohler, H. R., Kuch, B., Osterauer, R., Peschke, K., Richter, D., Scheurer, M. and Triebskorn, R. (2015). "Biological plausibility as a tool to associate analytical data for micropollutants and effect potentials in wastewater, surface water, and sediments with effects in fishes." *Water Research* **72**: 127-144.

Martinez-Rodriguez, H., Donkor, K., Brewer, S., Galar-Martinez, M., SanJuan-Reyes, N., Islas-Flores, H., Sanchez-Aceves, L., Elizalde-Velazquez, A. and Gomez-Olivan, L. M. (2018). "Metoprolol induces oxidative damage in common carp (*Cyprinus carpio*)." *Aquatic Toxicology* **197**: 122-135.

Massarsky, A., Trudeau, V. L. and Moon, T. W. (2011). "β-blockers as endocrine disruptors: the potential effects of human β-blockers on aquatic organisms." *Journal of Experimental Zoology Part A: Ecological Genetics and Physiology* **315A**(5): 251-265.

Maszkowska, J., Stolte, S., Kumirska, J., Lukaszewicz, P., Mioduszewska, K., Puckowski, A., Caban, M., Wagil, M., Stepnowski, P. and Bialk-Bielinska, A. (2014a). "Beta-blockers in the environment: Part II. Ecotoxicity study." *Science of the Total Environment* **493**: 1122-1126.

Maszkowska, J., Stolte, S., Kumirska, J., Łukaszewicz, P., Mioduszewska, K., Puckowski, A., Caban, M., Wagil, M., Stepnowski, P. and Białk-Bielińska, A. (2014b). "Beta-blockers in the environment: Part I. Mobility and hydrolysis study." *Science of The Total Environment* **493**: 1112-1121.

Maurer, M., Escher, B. I., Richle, P., Schaffner, C. and Alder, A. C. (2007). "Elimination of β-blockers in sewage treatment plants." *Water Research* **41**(7): 1614-1622.

McRae, N. K., Glover, C. N., Burket, S. R., Brooks, B. W. and Gaw, S. (2018). "Acute exposure to an environmentally relevant concentration of diclofenac elicits oxidative stress in the culturally important galaxiid fish *Galaxias maculatus*." *Environmental Toxicology and Chemistry* **37**(1): 224-235.

Mehinto, A. C., Hill, E. M. and Tyler, C. R. (2010). "Uptake and biological effects of environmentally relevant concentrations of the nonsteroidal anti-inflammatory pharmaceutical diclofenac in rainbow trout (*Oncorhynchus mykiss*)." *Environmental Science & Technology* **44**(6): 2176-2182.

Memmert, U., Peither, A., Burri, R., Weber, K., Schmidt, T., Sumpter, J. P. and Hartmann, A. (2013). "Diclofenac: new data on chronic toxicity and bioconcentration in fish." *Environmental Toxicology and Chemistry* **32**(2): 442-452.

Meyer, W., Reich, M., Beier, S., Behrendt, J., Gulyas, H. and Otterpohl, R. (2016). "Measured and predicted environmental concentrations of carbamazepine, diclofenac, and metoprolol in small and medium rivers in northern Germany." *Environmental Monitoring and Assessment* **188**(8): 16.

Moermond, C. T. A., Kase, R., Korkaric, M. and Ågerstrand, M. (2015). "CRED: Criteria for reporting and evaluating ecotoxicity data." *Environmental Toxicology and Chemistry*: n/a-n/a.

Moermond, C. T. A. and Smit, C. E. (2016). "Derivation of water quality standars for carbamazepine, metoprolol, and metformin and comparison with monitoring data." *Environmental Toxicology and Chemistry* **35**(4): 882-888.

Monserrat, J. M., Geracitano, L. A., Pinho, G. L. L., Vinagre, T. M., Faleiros, M., Alciati, J. C. and Bianchini, A. (2003). "Determination of lipid peroxides in invertebrates tissues using the Fe(III) xylenol orange complex formation." *Archives of Environmental Contamination and Toxicology* **45**(2): 177-183.

Monteiro, S. C. and Boxall, A. B. A. (2010). "Occurrence and fate of human pharmaceuticals in the environment." *Reviews of Environmental Contamination and Toxicology* **202**: 53-154.

Morimoto, R. I. (1993). "Cells in stress: Transcriptional activation of heat shock genes." *Science* **259**(5100): 1409-1410.

Nalecz-Jawecki, G. and Persoone, G. (2006). "Toxicity of selected pharmaceuticals to the anostracan crustacean *Thamnocephalus platyurus* - Comparison of sublethal and lethal effect levels with the 1h Rapidtoxkit and the 24h Thamnotoxkit microbiotests." *Environmental Science and Pollution Research* **13**(1): 22-27.

Näslund, J., Fick, J., Asker, N., Ekman, E., Larsson, D. G. J. and Norrgren, L. (2017). "Diclofenac affects kidney histology in the three-spined stickleback (*Gasterosteus aculeatus*) at low µg/L concentrations." *Aquatic Toxicology* **189**: 87-96.

Nassef, M., Matsumoto, S., Seki, M., Khalil, F., Kang, I. J., Shimasaki, Y., Oshima, Y. and Honjo, T. (2010). "Acute effects of triclosan, diclofenac and carbamazepine on feeding performance of Japanese medaka fish (*Oryzias latipes*)." *Chemosphere* **80**(9): 1095-1100.

Nieto, E., Corada-Fernández, C., Hampel, M., Lara-Martín, P. A., Sánchez-Argüello, P. and Blasco, J. (2017). "Effects of exposure to pharmaceuticals (diclofenac and carbamazepine) spiked sediments in the midge, *Chironomus riparius* (Diptera, Chironomidae)." *Science of The Total Environment* **609**: 715-723.

Noble, C., Mizusawa, K., Suzuki, K. and Tabata, M. (2007). "The effect of differing self-feeding regimes on the growth, behaviour and fin damage of rainbow trout held in groups." *Aquaculture* **264**(1-4): 214-222.

Novoa-Luna, K. A., Romero-Romero, R., Natividad-Rangel, R., Galar-Martinez, M., SanJuan-Reyes, N., Garcia-Medina, S., Martinez-Vieyra, C., Neri-Cruz, N. and Gomez-Olivan, L. M. (2016). "Oxidative stress induced in *Hyalella azteca* by an effluent from a NSAID-manufacturing plant in Mexico." *Ecotoxicology* **25**(7): 1288-1304.

Nunes, B., Campos, J. C., Gomes, R., Braga, M. R., Ramos, A. S., Antunes, S. C. and Correia, A. T. (2015). "Ecotoxicological effects of salicylic acid in the freshwater fish *Salmo trutta fario*: antioxidant mechanisms and histological alterations." *Environmental Science and Pollution Research* **22**(1): 667-678.

Oaks, J. L., Gilbert, M., Virani, M. Z., Watson, R. T., Meteyer, C. U., Rideout, B. A., Shivaprasad, H. L., Ahmed, S., Chaudhry, M. J. I., Arshad, M., Mahmood, S., Ali, A. and Khan, A. A. (2004). "Diclofenac residues as the cause of vulture population decline in Pakistan." *Nature* **427**(6975): 630-633.

OECD (1998). "Test No. 212: Fish, Short-term toxicity test on embryo and sac-fry stages", *OECD Publishing*.

OECD (2007). "Test No. 225: Sediment-Water *Lumbriculus* Toxicity Test Using Spiked Sediment", *OECD Publishing*.

OECD (2012). "Test No. 211: *Daphnia magna* Reproduction Test", *OECD Publishing*.

OECD (2016). "Test No. 242: *Potamopyrgus antipodarum* Reproduction Test", *OECD Publishing*.

Oldach, L. and Zhang, J. (2014). "Genetically encoded fluorescent biosensors for live-cell visualization of protein phosphorylation." *Chemistry & Biology* **21**(2): 186-197.

Owen, S. F., Giltrow, E., Huggett, D. B., Hutchinson, T. H., Saye, J., Winter, M. J. and Sumpter, J. P. (2007). "Comparative physiology, pharmacology and toxicology of β-blockers: Mammals versus fish." *Aquatic Toxicology* **82**(3): 145-162.

Prakash, V., Pain, D. J., Cunningham, A. A., Donald, P. F., Prakash, N., Verma, A., Gargi, R., Sivakumar, S. and Rahmani, A. R. (2003). "Catastrophic collapse of Indian white-backed *Gyps bengalensis* and long-billed *Gyps indicus* vulture populations." *Biological Conservation* **109**(3): 381-390.

Praskova, E., Plhalova, L., Chromcova, L., Stepanova, S., Bedanova, I., Blahova, J., Hostovsky, M., Skoric, M., Marsalek, P., Voslarova, E. and Svobodova, Z. (2014). "Effects of subchronic exposure of diclofenac on growth, histopathological changes, and oxidative stress in zebrafish (*Danio rerio*)." *The Scientific World Journal* **2014**: 645737.

Rand-Weaver, M., Margiotta-Casaluci, L., Patel, A., Panter, G. H., Owen, S. F. and Sumpter, J. P. (2013). "The read-across hypothesis and environmental risk assessment of pharmaceuticals." *Environmental Science & Technology* **47**(20): 11384-11395.

Ratcliffe, D. A. (1970). "Changes attributable to pesticides in egg breakage frequency and eggshell thickness in some british birds." *Journal of Applied Ecology* **7**(1): 67-+.

Ribeiro, S., Torres, T., Martins, R. and Santos, M. M. (2015). "Toxicity screening of diclofenac, propranolol, sertraline and simvastatin using *Danio rerio* and *Paracentrotus lividus* embryo bioassays." *Ecotoxicology and Environmental Safety* **114**: 67-74.

Rubirola, A., Llorca, M., Rodriguez-Mozaz, S., Casas, N., Rodriguez-Roda, I., Barcelo, D. and Buttiglieri, G. (2014). "Characterization of metoprolol biodegradation and its transformation products generated in activated sludge batch experiments and in full scale WWTPs." *Water Research* **63**: 21-32.

Sánchez, S., Martín, M. J., Ortiz, P., Motilva, V. and Lastra, C. A. D. L. (2002). "Effects of dipyrone on inflammatory infiltration and oxidative metabolism in gastric mucosa: Comparison with acetaminophen and diclofenac." *Digestive Diseases and Sciences* **47**(6): 1389-1398.

Sanders, B. M. (1993). "Stress proteins in aquatic organisms: An environmental perspective." *Critical Reviews in Toxicology* **23**(1): 49-75.

Santos, L., Araujo, A. N., Fachini, A., Pena, A., Delerue-Matos, C. and Montenegro, M. (2010). "Ecotoxicological aspects related to the presence of pharmaceuticals in the aquatic environment." *Journal of Hazardous Materials* **175**(1-3): 45-95.

Saucedo-Vence, K., Dublán-García, O., López-Martínez, L. X., Morachis-Valdes, G., Galar-Martínez, M., Islas-Flores, H. and Gómez-Oliván, L. M. (2014). "Short and long-term exposure to diclofenac alter oxidative stress status in common carp *Cyprinus carpio*." *Ecotoxicology* **24**(3): 527-539.

Scheurer, M., Ramil, M., Metcalfe, C. D., Groh, S. and Ternes, T. A. (2010). "The challenge of analyzing beta-blocker drugs in sludge and wastewater." *Analytical and Bioanalytical Chemistry* **396**(2): 845-856.

Schirling, M., Jungmann, D., Ladewig, V., Nagel, R., Triebskorn, R. and Kohler, H. R. (2005). "Endocrine effects in *Gammarus fossarum* (Amphipoda): Influence of wastewater effluents, temporal variability, and spatial aspects on natural populations." *Archives of Environmental Contamination and Toxicology* **49**(1): 53-61.

Schmidt, W., O'Rourke, K., Hernan, R. and Quinn, B. (2011). "Effects of the pharmaceuticals gemfibrozil and diclofenac on the marine mussel (*Mytilus spp*.) and their comparison with standardized toxicity tests." *Marine Pollution Bulletin* **62**(7): 1389-1395.

Schneider, I., Oehlmann, J. and Oetken, M. (2015). "Impact of an estrogenic sewage treatment plant effluent on life-history traits of the freshwater amphipod *Gammarus pulex*." *Journal of Environmental Science and Health Part a-Toxic/Hazardous Substances & Environmental Engineering* **50**(3): 272-281.

Schreiber, R., Gundel, U., Franz, S., Küster, A., Rechenberg, B. and Altenburger, R. (2011). "Using the fish plasma model for comparative hazard identification for pharmaceuticals in the environment by extrapolation from human therapeutic data." *Regulatory Toxicology and Pharmacology* **61**(3): 261-275.

Schröder, P., Helmreich, B., Skrbic, B., Carballa, M., Papa, M., Pastore, C., Emre, Z., Oehmen, A., Langenhoff, A., Molinos, M., Dvarioniene, J., Huber, C., Tsagarakis, K., Martinez-Lopez, E., Pagano, S., Vogelsang, C. and Mascolo, G. (2016). "Status of hormones and painkillers in wastewater effluents across several European states-considerations for the EU watch list concerning estradiols and diclofenac." *Environmental Science and Pollution Research* **23**(13): 12835-12866.

Schwaiger, J. (2001). "Histopathological alterations and parasite infection in fish: indicators of multiple stress factors." *Journal of Aquatic Ecosystem Stress and Recovery* **8**(3): 231-240.

Schwaiger, J., Ferling, H., Mallow, U., Wintermayr, H. and Negele, R. D. (2004). "Toxic effects of the non-steroidal anti-inflammatory drug diclofenac Part 1: histopathological alterations and bioaccumulation in rainbow trout." *Aquatic Toxicology* **68**(2): 141-150.

Schwaiger, J., Wanke, R., Adam, S., Pawert, M., Hönnen, W. and Triebskorn, R. (1997). "The use of histopathological indicators to evaluate contaminant-related stress in fish." *Journal of Aquatic Ecosystem Stress and Recovery* **6**(1): 75-86.

Schwarzenbach, R. P., Escher, B. I., Fenner, K., Hofstetter, T. B., Johnson, C. A., von Gunten, U. and Wehrli, B. (2006). "The challenge of micropollutants in aquatic systems." *Science* **313**(5790): 1072-1077.

Sies, H. (1993). "Strategies of antioxidant defense." *European Journal of Biochemistry* **215**(2): 213-219.

Sies, H. (1997). "Oxidative stress: oxidants and antioxidants." *Experimental Physiology* **82**(2): 291-295.

Simmons, D. L., Botting, R. M. and Hla, T. (2004). "Cyclooxygenase isozymes: the biology of prostaglandin synthesis and inhibition." *Pharmacological Reviews* **56**(3): 387-437.

Smith, V. H. (2003). "Eutrophication of freshwater and coastal marine ecosystems - A global problem." *Environmental Science and Pollution Research* **10**(2): 126-139.

Sørensen, J. G., Kristensen, T. N. and Loeschcke, V. (2003). "The evolutionary and ecological role of heat shock proteins." *Ecology Letters* **6**(11): 1025-1037.

Steinbach, C., Burkina, V., Fedorova, G., Grabicova, K., Stara, A., Velisek, J., Zlabek, V., Schmidt-Posthaus, H., Grabic, R. and Kocour Kroupova, H. (2014a). "The sub-lethal effects and tissue concentration of the human pharmaceutical atenolol in rainbow trout (*Oncorhynchus mykiss*)." *Science of the Total Environment* **497-498**: 209-218.

Steinbach, C., Burkina, V., Fedorova, G., Grabicova, K., Stara, A., Velisek, J., Zlabek, V., Schmidt-Posthaus, H., Grabic, R. and Kroupova, H. K. (2014b). "The sub-lethal effects and tissue concentration of the human pharmaceutical atenolol in rainbow trout (*Oncorhynchus mykiss*)." *Science of the Total Environment* **497**: 209-218.

Stepanova, S., Praskova, E., Chromcova, L., Plhalova, L., Prokes, M., Blahova, J. and Svobodova, Z. (2013). "The effects of diclofenac on early life stages of common carp (*Cyprinus carpio*)." *Environmental Toxicology and Pharmacology* **35**(3): 454-460.

Stoate, C., Báldi, A., Beja, P., Boatman, N. D., Herzon, I., van Doorn, A., de Snoo, G. R., Rakosy, L. and Ramwell, C. (2009). "Ecological impacts of early 21st century agricultural change in Europe - A review." *Journal of Environmental Management* **91**(1): 22-46.

Stoate, C., Boatman, N. D., Borralho, R. J., Carvalho, C. R., De Snoo, G. R. and Eden, P. (2001). "Ecological impacts of arable intensification in Europe." *Journal of Environmental Management* **63**(4): 337-365.

Stoschitzky, K. (2001). "Betablocker-aktuelle Indikationen." *Journal für Kardiologie-Austrian Journal of Cardiology* **8**(B): 3-6.

