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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

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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

Rita Triebskorn, Simon Schwarz, Hannah Schmieg, Heinz-R. Köhler
University of Tübingen, Animal Physiological Ecology

Rita Triebskorn
Steinbeis Transfer Center for Ecotoxicology and Ecophysiology, Rottenburg

Dirk Jungmann, Kristin Berg, Anna Buchberger
GWT-TUD GmbH, Dresden

Manfred Frey
Steinbeis Innovation Center Cell Culture Technology, Mannheim

Marco Scheurer, Frank Sacher
DVGW Water Technology Center, Karlsruhe

Matthias Oetken, Jörg Oehlmann
University of Frankfurt, Aquatic Ecotoxicology

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Wörlitzer Platz 1
06844 Dessau-Roßlau
Tel: +49 340-2103-0
Fax: +49 340-2103-2285
info@umweltbundesamt.de
Internet: www.umweltbundesamt.de

/umweltbundesamt.de
/umweltbundesamt

Study performed by:
University of Tübingen
Animal Physiological Ecology
Auf der Morgenstelle 5
72076 Tübingen
Germany

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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.

Kurzbeschreibung

Als Werkzeuge für das Biomonitoring von Arzneimitteln wurden Wirkmechanismus-basierte *in vitro*-Assays entwickelt, durch die der Nachweis von Wirkungen zweier Arzneimittelgruppen, entzündungshemmender Schmerzmittel, wie Diclofenac (NSAIDs) und Beta-Blocker, wie Metoprolol. möglich ist. Hierfür wurden stabile Zelllinien entwickelt, die Wirkmechanismus-basierte sensorische und signalgebende rekombinante Proteine exprimieren. Diese ermöglichen die schnelle Visualisierung von Fluoreszenzsignaländerungen in den lebenden Zellen. Darauf aufbauenden Mikroplattenformat-Assays wurden für die quantitative Bestimmung von Metoprolol- und Diclofenac-Aktivität in Kläranlagenabläufen erfolgreich eingesetzt

Um die Relevanz und Eignung dieser Tests zum Abbilden von Wirkungen bei Freiland-relevanten aquatischen Organismen zu zeigen, wurden parallel Wirkuntersuchungen mit Fischen (Bachforellen und deren Eier), Krebstieren (Flohkrebsen, Daphnien), Schnecken und mit im Sediment lebenden Würmern durchgeführt. Die Untersuchungen fanden (1) unter kontrollierten Laborbedingungen mit Einzelsubstanzen und binären Mischungen, (2) in aquatischen Mesokosmen (Fließrinnen) sowie im Freiland in einem Durchflusssystem, das vom Ablauf einer kommunalen Kläranlage gespeist war, statt. An den exponierten Organismen wurden sowohl populationsrelevante Endpunkte, wie Mortalität, Fruchtbarkeit oder Reproduktion, als auch Biomarker (Reaktionen von Stressproteinen und Parametern, die oxidativen Stress anzeigen sowie histologische Veränderungen) untersucht, die den Gesundheitsstatus der Individuen abbilden.

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List of Abbreviations

A. victoria	*Aequorea victoria*
ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
ATP	Adenosine triphosphate
β-AR	β-adrenoceptor
cAMP	Cyclic adenosine monophosphate
CBA	Cost-benefit analysis
CFP	Cyan fluorescent protein
CNG	Cyclic nucleotide-gated
COX	Cyclooxygenase
CRE	Cyclic adenosine monophosphate response element
CREB	cAMP response element binding protein
d	day(s)
D. magna	*Daphnia magna*
E.coli	*Escherichia coli*
EC50	half-maximal effective concentration
EGFP	Enhanced green fluorescent protein
EGS	Ecosystem Goods and Services
Epac	Exchange protein directly activated by cyclic adenosine monophosphate
f. *fario*	forma *fario*
FRET	Fluorescence Resonance Energy Transfer or Förster Resonance Energy Transfer
G. fossarum	*Gammarus fossarum*
GCa-MPs	M13 domain of a myosin light chain kinase and calmodulin based genetically encoded calcium indicator
GFP	Green fluorescent protein
GPCR	G protein-coupled receptor
HEK293	Human Embryonic Kidney 293 cells
HELCOM	Kommission zum Schutz der Meeresumwelt im Ostseeraum (Convention on the Protection of the Marine Environment of the Baltic Sea Area).
Hsp70	heat shock protein with about 70 kD
ICUE	Indicator of cAMP using Epac
L. variegatus	*Lumbriculus variegatus*
NSAID	Non-steroidal anti-inflammatory drug
P. antipodarum	*Potamopyrgus antipodarum*

roGFPs	Redoxsensitive fluorescence protein indicators
S. trutta	*Salmo trutta*
UBA	Umweltbundesamt
WWTP	Waste water treatment plant
YFP	Yellow fluorescent protein

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 1: Summary of the approaches followed in the EFF-Pharm project

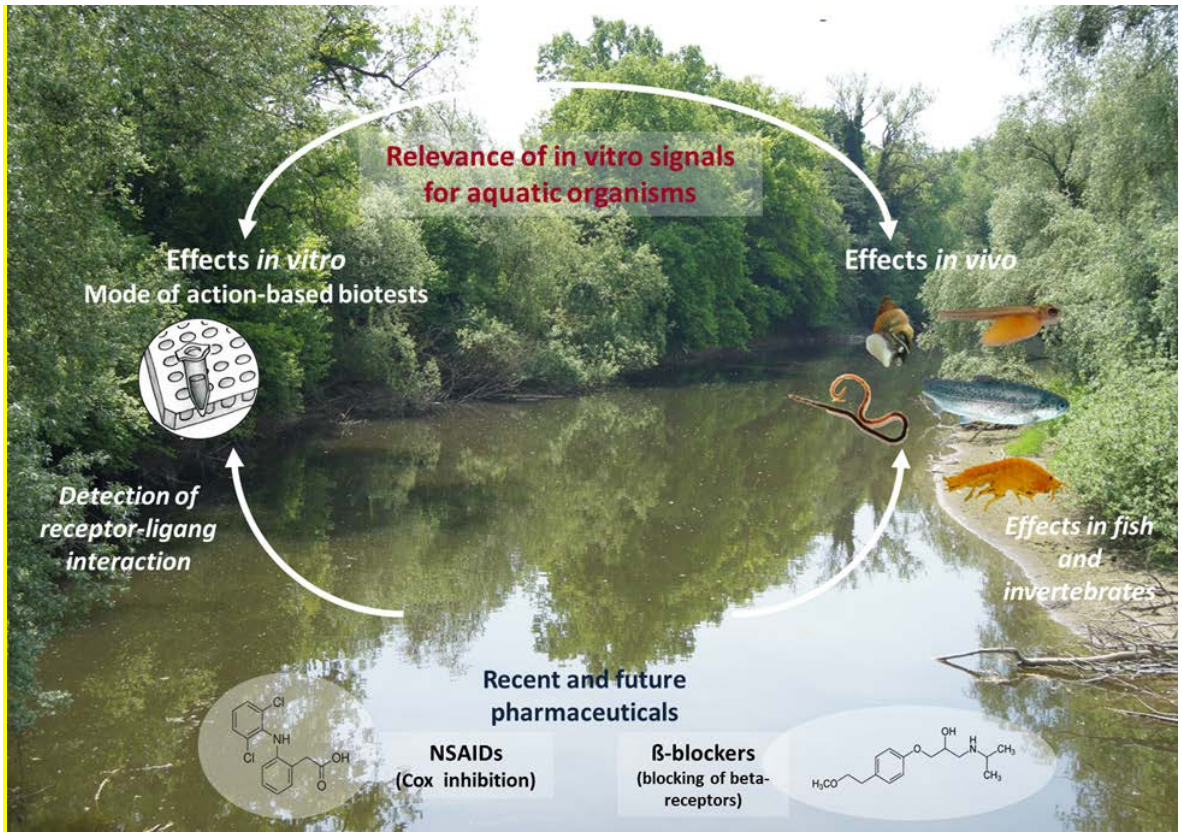
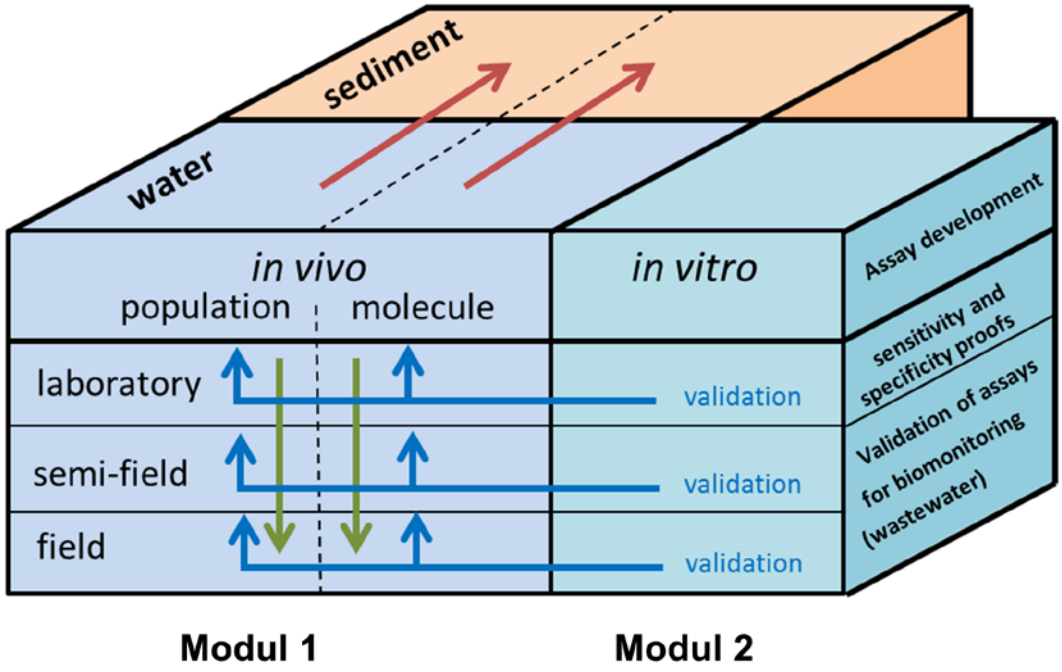


Figure 2 summarizes the project structure of Eff-Pharm: In two modules running in parallel, *in vivo*-testing and *in vitro*-test development and validation of methods were realized. By a stepwise approach from laboratory to field via semi-field mesocosms, results that are mandatory to validate *in vitro*-biotests 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 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 pre-copula 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 effects 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

	Species	Diclofenac	Metoprolol
	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.

Zusammenfassung

Eine Literaturstudie, "From theory to reality - Evaluation of suitable organisms and test systems for the biomonitoring of pharmaceuticals" (Triebskorn et al. 2013), erbrachte, dass eine Anwendung *Mode of Action* (MOA)-basierter Biotests im Kontext von Umweltrisikobewertung und Umwelt-Pharmakovigilanz für ein wirkungsorientiertes Biomonitoring von Pharmazeutika notwendig ist. Des Weiteren wurde darauf hingewiesen, dass eine Validierung derartiger Biotests durch Abgleich mit *in vivo*-Antworten von sensitiven und ökologisch relevanten Organismen parallel zu ihrer Entwicklung durchgeführt werden muss.

In Phase II dieses Projektes mit dem Akronym "Eff-Pharm" wurden *Mode of Action*-basierte Biotests (Bioassays) für Beta-blocker und nicht-steroidale Entzündungshemmer (NSAIDs) entwickelt. Parallel hierzu wurden *in vivo*-Experimente mit Fischen, Crustaceen, Mollusken und Anneliden durchgeführt. Diese Fallstudien konzentrierten sich auf Effekte von Diclofenac, einem repräsentativen Arzneimittel aus der Gruppe der NSAIDs, und Metoprolol, welches Beta-blocker repräsentierte. Sie hatten zum Ziel, Schwellenwerte für wirkungs- und nebenwirkungsabbildende Biomarker sowie für populationsrelevante Endpunkte bei diesen Organismen zu ermitteln. Die In Eff-Pharm realisierte Vorgehensweise ist in Figure 3.

Figure 3: Zusammenfassung des Forschungsansatzes von Eff-Pharm

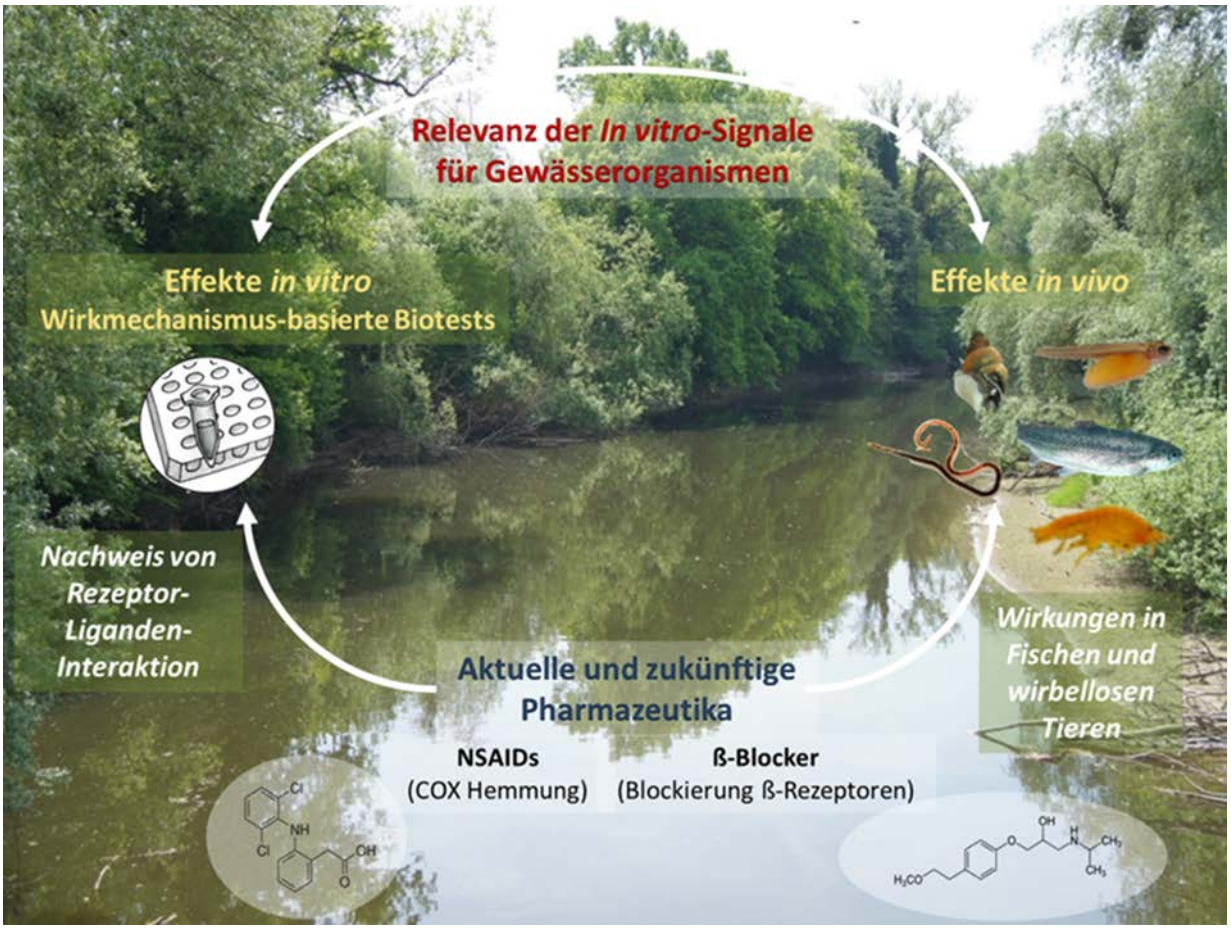
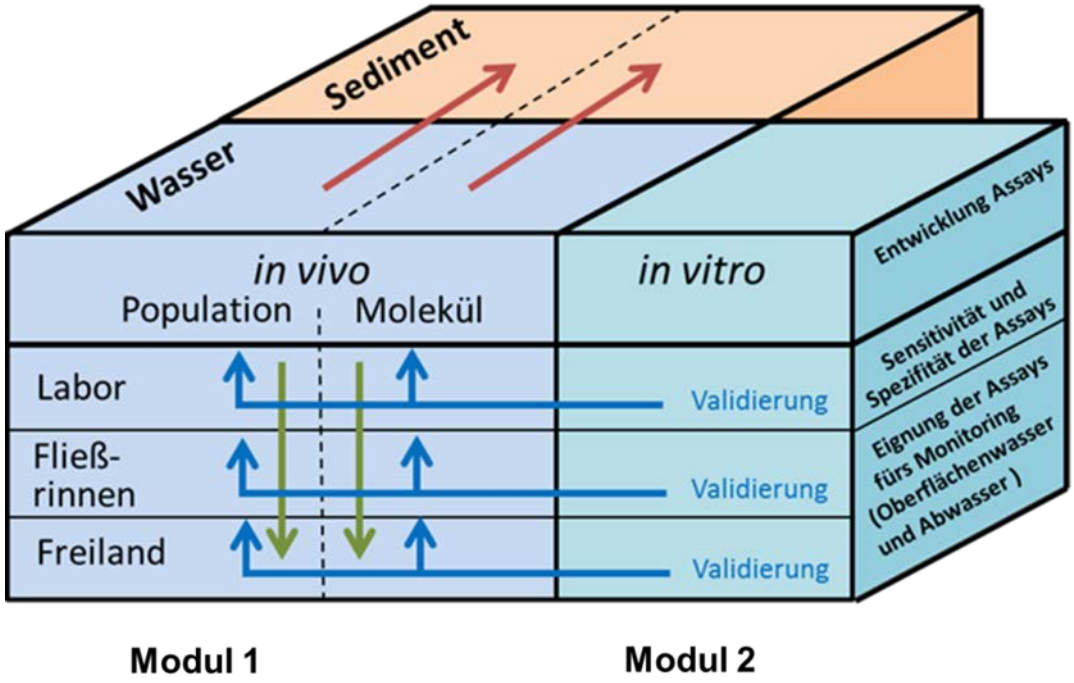


Figure 4 fasst die Projektstruktur von Eff-Pharm zusammen. In zwei parallel laufenden Modulen wurden *in vivo*-Tests durchgeführt und *in vitro*-Tests entwickelt und validiert. Über einen stufenweisen Ansatz vom Labor über Halbfreiland-Mesokosmen zum Freiland wurden für Schlüsselarten aus Fließgewässern und deren Sedimenten (Fischen, Gammariden, Schnecken und sedimentbewohnenden Anneliden) Daten erhoben, die zur Validierung der *in vitro*-Biotests notwendig waren. Auch die *Mode of*

Action-basierten Bioassays folgten dem stufenweisen Ansatz vom Labor ins Freiland: Ziel des Projektes war es, Testsysteme bereitzustellen, die einerseits sensitiv und spezifisch für die jeweiligen Arzneimittelgruppen, jedoch andererseits auch robust genug sind, um als Werkzeuge für ein Biomonitoring von Oberflächenwasser und Abwasser unter Freilandbedingungen zu fungieren.

Figure 4: Struktur des Projekt "Von der Theorie zur Wirklichkeit – Bewertung geeigneter Testorganismen und Testsysteme für das Biomonitoring von Arzneimitteln –Fallstudien" (Eff-Pharm)



Das Projekt bestand aus zwölf Arbeitspaketen, unter denen acht wissenschaftliche Fragestellungen bearbeitetet wurden (Table 3). Die Arbeitspakete wurden als " Eff-Pharm 2" und "Eff-Pharm 3" in zwei Stufen finanziert. Vom Umweltbundesamt wurden ergänzende Studien mit Daphnien durchgeführt (Teil 4).

Table 3: Arbeitspaktete (APs) von Eff-Pharm

AP	Inhalt
1 (Teil 2a)	Koordination, Projektmanagement
2 (Teil 2a)	Kick-off-Meeting
3A (Teil 2a)	Labortests Oberflächenwasser - Einzelsubstanzen
	Test 1: Biomarker bei juvenilen Bachforellen
	Test 2: Embryotests mit Bachforellen
	Test 3: Untersuchungen an Flohkrebsen
3B (Teil 3)	Labortests Oberflächenwasser - Stoffmischungen
4 (Teil 3)	Fließrinnenexperimente mit Flohkrebsen, Schnecken und Ringelwürmern
5 (Teil 2a)	Freilandstudien (Expositionen im Kläranlagenablauf)
	5A: Embryotests mit Bachforellen
	5B: Exposition von Flohkrebsen

AP	Inhalt
6 (Teil 2b)	Sedimenttoxizität: Einzelstoffe und Mischungen
7 (Teile 2a, b, 3)	Chemische Analytik
8 (Teil 2a)	Entwicklung von *in vitro*-Tests für NSAID und ß-Blocker
9 (Teil 2a)	Optimierung der *in vitro*-Tests
10A, B (Teile 2a, 3)	Validierung der *in vitro*-Tests
11	Berichte
12	Vorträge, Projekttreffen, Abschlusssymposium

Die unterschiedlichen Projektteile wurden von folgenden Einrichtungen bearbeitet:

- Projektkoordination, Management und Berichte: Steinbeis Transferzentrum für Ökotoxikologie und Ökophysiologie, Rottenburg und Universität Tübingen, Physiologische Ökologie der Tiere
- Tests mit Fischen: Universität Tübingen, Physiologische Ökologie der Tiere
- Tests mit Flohkrebsen: GWT-TUD GmbH, Dresden
- Sedimenttests mit Würmern und Tests mit Schnecken: Universität, Frankfurt, Aquatische Ökotoxikologie
- Tests mit *Daphnia magna*: Umweltbundesamt
- Chemische Analytik: DVGW- Technologiezentrum Wasser, Karlsruhe
- Biosensorentwicklung: Steinbeis Innovationszentrum für Zellkulturtechnologie, Mannheim

Für die Inhalte der einzelnen Kapitel dieses Berichts zu den o.g. Projektteilen sind die genannten Kooperationspartner verantwortlich.

Im Zentrum der Studien, die im Projekt Eff-Pharm durchgeführt wurden, standen Beta-blocker (repräsentiert durch Metoprolol) und NSAIDs (repräsentiert durch Diclofenac). Diese beiden Arzneimittelgruppen sind in Deutschland weit verbreitet, treten in relativ hohen Konzentrationen in aquatischen Ökosystemen auf und wurden dementsprechend als umweltrelevant eingestuft (Ebert et al. 2014). In Oberflächengewässern treten Diclofenac und Metoprolol im ng/L- bis µg/L-Bereich auf, im Abwasser von Kläranlagen erreichen beide Pharmazeutika den µg/L-Bereich. In Sedimenten von Flüssen wurden Konzentrationen von bis zu 52 µg/kg Diclofenac und 33 µg/kg Metoprolol nachgewiesen (Ramil et al. 2010, Camacho-Munoz et al. 2013).

Die in vivo-Studien mit den beiden Einzelsubstanzen erbrachten folgende Resultate:

In Konzentrationen von 0,1-1000 µg/Liter Diclofenac oder Metoprolol wurden keine Einflüsse der beiden Testsubstanzen auf die Entwicklung, die Überlebensrate, die Herzschlagfrequenz und das Körpergewicht von Bachforellenembryonen beobachtet. Im Gegensatz hierzu erhöhten Konzentrationen von ≥100 µg/L Diclofenac die Mortalität von juvenilen Bachforellen deutlich. Darüber hinaus zeigten diese Tiere ab 10 µg/Liter Diclofenac ein verstärktes Auftreten von Bissspuren, ein Zeichen vermehrten aggressiven Verhaltens. Für Metoprolol konnten solche Effekte nicht nachgewiesen werden. Ebenso wurden der Hsp70-Gehalt sowie der Grad an Lipidperoxiden bei juvenilen Bachforellen von beiden Pharmazeutika nicht beeinflusst. Die histologischen Studien deuten auf einen generell leicht erhöhten Stress durch die Laborexposition hin, ab einer Konzentration an Diclofenac von 10 µg/L war jedoch eine deutlich zunehmende Degradation des Nierengewebes zu beobachten. Generell zeigte sich, dass juvenile Individuen von Bachforellen sensitiver auf Pharmazeutika reagieren als die frühen embryonalen Stadien im Schutz des Chorions.

Gammarus fossarum wurde gegenüber Konzentrationen von 0,49 - 40 mg/L Diclofenac bzw. 5 - 450 mg/L Metoprolol exponiert und hinsichtlich Mortalität, des Verhältnisses von juvenilen zu adulten Tie-

ren, der Anzahl von Präkopula-Stadien und der Anzahl an Eiern pro Weibchen untersucht. Der sensitivste Endpunkt für eine Diclofenac-Wirkung war das Verhältnis von Juvenilen zu Adulti mit einer NOEC von 0,70 mg/L und einer LOEC von 2,62 mg/L. Als sensitivster Endpunkt für eine Wirkung von Metoprolol wurde ebenfalls das Verhältnis von juvenilen zu adulten Tieren und die Anzahl an Eiern pro Weibchen erkannt, jeweils mit einer NOEC von 5 mg/L und einer LOEC von 15 mg/L. Wie bei Forellen blieben die Level von Hsp70 und Lipidperoxiden von beiden Pharmazeutika unbeeinflusst.

Als Vertreter der Weichtiere wurde die Schnecke *Potamopyrgus antipodarum* gegenüber verschiedenen Konzentrationen von Metoprolol exponiert. Es traten signifikant erhöhte Hsp70 Levels bei Exposition gegenüber Konzentrationen von ≥ 3.2 mg/L des Wirkstoffs auf (NOEC: 1mg/L). Ab 10mg/L Metoprolol wurde eine 10%ige Reduktion der Nachkommenzahl beobachtet.

Bei *Daphnia magna* wurden die Mortalität, die Anzahl der Nachkommen, der Zeitpunkt der ersten Reproduktion und Verhaltensänderungen in Reaktion auf 1,9 - 50 mg/L Diclofenac bzw. 0,1 - 25 mg/L Metoprolol erfasst. Als sensitivster Parameter erwies sich die Reproduktion mit einer LOEC von 6.25 mg/L für Diclofenac bzw. 2.5 mg/L für Metoprolol.

Alle Versuche zur *Mischungstoxizität* von Diclofenac und Metoprolol, die mit Fischen, Gammariden oder Daphnien durchgeführt wurden, ergaben für keine der Kombinationen, Testarten oder untersuchten Endpunkte Hinweise auf synergistische Wirkungen dieser Pharmazeutika.

Toxizitätstests mit dem Sediment-bewohnenden Oligochaeten *Lumbriculus variegatus* erbrachten eine LOEC für die Reproduktion von 100 mg/kg (Sediment Trockengewicht) für Diclofenac und 255 mg/kg (Sediment Trockengewicht) für Metoprolol. In Mischungsversuchen mit beiden Substanzen wurde keine synergistische Wirkung nachgewiesen. Generell wurden auch Hsp70-Gehalte und Lipidperoxide nicht durch die beiden Stoffe beeinflusst.

Mesokosmos-Experimente, die in Fließrinnen durchgeführt wurden, adressierten den möglichen Einfluss von 0,47; 1,9; 7,5 und 30 mg/L Metoprolol (Nominalkonzentrationen) auf *G. fossarum*, *P. antipodarum* und *L. variegatus*, die entweder direkt in der fließenden Welle ("freilebend") oder in kleinen Expositionsgefäßen ("*Enclosures*") exponiert wurden. Die Resultate spiegelten weitgehend die Befunde aus den obengenannten Toxizitätsexperimenten mit den einzelnen Arten wieder. Für einige Parameter verstärkten jedoch die Bedingungen in den Fließrinnen (höchstwahrscheinlich bedingt durch den Wasserfluss) den Effekt von Metoprolol. So zeigten "freilebende" Gammariden in den Fließrinnen eine Reduktion der Anzahl von Präkopulastadien mit steigenden Metoprolol-Konzentrationen. Des Weiteren lag die ermittelte EC_{10} für die Reproduktion (28d) mit 0,594 mg/L niedriger als in den Einzelspeziestests. Auch für die anderen Arten wurde die stärkste Metoprolol- Toxizität bei "freilebenden" Individuen festgestellt (*L. variegatus*: EC_{10} Reproduktion (40d): 0,569 mg/L; *P. antipodarum*: EC_{10} Reproduktion (40d): 0,253 mg/L).

In Annäherung an *Expositionsbedingungen im Freiland* wurden sowohl Embryonen der Bachforelle als auch Gammariden gegenüber Wasser eines Kläranlagenauslaufs (Kläranlage Eriskirch am Bodensee), welches mittlere Konzentrationen von 1,3 µg/L Diclofenac und 1,4 µg/L Metoprolol aufwies, exponiert. In Embryonen der Bachforelle wurden signifikant reduzierte Lipidperoxid-Werte sowie ein verringertes Körpergewicht nachgewiesen, wohingegen bei Gammariden eine verminderte Anzahl an Eiern pro Weibchen, einhergehend mit einem höheren Körpergewicht, festgestellt werden konnte.

Die Resultate der *in vivo*-Studien sind in Table 4 zusammengefasst.

Table 4: Sensitivste Parameter und niedrigste LOECs (Realkonzentrationen)

	Testart	Diclofenac	Metoprolol
Labor Einzelsubstanz	Fische: *Salmo trutta* f. *fario*	Verhalten, Histologie: 10 µg/L	Histologie Niere > 745 µg/L
	Flohkrebse: *Gammarus fossarum*	Verhältnis Juvenile / Adulte: 2,6 mg/L	Verhältnis Juvenile / Adulte sowie Anzahl Eier/eitragendem Weibchen: 15 mg/L
	Wasserflöhe: *Daphnia magna*	Reproduktion: 6,25 mg/L	Reproduktion: 2,5 mg/L
	Schnecken: *Potamopyrgus antipodarum*		Proteotoxizität: 3,2 mg/L
	Rundwürmer: *Lumbriculus variegatus*	Reproduktion (28d): 100 mg/kg	Reproduktion (28d): 255 mg/kg
Labor Mischung	Fische: *Salmo trutta* f. *fario*	Histologie: 68,6 /676 µg/L (¾ TU DIC + ¾ TU MET)	
	Flohkrebse: *Gammarus fossarum*	Keine signifikante Effekte bei 1.97/11.25 mg/L (¾TU DIC + ¾ TU MET)	
	Wasserflöhe: *Daphnia magna*	Reproduktion: 4,69 / 1,88 mg/L (¾ TU DIC + ¾ TU MET)	
	Rundwürmer: *Lumbriculus variegatus*	Reproduktion: 203.6 mg/kg	
Mesokosmos	Flohkrebse: *Gammarus fossarum*		Eier pro eitragendem Weibchen: 3 mg/L
	Schnecken: *Potamopyrgus antipodarum*		Reproduktion Enclosure (28d): 0,22 mg/L
	Rundwürmer: *Lumbriculus variegatus*		Reproduktion frei in der Rinne EC50 (40d): 3,38 mg/L
Freiland / KA-Ablauf	Fische: *Salmo trutta f. fario*	Lipidperoxide (Abnahme), Körpergewicht (Abnahme)	
	Flohkrebse: *Gammarus fossarum*	Eier pro eitragendem Weibchen	

Für beide Arzneimittelklassen, Beta-blocker und NSAIDs, wurden sensitive *Biosensoren* entwickelt und etabliert.

Für den Effekt-basierten Nachweis von Beta-blocker-Wirkungen zeigte der Beta-blocker CEPAC Sensor Assay die beste Eignung und wurde deshalb für die weitere Entwicklung eines *Mode of Action*-basierten *in vitro*-Tests ausgewählt. Im Gegensatz hierzu zeigten einige publizierte roGFP Redox-Sensoren nach stabiler Transfektion in CHO-Zellen nur ein schwaches Fluoreszenzsignal und ein schlechtes Verhältnis von Signal und Hintergrundrauschen. Obwohl es möglich war, H_2O_2-induzierten, oxidativen Stress im Fluoreszenzmikroskop zu detektieren, war es aufgrund des schwachen Signals nicht möglich, diesen Assay im Mikroplattenformat zu etablieren. Aus diesem Grund war es nötig, eine neue roGFP Mutante, roGFP3, mit einem stark verbesserten Fluoreszenzsignal zu generieren. Hierbei resultierte eine Fusion von Grx-1 mit roGFP3 in einem stark verbesserten Redox-Sensor Grx-roGFP3. Dieser Sensor wurde im Weiteren für die Entwicklung von NSAID-Biosensor-Zelllinien verwendet. Auf der Basis dieses neuen Redox-Sensors wurde somit ein Biotest für Redox-basierte Messungen der Cox-1-Aktivität entwickelt.

Die Subklonierung gemischter Zellpopulationen von Beta-blocker- und NSAID-Biosensoren resultierte in Einzelzellklonen mit stark verbessertem Verhältnis von Signal und Hintergrundrauschen. Diese Klone wurden zur Bestimmung von IC_{50}-Konzentrationen für Metoprolol bzw. Diclofenac verwendet. Hierbei zeigten die sensitivsten Beta-blocker-Sensorklone eine halbmaximale Signalreduktion bei einer Metoprololkonzentration von ca. 15 nM. Die sensitivsten NSAID-Sensorklone zeigten eine halbmaximale Signalreduktion bei einer Diclofenac-Konzentration von ca. 2 nM. Hierbei erforderte das äußerst schnell erzeugte Signal bei beiden Assays nur eine sehr kurze Inkubationszeit der Sensorzellen mit den zu analysierenden Lösungen. Somit besteht bei der Anwendung dieser neuartigen Bioassays keine Notwendigkeit, unter sterilen Bedingungen während der Messungen zu arbeiten, was insbesondere gegenüber Reportergen-Assays von Vorteil ist.

Proben von Haushaltsabwässern, durch Festphase-Extraktion angereichert, wurden nach Verdünnung im Puffer mit den neu entwickelten Beta-blocker- und NSAID-Biotests analysiert. Hierbei konnten Beta-blocker- und NSAID-Aktivitäten in diesen Proben gemessen und mit Metoprolol- und Diclofenac-Konzentrationen, welche mittels LC-MS bestimmt wurden, verglichen werden. Aufgrund der hohen Sensitivität der neu entwickelten Biosensor-Assays war es möglich, auch NSAID- und Beta-blocker-Aktivitäten in Proben zu bestimmen, die nicht durch Festphase-Extraktion angereichert wurden. Es zeigte sich, dass die gemessenen Beta-blocker-Aktivitäten in Abwasserproben zwar signifikant höher lagen als die mit LC-MS bestimmten Metoprolol-Konzentrationen, dass diese Differenz jedoch durch ebenfalls mittels LC-MS quantifizierte andere Beta-blocker erklärt werden konnte. Ein derartiger Effekt trat bei der Bestimmung von NSAID-Aktivitäten nicht auf, die im Wesentlichen durch die Rückstände von Diclofenac in diesen Proben erklärt werden konnten. In weiteren Experimenten werden Validierungen mit internen Standards durchgeführt, um mögliche Matrixeffekte beurteilen zu können. Unterschiedliche Abwasserproben werden charakterisiert, und angereicherte sowie native Proben werden in diesem Zusammenhang miteinander vergleichen.

Die im Projekt Eff-Pharm erzielten Resultate zeigen unzweifelhaft die Präzision und die hohe Sensitivität beider Biosensor-Zelllinien, welche (a) die Konzentrationen von Beta-blockern und NSAIDs in der Umwelt erfassen und (b) die für die sensitivsten Arten und Endpunkte bekannten LOECs und NOECs abbilden. Somit sind diese Biosensoren als höchst vielversprechend für den Einsatz im Umweltmonitoring anzusehen.

RESULTS

1 WP3A Toxicity of pharmaceuticals - single substances (Part II)

1.1 Studies with juvenile brown trout

1.1.1 Introduction

Fish are a useful model for organisms of higher trophic levels in aquatic ecosystems. However, toxicity tests usually employ standard test species of comparably low environmental relevance. In our experiment we used brown trout *Salmo trutta* f. *fario*, a species of high ecological and economical relevance in Middle Europe.

Our aim was to identify the effect of two pharmaceuticals, the non-steroidal anti-inflammatory drug diclofenac and the beta-blocker metoprolol, on various parameters in juvenile brown trout. The test concentrations were aimed to be in a sublethal range, thus, mortality was not an original endpoint of interest. Instead, sublethal effects, like changes in the stress protein level or amount of lipid peroxides, as well as histological alterations of gills, liver, kidney and heart were of paramount importance.

Overall, the experiments provide new data on the potential toxicity of the investigated pharmaceuticals – thereby helping to create a framework of effect data, the responses in the *in vitro*-systems can be related to.

1.1.2 Materials and Methods

1.1.2.1 Fish exposure

Exposure of juvenile brown trout to diclofenac was started on May 21th 2014 and ended 27 days later on June 17th 2014. Fish were exposed to diclofenac in concentrations of 0 (control), 0.1, 0.5, 1, 10 and 100 µg/L. The substance was applied as diclofenac sodium salt (CAS 15307-79-6), which is readily soluble in water, so no additional organic solvents had to be used. Due to high mortality in the highest concentration (100 µg/L), the exposure in this group was terminated already after 14 days.

The re-run of the experiment was started on July 10th 2015 and ended 24 days later on August 3rd 2015. Except for the age of fish (comparable to that of fish used for the metoprolol exposure in 2014) and a slight modification of the test concentrations, the test setup was kept the same as in 2014. The diclofenac concentrations were: 0 (control), 0.1, 1, 10, 100 and 200 µg/L. The exposure time was slightly shortened (24 days) compared to the first experiment due to high mortality rates in the treatments with higher test concentrations.

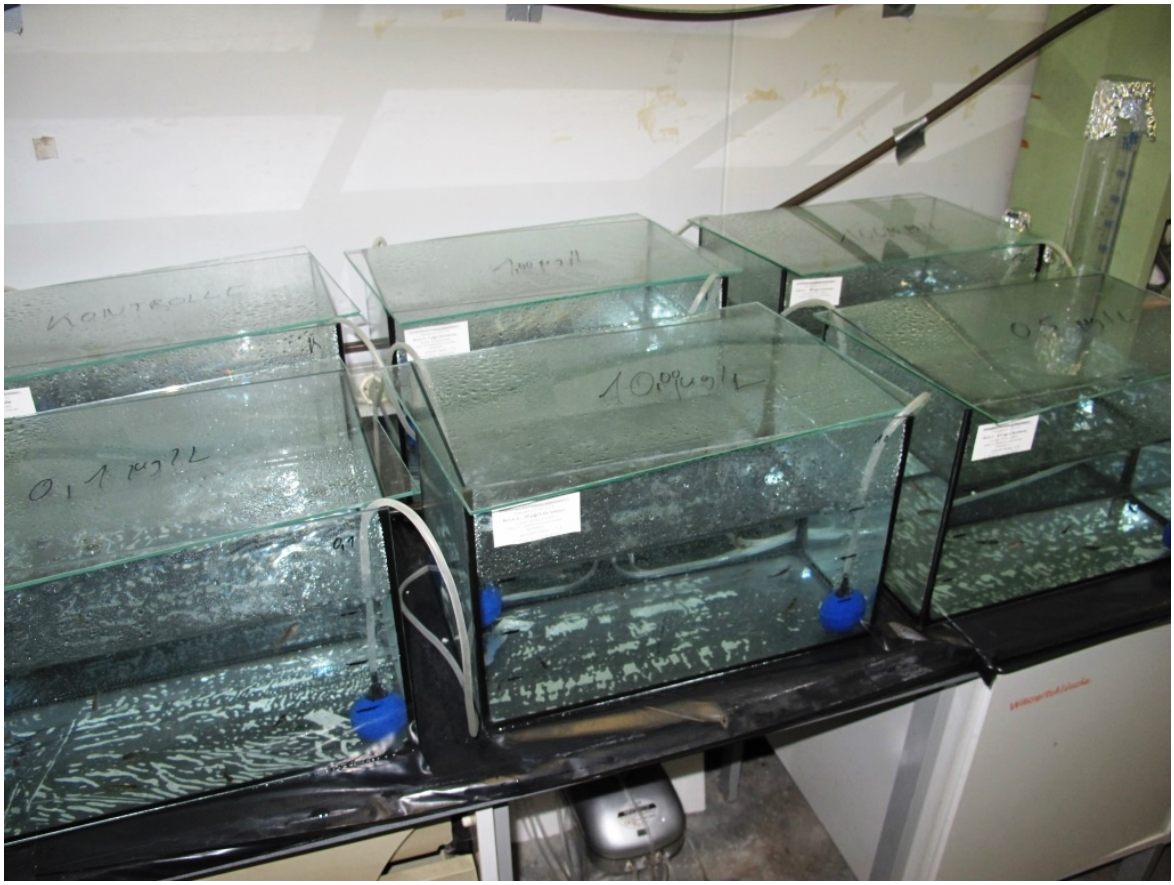
The exposure of juvenile brown trout to metoprolol was started on July 29th 2014 and ended 28/29 days later on August 26th and 27th 2014 (the sampling had to be done over two days because of the high sample size). The fish were exposed to metoprolol in concentrations of 0 (control), 0.1, 1, 10, 100 and 1000 µg/L. The substance was applied as metoprolol tartrate (CAS 56392-17-7), which is also soluble in water so no solvent had to be used.

For all three experiments, three 25 litre aquaria were used per concentration and 13 individual fish were exposed per aquarium (Figure 5). Per experiment, 234 animals were exposed in 18 different aquaria. The aquaria were grouped in three blocks, within each every concentration was represented. This experimental design was necessary to allow a statistical evaluations controlling for the aquarium/block as a confounding factor and reducing the problem of pseudo-replication (Hurlbert, 1984). The exposure took place in a thermo-constant-chamber set to 8 °C and a 12 h:12 h light-dark-cycle; animals were fed every day. Water exchanges were done every three- to four days, where one quarter to one third of the aquarium volume was removed and replaced with the same volume of freshly pre-

pared pharmaceutical solution. As references, and as a possibility to control for the effect of the lab exposure by itself, three samplings were realized directly at the fish farm in 2014 (May 9th, July 11th and September 3rd) and two in 2015 (July 7th and August 8th).

Mortality during the experiment was recorded. Additionally, water parameters of the aquaria, including temperature, pH, oxygen content, oxygen saturation and concentration of nitrite, were regularly documented. At the end of the exposure, all animals were euthanized by an overdose of tricaine mesylate (ethyl 3-aminobenzoate methane sulfonate, CAS 886-86-2), followed by a neck-cut. The recorded biometrical values included length [cm], standard length [cm] (only for the metoprolol exposure and the diclofenac re-run), mass [g], presence of ocular lesions, condition of the operculum, condition of the fins. The additional parameters for the metoprolol and the second diclofenac exposure were included because animals showed a more aggressive behaviour than fish in the first diclofenac exposure (since they were already older) – resulting in bite marks in fins and opercula. Tissue samples were taken to evaluate biochemical and histological parameters. For histology, samples were chemically conserved (see description below), for biochemical analyses, samples were directly frozen in liquid nitrogen. The tissue samples included gill, liver, head, kidney, heart (only for metoprolol and the re-run of diclofenac) and the remaining anterior part. Furthermore, the remaining posterior part of the animal was frozen and stored at -80 °C to allow chemical analysis of the pharmaceutical concentrations in the tissue. After consultation with UBA, we decided to measure the tissue concentration for the second diclofenac experiment. Per concentration, two pools of four to five individual animals were freeze dried and homogenized before further processing.

Figure 5: General test setup for the single substance exposure of brown trout: 18 aquaria were arranged in three blocks, each block containing one control and the five test concentrations. 13 fish were exposed per aquarium.



1.1.2.2 Determination of stress protein Hsp70

The Stress protein level was quantified in the liver of exposed fish. Samples were stored at -80 °C until processing. The frozen samples were mechanically homogenized on ice in an appropriate volume (50 to 300 µL) of extraction medium (consisting of 96 % concentrated extraction buffer (80 mM potassium acetate, 5 mM magnesium acetate, 20 mM Hepes in bidestilled water, adjusted to a pH of 7,5 and 2 % protease inhibitor). Homogenized samples were centrifuged at 4 °C and 20000 rcf for 10 min. 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 (20 % glycerine, 3 % sodium dodecyl sulphate, 0.3 % β-mercaptoethanol, 10 mM Tris pH 7 and 0.005 % bromophenol blue in bidestilled water) in a proportion of 2 parts supernatant to 1 part SDS buffer, and boiled at 95-100 °C for 5 min. Both mixtures were stored at -20 °C until further usage.

The protein content was quantified according to Bradford (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 (0.001 % Coomassie brilliant blue G-250, 4.75 % ethanol, 8.5 % phosphoric acid in bidestilled water) 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) were applied to the gels, following the results of the Bradford analysis. To allow for a comparison between different gels, a standard (brown trout full body homogenate) in duplicate was added to every gel. Gel chambers were placed in E-buffer + SDS (0.19 M glycin, 25 mM $Tris_{base}$ and 0.1 % SDS). Gels were run for 15 min at a voltage of 80 V, followed by 1 h at 120 V to separate the proteins.

Protein transfer from the gel to a nitrocellulose membrane was performed via semi-dry Western blot. The membrane was fitted to the gel, encased in Whatman-papers soaked in transfer buffer (0.0076 % $Tris_{HCl}$, 0.5072 % $Tris_{base}$, 0.2904 % glycine and 0.00368 % SDS 20 % methanol in bidestilled water, adjusted to a pH of 9) and placed in a blotting chamber. For each gel used in the blotting, an amperage of 90 mA was applied, with the voltage not exceeding 10 V. The blotting process was done over a time period of 2 hours.

After the blotting process, the filter was blocked in blocking solution (TBS (0.88 % sodium chloride, 0.635 % Tris-HCl and 0.118 % Tris-base in bidestilled water, adjusted to a pH of 7.5) with horse serum in a mixture of 1:2) for one hour. 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 (1 mM 4-chloro-1-naphtol, 6 % methanol, 0.015 % hydrogen peroxide in 30 mM Tris pH 8.5) 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 an Hsp70 standard (prepared from full body homogenate of *Salmo trutta* f. *fario*).

1.1.2.3 Determination of lipid peroxides

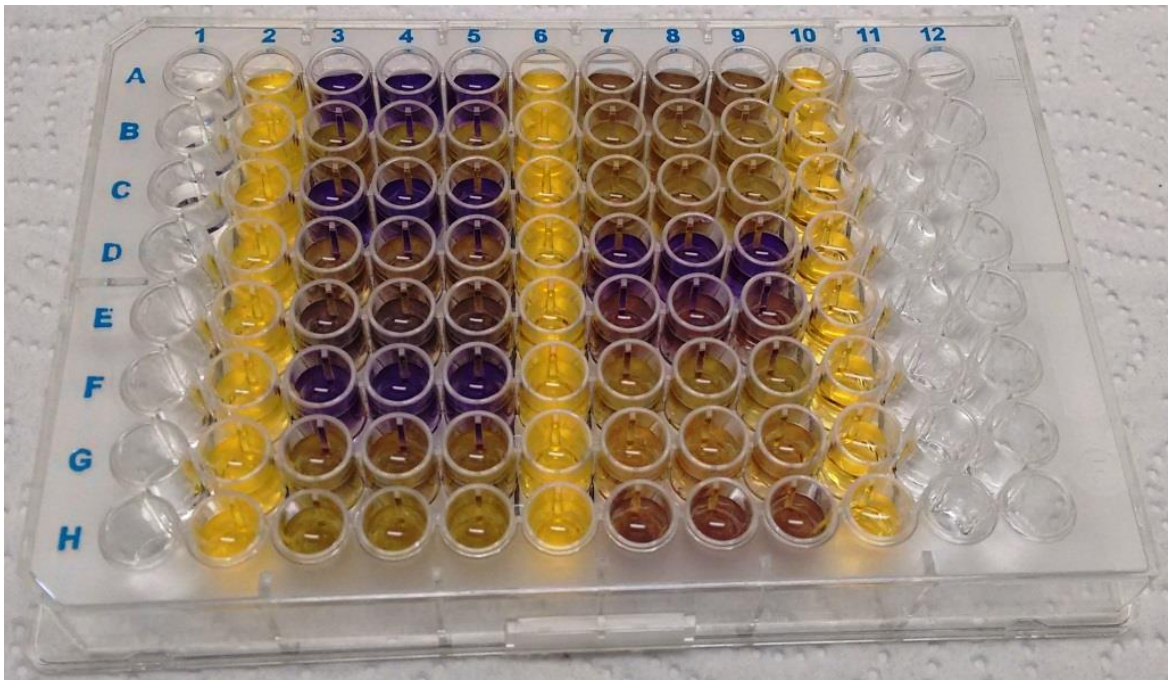
The FOX assay procedure was conducted according to a modified version of the protocol proposed by HERMES-LIMA ET AL. (1995). The head of the sampled trout were used for lipid peroxide quantification. Samples were stored at -80 °C until further processing. The samples were weighed, diluted in a ratio of 1:3 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 (Figure 6). 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 bidestilled water. Each sample was tested in triplicates, 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 150 min 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 another incubation period of 30 min, 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 ($CHP_{equiv.}$/mg wet weight) were calculated using the following equation:

$$\frac{A580nm}{A580nmCHP} * volumeCHP * \frac{total\ volume}{sample\ volume} * dilution\ factor = \frac{A580nm}{A580nmCHP} * 1 * \frac{200}{20} * 3$$

Figure 6: 96h well plate used for ferrous oxidation xylenol orange assay



After the first experiment was evaluated, the target organ was switched to kidney for the repeated experiment. We hoped that this switch to a more homogenous organ would yield results with lower variability. For the second diclofenac exposure, the dilution was adjusted to 1:7, and the first incubation time to 135 min. Otherwise, the procedure remained the same.

1.1.2.4 Histopathological examinations

Histological examinations were done for samples of the gill, kidney and liver. Additionally, the hearts of the metoprolol-exposed animals were investigated. The tissue samples were fixed in 2 % glutardialdehyde in 0.1 M cacodylate buffer (dimethylarisinic acid sodium salt, pH 7.6), stored at 4 °C for several weeks to guarantee a complete fixation of the tissue. Since the scope of the study included only an exemplary overview on the histological effects, three samples per block and treatments were investigated, totalling to nine samples per concentration.

Before the 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 12 h). Samples were placed in tissue embedding cassettes; the dehydration and embedding in paraffin took place in an automated tissue infiltrator (TP 1020, Leica).

Histological sections were cut with a sledge microtome (SM 2000 R, Leica) set to a thickness of 3 µm. One part of the slices was stained with hematoxilin-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).

1.1.2.5 Statistical analysis

Statistical analysis was conducted using SAS JMP 11.0 and R 3.2.1. Data were checked for normal distribution using the residual histogram and quantile-quantile plot. If necessary, data were transformed to fit the assumptions of parametric testing – if not stated otherwise, the analyses were done on the original data. Data were checked for homogeneity of variance using the Fligner-Killeen test.

Mortality data was analysed using COX-regression. The biometrical data on length and mass, as well as the Hsp70 level and level of lipid peroxides were analyzed using a nested ANOVA including the aquarium as a nested factor. The significance level was set to α=0.05.

Since the histological data was only assessed in an exemplary manner, this data was evaluated qualitatively without statistical testing.

1.1.3 Results

1.1.3.1 Chemical analysis

The measured concentrations of diclofenac in the first run were: <detection limit for the control, 0.095, 0.498, 0.942, 10.5 and 100.2 µg/L. In the re-run, the measured concentrations were <detection limit for the control, 0.09, 0.95, 9.2, 98 and 200 µg/L. All concentrations were in a close range of the nominal concentrations. The measured concentrations of metoprolol were <detection limit for the control, 0.094, 0.95, 6.9, 86 and 745 µg/L, slightly deviating from the nominal concentrations. In all following graphs, measured concentrations are displayed.

1.1.3.2 First diclofenac exposure

All physico-chemical water parameters were within the normal range (Table 5). During the exposure an unexpected high mortality was observed, especially in the group exposed to the highest concentration of diclofenac (100 µg/L]. After 14 d, the mortality rate had reached 64 %, so the exposure of this group was ended and the animals were sampled to assure a sufficient sample size for the further analyses. The mortality at 14 d was significantly increased in the 100 µg/L exposure compared to all other exposures (COX-regression, df=5, χ^2=43.54, p<0.0001). In the other exposure groups, including the control, a relatively high mortality became obvious as well, especially in the second half of the exposure period. On day 27, the mortality had reached 46 % in the control and in the 10 µg/L treatment. No

significant difference in mortality could be observed between the different treatments (COX-regression, df=4, χ^2=1.13, p=0.8896). The results are displayed in Figure 7 and

Table 6.

Table 5: Physico-chemical parameters at the end of the first diclofenac exposure experiment of juvenile brown trout.

Block	Exposure group	temperature [°C]	pH	oxygen saturation [%]	oxygen content [mg/L]
1	control	8.1	8.42	95.70	10.74
1	0.1 µg/L DIC	8.3	8.49	95.80	10.72
1	0.5 µg/L DIC	8.0	8.49	96.80	10.82
1	1 µg/L DIC	8.2	8.50	96.40	10.84
1	10 µg/L DIC	8.1	8.50	97.30	10.78
2	control	8.3	8.50	95.20	10.61
2	0.1 µg/L DIC	8.3	8.49	96.10	10.77
2	0.5 µg/L DIC	8.1	8.50	96.10	10.71
2	1 µg/L DIC	8.3	8.50	95.60	10.66
2	10 µg/L DIC	8.2	8.50	96.90	10.78
2	100 µg/L DIC	8.2	8.50	96.70	10.78
3	control	7.3	8.45	95.00	10.83
3	0.1 µg/L DIC	7.2	8.46	95.20	10.94
3	0.5 µg/L DIC	7.7	8.48	96.80	10.75
3	1 µg/L DIC	7.3	8.49	95.90	10.97
3	10 µg/L DIC	7.4	8.49	96.50	10.94
3	100 µg/L DIC	7.5	8.47	95.80	10.94

Figure 7: Cumulative mortality of juvenile brown trout exposed to diclofenac in different concentrations over 27 days. At 100 µg/L, a significant increase in mortality was observed (COX-regression, df=5, $\chi 2=43.54$, $p<0.0001$), so this exposure was terminated after 14 days. The mortality in the other exposures increased especially in the second half of the experiment, but there was no significant difference between the treatments (COX-regression, df=4, $\chi 2=1.13$, $p=0.8896$)

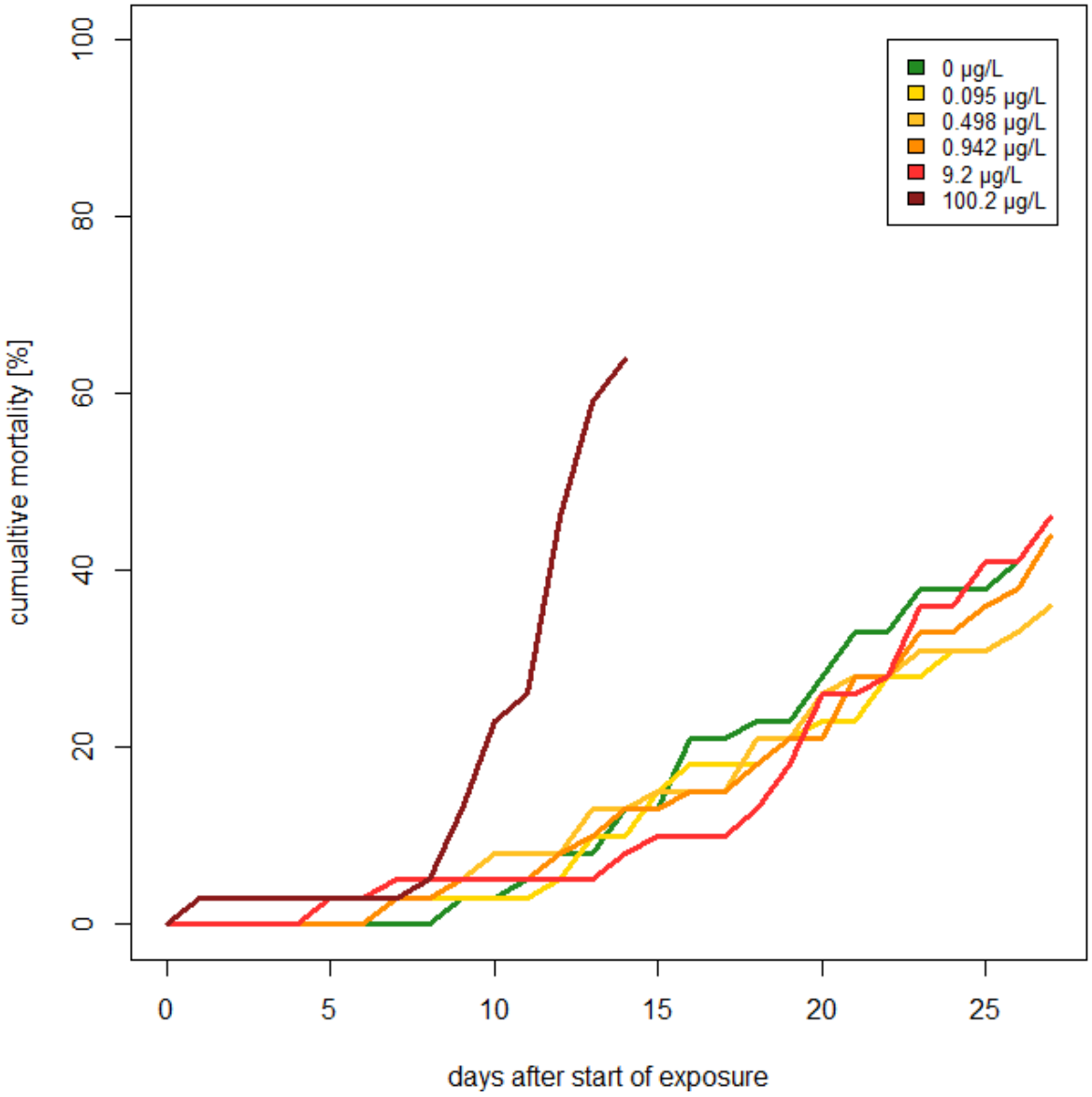


Table 6: Overview of the mortality rate of diclofenac-exposed juvenile brown trout after 27 days. All treatments, including the control, showed an apparently increased mortality, ranging between 23 % and 69 %. Mortality after 27 days of diclofenac exposure

Exposure group	Block 1	Block 2	Block 3	Overall
control	31	69	38	46
0.1 µg/L	31	38	38	36
0.5 µg/L	38	31	38	36
1 µg/L	38	69	23	44

Exposure group	Block 1	Block 2	Block 3	Overall
10 µg/L	38	62	38	46

The comparison of the biometrical measurements showed no significant differences in body length and body mass between the diclofenac treatments (body length: nested ANOVA, df=5, F=1.3698, p=0.2412; body mass: nested ANOVA, df=5, F=1.6892, p=0.1431). The slightly smaller size of the animals in treatment group "100.2 µg/L" is due to the fact that those were sampled 13 days before the others. The results are displayed in Figure 8 and Figure 9.

Figure 8: Length of juvenile brown trout after 27 days exposure to diclofenac. The treatment group "100.2" was exposed for only 14 days. The first hatchery control (hc1), illustrates the length of the animals at the beginning of the experiment and was not included in the analysis. Bold lines display the median values, boxes the 25 % to 75 % quantiles, whiskers the minimum and maximum values. There were no significant differences between the different treatment groups (nested ANOVA, df=5, F=1.3698, p=0.2412).

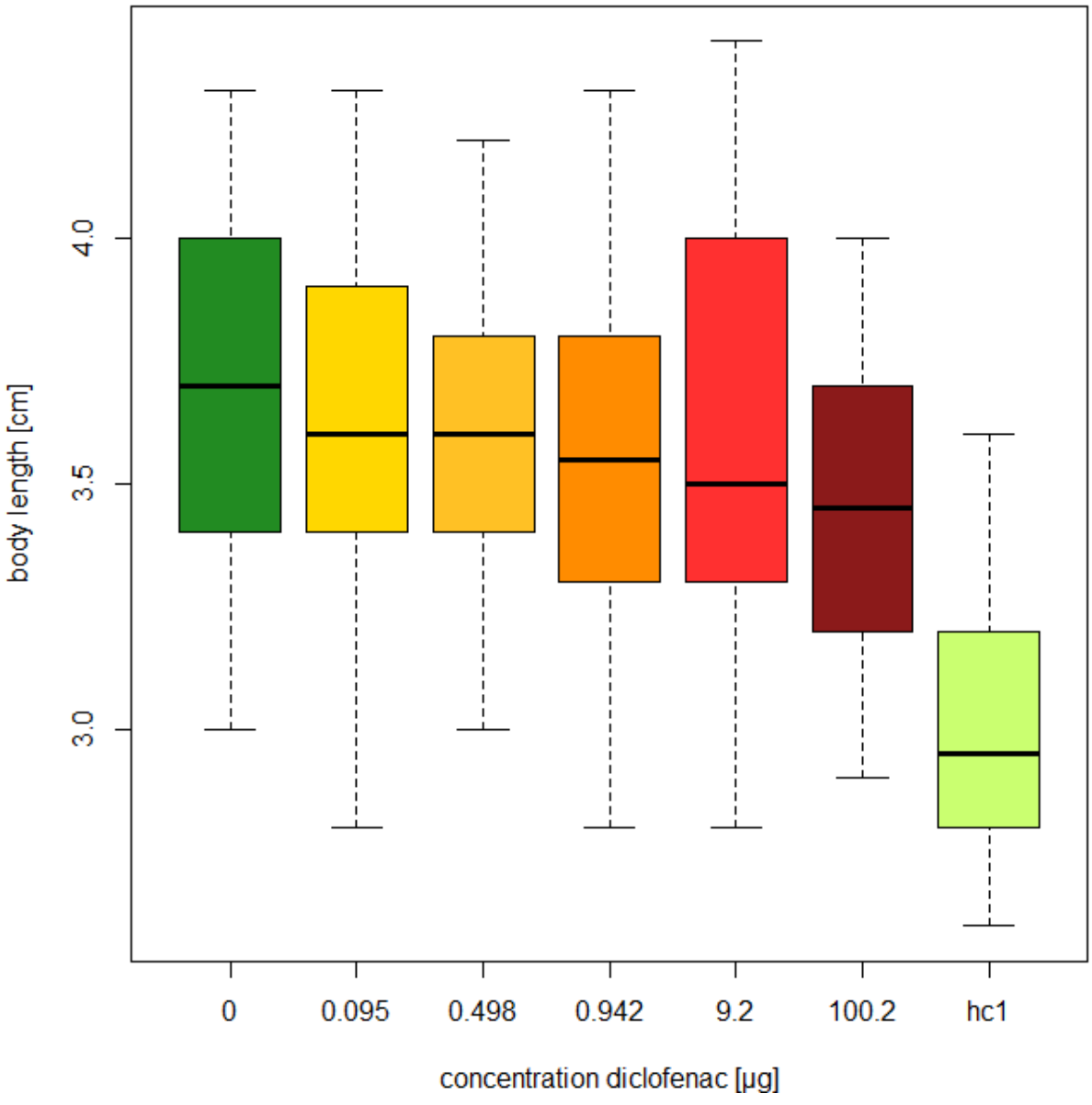
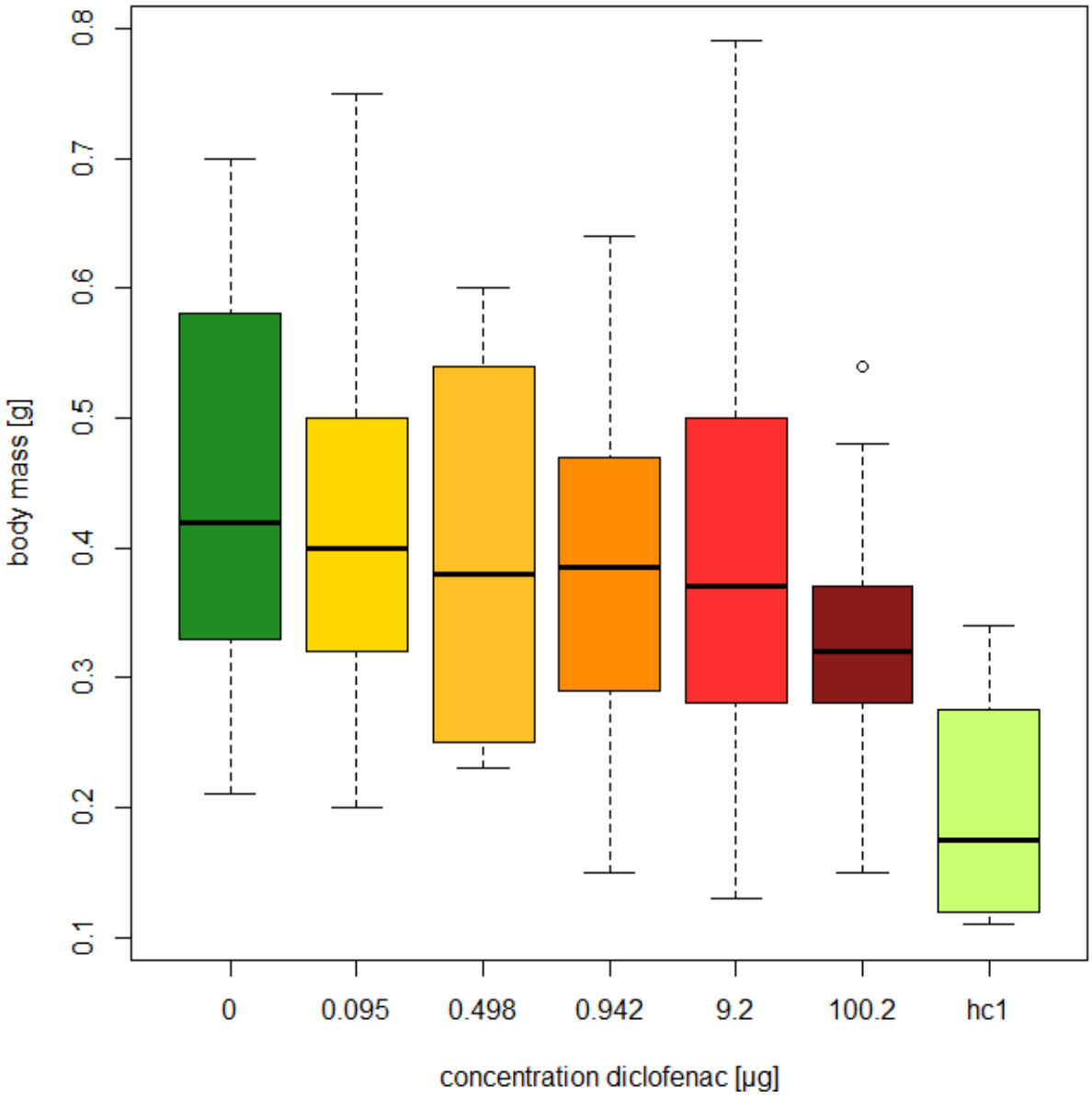
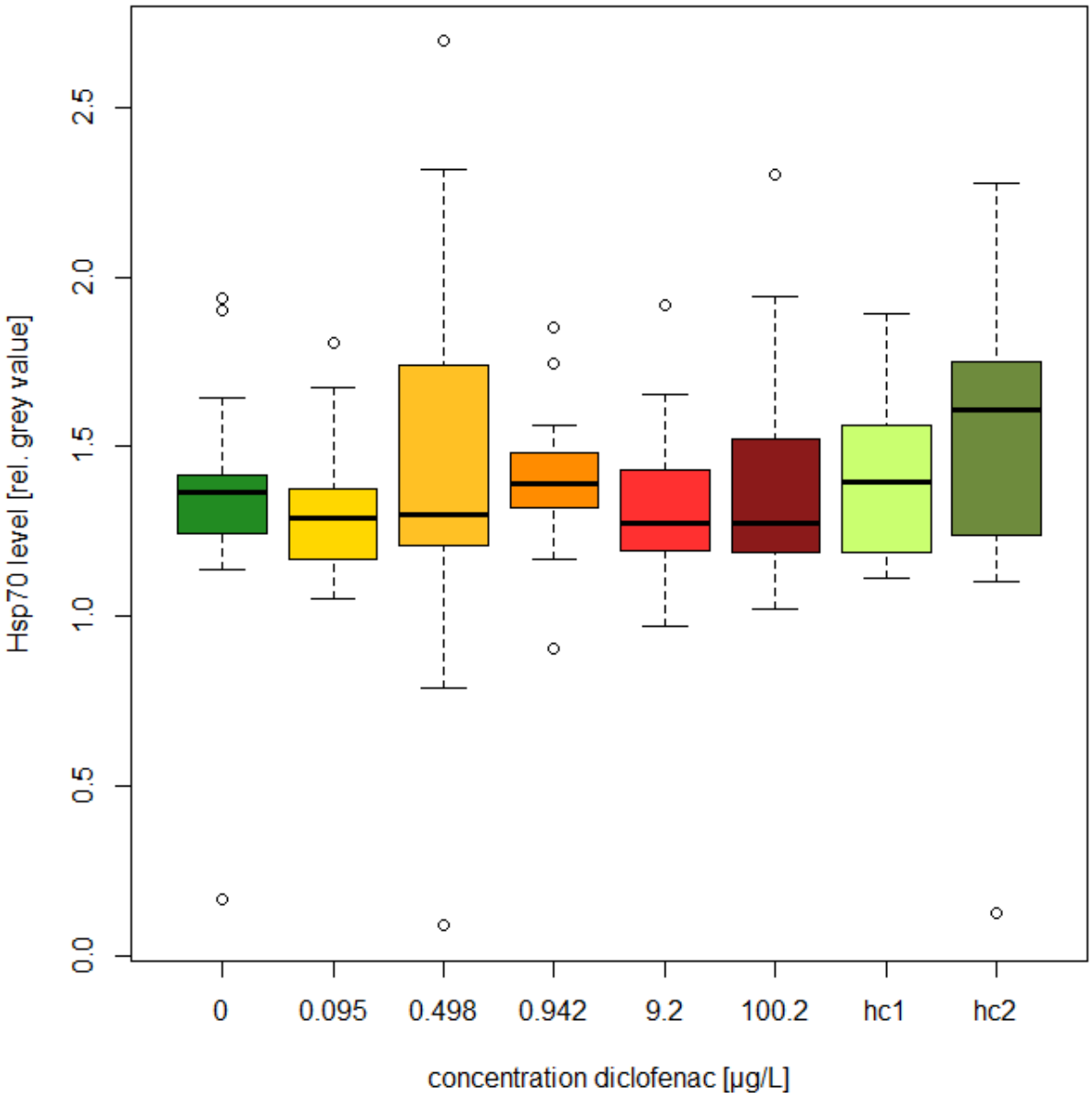


Figure 9: Body mass of juvenile brown trout after 27 days exposure to diclofenac. The treatment group "100.2" was exposed for only 14 days. The first hatchery control (hc1), illustrates the mass of the animals at the beginning of the experiment and was not included in the analysis. Bold lines display the median values, boxes the 25 % to 75 % quantiles, whiskers the minimum and maximum values, circles potential outliers. There were no significant differences between the different treatment groups (nested ANOVA, df=5, F=1.6892, p=0.1431).



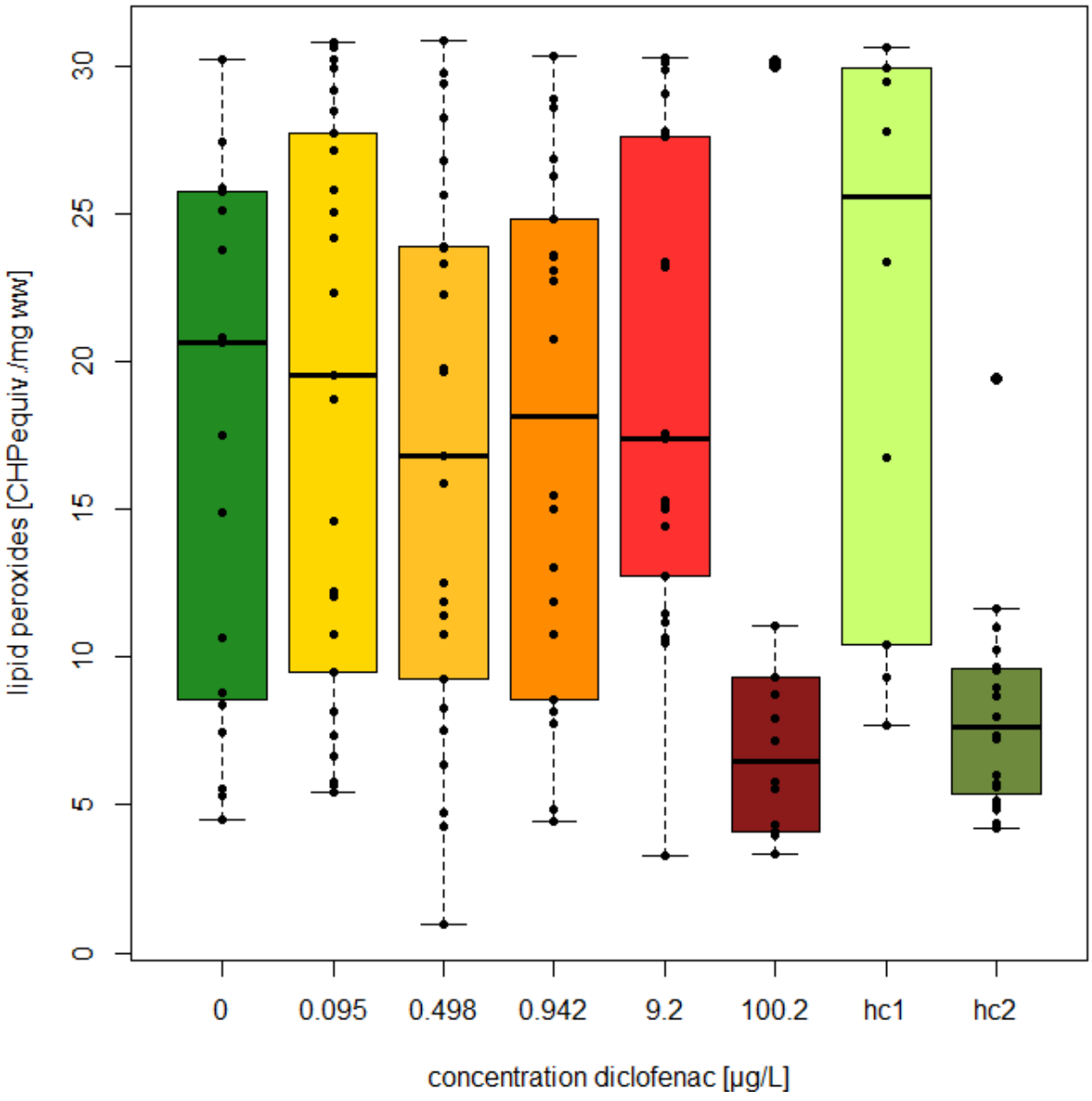
The Hsp70 level did not show an apparent difference between fish exposed to the different concentrations (linear model, df=5/103, F=0.4174, p=0.8357). Results are shown in Figure 10.

Figure 10: Hsp70 level of juvenile brown trout exposed to diclofenac for 27 days. “hc1“ and ”hc2“ depict the first and second sampling directly at the fish hatchery. Bold lines display the median values, boxes the 25 % to 75 % quantiles, whiskers the minimum and maximum values, circles potential outliers.



The FOX assay revealed an unusual distribution of the data *for* lipid peroxidation. Each treatment seemed to be separated into two different groups, one with high and one with low degree of lipid peroxidation. Sole exception is the second sampling at the fish hatchery, which showed generally low values of lipid peroxidation. Further statistical analysis revealed an effect of the sample mass, which was taken into account as covariate for the calculations. Only the highest concentration of diclofenac led to a significantly lower degree of lipid peroxidation (linear model with sample mass as covariate, df=6/119, F=11.1, p=0.0001). The results are shown in Figure 11.

Figure 11: Lipid peroxidation (in cumene hydroperoxide equivalents per mg wet weight) of head of juvenile brown trout exposed to diclofenac for 27 days. ”hc1“ and ”hc2“ depict the first and second sampling directly at the fish farm. Bold lines display the median values, boxes the 25 % to 75 % quantiles, whiskers the minimum and maximum values, original data points as black dots.



Since the experiment was repeated to achieve more reliable results, the evaluation of the histological samples was omitted for the first diclofenac exposure.

1.1.3.3 Second diclofenac exposure

In the second experiment with diclofenac, all physico-chemical water parameters were within the normal range (Table 7). An unexpectedly high mortality rate became obvious as clear effect of the treatment. While the overall control mortality did not exceed 10 %, survival was affected already at 0.1 µ/L diclofenac. Overall, a significant effect of diclofenac treatment could be identified (nested COX-analysis, df=5, χ^2=13.457, p=0.0194), while the aquaria themselves (nesting factor) did not show differences (df=12, χ^2=14.263, p=0.2842) (Figure 12). Already at 0.1 µg/L, the overall mortality rate was 33 %.

However, 1 µg/L showed a slightly lower mortality of 21 %. Higher concentrations increased the mortality rate even further, with the highest values being recorded for 100 µg/L and 200 µg/L (Table 8). A sequential analysis, omitting the block as irrelevant factor, yielded a LOEC of 100 µg/L and a NOEC of 10 µg/L.

Table 7: Physico-chemical parameters at the end of the second diclofenac exposure experiment of juvenile brown trout.

Block	Exposure group	Temperature [°C]	pH	Oxygen saturation [%]	Oxygen content [mg/L]
1	control	6.7	8.41	95.9	11.08
1	0.1 µg/L DIC	6.6	8.46	96.1	11.11
1	1 µg/L DIC	6.9	8.54	95.9	11.03
1	10 µg/L DIC	6.8	8.55	95.8	11.04
1	100 µg/L DIC	6.8	8.55	95.8	11.05
1	200 µg/L DIC	6.9	8.55	95.7	11.03
2	control	7.1	8.55	95.6	10.95
2	0.1 µg/L DIC	7.1	8.56	95.7	10.98
2	1 µg/L DIC	7.1	8.55	95.7	10.97
2	10 µg/L DIC	7.1	8.55	95.6	10.94
2	100 µg/L DIC	7.1	8.56	95.4	10.91
2	200 µg/L DIC	7.2	8.44	91.2	10.42
3	control	6.8	8.52	94.8	10.94
3	0.1 µg/L DIC	6.7	8.54	95.1	10.99
3	1 µg/L DIC	7.3	8.49	95.9	10.97
3	10 µg/L DIC	7.4	8.49	96.5	10.94
3	100 µg/L DIC	7.5	8.47	95.8	10.94

Figure 12: Cumulative mortality of juvenile brown trout exposed to diclofenac (rerun in 2015) in different concentrations over 24 days. Overall, the mortality rate was strongly increased by the treatment, especially in high concentrations (nested COX-analysis, df=5, χ^2=11.90, p=0.0361).

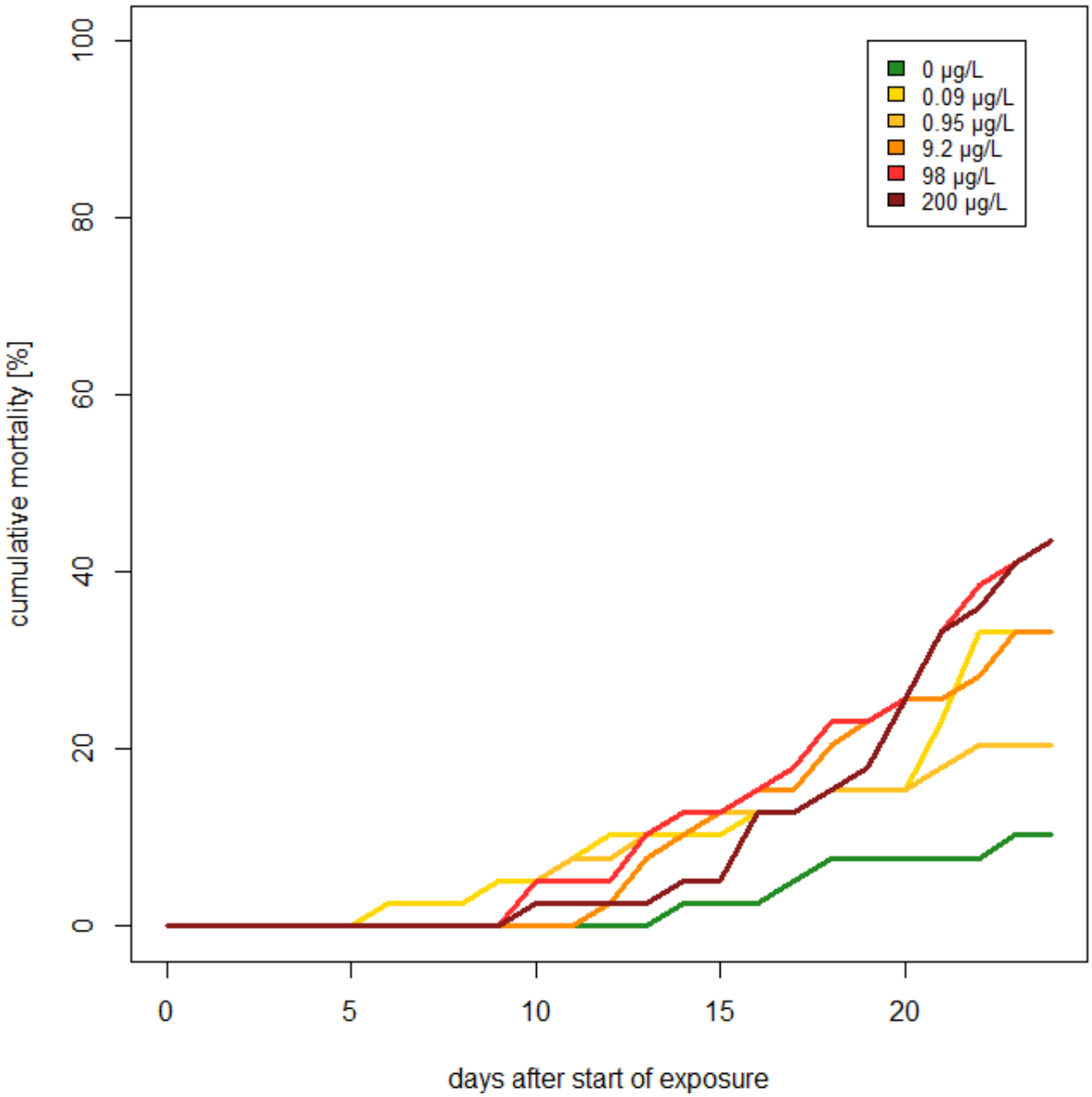


Table 8: Overview on the mortality rate of diclofenac-exposed juvenile brown trout after 24 days. All diclofenac treatments showed an increased mortality, ranging between 15 % and 62 %.

Exposure group	Block 1	Block 2	Block 3	Overall
control	8	15	8	10
0.1 µg/L	38	23	38	33
1 µg/L	15	15	31	21
10 µg/L	23	31	46	33
100 µg/L	54	54	23	44

Exposure group	Block 1	Block 2	Block 3	Overall
200 µg/L	15	54	62	44

The analysis of fish tissue concentration showed increasing concentrations of diclofenac in muscle with increasing water concentrations. While the concentrations were below the detection limit of 5 µg/kg dw in the control and the two lowest exposures, they rose up to a mean of 169.5 µg/kg dw in the highest tested exposure concentration (Table 9).

Table 9: Tissue concentration (muscle) of diclofenac in brown trout exposed to the substance for 24 days. The value refers to the mean of two pools (overall 10 individuals).

Exposure group	conc. water [µg/L]	Conc. Muscle [µg/kg dw]
control	0	< 5
0.1 µg/L	0.09	< 5
1 µg/L	0.95	< 5
10 µg/L	9.2	10.16
100 µg/L	98	84.60
200 µg/L	200	169.50

Additionally, it could be observed that fish exposed to higher concentrations of diclofenac showed marks of aggressive behaviour (bite marks on fins and body) more frequently than control animals (Figure 13 and Figure 14). While this difference could only be seen in tendency for the body injuries (Likelihood-ratio, n=161, df=5, χ^2=8.48, p=0.1315), the fin damages were significantly higher at 10, 100 and 200 µg/L diclofenac (Likelihood-ratio, n=161, df=10, χ^2=30.50, p=0.0007). Results are shown in Figure 12 and Figure 13.

Figure 13: Percentage of brown trout showing bite marks on their body (usually head, back or side). With increasing diclofenac concentrations, a higher proportion of animals was injured. This trend was not statistically significant (Likelihood-ratio, n=161, df=5, χ^2=8.48, p=0.1315).

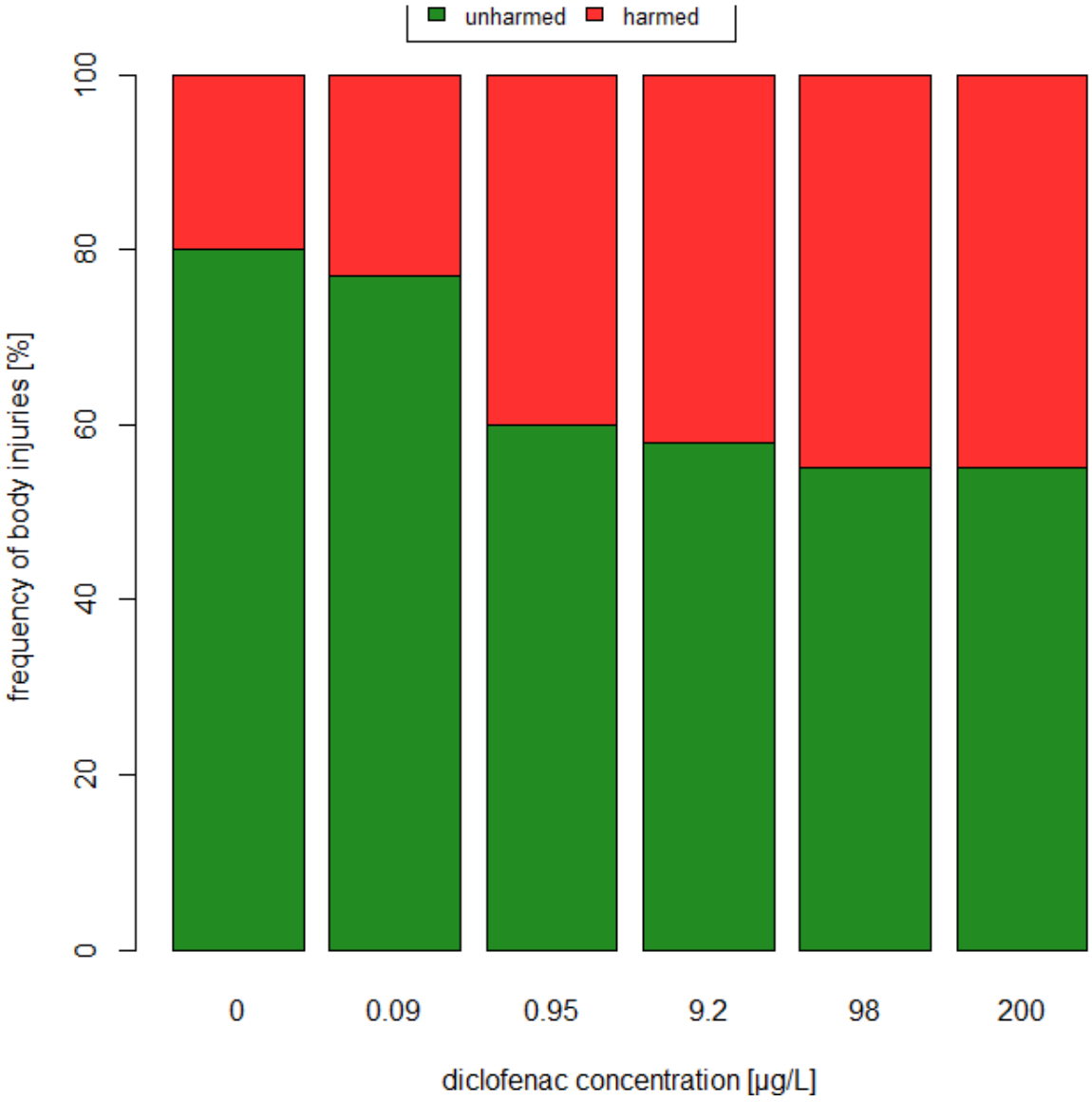
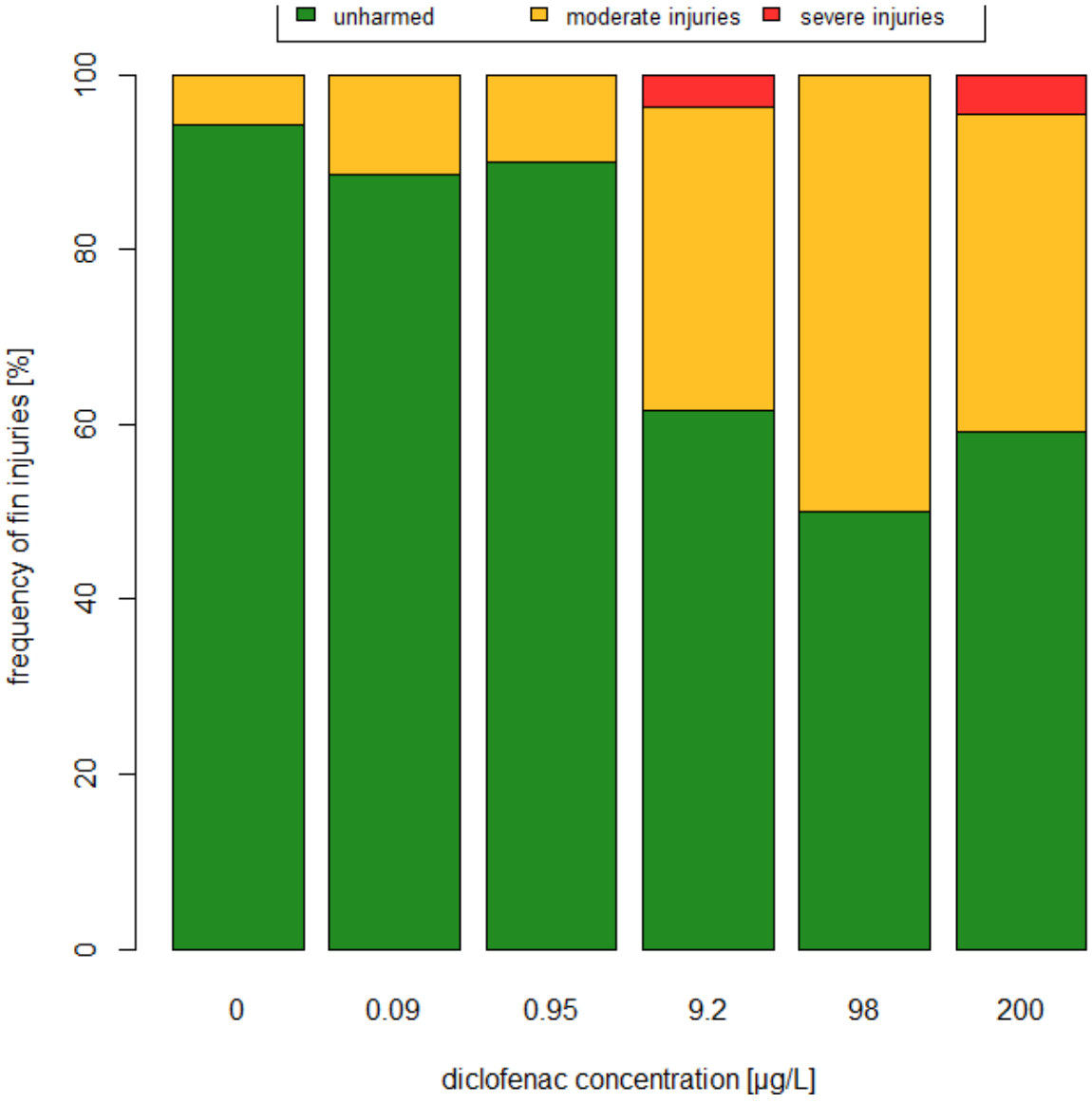


Figure 14: Percentage of brown trout exposed to diclofenac for 24 days showing bite marks on fins. Especially at the three highest concentrations, a large proportion of animals showed fin damages (Likelihood-ratio, n=161, df=10, X^2=30.50, p=0.0007).