Stumpf, M., Ternes, T. A., Haberer, K., Seel, P. and Baumann, W. (1996). "Nachweis von Arzneimittelrückständen in Kläranlagen und Fließgewässern." *Vom Wasser* **86**: 291-303.

Sun, L. W., Xin, L. H., Peng, Z. H., Jin, R., Jin, Y. X., Qian, H. F. and Fu, Z. W. (2014). "Toxicity and enantiospecific differences of two beta-blockers, propranolol and metoprolol, in the embryos and larvae of zebrafish (*Danio rerio*)." *Environmental Toxicology* **29**(12): 1367-1378.

Takashima, F. and Hibiya, T. (1995). "An atlas of fish histology: normal and pathological features". Tokyo, *Kodansha ltd. - Gustav Fischer Verlag*.

Ternes, T. A. (1998). "Occurrence of drugs in German sewage treatment plants and rivers." *Water Research* **32**(11): 3245-3260.

Tixier, C., Singer, H. P., Oellers, S. and Muller, S. R. (2003). "Occurrence and fate of carbamazepine, clofibric acid, diclofenac, ibuprofen, ketoprofen, and naproxen in surface waters." *Environmental Science & Technology* **37**(6): 1061-1068.

Triebskorn, R. (2017). "Weitergehende Abwasserreinigung: ein wirksames und bezahlbares Instrument zur Verminderung von Spurenstoffen und Keimen im Wasserkreislauf: gemeinsamer Schlussbericht der vom Bundesministerium für Bildung und Forschung sowie vom Ministerium für Umwelt, Klima und Energiewirtschaft Baden-Württemberg geförderten Projekte SchussenAktiv, SchussenAktiv*plus* und SchussenAktiv*plus+*", *Universitätsbibliothek Tübingen*.

Triebskorn, R., Adam, S., Casper, H., Honnen, W., Pawert, M., Schramm, M., Schwaiger, J. and Köhler, H.-R. (2002). "Biomarkers as diagnostic tools for evaluating effects of unknown past water quality conditions on stream organisms." *Ecotoxicology* **11**(6): 451-465.

Triebskorn, R., Berg, K., Ebert, I., Frey, M., Jungmann, D., Oehlmann, J., Oetken, M., Sacher, F., Scheurer, M., Schmieg, H., Schwarz, S. and Köhler, H. R. (2015). "Monitoring primary effects of pharmaceuticals in the aquatic environment with mode of action-specific *in vitro* biotests." *Environmental Science & Technology* **49**(5): 2594-2595.

Triebskorn, R., Böhmer, J., Braunbeck, T., Honnen, W., Köhler, H.-R., Lehmann, R., Oberemm, A., Schwaiger, J., Segner, H., Schüürmann, G. and Traunspurger, W. (2001). "The project VALIMAR (VALIdation of bioMARkers for the assessment of small stream pollution): objectives, experimental design, summary of results, and recommendations for the application of biomarkers in risk assessment." *Journal of Aquatic Ecosystem Stress and Recovery* **8**(3): 161-178.

Triebskorn, R., Casper, H., Heyd, A., Eikemper, R., Köhler, H. R. and Schwaiger, J. (2004). "Toxic effects of the non-steroidal anti-inflammatory drug diclofenac Part II. Cytological effects in liver, kidney, gills and intestine of rainbow trout (*Oncorhynchus mykiss*)." *Aquatic Toxicology* **68**(2): 151-166.

Triebskorn, R., Casper, H., Scheil, V. and Schwaiger, J. (2007). "Ultrastructural effects of pharmaceuticals (carbamazepine, clofibric acid, metoprolol, diclofenac) in rainbow trout (*Oncorhynchus mykiss*) and common carp (*Cyprinus carpio*)." *Analytical and Bioanalytical Chemistry* **387**(4): 1405-1416.

Triebskorn, R., Schwarz, S., Köhler, H.-R., Berg, K., Jungmann, D., Frey, M., Oehlmann, J. and Oetken, M. (2014). "From theory to reality – Evaluation of suitable organisms and test systems for the biomonitoring of pharmaceuticals - Part I: Literature review". *UBA Texte*. **64/2014**.

Triebskorn, R., Schwarz, S., Schmieg, H., Köhler, H.-R., Jungmann, D., Berg, K., Buchberger, A.-K., Frey, M., Scheurer, M., Sacher, F., Oetken, M. and Oehlmann, J. (2017). "EFF-Pharm: Effects of pharmaceuticals (nonsteroidal anti-inflammatory drugs and beta-blockers) in fish and invertebrates and their detection by newly developed *in vitro*-bioassays". FKZ 3713 63 4102/3715 63 4120. Umweltbundesamt. *UBA Texte*. **44/2017**.

Triebskorn, R., Telcean, I., Casper, H., Farkas, A., Sandu, C., Stan, G., Colărescu, O., Dori, T. and Köhler, H.-R. (2008). "Monitoring pollution in River Mureş, Romania, part II: Metal accumulation and histopathology in fish." *Environmental Monitoring and Assessment* **141**(1): 177-188.

Valavanidis, A., Vlahogianni, T., Dassenakis, M. and Scoullos, M. (2006). "Molecular biomarkers of oxidative stress in aquatic organisms in relation to toxic environmental pollutants." *Ecotoxicology and Environmental Safety* **64**(2): 178-189.

van den Brandhof, E.-J. and Montforts, M. (2010). "Fish embryo toxicity of carbamazepine, diclofenac and metoprolol." *Ecotoxicology and Environmental Safety* **73**(8): 1862-1866.

van Leeuwen, J. S., Vredenburg, G., Dragovic, S., Tjong, T. F. J., Vos, J. C. and Vermeulen, N. P. E. (2011). "Metabolism related toxicity of diclofenac in yeast as model system." *Toxicology Letters* **200**(3): 162-168.

Vane, J. R. and Botting, R. M. (1998). "Anti-inflammatory drugs and their mechanism of action." *Inflammation Research* **47**(2): 78-87.

Verbruggen, B., Gunnarsson, L., Kristiansson, E., Österlund, T., Owen, S. F., Snape, J. R. and Tyler, C. R. (2018). "ECOdrug: a database connecting drugs and conservation of their targets across species." *Nucleic Acids Research* **46**(D1): D930-D936.

Wang, J., He, B., Yan, D. and Hu, X. (2017). "Implementing ecopharmacovigilance (EPV) from a pharmacy perspective: A focus on non-steroidal anti-inflammatory drugs." *Science of The Total Environment* **603-604**(Supplement C): 772-784.

Wenger, M., Ondračková, M., Machala, M., Neča, J., Hyršl, P., Šimková, A., Jurajda, P., Von Der Ohe, P. and Segner, H. (2010). "Assessing relationships between chemical exposure, parasite infection, fish health, and fish ecological status: A case study using chub (*Leuciscus cephalus*) in the Bílina River, Czech Republic." *Environmental Toxicology and Chemistry* **29**(2): 453-466.

Wigh, A., Geffard, O., Abbaci, K., Francois, A., Noury, P., Berge, A., Vulliet, E., Domenjoud, B., Gonzalez-Ospina, A., Bony, S. and Devaux, A. (2017). "*Gammarus fossarum* as a sensitive tool to reveal residual toxicity of treated wastewater effluents." *Science of the Total Environment* **584**: 1012-1021.

Wilhelm, S., Henneberg, A., Kohler, H. R., Rault, M., Richter, D., Scheurer, M., Suchail, S. and Triebskorn, R. (2017). "Does wastewater treatment plant upgrading with activated carbon result in an improvement of fish health?" *Aquatic Toxicology* **192**: 184-197.

Wolf, J. C., Baumgartner, W. A., Blazer, V. S., Camus, A. C., Engelhardt, J. A., Fournie, J. W., Frasca, S., Jr., Groman, D. B., Kent, M. L., Khoo, L. H., Law, J. M., Lombardini, E. D., Ruehl-Fehlert, C., Segner, H. E., Smith, S. A., Spitsbergen, J. M., Weber, K. and Wolfe, M. J. (2015). "Nonlesions, misdiagnoses, missed diagnoses, and other interpretive challenges in fish histopathology studies: A guide for investigators, authors, reviewers, and readers." *Toxicologic Pathology* **43**(3): 297-325.

Wolf, J. C., Ruehl-Fehlert, C., Segner, H. E., Weber, K. and Hardisty, J. F. (2014). "Pathology working group review of histopathologic specimens from three laboratory studies of diclofenac in trout." *Aquatic Toxicology* **146**: 127-136.

Wright-Walters, M., Volz, C., Talbott, E. and Davis, D. (2011). "An updated weight of evidence approach to the aquatic hazard assessment of Bisphenol A and the derivation a new predicted no effect concentration (Pnec) using a non-parametric methodology." *Science of The Total Environment* **409**(4): 676-685.

Yokota, H., Eguchi, S., Hasegawa, S., Okada, K., Yamamoto, F., Sunagawa, A., Tanaka, M., Yamamoto, R. and Nakano, E. (2016). "Assessment of in vitro antiovulatory activities of nonsteroidal anti-inflammatory drugs and comparison with *in vivo* reproductive toxicities of medaka (*Oryzias latipes*)." *Environmental Toxicology* **31**(12): 1710-1719.

Zimmerli, S., Bernet, D., Burkhardt-Holm, P., Schmidt-Posthaus, H., Vonlanthen, P., Wahli, T. and Segner, H. (2007). "Assessment of fish health status in four Swiss rivers showing a decline of brown trout catches." *Aquatic Sciences* **69**(1): 11-25.

Publication IV: EFF-Pharm: Effects of pharmaceuticals (nonsteroidal anti-inflammatory drugs and beta-blockers) in fish and invertebrates and their detection by newly developed *in vitro*-bioassays

From theory to reality – Evaluation of suitable organisms and test systems for the biomonitoring of pharmaceuticals.

Part 2: Case studies

Part 3: Validation of methods

Part 4: Tests with Daphnia magna

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Study report on behalf of the Federal Environment Agency, published in UBA Texte 44/2017

Environmental Research of the Federal Ministry for the Environment, Nature Conservation, Building and Nuclear Safety

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Here, only the abstract and summary are shown. The study report is too long to be included in this section and is available on the attached CD.

Abstract

As tools for environmental monitoring of pharmaceuticals, mode of action (MOA)-based *in vitro*-assays were developed for beta-blockers, as e.g. metoprolol, and non-steroidal anti-inflammatory drugs (NSAIDs), as e.g. diclofenac. For this purpose stable cell lines were generated which expressed recombinant MOA-based sensing and reporting units allowing for rapid live-cell visualization of immediate fluorescence signal changes. Sensitive cell based assays developed in microtiter plate format facilitated the quantitative determination of metoprolol and diclofenac activities in effluents of wastewater treatment plants.

In order to validate these tests for their suitability to reflect *in vivo*-effects in environmentally relevant aquatic organisms, toxicity tests and biomarker studies were conducted with fish (brown trout), crustaceans (gammarids, daphnia), snails and sediment-dwelling invertebrates (annelids). Test were conducted (1) in the laboratory under controlled experimental conditions with isolated substances and binary mixtures, (2) in aquatic mesocosms and (3) under field conditions in a bypass-system connected to the effluent of a municipal wastewater treatment plant. In addition to population relevant endpoints as e.g. reproduction, development or fertility, also individual health parameters were investigated by means of stress protein analyses, histological investigations and studies revealing the oxidative stress status of the exposed organisms.

Summary

A literature review conducted in phase I of the project "From theory to reality – Evaluation of suitable organisms and test systems for the biomonitoring of pharmaceuticals" (Triebskorn et al., 2013) made evident that mode of action (MOA)-based *in vitro*-biotests are necessary for an effect-directed biomonitoring of pharmaceuticals in the context of environmental risk assessment and eco-pharmacovigilance. It has also been emphasized that validation of such biotests with respect to *in vivo*-responses in sensitive and ecologically relevant organisms should be realized in parallel to their establishment.

In phase II of this project with the acronym "*Eff-Pharm*", MOA-based biotests were developed for Beta-blockers and nonsteroidal anti-inflammatory drugs (NSAIDs). In parallel, *in vivo*-experiments were conducted with fish, crustaceans, molluscs and annelids. These case studies focussed on effects of diclofenac as a representative pharmaceutical of NSAIDs and metoprolol representing beta-blockers aiming at determining threshold values for mode of action- and side effect-driven biomarkers as well as for population-relevant endpoints in these organisms. The approaches of *Eff-Pharm* are illustrated in Figure 1.

Figure 2 summarizes the project structure of *Eff-Pharm*: In two modules running in parallel*, in vivo*-testing and *in vitro*-test development and validation of methods were realized. By a stepwise approach from laboratory to field via semi-field mesocosms, results that are mandatory to validate *in vitro*-biotests have been generated for key biota of surface water and sediments, i.e. fish, gammarids, molluscs and sediment- dwelling annelids. Also the MOA-based biotests followed the steps from the laboratory to the field: it was the aim to provide test systems which are, on one hand, sensitive and specific for the respective groups of pharmaceuticals but also robust enough to serve as tools for wastewater and surface water biomonitoring in the field.

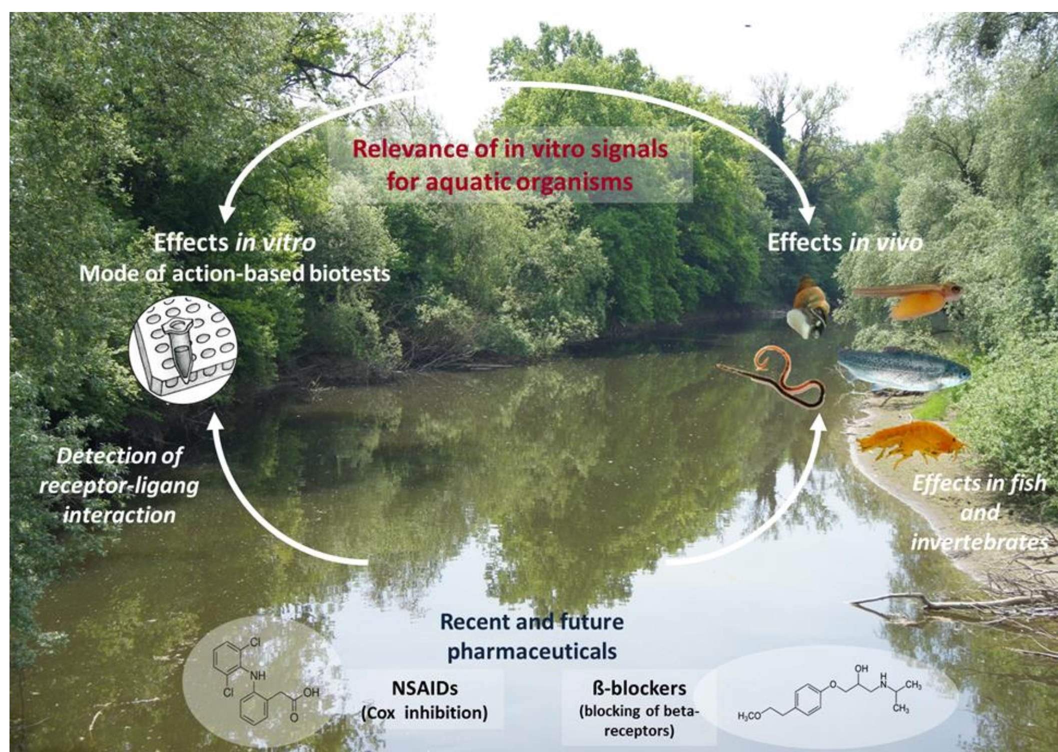

Figure 1: Summary of the approaches followed in the EFF-Pharm project

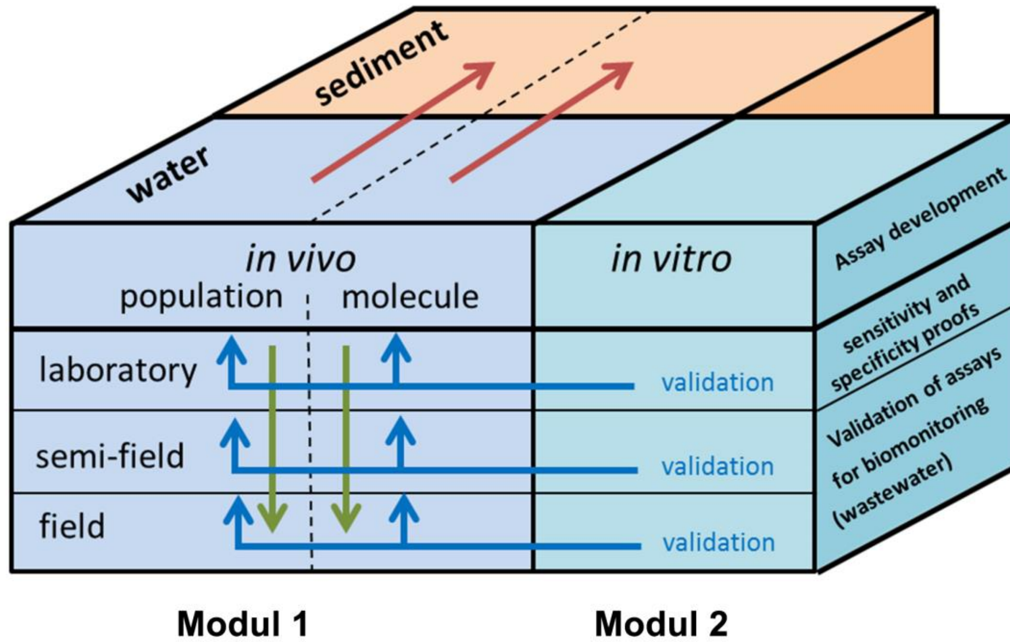

Figure 2: Structure of the project "From theory to reality – Evaluation of suitable organisms and test systems for the biomonitoring of pharmaceuticals – case studies" (Eff-Pharm)

The project consisted of 12 work packages among which 8 addressed scientific questions (Table 1). The work packages were funded in two steps as EFF-Pharm 2 and 3. Supplementary experiments with *Daphnia magna* were conducted by the German Environmental Agency (part 4).

Table 1: Work packages (WPs) of Eff-Pharm

WP	Content
1 (*Eff-Pharm* 2, 3)	Coordination, project management
2 (*Eff-Pharm* 2)	Kick-off-meeting
3A (*Eff-Pharm* 2)	Laboratory tests surface water: single substances *Test 1. Biomarker studies with juvenile trout* *Test 2. Embryo tests with trout* *Test 3: Tests with gammarids*
3B (*Eff-Pharm* 3)	Laboratory tests surface water: mixtures
4 (*Eff-Pharm* 3)	Semi-field mesocosm experiments surface water
5 (*Eff-Pharm* 2)	Field experiments (waste water effluents) 5A: Fish embryo tests 5B: gammarids
6 (*Eff-Pharm* 2b)	Sediment toxicity: single substances and mixtures
7A (*Eff-Pharm* 2)	Chemical analyses in obligatory tests
7B (*Eff-Pharm* 2b and 3)	Chemical analyses in optional tests
8 (*Eff-Pharm* 2)	Development of *in vitro*-test systems
9 (*Eff-Pharm* 2)	Verification of *in vitro*-test systems
10A (*Eff-Pharm* 2)	Validation of *in vitro*-test systems
10B (*Eff-Pharm* 3)	Further validation of *in vitro*-test systems
11 (*Eff-Pharm* 2, 3)	Interim reports, final report
12 (*Eff-Pharm* 2, 3)	Presentation of results, organization final symposium

The different tasks of the project were allocated as follows:

Project coordination, management and reports: Steinbeis Transfer Center for Ecotoxicology and Ecophysiology, Rottenburg and University of Tübingen, Animal Physiological Ecology

- Tests with fish: University of Tübingen, Animal Physiological Ecology
- Tests with gammarids: GWT-TUD GmbH, Dresden
- Tests with sediment-dwelling worms and snails: University of Frankfurt, Aquatic Ecotoxicology
- Tests with *Daphnia magna:* German Environmental Agency
- Chemical analyses: DVGW Water Technology Center, Karlsruhe
- Biosensor development: Steinbeis Innovation Center Cell Culture Technology, Mannheim
- The cooperation partners are responsible for their respective parts of the present report.

As mentioned above, *Eff-Pharm* focussed on effects of beta-blockers (represented by metoprolol) and nonsteroidal anti-inflammatory drugs (represented by diclofenac), both representing pharmaceutical classes which are frequently used in Germany, which occur in relatively high concentrations in aquatic ecosystems and which therefore were classified as to be environmentally relevant (Ebert et al., 2014). In surface waters, diclofenac and metoprolol occur in the ng/L - µg/L range, in sewage treatment plant effluents concentrations of both pharmaceuticals reach the µg/L range. In river sediments, concentrations of up to 52 µg/kg diclofenac and 33 µg/kg metoprolol have been reported (Ramil et al., 2010; Camacho-Muñoz et al., 2013).

Single substance in vivo-tests with these two pharmaceuticals revealed the following results:

In a range of different concentrations (0.1 – 1000 µg/L diclofenac or metoprolol) embryos of brown trout (*Salmo trutta* f. *fario*) did not show any alterations of development, survival, heart rate and body mass. In contrast, concentrations of 100 µg/L diclofenac or higher drastically increased the mortality of juvenile brown trout. Furthermore, these juveniles displayed an increased number of bite marks, indicative for elevated aggressiveness, following exposure to 10 µg/L diclofenac or higher. Metoprolol did not cause such effects. As well, the Hsp70 and lipid peroxidation levels in juvenile brown trout were

not affected by the two pharmaceuticals. Histopathology pointed in the direction of moderate stress experienced by the fish during lab exposure plus indicated proceeding degradation of renal tissue diclofenac concentrations of 10 µg/L and higher. In general, juvenile individuals of brown trout were shown to respond more sensitively to pharmaceuticals than the early embryonic stages inside the chorion.

Gammarus fossarum was exposed to a range of 0.49 – 40 mg/L diclofenac or 5 – 405 mg/L metoprolol and investigated for mortality, juvenile/adult ratio, the number of precopula stages and the number of eggs per female. The most sensitive endpoint for diclofenac effect was the juvenile/adult ratio with a NOEC of 0.79 mg/L and a LOEC of 2.62 mg/L. The most sensitive endpoint for metoprolol effect also was the juvenile/adult ratio and the number of eggs per female, each with a NOEC of 5 mg/L and a LOEC of 15 mg/L. As in trout, Hsp70 and lipid peroxidation levels in gammarids remain unaffected by both pharmaceuticals.

The snail *Potamopyrgus antipodarum* was exposed to a series of concentrations of metoprolol only. Significantly elevated Hsp70 levels were found in response to 3.2 mg/L metoprolol (NOEC: 1mg/L) or higher. After exposure to 10 mg/L metoprolol a 10% reduction in offspring production became obvious.

In *Daphnia magna*, mortality, number of offspring, time until reproduction and behaviour was monitored following exposure to either 1.9 – 50 mg/L diclofenac or 0.1 - 25 mg/L metoprolol. The most sensitive parameter in this species was reproduction with a LOEC of 6.25 mg/L for diclofenac and 2.5 mg/L for metoprolol.

Mixture toxicity experiments, conducted with fish, gammarids, and daphnids, failed to reveal synergistic (more-than-additive) effects for all combinations, test species, and investigated endpoints.

In sediment toxicity tests with the sediment-dwelling oligochaete worm *Lumbriculus variegatus , the* LOEC for reproduction was 100 mg/kg sediment dry wt (diclofenac), and 255 mg/kg sediment dry wt (metoprolol). A mixture of both substances did not exert synergistic effects. Consistent with the above-mentioned results, Hsp70 and lipid peroxidation levels were not altered by the pharmaceuticals.

Mesocosm experiments conducted in artificial indoor streams investigated the impact of 0,47; 1,9; 7,5; and 30 mg/L metoprolol (nominal) on *G. fossarum*, *P. antipodarum*, and *L. variegatus*, either exposed directly to the running water ("free living") or sheltered in enclosures. Results largely resembled the findings obtained in the above-mentioned single species toxicity experiments. For some parameters, however, the conditions in the indoor streams (and here, most likely, the water current) fortified the metoprolol effects. Thus, "free-living" gammarids in the indoor streams exhibited a reduction in precopula number with increasing metoprolol concentrations, and the EC_{10} for reproduction impairment (28d) was found to be lower (0,594 mg/L) than in the single species experiment. Also for the other species, highest metoprolol toxicity was found for "free living"individuals (*L. variegatus*: EC_{10} reproduction (40d): 0,569 mg/L; *P. antipodarum*: EC_{10} reproduction (40d): 0,253 mg/L).

In order to approach conditions of environmental exposure, brown trout embryos and gammarids were exposed *to effluent water* from a sewage treatment plant (Eriskirch, close to Lake Constance), containing mean concentrations of 1,3 µg/L diclofenac and 1,4 µg/L metoprolol. Embryos of brown trout exhibited reduced lipid peroxides and body mass reduction after exposure to the effluent, wheras in gammarids, the effluent caused a decreased number of eggs per female in parallel to a higher body weight.

The results of the *in vivo*-tests (LOECs and most sensitive parameters) are summarized in Table 2

Table 2: Most sensitive parameters and lowest LOECs (Real concentrations)

	Species	Diclofenac	Metoprolol
Single substances (laboratory studies)	Fish*: Salmo trutta f. fario*	Behaviour, histology: 10 µg/L	Histology kidney > 745 µg/L
	Crustacea: *Gammarus fossarum*	Ratio juveniles / adults: 2.6 mg/L	Ratio juveniles / adults and number eggs /egg-bearing female: 15 mg/L
	Crustacea: *Daphnia magna*	Reproduction: 6.25 mg/L	Reproduction: 2.5 mg/L
	Mollusc: *Potamopyrgus antipodarum*		Proteotoxicity 3.2 mg/L
	Annelids: *Lumbriculus variegatus*	Reproduction (28d): 100 mg/kg	Reproduction (28d): 255 mg/kg
Mixture toxicity (laboratory studies)	Fish: *Salmo trutta* f. *fario*	Histology: 68.6/676 µg/L (¾ TU DIC + ¾ TU MET)	
	Crustacea: *Gammarus fossarum*	No significant effecs at 1.97/11.25 mg/L (¾TU DIC + ¾ TU MET)	
	Crustacea: *Daphnia magna*	Reproduction: 4.69 / 1.88 mg/L (¾ TU DIC + ¾ TU MET)	
	Annelids: *Lumbriculus variegatus*	Reproduction: 203.6 mg/kg	
Mesocosm experiments	Crustacea: *Gammarus fossarum*		number eggs /egg-bearing female: 3 mg/L
	Snail: *Potamopyrgus antipodarum*		Reproduction enclosures (28d): 0.22 mg/L
	Annelids: *Lumbriculus variegatus*		Reproduction (free living animals): EC50 (40d): 3.38 mg/L
Field experiments	Fish: *Salmo trutta f. fario*	Lipid peroxides (reduction), body mass reduction	
	Crustacea: *Gammarus fossarum*	Number eggs / egg-bearing female	

For both classes of pharmaceuticals, *beta-blockers and NSAIDs, sensitive biosensors* were raised and established.

For the effect-based detection of beta-blocker effects, the beta-blocker CEPAC sensor assay showed the best test performance and was therefore used for further development of a mode of action based *in vitro*-assay. In contrast, several published roGFP redox sensors, which were tested in CHO cells after stable transfection, only showed a weak fluorescence signal and a poor signal-to-noise ratio. Although H_2O_2 induced oxidative stress could be measured by fluorescence microscope measurements it was not possible to transfer this assays into a microplate format due to low signal intensity. Therefore, the new roGFP mutant roGFP3 with a strongly improved fluorescence signal was created. Here the fusion of Grx-1 to roGFP3 resulted in the improved redox sensor Grx-roGFP3. This redox sensor was used for further development of NSAID biosensor cell lines. Based on this new redox sensor an assay was developed for redox based measurement of Cox-1 activity.

Subcloning of beta-blocker and NSAID biosensor mixed cell populations resulted in single cell clones with enhanced signal to noise ratio. Such clones were used for measuring IC_{50} concentrations of the beta-blocker metoprolol or the NSAID diclofenac. Best beta-blocker biosensor clones showed a half maximal signal reduction at a metoprolol concentration of around 15nM. Best NSAID biosensor clones showed a half maximal signal reduction at a diclofenac concentration of around 2nM. The fast signal output of both assay formats requires only short incubation times of sensor cells with analyte solutions. Consequently there is no need for working under sterile conditions during the measurements which are particularly advantageous over reporter gene assays.

Municipal wastewater samples, enriched by solid phase extraction, were diluted in assay buffer and tested in beta-blocker and NSAID assays. Beta-blocker and NSAID activities of SPE enriched wastewater samples could be measured and were comparable with concentrations of metoprolol and diclofenac concentrations determined by LC-MS.

Due to the high sensitivities of the *in vitro*-assays it is possible to directly measure NSAID and beta-blocker activities in samples that are not enriched by solid phase extraction. It turned out that beta-blocker activities of wastewater samples were significantly higher than metoprolol concentrations measured by LC-MS of corresponding SPE enriched samples, but the difference could be explained by LC-MS-based proofs of beta-blockers other than metoprolol in these wastewater samples. Such effect was not found for NSAID activities that could largely be related to diclofenac residues in these samples. During further experiments validations with internal standards for checking possible matrix effects will be performed. Different wastewater samples will be characterized and undiluted as well as SPE enriched samples will be compared.

The results of this project undoubtedly revealed the precision and the high sensitivities of both biosensors which (a) correspond to environmental concentrations of beta-blockers and NSAIDs and (b) cover the known LOEC and NOEC ranges even for the most sensitive species and endpoints. Such biosensors are thus highly promising in view to their integration in environmental monitoring.

Publication V: Health effects of metoprolol in epibenthic and endobenthic invertebrates—A basis to validate future *in vitro* biotests for effect-based biomonitoring

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Abstract

The aim of this study was to determine the effect data for metoprolol as a model substance for betablockers in aquatic invertebrates. The results will be used as a basis for the validation of future mode of action-based *in vitro* test systems targeting this class of pharmaceuticals. Effects of metoprolol were investigated in two autochthonous species with high relevance in stream ecology: the amphipod *Gammarus fossarum* and the oligochaete *Lumbriculus variegatus*. Mortality in *G. fossarum* was not observed in acute toxicity testing (48 h), and a significant increase of mortality at 45 mg/L was found when amphipods were exposed chronically (40 days). The most sensitive population-relevant endpoints were the juvenile-adult ratio and number of egg-bearing females with NOEC/LOEC-values of 5/15 mg/L. No proteotoxic effects were identified in *G. fossarum*. The sediment toxicity test with *L. variegatus* according to the OECD Guideline 225 with an exposure time of 28 days resulted in EC10-values of 92.5 and 126.1 mg/kgdw for the endpoints reproduction and biomass, respectively. In *L. variegatus* the response kinetics of Hsp70 showed no significant difference between the treatments. A tendency for rising lipid peroxide concentrations was found between 0.03 and 10 mg/kg dw, which were significant between the treatments, but not to the control.

Introduction

The increasing use of human and veterinary pharmaceuticals and their incomplete removal in wastewater treatment plants (WWTP) has led to increased concentrations of these compounds in the environment.[1] As a consequence, concerns about possible negative effects on organisms, particularly in aquatic ecosystems, have been expressed. Exposure monitoring in WWTP effluents as well as in receiving surface waters have detected an increasing number of pharmaceuticals over time and in all investigated aquatic habitats.[2–7]

The calculation of the trigger value for a possible environmental risk is the quotient of exposure (Predicted Environmental Concentration, PEC; Measured Environmental Concentration, MEC) and hazard (Predicted No Effect Concentration; PNEC). Therefore, with increasing environmental concentrations, the risk quotient (RQ) increases and risk becomes more probable. Up to now, data for the identification of risk are scarce for many pharmaceuticals.[8]

In order to address risks posed by pharmaceuticals it is important to keep in mind that pollution with human pharmaceuticals mainly originates from point sources (e.g. WWTP). On the contrary, veterinary pharmaceuticals often reach the aquatic environment from diffuse sources of input (e.g. by runoff from pasture and from industrialised stock farming).

Currently, chemical analysis is the method of choice to trace pharmaceuticals in the environment. The methodology is highly specific, and modern techniques are very sensitive down to the ng/L range. A considerable drawback of this approach, however, is the necessity to develop and establish a new analytical method for every novel, released pharmaceutical.

Bioassays could be a supplemental method to complement the chemical analysis for water quality assessment.[9] Their availability for many applications in medicine and ecotoxicology targeting health-relevant biological endpoints makes them appropriate tools for mode of action-based investigations. In this context, a bioassay targeting the mode of action of e.g. beta-blockers will detect not only a single compound but the entirety of all beta receptor-binding compounds in the sample. This is a considerable advantage compared to the chemical analysis that detects solely specific compounds under investigation. With the exception of synthetic steroid hormones the majority of such approaches to date have mainly focused on non-pharmaceutical compounds. However, environmental monitoring of the multitude of other pharmaceuticals still remains to be mainly based upon highly developed analytical techniques.[8] A detailed description of mode of action based bioassays, cell based approaches, and currently established measures for bioassays are to be found in Triebskorn et al.[8] and Escher et al.[10]

Before efforts are made that consider the development of new bioassays as a part of risk assessment, it is essential to characterise effect concentrations of pharmaceuticals for hazard identification. Despite this need, publicly available data from effect analyses are scarce for many pharmaceuticals. Recently, Triebskorn et al.[8] made a plea for developing a concept for such effect-oriented biomonitoring of pharmaceuticals by using a new mode of action-based *in vitro* test systems validated by *in vivo* effects in fish and invertebrates. Here we aim at investigating the effects of metoprolol, a pharmaceutical of high priority according to Triebskorn et al.,[11] in epi- and endobenthic invertebrates. Invertebrates have, compared e.g. to fish, a faster reproductive cycle and are therefore useful to investigate impacts of chemicals on reproduction. Consequently, two autochthonous organisms with high relevance in stream ecology were chosen.