Neither body length (standard length) nor body mass were affected by the treatments (body length: nested ANOVA, df=5, F=1.4084, p=0.2224; body mass: nested ANOVA, df=5, F=1.1950, p=0.3127). Data are illustrated in Figure 15 and Figure 16.

Figure 15: Standard body length (from snout to base of the tail) of juvenile brown trout after 24 days exposure to diclofenac. The fourth hatchery control (hc4), illustrates the length of the animals at the beginning of the experiment and was not included in the analysis. Bold lines display the median values, boxes the 25 % to 75 % quantiles, whiskers the minimum and maximum values. There were no significant differences between the different treatment groups (nested ANOVA, df=5, F=1.4084, p=0.2224).

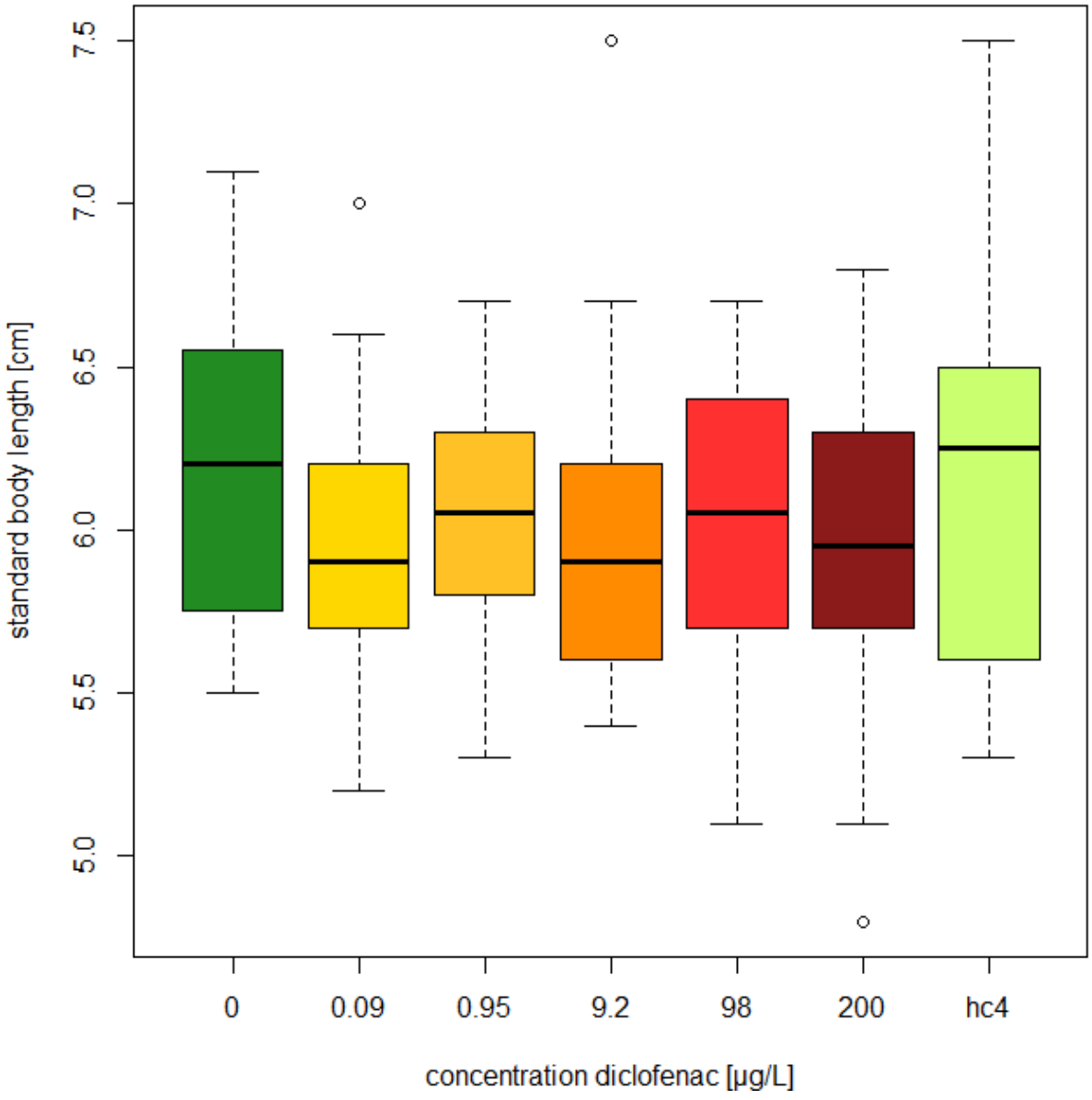
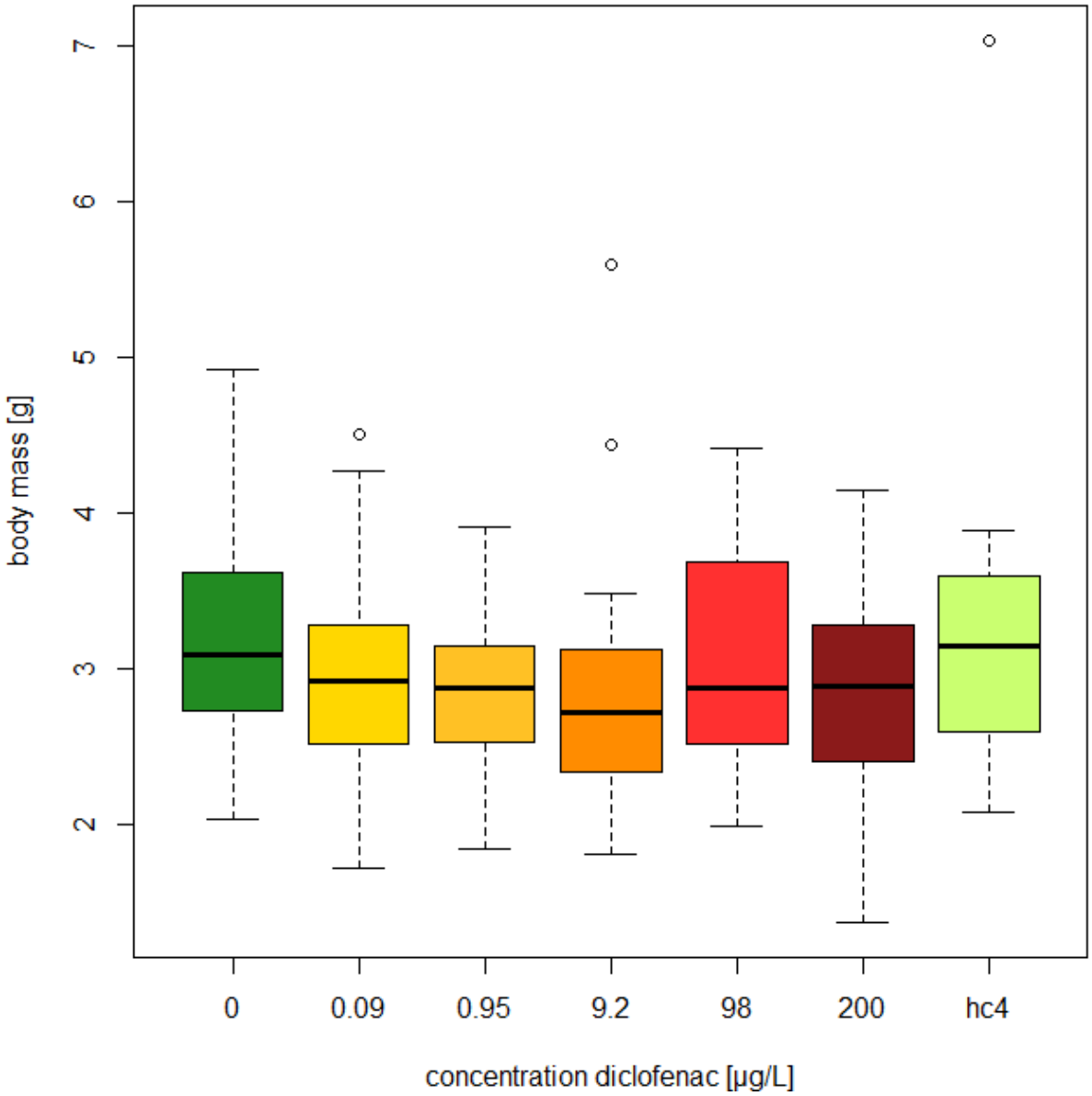


Figure 16: Body mass of juvenile brown trout after 24 days exposure to diclofenac. The fourth hatchery control (hc4), illustrates the mass of the animals at the beginning of the experiment and was not included in the analysis. Bold lines display the median values, boxes the 25 % to 75 % quantiles, whiskers the minimum and maximum values, circles potential outliers. There were no significant differences between the different treatment groups (nested ANOVA, df=5, F=1.1950, p=0.3127).



Considering the evaluated biochemical markers, no effect of diclofenac on the stress protein Hsp70 could be observed (linear model, df=5/151, F=1.046, p=0.3928). Again, the evaluation of lipid peroxide data proved to be complicated. Data were third-root transformed to allow evaluation. Again, the sample mass had an influence on the final data. A linear model including the block and sample mass as additional factor revealed a slight significance of the overall model (linear model, df=35/123, F=1.639, p=0.02581), but no influence of the test concentrations. Data are illustrated in Figure 17 and Figure 18.

Figure 17: Hsp70 level of juvenile brown trout exposed to diclofenac for 24 days. ”hc4“ and ”hc5“ depict the fourth and fifth sampling directly at the fish hatchery. Bold lines display the median values, boxes the 25 % to 75 % quantiles, whiskers the minimum and maximum values, circles potential outliers. There were no significant differences between the treatment groups (linear model, df=5/151, F=1.046, p=0.3928).

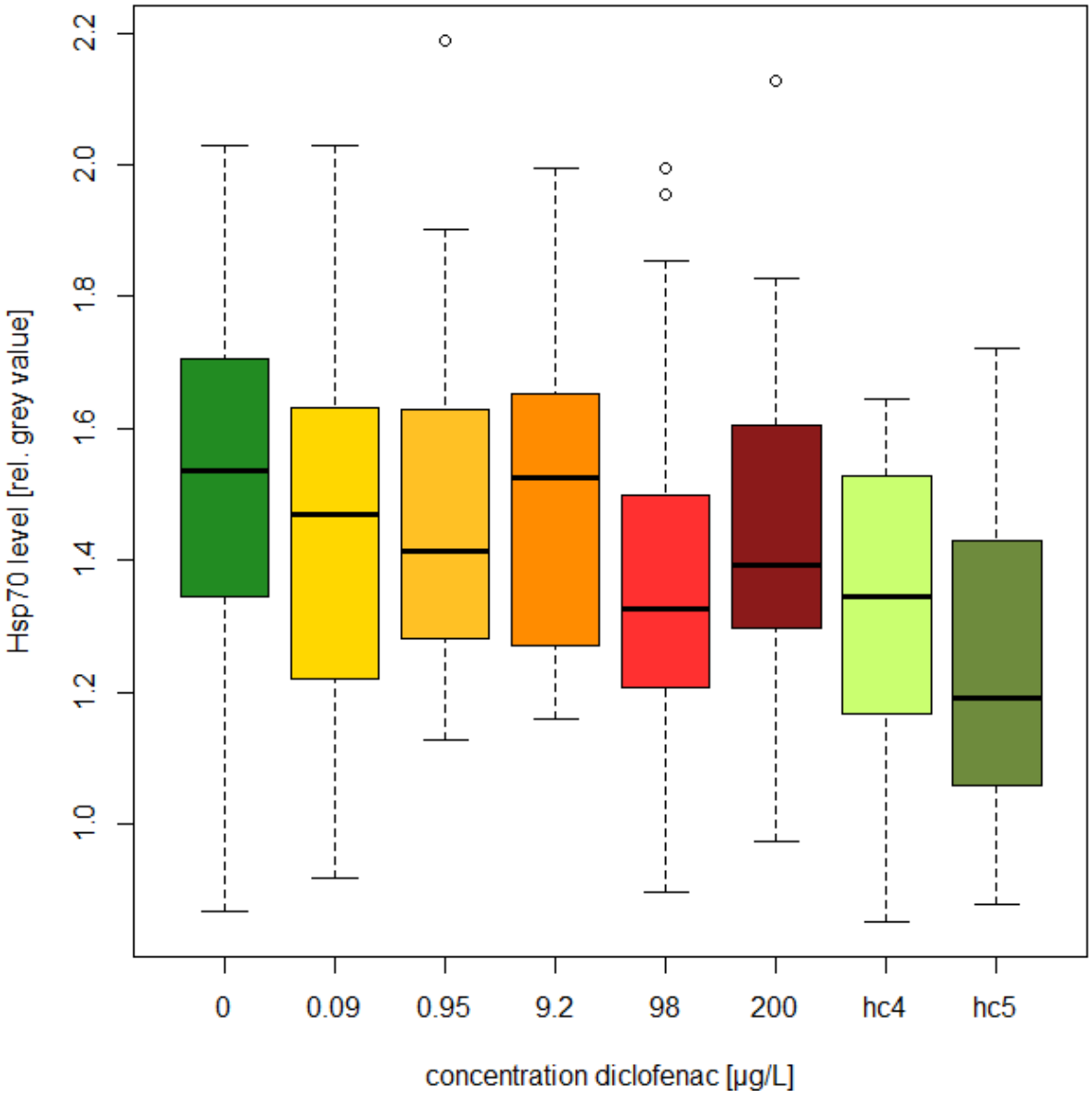
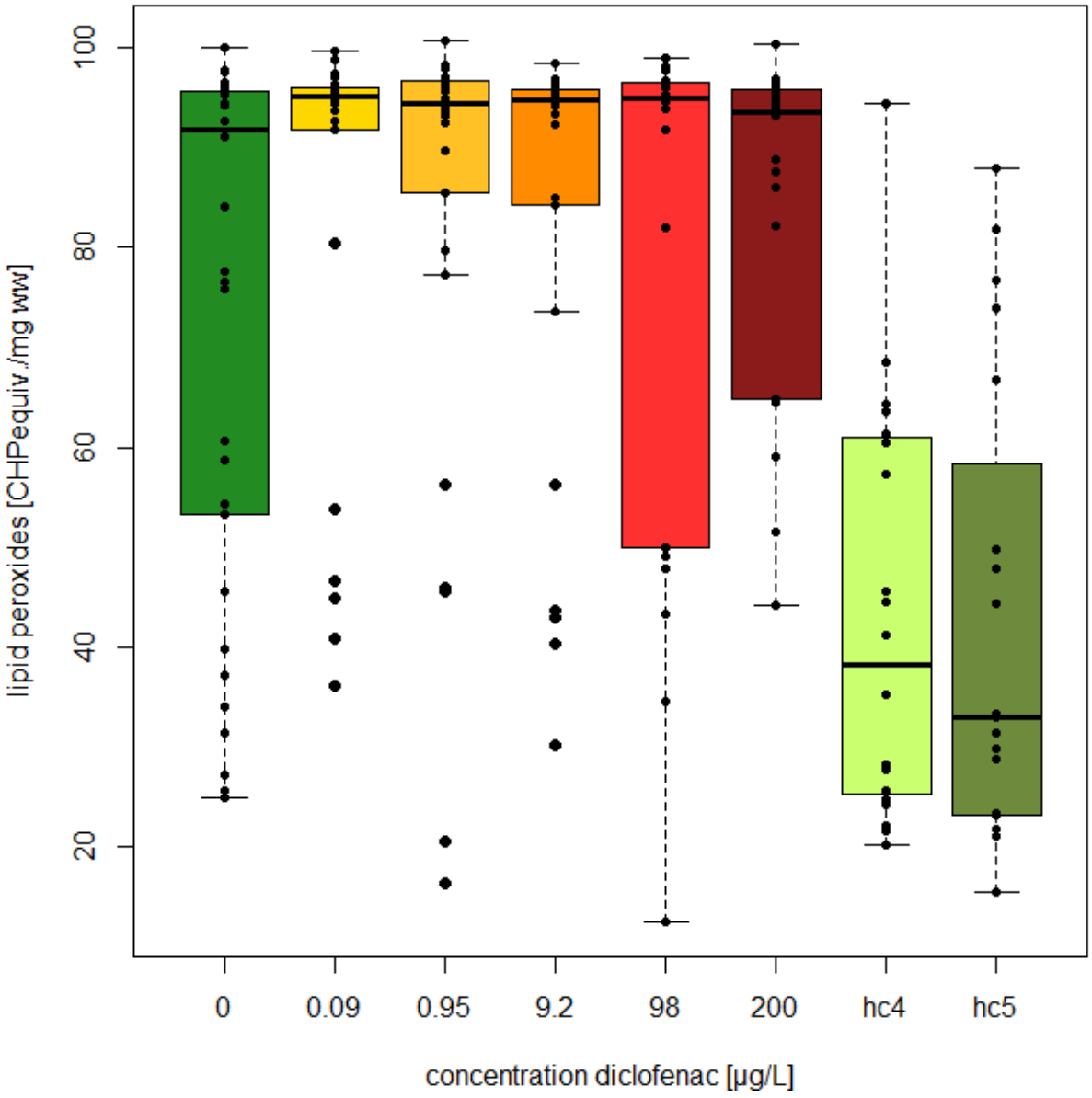


Figure 18: Lipid peroxidation (in cumene hydroperoxide equivalents per mg wet weight) of kidney of juvenile brown trout exposed to diclofenac for 24 days. ”hc4“ and ”hc5“ depict the fourth and fifth sampling directly at the fish farm. Bold lines display the median values, boxes the 25 % to 75 % quantiles, whiskers the minimum and maximum values, original data points as black dots.



The histological examination revealed overall reactive patterns in all exposed fish, including the control. The exposure to diclofenac induced further pathologies:

In the liver, reduced glycogen storage in hepatocytes was visible for almost all individuals kept in the lab. As a direct consequence, vacuolisations and vesicularisations in hepatic cells were a common finding. Light inflammative reactions, often associated with aggregations of macrophages, were further reactive patterns. Diclofenac led to severe reaction and destructions in the hepatic tissue. Besides the frequent occurrence of heavy inflammations, necrotic events, conjoined with nuclear pycnosis and complete cellular degeneration, were common findings in diclofenac-treated individuals. A concentration-dependent increase of severe reactions was visible from concentrations higher than 9.2 µg/L.

The most common alteration found in kidney was the presence of protein inclusions in tubular cells. These hyaline droplets were frequently found in the proximal convoluted elements of tubuli, and occasionally in the distal part. The glomerular structures were, in most cases, in normal shape. Occasional pathologies included the shrinkage of the glomerulus and dilatation of the Bowman space. Diclofenac in concentrations higher than 98 µg/L led to severe reactions and destructions in kidney tissue: severe hyaline droplet degeneration, focal necrosis and tubular degenerations were commonly found pathologies. These changes were reflected in a conspicuous alteration of the overall appearance of the hematopoietic tissue: the tissue appeared perforated and multiple samples showed increased bleeding. All observed pathologies were also found, in lower frequency, in the control animals and those exposed to low diclofenac concentrations.

Gill structure was affected in all animals kept in the lab. The normal structure of gills, seen in the hatchery control, features a thin primary filament and delicate secondary lamellae, covered only by a single layer of thin epithelial cells. All exposed animals showed a thickened gill structure, caused by hyperplasia of interlamellar cells and hypertrophy of epithelial plaster cells. The dimension of thickening varied from slight hypertrophy to near-complete fusion of secondary lamellae. Further pathologies were edema and slight epithelial lifting. Overall, the reactions were more pronounced in diclofenac-treated animals, with visible differences at concentrations higher than 0.95 µg/L.

An overview on the examined organs is given in Figure 19 and the results of the semi-quantitative assessment are shown in Figure 20.

Figure 19: Histopathological sections of diclofenac treated brown trout. a) liver, hatchery control (HE): normal hepatic structure with well-defined nuclei and glycogen-depositions (unstained cell-parts). b) liver, 200 µg/L diclofenac (HE): destruction state featuring reduced glycogen, pycnotic nuclei and a large necrotic area. c) kidney, control (HE): visible protein inclusions in tubulus cells (hyaline droplets). d) kidney, 200 µg/L diclofenac (HE): degeneration state of kidney, hyaline inclusion and necrosis in hematopoietic tissue. e) gill, hatchery control (HE): normal gill structure, featuring thin epithelial cells. f) gill, 200 µg/L diclofenac (HE): thickening of gill structure, including hyperplasia of interlamellar cells and hypertrophy of epithelial cells.

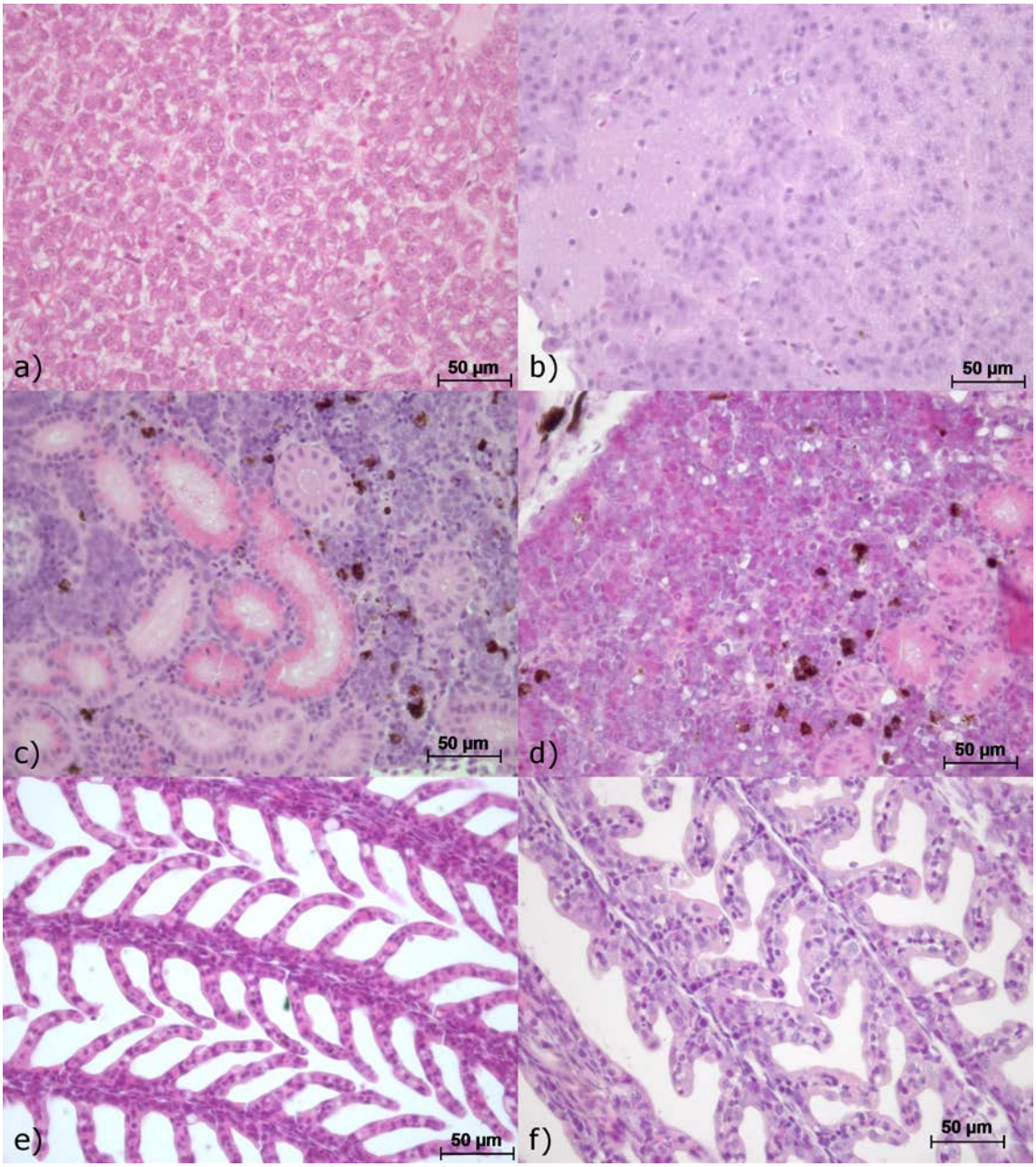
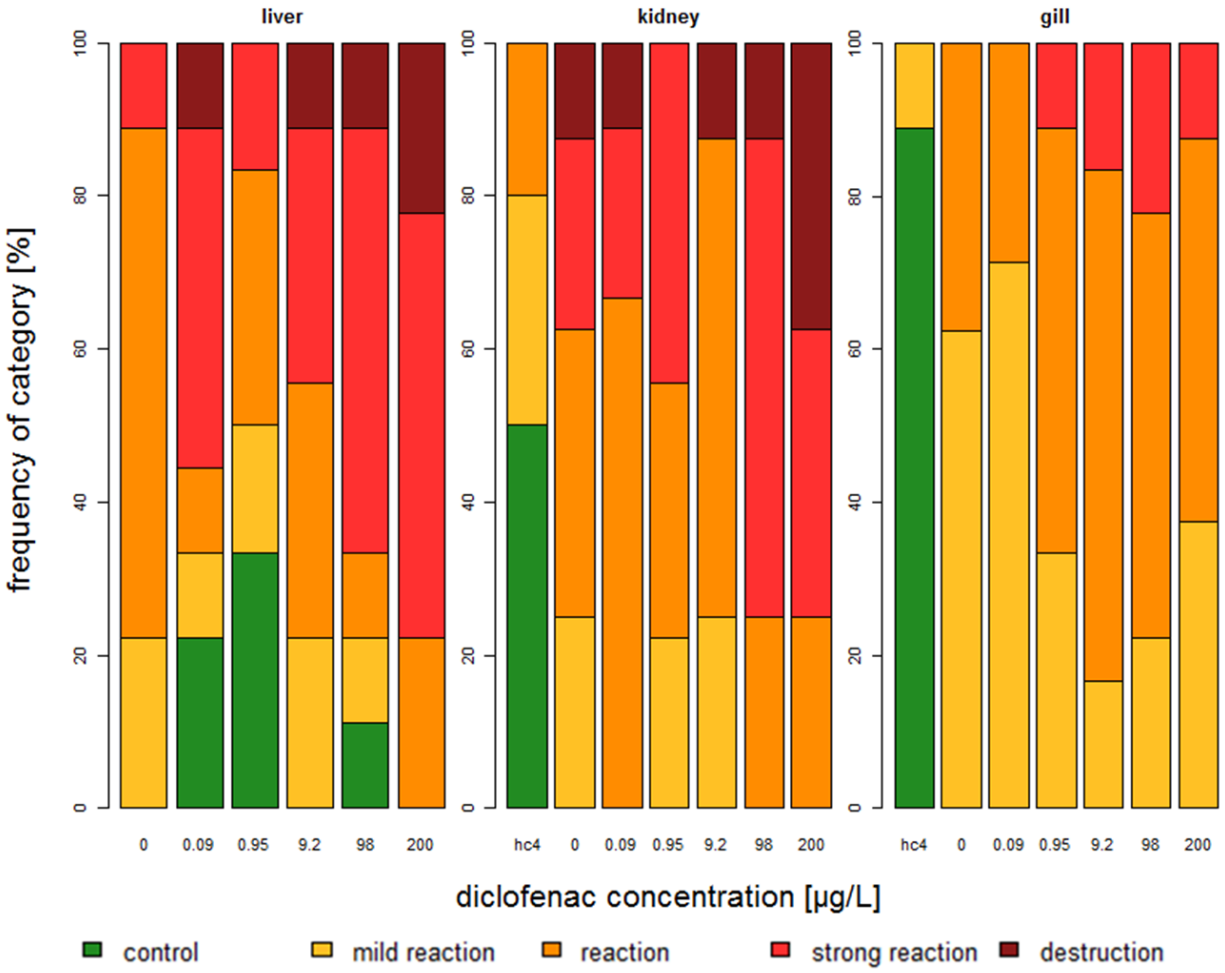


Figure 20: Semi-quantitative assessment of histological condition of brown trout exposed to diclofenac: All investigated organs showed reactions already in the control and worsening histological state with increasing diclofenac concentrations.



1.1.3.4 Metoprolol exposure

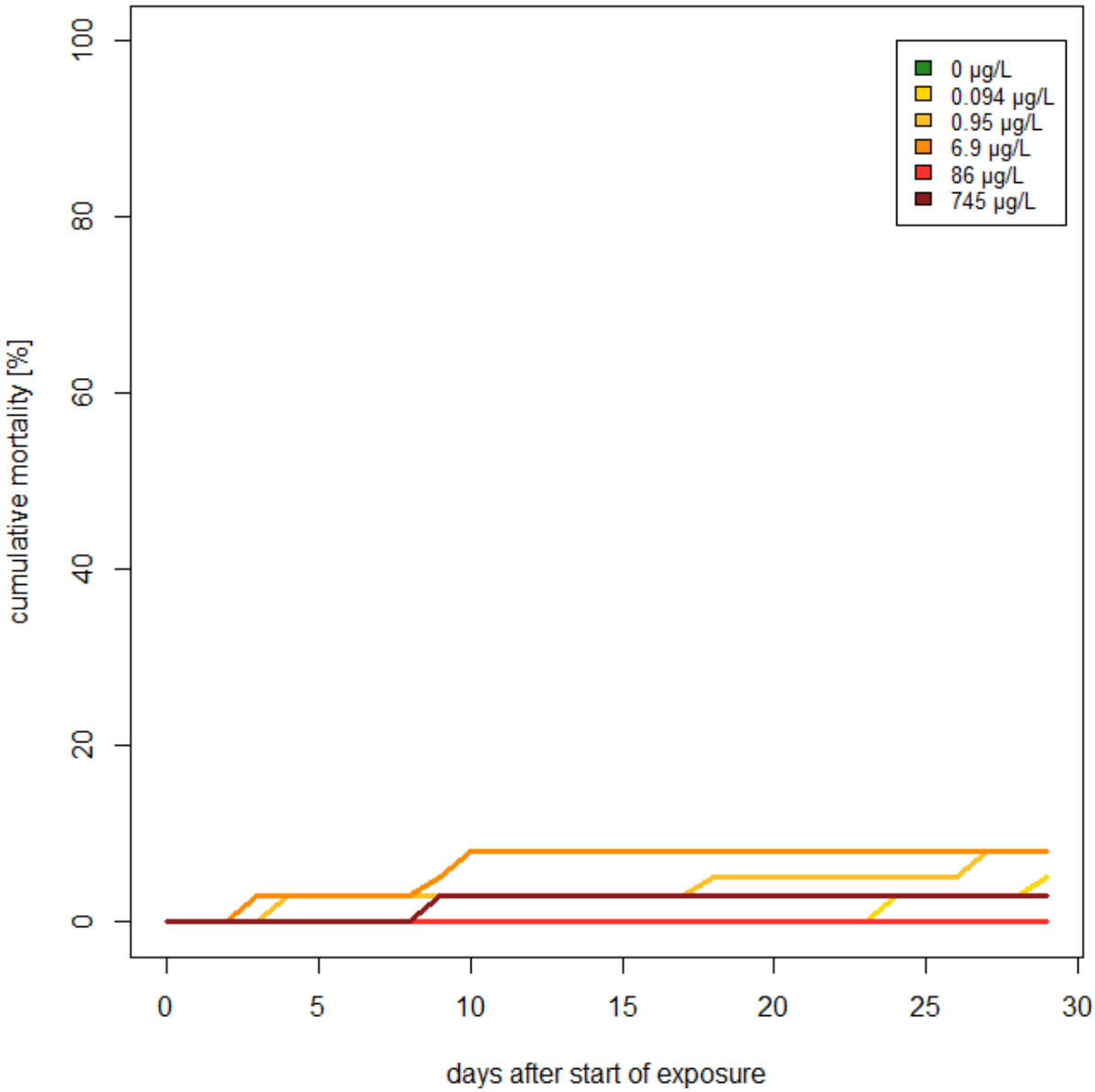
Physico-chemical parameters were within the normal range (Table 10). Mortality in the metoprolol-exposure experiment was very low, the highest rate was found for 0.95 µg/L and 6.9 µg/L with 8 %. There were no differences between the treatment groups (COX-regression, df=5, χ^2=0.0319, p=1.0). The data are displayed in Figure 21.

Table 10: Physico-chemical parameters at the end of the metoprolol exposure experiment of juvenile brown trout

Block	Exposure group	temperature [°C]	pH	oxygen saturation [%]	oxygen content [mg/L]
1	control	7.8	8.19	95.2	10.56
1	0.1 µg/L MET	7.8	8.19	95.2	10.57
1	1 µg/L MET	7.9	8.20	95.8	10.68
1	10 µg/L MET	7.8	8.20	94.8	10.69
1	100 µg/L MET	7.9	8.21	95.1	10.54
1	1000 µg/L MET	8.1	8.21	94.5	10.50

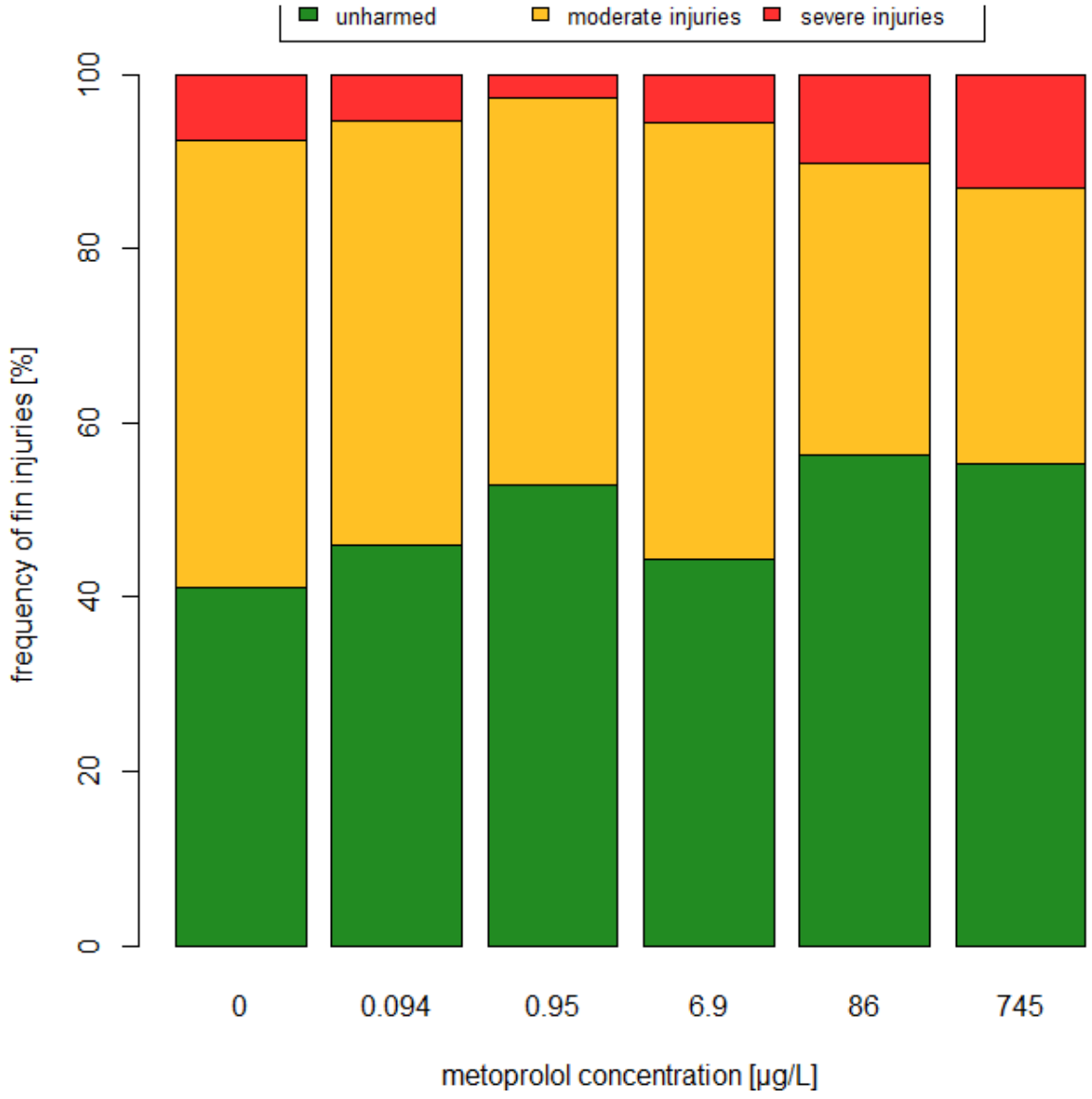
Block	Exposure group	temperature [°C]	pH	oxygen saturation [%]	oxygen content [mg/L]
2	control	8.1	8.22	94.1	10.50
2	0.1 µg/L MET	8.0	8.23	95.5	10.65
2	1 µg/L MET	7.9	8.24	94.4	10.68
2	10 µg/L MET	8.0	8.27	96.4	10.69
2	100 µg/L MET	8.0	8.24	95.2	10.50
2	1000 µg/L MET	7.6	8.22	94.4	10.52
3	control	7.8	8.27	94.9	10.56
3	0.1 µg/L MET	7.6	8.25	94.7	10.68
3	1 µg/L MET	7.6	8.24	95.3	10.64
3	10 µg/L MET	7.5	8.23	93.8	10.58
3	100 µg/L MET	7.7	8.23	94.9	10.55

Figure 21: Cumulative mortality of juvenile brown trout exposed to metoprolol in different concentrations over 29 days. Overall, the mortality rate was very low and did never exceed 10% and there was no difference between the treatment groups (COX-regression, df=5, χ^2=0.0319, p=1.0).



The occurrence of fin injuries was high, ranging around 50 % in all experimental groups. However, there was no clear difference between the tested groups (likelihood-ratio, df=10, χ^2=8.546, p=0.5757). Data are shown in Figure 22.

Figure 22: Percentage of animals showing bite marks on fins. A high proportion of animals were injured, but there were no differences between the tested groups (likelihood-ratio, df=10, χ^2=8.546, p=0.5757).



Also the comparison of the biometrical measurements showed no differences between the groups (body length: nested ANOVA, df=5, F=0.3146, p=0.9039; body mass: nested ANOVA, df=5, F=0.0519, p=0.9983). Data are shown in Figure 23 and Figure 24.

Figure 23: Standard body length (from snout to base of the tail) of juvenile brown trout after 29 days exposure to metoprolol. Bold lines display the median values, boxes the 25 % to 75 % quantiles, whiskers the minimum and maximum values. There were no significant differences between the different treatment groups (nested ANOVA, df=5, F=0.3146, p=0.9039).