In the following sections, results from toxicity tests with *Gammarus fossarum* as representative for epibenthic freshwater invertebrates and *Lumbriculus variegatus* as model organism for the endobenthic fauna are presented. We report observations on population-relevant endpoints in *G. fossarum* and in *L. variegatus*. In addition, results from biomarker tests (alterations of stress proteins and the formation of lipid peroxides) both serve as health markers and indicators for proteotoxic and oxidative stress, respectively.

Materials and methods

Metoprolol properties and chemical analysis

The pharmaceutical metoprolol is a selective blocker of the beta-1 adrenergic receptor and was purchased from Sigma Aldrich as tartrate (CAS 56392-17-7) with purity >98%. Log K_{ow} is 1.9 and water solubility is unknown, however higher than 1.0 g/mL.

An external calibration with directly injected standards was setup to determine linearity of detection. The injector needle and the injection port were washed with methanol after every injection to avoid potential carryover. Instrumental and sample preparation contaminations were controlled by measuring blanks at regular intervals of every ten injections. With retention time for metoprolol of 10.5 min the compound eluted several minutes after potential inorganic contaminants, which normally elute in the void volume of the analytical column. The limit of detection (LOD) was 1 mg/kg if 1 g of sediment was used for extraction and increased by a factor of ten if 0.1 g sediment was used. Peaks at the LOD level still showed a signal- to-noise ratio that is higher than ten.

At respective sampling times during exposure, 1.5 mL water samples were taken, transferred to glass vessels (1.5 mL short thread brown glass flask, Fisher Scientific) and immediately frozen and stored at -20°C. Collected samples were sent to TZW and analysed within 48 h after thawing. Metoprolol was analysed by high performance liquid chromatography (HPLC, Agilent 1290, Waldbronn, Germany)

coupled to a tandem mass spectrometer (Tandem-MS, API 5500, AB Sciex, Foster City, CA, USA) including an electrospray interface with positive ionization in the respective experiments to verify the real concentration. Samples were injected on an Ultra Aromax HPLC column (150 x 2.1 mm, 3 mm, Restek, Bad Homburg) operated at a flow rate of 220 mL/min. The used solvents were (1) ultrapure water provided by an Arium 611 laboratory water

purification system (Sartorius AG, Göttingen, Germany) and (2) HPLC-grade methanol/acetonitrile (33%/66%, Promochem LGC Standards, Wesel, Germany/VWR, Fontenay-sous-Bois, France) both with 5 mM ammonium formate (purity >99%, Sigma-Aldrich, Steinheim Germany). The gradient started with 20% B, increased within 5 min to 100% B and was held for 6 min before returning to the initial conditions within 1 min. Depending on the spiked concentration 0.1 g (for 20 and 320 mg/kg) or 1 g (for 0.03 mg/kg) of sediment was extracted twice with 5 mL methanol in an ultrasonic bath (Sonorex RK 510H, Bandelin electronic, Berlin, Germany). After the first extraction, the samples were centrifuged for 30 min at 3,000 rpm. The supernatant was removed and combined afterwards with the one obtained after the second extraction. An aliquot of 5 mL was diluted with 500 mL water and cleaned-up with solid phase extraction (SPE). For SPE 200 mg polymeric sorbent was used (Strata-X, phenomenex, Aschaffenburg, Germany). After drying the sorbent material under a gentle stream of nitrogen, it was eluted with organic solvent. The eluate was blown down with nitrogen to dryness and reconstituted with the buffers used for HPLC. Geometric means were calculated as described in the OECD guideline.[12]

Test organisms and test design

Acute and chronic tests with *Gammarus fossarum*

G. fossarum was collected at the Tännichtgrundbach, a small second-order mountain stream that joins the river Elbe downstream of the city Dresden (Germany).[13] Organisms were obtained by kick sampling,[14] transferred to plastic boxes filled with stream water and transported immediately to the laboratory. In the laboratory, gammarids were cultured until use in modified Borgmann media [15] (LO4-S and additives E C H). Ingredients were solved in particle free and activated carbon filtered tap water for culturing purposes and in double-deionized water (Milli-Q, Millipore) for toxicity experiments. Further modifications were made concerning the original $CaCO_3$ concentration in the media, which was reduced to 12.5%. The gammarids were kept in enclosures in artificial indoor streams at 15°C at low flow and fed with leaves as described in detail by Ladewig et al.[16]

Sorption of metoprolol to organic food source

Leaves from alder (Alnus glutinosa) which were handled and conditioned as described in detail by Jungmann et al.[17] were used as food. Discs with a diameter of 28 mm were punched out of conditioned leaves and served as a food source for gammarids during exposure. Sorption of metoprolol from the water phase to leaf discs was investigated in 50 mL Borgmann medium [15] in 100 mL glass beaker with 5 and 45 mg/L metoprolol over 48 h in a greenhouse. One disc was exposed per beaker and tests were run in triplicate per treatment. Metoprolol in the water was analysed at the beginning and the end of the exposure. Water samples of 500 mL per beaker were sampled (Eppendorf-pipette) and the 3 samples per treatment were pooled in 1.5 mL short thread brown glass flask (Fisher Scientific). After 48 h leaf discs were freeze-dried (Alpha 1–2, Christ) and metoprolol analyses were carried out as described above. Water samples were diluted 100 and 200 times before HPLC analysis. Leaves were extracted with ultrapure water and adjusted to pH 5 with formic acid (Sigma-Aldrich) in an ultrasonic bath for 15 min. Water content was calculated as the difference in weight between wet and freeze-dried leaf discs.

Acute toxicity of metoprolol to *Gammarus fossarum*

Ten gammarids of similar size (7 ± 0.5 mm) per treatment were individually exposed in 15 mL glass beakers filled with 10 mL of Borgmann medium at 15 ± 1◦C in a greenhouse. Five nominal concentrations of metoprolol (3.1, 12.5, 50, 200 and 800 mg/L) with a spacing factor of 4 were investigated. The 7 ± 0.5 mm sized animals were sorted using reference organisms which were accurately measured with a binocular (Thalheim Spezial Optik, Germany), and were then transferred to a glass beaker. Equally sized animals were selected from the population of the field sample with bare eye by comparison with the reference organisms. Chemical analysis was conducted at the beginning of the experiment and after 48 h of exposure. Each beaker was sampled (150 mL) and replicates were pooled in each treatment. Analysis was carried out as described above.

Chronic test with *Gammarus fossarum*

Chronic exposure was carried out in 2 L glass beakers with five different treatments (5–405 mg /L, spacing factor 3) and a control with four replicates in 1 L Borgmann medium. Twenty animals were exposed per treatment. Each beaker contained two unglazed ceramic tiles (5.5 x 4.1 cm) and a pebble stone (3 x 5 cm) as artificial substrate. Five conditioned leaf discs per beaker served as food source. The leaf discs were exposed for 2 days in the same concentration of metoprolol before they were transferred to the exposure vessels. After 7 days evaporated water was replaced by double-deionised water (-18.2 MΩ; Millipore). Subsequently 80% of the medium was exchanged, the old leaf discs substituted and the medium was than filled up to the original volume with the respective metoprolol concentrations in Borgmann medium. To ensure survival of the gammarids during the experiments, oxygen was supplied by aeration with compressed air. Oxygen saturation and concentration was analysed 3 times a week, soluble reactive phosphorus [18] nitrate (LCK 339, Hach Lange) and ammonium [19] were determined on day 28 before and after medium exchange. For the quantification of nitrogen and phosphorus a 100 mL water sample per treatment was taken and stored in the freezer at ¡18_C until analysis.

To determine Hsp 70 responses, 47 gammarids were randomly taken from the experimental population prior to the start of the exposure, subsequently shock frozen in liquid nitrogen, and stored at -80◦C (Sanyo Ultra Low) until further processing. The same was done with 33 individuals per treatment at the end of the experiment. Samples were sent to Tübingen University overnight on dry ice. After arrival samples were stored at -80◦C until further processing. The biomarker tests were carried out according to the protocols given in detail below.

Toxicological endpoints on the individual level were mortality, number of precopulae, number of egg-bearing females, number and size of juveniles and number of eggs per eggbearing female.

Water samples for chemical analyses were taken as described above at day 0, day 7, and day 35 after replacing evaporated water but before water renewal, and at day 28 after water renewal. Due to limitations in capacity of chemical analysis samples were taken only in the control, the 5 and 15 mg/L treatment. The latter are those two concentrations at which relevant effects occurred.

Chronic test with *Lumbriculus variegatus*

L. variegatus has been used as a standard organism [20] in sediment toxicity tests.[21–24] The oligochaetes were established as an in-house culture and were kept in continuously aerated tap water in 10-L glass aquaria at 20 ± 1◦C under a 16 h light to 8 h dark photoperiod. For the breeding culture, artificial sediment with a mean particle size of 175 mm was used. Worms were fed once a week with TetraMin. Reproduction is mainly asexual by self-fragmentation.[25,26] In the experiment we used synchronised worms only. Therefore, fragmentation was induced by cutting the worms in the middle of their body. The posterior end will regenerate a new head within 2–3 weeks before start of the test to ensure that

all worms were in a similar physiological status at the start of the experiment. Thus an increased variance in the data set was avoided (for detail cf.[14]).

Two kinds of 28-day sediment toxicity tests were conducted: (1) using high but still sublethal concentrations (high concentration tests) for population-relevant endpoints according to OECD guideline 225 and (2) using low concentrations for biomarker studies (low concentration tests). For the high concentration tests 0, 20, 40, 80, 160, and 320 mg/kg_{dw} were investigated and effects on reproduction and biomass were studied. For the low concentration tests 0, 0.039, 0.078, 0.156, 0.312, 0.62, 1.25, 2.5, 5 and 10 mg/kg_{dw} were investigated and solely their effects on biomarkers were analysed. According to the OECD guideline 225, the test included a water control, a solvent control and five concentrations of the pharmaceutical in four replicates each.[20] Sediment was prepared as described by [22] with the exception that methanol was used as solvent. The tests were conducted in 250 mL beakers containing 2 cm of spiked artificial sediment, 150 mL of reconstituted water [20] and aerated with compressed air (2 bubbles/s).

The artificial sediment was prepared according to [20]. Peat (5% dry weight, Floragard, Oldenburg) and powdered leaves of Stinging Nettle (0.5% (dry weight), powder manufactured by Ceasar & Loretz, Hilden) were treated at the beginning with the appropriate volume of reconstituted water. This mixture was put on a shaker for 1 day (Society for Laboratory Technology (GFL) 3017, Großburgwedel). Thereafter, the other ingredients kaolin (19.8%, dry weight, Merck, Darmstadt) and cellulose (0.5%, dry weight, Sigma-Aldrich, Steinheim) were added, and then shaken for another day. The pH value was adjusted to 6.5–7.5 using calcium carbonate (VWR, Darmstadt, Germany). First, each 250 mL beaker was filled with 10 g of quartz sand. After that, metoprolol was dissolved in 50 mL methanol (Merck, Darmstadt) to produce a concentrated stock solution. The quartz sand was spiked with an appropriate amount of metoprolol, and the same volume of solvent was used for every treatment. The solvent control was spiked with an appropriate volume of solvent only. After the solvent had evaporated overnight, each beaker was filled with the remaining 27.5 g of quartz sand as well as 20 mL of the sediment components that was taken from the shaker. Finally, the whole sediment was mixed to obtain a homogenous solution. Each beaker was filled carefully with 150 mL reconstituted water.[20] As recommended in the OECD guideline 225, a positive control with 30 mg/kg_{dw} pentachlorophenol (Sigma-Aldrich, Steinheim) was used. The beakers were arranged in a controlled-climate room at constant room temperature (20 ± 1°C) and at a light/dark period of 16 to 8 h with a light intensity of 500–1000 lux. Under these conditions, the sediment was aged for 2 days to ensure equilibrium. Before starting, water conditions (temperature, pH, conductivity and dissolved oxygen) were measured with a Multi340i (WTW, Weilheim). These measurements were repeated once a week during the 28-days toxicity tests. In addition, the ammonium concentration was measured in the water at the start (day 0) and end (day 28) of the test using an ammonium test (Merck Darmstadt). At the start of the experiment, 10 synchronized [20] worms were added to each beaker. During the tests, aeration was checked daily. After 28 days, the number of individuals per beaker was recorded. In addition, the biomass per concentration (dry weight) was determined. In case of the low concentration experiments, worms were frozen in liquid nitrogen and sent to the University of Tübingen on dry ice for further analyses of biomarkers (Hsp70 and lipid peroxidation).

Biomarker tests

Hsp70 quantification

The level of the stress protein family, Hsp70, was quantified according to Köhler et al.[27] and Dietrich et al.[28] Frozen samples were homogenized on ice (*G. fossarum* samples were analysed individually, *L. variegatus* samples were analysed in pools of three individuals (because of their smaller size)) in 50 mL extraction buffer (80 mM potassium acetate, 5 mM magnesium acetate, 20 mM HEPES and 2% protease inhibitor at pH 7.5) in case of *G. fossarum*, or 40 mL in case of *L. variegatus*. Following

centrifugation (10 min at 20,000 relative centrifugal force (RCF) at 4◦C in an Eppendorf 5804 R centrifuge), one part of the supernatant was used for total protein quantification according to Bradford.[29] The other part was processed for minigel SDS-PAGE (12% acrylamide, 0.12% bisacrylamide, 30 min at 80 V plus 90 min at 120 V) where 40 mg of total protein from *G. fossarum* and 60 mg from *L. variegatus* was used for each sample. Proteins were transferred to a nitrocellulose membrane via semi-dry blotting, incubated with the first antibody (mouse anti-human Hsp70, Dianova, Hamburg, Germany, dilution 1:5000 in 10% horse serum/TBS) overnight, rinsed and incubated with the secondary antibody (goat anti mouse IgG conjugated to peroxidase, Jackson Immunoresearch, West Grove, PA, dilution 1:1000 in 10% horse serum/TBS) for 2 h at room temperature. The membranes were stained (1 mM 4-chloro(1)naphthol, 0.015% H_2O_2, 30 mM TRIS pH 8.5 and 6% methanol), scanned, and the optical volume (band area x average grey scale value) of each protein band was determined using E.A.S.Y.Win 32 (Herolab, Wiesloch, Germany). To allow comparability between the minigels, each optical volume was related to an internal Hsp70 standard (full body homogenate of *Salmo trutta* f. *fario*) and was run in duplicate on each gel.

Quantification of lipid peroxides

To quantify the amount of lipid peroxidation, a modified version of the FOX assay described by Hermes-Lima [30] was employed. Pools consisting of 3 *L. variegatus* individuals were homogenized in ice-cold HPLC-grade methanol (dilution 1:10) and centrifuged at 14,000 rpm (Eppendorf centrifuge 5804 R with rotor F-45-30-

11) at 4◦C. The supernatants were stored at -80◦C until further usage. The assay was conducted in 96-well plates; each well was filled with 50 mL of 0.25 mM$FeSO_4$, 25 mMH_2SO_4 and 0.1 mM xylenol orange; 20 mL of supernatant was added and the volume adjusted to 200 mL using bidistilled water. Each sample was tested in triplicate, and a blank sample in which the $FeSO_4$ solution had been added was substituted with water. All data were related to a master blank, which consisted of bidistilled water. The samples were incubated for 48 h at room temperature. The absorbance was measured at 580 nm using an automated microplate reader (Bio-Tek Instruments, Winooski VT, USA). After the first measurement, 1 mL of 1 mM cumene hydroperoxide solution was added to each well. After another incubation period of 1 h, the absorbance was measured again at 580 nm. Cumene hydroperoxide equivalents ($CHP_{equiv.}$/mg wet weight) were calculated according to Eq. (1):

$$\frac{A580\,nm}{A\,580\,nm\,CHP} * volume\ CHP * \frac{total\ volume}{sample\ volume} * dilution\ factor \quad (1)$$

Statistical analysis

Statistical analysis was conducted using SAS JMP 11.0 and Tox- Rat software (ToxRat Solutions, Version 2.10.05, Aachen, Germany). Data were checked for normal distribution using the Pearson-D'Agostino Omnibus test. If necessary, data were transformed using square root or third root transformations to fit normal distribution. Data were checked for homogeneity of variance using Levene's test. If the requirements for parametric tests were fulfilled, an ANOVA (if necessary, combined with Tukey-Kramer HSD or Dunnett's post hoc test) was used to compare the means between the different treatment groups. If the data did not show homogeneity of variance a Welch- ANOVA was used instead. In cases of obvious trends of data to increase or decrease with rising test concentrations, a correlation analysis was conducted using Kendall's Tau test. ECx-values (EC_{10} and EC_{50}, respectively) were computed using probit and Weibull analysis depending on the best fit to distribution, respectively. The significance level was set to α = 0.05 for all tests. Figures were made by software R (version 3.1.1).

Results

Chemical analysis of metoprolol

Metoprolol concentrations in the water phase

In water samples from the natural habitat (Tännichtgrundbach, near Dresden, Germany) of *G. fossarum*, metoprolol was not detected (limit of detection <0.01 mg/L). In the acute toxicity experiment with *G. fossarum* the relative difference between nominal and measured concentrations in the three analysed approaches at the beginning of the experiment fitted well and only differed approximately by 13%. The relative difference between the measured concentration at the beginning and at the end of the experiment was 12% (cf. Table A1). Hence the calculation of the effect concentration was based on nominal concentrations.

Sorption of metoprolol to the organic fraction, especially to the leaf discs, was estimated. The decrease of the water concentration was calculated to be 5% within 48 h. At the beginning the measured concentration of 44.5 mg/L (2.23 mg total) fitted well to the nominal concentration of 45 mg/L. After 48 h, the analysed concentration was 42.6 mg/L (2.13 mg total). Within 48 h 1.8% of the total metoprolol amount was sorbed to the leaf discs which corresponds to 0.041 mg per leaf disc.

The lowest and medium concentrations from the chronic exposure experiment were analysed. Table 1 shows the analysed concentrations and the relative decrease over time. At t_0 the metoprolol concentrations fit well with the nominal concentrations with a relative difference of 10.0% and 11.3%, respectively. Analyses at day 7 and 35 reflected the dissipation of metoprolol over 7 days of exposures from the water phase at an early and later period of the experiment, respectively. In both analyses, data showed a minor concentration dependent decrease of 4% in the lower and 12% in the higher concentration compared to the nominal concentration. The concentrations in the renewed medium are represented by the results of the analyses at day 28 and Table 1 depicts that deviations from nominal concentrations were again low with 10% and 11%, respectively. Hence, the following calculation of the effect concentrations was based on nominal concentrations. In the control 0.0002 mg/L metoprolol was detected, close to the limit of quantification. The test setups were aerated and a weekly loss of 50–100 mL of the exposure medium was observed and we assume that metoprolol contamination in the control took place via aerosol transfer.

Table 1. Nominal and measured concentrations of metoprolol in the chronic experiment with G. fossarum.

	mg/L				%		
Nominal	t_0	t_7	t_{20}	t_{25}	Delta$_{(n/0)}$	Delta$_{(n/28)}$	Delta$_{(n/7;35)}$
5.0	4.5	4.4	4.5	4.4	10.0	10.0	4.4; 4.4
15.0	13.3	12.4	13.3	12.7	11.3	11.3	12.4; 12.7

t: time (with numbers indicating the respective day). t_0 was the beginning of the experiment and t_7 samples were taken after balancing evaporated water but before water replacement. Delta: relative decrease of measured concentrations after 7 days compared to the nominal concentration.

Metoprolol concentrations in the sediment

The measured concentrations in the sediment at day 0 showed major differences compared to the nominal concentration (Table 2). The highest difference was found for the lowest concentrations with 82%. For the highest concentration, the difference was at least 20%. After 28 days of exposure, the differences even increased to 98% for the lowest, 85% for the medium, and 51% for the highest concentration. Therefore, the geometric mean was calculated according to the polynomial function (y D $0.0011x^2$ C 0.2788x - 0.0079) to estimate the effective concentration of sediment exposure to *L.*

variegatus depicted in Table 2 with 0.003, 6, and 200 mg/kg, respectively. For 160 mg/kg exposure, the extrapolation of a geometric mean led to 72.8 mg/kg.

Table 2. Nominal and measured concentrations in the sediment and the overlaying water in the exposure test with L. variegatus according to OECD 225.

	Sediment					Water (mg/L) measured	
Nominal	Measured		Geom.	Delta (%)			
	0	28		(n/0)	(n/28)	0	28
SC	<0.001	n.d.	-			0.00005	<0.00005
0.039	0.0068	<0.001	0.003	82	98	0.00055	0.0003
20	12.5	3.1	6	38	85	3.1	n.d.
320	255	156	200	20	51	7.4	11

geoM: geometric mean. n/0: relative decrease between nominal and measured concentration at day 0. n/28: relative decrease between nominal and measured concentration at day 28. n.d.: not determined.

Effects of metoprolol

Gammarus fossarum

Up to the highest concentration of 800 mg/L no significant immobility of the gammarids was detected after 48 h in the acute toxicity experiment.

In Table A2 the median values of pH, conductivity, and oxygen concentration in the chronic test with *G. fossarum* are shown. Neither of the values were significantly different to the control, nor were any values detected that raised issues of concern for the development of the gammarids.

In the chronic exposure experiment with *G. fossarum* elevated mortality occurred. In the control the median mortality was 35% which was not significantly different from the two lowest test concentrations with 30% and 5%, respectively. Significant differences to the control occurred at concentrations of 45, 135, and 405 mg/L with 88%, 100% and 100%, respectively. Figure 1 shows the concentration-response curve for mortality in the chronic experiment. The calculated EC_{10} was 17 mg/L. Due to the non-monotonic character of data distribution data obtained for 5 and 15 mg/L metoprolol had to be excluded from ToxRat analysis. Table 3 shows the investigated endpoints and the respective no/lowest observed effect concentrations (NOEC/LOEC). The most sensitive endpoints were the “ratio juvenile to adults” and “egg number per egg-bearing female,” both with NOEC/LOEC values of 5/15 mg/L, respectively. Figures 2 and 3 show the results for the most sensitive endpoints in the chronic exposure test with *G. fossarum*. Both, the ratio of juveniles to adults as well as the egg number per egg bearing female, showed a clear concentration-effect relationship with NOEC/LOEC values of 5/15 mg/L, respectively. The relative abundance of precopulae were not affected below 135 mg/L at which 100% mortality occurred.

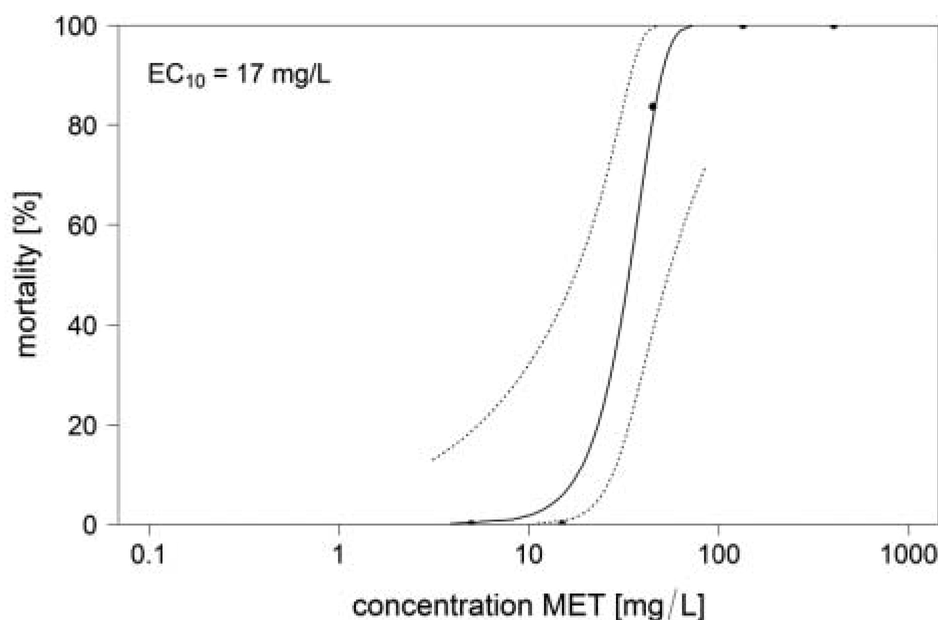

Figure 1. Concentration-response relationship (black line) for mortality of gammarids exposed to metoprolol for 40 days. Dashed line is the 95% confidence interval. For calculations of metoprolol toxicity lowest concentrations were excluded because the concentration-response relation deviated from the concentration-response model.

Table 3. Investigated endpoints in the chronic exposure experiment with G. fossarum and the corresponding no/lowest observed effect concentrations (NOEC/LOEC).

	Metoprolol	
Endpoint	NOEC (mg/L)	LOEC (mg/L)
Ratio juvenile/adults	5	15
Egg number/ egg bearing female	5	15
Relative number of egg bearing females*	15	45
Relative abundance of precopula*	≥135	>135

*Additional figures in Appendix.

Lumbriculus variegatus

In Figure 4, the effect of metoprolol on the endpoint reproduction is shown. The number of individuals hardly changes with increasing beta-blocker concentration, however, in the highest concentration (nominal 320 mg/kg$_{dw}$) a maximal reduction of 24.3% of the reproduction (compared to the solvent control) was observed. Thus only a nominal EC_{10} value of 189.8 mg/kg$_{dw}$ was computable resulting in an effective concentration of 92.5 mg/kg$_{dw}$. For the endpoint reproduction NOEC and LOEC values were nominal 160 and 320 mg/kg$_{dw}$ resulting in effective concentrations of 72.8 and 202 mg/kg$_{dw}$., respectively (One-way ANOVA, Dunnett_s test). Also, the total biomass decreased slightly with increasing metoprolol concentration (cf. appendix). The EC_{10} was 126.1 mg/kg$_{dw}$ as effective concentrations (confidence intervals could not be calculated).

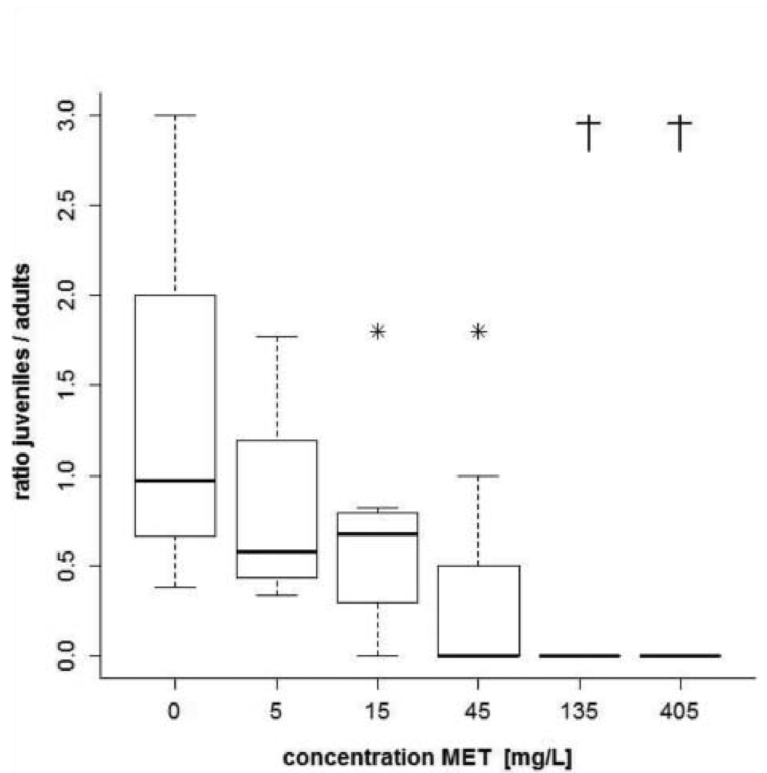

Figure 2. Box-Whisker plot of the ratio between juveniles and adults in the chronic exposure of G. fossarum with metoprolol. Asterisk: statistically significant different from the control (α = 0.05); †100% mortality. MET: metoprolol (α = 0.05).

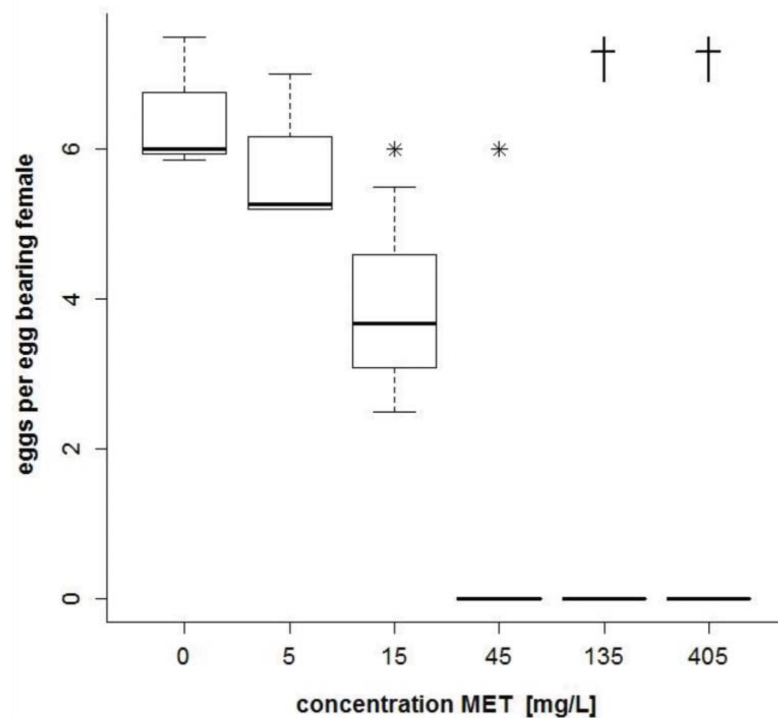

Figure 3. Box-Whisker plot of the egg number per egg bearing female in the chronic exposure of G. fossarum with metoprolol. Mean number of individuals (±75 interval and minimum and maximum values, n = 4 replicates). Asterisk: statistically significant different to the control (α = 0.05); †100% mortality. MET: metoprolol (α = 0.05).

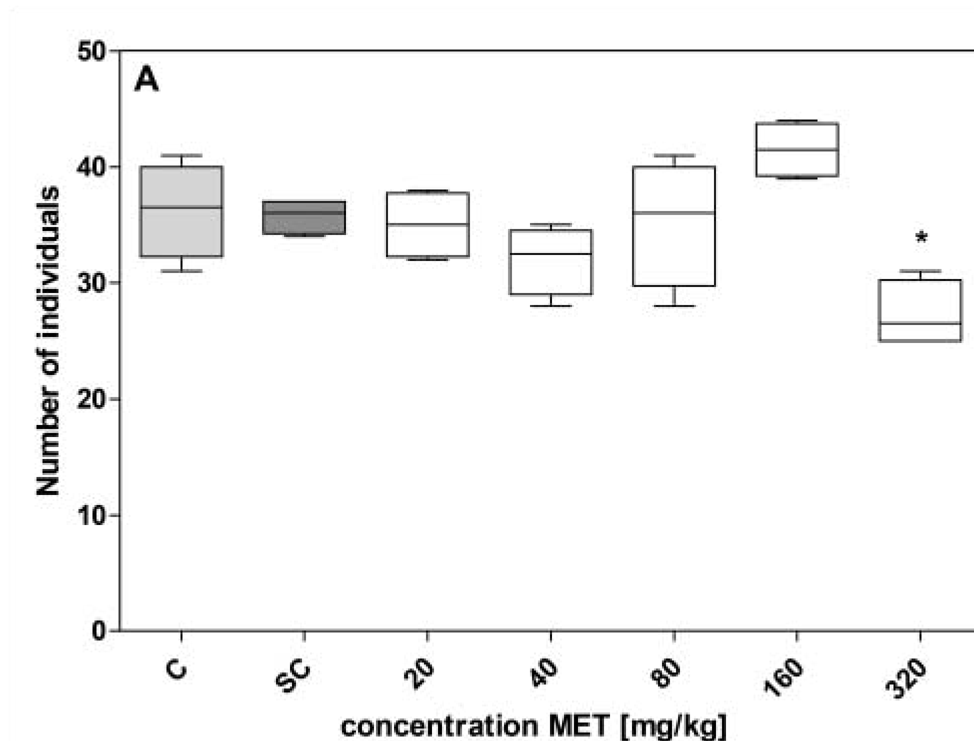

*Figure 4. 28-day sediment toxicity test with the oligochaete Lumbriculus variegatus and metoprolol according to the OECD guideline 225. Number of individuals (25-percentile, median, 75-percentile, min, max, n = 4 replicates). At the start of the experiment 10 synchronized worms were added to each beaker. One-way ANOVA with Dunnett's post hoc test.*P < 0.05.*

Biomarkers Hsp70 and lipid-peroxidation

Hsp70 levels in *Gammarus fossarum*

In Figure A2 the Hsp70 levels of Gammarus are depicted. The data showed normal distribution after transformation using the second root (Pearson-D'Agostino-Omnibus, n = 102, P = 0.406), homogeneity of variance was obtained after transformation (Levene, df = 3, F = 1.4176, P = 0.2423). No statistically significant difference was observed between the treatments (ANOVA, df = 3, F = 0.1333, P = 0.94). However, Hsp70 levels of gammarids taken from the basic population from field samples showed a significantly lower Hsp70 level compared to the control treatment after 40 days of exposure (t-test, df = 58, t = -2.506, P = 0.015).