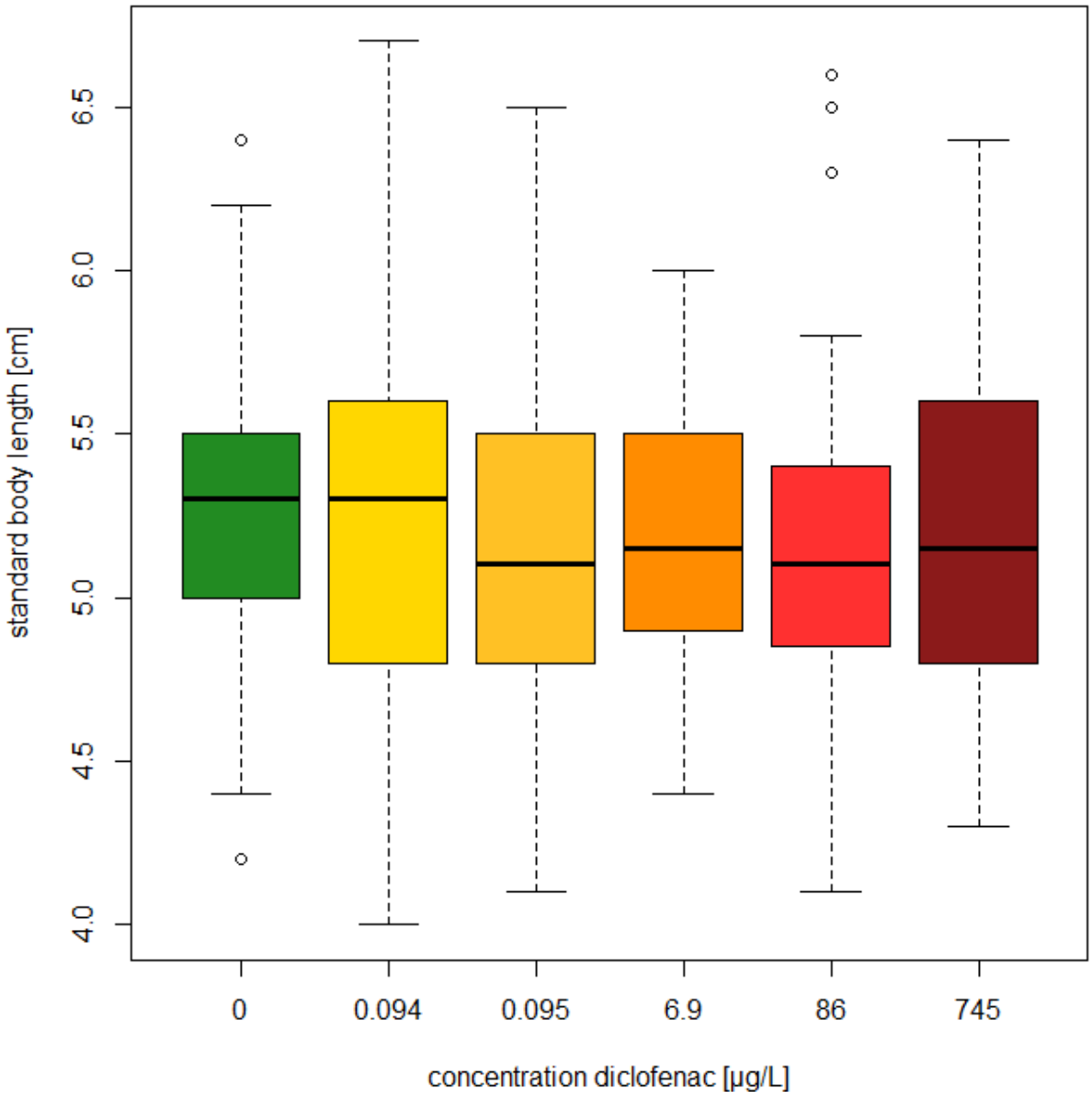
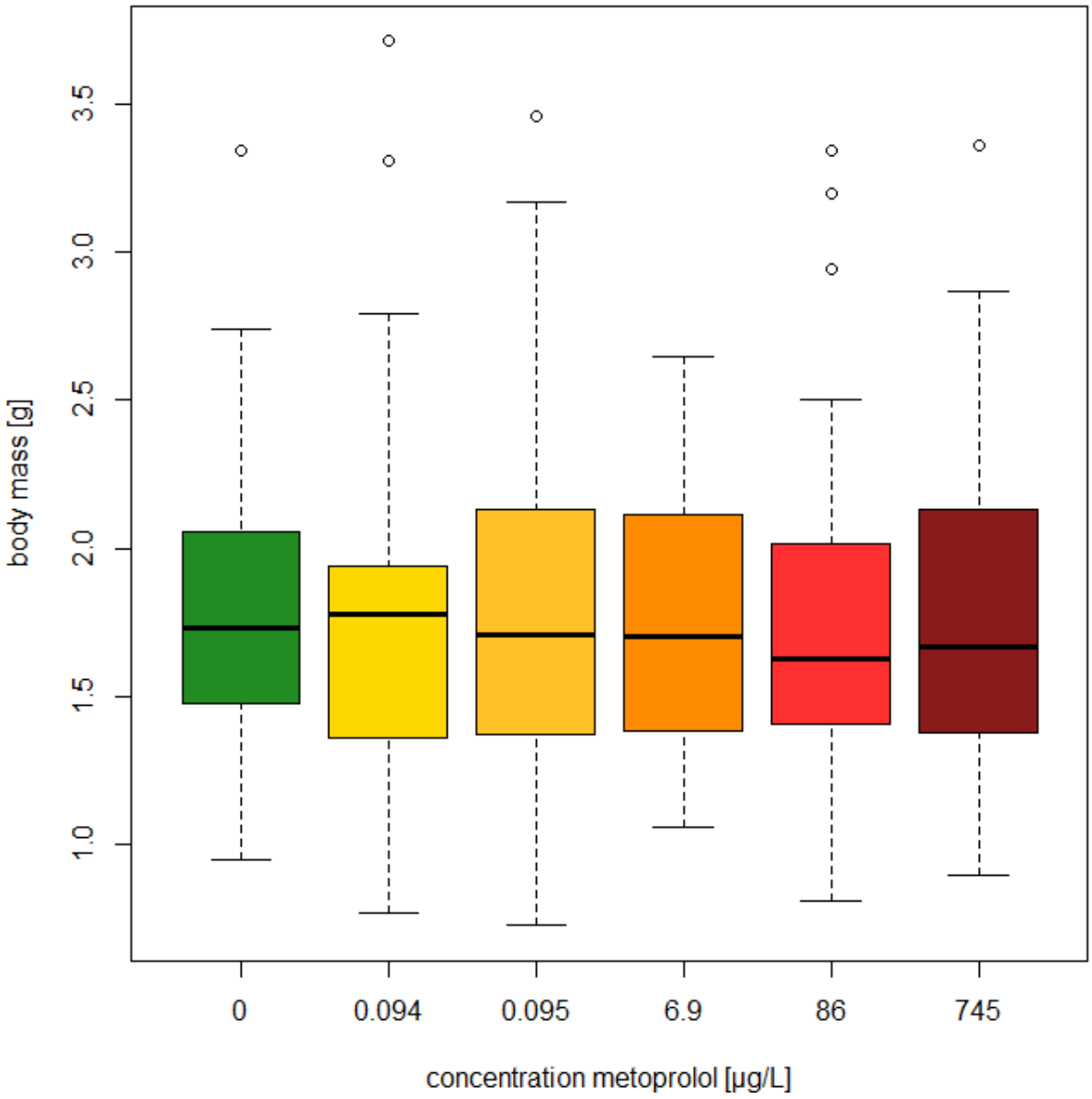
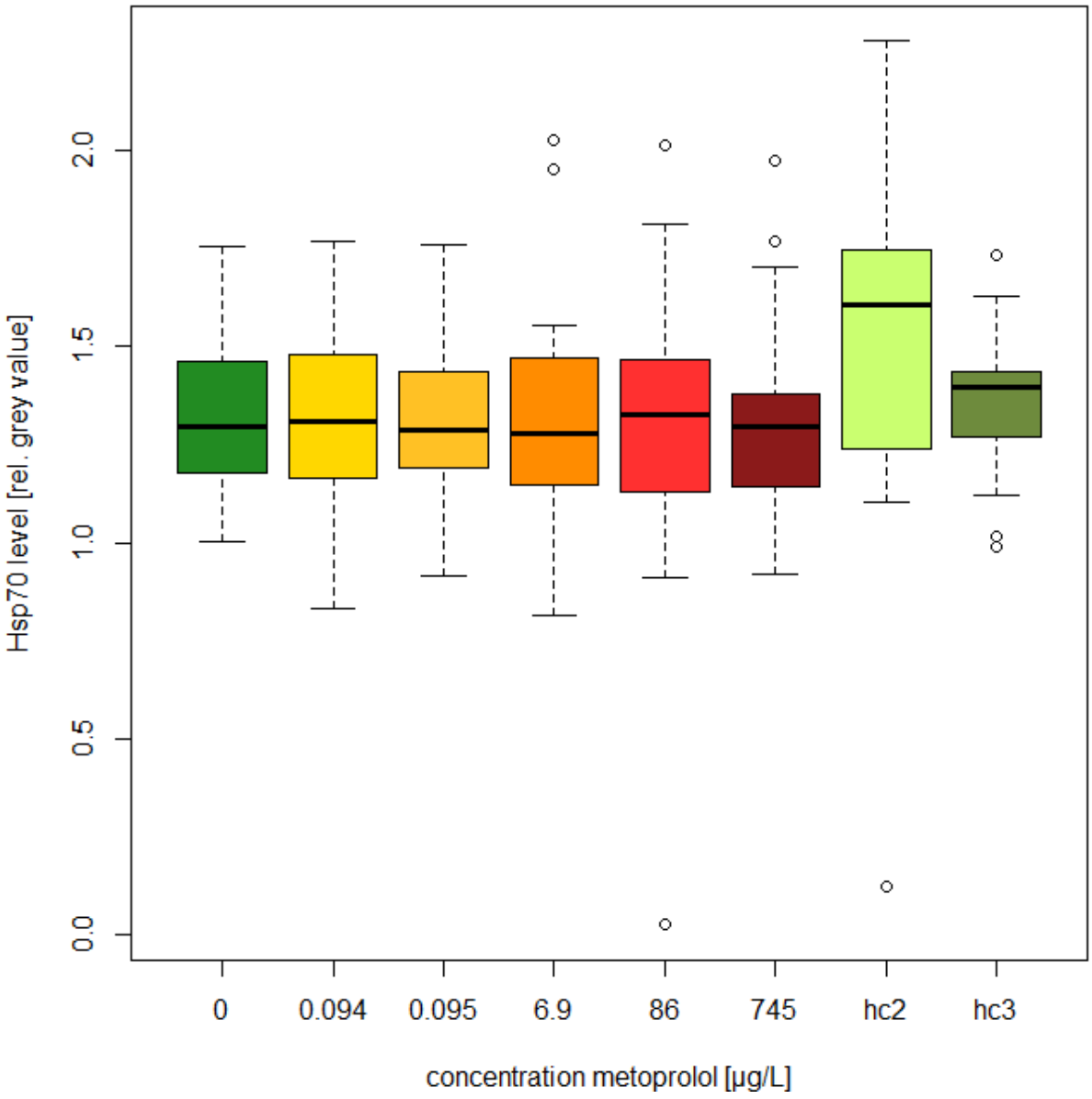


Figure 24: Body mass of juvenile brown trout after 29 days exposure to metoprolol. Bold lines display the median values, boxes the 25 % to 75 % quantiles, whiskers the minimum and maximum values. There were no significant differences between the different treatment groups (nested ANOVA, df=5, F=0.0519, p=0.9983)



The Hsp70 level did not show differences between the control and any of the metoprolol-exposures (linear model, df=5/202, F=0.1355, p=0.984). Results are shown in Figure 25.

Figure 25: Hsp70 level of juvenile brown trout exposed to metoprolol for 29 days. ”hc2“ and ”hc3“ depict the second and third sampling directly at the fish hatchery. Bold lines display the median values, boxes the 25 % to 75 % quantiles, whiskers the minimum and maximum values, circles potential outliers. There were no significant differences between the treatment groups (linear model, df=5/202, F=0.1355, p=0.984).



In a healthy status, the liver cells of brown trout are regularly shaped and appear relatively bright due to high glycogen content. The nuclei have a round shape and are usually circa one fifth of the size of the hepatocytes. In the investigated liver sections, reduced glycogen content and slightly enlarged intercellular spaces occurred. Besides, inflammations and large lipid vacuoles were found. Furthermore, the sizes of the hepatocytes of some fish were reduced, which is also mirrored in the proportion of the size of the cells to the size of the nuclei. The histological status of the liver was better in the hatchery control than in the control group and the fish exposed to 745 µg/L metoprolol. This difference is mostly caused by the reduced size of the hepatocytes, the enlarged intercellular space and the lower glycogen content of the control group and the fish exposed to 745 µg/L metoprolol. Between the laboratory con-

trol group and fish exposed to 745 µg/L metoprolol no difference occurred. Since there was no difference between the highest concentration and the control group, the intermediate concentrations were not examined.

Again, the kidney showed visible reactions already in the lab control – light inclusions of hyaline material in proximal tubuli were a frequent finding, joined by occasionally altered structure of the hematopoietic tissue. In higher metoprolol concentrations, these reactions were more pronounced: a larger portion of proximal, and in several cases distal, tubulus elements showed hyaline droplets. Furthermore, multiple samples featured reactions of the glomerular structures, reaching up to occasional degenerations and necrosis of the renal elements. The changes were not fully concentration dependent, since the highest proportion of severe reactions was found at 6.9 µg/L metoprolol. Yet, all metoprolol exposures showed a clear difference compared to the lab control.

Gill structure was affected in a comparable manner as in the diclofenac experiment. The overall structure was thickened by hyperplasia and hypertrophy of epithelial and interlamellar cells. Furthermore, lamellar fusions, edema, increased proportion of mucus cells and occasional epithelial lifting were the most common findings. All reactions could be seen in the control, as well as in the highest and intermediate concentration. Since there were no differences between the highest concentration, 6.9 µg/L metoprolol and the control group, no further intermediate concentrations were investigated.

The heart of a brown trout is structured in an atrium and a ventricle. From the ventricle the blood flows through the bulbus arteriosus in the circulatory system. The ventricle consists of an external membrane (epicardium), an intermediate layer of cardiac muscle fibers and an internal membrane (endocardium). No pathologic alterations were found in the examined hearts.

During the histopathological investigation of the heart, however, different proportions of erythrocytes and lymphocytes in the sections were noticed. The quotient of erythrocytes to lymphocytes was calculated to consider the varying amount of cells in the heart. A significant difference occurred between the hatchery control with a ratio of 1.48 erythrocytes to lymphocytes and the animals that were exposed to 1000 µg/L metoprolol with a ratio of 0.50 erythrocytes to lymphocytes (Kruskall-Wallis + Steel-Dwass post hoc, Z=-2.36184; q=2.34370; p=0.0477). This means that in the heart of the trout exposed to 1000 µg/L metoprolol in comparison to the amount of erythrocytes significantly more lymphocytes were found than in the hearts of the trout of the hatchery control. No significant difference occurred between the control group and the trout exposed to 1000 µg/L.

An overview of the examined organs in shown in Figure 26, the results of the semi-quantitative assessment are given in Figure 27 and the ratio of erythrocytes to lymphocytes is illustrated in Figure 28.

Figure 26: Histological sections of brown trout exposed to metoprolol: a) liver, hatchery control (AB-PAS): large amounts of glycogen stored in hepatocytes. b) liver, control (AB-PAS): reduced amount of glycogen in hepatocytes and high frequency of lipid vacuoles. c) kidney, 10 µg/L metoprolol (HE): hyaline inclusions in tubuli and reaction state of glomeruli. d) kidney, 1000 µg/L metoprolol (HE): reaction of glomerular structure, featuring enlarged Bowman space. e) gill, control (HE): thickening of primary filament and secondary lamellae. f) gill, control (PAS): thickened gill structure and epithelial edema.

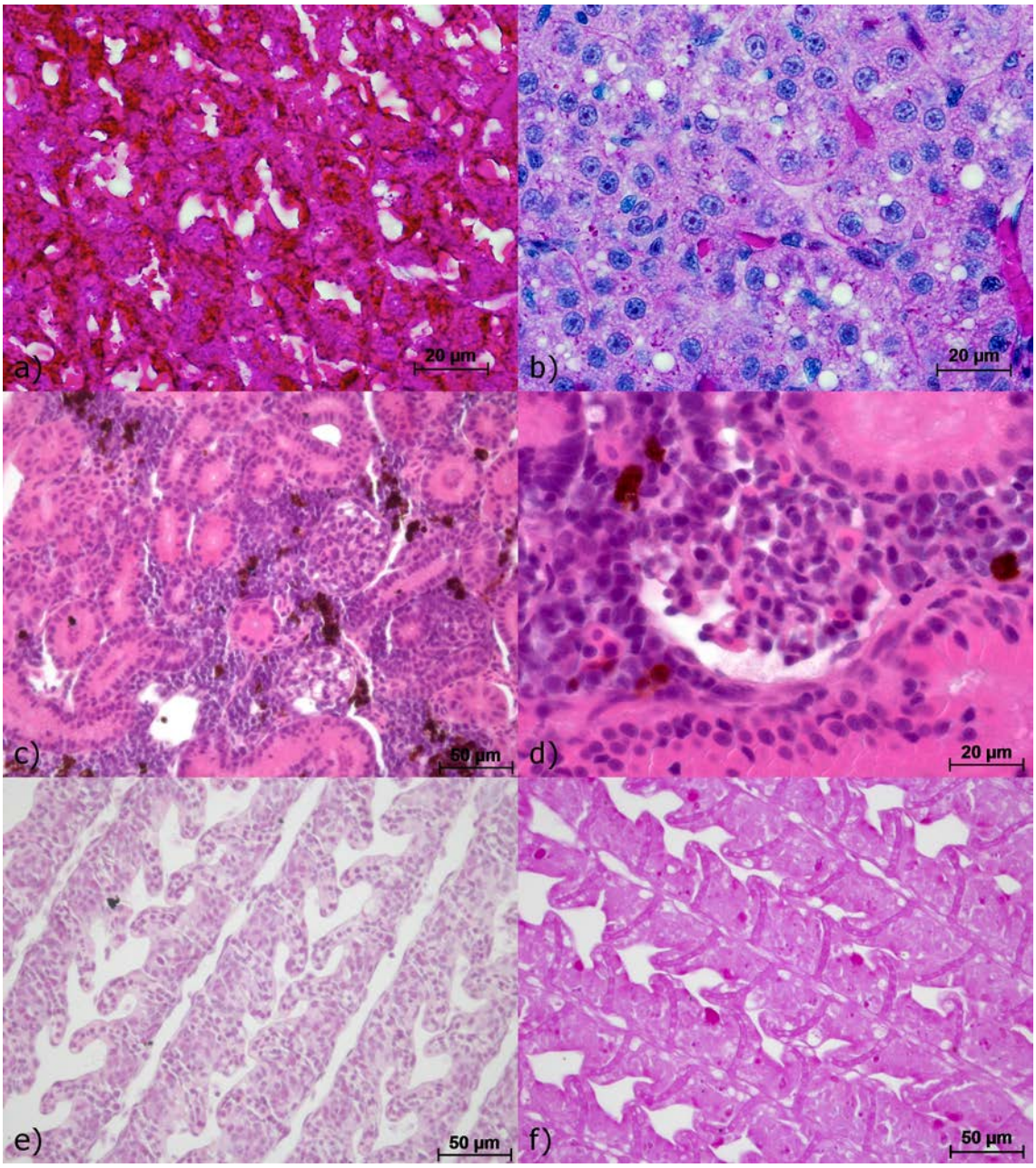


Figure 27: Semi-quantitative assessment of histological condition of brown trout exposed to metoprolol. All investigated organs showed reactions already in the control. For liver and gill there was no effect of the metoprolol treatment. The histological state of kidney worsened with increasing concentrations of metoprolol.

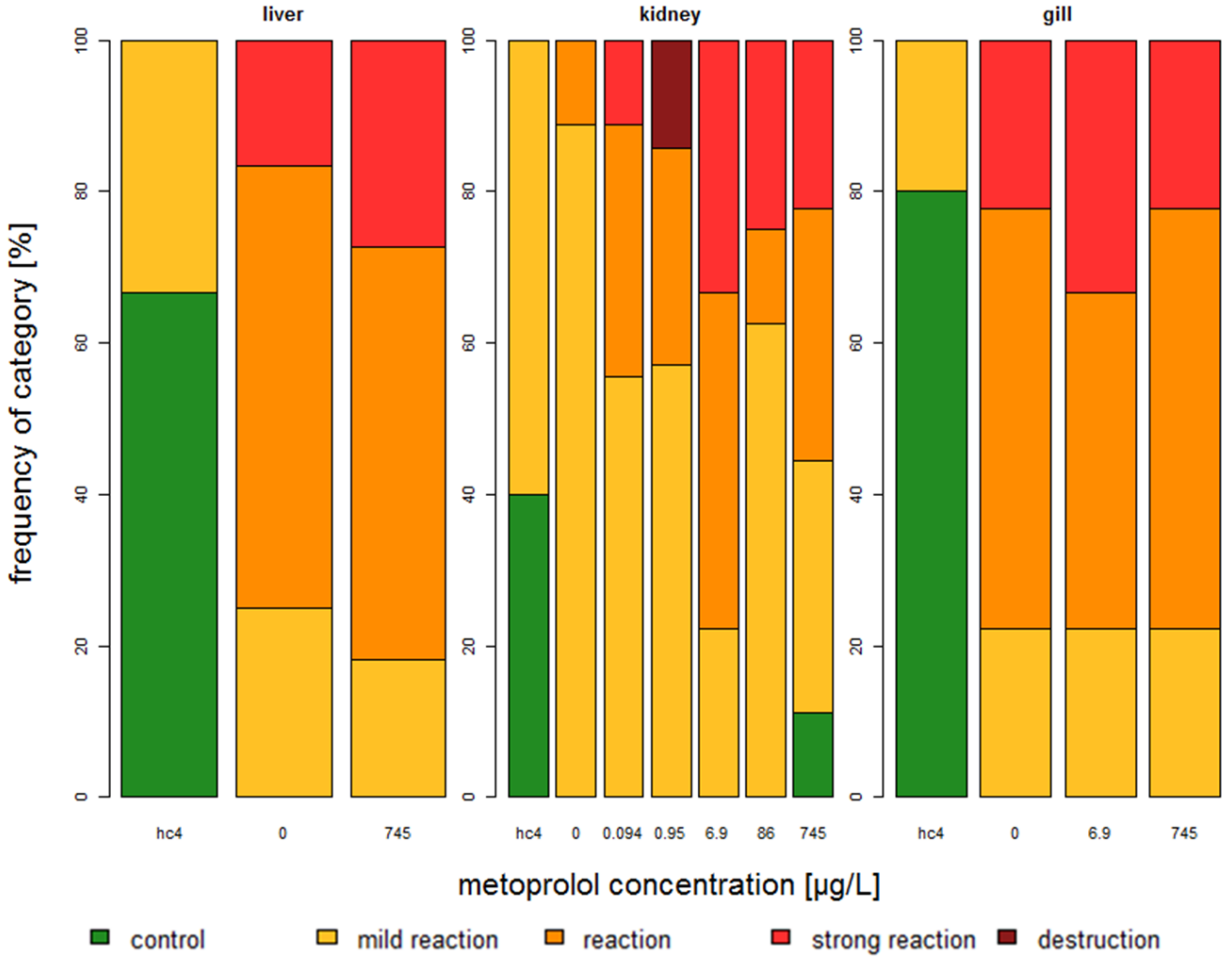
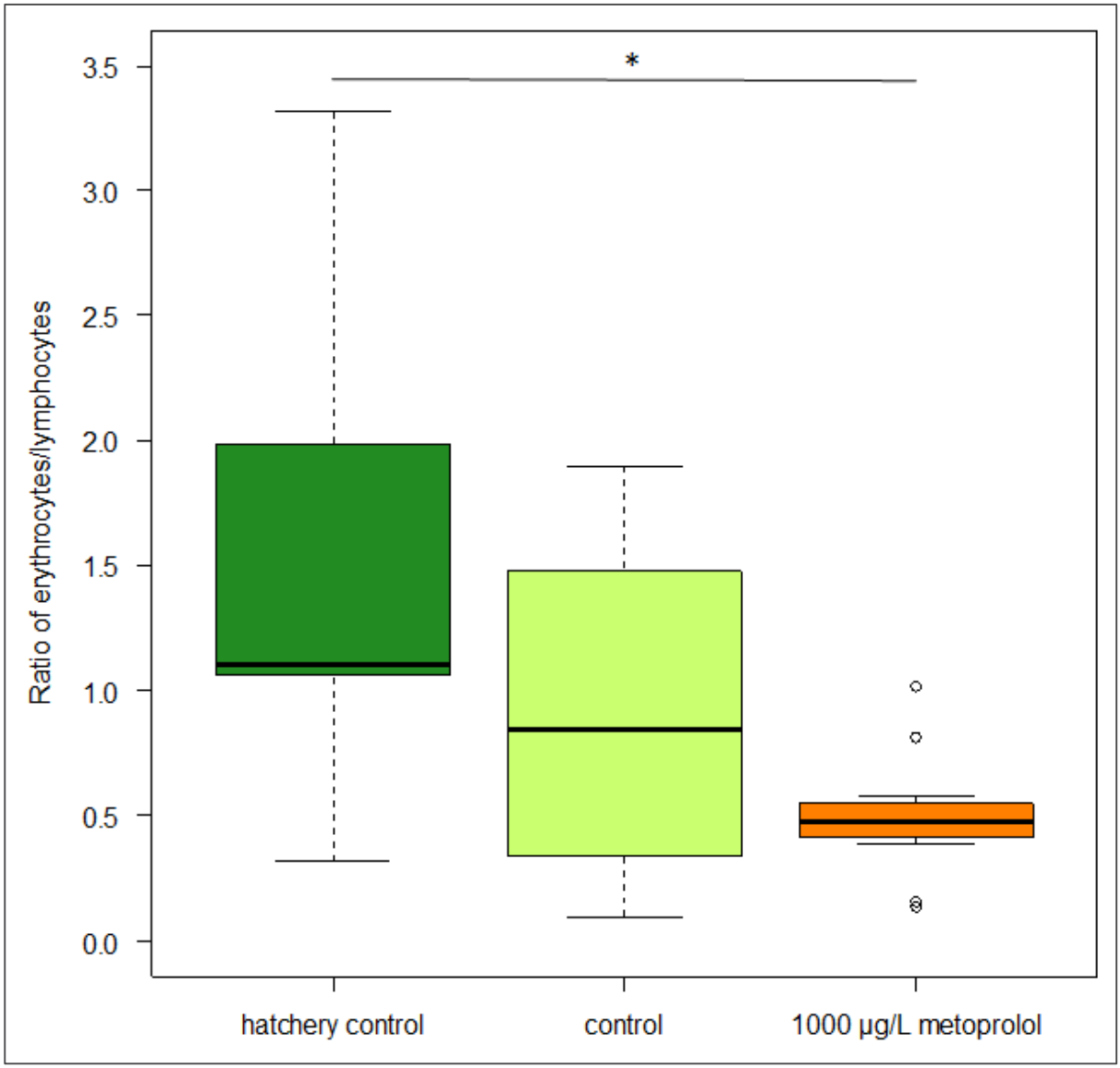


Figure 28: Ratio of erythrocytes to lymphocytes in the heart of juvenile brown trout. The box plots display the median and the quantiles, the dots indicate outliers. The ratio of erythrocytes to lymphocytes was significantly lower in trout exposed to 1000 µg/L metoprolol than in fish of the hatchery control. (Kruskall-Wallis + Steel-Dwass post hoc, Z=-2.36184; q=2.34370; p=0.0477). The visually recognizable difference between the control and the animals that were exposed to 1000 µg/L metoprolol is not confirmed by the statistical analysis (Kruskall-Wallis + Steel-Dwass post hoc Z=-1.24764, q=2.34370; p=0.4251).



1.1.4 Discussion

1.1.4.1 Influence of diclofenac on juvenile brown trout

Overall, the most prominent result in the first diclofenac experiment was the greatly increased mortality in fish exposed to the highest concentration of diclofenac. However, this result is relativized by the fact that the mortality in this experiment was generally very high. In contrast, the mortality in the metoprolol-exposure was very low (only 9 of 234 exposed animals). Here, fish were slightly older, while the other environmental conditions were the same. Consequently, it can be assumed that the used life stage of *Salmo trutta* in the first diclofenac exposure was very sensitive to the conditions in the laboratory experiment. These conditions include the keeping in a relatively small volume (com-

pared to big basins under flow-through conditions they are kept in for fish breeding) or slight fluctuations in the nitrite concentrations (in the employed, semi-static study design, the nitrite content was only regulated by water changes). It can be hypothesized that the used, very young life stage of *Salmo trutta* is very susceptible to these conditions, which usually pose no problems for older individuals, which were used in the metoprolol and in the second diclofenac experiment. In the first run, the high diclofenac concentrations seem to act as an additional stressor, which cannot be handled by the fish – leading to drastically increased mortality rates, even compared to the generally high mortality in controls.

Consequently, the second experiment with diclofenac was repeated in 2015, with individuals of comparable age as in the metoprolol exposure.

In the first diclofenac exposure, the difference between the "100.2 µg/L" exposure and the other treatments is also reflected in the biometrical data, where body length and body mass were obviously lower. However, this effect cannot be reliably attributed to the pharmaceutical exposure, since the sampled animals were younger than the ones of the other experimental groups.

The high degree of stress in the first diclofenac experiment was neither reflected by the level of Hsp70, nor in the degree of lipid peroxidation. There were no obvious differences between the experimental groups. These results suggest that diclofenac does not exhibit a proteotoxic effect on brown trout in the tested concentrations.

The low degree of lipid peroxidation in the "100.2 µg/L Diclofenac" exposure could either be a sign of an anti-oxidative capacity of diclofenac, but also be due to the fact that all animals exhibiting high lipid peroxidation might already have died at the sampling time. In general, the animals from the lab, including the control, showed much higher variability and higher levels of lipid peroxides than the animals sampled at the breeder. The exposure in the lab itself seems to induce oxidative stress. The fact that the sample mass had an obvious effect on the CHP-equivalents suggests that the head might not be the optimal sample type for this kind of analysis. For the re-run of the experiment, lipid peroxidation was examined in kidney samples. They should pose a more homogenous kind of sample with less effect of the overall sample mass. It was not possible to do this for the samples from the first trial, since these animals were too small to yield a kidney sample of sufficient size.

The second diclofenac exposure gave further indications that this substance has the potential to negatively affect the survival of brown trout. Mortality was significantly increased in a concentration-dependent manner (except the lowest tested concentration, which provoked a higher mortality than the second-lowest). The increase of mortality in high diclofenac exposures was still present, and could be seen in even lower concentrations than at the first experiment. Mortality increases in this low concentration range have not been reported for diclofenac so far. Acute tests on adult fish reported EC50 of 71 mg/L for carp (Islas-Flores et al. 2013, Saucedo-Vence et al. 2014), and none of the studies on trout examining a similar concentration range as in this study (Hoeger et al. 2005, Mehinto et al. 2010, Schwaiger et al. 2004, Triebskorn et al. 2004) reported significant mortality increases.

The biggest influences on lipid peroxidation were present between the hatchery controls and experimental groups. Despite the more homogenous tissue type that was used for the analysis, a high variability and significant influence of sample mass was still present. Yet, the analyses considering these factors did not yield any hint on an influence of the diclofenac treatment. 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. However, since this was the only concentration tested, no effect concentration can be derived. On the contrary, Praskova et al. (2014) found a decrease of lipid peroxidation in *Danio rerio* at 20 µg/L, without other parameters of the oxidative stress response being affected at concentrations up to 60 mg/L. Ghelfi et al. (2016) found an increase of the enzyme superoxide dismutase in catfish kidney at 0.2 µg/L, but no influence on lipid peroxidation. For the interpretation it has to be considered that the FOX-assay only quantifies lipid peroxides and not the overall oxidative

stress. Therefore, a comparison with effect values obtained by other methods is generally difficult. Our results showed no signs of any influence of diclofenac on the induction of lipid peroxides.

The lack of Hsp70-induction is in accordance with studies on zebrafish, which found no effect in concentrations up to 2 mg/L (Hallare et al., 2004). We can draw the conclusion that the proteotoxic effect of diclofenac is negligible in fish.

It was also assured via chemical analysis that the substance was taken up into the organism. The concentrations in muscle tissue were within the range of the water concentration and showed no indication of large-scale bioaccumulation in this type of organ. However, no such statements can be made for other organ types like liver or kidney. Previous studies revealed a much higher potential of these metabolically active organs to accumulate diclofenac (Memmert et al. 2013, Schwaiger et al. 2004). In our case, the animals were too small to yield a sufficient amount of organ sample for such analyses.

Based on the results of both the first and second trials, a LOEC of 100 µg/L (with 10 µg/L being the corresponding NOEC) is calculated for diclofenac, with relevant endpoints being mortality and lipid peroxidation. However, due to the stated reasons the lipid peroxidation results should be viewed with care.

For bite marks on fins, as a proxy for increased aggressive behaviour, the LOEC was even lower, with 10 µg/L. However, it was no primarily investigated endpoint – and the cause cannot be traced unequivocally. On the one side, diclofenac might lead to increased territoriality and aggression in brown trout. On the other side, the medication may dull the senses of treated animals, so that they cannot react quickly enough to attacks and suffer greater injuries. The increased mortality might also be, at least in parts, due to secondary effects of this intraspecific aggression. Injured animals are weakened and more prone to further harm. On the other hand, the increased intraspecific aggression could also be a secondary effect of higher mortality: brown trout show a peaceful swarming behaviour only as long as they are kept in sufficient densities. Low densities lead to the development of territorial behaviour – which results in intraspecific aggression (personal correspondence with J. Schindler). Occurrence of fin nips is a common problem in commercial fish farming. Studies have related the effects to stocking densities, feeding conditions and general water qualities (Bosakowski & Wagner 1994, Cañon Jones et al. 2010, Jones et al 2011, Noble et al. 2007). Effects of a non-psychoactive substance on this kind of behaviour have, to our knowledge, not been reported so far. Ajima et al. (2015) reported on behavioural changes on African catfish at 25 mg/L diclofenac – which included respiratory distress, loss of balance and erratic swimming, but no signs of aggression. Undoubtedly, this is a very interesting finding, which should be examined more closely. One possibility for further analyses would be a tracking experiment investigating the social actions and reactions of several individuals in a small group.

The higher frequency of body injuries in all treatment groups, which showed no significant differences, need not be related to intraspecific aggression. It is a common finding that caged trout, and animals kept in tanks, may injure their snout when colliding with the walls. However, since there is a visible difference between the control and fish exposed to concentrations higher than 0.95 µg/L, a treatment effect (as seen in the fin bites) is also likely.

The histological analyses shed further light on the reason of the high mortality. All exposed animals showed histological reactions in all investigated organs – likely an adaptation to the laboratory conditions. In the hatchery, fish are kept in great flow-through basins and receive high amounts of food to fit the demands of commercial aquaculture. Furthermore, they are adapted to the local water conditions (pH, salinity, electrolyte composition). In contrast, the lab experiment is performed in aquaria. Water was changed in a semi-static way instead of flow-through, since this would not be technically possible for the number of replicates required for this study. Lab fish received a defined amount of food that allowed them to maintain their normal metabolism, but avoid excess feeding and risk of increasing ni-

trite concentrations in aquarium water. Furthermore, the fish had to adapt to new environmental conditions, especially concerning the water conditions. All conditions (Table 7) were within the tolerable range of brown trout, but still different to the original water of the hatchery.

Therefore, the thickening of gill filaments in control fish can likely be seen as an adaptation to changed osmotic conditions of the water. These disturbances in electrolyte metabolism are possibly also the explanation for the peculiar findings in renal tubuli. The reduced amount of glycogen is a result of the lab feeding regiment that reduced the storage of energetic reserves. Nevertheless, the findings point out that the animals were in an overall stressed state, which might influence further reactions to other stressors. This is hardly avoidable when working with environmentally relevant species that are not adapted to aquarium conditions. It points out the importance of conducting a hatchery control, to assess the "normal" state of the animals, as well as a lab control, to assess the state of the animal under lab conditions.

Diclofenac led to further reactions and destructions in all three investigated organs. Especially liver and kidney were gravely affected, oftentimes resulting in degenerations of the organ. The deceased individuals could not be investigated, but the condition of the survivors suggests multiple organ failure as the reason of the increased mortality. It can be assumed that the mortality rate would have increased even more in a longer exposure. Histology as biomarker reacted sensitively at concentrations that did not yet lead to significantly increased mortality. Furthermore, the concentration-dependent increase of mortality is well reflected in the histological results. The determination of definite LOECs and NOECs for histological endpoints would have required a larger scale examination of high sample sizes, which was not within the scope of our study. Histological endpoints are among the most sensitive endpoints reported for diclofenac so far (Hoeger et al. 2005, Mehinto et al. 2010, Schwaiger et al. 2004, Triebskorn et al. 2004) and ultrastructural studies revealed damages even on the cytological level (Triebskorn et al. 2007). A recent publication comparing several studies on diclofenac criticized the method of histopathology as vulnerable to personal interpretation (Wolf et al. 2014), relativizing certain published LOECs. In our case, the histological findings are in accordance with the results we obtained on the organismic level.

It is possible that the overall reactions mask the extent of the alterations induced by diclofenac. Since the assessment is already starting in a range of mild to severe reactions, fine nuances of changes indicating a starting effect at low concentration might well be overlooked. Therefore, only severe changes can be visualized. On the other side, the already stressed condition of the trout may have left them more prone to further stressors. Consequently, the observed effects can be seen as the combined results of overall stress and further chemical influence. Under other conditions, the observed effects might not have been evoked at concentrations as low as in our findings.

1.1.4.2 Influence of metoprolol on juvenile brown trout

In contrast to diclofenac, metoprolol had no influence on animal survival at all. The few cases of mortality are within the natural variability and show no relation to the test concentration. Likewise, neither body mass nor body length was affected by any treatment - indicating that metoprolol does not gravely influence basic physiological ways, at least in the tested concentration range. Acute toxicity tests with concentrations higher than those examined in our study found no effect on Japanese medaka (Huggett et al. 2002) or *Danio rerio* after 72 h (van den Brandhof & Montforts 2010). Another study observed an increased mortality at a 96 h exposure of zebrafish embryos at 16 mg/L metoprolol (Sun et al. 2014).

Like in the second diclofenac experiment, the fish showed signs of intraspecific aggression, manifesting in bite marks on fins. However, the classifications used for both experiments are not directly comparable. The described moderate injuries were usually small nips on the dorsal or tail fin. Those also occurred, to a certain percentage, in the hatchery control. In a qualitative way, the bite marks were overall less severe than those observed in the diclofenac experiment. When comparing the biometric

measurements, it stands out that the animals used for the diclofenac experiment were substantially larger than the ones for the metoprolol experiment. The experiments took place in two separate years, so this is due to the overall conditions (hatching date, ambient temperature, feeding) while trout were bred. This stresses out how differently the animals can react to lab keeping, depending on their general condition. Still, the results show no indications for any modulation of aggression behaviour through metoprolol.

The stress protein Hsp70 remained unaffected by the treatment, indicating no signs of proteotoxic potential for metoprolol. Contardo-Jara et al. (2010) found an up to four fold increase of *hsp70*-mRNA after four day exposure of the zebra mussel *Dreissena polymorpha* to 0.534 µg/L metoprolol. Furthermore, Sun et al. (2014) observed a qualitative increase of *hsp70*-mRNA in *Danio rerio* after 96 h exposure to 0.01 mg/L metoprolol. These studies examined mRNA expression, while our study quantified the amount of protein, which has to be considered when comparing the results.

Histological results of control fish are comparable to the controls of the diclofenac experiment - possible explanations are given in 1.1.4.2. Metoprolol did not alter the condition of liver and gills, but led to changes in kidney. Those alterations were within the range of mild to severe reactions; only in one examined case destructive. Overall, these reactions were less severe than the ones found for diclofenac, seldom featuring focal necrosis and degeneration of the hematopoietic tissue. In contrast, reactions of the glomerular structures were mainly found for metoprolol. The better histological state explains the much lower mortality in the metoprolol exposure experiment. Previous studies found ultrastructural effects on tissue integrity of rainbow trout at concentrations as low as 1 µg/L. On the one side, the sensitivity of the applied ultrastructural analysis is higher than our light microscopical approach. On the other side, the age and species of the investigated animals was different, which may also account for the different results. In the present study no histopathological alterations were found in the heart of any investigated group. Steinbach et al. (2014) examined the effects of the beta blocker atenolol on the heart of rainbow trout: they observed more pronounced signs of inflammation in the pericardium of fish exposed to 1000 µg/L atenolol compared to the control. In addition, they found mild to moderate histological alterations like oedematous pericardial fibrous tissue, infiltration in the sub-endocardial space, and myocardial edema in trout exposed to atenolol. It is possible that atenolol is more harmful to trout than metoprolol and therefore leads to stronger histopathological alterations of the fish heart. Another explanation is, again, that rainbow and brown trout are differently sensible to beta blockers. In the present study, epicardium, myocardium and endocardium of the brown trout were examined. Steinbach et al. (2014) found histopathological alterations in the pericardium. It cannot be excluded that metoprolol leads to pathological alterations in the pericardium. Thus, in further experiments the pericardium should be considered by the investigation of histopathological alterations of the fish heart.

The finding of an altered ratio of erythrocytes to lymphocytes was an interesting finding, which has to be interpreted with care. Blood characteristics can change due to various stressors and can indicate toxicological effects (Clauss et al. 2008, Velisek et al. 2010). Our samples were, however, not originally designed to evaluate blood samples. Our finding can therefore be seen as a starting point for further studies – but those should apply suitable methods for evaluating the blood composition.

1.2 Studies with brown trout embryos

1.2.1 Introduction

Embryonic development is a crucial step in individual life history – disturbances can lead to grave and permanent impairments of individual health, which can result in population instability. Embryonic development, therefore, can be used as a toxicity endpoint linking individual health to population integrity.

Embryo tests with fish, as e.g. zebrafish *Danio rerio*, are standard toxicity tests that are frequently used in this context. In contrast, embryo tests with feral fish species are less common. In our experiment, we exposed eggs of brown trout to the pharmaceuticals diclofenac and metoprolol. Compared to the test with *Danio rerio*, these tests are more time-consuming and need more elaborated experimental requirements. Brown trout live and breed in cold, oxygen-rich waters – the embryonic development takes around 100 days under normal conditions; additionally, the eggs are extremely sensible during the first weeks of development.

To our knowledge, up to know, no data exist for possible effects of diclofenac and metoprolol in developing brown trout from the point of fertilization. Hence, we wanted to produce further toxicity data *in vitro*-systems can be related to.

1.2.2 Materials and Methods

The exposure experiment with brown trout eggs was started on December 1st 2014 and was ended on April 7th 2015, totalling to an exposure time of 127 days. It was conducted according to guideline OECD 212 (short term toxicity test on embryo and sac-fry stages of fish). Due to the fact that the brown trout is no standard test organism, some adaptations had to be made.

The experiment was realized in triplicates: three blocks, each containing one control and five concentrations of diclofenac (0.1, 0.5, 1, 10 and 100 µg/L) and five concentrations of metoprolol (0.1, 1, 10, 100 and 1000 µg/L) were placed on a shelf in randomized order (Figure 29). All pharmaceutical solutions were freshly prepared by adding an appropriate volume of stock solution to aerated artificial water (294 mg/L $CaCl_2$ x2 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 Millipore-filtered deionized water). Glass petri dishes (diameter: 15 cm) served as experimental vessels, each containing 200 mL of solution. 30 eggs were added to each test vessels within 24 h after fertilization. The whole experiment was conducted in a thermo-constant chamber where temperature was set to 7 °C. Until the developmental stage determined by eye development was reached, the test was conducted in complete darkness and any movement / agitation of the eggs had to be avoided. After the embryos had developed eyes, the light/dark cycle was set to 10 h/14 h. Water exchanges took place three times a week, every time removing 150 mL of old medium and replacing it with freshly prepared solution. The frequent water changes were necessary, because the test vessels cannot be aerated, since this would disturb developing eggs too much.

The recorded parameters included fertilization rate, mortality of eggs and fry, time until eye pigmentation, time until hatching, heart beat rate and time until resorption of the yolk sac. Heart beat rate was determined by counting the number of beats in 20 s for 10 individuals in each vessel.

Water samples for chemical analyses were taken at the beginning of the experiment, at the time point of eye development, at the time point of hatching, and when the experiment was terminated.

Figure 29: Experimental setup of the embryo test using brown trout. Five concentrations of diclofenac (0.1, 0.5, 1, 10 and 100 µg/L), five concentrations of metoprolol (0.1, 1, 10, 100 and 1000 µg/L) and one control treatment (artificial water only) were tested in triplicates.

1.2.3 Results

1.2.3.1 Chemical analysis

Measured concentrations revealed some deviations from nominal values. Concentrations for diclofenac were 0, 0.06, 0.51, 0.75, 7.8 and 74.6 µg/L for diclofenac and 0, 0.06, 1.0, 9.8, 70.1 and 998 µg/L for metoprolol. In all following graphs, measured concentrations are displayed. Oxygen saturation was, in several cases, slightly reduced but still within the tolerable range (Table 11).

Table 11: Physico-chemical parameters at the end of the pharmaceutical exposure experiment of brown trout embryos and sac-fry stages.

Block	Exposure group	Temperature [°C]	pH	Oxygen saturation [%]	Oxygen content [mg/L]
1	control	7.6	7.30	60.2	7.10
1	0.1 µg/L DIC	7.3	7.06	59.6	7.37
1	0.5 µg/L DIC	7.3	6.60	64.7	7.61
1	1 µg/L DIC	7.5	6.69	69.7	8.01
1	10 µg/L DIC	7.3	6.70	73.4	8.51

Block	Exposure group	Temperature [°C]	pH	Oxygen saturation [%]	Oxygen content [mg/L]
1	100 µg/L DIC	7.4	6.76	72.4	8.37
1	0.1 µg/L MET	7.3	6.81	74.2	8.61
1	1 µg/L MET	7.3	6.82	71.3	8.41
1	10 µg/L MET	7.4	6.76	74.0	8.33
1	100 µg/L MET	7.3	6.67	77.5	9.15
1	1000 µg/L MET	7.1	6.64	78.9	9.17
2	control	7.0	7.15	77.0	8.99
2	0.1 µg/L DIC	7.2	7.00	79.0	9.19
2	0.5 µg/L DIC	7.0	6.99	74.8	8.64
2	1 µg/L DIC	7.2	6.91	80.3	9.33
2	10 µg/L DIC	7.0	6.92	76.0	8.91
2	100 µg/L DIC	7.0	6.87	76.5	8.93
2	0.1 µg/L MET	6.8	6.82	81.4	9.54
2	1 µg/L MET	7.1	6.80	74.1	8.64
2	10 µg/L MET	6.9	6.83	73.5	8.65
2	100 µg/L MET	6.7	6.90	78.9	9.23
2	1000 µg/L MET	6.9	6.69	72.8	8.55
3	control	7.0	6.46	63.4	7.46
3	0.1 µg/L DIC	7.3	6.68	73.6	8.53
3	0.5 µg/L DIC	7.4	6.70	73.4	8.55
3	1 µg/L DIC	7.1	6.69	70.1	8.15
3	10 µg/L DIC	7.1	6.78	79.7	9.28
3	100 µg/L DIC	7.1	6.73	74.2	8.77
3	0.1 µg/L MET	6.9	7.00	68.2	7.99
3	1 µg/L MET	7.0	6.70	73.2	8.55
3	10 µg/L MET	7.1	6.62	76.8	8.93
3	100 µg/L MET	6.7	6.72	73.0	8.58
3	1000 µg/L MET	6.9	6.62	76.4	8.93

1.2.3.2 Mortality

Mean percentage of coagulated eggs was between 6.7 and 17.8 %. The overall embryo mortality during the whole experiment was very low. Mean survival (excluding the unfertilized eggs) varied between 94.6 and 100%.

1.2.3.3 Eye pigmentation

The first signs of eye pigmentation could be observed after 29 days of exposure; after 33 days, all embryos showed developed eyes. No obvious differences could be observed between the treatment groups.

1.2.3.4 Hatching

Hatching of larvae began 63 days after the start of the exposure. All treatments showed high hatching success, only a slight decrease could be seen at 1 µg/L diclofenac and a slight delay at 1000 µg/L metoprolol (Figure 30 and Figure 31)

Figure 30: Hatching rate over time for brown trout embryos exposed to diclofenac. All values are given relative to the number of originally exposed eggs. After day 70, all surviving larvae had hatched.