Response of Hsp70 and lipid-peroxidation levels in *Lumbriculus variegatus*

Figure 5 depicts the levels of Hsp70 after exposure of *L. variegatus* to metoprolol. Data showed normal distribution after transformation using the third root (Pearson-D'Agostino-Omnibus, n = 26, P = 0.2364); homogeneity of variance was obtained after transformation (Levene, df = 5, 20, F = 0.5410, P = 0.7431).The stress protein level of *L. variegatus* exposed to metoprolol was not significant different between the approaches (ANOVA, df = 5, 20, F = 2.2054, P = 0.0942).

Lipid-peroxidation levels

Figure 6 shows the results of lipid-peroxidation levels after exposure of *L. variegatus* to metoprolol. Data showed a normal distribution after transformation using the square root (Pearson- D'Agostino-Omnibus, n = 30, P = 0.6766), homogeneity of variance was not obtained (Levene, df = 5.24, F = 3.2112, P = 0.0233). There was a significantly positive correlation of the degree of lipid peroxidation with increasing metoprolol concentration (Kendall's Tau, n = 30, t = 0.2847, P = 0.0371). The means showed a significant overall difference (Welch-ANOVA, df = 5, 10.9, F = 5.2488, P = 0.0107); however, there was no significant difference between any of the compared treatment groups and the control (Fig. 6).

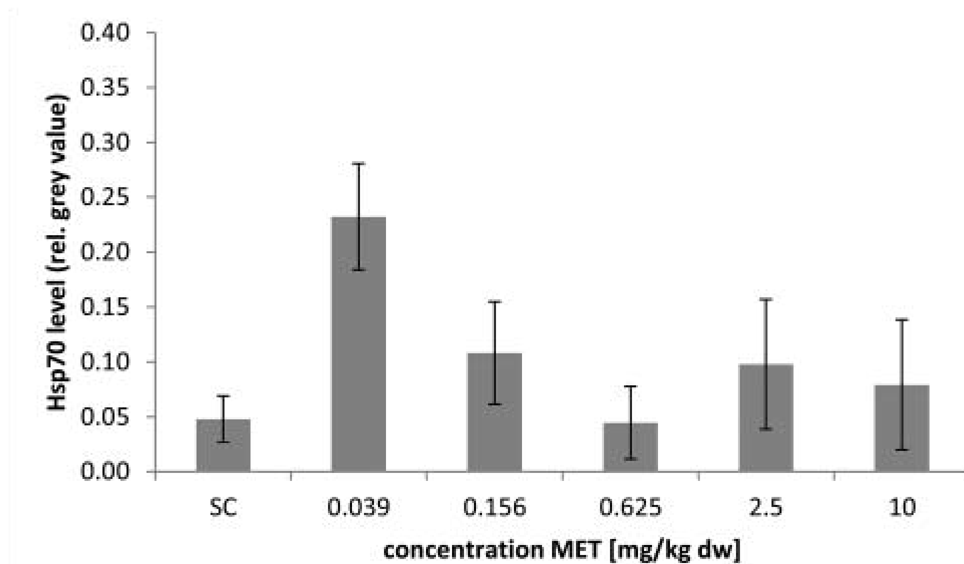

Figure 5. Relative Hsp70 level (quantified as optical volume relative to standard compared to the standard) of L. variegatus exposed to increasing concentrations of metoprolol. SC, solvent control. The bar chart depicts arithmetic means plus/minus standard errors. There is a trend towards an increased Hsp70 level at the concentration of 0.039 mg/L, but not at the other tested concentrations.

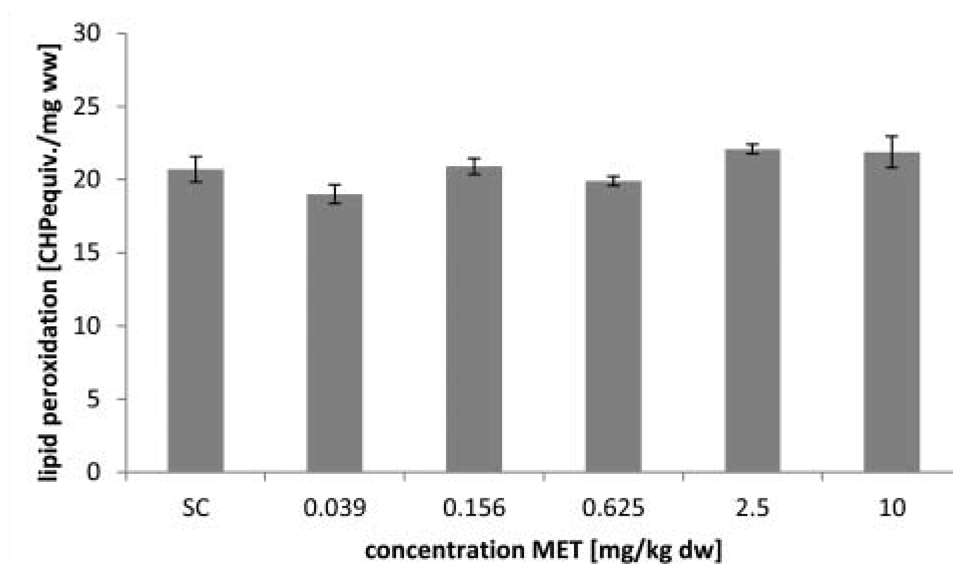

Figure 6. Lipid peroxidation (in cumene hydroperoxide equivalents per mg wet weight) of L. variegatus exposed to increasing concentrations of metoprolol. SC, solvent control. The bar chart depicts arithmetic means plus/minus standard errors. The degree of lipid peroxidation increased with rising metoprolol concentration (Welch-ANOVA).

Discussion

The aim of the present study was to provide effect data for metoprolol as a model substance for beta-blockers in ecologically relevant aquatic invertebrates as a basis for the validation of future mode of action-based *in vitro* test systems targeting this class of pharmaceuticals. In the aquatic environment average metoprolol concentrations were reported to reach 404 ng/L in the water phase of sediments, 33 mg/kg dw in sediments,[31] and up to 2.2 mg/L in surface waters.[32] The metoprolol concentrations in the Tännichtgrundbach, where gammarids were sampled, were below the limit of detection (<0.01 mg/L).

When metoprolol was applied via the water phase in our experiment with gammarids, the real concentrations were close to the nominal concentrations only differing by 10% and 13%, respectively. Water solubility of metoprolol is rather high with 1,000 mg/L (Merck Index 14: 6151), which in general

causes minor problems to achieve nominal concentrations. Dissipation from the water phase was low, between 12% and 10% over 2 and 7 days, respectively. The slightly higher dissipation of metoprolol in the 2 day acute toxicity experiment might be a result of a higher surface:volume ratio resulting from smaller vessels. The half-life time of metoprolol under natural conditions was estimated with 24 days, and direct photolysis follows pseudo-first-order kinetics under solar simulation and is strongly influenced by daylight surface conditions, light intensity of the light source or the sun at different latitudes and seasons.[33,34] In a greenhouse, which is shaded to simulate natural conditions of lower mountain streams,[35] a higher half-life time due to lower radiation intensity was observed, which is in agreement with our findings. To calculate the effect concentrations in the experiments with *G. fossarum*, the nominal concentrations were used. The extremely low amount of contamination with metoprolol in the control was assumed by transfer via aerosols. The detected concentration was 2.500 times lower compared to the lowest LOEC, hence we suppose no impact on the results from the control.

During short-term exposure to metoprolol with leaf discs as organic food source, only minor dissipation from the water phase was determined. Sorption processes of chemicals in the aquatic environment to organic material are well known and mainly depend on K_{ow} or K_{oc} values.[36] As the log K_{ow} of metoprolol is 1.9 [37] a high sorption potential of metoprolol is not expected. Thus the major exposure pathway in the experiments with gammarids was via the water phase.

In the experiment with *L. variegatus* exposure via the sediment showed a comparable pattern to water exposure, however, with strong impact on the sediment concentration over time. The measured concentrations in the sediment were out of the aimed range for recovery of 80–120%.[20] Due to the high water solubility and low log K_{ow} value, metoprolol dissipated from the sediment, and its concentration in the water increased quickly. This became more obvious when comparing the mass balance, e.g. between the sediment and water at day 0 directly after the tests were started. In the experiment with the highest concentration the total mass of applied metoprolol was 2.5 mg in the 10 g of sediment, and 1.1 mg in the 150 mL overlaying water. This means that approximately one-third of the applied metoprolol was found in the overlaying water. After 28 days of exposure the total mass in the overlaying water increased to 50% of the metoprolol concentration measured in the respective beakers. This was demonstrated in other experiments too, when sorption to different sediments,[38] mobility,[39] or dissipation under varying environmental conditions were observed.[34] Hence, nominal sediment concentrations are not suitable to express effective concentrations.[20] In this case the calculation of the geometric mean is recommended [12] and was applied.

Acute toxicity tests with G. fossarum

Acute toxicity of metoprolol to gammarids was not detected up to 800 mg/L. In the control no mortality occurred. Thus acute toxicity of metoprolol to *G. fossarum* can be regarded as low. In acute toxicity tests with Daphnia magna EC_{50} values for metoprolol ranged between 70 and 438 mg/L.[9,40,41] *Hyalella azteca*, another amphipod tested, showed an EC_{50} of 100 mg/L after 48 h of exposure. Hence, gammarids seem to be less sensitive to metoprolol compared to daphnids and *Hyalella*; however, all data lie in the upper milligram range which means that all the organisms tested are relatively insensitive to this compound under acute exposure.

Chronic toxicity tests with G. fossarum

The acute toxicity values from acute tests were taken to establish a suitable concentration range for the chronic exposure of *G. fossarum* over 40 days. At the end of the test, mortality in the control was 35%. As no guideline and, therefore, no validity criteria for a chronic test with *Gammarus* exist, the mortality in the control could only be compared with results from other experiments. Schneider et al.[42] performed a flow-channel experiment in the lab (6.5_C) with G. pulex and found a mean mortality

rate of 7.1% in the control after 30 days. Oskarsson et al.[43] observed a mortality rate of 28–70% during 8 weeks at a temperature of 10–11_C in controls, which even increased, whenever individuals were exposed in the same vessel. Dietrich et al.[44] determined a mortality of 40% in controls at 12_C after 100 days of exposure. After 103 days of exposure at 15_C, *G. fossarum* showed a mortality of 76% in controls.[45] Mortality rates that have been reported in other experiments and ours did not give reason to assume an exceptional situation in the study reported here, even though all these studies differ regarding boundary conditions like temperature, age of individuals, and exposure medium. In general, none of the measured physico-chemical parameters in the water gave rise to concern for the survival of the gammarids, neither in the control nor in the treatments. In the two highest tested concentrations 100% mortality occurred.

At lower concentrations endpoints of relevance for population development were affected. The most sensitive endpoints were the ratio of juveniles to adults and the egg number per egg-bearing female with NOEC/LOEC values of 5 and 15 mg/L. To the best of our knowledge only chronic exposure experiments to metoprolol with other amphipods have been carried out by Dzialowski et al.[4] They found in Daphnia magna NOEC/LOEC values for reproduction of 3.1/6.2 mg/L and the heart beat rate was affected at the lowest tested concentrations of 3.1 mg/L. These results are of the same order of magnitude as in the chronic experiment with *G. fossarum*. In contrast to the results from acute toxicity tests, gammarids and daphnids showed similar sensitivity for population relevant endpoints. In a multi-generation study with D. magna effects on the number and body length of neonates were found at lower concentrations of 0.012 mg/L.[6] This is the lowest effect concentration found in the literature for invertebrate species. In general a lower toxicity of metoprolol to invertebrate crustacean species is not surprising as these organisms use different hormones.[46]

The Hsp70 level in *G. fossarum* did not show any concentration- dependent difference after exposure to metoprolol for 40 days. In general the stress level was higher in the control at the end of the experiment compared to the situation at the start of the experiment. Food shortage may not be the reason for intraspecific stress because food was renewed every 7 days during water exchange and it was never found to be consumed completely. Potentially the handling itself or the difference in age could be a reason for the increase in Hsp70 level, because this was a constant factor in all treatments. Possibly the concentrations of metoprolol were too high to meet the phase of Hsp70 elevation but rather reflect the breakdown of the Hsp70 response system in situations of high stress intensity (for Hsp70 induction kinetics see [47]).

Toxicity tests with L. variegatus

Effect data for metoprolol on endobenthic or sediment-associated invertebrates are currently not available in the literature. Initially, this is not surprising because the physico-chemical properties of metoprolol, specifically the log K_{ow} and log K_{oc} values do not implicate concern derived from sorption of metoprolol to the sediment. On the other hand the results of the experiment with *L. variegatus* show that a smaller, but nevertheless significant amount of metoprolol remained in the sediment. Hence, it may be good advice to address sediment as a compartment in pharmaceutical risk assessment. The sediment toxicity test with *L. variegatus* fulfills all validity criteria mentioned in the OECD guideline 225.[20] These are the first results for a sediment-dwelling organism exposed to metoprolol via sediment resulting in EC_{10} values of 126.1 mg/kg_{dw} for biomass reduction and NOEC/LOEC-values of 72.8/202 mg/kg for decrease of reproduction. From these data it remains unknown, whether effects are driven via water exposure or sediment exposure. For Lumbriculus it was also shown that lipid peroxide levels did not differ compared to the control when exposure took place at lower concentrations.

For the environmental risk assessment the lowest effect concentration is implemented after a respective assessment factor was applied, which leads to the PNEC.[48] For our estimations an assessment factor of 50 was applied.[48] This leads to PNECs of 0.1 mg/L for *G. fossarum* and 1.45 mg/kg$_{dw}$ for *L. variegatus*, in respect to organismic endpoints. Based on the results obtained in the present study, we demand a sensitivity of future *in vitro* tests for effect-based monitoring of beta-blockers in the range of 0.374 up to 5.42 mmol in order to reflect effects of metoprolol in epibenthic and sediment-dwelling freshwater invertebrates. All determined values are far from environmentally relevant concentrations (MEC: 0.0022 mg/L[49]). Based on the results obtained in the present study the potential risk of metoprolol for gammarids and sediment dwelling organisms like Lumbriculus is low. However, in consideration of an effect concentration as low as 0.001 mg/L that has been reported for fish,[50] and an estimated PNEC of 0.0001 mg/L, metoprolol nevertheless seems to pose a risk in the aquatic environment. As fish species belong to vertebrates they use norepinephrine as neurotransmitter and the target specific β adrenergic receptor is present.[51] Therefore a higher sensitivity of vertebrate fish species compared to invertebrate crustacean is comprehensible. Hence, further investigations with fish as the most sensitive organism are recommended, in particular concerning population relevant endpoints to provide a basis for benchmarking future effect-based *in vitro* test systems for beta-blockers.

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References

[1] **Lawton, J.** Demographic Change and the Environment. *The Royal Commission on Environmental Pollution*: London, UK, 2011.

[2**] Boxall, A.B.A.** The environmental side effects of medication—How are human and veterinary medicines in soils and water bodies affecting human and environmental health? *Embo Rep.* 2004, 5, 1110–1116.

[3] **Daughton, C.G.; Ternes, T.A.** Pharmaceuticals and personal care products in the environment: Agents of subtle change? *Environ. Health Perspect.* 1999, 107, 907–938.

[4] **Dzialowski, E.M.; Turner, P.K.; Brooks, B.W.** Physiological and reproductive effects of beta adrenergic receptor antagonists in Daphnia magna*. Arch. Environ. Contam. Toxicol.* 2006, 50, 503–510.

[5] **Barber, L.B.; Keefe, S.H.; Brown, G.K.; Furlong, E.T.; Gray, J.L.; Kolpin, D.W.; Meyer, M.T.; Sandstrom, M.W.; Zaugg, S.D.** Persistence and potential effects of complex organic contaminant mixtures in wastewater-impacted streams. *Environ. Sci. Technol.* 2013, 47, 2177–2188.

[6] **Dietrich, S.; Ploessl, F.; Bracher, F.; Laforsch; C.** Single and combined toxicity of pharmaceuticals at environmentally relevant concentrations in Daphnia magna—A multigenerational study. *Chemosphere* 2010, 79, 60–66.

[7] **Monteiro, S.C.; Boxall, A.B.A.** Occurrence and fate of human pharmaceuticals in the environment. In *Reviews of Environmental Contamination and Toxicology*; **Whitacre, D.M.,** Ed.; Springer: New York, 2010; Vol. 202, 53–154.

[8] **Triebskorn, R.; Berg, K.; Ebert, I.; Frey, M.; Jungmann, D.; Oehlmann, J.; Oetken, M.; Sacher, F.; Scheurer, M.; Schmieg, H.; Schwarz,S.; Köhler, H.** Monitoring primary effects of pharmaceuticals in the aquatic environment with mode of action-specific *in vitro* biotests. *Environ. Sci. Technol.* 2015, 49(5), 2594–2595.

[9] **Cleuvers, M.** Initial risk assessment for three beta-blockers found in the aquatic environment. *Chemosphere* 2005, 59, 199–205.

[10] **Escher, B.I.; Allinson, M.; Altenburger, R.; Bain, P.A.; Balaguer, P.; Busch, W.; Crago, J.; Denslow, N.D.; Dopp, E.; Hilscherova, K.; Humpage, A.R.; Kumar, A.; Grimaldi, M.; Jayasinghe, B.S.; Jarosova, B.; Jia, A.; Makarov, S.; Maruya, K.A.; Medvedev, A.; Mehinto, A.C.; Mendez, J.E.; Poulsen, A.; Prochazka, E.; Richard, J.; Schifferli, A.; Schlenk, D.; Scholz, S.; Shiraish, F.; Snyder, S.; Su, G.; Tang, J.Y.M.; van der Burg, B.; van der Linden, S.C.; Werner, I.; Westerheide, S.D.; Wong, C.K.C.; Yang, M.; Yeung, B.H.Y.; Zhang, X.; Leusch, F.D.L.** Benchmarking organic micropollutants in wastewater, recycled water and drinking water with *in vitro* bioassays. *Environ. Sci. Technol.* 2014, 48, 1940–1956.

[11] **Triebskorn, R.; Jungmann, D.; Köhler, H.-R.; Ladewig, V.; Ludwichowski, K.-U.; Mösenfechtel, A.; Scheil, V.; Schirling, M.; Nagel, R.** Das Projekt Xehogamm: Endokrine Effekte bei Flohkrebsen auf unterschiedlichen biologischen Ebenen. In *Pharmaka und Hormone in der aquatischen Umwelt—eine Bedrohung*; **Metzger, J.W.** Ed.; Stuttgarter: *Berichte zur Siedlungswasserwirtschaft*, 2002; 103–125.

[12] **OECD-23.** OECD Series on Testing and Assessment 23. Series on Testing and Assessment 23. In: Guidance Document on aquastic Toxicity Testing of difficult Substances and Mixtures; Directorate, Ed. OECD: Paris, 2000; 53 p.

[13] **Winkelmann, C.; Petzoldt, T.; Koop, J.H.E.; Matthaei, C.D.; Benndorf, J.** Benthivorous fish reduce stream invertebrate drift in a largescale field experiment. *Aquat. Ecol.* 2008, 42, 483–493.
[14] **Whitehurst, I.T.** The effects of sampling techniques on the *Gammarus*: *Asellus* ratio. *Wat. Res.* 1991, 25, 745–748.
[15] **Borgmann, U.** Systematic analysis of aqueous ion requirements of *Hyalella azteca*: A standard artificial medium including the essential bromide ion. *Arch. Environ. Contam. Toxicol.* 1996, 30, 356–363.
[16] **Ladewig, V.; Jungmann, D.; Köhler, H.-R.; Licht, O.; Ludwichowsk, K.U.; Schirling, M.; Triebskorn, R.; Nagel, R.** Effects of Bisphenol A on *Gammarus fossarum* and *Lumbriculus variegatus* in artificial indoor streams. *Toxicol. Environ. Chem.* 2006, 88, 649–664.
[17] **Jungmann, D.; Ladewig, V.; Ludwichowski, K.U.; Petzsch, P.; Nagel, R.** Intersexuality in *Gammarus fossarum* KOCH—A common inducible phenomenon? *Arch. Hydrobiol.* 2004, 159, 511–529.
[18] **DIN38406**. Deutsche Einheitsverfahren zur Wasser-, Abwasser- und Schlammuntersuchung: Kationen (Gruppe E) Bestimmung des Ammonium-Stickstoffs (E5). Wasserchemische Gesellschaft—Fachgruppe in der Gesellschaft Deutscher Chemiker in Gemeinschaft mit Normenausschuß Wasserwesen (NAW) im DIN Deutsches Institut für Normung e.V. In 38406. *Deutsches Institut für Normung e.V.*; Beuth, Germany; 1983.
[19] **DIN38405.** Deutsche Einheitsverfahren zur Wasser-, Abwasser- und Schlammuntersuchung: Kationen (Gruppe E) Bestimmung des Ammonium-Stickstoffs (E5). Wasserchemische Gesellschaft—Fachgruppe in der Gesellschaft Deutscher Chemiker in Gemeinschaft mit Normenausschuß Wasserwesen (NAW) im DIN Deutsches Institut für Normung e.V. In 38408. *Deutsches Institut für Normung e.V.*; Beuth, Germany; 1983.
[20**] OECD-225**. Sediment-water *Lumbriculus* toxicity test using spiked sediment. Guidelines for the Testing of Chemicals; Directorate, Ed. OECD: Paris, 2007; 31 p.
[21] **Phipps, G.L.; Ankley, G.T.; Benoit, D.A.; Mattson, V.R.** Use of the aquatic oligochaete *Lumbriculus variegatus* for assessing the toxicity and bioaccumulation of sediment-associated contaminats. *Environ. Toxicol. Chem.* 1993, 12, 269–279.
[22] **Oetken, M.; Nentwig, G.; Loffler, D.; Ternes, T.; Oehlmann, J.** Effects of pharmaceuticals on aquatic invertebrates. Part I. The antiepileptic drug carbamazepine. *Arch. Environ. Contam. Toxicol.* 2005, 49, 353–361.
[23] **Nentwig, G.** Effects of pharmaceuticals on aquatic invertebrates. Part II: The antidepressant drug fluoxetine. *Arch. Environ. Contam. Toxicol.* 2007, 52, 163–170.
[24**] Galluba, S.; Oetken, M.; Oehlmann, J.** Comprehensive sediment toxicity assessment of Hessian surface waters using *Lumbriculus variegatus* and Chironomus riparius. *J. Environ. Sci. Health A-Tox. Hazard. Subs. Environ. Engin.* 2012, 47, 507–521.
[25] **Brinkhurst, R.O.; Jamieson, B.G.M.** Aquatic Oligochaeta of the World. Oliver & Boyd: Edinburgh, UK, 1971.
[26] **Drewes, C.D.; Rourtner, C.R.** Morphallaxis in an aquatic oligochaete, *Lumbriculus variegatus*: Reorganization of escape reflexes in regenerating body fragments. *Dev. Biol.* 1990, 138(1), 94–103.
[27] **Köhler, H.-R.; Lazzara, R.; Dittbrenner, N.; Capowiez, Y.; Mazzia, C.; Triebskorn, R.** Snail phenotypic variation and stress proteins: Do different heat response strategies contribute to Waddington's widget in field populations? *J. Exp. Zool. B-Mol. Develop. Evol.* 2009, 312B, 136–147.
[28] **Dietrich, A.; Troschinski, S.; Schwarz, S.; Di Lellis, M.A.; Henneberg, A.; Fischbach, U.; Ludwig, M.; Gaertner, U.; Triebskorn, R.; Köhler, H.R.** Hsp70 and lipid peroxide levels following heat stress in *Xeropicta derbentina* (Krynicki 1836) (Gastropoda, Pulmonata) with regard to different colour morphs. *Cell Stress Chaperones* 2015, 20, 159–168.
[29] **Bradford, M.M.** Rapid and sensitive method for quantitation of microgram quantities of protein utilizing principle of protein-dye binding. *Anal. Biochem.* 1976, 72, 248–254.
[30] **Hermes-Lima, M.; Willmore, W.G.; Storey, K.B.** Quantification of lipid-peroxidation in tissue-extracts based on Fe(III)xylenol orange complex formation. *Free Radic. Biol. Med.* 1995, 19, 271–280.
[31] **Ramil, M.; El Aref, T.; Fink; G.; Scheurer, M.; Ternes, T.A.** Fate of Beta Blockers in Aquatic-Sediment Systems: Sorption and Biotransformation. *Environ. Sci. Technol.* 2010, 44, 962–970.
[32] **Ternes, T.A.** Analytical methods for the determination of pharmaceuticals in aqueous environmental samples. *TrAC Trends Anal. Chem.* 2001, 20, 419–434.
[33] **Liu, Q.-T.; Williams, H.E.** Kinetics and degradation products for direct photolysis of beta-blockers in water. *Environ. Sci. Technol.* 2007, 41, 803–810.
[34] **Radke, M.; Maier, M.P.** Lessons learned from water/sediment-testing of pharmaceuticals. *Wat. Res.* 2014, 55, 63–73.
[35] **Jungmann, D.; Brust, K.; Licht, O.; Mählmann, J.; Schmidt, J.; Nagel, R.** Artificial indoor streams as a method to investigate the impact of chemicals on lotic communities. *Environ. Sci. Pollut. Res. Int.* 2001, 8, 49–55.
[36] **Lamoureux, E.M.; Brownawell, B.J.** Chemical and biological availability of sediment-sorbed hydrophobic organic contaminants. *Environ. Toxicol. Chem.* 1999, 18, 1733–1741.
[37] **Hansch, C.; Leo, A.; Hoekmann, D.** Exploring QSAR—Hydrophobic, electronic, and steric constants. *The American Chemical Society*: Washington, DC, 1995; 137.
[38] **Burke, V.; Treumann, S.; Duennbier, U.; Greskowiak, J.; Massmann, G.** Sorption behavior of 20 wastewater originated micropollutants in groundwater—Column experiments with pharmaceutical residues and industrial agents. *J. Contam. Hydrol.* 2013, 154, 29–41.
[39] **Maszkowska, J.; Stolte, S.; Kumirska, J.; Lukaszewicz, P.; Mioduszewska, K.; Puckowski, A.; Caban, M.; Wagil, M.; Stepnowski, P. and Bialk-Bielinska, A.** Beta-blockers in the environment: Part I. Mobility and hydrolysis study. *Sci. Total Environ.* 2014, 493, 1112–1121.
[40] **Hernando, M.D.; Petrovic, M.; Fernandez-Alba, A.R.; Barcelo, D.** Analysis by liquid chromatography-electro spray ionization tandem mass spectrometry and acute toxicity evaluation for beta-blockers and lipid-regulating agents in wastewater samples. *J. Chromatogr. A* 2004, 1046, 133–140.
[41] **Huggett, D.B.; Brooks, B.W.; Peterson, B.; Foran, C.M.; Schlenk, D.** Toxicity of select beta adrenergic receptor-blocking pharmaceuticals (β-blockers) on aquatic organisms. *Arch. Environ. Contam. Toxicol.* 2002, 43, 229–235.
[42] **Schneider, I.; Oehlmann, J.; Oetken, M.** Impact of an estrogenic sewage treatment plant effluent on life-history traits of the freshwater amphipod *Gammarus pulex*. *J. Environ. Sci. Health A-Tox. Hazard. Subs. Environ. Eng.* 2015, 50, 272–281.

[43] **Oskarsson, H.; Wiklund, A.K.E.; Lindh, K.; Kumblad, L.** Effect studies of human pharmaceuticals on *Fucus vesiculosus* and *Gammarus spp*.. *Mar. Environ. Res.* 2012, 74, 1–8.
[44] **Dietrich, S.; Dammel, S.; Ploessl, F.; Bracher, F.; Laforsch, C.** Effects of a pharmaceutical mixture at environmentally relevant concentrations on the amphipod *Gammarus fossarum*. *Mar. Freshwater Res.* 2010, 61, 196–203.
[45] **Schirling, M.; Jungmann, D.; Ladewig, V.; Ludwichowski, K.U.; Nagel, R.; Köhler, H.R.; Triebskorn, R.** Bisphenol A in artificial indoor streams: II. Stress response and gonad histology in *Gammarus fossarum* (Amphipoda). *Ecotoxicology* 2006, 15, 143–156.
[46] **Pfluger, H.J.; Stevenson, P.A.** Evolutionary aspects of octopaminergic systems with emphasis on arthropods. *Arthropod Struct. Dev.* 2005, 34, 379–396.
[47] **Eckwert, H.; Köhler, H.R.** The indicative value of the hsp70 stress response as a marker for metal effects in *Oniscus asellus* (Isopoda) field populations: Variability between populations from metal-polluted and uncontaminated sites. *Appl. Soil Ecol.* 1997, 6, 275–282.
[48] **EC Technical Guidance Document on Risk Assessment.** European Commission Joint Research Centre, 2003; Part I-IV.
[49] **Ternes, T.A.** Demographic Change and the Environment. *Wasser Boden* 2001, 53(4), 9–14.
[50] **Triebskorn, R.; Casper, H.; Scheil, V.; Schwaiger, J.** Ultrastructural effects of pharmaceuticals (carbamazepine, clofibric acid, metoprolol, diclofenac) in rainbow trout (*Oncorhynchus mykiss*) and common carp (*Cyprinus carpio*). *Anal. Bioanal. Chem.* 2007, 387, 1405–1416.
[51] **Steele, S.L.; Yang, X.; Debiais-Thibaud, M.; Schwerte, T.; Pelster, B.; Ekker, M.; Tiberi, M.; Perry, S.F.** *In vivo* and *in vitro* assessment of cardiac beta-adrenergic receptors in larval zebrafish (*Danio rerio*). *J. Exp. Biol.* 2011, 214, 1445–1457.

Appendix

Table A 1. Nominal and measured (m) concentrations of metoprolol in the water in the acute toxicity test over 48 h of exposure.

	mg/L			%	
Nominal	Measured (0)	Measured (48)	Rel. diff. (n/0)	Rel. diff. (n/48)	Rel. diff. (0/48)
3.1	2.63	2.3	15	26	13
50.0	45.1	39.8	10	20	12
800.0	691	620	14	23	10

Number in brackets: time; n: nominal concentration; rel. diff.: relative difference.

Table A 2. Nominal concentrations, mortality and relative mortality (rel. mortality) in the acute toxicity test with G. fossarum after 48 h of exposure with metoprolol.

Concentration mg/L		C	3.1	12.5	50	200	800
Mortality	48 h	0	0	0	0	0	0
Rel. mortality (%)		0	0	0	0	0	0

Table A 3. Median values of pH, conductivity and oxygen concentration in the prolonged test with G. fossarum.

	Unit	Control	5	15	45	135	405
pH-Wert (min; max)	-	7.88	7.84	7.84	7.86	7.9	7.9
		(7.74; 8.15)	(7.76; 8.1)	(7.71; 8.1)	(7.73, 7.86)	(7.74; 8.14)	(7.71; 7.97)
Conductivity (min; max)	µS/cm	424	428	428	432	426	453
		(385; 696)	(385; 676)	(386; 676)	(391; 686)	(403; 698)	(446; 462)
Oxygen (min; max)	mg/L	10.1	10.1	10.1	10	9.9	8.3
		(8.0; 10.7)	(7.9; 10.7)	(7.7; 10.5)	(7.5; 10.4)	(7.4; 10.4)	(7.8; 10.2)

Min: minimum; max: maximum; S: Siemens.

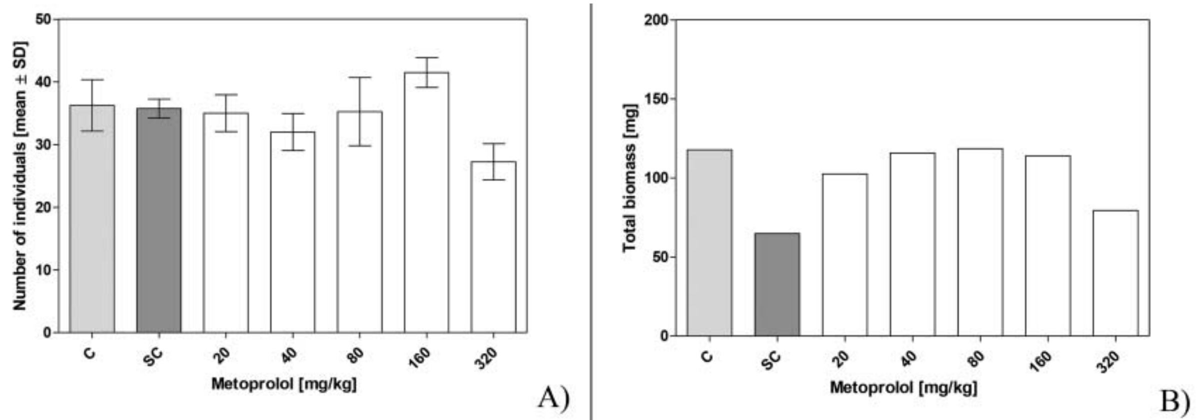

Figure A 1. Definitive 28-day sediment toxicity test with the oligochaete Lumbriculus variegatus and metoprolol according to the OECD guideline 225. Mean number of individuals (±SD, n = 4 replicates, probit analysis) (A) and total biomass of L. variegatus (mean, data were pooled from four replicates, probit analysis) (B). At the start of the experiment 10 synchronized worms were added to each beaker.