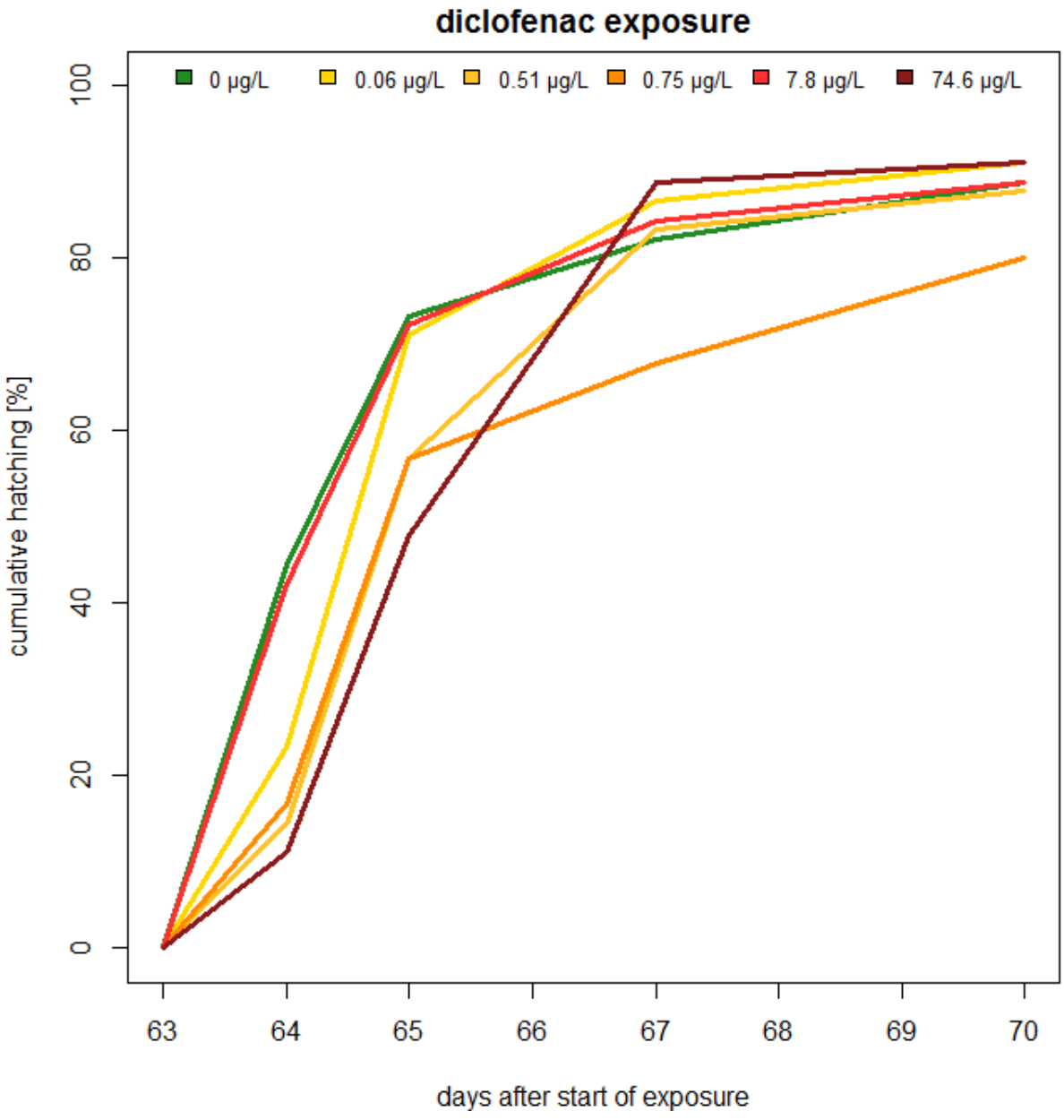
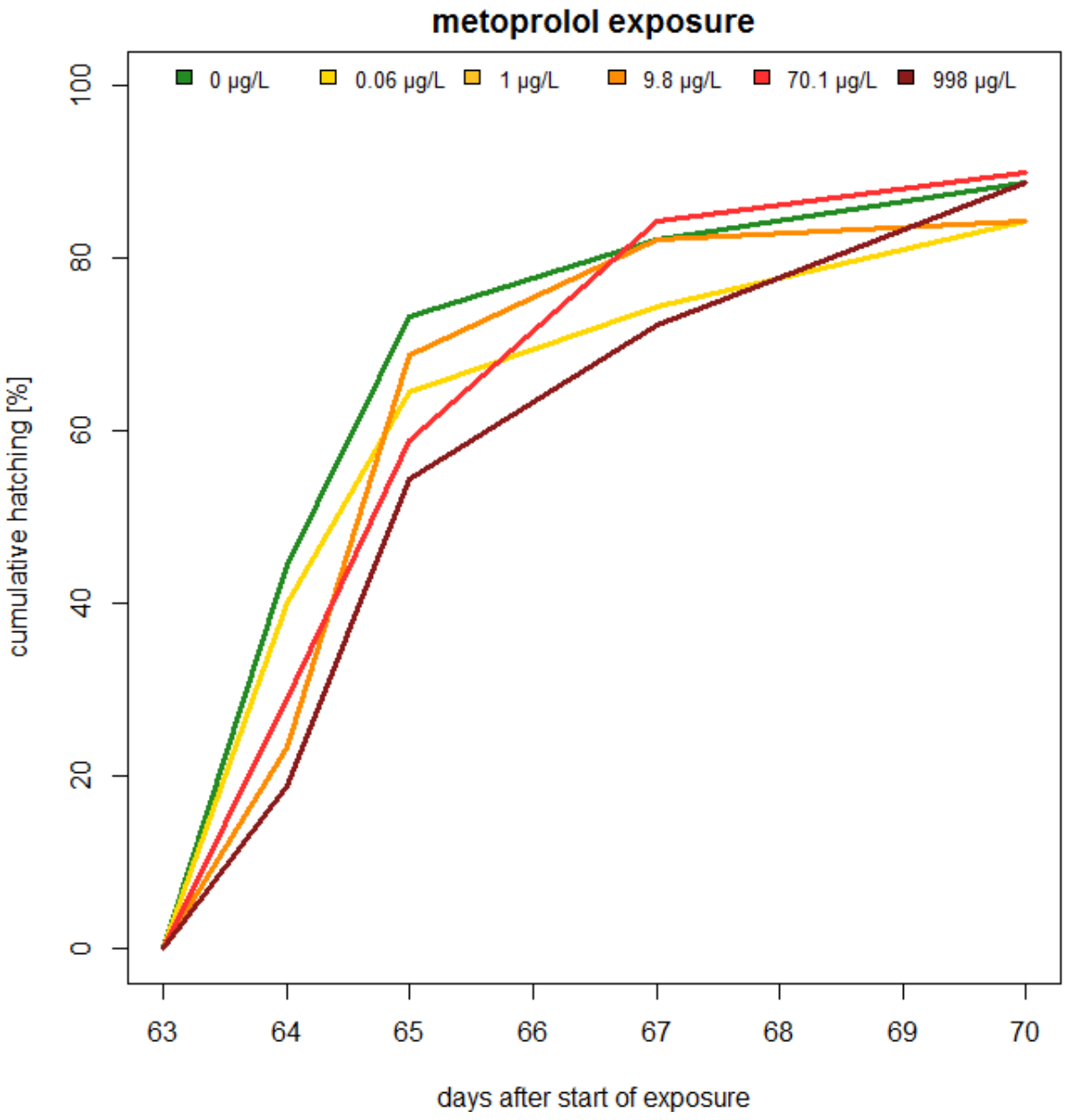


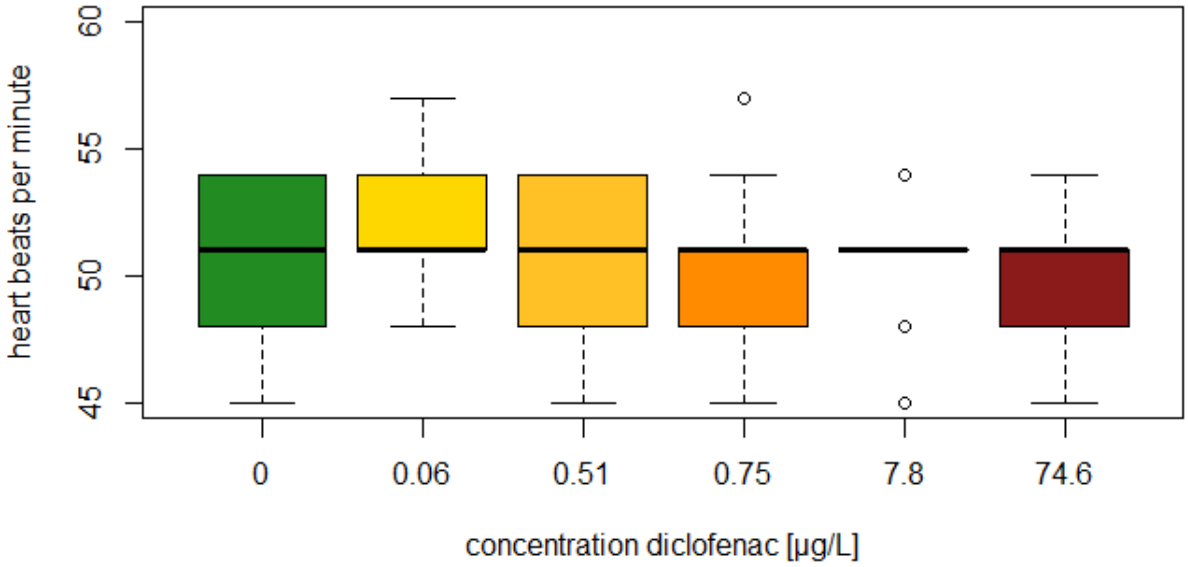
Figure 31: Hatching rate over time for brown trout embryos exposed to metoprolol. All values are given relative to the number of originally exposed eggs. After day 70, all surviving larvae had hatched.

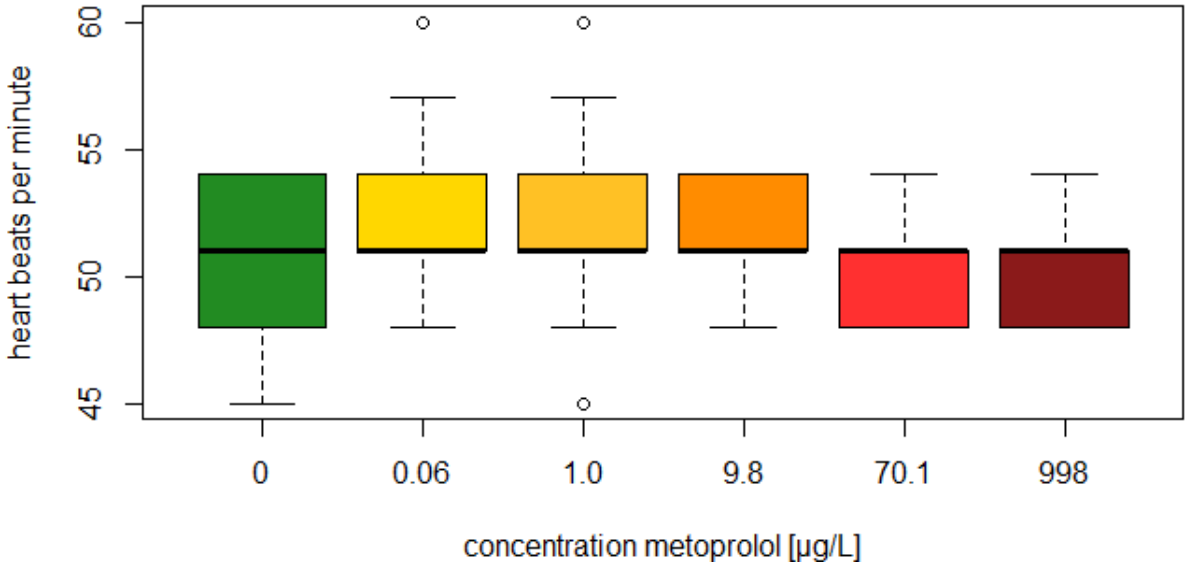


1.2.3.5 Heart rate

The heart rate showed only minor differences between the exposures. Overall, there were no visible modulations of heart rate through any of the exposure treatments (Figure 32).

Figure 32: Heart rate (beats per minute) of brown trout larvae exposed to diclofenac (top) and metoprolol (bottom). Bold lines display the median values, boxes the 25 % to 75 % quantiles, whiskers the minimum and maximum values. Overall, the heart rate was similar throughout all treatments, with little variability.



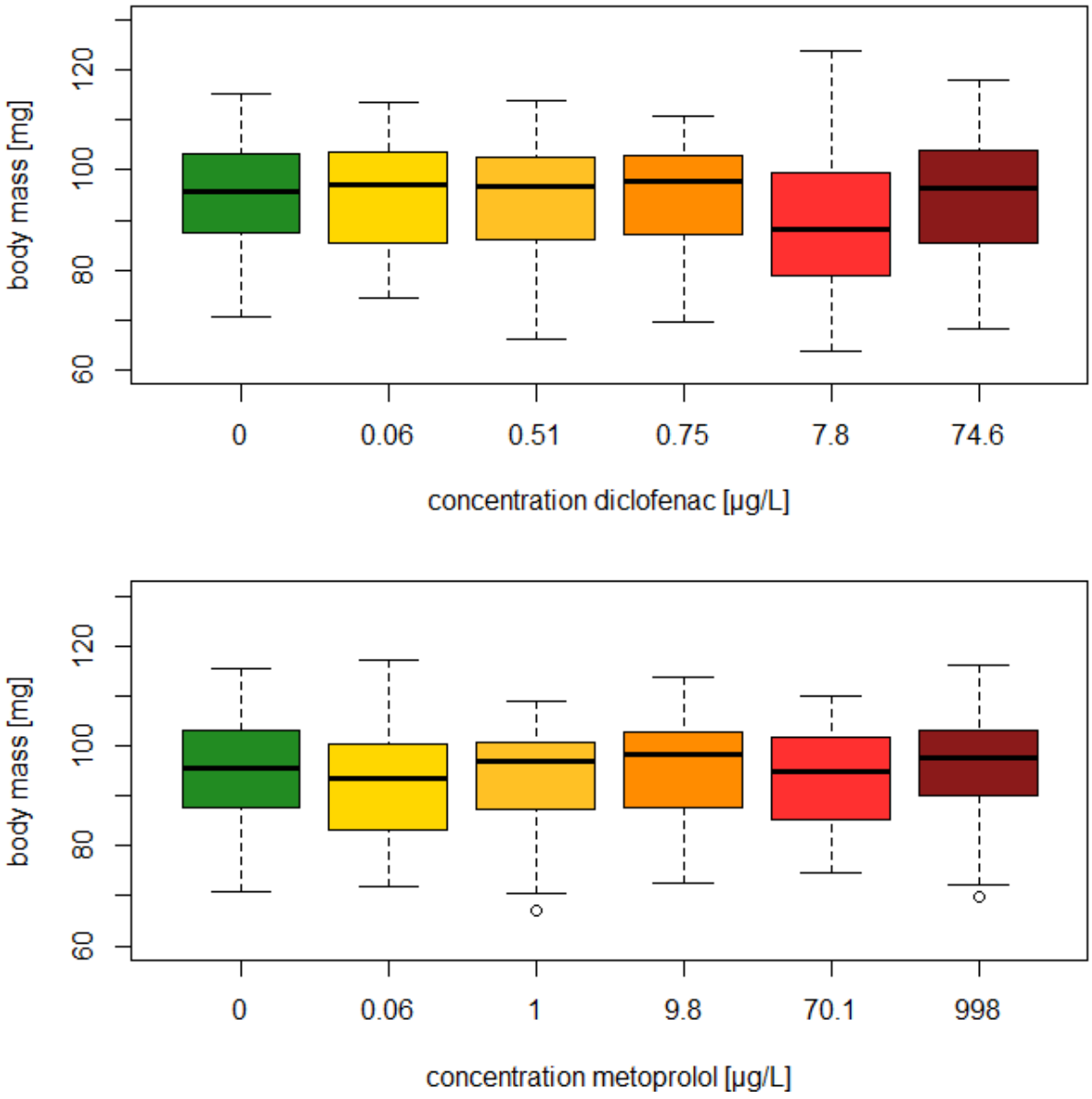


1.2.3.6 Body mass

Body mass showed some slight differences for diclofenac: larvae exposed to the second highest concentration (7.8 µg/L) had a slightly lower body mass. However, the results were clear only in one of three experimental blocks. Overall, the block seemed to have a stronger influence than the treatment itself (nested ANOVA on data raised to the power of 3: treatment df=5, F=3.4504, p=0.0046; Block[Treatment] df=12, F=4.2617, p<0.0001), and there was no visible cause-effect-relationship.

For metoprolol, the block was also of greater influence than the treatment itself (nested ANOVA on data raised to the power of 3: treatment df=5, F=2.0237, p=0.0744; block [treatment] df=12, F=2.7347, p=0.0014). Data are shown in Figure 33.

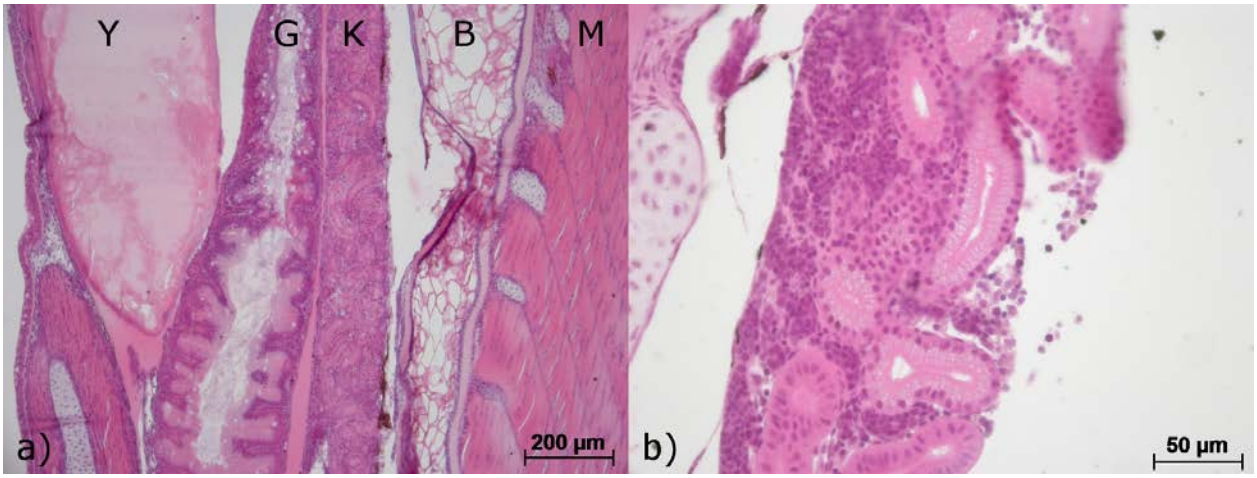
Figure 33: Body mass of brown trout larvae exposed to diclofenac (top) and metoprolol (bottom) for 127 days. Bold lines display the median values, boxes the 25 % to 75 % quantiles, whiskers the minimum and maximum values. The only significant effect was a slightly lower mass of larvae exposed to 7.8 µg/L diclofenac, which could only been seen in one of three experimental blocks.



1.2.3.7 Histopathological examinations

Since trout larvae in the examined life stage are still developing their gills an investigation of this organ was not meaningful. Instead, we focused on the histological state of kidney. Only few individuals showed peculiarities in respect to their renal structure. A common finding was the occurrence of large vacuoles (unstained in HE) in the tubular cell cytoplasm – which was regarded as normal finding in individuals of the used age class. Further peculiarities were occasional reactions of glomerular structures and increased vesicularizations in tubulus cells. The semi-quantitative assessment showed all individuals to be in the range of control state to (mild) reactions. There were no differences between the control and the metoprolol and diclofenac exposure.

Figure 34: Histological sections of brown trout embryos exposed to diclofenac and metoprolol: a) control (HE): overview on general anatomy, arranged from ventral (left) to dorsal (right, featuring yolk sac (Y), gut (G), kidney (K), bone structures (B) and the dorsal muscles (M). b) kidney, 1000 µg/L metoprolol (HE): only few reactions were found in embryonic brown trout. Several individual displayed increased vesicularization and protein inclusions in tubular cells.



1.2.4 Discussion

There were no signs indicating embryotoxic potential of diclofenac and metoprolol. Neither the time until eye pigmentation, nor the time until hatching was delayed. Furthermore, the heart beat rate was not influenced by the pharmaceutical treatment. This is especially unexpected for metoprolol, which was expected to influence the heart at least in higher concentrations. A previous study reported a NOEC of 500 µg/L diclofenac for mortality, hatching, development and teratogenicity (LfW 2004). Comparable results were found for embryonic stages of *Danio rerio* (Hallare et al. 2004, van den Brandhof & Montforts 2010, Memmert et al. 2013, Sun et al. 2014), *Oncorhynchus mykiss* (Memmert et al. 2013) or *Cyprinus carpio* (Stepanova et al. 2013), where significant effects were only found in concentrations higher than 1 mg/L.

Overall, the eggs showed to be of high quality with a very high fertilization rate. In addition, most of the embryos reached the stage of eye pigmentation and hatched. For the assessment of the developmental time is has to be considered, that fish development is strongly influenced by ambient temperature. A comparison to literature data already shows that our data are in the range of normal brown trout development described by Killeen et al. (1999a) and Killeen et al. (1999b).

There were no signs of increased mortality through any of the tested treatments – even at the high diclofenac concentrations that exhibited a strong effect in juvenile animals. This was reflected in the inconspicuous findings of the histological examination. Since the fish were exposed to the chemicals for their whole life, it may be the case that they are better acclimatized to this kind of stress. In contrast, the juvenile fish from 4.3.1 were reared in very pristine water at the fish farm – so the pharmaceutical exposure could be a much greater shock and stress for these animals.

1.3 Studies with *Gammarus fossarum*

1.3.1 Introduction

Single substance tests with diclofenac and metoprolol were conducted with the epibenthic amphipod *Gammarus fossarum* over 40 days. Crustaceans were chosen as test organisms, because they represent an important organismic group in German freshwaters, thus, they are of high ecological relevance (Triebskorn et al., 2013).

It is the aim of the study to provide effect data for population relevant endpoints and biomarkers for diclofenac and metoprolol as benchmarks for the mode of action-based *in vitro*-tests realized in Eff-Pharm, and as a basis for the characterization of possible risk posed by the two pharmaceuticals.

Data on effects of diclofenac and metoprolol in gammarids are scarce. In one study, a NOEC of 1 mg/L was determined for *Gammarus spp*. exposed to diclofenac. Here the endpoints were respiration and NH_4^+-excretion (Oskarsson, 2012). No studies were found for effects of metoprolol on gammarids in single substance tests.

To determine exposure concentration in chronic experiments, preliminary tests were required for identification of acute toxic effect concentrations. In preliminary investigations sorption processes of the pharmaceuticals to organic matter in form of the food source was investigated.

1.3.2 Material and Methods

1.3.2.1 Effect data from literature

Publications on effect data with crustaceans exposed to diclofenac and metoprolol were reviewed by Triebskorn et al. (2013) and Bergmann (2011).

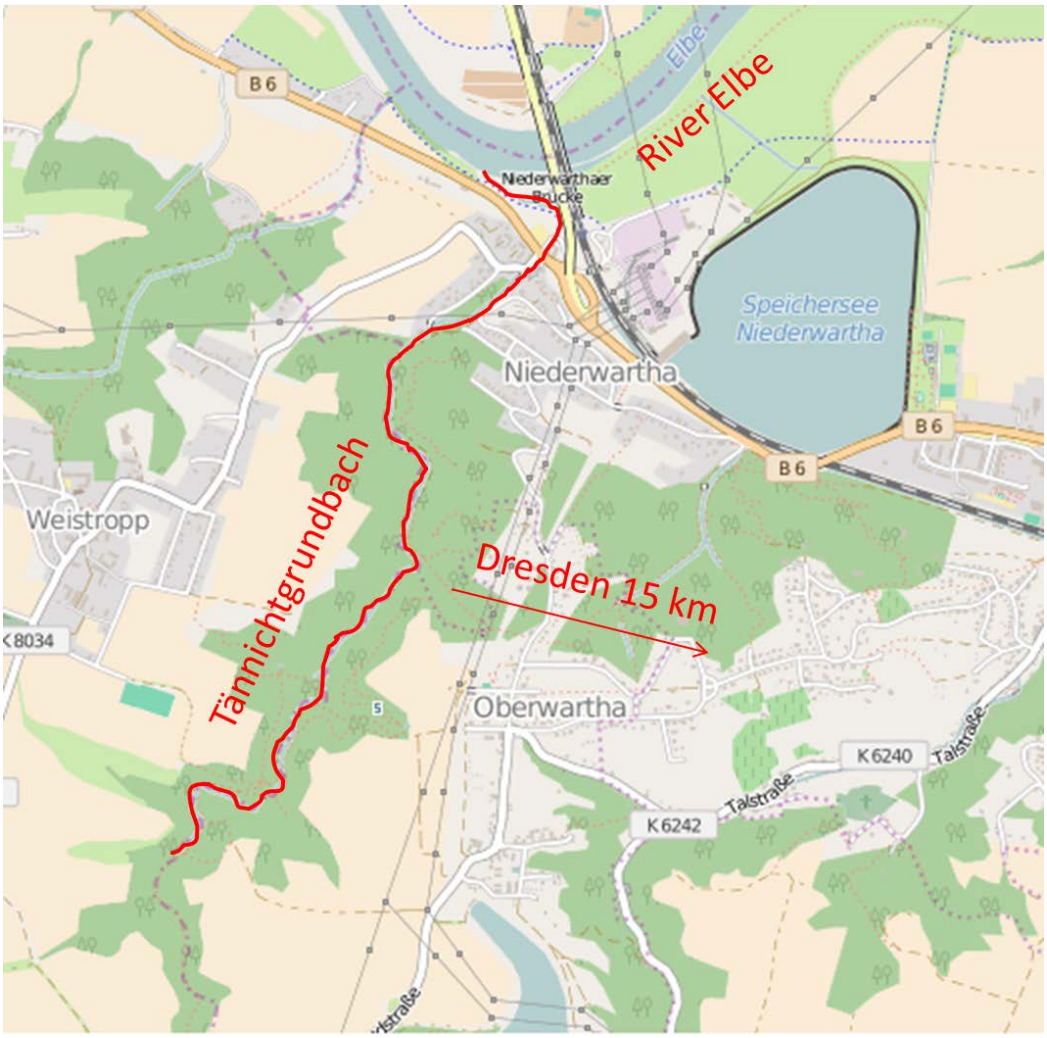
1.3.2.2 Analysis of pre-incubated leaf matter of *Alnus glutinosa*

Air-dried leaves from alder (*Alnus glutinosa*) were weighed and conditioned for 14 days in aerated stream water from Tännichtgrundbach in an aquarium. Dry weight and wet weight after conditioning were examined to determine the water content. Leaf discs with a diameter of 28 mm were punched from conditioned leaves and weighed. Each leaf disc was incubated in 50 ml exposure medium (100 ml glass beaker) for 48 h with 3 replicates each for diclofenac and metoprolol, respectively, at concentrations of 5 mg/L and 45 mg/L. At the beginning and end of the experiments, samples of the respective medium were taken as a composed sample of 500 µl per replicate with an Eppendorf pipette into 1,5 ml short thread vials (Fisher Scientific) for chemical analysis of pharmaceutical concentrations, and deep frozen. Subsequently, leave matter was freeze-dried for 24 h (Alpha 1-2, Fa. Christ) and sent to TZW Karlsruhe together with the aqueous samples of exposure media for analysis. Pharmaceutical concentrations in the aqueous phase were determined at the beginning and the end of exposure. Data are shown in Chapter 6.3.

1.3.2.3 Screening of pharmaceutical natural background exposure in original habitat water of gammarids

Individuals of *G. fossarum* used for experiments originated from Tännichtgrundbach. Water samples taken at two days were analyzed for pharmaceuticals. Tännichtgrundbach is a first order stream situated northwest of Dresden, left side of river Elbe (Figure 35). After a distance of 3.2 km the stream flows into the Lotzebach in Niederwartha, which flows into river Elbe after short distance. Along its course no settlement or other construction exists, which keeps the Tännichtgrundbach free from domestic or industrial wastewaters.

Figure 35: Course of the Tännichtgrundbach (source: OpenStreetMap).



At two days of sampling, March 31, 2014 and April 15, 2014 water was taken from Tännichtgrundbach, deep frozen (Bosch, -22 °C), and analyzed by TZW for 28 pharmaceuticals, including the test substances metoprolol and diclofenac. Data are shown in Chapter 6.3.

Compared with other surface waters the Tännichtgrundbach is less polluted (Aydin and Talinli, 2013; Wang and Gardinali, 2012) and the concentrations are three orders of magnitude lower than effect concentrations found in our experiments. Hence, an impact on the gammarids sampled from the Tännichtgrundbach for the experiments can be neglected.

1.3.2.4 Acute tests

Acute tests (duration: 48 h) were conducted with 5 concentrations of diclofenac or metoprolol. Per concentration and control, 10 individuals were individually exposed in 10 mL medium in 15 mL welted glasses with snap-on lids at 15 ± 1 °C in a greenhouse (Figure 36).

Metoprolol tartrate (2:1, CAS 56392-17-7, purity >= 98%) was purchased from Sigma Aldrich, and diclofenac sodium salt (CAS 15307-79-6, purity >= 99%) was obtained from Cayman Chemical Company.

Stock solutions were established in Borgmann medium (Borgmann, 1996) and subsequently diluted to achieve respective exposure concentrations. Diclofenac was tested at concentrations of 1mg/L to 256 mg/L with spacing factor 4, while metoprolol was tested at concentrations of 3.1 mg/L to 800 mg/L with spacing factor 4 (Table 12).

Table 12: Nominal concentrations of diclofenac and metoprolol in acute tests with *Gammarus fossarum.*

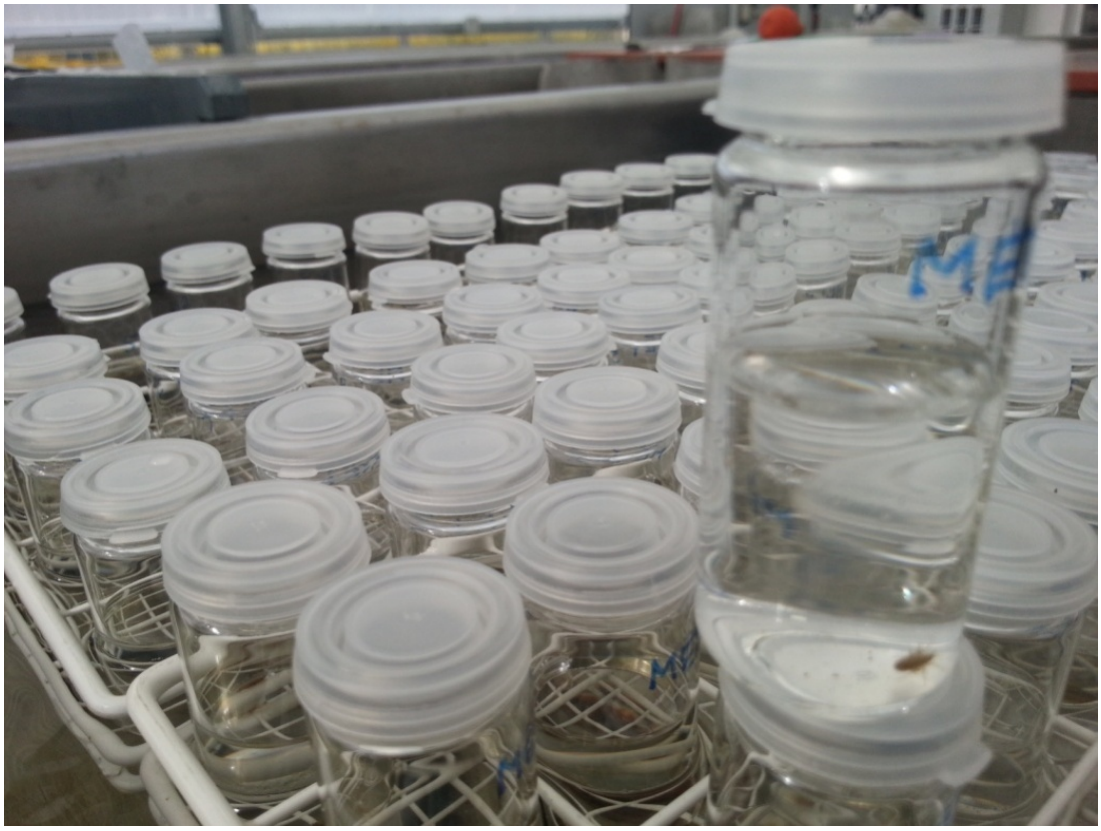
Pharmaceutical	Conc. 1	Conc. 2	Conc. 3	Conc. 4	Conc. 5
Diclofenac [mg/L]	1.0	4.0	16.0	64.0	256.0
Metoprolol [mg/L]	3.1	12.5	50.0	200.0	800.0

Test organisms were obtained by field sampling from the stream Tännichtgrundbach in the vicinity of the city of Dresden. The gammarids were kept in an artificial indoor stream since autumn 2013 at 15 ± 1 °C with conditioned foliage from *Alnus glutinosa* as food source as described by Ladewig et al. (2006).

For exposure, individuals with body lengths of 0.7 cm ± 0.5 mm were selected. Selection was based on measures of selected individuals by determination of their exact length using a stereomicroscope (Thalheim Spezial Optik). Further selection was carried out by macroscopic comparison of these individuals to the other potential test organisms.

Exposure conditions for gammarids were 15 ± 1 °C and seasonal light conditions. Welted glasses with snap-on lid (15 ml) were cooled in the artificial indoor stream. The physico-chemical parameters pH, conductivity, and oxygen content were measured prior and after exposure. For diclofenac, pH was measured exclusively prior to exposure. Throughout the entire exposure, no food was supplied.

Figure 36: Exposure of *G. fossarum* with metoprolol in the acute test.



After 48 h, surviving gammarids were counted at each concentration. For metoprolol, exposure was prolonged to 144 h, and organisms were controlled daily.

Samples for chemical analysis of the pharmaceuticals were taken at the start of exposure and after 48 h as composite sample per concentration (150 µl per exposure vial) with an Eppendorf pipette and

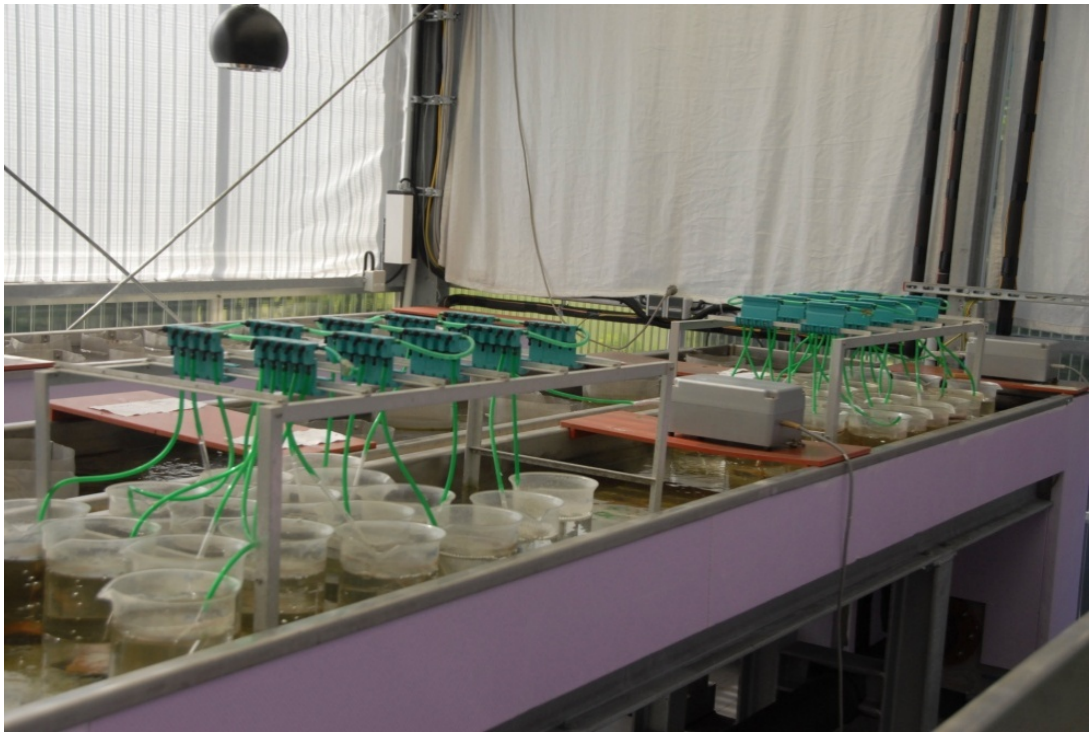
deep-frozen (Bosch, -22 °C). Samples from the lowest, medium, and highest exposure concentration were sent to TZW Karlsruhe for analyses regarding pharmaceutical concentrations.

For computation of EC_{10}, EC_{20}, and EC_{50} values, ToxRat software (Version 2.1, ToxRat Solutions GmbH) was deployed.

1.3.2.5 Chronic experiments with diclofenac and metoprolol

Over a period of 40 days, 20 individuals of *G. fossarum* per concentration were exposed to 5 concentrations of either diclofenac (CAS 15307-79-6) or metoprolol (CAS 56392-17-7) in 2 L beakers (glassware). All treatments and the controls were run in quadruplicate (Figure 37).

Figure 37: Chronic exposure of gammarids to diclofenac and metoprolol.



1.3.2.6 Applied pharmaceuticals

Concentrations of pharmaceuticals selected for the chronic exposure experiments derived from EC_{50} values obtained from previous acute tests with *G. fossarum* (Table 13).

Table 13: Nominal concentrations for chronic exposure of *G. fossarum* to diclofenac and metoprolol (spacing factor 3).

Pharmaceutical	Conc. 1	Conc. 2	Conc. 3	Conc. 4	Conc. 5
Diclofenac [mg/L]	0.49	1.80	4.44	13.33	40.00
Metoprolol [mg/L]	5.00	15.00	45.00	135.00	405.00

Stock solutions were prepared, at respective highest concentrations, in Borgmann medium (particle free and active carbon filtered tap water with additives LO4-S and E + H as described BY BORGMANN (1996) and subsequently diluted with Borgmann-medium to the respective exposure concentrations. Metoprolol tartrate and diclofenac as sodium salt were purchased from Sigma Aldrich. The given concentrations refer to the active ion of the pharmaceuticals (without tartrate or sodium ions).

1.3.2.7 Exposure of gammarids

Individuals of *G. fossarum* were collected in Tännichtgrundbach (Winkelmann et al., 2008) and adapted to laboratory conditions in Borgmann-Medium at 15 ± 1 °C for 2 to 3 days prior to exposure.

For exposure, individuals with lengths of 7 mm ± 0.5 mm were chosen. Selection was based on measures of selected individuals by determination of exact length using a stereomicroscope (Thalheim Spezial Optik). In detail, distances between the basis of the first antenna and the basis of the telson were determined (Ladewig et al., 2005). Further selection of individuals was carried out by macroscopic comparison of measured individuals to the other potential test individuals.

In each 2 L beaker (VWR, glassware) 20 individuals of gammarids were introduced, along with two unglazed ceramic tiles (size 5.5x4x1 cm) and one crushed stone (ca. 3x5 cm), into 2 L Borgmann medium with the respective concentrations of pharmaceuticals, and exposed at 15 ± 1 °C in a greenhouse. For oxygen supply, beakers were aerated (Pasteur pipette, glassware, 3 air bubbles per second). Gammarids were fed weekly by adding 5 fresh *Alnus glutinosa* leaf discs (d=28 mm) per beaker after removing remainders of fodder. Whole leafs collected and dried in fall 2013, were conditioned for 14 days in aerated stream water (see above) and leaf discs were punched subsequently. Leaf discs were incubated at respective pharmaceutical concentrations for 2 days before they served as food for the gammarids.

80% of the medium were exchanged weekly. Evaporated medium was filled up using fully deionized water (18.2 MOhm) before exchange of medium. Stock solutions were prepared freshly as described above and diluted to working concentration with Borgmann medium. Old medium was extracted from beakers using a flexible hose (d=0.35cm) the opening of which was covered with gauze at the side. Leaf disc material that was not consumed by gammarids was removed and 5 fresh discs (see above) were added. Dead individuals were removed.

During exposure, the oxygen content and saturation were examined every 2 to 3 days. Conductivity and pH values were measured weekly.

The weekly exchange of 80% of medium, deviating from the Eff-Pharm proposal (27.10.2014), was agreed at the Kick-Off-Meeting on 14.03.2014. For the reason of this variation a single time samples were drawn immediately before medium exchange for analysis of concentrations of nitrate, dissolved organic phosphorus (SRP) and ammonium. Samples were taken after 35 days from diclofenac exposure experiments, and after 28 days from metoprolol experiments as collective samples for each concentration (100 ml per replicate, pipette Fisherbrand). Nitrate was analyzed using rapid test cuvettes (LCK 339, Hach Lange). Ammonium and SRP (Legler, 1976) were determined photometrically. Samples were stored in a freezer (Bosch, -22 °C) until analytical processing.

For the analysis of stress proteins and lipid peroxides individuals from the original population of gammarids were frozen in liquid nitrogen at the beginning of the experiment: 57 individuals for diclofenac and 47 for metoprolol were frozen and stored at -80 °C (Sanyo Ultra Low). At the end of the experiment 32 to 34 individuals per concentration were stored in the same manner for further analysis. Samples were shipped on dry ice to the University of Tübingen.

At the end of the experiment, mortality, the number of precopulae and the number of egg-bearing females per exposure replicate were determined. Subsequently, individuals were fixed for biomarker analysis (see above). Remaining gammarids including juveniles of the F1 generation were fixed in formaldehyde.

With a stereomicroscope (Thalheim Spezial Optik) the number of eggs per egg bearing female and the body length of juveniles were measured for up to 20 or, whenever less, for all individuals per replicate.

1.3.2.8 Sampling for chemical analysis

At the beginning and end of experiments, as well as before and after each exchange of medium from every exposure vessel, the medium was sampled for chemical analysis of pharmaceutical concentrations (1.5 ml, Eppendorf pipette). Samples were deep frozen (Bosch, -22 °C) in HPLC glass bottles (1.5 ml short thread bottles, Fisher Scientific). Samples were analyzed at TZW Karlsruhe.

Collective samples of the concentration replicates from the beginning of experiment, from day 7 before medium exchange, from day 28 after medium exchange, and from day 35 before medium exchange were analyzed. Controls were analyzed at the beginning of the experiment and after 35 days.

For the approaches in which the lowest LOEC/NOEC values were determined (most sensitive endpoints) water samples were analyzed. For diclofenac, samples from the 1.48 and 4.44 mg/L set-up, and for metoprolol samples from the 5 and 15mg/L set-up were analyzed.

1.3.2.9 Statistical Evaluation

William test (α=0.05) for determination of NOEC and LOEC values and Weibull regression for determination of EC_x values were applied, using ToxRat software (Version 2.1, ToxRat Solutions GmbH).

1.3.2.10 Determination of biochemical markers

For identifying the level of stress proteins in gammarids, the same protocol as described in 1.1.2.2 was used with only minor changes. The samples were homogenized in a standard sample volume of 50 µL extraction medium, since all samples were of comparable size.

Between 16 and 19 samples per treatment of the diclofenac exposure were used for stress protein quantification. For the metoprolol treatment, between 30 and 34 samples (exception: concentration 3 with 13 samples) per group were analyzed. The lower sample size for the diclofenac treatment is due to the fact that half of the samples were used for the FOX-Assay.

The quantification of lipid peroxides followed the same protocol as described in 1.1.2.3, with some adjustments in parameters. Samples were diluted in a ratio of 1:10 to gain a sufficient volume for the assay. As for the trout, the first incubation was done over a time period of 150 min, but the incubation after the addition of cumene hydroperoxide was increased to 60 min. Sample sizes ranged between 13 and 17 individual gammarids.

1.3.3 Results

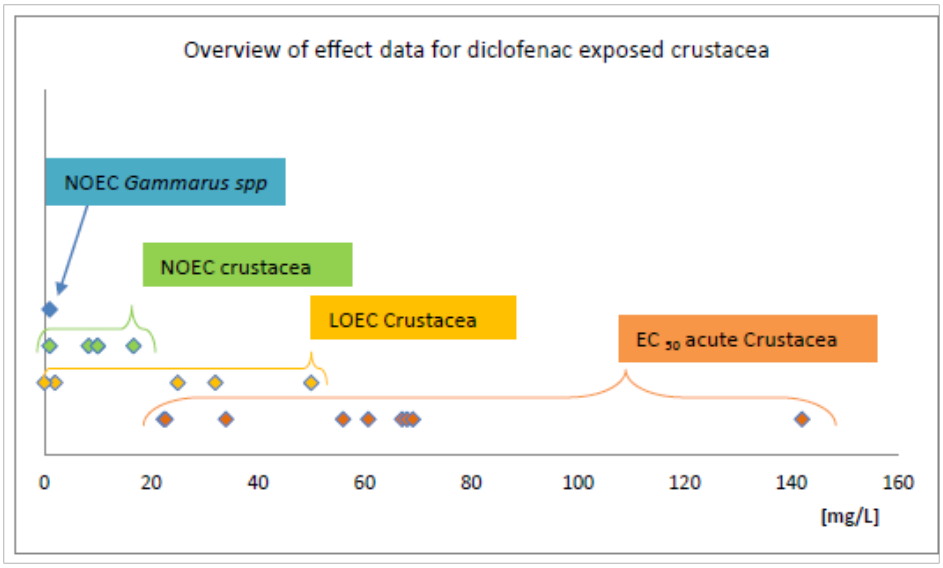
1.3.3.1 Effect data for crustaceans from the literature

For effects of diclofenac and metoprolol in gammarids, only very few data are currently available from the literature (Triebskorn et al. 2014). In a single study, a NOEC of 1 mg/L was determined for *Gammarus spp.* exposed to diclofenac, regarding the endpoints respiration and NH_4^+-excretion (Oskarsson, 2012). No studies were found for effects of metoprolol on gammarids in single substance tests. For the determination of exposure concentration in chronic experiments, preliminary tests were required for the identification of acute effect concentrations.

An overview of studies with crustaceans is given in Annex 1 and Annex 2. Most investigations with crustaceans were realized with the planktonic species *Daphnia magna* and *Ceriodaphnia dubia*, whereby the latter was found to be more sensitive.

Effect data for crustaceans exposed to diclofenac are shown in Figure 38.

Figure 38: LOEC, NOEC and EC_{50} data for effects of diclofenac in crustaceans after acute and chronic exposure (data taken from literature).