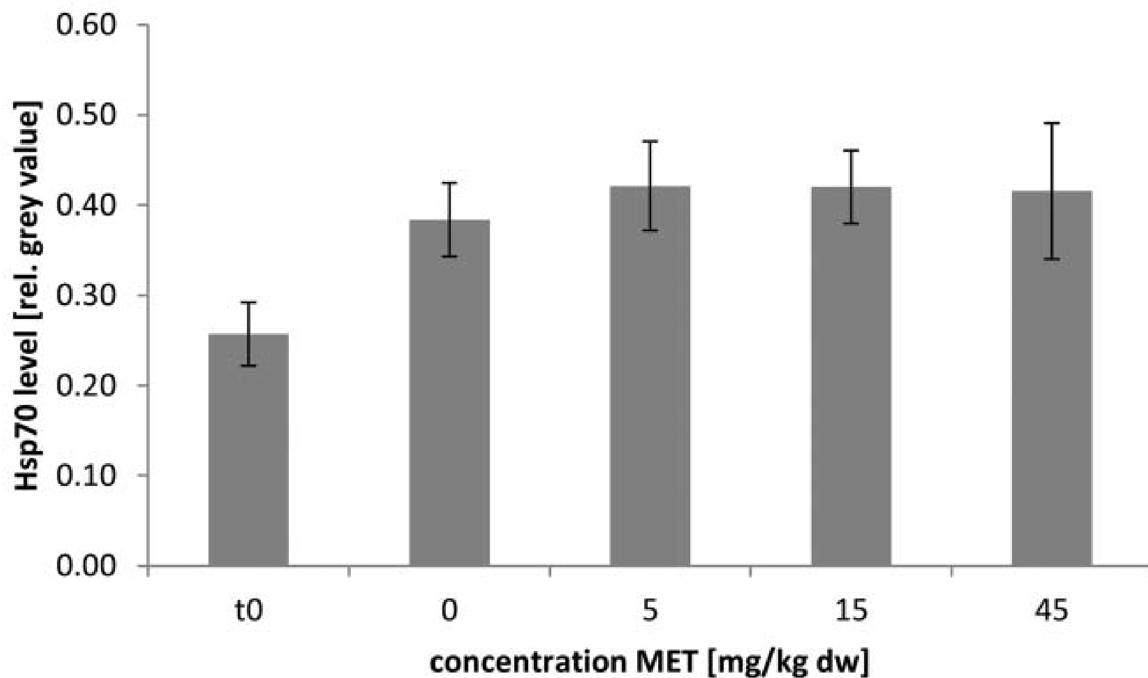

Figure A 2. Relative Hsp70 level (quantified as relative grey value compared to the standard) of in Gammarus after exposure to metoprolol. The two highest concentrations are excluded due to 100% mortality. Data showed normal distribution after transformation using the second root (Pearson-D'Agostino-Omnibus, n = 102, P = 0.406), homogeneity of variance was given (Levene, df = 3, F = 1.4176, P = 0.2423).

Appendix

Artificial water for brown trout embryos and sac-fry stages

- 294 mg/L $CaCl_2$ x 2 H_2O,
- 123.25 mg/L $MgSO_4$ x 7 H_2O
- 64.75 mg/L $NaHCO_3$
- 5.75 mg/L KCl
- Added to deionized Millipore-filtered water

Stress protein analysis

Concentrated extraction buffer for Hsp70 analysis

80 mM potassium acetate, 5 mM magnesium acetate, 20 mM Hepes in double-distilled water, adjusted to a pH of 7.5

SDS buffer

20 % glycerine, 3 % sodium dodecyl sulphate, 0.3 % β-mercaptoethanol, 10 mM Tris pH 7 and 0.005 % bromophenol blue in double-distilled water

Bradford mixture

0.001 % Coomassie brilliant blue G-250, 4.75 % ethanol, 8.5 % phosphoric acid in double-distilled water

E-buffer + SDS

0.19 M glycin, 25 mM $Tris_{base}$ and 0.1 % SDS in double-distilled water

Blocking solution

TBS (0.88 % sodium chloride, 0.635 % Tris-HCl and 0.118 % Tris-base in double-distilled water, adjusted to a pH of 7.5) with horse serum in a mixture of 1:2

Staining solution

1 mM 4-chloro-1-naphtol, 6 % methanol, 0.015 % hydrogen peroxide in 30 mM Tris pH 8.5

Minigels

Reagents

- 30 % acrylamide, 0.8 % bisacrylamide in double-distilled water - ready for use preparation by Carl Roth GmbH + Co. KG, Karlsruhe
- Separation gel buffer (4x): 18.5 g $Tris_{HCl}$ + 76.95 g $Tris_{base}$ + 2 g SDS in 500 mL double-distilled water, adjusted to pH 8.8
- Collection gel buffer (4x): 17.55 $Tris_{HCl}$ + 1.68 g $Tris_{base}$ + 2 g SDS in 500 mL double-distilled water, adjusted to pH 6.8
- 10 % ammonium persulfate (APS)
- Tetramethyleethylenediamine (TEMED) – ready for use by Merck Schuchardt OHG, Hohenbrunn

Procedure

- For two separation gels: 2.65 mL double-distilled water, 3 mL acylamide/bisacrylamide, 1.87 mL separation gel buffer (4x), 40 µL APS, 20 µL TEMED
- For two collection gels: 1.4 mL double-distilled water, 1 mL acylamide/bisacrylamide, 0.6 mL collection gel buffer (4x), 20 µL APS, 20 µL TEMED

Histology

Embedding process

Step	Medium	Duration
Washing (three times)	0.1 M cacodylate-buffer	3 x 10 min
Decalcification (only for gill and kidney)	Formic acid 1:2 in 70 % ethanol	30 min
Washing (three times)	70 % ethanol	3 x 15 min
Dehydration 1	70 % ethanol	30 min
Dehydration 2	70 % ethanol	90 min
Dehydration 3	80 % ethanol	60 min
Dehydration 4	90 % ethanol	60 min
Dehydration 5	96 % ethanol	60 min
Dehydration 6	100 % ethanol	60 min
Dehydration 7	100 % ethanol	60 min
Intermedium 1 – 1	Isopropyl alcohol	90 min
Intermedium 1 – 2	Isopropyl alcohol	120 min
Intermedium 2	Isopropyl alcohol/paraffin 1:2	180 min
Infiltration 1	Paraffin	180 min
Infiltration 2	Paraffin	480 min

Staining Hematoxilin-Eosin (for paraffin-embedded samples)

- Roti®-Histol: ready-to-use solution by Carl Roth GmbH + Co. KG, Karlsruhe
- Hematoxilin (Mayer): ready-to-use solution by Carl Roth GmbH + Co. KG, Karlsruhe

Medium	Duration
Roti®-Histol	5 min
Roti®-Histol	5 min
100 % ethanol	5 min
90 % ethanol	5 min
80 % ethanol	5 min
70 % ethanol	5 min
Distilled water	5 min
Hematoxilin (Mayer)	4 min
Distilled water	4 sec
Tap water (flow-through)	20 min
Eosin (5 g/L)	5 min
70 % ethanol	10 sec
80 % ethanol	5 min
90 % ethanol	5 min
96 % ethanol	5 min
100 % ethanol	5 min
Roti®-Histol	5 min
Roti®-Histol	5 min

Staining Alcian blue – PAS (for paraffin-embedded samples)

- Alcian blue solution: 10 g/L in 3 % acetic acid
- Schiff reagent: ready-to-use solution by Sigma-Aldrich
- Sulfite water: 22.5 mL HCl (1 M) + 11.25 g sodium disulfite + 2227.5 mL distilled water

Medium	Duration
Roti®-Histol	3 min
96 % ethanol	2 min
80 % ethanol	2 min
70 % ethanol	2 min
Distilled water	5 min
3 % acetic acid	3 min
Alcian blue	30 min
3 % acetic acid	3 min
Distilled water	5 min
1 % periodic acid	10 min
Distilled water	5 sec
Distilled water	5 sec
Distilled water	5 sec
Schiff reagent	5 min
Sulfite water	2 min
Sulfite water	2 min
Tap water (flow-through)	15 min
Distilled water	5 sec
70 % ethanol	5 min
80 % ethanol	5 min
90 % ethanol	5 min
100 % ethanol	5 min
Roti®-Histol	5 min

Contents of the attached CD

- Electronic version of this thesis
- Electronic version of the graphical abstract
- List of reviewed literature for Publication I
- Manuscript Publication IV: Triebskorn *et al.* (2017) EFF-Pharm: Effects of pharmaceuticals (nonsteroidal anti-inflammatory drugs and beta-blockers) in fish and invertebrates and their detection by newly developed *in vitro*-bioassays.

Publications and contributions to congresses

Written publications

- **Schwarz S, Schmieg H, Scheurer M, Köhler H-R, Triebskorn R** (2017) Impact of the NSAID diclofenac on survival, development, behaviour and health of embryonic and juvenile stages of brown trout, *Salmo trutta* f. *fario*. *Science of the Total Environment*, 607-608, 1026-1036
- **Triebskorn R. Schwarz S, Schmieg H, Köhler H-R, Jungmann D, Berg K, Buchberger A, Frey M, Scheurer M, Sacher F** (2017) EFF-Pharm: Effects of pharmaceuticals (nonsteroidal anti-inflammatory drugs and beta-blockers) in fish and invertebrates and their detection by newly developed *in vitro*-bioassays. *UBA-Texte* 44/2017
- **Triebskorn *et al.*** (2017) Weitergehende Abwasserreinigung: Ein wirksames und bezahlbares Instrument zur Verminderung von Spurenstoffen und Keimen im Wasserkreislauf. Universität Tübingen, ISBN 978-3946552116
- **Jungmann D, Berg K, Dieterich A, Frank M, Gräf T, Scheurer M, Schwarz S, Siewert C, Oetken M** (2017) Health effects of metoprolol in epibenthic and endobenthic invertebrates—A basis to validate future *in vitro* biotests for effect-based biomonitoring. *Journal of Environmental Science and Health, Part A,* 52/3, 189-200
- **Werner *et al.*** (2016) The 2015 Annual Meeting of SETAC German Language Branch in Zurich (7–10 September 2015): Ecotoxicology and environmental chemistry—from research to application. *Environmental Sciences Europe,* 28/1, Article Number 20
- **Schwarz S, Rackstraw A, Behnisch PA, Brouwer A, Köhler H-R, Kotz A, Kuballa T, Malisch R, Neugebauer F, Schilling F, Schmidt D, von der Trenck K** (2016) Peregrine falcon egg pollutants mirror Stockholm POPs list including methylmercury. *Toxicological & Environmental Chemistry,* 98/8, 886-923
- **Haap T, Schwarz S, Köhler H-R** (2016) Metallothionein and Hsp70 trade-off against one another in *Daphnia magna* cross-tolerance to cadmium and heat stress. *Aquatic Toxicology,* 170, 112-119
- **Thellmann P, Köhler H-R, Rößler A, Scheurer M, Schwarz S, Vogel H-J, Triebskorn R** (2015) Fish embryo tests with *Danio rerio* as a tool to evaluate surface water and sediment quality in rivers influenced by wastewater treatment plants using different treatment technologies. *Environmental Science and Pollution Research*, 22:21
- **Triebskorn R, Berg K, Ebert I, Frey M, Jungmann D, Oehlmann J, Oetken M, Sacher F, Scheurer M, Schmieg H, Schwarz S, Köhler H-R** (2015) Monitoring Primary Effects of Pharmaceuticals in the Aquatic Environment with Mode of Action-Specific *in Vitro* Biotests. *Environmental Science & Technology*, 49, 2594-2595
- **Dieterich A, Troschinski S, Schwarz S, Di Lellis M, Henneberg A, Fischbach U, Ludwig M, Gärtner U, Triebskorn R, Köhler H-R** (2015) Hsp70 and lipid peroxide levels following heat stress in *Xeropicta derbentina* (Krynicki 1836) (Gastropoda, Pulmonata) with regard to different colour morphs. *Cell Stress and Chaperones*, 20/1, 159-168
- **Triebskorn R, Schwarz S, Köhler H-R, Berg K, Jungmann D, Frey M, Oehlmann J, Oetken M** (2014) From theory to reality – Evaluation of suitable organisms and test systems for biomonitoring of pharmaceuticals – Part I: Literature review. *UBA-Texte* 64/2014

Platform and poster presentations

- **Schwarz S, Bachmann J, Brandt U**: Environmental risk assessment of human pharmaceuticals – what can we learn from regulatory effect data so far? *Poster presentation at SETAC Europe 28th annual meeting, Rome, 2018/05/13-17*
- **Rönnefahrt I, Schwarz S, Hein A, Konradi S, Westphal-Settele K, Ebert I**: Prioritisation of human pharmaceutical substances – a regulatory perspective. *Poster presentation at SETAC Europe 28th annual meeting, Rome, 2018/05/13-17*
- **Schwarz S**: "Moderne" Rückstände in Gewässern – Medikamente, Hormone, Endokrine Disruptoren. *Platform presentation at Transnationales Seminar: Aktuelle Herausforderungen für den Schutz von Feuchtgebieten, Radolfzell, 2017/11/30*
- **Schwarz S**: From theory to reality – Evaluation of suitable organisms and test systems for the biomonitoring of pharmaceuticals. *Platform presentation EvE seminar, Tübingen, 2016/11/30*
- **Schwarz S, Schmieg H, Scheurer M, Köhler H-R, Triebskorn R**: Effekte des Beta-Blockers Metoprolol und des NSAIDs Diclofenac auf die Embryonalentwicklung und den Gesundheitszustand der Bachforelle *Salmo trutta* f. *fario. Platform presentation at SETAC GLB annual meeting, Tübingen, 2016/09/08*
- **Schwarz S, Schmieg H, Scheurer M, Köhler H-R, Triebskorn R**: Effects of the NSAID diclofenac on the survival, health and behaviour of embryonic and juvenile brown trout *Salmo trutta* f. *fario*. *Platform presentation at SETAC Europe 26th annual meeting, Nantes, 2016/05/23*
- **Schmieg H, Schwarz S, Scheurer M, Köhler H-R, Triebskorn R:** Effects of the beta-blocker metoprolol and the NSAID diclofenac on the embryonic development and health of brown trout *Salmo trutta* f. *fario*. *Poster presentation at SETAC Europe 26th annual meeting, Nantes, 2016/05/23*
- **Schwarz S, Berg K, Ebert I, Frey M, Jungmann D, Oehlmann J, Triebskorn R:** Effects of pharmaceuticals in aquatic organisms and their relevance for ecosystems – a literature review. *Poster presentation at SETAC-GLB annual meeting, Zurich, 2015/09/07-10*
- **Schmieg H, Schwarz S, Scheurer M, Köhler H-R, Triebskorn R**: Effects of the beta-blocker metoprolol on the embryonic development and health of brown trout *Salmo trutta* f. *fario*. *Poster presentation at SETAC-GLB annual meeting, Zurich, 2015/09/07-10*
- **Schwarz S, Schmieg H, Scheurer M, Sacher F, Köhler H-R, Triebskorn R:** Effects of the beta-blocker metoprolol and the NSAID diclofenac on the embryonic development and health of brown trout *Salmo trutta* f. *fario*. *Poster presentation at SETAC Europe 25th annual meeting, Barcelona, 2015/05/03-07*
- **Schwarz S, Triebskorn R, Jungmann D, Berg K, Frey M, Oehlmann J, Oetken M, Ebert I:** From theory to reality – Evaluation of suitable organisms and test systems for the biomonitoring of pharmaceuticals - Literature review. *Platform presentation at SETAC Europe 24th annual meeting, Basel, 2014/05/11-15*
- **Schwarz S:** Untersuchungen zur Schalendicke von Eiern des Wanderfalken *Falco peregrinus* in Baden-Württemberg mit Bezug zur Belastung durch Organohalogenverbindungen und Quecksilber. *Platform presentation at NABU-AGW annual meeting, Blaubeuren, 2010/10/17*

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