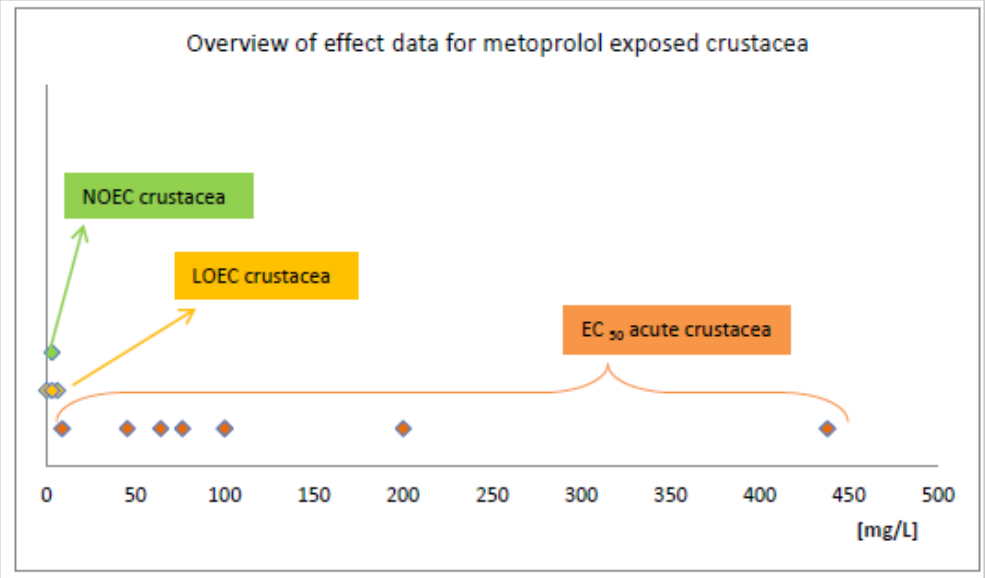
In total, 9 studies with diclofenac were found, revealing 19 acute and chronic effect concentrations (see Annex 1 for data). As displayed in Figure 38 and Table 14, effect concentrations from acute and chronic tests overlap distinctly. Especially remarkable is a multi-generation test with six generations, which determined negative effects of diclofenac on reproduction of Daphnia at a concentration of 0.36 µg/L (Dietrich et al. 2010). This effect concentration is 5,000-fold lower than the closest LOEC found in other studies, although results were not fully reproducible in all multi generation tests conducted by Dietrich et al. (2010).

Table 14: Overview of published effect data for diclofenac tests on crustaceans; A – acute test, C-chronic test, SA-sub-acute test.

Test	Testduration [h]	Number of studies	Test organism	Endpoint	Effect conc. MIN [mg/L]	Effect conc. MAX [mg/L]
A	24 – 48	9	crustaceans, daphnids	mortality	EC_{50} 22.40	EC_{50} 69.05
A	48	1	crustaceans, moinidae	mortality		EC_{50} 142.60
C	168 – 504	1	crustaceans, daphnids	mortality	NOEC 10.00	LOEC 32.00
C	168 – 504	4	crustaceans, daphnids	reproduction	NOEC 1.00	NOEC 10.00
C	168 – 504	1	crustaceans, moinidae	reproduction	NOEC 16.70	LOEC 50.00
C	168 – 684	2	crustaceans, daphnids	reproduction	LOEC 0.00036	LOEC 25.00
SA	1344	1	crustaceans, *Gammarus spp*.	respiration, NH_4^+ -excretion		NOEC 1.00

Concerning the impact of metoprolol on crustaceans fewer studies were published. In 8 studies (acute and chronic exposure) 11 effect concentrations were determined (Table 14). As visible from Figure 39 and Table 15, effect concentrations for acute and chronic exposure overlap strongly. Remarkably, EC_{50}-values for crustaceans vary by a factor of 50.

Figure 39: LOEC, NOEC and EC50 data for effects of metoprolol in crustaceans after acute and chronic exposure /data taken from literature).



In the study of Dietrich (2010), a very low LOEC was also determined for metoprolol (0.012 mg/L) with negative effects on the reproduction of Daphnia, which again differed significantly from LOECs determined in other studies.

Table 15: Overview of published effect data for metoprolol tests on crustacea; A – acute test, C-chronic test, SA-sub-acute test.

Test	Test duration [h]	Number of studies	Test organism	Endpoint	Effect conc. MIN [mg/L]	Effect conc. MAX [mg/L]
A	24 – 48	7	crustaceans, daphnids	mortality	EC_{50} 8.8	EC_{50} 438.0
A	0,5	1	crustaceans, daphnids	heart rate		LOEC 32.0
C	216 – ca. 684	1	crustaceans, daphnids	reproduction		NOEC 3.1
C	216 – ca. 684	2	crustaceans, daphnids	reproduction growth	LOEC 0.0012	LOEC 6.2

1.3.3.2 Acute tests with G. fossarum

Detected concentrations of the pharmaceuticals at lowest, medium and highest exposure concentration were accounting for 85 to 90% of nominal concentrations for metoprolol and 85 to 100% for diclofenac at the beginning of acute tests. Decline of concentrations over 48 h amounted 10 to 12% compared to measured concentration in the metoprolol assay and 1 to 23% in the diclofenac assay. At the lowest nominal concentrations, the decline was stronger than at the higher concentrations. Hence,

nominal water concentrations are suitable to express effect concentrations (OECD-23 2000) (Table 16).

Table 16: Nominal and measured concentrations of diclofenac and metoprolol in acute tests at the beginning and the end of the exposure, as well as recovery rates and chronological decline of concentration over 48 h.

Diclofenac Pharmaceutical	Nominal conc. [mg/l]	Sampling time[h]	Determined conc. [mg/L]	Recovery [%]	Recovery 48 h [%]
Diclofenac	1	0	0,85	85	82
		48	0,7		
	16	0	15,8	99	77
		48	12,1	…	
	256	0	257	100	99
		48	254	….	
Metoprolol	3,1	0	2,6	85	88
		48	2,3	…	
	50	0	45,1	90	88
		48	39,8	…	
	800	0	691	86	90
		48	620	…	

In the diclofenac assay, determined pH values were 7.1 to 7.9, conductivity was 482 to 632 µS/cm, and oxygen saturation at the end of the test was 76 to 84%. Lowest oxygen saturation was measured after 48 h at the highest concentration.

In the acute test with metoprolol, pH values were between 7.8 and 8.0, conductivities between 728 and 967 µS/cm and oxygen saturation at the end of the test between 54 and 85%. Again, lowest oxygen saturation was measured after 48 h at the highest concentration. Detailed data are displayed in Annex 3 and Annex 4.

Results of the acute toxicity test with diclofenac are shown in Table 17. Exposure at 256 mg/L diclofenac caused 100% mortality after 24 h.

Table 17: Mortality in acute tests with diclofenac after 24 and 48 h.

Nominal conc. [mg/l]	No. of dead animals after 24 h	No. of dead animals after 48 h	Rel. Mortality [%] after 48 h
control	0	0	0
1	1	2	20
4	0	1	10
16	0	0	0
64	2	6	60
256	10	10	100

In the test with metoprolol, all individuals tested survived 48 h of exposure (Table 18). After prolonged exposure for 144 h, considerable mortality occurred (Table 19).

Table 18: Mortality in acute test with metoprolol after 48 h.

Nominal conc. [mg/l]	No. of dead animals after 48 h	Rel. Mortality [%] after 48 h
control	0	0
3,1	0	0
12,5	0	0
50	0	0
200	0	0
800	0	0

Table 19: Mortality in acute test with metoprolol after 96, 120 and 144 h. n.i.: not investigated.

Nominal conc. [mg/l]	No. of dead animals after 96 h	No. of dead animals after 120 h	No. of dead animals after 144 h	Rel. Mortality [%] after 144 h
control	0	0	0	0
3,1	0	n.i.	2	20
12,5	0	n.i.	0	0
50	0	n.i.	0	0
200	1	n.i.	1	10
800	4	6	7	70

Using Weibull regression, EC_x-values were determined for both pharmaceuticals (Table 20).

Table 20: EC_x values calculated from acute tests for diclofenac and metoprolol; l. C.: lower confidence interval; u. C.: upper confidence interval. -: no mortality observed.

Pharmaceutical		EC_{10} [mg/l]	EC_{20} [mg/l]	EC_{50} [mg/l]
Diclofenac	EC_x (48 h)	30	39	58
	l. C. 95% /u. C. 95%	4 /43	11 /52	39 /92
Metoprolol	EC_x (48 h)	-	-	-
	EC_x (144 h)	278	324	593
	l. C. 95% / u. C. 95%	8 / 381	37 / 503	284 / 955

Figure 40: Concentration response relationship for *G. fossarum* exposed to diclofenac for 48 h.

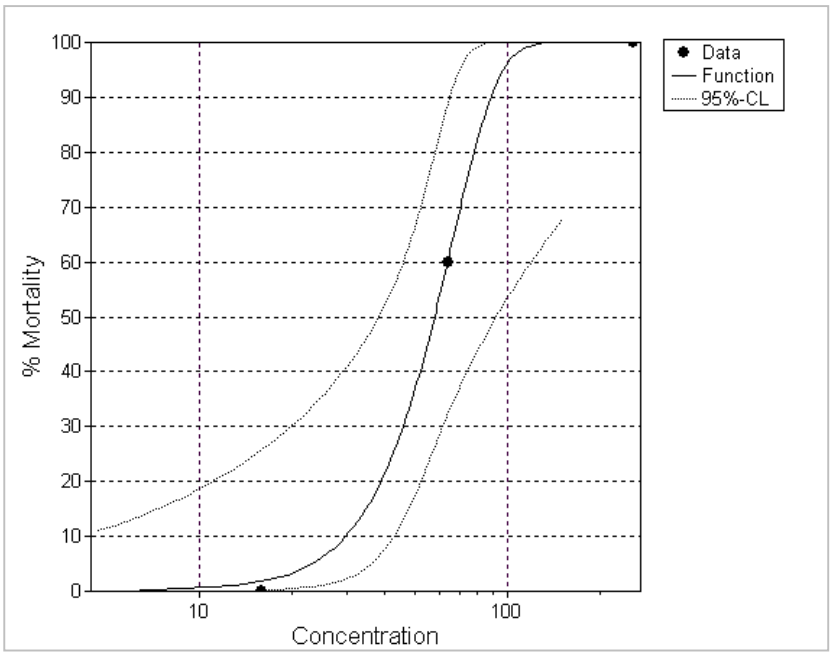
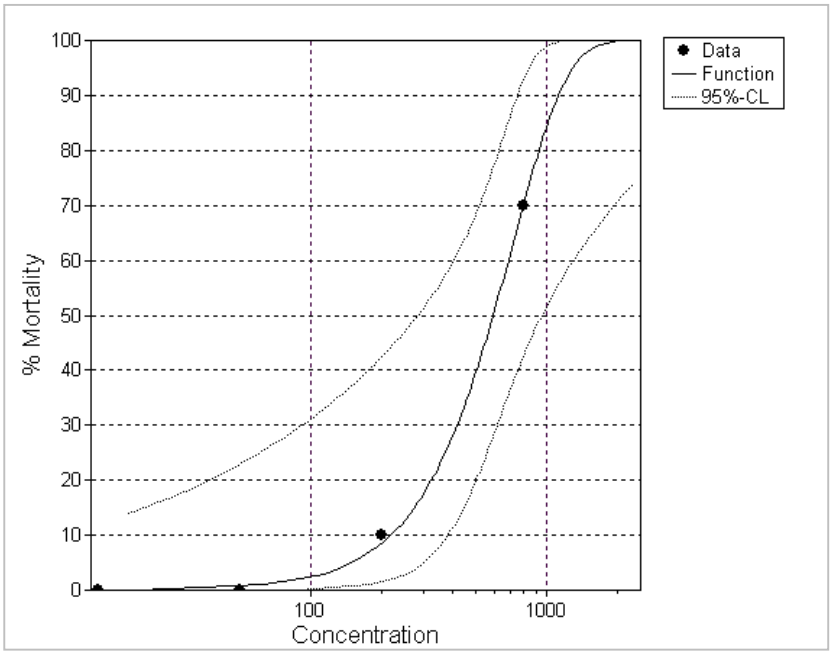


Figure 41: Concentration response relationship for *G. fossarum* exposed to metoprolol for 144 h.



1.3.3.3 Chronic exposure to diclofenac

Chemical analysis of exposure concentrations

At the beginning of exposure, the concentration of diclofenac was below 0.001 mg/L in controls. After 35 days, a concentration of 0.003 mg/L was determined in controls.

Real concentrations of the pharmaceuticals were determined in the experiments with nominal concentrations of 1.48 and 4.44 mg/L. In these experiments, effects were visible in gammarids. A total of 81% of the nominal concentrations were detected in the two treatments at the beginning of the experiment (Table 21). Concentrations decreased throughout the first week of exposure by 33 and 41%, respectively. After 28 days, 65 and 72% of nominal concentrations were detected after exchange of 80% of

the medium. This decline was caused by the weekly exchange of medium, where one fifth of old medium, presumably with decreasing concentrations of the pharmaceuticals remained in the vessels. This effect could have been avoided by an exchange of the entire medium, however, this is not recommended in order to avoid handling stress for the test organisms.

Between days 28 and 35 of exposure, concentrations were decreased by 47 and 53%, respectively. The relative decline was higher at lower exposure concentration (1.48 mg/L) in both time frames investigated. In this second period of investigation (day 28 to day 35) a 12 and 15% larger decline of diclofenac concentration was observed than in the first period (day 1 to day 7). Diclofenac is prone to photodegradation, which would be stronger in June than in May, probably due to higher radiation intensity resulting in higher degradation rates in the last period of the experiment.

Table 21: Analysis of concentrations during 40 days of exposure to diclofenac; ME - medium exchange.

Sampling time [d]	Nominal conc. [mg/l]	Determined conc. [mg/L]	Recovery rate [%]	Decline per 7 days [%]
0	0.00	< 0.001	-	
0	1.48	1.20	81	
0	4.44	3.60	81	
7 before ME	1.48	0.71	48	41
7 after ME	4.44	2.40	54	33
28 before ME	1.48	0.97	65	
28 after ME	4.44	3.20	72	
35 before ME	0.00	0.003		
35 before ME	1.48	0.46	31	53
35 after ME	4.44	1.70	38	47

The concentrations measured in the water were out of the desired range for recovery of 80 – 120%. Due to the high photocatalytic degradation of diclofenac in this experiment due to daylight conditions (in contrast to the fish experiments, which were conducted at artificial light conditions), nominal water concentrations are not suitable to express effective concentrations (OECD-23 2000). In this case, the calculation of the geometric mean is recommended to estimate effective concentrations (OECD-23 2000). When applied to our dataset, these effective concentrations were 0.79 and 2.62 mg/L for the nominal concentrations of 1.48 and 1.44 mg/L, respectively. The factors between nominal and calculated effective concentrations thus were 1.87 and 1.66, respectively. These factors were subsequently used to calculate effective concentrations also for the other set-ups, for which no analytical data were present: Resulting estimated effective concentrations thus were 0.26, 0.79 (factor 1.87), 2.62, 8.0 and 24.1 (factor 1.66) mg/L. These were used for further calculation of effect concentrations.

At day 35 of exposure, a concentration of 0.003 mg/L was measured in controls. During the exchange of the medium, a synthetic flexible hose was used for the extraction of old medium. Despite thorough cleaning of these tubes, contamination of samples, particularly of controls, could not be excluded. However, the concentration of pharmaceuticals in controls was extremely low and even lower than the reported lowest effect concentrations by a factor of 1000. Moreover, it was higher (by a factor of 10) than the concentration in Tännichtgrundbach, where test organisms originated from. Therefore, it can be assumed that these low concentrations of diclofenac did not cause negative effects on gammarids in controls.

Physico-chemical parameters

pH values were in the range of 7.7 to 8.2. Measured pH values were comparable for controls and treatments (Table 22).

Conductivity ranged between 383 and 463 µS/cm in the controls and exposures. Throughout one week of exposure, conductivity in controls and exposures ranged from 656 µS/cm to 688 µS/cm. This is probably caused by a mistake during preparation of the Borgmann medium. Conductivity also at these higher ranges is considered as harmless for gammarids. The median values for the oxygen concentrations was 10 mg/L, the lowest measured value was 7.7 mg/L, and the highest 10.6 mg/L. Minimum values resulted from calibration error of the probe at beginning of the experiment. As a continuous aeration of the exposure vessels was realized, a sufficient oxygen supply has to be assumed.

Table 22: Overview of physico-chemical parameters during long term exposure with diclofenac. Median (Minimum; Maximum) are shown for every concentration.

Diclofenac							
Parameter	**Unit**	**Controls**	**0.26 mg/L**	**0.79 mg/L**	**2.62 mg/L**	**8.0 mg/L**	**24.1 mg/L**
pH	-	7.85	7.86	7.86	7.85	7.83	7.88
		(7.68; 8,23)	(7.60; 8,06)	(7.70; 8.19)	(7.73; 8.17)	(7.71; 8.14)	(7.72; 8.19)
Conductivity	µS/cm	399	393	395	395	396	403
		(384; 447)	(383; 446)	(385; 449)	(386 ; 452)	(388; 450)	(393; 463)
Oxygen	mg/L	10	10.1	10,1	10	10,1	10
		(7.8 ; 10,6)	(7.8; 10.6)	(7.9; 10.6)	(7.9; 10.8)	(7.7; 10.6)	(7.9; 10.6)
Oxygen saturation	%	100	101	101	100	101	100

Determination of nitrate, ammonium and dissolved organic phosphor (SRP) concentrations

The nitrate content was 2.0 mg/L NO_3-N (Table 14) on day 35 of exposure for controls. For the different diclofenac concentrations, nitrate contents ranged from 1.9 to 2.3 mg/L (Table 23).

The ammonium concentration was higher in controls (25 µg/L) than in diclofenac treatments (6.6 to 17.1µg/L). Within controls of the diclofenac and metoprolol treatments, NH_4^+ -contents were comparable.

The soluble organic phosphor concentration was 0.78 µg/L in the control. With increasing diclofenac concentrations SRP increased up to 12.63 µg/L for the highest concentration. An influence of an observed discoloration of the test solutions in the setups with the high pharmaceutical concentrations on the SRP measurements cannot be excluded. The reason for this, however, is unknown.

Table 23: Concentrations of nitrate (NO_3-N), ammonium (NH_4^+) and SRP during exposure to diclofenac.

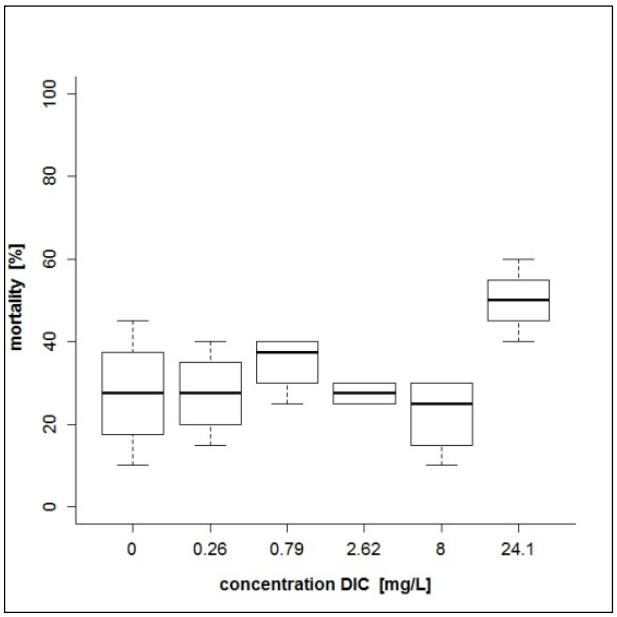
Diclofenac [mg/L]	NO_3-N [mg/L]	NH_4^+ [µg/L]	SRP [µg/L]
control	2.00	25.0	0.78
0.26	1.95	6.6	0.87
0.79	2.06	8.4	0.81
2.62	2.03	8.1	1.46

Diclofenac [mg/L]	NO_3-N [mg/L]	NH_4^+ [µg/L]	SRP [µg/L]
8.0	2.35	13.9	4.72
24.1	2.14	17.1	12.63

Effects on mortality and reproduction

The median of mortality was 28% in controls with a minimum of 10 and maximum of 45% (Figure 42). For increasing concentrations between 0.26 and 8.0 mg/L diclofenac, mortality was not significantly different from controls. Significant increase of mortality with a median of 50% was observed at the highest diclofenac concentration (24.1 mg/L). A calculation of EC_x values was not possible according to the obtained data set, as the experimental design was not preferentially focused on the evaluation of mortality as an endpoint.

Figure 42: Mortality of *G. fossarum* following exposure to diclofenac. Medians along with 25 and 75% percentiles and minimal and maximal values. DIC: diclofenac.

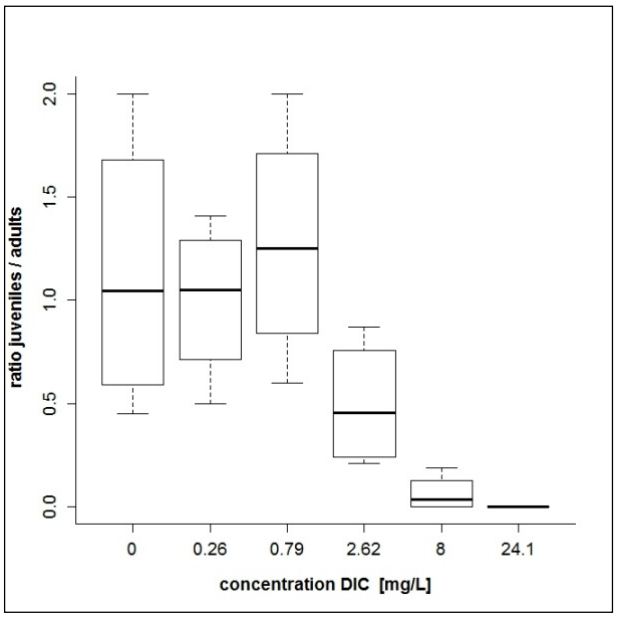


Effects of diclofenac on reproduction of *G. fossarum* were obvious according to relative numbers of juveniles, as derived from the ratio of number of juveniles to number of adults (Figure 43). Absolute numbers are depicted in Annex 6.

In controls this quotient ranged from 0.4 to 2.0 with a median of 1.0. For exposures of 0.26 and 0.79 mg/L diclofenac statistically no significant effect was found.

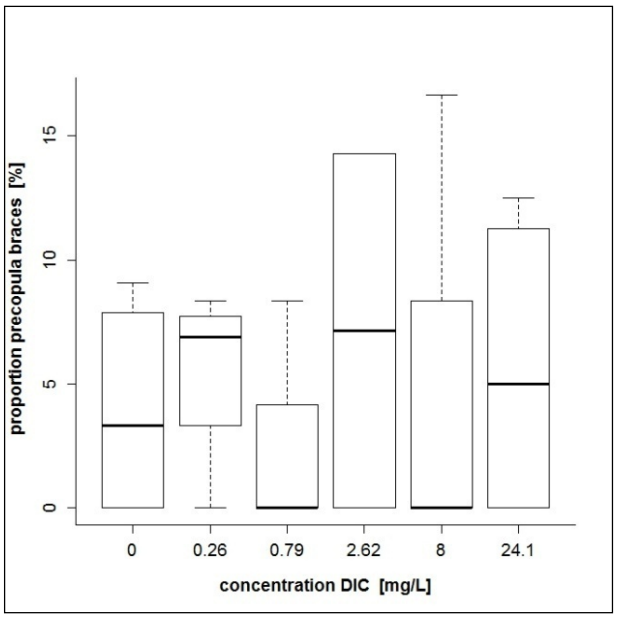
A concentration-dependent significant reduction of relative numbers of juveniles was observed at a concentration of 2.62 mg/L with a median of 0.5. At a concentration of 8.0 mg/L the median was 0.05. At 24.1 mg/L diclofenac no juveniles were observed. For this effect endpoint NOEC was 0.79 mg/L and LOEC was 2.62 mg/L according to Williams test (α=0.05).

Figure 43: Ratio of juveniles to adults for exposure with diclofenac. Medians are displayed along with 25 and 75% percentiles and minimum and maximum values. Conc.: Concentration.



The relative number of precopulae was statistically not significantly different between controls and all diclofenac concentrations (Williams test, α=0.05), (Figure 44).

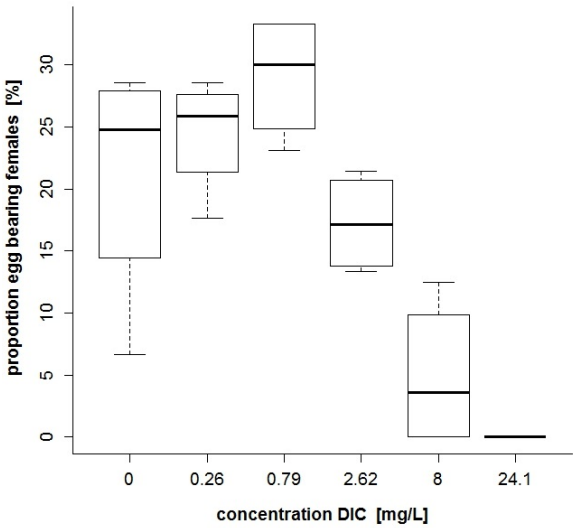
Figure 44: Relative number of precopulae braces of *G. fossarum* at exposure to diclofenac. Medians are displayed along with 25 and 75% percentiles and minimum and maximum values.



For the relative number of egg bearing females, the median in controls was 25%, the minimum was 7% and the maximum was 29%. At concentrations of 0.26 and 0.79 mg/L diclofenac, the proportions of egg bearing females with median values of 26% and 30% were statistically not significantly different from controls. Relative number of egg bearing females decreased with 2.62 mg/L diclofenac significantly, as indicated by Williams test (α=0.05). Median at this concentration was 17%. For proportion

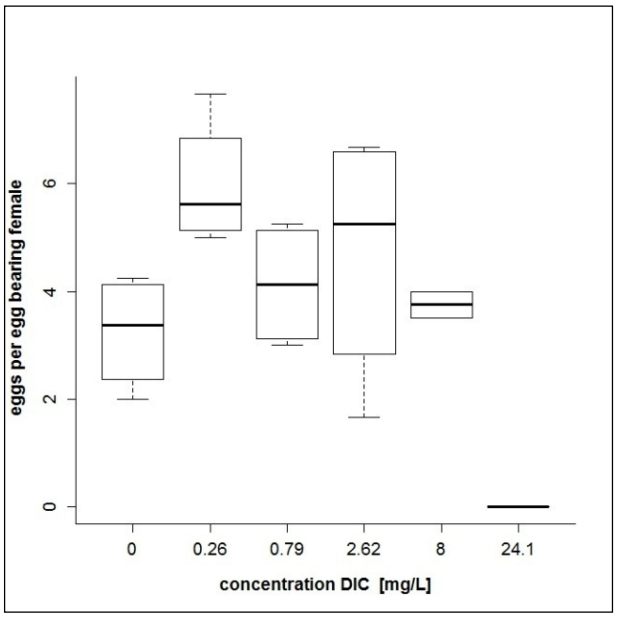
of egg bearing females of *G. fossarum* NOEC was 2.62 mg/L and LOEC was 8.0 mg/L (Figure 45; absolute numbers are depicted in Annex 6).

Figure 45: Relative number of egg bearing females of *G. fossarum* after exposure to diclofenac. Medians are displayed along with 25 and 75% percentiles and minimum and maximum values.



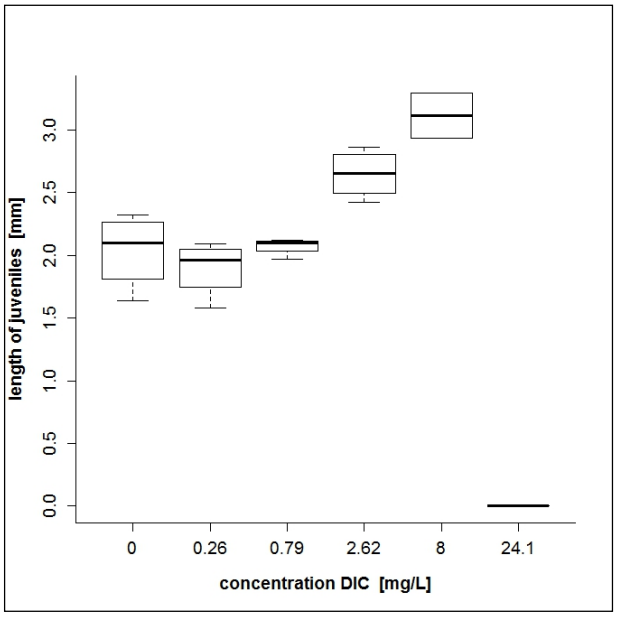
The absolute number of eggs per egg bearing female was between 2 and 4.25 in controls with a median of 3.4 (Figure 46). Per concentration tested, 1 to 2 eggs were released from some females during fixation which could not be reassigned to the respective individual. At lower diclofenac concentrations up to 2.62 mg/L, the number of eggs was higher with medians of 5.6 at 0.26 mg/L, 4.1 at 0.79 mg/L and 5.3 at 2.62 mg/L diclofenac. However, these differences were not significant. After exposure at 24.1 mg/L diclofenac, no egg bearing females were detected. For relative numbers of eggs per egg bearing female, the NOEC was 8.0 mg/L and the LOEC was 24.1 mg/L according to the Williams test (α=0.05).

Figure 46: Number of eggs per egg bearing females of *G. fossarum* after exposure to diclofenac. Medians are displayed along with 25 and 75% percentiles and minimum and maximum values.



In controls, the median of juvenile body length was 2.1 mm, the minimum was 2.0 mm and the maximum was 2.4 mm. At 2.62 mg/L, the size of juveniles increased (Figure 47), while the number of juveniles decreased significantly. Due to differing numbers of replicates at higher concentrations, for this endpoint no significance could be determined.

Figure 47: Length of juveniles of *G. fossarum* at exposure to diclofenac. Medians are displayed along with 25 and 75% percentiles and minimum and maximum values.



By categorization of juveniles into three size classes, juveniles ≥ 2.5 mm were observed in controls and the two lowest concentrations, whereas at these concentrations the majority of juveniles was ≤ 2.0 mm (Table 24). At 2.62 mg/L median was distinctly higher, as no juveniles were found in smallest size class.

As a conclusion, after diclofenac exposure, the relative number of juveniles per adults was the most sensitive endpoint. For this parameter, the LOEC was 2.62 mg/L, the NOEC was 0.79 mg/L.

Table 24: Number of juveniles at three size classes after exposure to diclofenac.

Size classes of juveniles			
Conc. [mg/L]	≤ 2.0 mm	2.0≤juv.≤2.5 mm	> 2,5 mm
control	33	10	10
0.26	36	15	4
0.79	32	16	9
2.62	1	8	20
8.00	0	0	4
24.10	0	0	0

Table 25: Overview on effect concentrations for *G. fossarum* after exposure to diclofenac (Williams test, α=0.05).

Diclofenac		
Effect endpoint	NOEC [mg/L]	LOEC [mg/L]
ratio juveniles/adults	0.79	2.62
relative number of precopulae	≥ 24.10	> 24.10
Relative number of egg bearing females	2.62	8.00
Number of eggs/egg bearing female	8.00	24.10

1.3.3.4 Chronic exposure to metoprolol

Chemical analyses of exposure concentrations

At the beginning of the exposure, the concentration of metoprolol was below 0.001 mg/L in controls. After 35 days, 0.002 mg/L metoprolol were determined in controls.

Measured concentrations were in the range of 89% to 90% of the nominal concentrations in the two treatments at the beginning of the experiment (Table 26). Concentrations decreased during the first week by 2 and 7%, respectively. 28 days after medium exchange, 90 and 89% of nominal concentrations were detected.

Between day 28 and 35, measured concentrations decreased by 2 and 5%, respectively. The percental decline was lower for lower exposure concentration (5 mg/L) than for higher ones (15 mg/L). This was true for both timeframes investigated.

At day 35, 0.002 mg/L metoprolol were measured in controls. During the exchange of the medium, a synthetic flexible hose was used for extraction of the old medium. Despite thorough cleaning of these tubes, a carryover of metoprolol cannot be excluded.

Table 26: Analyses of metoprolol concentrations during the 40 days exposure experiment (ME – medium exchange).

Sampling time [d]	nominal conc. [mg/l]	Determined conc. [mg/L]	Recovery rate [%]	Decline [%]
0	0	< 0.001		

Sampling time [d]	nominal conc. [mg/l]	Determined conc. [mg/L]	Recovery rate [%]	Decline [%]
0	5	4.5	90	
0	15	13.3	89	
7 before ME	5	4.4	88	2.00
7 before ME	15	12.4	83	7.00
28 after ME	5	4.5	90	
28 after ME	15	13.3	89	
35 before ME	0	0.002		
35 before ME	5	4.4	88	2.22
35 before ME	15	12.7	85	4.50

Physico-chemical parameters

During the exposure of gammarids to metoprolol, medians of measured pH values varied between 7.84 and 7.9 (Table 27). The total range of measured pH was between 7.7 and 8.2.

Conductivity ranged between 385 µS/cm and 466 µS/cm, with medians between 424 µS/cm and 453 µS/cm. Concomitant to the experiments with diclofenac, conductivity ranged from 663 to 688 µS/cm during a single week, suggesting a singular mistake in culture medium preparation. A variation in conductivity in this range, however, is considered harmless to gammarids.

Medians of oxygen concentration varied between 8.3 mg/L and 10.1 mg/L. As already explained for the experiments with diclofenac, a calibration error of the used gas potentiometric oxygen probe at the beginning of the experiment probably was responsible for the rather low values measured. With proceeding time the error was corrected. A particularly (and probably artificially) low median of 8.3 mg O_2/L resulted in the experiment with the highest metoprolol concentration, because after 2 weeks of exposure no further O2 measurements were undertaken, due to 100% mortality in this experiment.

Table 27: Physico-chemical parameters recorded during long-term exposure to metoprolol. Medians are displayed along with minima and maxima (in parentheses).

Metoprolol [mg/L]							
Parameter	Unit	Controls	5	15	45	135	405
pH	-	7.88 (7.74; 8.15)	7.84 (7.76; 8.1)	7.84 (7.71; 8.1)	7.86 (7.73; 7.86)	7.9 (7.74; 8.14)	7.9 (7.71; 7.97)
Conductivity	µS/cm	424 (385; 461)	428 (385;464)	428 (386; 464)	432 (391;457)	426 (403; 466)	453 (446 462)
Oxygen	mg/L	10.1 (8.0; 10.7)	10.1 (7.9; 10.7)	10.1 (7.7; 10.5)	10 (7.5; 10.4)	9.9 (7.4;10.4)	8.3 (7.8; 10.2)
Oxygen saturation	%	102 (81; 107)	102 (79; 107)	101 (77; 105)	101 (83; 104)	101 (82; 104)	85 (82; 103)

Nitrate, ammonium, and soluble reactive phosphorus (SRP)

The nitrate concentration in the controls of the metoprolol experiment was 2.05 mg/L on day 28, similar to the nitrate concentration in diclofenac controls (Table 28). In the onsets with metoprolol, nitrate medians were found to vary between 1.9 and 2.2 mg/L.

The median ammonium concentration in the controls was 23.4 µg/L, lower than in the onsets with metoprolol. In the experiments with the lowest metoprolol concentration (5 mg/L), the ammonium concentration was 7fold higher than in the controls. In comparison of the data obtained for the experiments with 5 mg/L and 15 mg/L metoprolol as well as in comparison of 15 mg/L and 45 mg/L, the concentration of ammonium was increased by factor 1.7 in each case.

The median SRP concentration in controls was 1.71 mg/L. In the other onsets concentrations ranged between 1.11 and 2.05 µg/L and thus did not significantly differ from the concentration in controls.

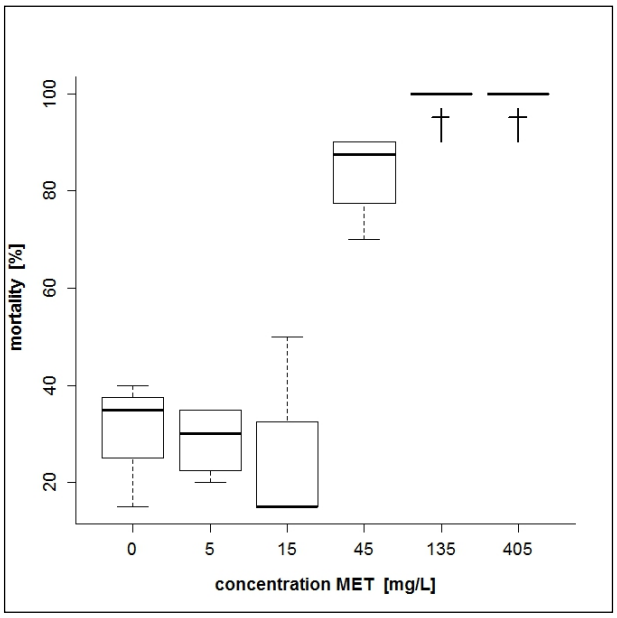
Table 28: Concentrations of nitrate (NO_3-N), ammonium (NH_4^+) and SRP during exposure to metoprolol (n.d.: not determined due to high mortality).

Metoprolol [mg/L]	NO_3-N [mg/L]	NH_4^+ [µg/L]	SRP [µg/L]
control	2.05	23.4	1.71
5	2.09	166.4	2.05
15	2.16	291.8	1.11
45	2.11	481.3	1.68
135	1.94	179.5	1.77
405	n.d.	n.d.	n.d.

Effects on mortality and reproduction

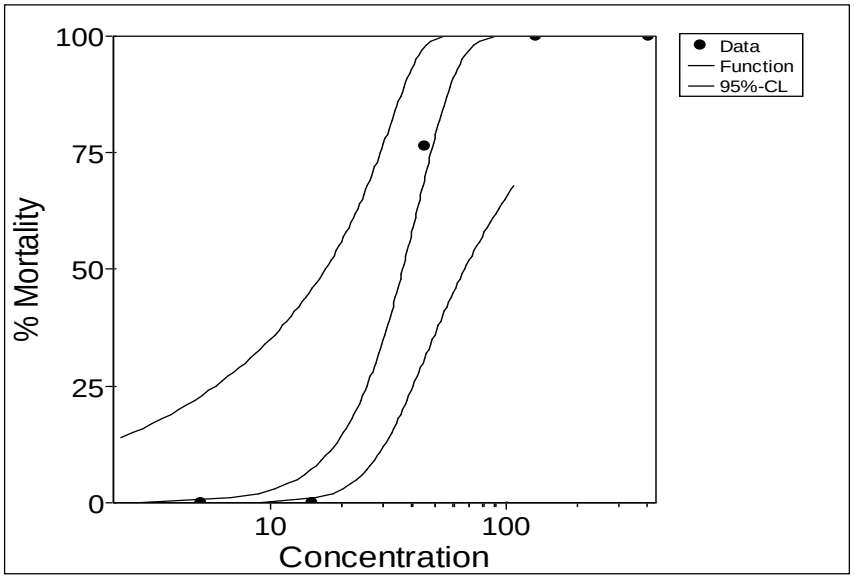
Median mortality was 35% in controls, with a minimum of 30% and a maximum of 36%. For the two lowest concentrations, 5 mg/L and 15 mg/L, the mortality with medians of 30% and 15%, respectively, did not significantly differ from controls (Figure 48). At a concentration of 45 mg/L metoprolol mortality increased to 88%. In the experiments with the two highest test concentrations, 135 and 405 mg/L, mortality was 100%.

Figure 48: Mortality in *G. fossarum* after exposure to metoprolol. Medians are displayed along with 25 and 75% percentiles and minimum and maximum values. †: 100% mortality.



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. EC_{10} and EC_{50} values for mortality were calculated using Weibull regression (Figure 49). The EC_{10} for metoprolol was calculated as 17 mg/L, with a 95% confidence interval of 1 to 28 mg/L, the EC_{50} was determined as 36 mg/L with a 95% confidence interval between 17 and 67 mg/L.

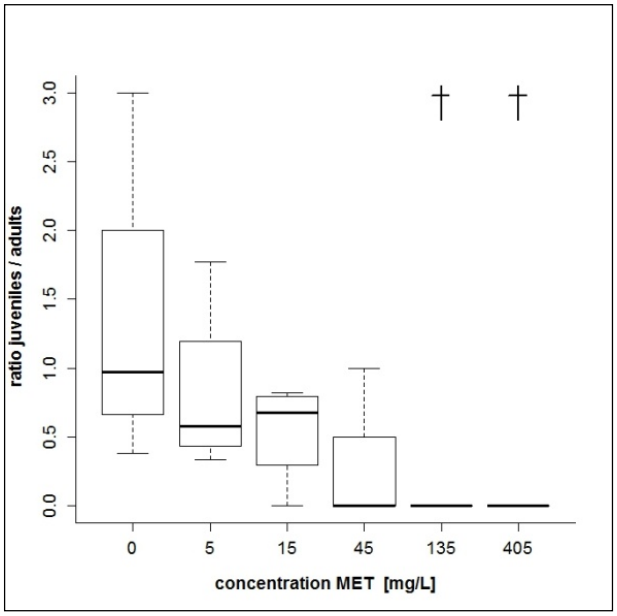
Figure 49: Concentration response relationship for mortality in *G. fossarum* after exposure to metoprolol.



Data recorded for the relative number of juveniles, as determined by the juvenile/adult ratio indicated adverse effects of metoprolol on the reproduction of *G. fossarum* (Figure 50). While in controls this ratio ranged between 0.4 and 3 with a median of 1.0, metoprolol exposure decreased the relative numbers of juveniles. At a concentration of 45 mg/L the median was 0. Following exposure to concentrations of 135 mg/L and 405 mg/L all individuals died. Using Williams test (α=0.05), a NOEC of 5 mg/L

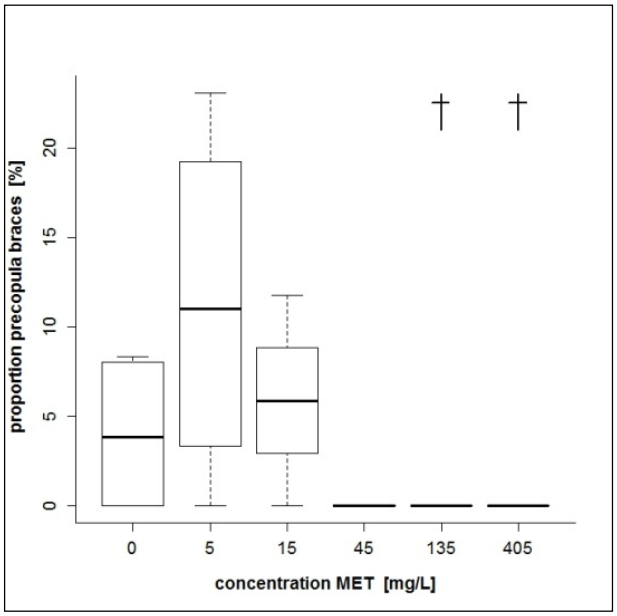
and a LOEC of 15 mg/L was determined for this endpoint. (Absolute numbers are depicted in Annex 6).

Figure 50: Juvenile/adult ratio in *G. fossarum* following exposure to metoprolol. Medians are displayed along with 25 and 75% percentiles and minimum and maximum values. †: 100% mortality.



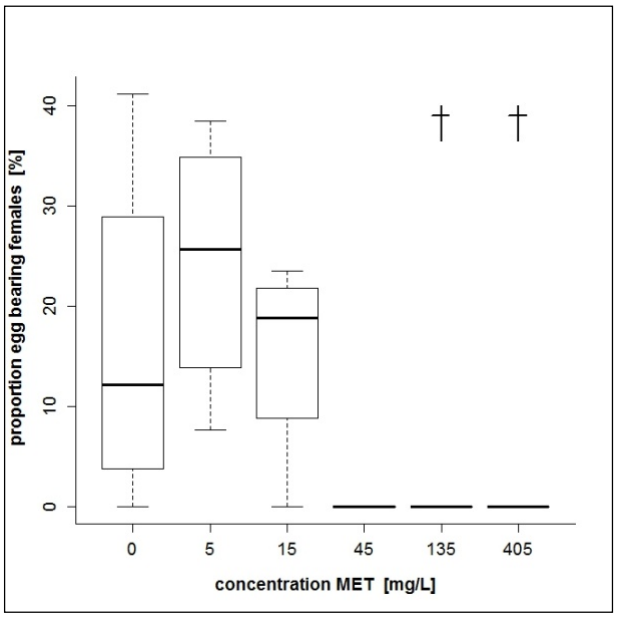
The relative number of precopulae in controls was 4%, the minimum was 0% and the maximum 8% (Figure 51). In the experiments with concentrations of 5 mg/L and 15 mg/L metoprolol, the medians were 11% and 6%, respectively. Following exposure to 45 mg/L, no precopulae pairs were observed. Data recorded for this endpoint were not significantly different between metoprolol exposure up to 45 mg/L and controls (Williams test, α=0.05).

Figure 51: Relative number of *G. fossarum* precopulae pairs after exposure to metoprolol. Medians are displayed along with 25 and 75% percentiles and minimum and maximum values. †: 100% mortality.



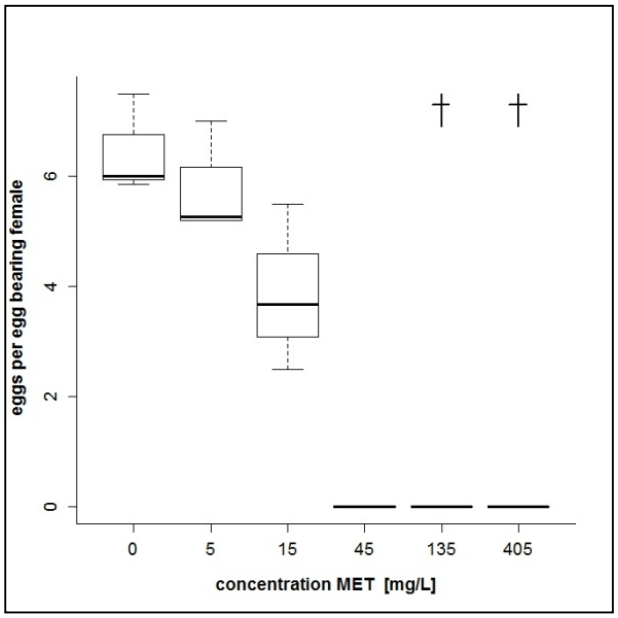
Relative numbers of egg-bearing females ranged between 0 and 41% in controls, with a median of 12% (Figure 52). In the experiments with the lowest test concentrations of metoprolol, the relative number of egg-bearing females was higher. For 5 mg/L, the median was found to be 26%, for 15 mg/L metoprolol it was 19%. No egg-bearing females were observed after exposure to 45 mg/L metoprolol. For this endpoint, a NOEC of 15 mg/L and a LOEC of 45 mg/L were calculated (Williams test, α=0.05). Absolute numbers are depicted in Figure 53.

Figure 52**:** Relative number of egg-bearing females of *G. fossarum* after exposure to metoprolol. Medians are displayed along with 25 and 75% percentiles and minimum and maximum values. †: 100% mortality.



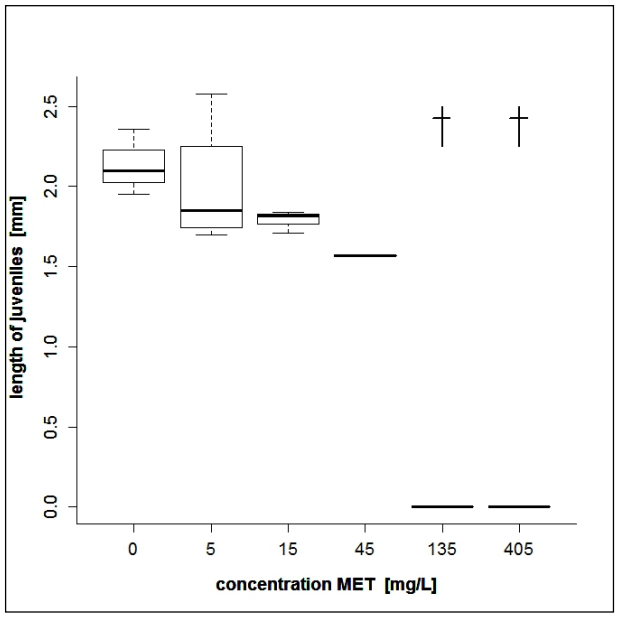
The number of eggs per egg-bearing female ranged from 5.9 to 7.5, with a median of 6, in controls. Following metoprolol exposure, this number was found to be reduced (Figure 53). At 5 mg/L metoprolol, the median was 5.3 eggs per female and at 15 mg/L it was 3.7. The observed reduction in number, however, was not significant at α=0.05 (Williams test). At concentrations ≥45 mg/L no egg-bearing females were observed.

Figure 53: Number of eggs per egg-bearing female of *G. fossarum* after exposure to metoprolol. Medians are displayed along with 25 and 75% percentiles and minimum and maximum values. †: 100% mortality.



While the median observed for the length of juveniles in the controls was 2.1 mm (minimum 2.0 mm and maximum 2.4 mm), juvenile length was found to be progressively diminished with increasing concentrations of metoprolol (Figure 54).

Figure 54: Juvenile length in *G. fossarum* after exposure to metoprolol. Medians are displayed along with 25 and 75% percentiles and minimum and maximum values. †: 100% mortality.



Categorization of juveniles into three size classes revealed the largest size class (> 2.5 mm) to be absent from experiments with metoprolol concentrations ≥ 15 mg/L, and size distribution was shifted to lower size classes (Table 29). Probable reasons for this may be a decreased hatching size of juveniles, growth inhibition, or reduced survival of (larger) juveniles exposed to higher concentrations of metoprolol.

In summary, parameters relevant for reproduction which were investigated in *G. fossarum* revealed significant effects of metoprolol at a concentration of 15 mg/L (LOEC). A NOEC of 5 mg/L metoprolol was determined (Table 30). Most sensitive endpoints in this context were the juvenile/adult ratio as well as the number of eggs per egg-bearing female.

45 mg/L metoprolol or higher concentrations terminated reproduction of *G. fossarum* during exposure. At the end of the experiment, no precopula pairs and no egg-bearing females could be observed. Nevertheless, in the experiment testing a concentration of 45 mg/L, a small number of juveniles were present, probably due to the introduction of at least one egg-bearing female at the beginning of the experiment. No significant effects were found for diclofenac and metoprolol regarding the relative numbers of precopulae.

Table 29**:** Numbers of juveniles categorized into three size classes following exposure to different concentration of metoprolol.

Size classes of juveniles			
Conc. [mg/L]	≤ 2.0 mm	2.0 ≤ juv. ≤ 2.5 mm	> 2,5 mm
control	35	4	15
5	26	10	7
15	34	4	0
45	3	0	0
135	-	-	-
405	-	-	-

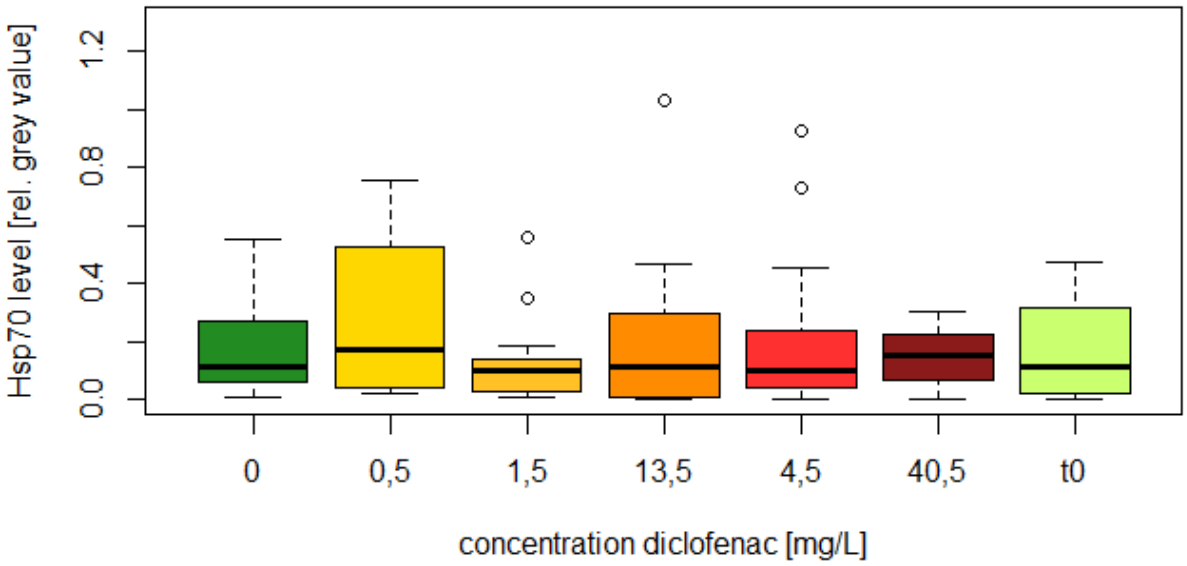
Table 30**:** Metoprolol effect concentrations for *G. fossarum* (Williams test, α=0.05).

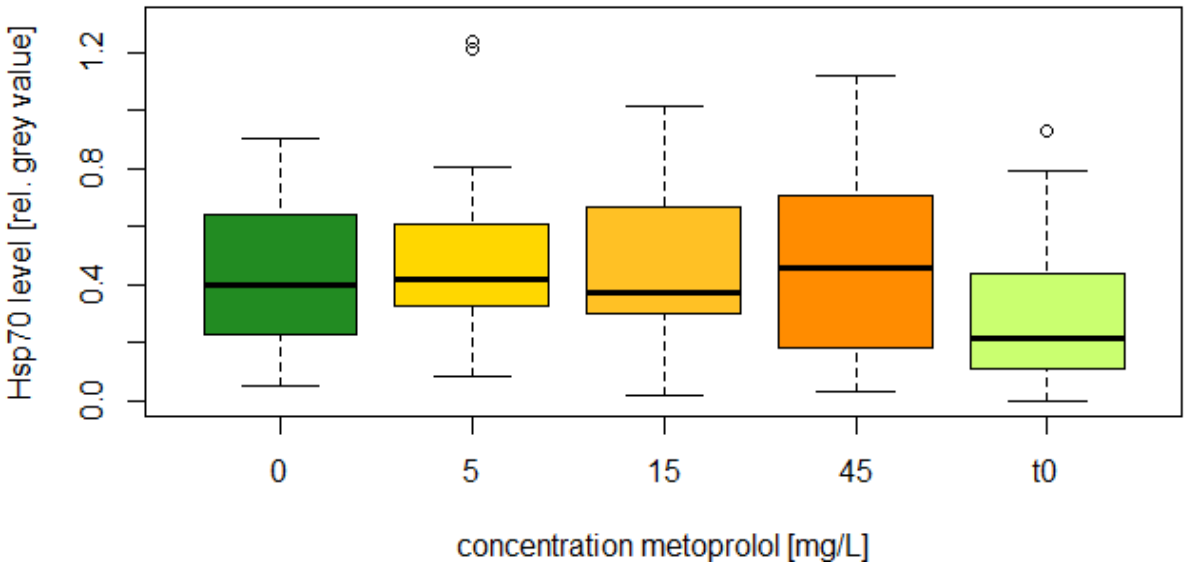
Metoprolol		
Effect endpoint	NOEC [mg/L]	LOEC [mg/L]
Juvenile/adult ratio	5	15
Relative number precopulae	≥ 405	> 405
Relative number of egg-bearing females	15	45
Number of eggs per egg-bearing female	5	15

1.3.3.5 Biochemical markers in response to diclofenac and metoprolol

Data for stress protein levels of the diclofenac-experiment had to be transformed with the 3rd root function to fit normal distribution. For the metoprolol data, a square-root function had to be applied. Homogeneity of variance was given in both cases. The level of stress proteins in metoprolol- and diclofenac-exposed gammarids did not show a significant difference between the tested concentrations or the control (diclofenac: ANOVA, df=5/97, F=0.7660, p=0.5765; metoprolol: ANOVA, df=3/98, F=0.1333, p=0.9400), but stress proteins were significantly higher in the metoprolol-control than in the t0-individuals (t-test, df=58, t=-2.506, p=0.015). Data are shown in Figure 55. In general, the stress protein levels were lower in the diclofenac exposure than the metoprolol exposure.

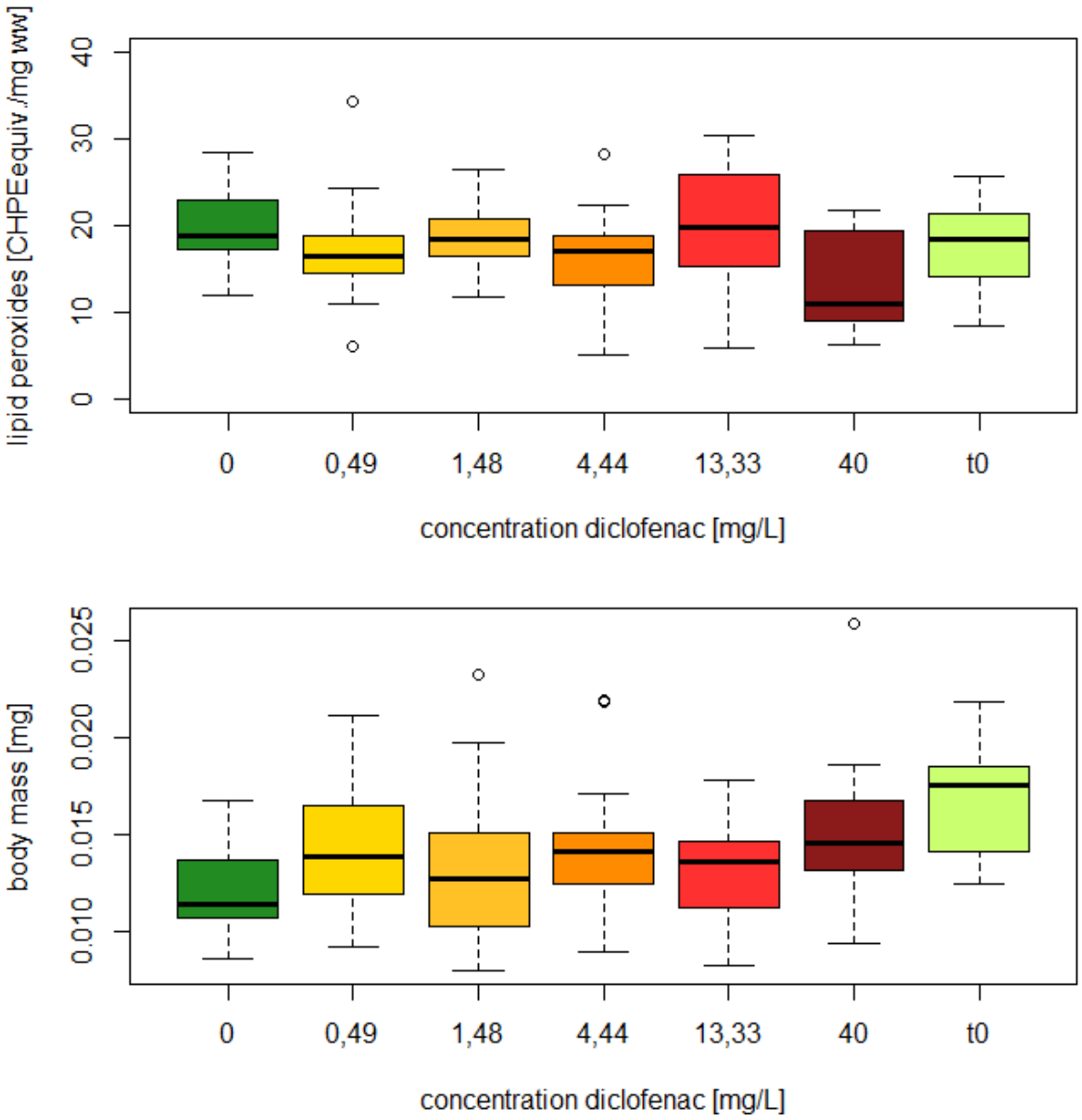
Figure 55: Stress protein level of *Gammarus fossarum* exposed to diclofenac (top) and metoprolol (bottom). The condition of the animals at the start of the exposure is seen in "t0". Bold lines display the median values, boxes the 25% to 75% quantiles, whiskers the minimum and maximum values, open circles potential outliers. The only significant effect could be seen between "t0" and the control in the metoprolol exposure.





Data of the FOX-assay had to be transformed with a fourth root function to fit normal distribution; however, homogeneity of variances was not given. The degree of lipid peroxidation showed a tendency to decrease with increasing diclofenac concentration but there were no significant differences to be found between the treatments (Welch-ANOVA, df=5, F=2.0571, p=0.0898). The body mass tended to increase with increasing diclofenac concentrations, but the biggest difference was found between the control and the t0 individuals, which were significantly heavier (ANOVA, df=6/105, F=3.3023, p=0.0051). Results are visualized in Figure 56.

Figure 56: Lipid peroxide content (top) and body mass (bottom) of *Gammarus fossarum* exposed to diclofenac. The condition of the animals at the start of the exposure is seen in "t0". Bold lines display the median values, boxes the 25% to 75% quantiles, whiskers the minimum and maximum values, open circles potential outliers. "t0" individuals were significantly heavier than the exposed individuals (ANOVA, df=6/105, F=3.3023, p=0.0051).



1.3.4 Discussion

1.3.4.1 Acute tests

Diclofenac and metoprolol concentrations determined at the beginning of the acute tests were in good agreement with nominal concentrations. Nevertheless, deviations were by trend larger at lower concentrations of the pharmaceuticals. Moreover, a decline of less than 12% metoprolol and less than 18 % diclofenac concentrations was detected within 48 h in the exposure media. Experiments were conducted in a greenhouse at realistic seasonal light conditions with particular focus on diclofenac, which is prone to photocatalytic degradation, a feature likely responsible for the observation that the decline of diclofenac concentrations exceeded that of metoprolol. Because diclofenac concentrations in

the chronic exposure experiment thus were out of the benchmark (80 – 120%), geometric means were calculated as effective diclofenac concentrations (OECD, 2000).

An EC_{50} of 58 mg/L was determined for diclofenac in the acute test which is in good agreement with the range of toxicity data published for crustaceans (22 to 69 mg/L). In this context, *G. fossarum* and Daphnia (Dietrich et al. 2010) appeared to be of similar sensitivity to diclofenac.

An EC_{50} (144h) of 593 mg/L was calculated for metoprolol. After 48 h of exposure to metoprolol no effects were detected at the highest tested concentration. In comparison of these data to the EC_{50} values reported for other Crustacea (Table 32, with acute effects already at 438 mg/L), gammarids have to be regarded less sensitive to metoprolol than other crustaceans.

1.3.4.2 Chronic tests: diclofenac

In controls, a mortality of 28% was observed which, however, must be regarded rather low compared to literature data with mortality in controls up to 70% (Schirling et al. 2006, Dietrich et al. 2010, Oskarsson et al. 2012).We therefore accepted the present experiment as reliable.

The analytically determined diclofenac concentrations at the beginning of the experiment were in good agreement with the nominal concentrations (81%). During 7 days of exposure, diclofenac concentrations decreased by 33 to 53%. Accordingly, the "effective" concentrations in our onsets were approached by the calculation of geometric means, which were lower than the nominal values by factors of 1.66 to 1.87.

Regular determination of the physico-chemical parameters pH, oxygen concentration, and conductivity showed that potential effects on gammarids by confounding factors (eventually exerted by the boundary conditions of the exposure experiments) are negligible.

Concentration response relationships were observed for the majority of the investigated endpoints. Regarding reproduction, the juvenile/adult ratio was shown as most sensitive parameter, with NOEC and LOEC values of 0.79 and 2.62 mg/L, respectively.

Reproduction was not terminated during exposure, but the relative number of egg-bearing females decreased significantly at concentrations of 8.0 mg/L and higher. Also the relative number of juveniles was significantly lowered at 2.62 mg/L diclofenac and all higher concentrations. The number of eggs per egg-bearing female did not change significantly up to a concentration of 40 mg/L. The reasons for the observed changes in juvenile size with increasing concentration are not clear. Probably, at concentrations of 2.62 mg/L and higher, less juveniles with larger size were released by females. Eventually also a lethal impact of diclofenac on the youngest and thus smallest juveniles may have decreased the abundance of individuals resulting in lower competition for food which then could have resulted in an enhancement of growth in surviving juveniles.

In the frame of this project particular focus was laid on ecologically relevant effects. For risk assessment, NOECs are utilized, which use the highest tested concentration at which no effects have been observed. Within the present project, also effect concentrations (LOECs) have been determined to provide information on the required sensitivity of the *in vitro*- tests to be developed. For the purpose to biomonitor pharmaceuticals, these limits of detection have to be met. The study of Dietrich et al. (2010) is not depicted in Table 31, because no chemical analyses of the exposure concentrations have been undertaken, and no concentration-response behaviour was found. Therefore in an earlier literature survey this study was rejected from risk assessment (Triebskorn et al. 2014).

Table 31: Derivation of detection limits for biomonitoring from effect data from the literature and this study with diclofenac. LOEC - lowest observed effect concentration.

Species	LOEC [mg/L]	citation
Gammarus spp.	2.62	this study

Species	LOEC [mg/L]	citation
Daphnia magna	1.90	This study (Part IV: Studies with *Daphnia magna*)
Ceriodaphnia spp.	2.00	Ferrari et al., 2003
Oncorhynchus spp.	1.00	Memmert et al., 2013

The predicted lowest effect concentration for diclofenac, derived from the LOEC and an assessment factor of 10, was estimated as 0.26 mg/L for *G. fossarum*. Table 31 makes evident that *G. fossarum* does not rank among the species with highest sensitivity to diclofenac – at least not for the endpoints investigated in this study. However, due to the high ecological relevance of this species for Central European freshwater ecosystems, toxicity data for *G. fossarum* are nevertheless of great importance. Hence, for *in vitro*-tests, a detection limit of 0.9 µmol diclofenac would be required to attest harmlessness of a given water sample to *G. fossarum*. This is also clue for the results derived from chronic tests with *Daphnia magna* which reveal effect concentrations in the same order of magnitude with a LOEC of 1.9 mg/L. However, other organisms have been reported to be more sensitive to diclofenac than this gammarid. Nevertheless, gammarids should be included in further investigations e.g. when interactions within the food web are investigated for instance in higher-tier studies, because it was shown by Brust et al. (2001) that stream communities can respond more sensitive to chemical exposure than single organisms.

1.3.4.3 Chronic tests: metoprolol

In controls, a mortality of 31% was observed which, as mentioned before, must be regarded rather low compared to literature data with mortality in controls up to 70% (Schirling et al. 2006, Dietrich et al. 2010, Oskarsson et al. 2012). We therefore accepted also the present experiment on metoprolol to be reliable.

Also here, the analytically determined metoprolol concentrations at the beginning of the experiment were in good agreement with the nominal concentrations (about 90%). Dissipation from the water phase was low, between 12 - 10% over 2 and 7 days, respectively. The slightly higher dissipation of metoprolol in the 48h 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 is 24 days. Direct photolysis follows a pseudo first order kinetic under solar simulation and is strongly influenced by daylight surface conditions, light intensity of the lamp, or the sun at different latitudes and seasons (Liu and Williams 2007, Radke and Maier 2014). In a green house shaded to simulate natural conditions of lower mountain streams (Jungmann et al. 2001) a higher half-life time due to lower radiation intensity is in agreement with these findings. To calculate the effect concentrations in the experiments with *G. fossarum*, however, the nominal concentrations were used.

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 to organic material are well known for aquatic environments and mainly depend on K_{ow} or K_{oc} values (Lamoureux and Brownawell 1999). As the log K_{ow} of metoprolol is 1.88 (Hansch et al., 1995) a high sorption potential of metoprolol is not expected. Thus, the major exposure pathway in the experiments with gammarids is strongly expected to have been via the water phase.

Already at low concentrations of metoprolol endpoints being of relevance for population development were affected, and the most sensitive ones were the juvenile/adult ratio and the egg number per egg-bearing female with NOEC/LOEC values of 5 and 15 mg/L, respectively. To the best of our knowledge no chronic exposure experiments with metoprolol have been carried out on other amphipods, and solely Dzialowski et al. (2006) and Dietrich et al. (2010a) have exposed *Daphnia sp.* in an extended (chronic) test. Dzialowski reported NOEC/LOEC values of 3.1/6.2 mg/L, respectively, for the reproduction of Daphnia, and their heart rate was also affected at the lowest tested concentrations of 3.1 mg/L.

In our study the endpoint time to first hatch in the chronic *Daphnia* test showed a LOEC value of 0.1 mg/L. this implies that the endpoint time to first hatch is a more sensitive concerning the exposure to metoprolol. Nevertheless, these data are in accordance with those derived from our chronic experiment with *G. fossarum*. In contrast to the results obtained from acute toxicity test, gammarids and daphnids ostensibly showed similar sensitivity to metoprolol for population-relevant endpoints in the chronic tests. However, in fact, exposure time differed, being 10 days for the daphnids and 40 days for the gammarids and, hence, daphnids have to be considered as slightly more sensitive than Gammarus, even though their sensitivity may be, roughly spoken, in the same range. Effects on the number and body length of neonates were found in a multi-generation study with Daphnia magna at 0.012 mg/L metoprolol (Dietrich et al. 2010b). This is the lowest effect concentration present in the literature for an invertebrate species.

Possible influences exerted by the boundary conditions of the experiments with metoprolol are negligible, as they were in the experiments with diclofenac.

Table 32 depicted the comparison of effect data for metoprolol with data from the literature. Also in respect to metoprolol toxicity, *G. fossarum* is not among the most sensitive species known. However, as discussed above should be implemented in further research for hazard identification. For *in vitro*-tests, a detection limit of 5.0 µmol metoprolol would be required to attest harmlessness of a given water sample to *G. fossarum*.

Table 32: Derivation of detection limits for biomonitoring from effect data from literature and this study with metoprolol.

Species	LOEC [mg/L]	citation
Gammarus spp.	15	this study
Daphnia magna	0.10	this study (Part IV: Studies with *Daphnia magna*)
Daphnia spp.	6.2	Dzialowski et al., 2006
Daphnia spp.	0.001	Dietrich et al., 2010
Oncorhynchus spp.	0.001	Triebskorn et al., 2007

1.3.4.4 Biochemical markers

Overall, stress proteins in gammarids did not seem to be affected by the treatment with diclofenac and metoprolol. Even the animals in groups that already showed high mortality (45 mg/L metoprolol) displayed no signs of elevated or decreased Hsp70 levels. However, the Hsp70 level was generally increased in the experimental animals compared to the t0 control. On the one side, this could mean that the handling and experimental procedure by itself increased the level of stress proteins in gammarids. On the other side these difference can also be attributed to differences in age and reproductive state between animals at two different sampling times. This effect was only found in the metoprolol-exposure – not for the diclofenac exposure.

The level of lipid peroxides showed an almost significant tendency to decrease with increasing diclofenac-concentrations. Especially the highest concentration showed several very low values – indicating a beneficial effect of diclofenac on oxidative stress. It is known that the mode of action of diclofenac can reduce the amount of lipid peroxides. However, the overall variation in the data was rather high, so no clear relationship between the two parameters can be shown. One methodological problem was that the samples had to be highly diluted, increasing variability of data. Future approaches should use pools of two or three individuals in order to increase the precision of the measurements. This would consequently require a larger number of test animals.

The higher body mass of animals treated with diclofenac is attributable to the lower proportion of adult individuals and reduced number of egg-bearing females. There were less juvenile individuals, who are smaller than adults, and the females invested less into the production of offspring – conserving their energy reserves.

In summary, there was no sign of proteotoxic action of diclofenac and metoprolol, even in concentrations leading to drastic mortality and affection 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, we are restricted to a conclusion concerning lipid peroxides, since no further tests concerning oxidative parameters (e.g. catalase or superoxide-dismutase activity) were within the scope of this study.

1.4 Studies with snails

1.4.1 Introduction

A 28 day-batch experiment with the snail *Potamopyrgus antipodarum* and metoprolol was performed according to the OECD Draft guideline (OECD, 2015) (as endpoints the number of embryos in the maternal brood pouch as well as the energy status of the snails were considered). By doing this experiment, we were able to compare the results from the laboratory test results with those from the flow channels. Moreover, we could compare sensitivity of both the snail *P. antipodarum* and the worm *Lumbriculus variegatus* (see work package 6). Secondly, we exposed caged *P. antipodarum* in the artificial indoor streams for 28 and 40 days to determine offspring (see work package 3).

1.4.2 Materials and Methods

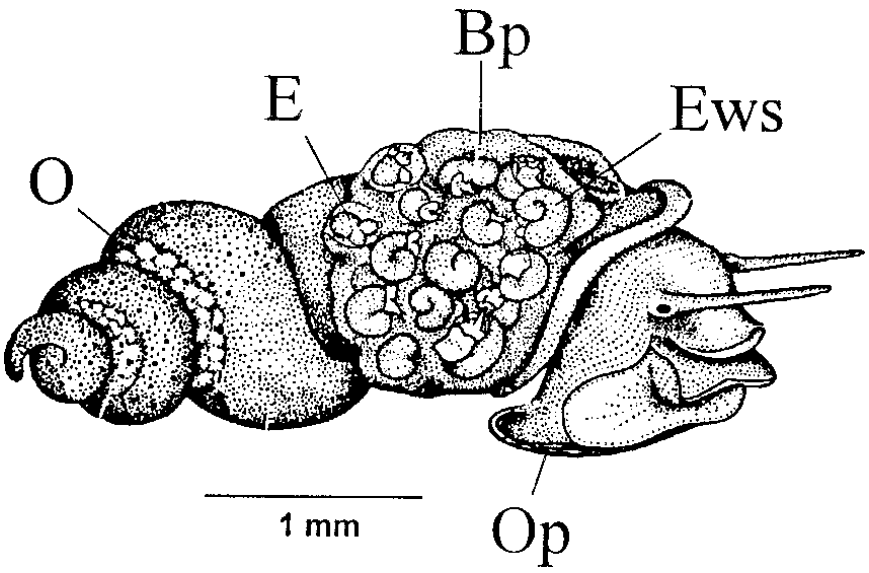
1.4.2.1 28-day reproduction test

The experiment was based on the proposal for an OECD guideline (OECD, 2015), which is currently accepted by the OECD. The semi-static test lasted for 28 days and was performed under controlled conditions (light:dark rhythm of 16:8 hours, 16 ± 1 °C). We used 5 concentrations of metoprolol (0.1; 0.32; 1; 3.2 and 10 mg/L), one positive control (25 ng/L EE2), a solvent control (10 µg/L DMSO) and a control (reconstituted water, OECD, 2015) in a 6-fold repetition. 16 snails with a size between 3.4-4.5 mm were inserted in 500 ml glass vessels. Feeding (0.06 mg per animal per day) was performed three times a week using crushed TetraPhyll Flakes (Tetra GmbH, Melle). During the test period three times per week test water was completely renewed and at each time temperature, pH, conductivity and oxygen content were measured.

After 28 days, animals were killed in liquid nitrogen and following the shell of 6 animals per replicate was removed carefully in order to determine the reproduction (number of embryos in the brood pouch, Figure 57). In addition, the energy status (lipid, protein and glycogen content) from 6 animals per replicate were measured with the help of micro-separation method according to Van Handel (1965; 1985).

The analysis of aqueous samples (lowest, middle and highest metoprolol concentration) was performed by HPLC-MS/MS by the project partner DVGW Water Technology Center Karlsruhe (TZW). Detailed information on the analytical method can be found in work package 7.

Figure 57: *Potamopyrgus antipodarum*, female, after removal of the shell (Duft et al. 2001, modified after Fretter and Graham, 1994). O ovary, Bp brood pouch, E new embryo without shell (unshelled), Ews embryo with shell, Op operculum.



1.4.2.2 Biochemical markers

For identifying the level of stress proteins in *Potamopyrgus*, the same protocol as described in 1.1.2.2 was used with minor changes. The samples had to be pooled (4 to 7 individuals per pool) to yield a sufficient amount of protein. They were homogenized in a standard sample volume of 60-90 µL extraction medium, depending on the animal and pool size.

1.4.3 Results and Discussion

1.4.3.1 28-day reproduction test

The mean reproduction in controls was more than five embryos per female at the end of the test, the dissolved oxygen content was higher than 60% and temperature was 16 ± 1.5 °C throughout the test in both control and exposure groups. Hence, all validity criteria stated in the OECD guideline with *P. antipodarum* were fulfilled.

Physico-chemical parameters

As shown in Table 33 mean pH values ranged from 8.2 (negative control) to 8.31 (two highest concentrations). Conductivity was between 797 µS/cm and 806 µS/cm and was very similar between all treatments. Oxygen concentration was also very comparable; the lowest level was found in the positive control with 91.7%. The temperature ranged between 16 and 17 °C. Hence, all validity criteria stated in the OECD guideline with *Potamopyrgus* were fulfilled.

Table 33: 28-day reproduction test with *Potamopyrgus antipodarum* and metoprolol. Mean values (min, max in parenthesis) of several physico-chemical parameters.

	Negative control	Solvent control	Positive control	Metoprolol [mg/L]				
	Reconst. Water	10 µg/L DMSO	25 ng/L EE2	0.1	0.32	1	3.2	10
pH	8.20 (8.0/8.5)	8.24 (8.03/8.5)	8.23 (7.99/8.36	8.25 (8.02/8.36)	8.29 (8.16/8.37)	8.29 (8.19/8.37)	8.31 (8.23/8.41)	8.31 (8.22/8.43)

	Negative control	Solvent control	Positive control	Metoprolol [mg/L]				
	Reconst. Water	10 µg/L DMSO	25 ng/L EE2	0.1	0.32	1	3.2	10
Conductivity [µS/cm]	799.00 (748/854)	799.00 (746/853)	798.00 (742/853)	799.00 (748/856)	797.00 (742/852)	797.00 (742/857)	800.00 (743/856)	806.00 (751/867)
O2 [%]	93.50 (92/96)	92.10 (89/95)	91.70 (87/95)	91.00 (86/95)	92.40 (91/95)	93.50 (89/96)	92.30 (89/95)	93.10 (87/96)
T [°C]	16.80 (16.6/16.9)	16.80 (16.5/17)	16.80 (16.6/17)	16.80 (16.5/17)	16.80 (16.7/17)	16.90 (16.7/16.9)	16.90 (16.7/17)	16.80 (16/17)

Chemical analysis

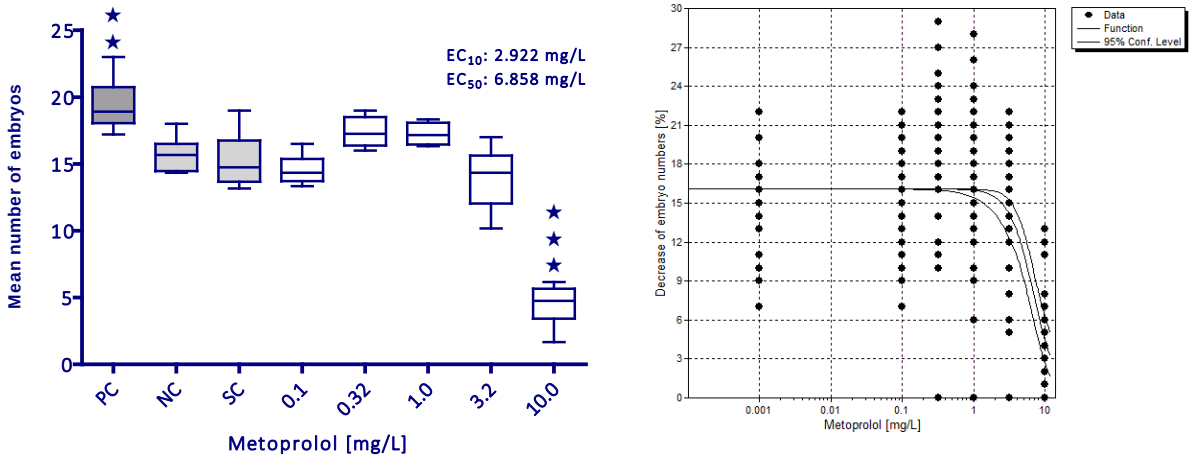
The measured concentrations of metoprolol are in the range of the nominal concentrations. Metoprolol was not found in the water of the negative control and the solvent control (Table 34). While two days before medium exchange between 94% (1 mg/L) and 98% (0.1 mg/L) of the nominal concentration were measured, directly after medium exchange between 96% (1 mg/L) and 98% (0.1 mg/L, 10 mg/L) were found. This means that a maximum of 6% of the test substance disappears within two days. Only the measured concentration of the positive control (25 ng/L EE2) was by a factor of 10 higher compared to the nominal concentration.

Table 34: Analyses of metoprolol concentrations during the 28 days exposure experiment. Three times per week medium was completely exchanged (ME).

Sampling time	nominal conc. [mg/l]	Determined conc. [mg/L]	Recovery rate [%]	Decline [%]
0	0 (control)	< 0.0020	-	-
0	0 (solvent control)	< 0.0020	-	-
before ME	0.1	0.0980	98	2
before ME	1.0	0.9400	94	6
before ME	10.0	9.6000	96	4
after ME	0.1	0.0980	98	2
after ME	1.0	0.9600	96	4
after ME	10.0	9.8000	98	2

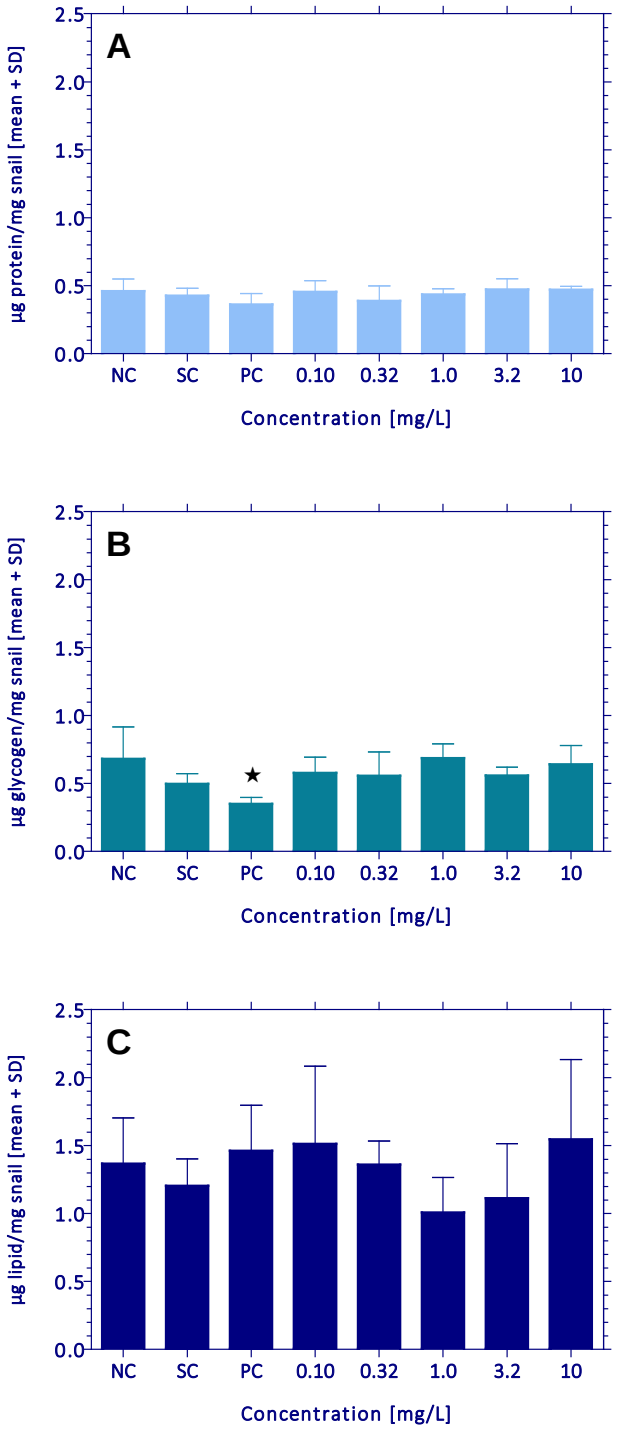
Throughout the batch experiment no mortality was observed. Reproduction of gastropods is shown in Figure 58. The positive control (EE2) indicates a significant increase in the mean number of embryos (mean of 19.4 individuals/female) compared with the control (mean of 15.4 individuals/female). The increase of reproduction of *P. antipodarum* was already shown when this species was exposed to EE2 (Jobling et al. 2003; Völker et al. 2014). The mean offspring in the treatments 0.1-3.2 mg/L was at a comparable level, while the highest concentration indicates a significant decrease (4.5 embryos per snail). Using a logistic non-linear regression model (ToxRat®), an EC_{10} value of 2.922 mg/L (lower/upper confidence interval of 1.762/3.859 mg/L) was calculated (Figure 58).

Figure 58: *Potamopyrgus antipodarum* reproduction test with the beta-blocker metoprolol according to OECD Draft guideline (OECD 2015). Mean total number of embryos after 28 days of exposure to metoprolol (left). NC and SC (no difference, t-test) were merged and tested against metoprolol (one-way ANOVA, ***p < 0,001. PC (25 ng/L EE2) was significant to SC (t-test, **p < 0.01). (median, 25th to 75th percentiles, min, max). N = 36, logistic non-linear regression model, EC10: 2.922 mg/L, lower/upper 95% CI: 1.762/3.859 mg/L, EC50: 6.858 mg/L, lower/upper 95% CI: 5.600/8.146mg/L (right).



The 28-day treatment with the beta-blocker metoprolol showed no concentration effect relationship between the snails' energy status (glycogen, lipid and protein content) and the metoprolol concentration (Figure 59). While the protein content ranged between 0.363 µg/mg snail (PC) and 0.472 µg/mg snail (3.2 mg/L) (Figure 59A), the glycogen content of the positive control showed a significant reduction compared to the control (one-way ANOVA, Dunnett's test) (Figure 59B). Moreover, a constant glycogen level was apparent in all metoprolol treatments assuming that glycogen is stored only at a certain level and then it was used for lipid synthesis. The lipid reserves are closely linked to the fecundity of *P. antipodarum* (Gust et al., 2011). As shown in Figure 59C it is obvious that the lipid content decreased slightly at concentrations between 0.32 mg/L and 3.2 mg/L, but the number of embryos increased slightly (Figure 58). Possibly, despite the increasing metoprolol concentration lipid reserves needed partly to maintain the reproduction. At the highest concentration (10 mg/L) the lipid content increased to an average of 1.55 µg/mg snail, while the gastropods showed a massive decrease in reproduction (4.47 embryos per female).

Figure 59: *Potamopyrgus antipodarum* Reproduction Test (OECD 2010). Energy status (protein (A), glycogen (B) and lipid content (C)) in *P. antipodarum* after 28 days of exposure to metoprolol (batch experiment). NC and SC (no difference, t-test) were merged and tested against metoprolol treatments (one-way ANOVA, *p < 0.05. PC (25 ng/L EE2). N = 6.

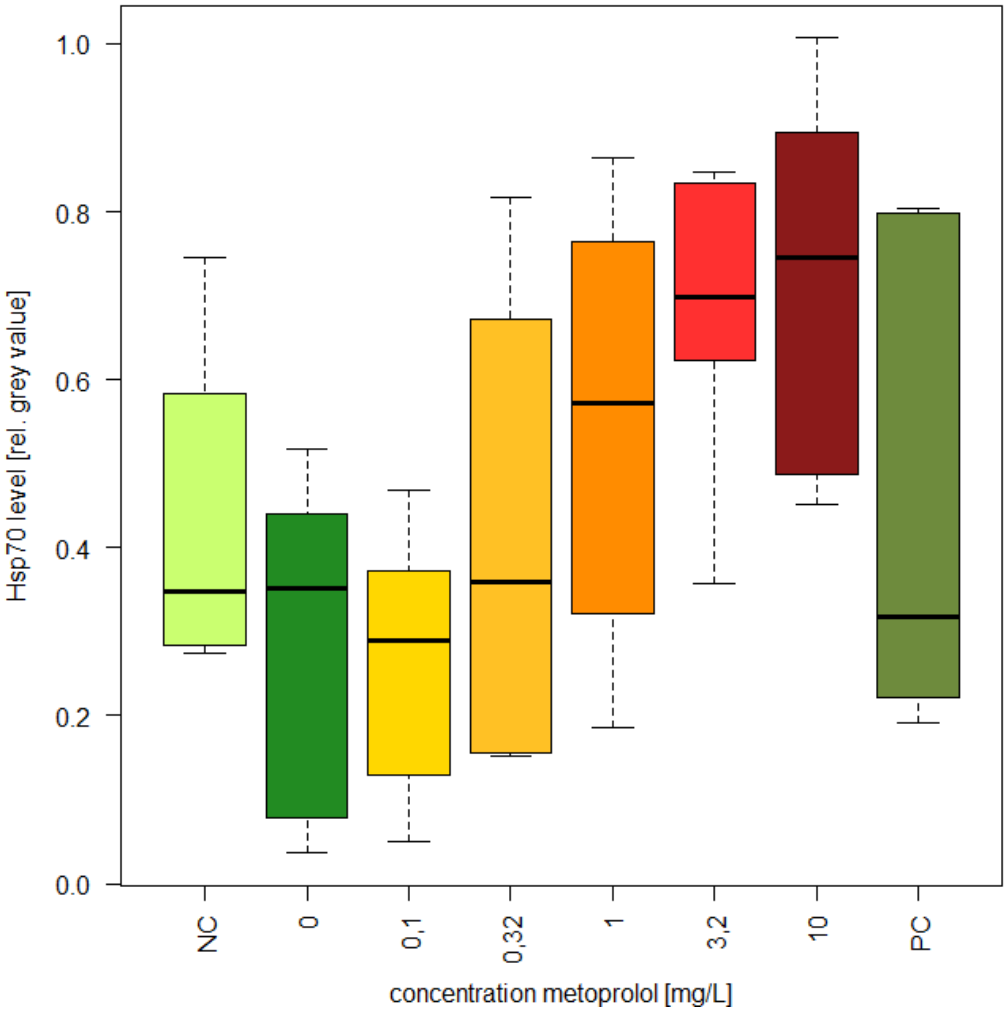


1.4.3.2 Biochemical markers

The level of the stress protein Hsp70 was increased by metoprolol in a concentration-dependent manner. Data were squared to fit the assumptions of parametric testing. The increase was significant for the two highest concentrations, 3.2 and 10 mg/L, compared to the solvent control (linear model,

df=7/40, F=2.965, p=0.01336). The positive control did not show any peculiarities in respect to the stress protein level. The data show proteotoxic action of metoprolol in *Potamopyrgus* (Figure 60). 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. This clear response was only visible for *Potamopyrgus* and none of the other investigated organisms. 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 - increasing sensitivity by detecting physiological changes before the alterations manifest in reduced reproduction. Contardo-Jara et al. (2010) found an increase of *hsp70*-mRNA in the mussel *Dreissena polymorpha* at concentrations as low at 0.534 µg/L. It may be that the stress protein system of molluscs reacts to metoprolol in a more sensitive manner than other taxa. Further studies with other species of gastropods or bivalves could help shedding light on this manner.

Figure 60: Stress protein Hsp70 levels of *Potamopyrgus antipodarum* exposed to metoprolol for 28 days. Bold lines display the median values, boxes the 25% to 75% quantiles, whiskers the minimum and maximum values, open circles potential outliers. “NC” refers to the negative control without solvent, “PC” to the positive control. The stress protein level is significantly increased by metoprolol in a concentration-dependent manner (linear model, df=7/40, F=2.965, p=0.01336).



2 WP3B Toxicity of pharmaceuticals – mixtures (Part II)

2.1 Studies with juvenile trout

2.1.1 Introduction

2.1.2 Materials and Methods

The exposure of juvenile brown trout to mixtures of diclofenac and metoprolol started on September 15th 2015 and was finished on October 13th 2015, after 28 days of exposure. Treatments based on toxic units (TU) derived from the single substance exposures – with 1 TU corresponding to 100 µg/L diclofenac or 1000 µg/L metoprolol, respectively. The final mixture concentrations were set, according to the agreement with UBA, as 25 µg/L diclofenac + 250 µg/L metoprolol, 25 µg/L diclofenac + 750 µg/L metoprolol, 75 µg/L diclofenac + 250 µg/L metoprolol, 75 µg/L diclofenac + 750 µg/L metoprolol and 100 µg/L diclofenac + 1000 µg/L metoprolol. Similar to the single substance experiments, diclofenac was applied as sodium salt (CAS 15307-79-6) and metoprolol as tartrate (CAS 56392-17-7).

In the experimental setup, each mixture and the control were tested in triplicates, with 10 individual fish exposed per replicate – summing up to a total of 180 animals. Fish were acclimatized to lab conditions for 38 days. This elongated time was due to delays in the experimental start. To check for possible effect of the keeping, a small sample of four individual fish was examined right at the start of the experiments. Apart from the reduced sample size and the elongated acclimation period, the experimental procedure was similar to the single substance experiments described in 1.1.2.1. One sampling event at the fish hatchery (August 8th) was used as a reference. We did not conduct at second hatchery control corresponding to the end of the experiment. Trout develop much faster in hatcheries than they do at lab keeping, and the experiment was delayed by a month. Therefore, the size difference between the experimental animals and the hatchery trout would have been too big to allow a meaningful comparison.

The battery of samples taken and parameters tested was the same as described in 1.1.2.1.

2.1.3 Results

All measured concentrations were within a close range of the nominal concentrations: control – 0 µg/L, “75D/250M” – 69.7 µg/L D and 228 µg/L M, “25D/750M” – 23 µg/L D and 706 µg/L M, “75D/750M” – 68.6 µg/L D and 676 µg/L M, “25D/250M” – 23.2 µg/L D and 234 µg/L M, “100D/1000M” – 89 µg/L D and 912 µg/L M. In all following graphs, measured concentrations are displayed.

Mortality remained low in all experimental groups throughout the whole time (Figure 61).

Considering standard body length and body mass, there was a slightly significant interaction of treatment with the experimental block, but no significant effect of the pharmaceutical treatment (Nested ANOVA: length - treatment: df=5, F=0.21156, p=0.0663, Block[treatment]: df=12, F=1.9058, p=0.0373; mass – treatment: df=5, F=1.7249, p=0.1319, Block[treatment]: df=12, F=2.1089, p=0.0191). Data are shown in

Figure 62 and Figure 63.

Like in the other experiments, several animals showed bite marks on their fins. Overall, these differences were weakly significant (Likelihood-Ratio, df=10, χ^2=18.963, p=0.0407). Post-hoc pairwise comparisons revealed the only significant difference to be between 25D/750M and 75D/250M (Figure 64).

The data on lipid peroxides showed high variability. Sample mass and experimental block were no factors of influence, but the analysis also revealed no differences between the tested mixture con-centrations (linear model, df=5/167, F=0.6094, p=0.6928). Data are shown in Figure 63.

Hsp70 was neither affected by the experimental block, nor by the mixture treatment (linear model, df=5/166, F=1.18, p=0.3213). Data are shown in Figure 66.

Histological examination revealed pathologies similar to those observed in the single substance exposures. Control fish were already in a reaction state, but exposure to the mixtures led to further deterioration of the histological condition.

The glycogen storage in liver reached from normal condition with plenty of storage substance up to the complete depletion of glycogen. Inflammations, often associated with macrophage aggregations, were another commonly found reaction state. Macrophage aggregations were more frequently found in individuals exposed to the mixtures. Several individuals showed severe degenerations of the hepatic tissue, with large-scale necrosis. Yet, only the mixture of ¾ TU diclofenac and ¼ TU metoprolol showed a visible overall difference to the control treatment.

In general, the kidneys of animals in the mixture experiment featured a very high abundance of melanomacrophages, compared to the single substance exposures. Control animals showed hyaline droplets in proximal and distal tubular elements, and occasional alterations of glomerular elements and hematopoietic tissue. In the mixture treatments, many samples revealed degenerations of renal tissue, including focal to large-scale necrosis, visible debris in tubular lumina and a perforated structure of the hematopoietic tissue. Especially in the two highest mixture treatments, the occurrence of severe degradations was much higher.

Again, the gill reactions were characterized by visible thickening through hyperplasia and hypertrophy of epithelial and interlamellar cells. Furthermore, edema were a frequent finding, as well as partial lamellar fusion. These reactions were more pronounced in animals exposed to the mixture treatment. Visible differences were present for the two highest mixtures.

The observed pathologies are shown in Figure 67, and the results of the semi-quantitative assessment are summarized in Figure 68.

Figure 61: Cumulative mortality of juvenile brown trout exposed to binary mixtures of diclofenac and metoprolol over 28 days. Mortality remained low throughout the entire experimental period.

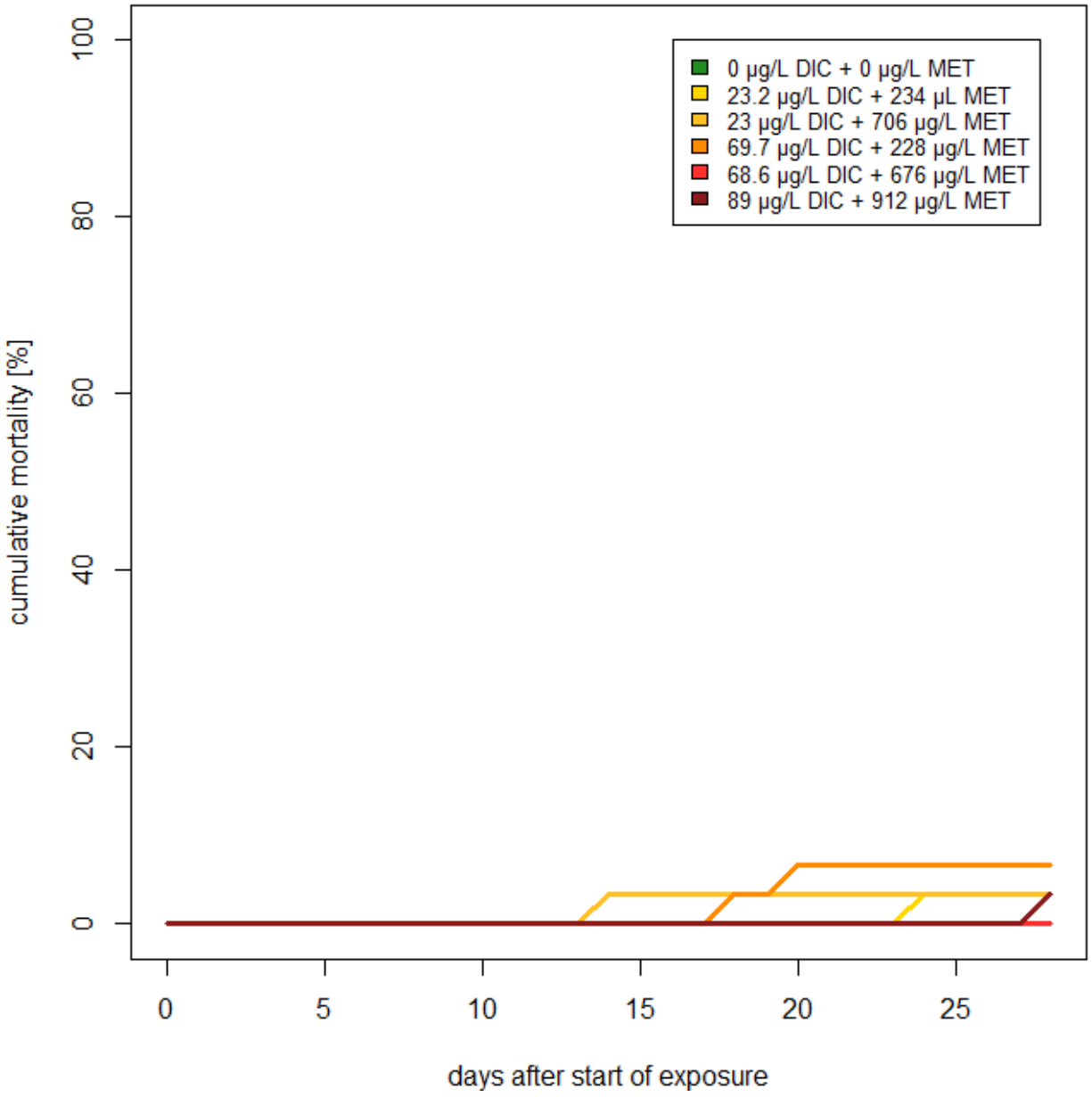


Figure 62: Standard body length (from snout to base of the tail) of juvenile brown trout after 28 days exposure to binary mixtures of diclofenac (D) and metoprolol (M). Bold lines display the median values, boxes the 25 % to 75 % quantiles, whiskers the minimum and maximum values, circles potential outliers. Treatments are given in fractions of toxic units (TU): 1 TU corresponds to 100 µg/L diclofenac and 1000 µg/L metoprolol.

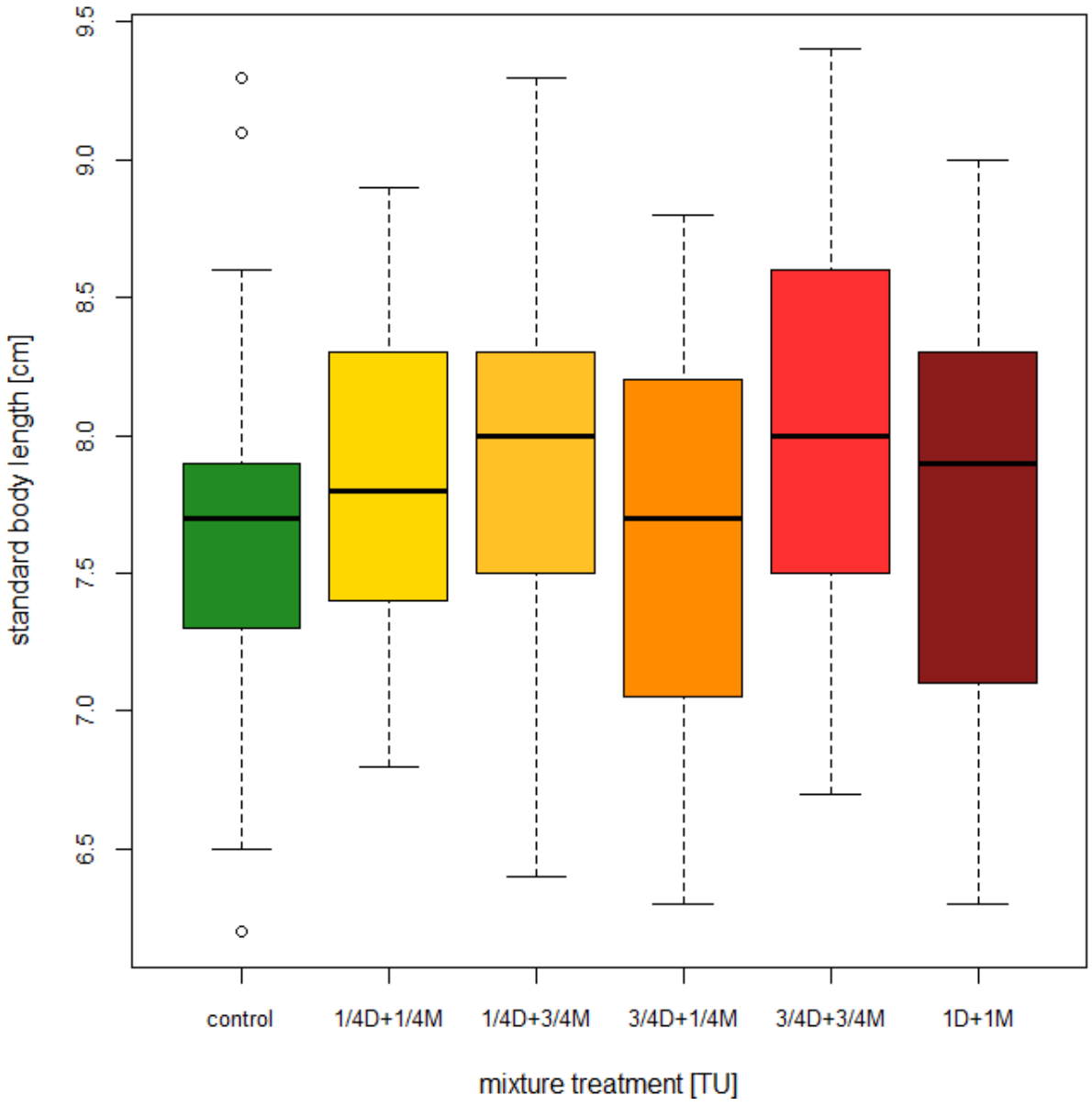


Figure 63: Body mass of juvenile brown trout after 28 days exposure to binary mixtures of diclofenac (D) and metoprolol (M). Bold lines display the median values, boxes the 25% to 75% quantiles, whiskers the minimum and maximum values, circles potential outliers. Treatments are given in fractions of toxic units (TU): 1 TU corresponds to 100 µg/L diclofenac and 1000 µg/L metoprolol.

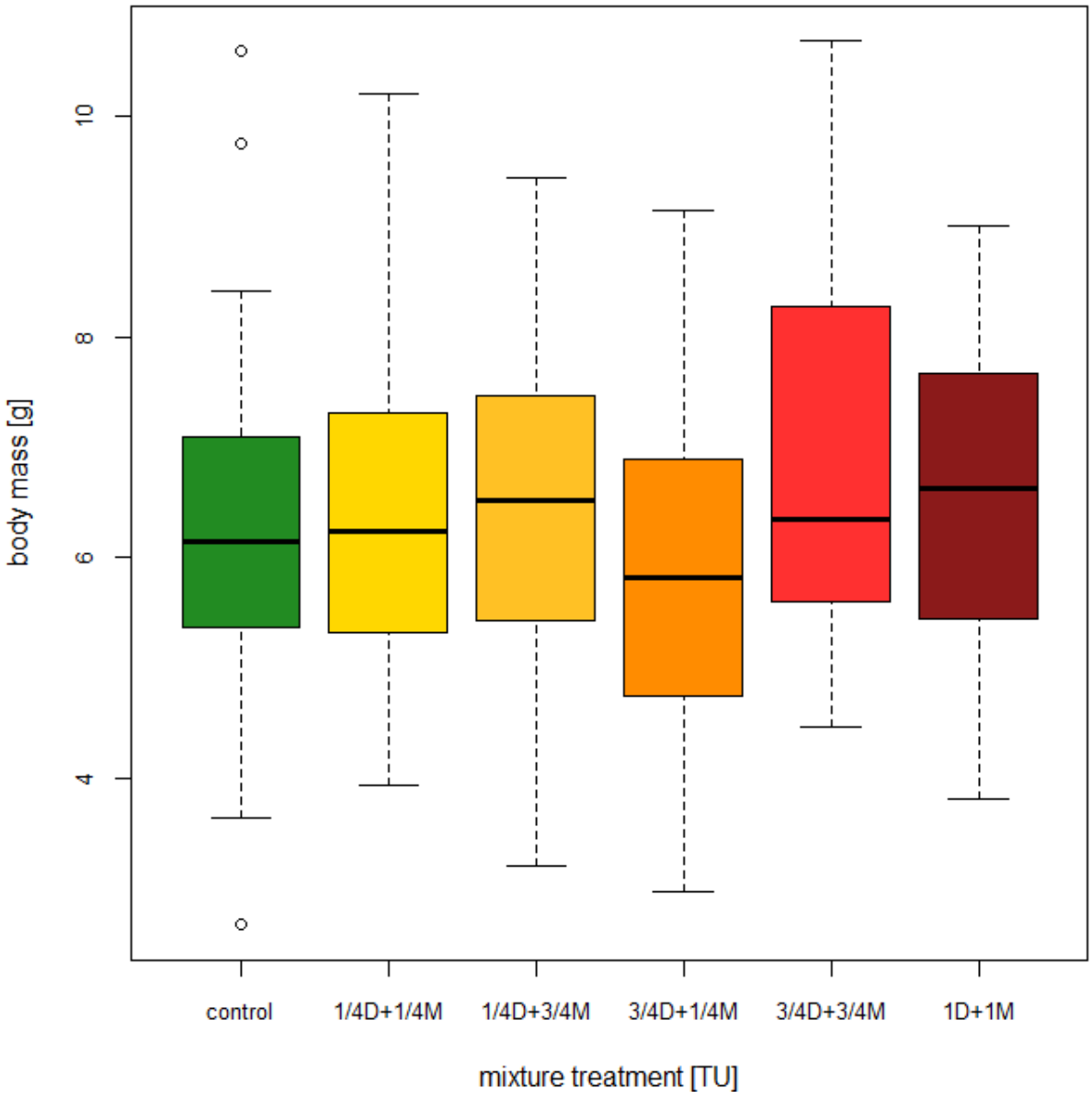


Figure 64: Percentage of brown trout exposed to binary mixtures of diclofenac (D) and metoprolol (M) showing bite marks on fins. The animals exposed to a mixture of 25 µg/L diclofenac and 750 µg/L metoprolol had the lowest proportion of injuries. Treatments are given in fractions of toxic units (TU): 1 TU corresponds to 100 µg/L diclofenac and 1000 µg/L metoprolol.

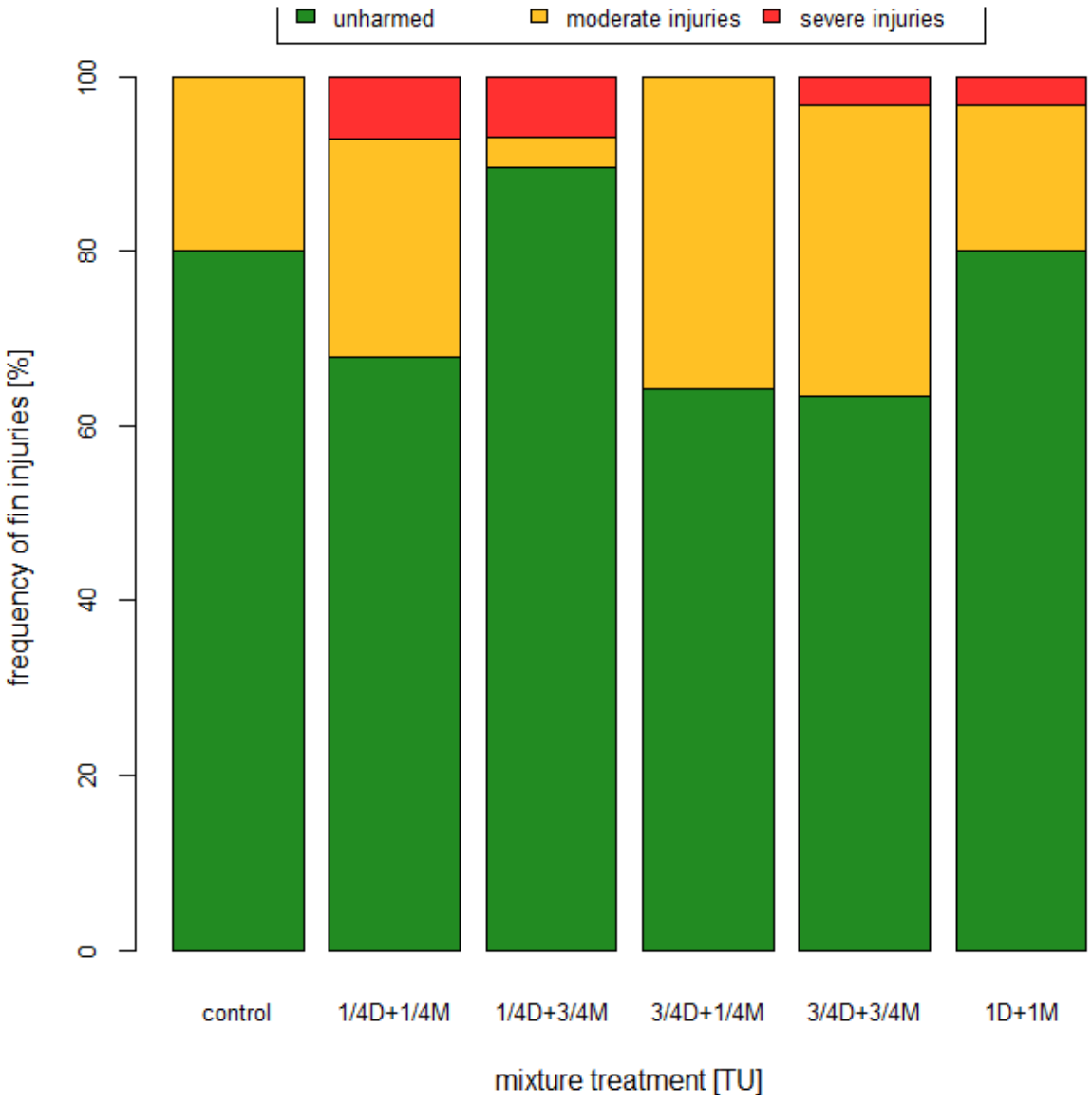


Figure 65: Lipid peroxidation (in cumene hydroperoxide equivalents per mg wet weight) of kidney of juvenile brown trout exposed to mixtures of diclofenac and metoprolol for 28 days. Bold lines display the median values, boxes the 25% to 75% quantiles, whiskers the minimum and maximum values, original data points as black dots. Treatments are given in fractions of toxic units (TU): 1 TU corresponds to 100 µg/L diclofenac and 1000 µg/L metoprolol.

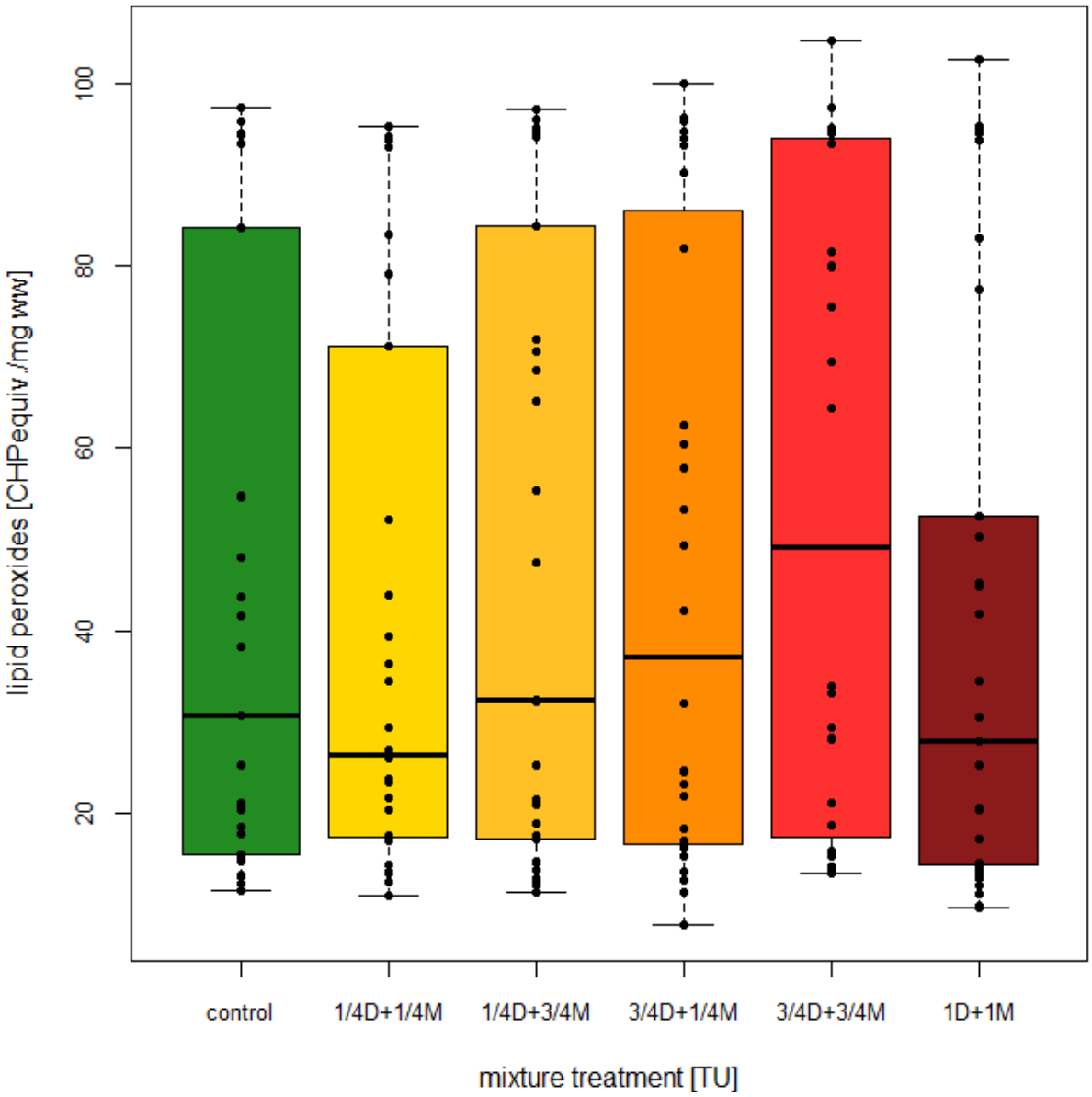


Figure 66: Hsp70 level (given in relative grey value) in liver of juvenile brown trout exposed to mixtures of diclofenac and metoprolol for 28 days. Bold lines display the median values, boxes the 25% to 75% quantiles, whiskers the minimum and maximum values, original data points as black dots. Treatments are given in fractions of toxic units (TU): 1 TU corresponds to 100 µg/L diclofenac and 1000 µg/L metoprolol.

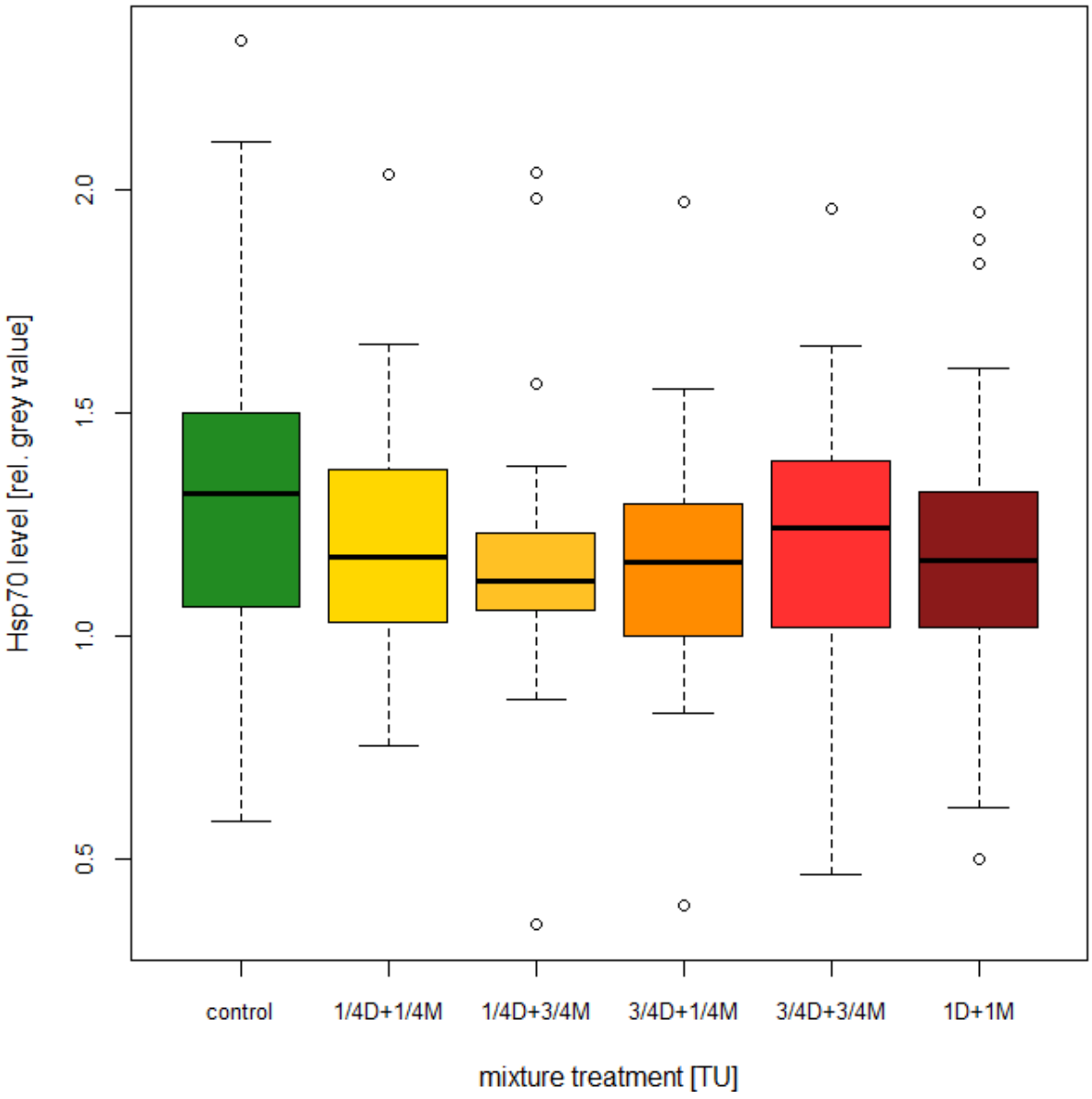


Figure 67: Histological sections of brown trout exposed to mixtures of diclofenac and metoprolol: a) liver, control (HE): reduced glycogen content and macrophage aggregations in hepatic tissue. b) liver, 1 TU diclofenac and 1 TU metoprolol (AB-PAS): reduced glycogen content and severe necrotic degeneration of hepatocytes. c) kidney, control (HE): dilation of glomerular structure. d) kidney, 1 TU diclofenac and 1 TU metoprolol (HE): degeneration of tubular structures and hematopoietic tissue. e) gill, control (AB-PAS): hyperplasia and hypertrophy of epithelial cells. f) gill, 1 TU diclofenac and 1 TU metoprolol (AB-PAS): hyperplasia and hypertrophy of epithelial cells, edema and epithelial lifting.

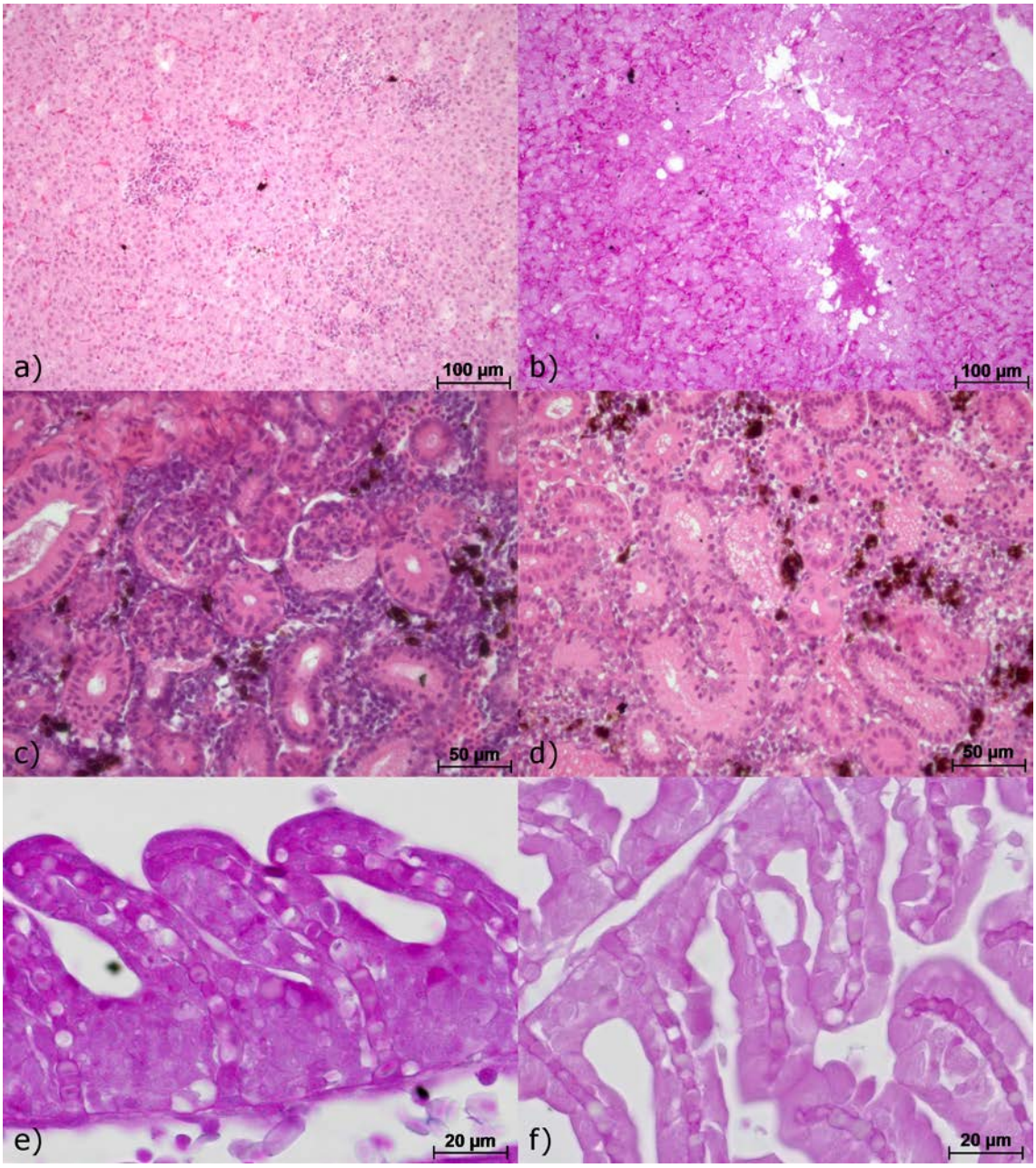
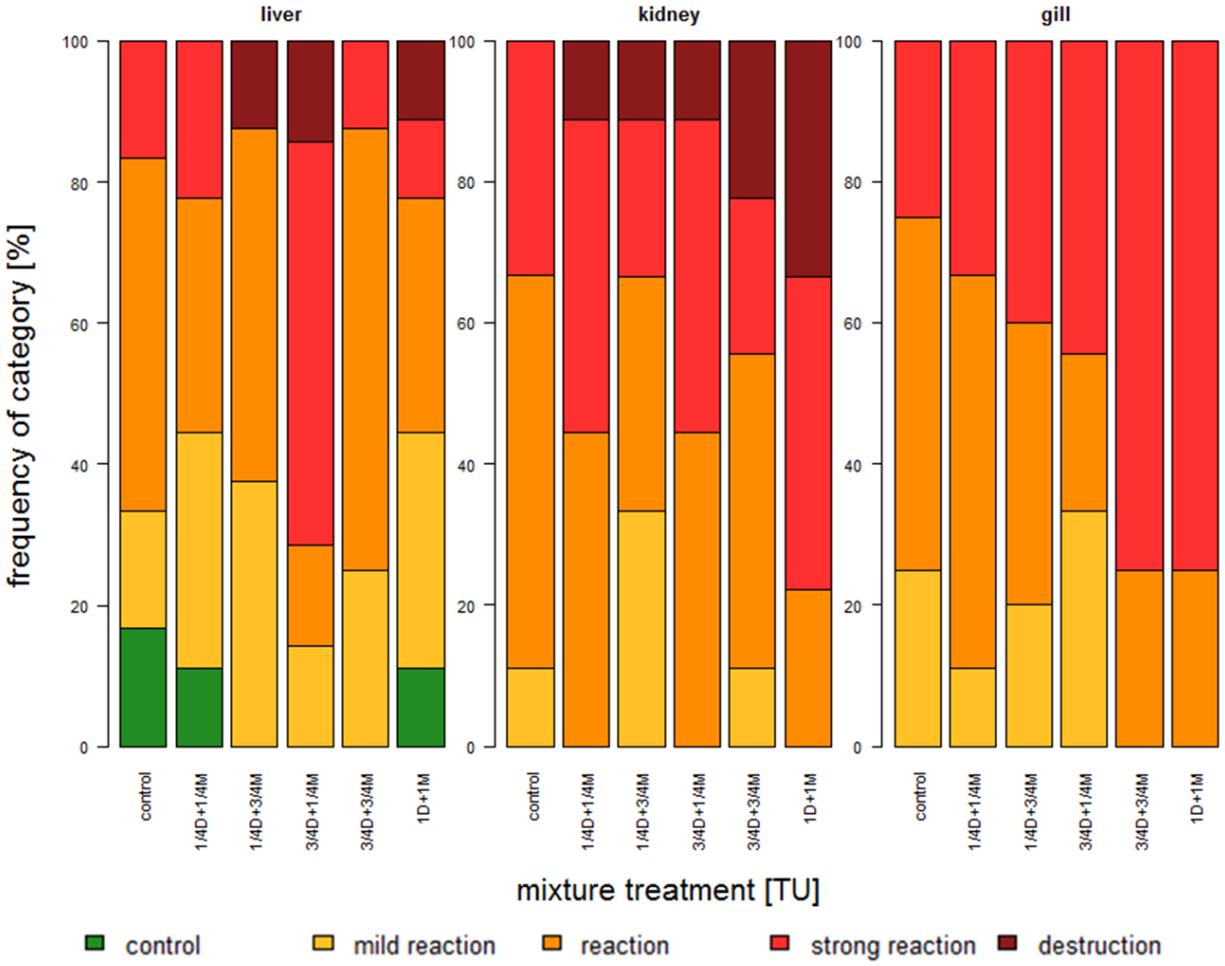


Figure 68: Semi-quantitative assessment of histological condition of brown trout exposed to mixtures of diclofenac and metoprolol. Treatments are given in fractures of toxic units (TU): 1 TU corresponds to 100 µg/L for diclofenac (D) and to 1000 µg/L for metoprolol (M). For all investigated organs, the controls were already in a reaction state. The mixture treatments lead to worsened histological condition of kidney and gill. In liver, only the mixture of ¾ TU diclofenac and ¼ TU metoprolol could invoke a stronger reaction.



2.1.4 Discussion

Only few animals died throughout the exposure period. The observed mortality rates show an obvious contrast to the data observed for diclofenac alone, but match with the data from the metoprolol exposure. There are several possible explanations for this finding: Since the whole experiment was conducted after the diclofenac exposure, the animals were approximately 2 months older. When comparing data on juvenile and embryonic/larval exposure, it is seems obvious that the life stage of brown trout plays a crucial role in its susceptibility to the pharmaceutical. Therefore, it is possible that the older fish were stronger and more resilient towards stressful conditions. Furthermore, the strongest, and significant, effects on mortality were visible for the highest concentrations in the single substance diclofenac exposure. In the mixture experiment, only one treatment group included diclofenac in a concentration close to the defined LOEC of 100 µg/L. Moreover, an antagonistic effect of diclofenac and metoprolol in combination would be another possible explanation. Yet, a clear statement concerning this interaction can hardly be made, since the age of the animals differed slightly between the single substance and mixture exposures.

The weak differences in length and mass, depending on the experimental block, is most likely due to small temperature variations. Although all experiments took place in the same thermoconstant chamber, the measured aquarium temperature is affected by the exact position of the tank in the room. Fish from warmer aquaria grow faster due to higher metabolic activity. Still, it needs to be stressed out that the effects were in no case highly pronounced, and only marginally significant. The blocked design allowed to correct for this influence, and revealed no effect of the binary mixture on growth during the exposure period.

The variable bite marks showed a complicated reaction pattern. Unlike the diclofenac exposure, where there was a clear threshold level after which the number of fin bites increased, and the metoprolol exposure, which revealed a generally high frequency of fin bites in all treatments. Especially the treatment group exposed to 23 µg/L diclofenac and 706 µg/L metoprolol had an obviously lower proportion of injured animals. This is the only treatment, where a low diclofenac concentration is connected to a high metoprolol concentration – hinting on a possible antagonistic effect. Yet, the effect was only weakly significant, and the other exposures showed frequencies close to the control treatment. Therefore, this single finding is interesting information, but does not need be of actual biological meaning.

Lipid peroxidation showed, like in the other examinations, a high variability of results. Again, the obtained values were distributed bimodally, hinting on the existence of two different reaction types. The treatment itself was of no influence, corresponding with the results obtained for the single substance diclofenac exposure. Like for the single substances, no signs of proteotoxic action were seen in the binary mixtures of diclofenac and metoprolol.

The histological reactions evoked by the mixtures were comparable to those seen in the single substance exposures of diclofenac. In liver, the reactions were less pronounced, while the gill showed stronger reactions. One possible explanation for this finding is, again, the higher age of the animals. Due to their longer time of growth in the fish hatchery, the animals could accumulate larger energy reserves, rendering them more resilient to stress. Based on the histological condition it seems likely that a longer exposure period would have led to increases in mortality, as they were observed in the single substance exposure. Nevertheless, the toxicity of the mixture seems to be mainly driven by diclofenac rather than metoprolol. There were no clear signs of synergistic effects of both pharmaceuticals.

2.2 Studies with gammarids

2.2.1 Introduction

In the aquatic environment a diversity of chemicals e.g. pharmaceuticals are present and can cause adverse effects in organisms. Effect concentrations from single substance tests only provide information solely about the toxicity of the investigated pharmaceutical. As a second step characterizing risks in the environment the toxicity of mixtures of the NSAID diclofenac and the ß-blocker metoprolol was investigated in *Gammarus fossarum*. According to the mode-of-action of the pharmaceuticals toxicity of a mixture can act additive, synergistic or antagonistic compared to the single substance toxicity. To enable comparisons between single substance and mixture toxicity tests the exposure conditions and investigated endpoints were identical in both chronic tests.

2.2.2 Material and Methods

2.2.2.1 Applied pharmaceuticals

Nominal mixture concentrations were determined using the concept of Toxic Units (TU), hereby 1 TU = LOEC (derived from single substance test). In WP 3A the LOEC for diclofenac was determined with 2.6 mg/L, for metoprolol with 15 mg/L for the most sensitive effect endpoint, the juvenile/adult ratio, respectively. According to “Angebot UFOPLAN 2013” the following mixture ratios and calculated concentrations were investigated (Table 35).

Table 35: Mixing ratios and nominal concentrations of diclofenac and metoprolol in chronic exposure to a mixture; TU - toxic unit; 1 TU (DIC) = 2.62 mg/L, 1 TU (MET) = 15 mg/L; DIC- Diclofenac; MET – Metoprolol.

Sample identification	Mixture ratio		Nominal concentrations of the pharmaceuticals in the mixture [mg/L]	
	TU (DIC)	TU (MET)	DIC	MET
Control	0	0	0.00	0.00
CM1	¼	¼	0.66	3.75
CM2	½	½	1.31	7.50
CM3	¼	¾	0.66	11.25
CM4	¾	¼	1.97	3.75
CM5	¾	¾	1.97	11.25

As described in Chapter 1.3 stock solutions of diclofenac and metoprolol were prepared in 5.6 fold concentration of highest exposure concentration and diluted to the respective exposure concentrations. Metoprolol tartrate and diclofenac sodium salt were again purchased from Sigma Aldrich. The nominal concentrations refer to the active substance of the pharmaceuticals.

2.2.2.2 Sampling for chemical analysis

For chemical analysis, individual samples for each replica were taken with a volume of 1.5ml. Additionally, samples with 0.188 ml of each replica of a respective concentration were taken and pooled, further referred to as pooled samples. Thus, the volume of the pooled samples was also 1.5 ml. Since day 23 of the exposure, 0.214 ml was taken from each control replicate for the mixed samples due to the loss of the replicate K6. All samples were stored in HPLC glass bottles (1.5 ml short thread bottles; FISHER SCIENTIFIC) and frozen at - 22 °C.

The pooled samples of the controls from day 0 and day 35 before the medium exchange were analyzed as well as the mixtures CM1 and CM5 of the pooled samples from day 0, day 7 before, day 28 after and day 35 before medium exchange (ME). The analysis was conducted by TZW Karlsruhe.

2.2.2.3 Exposure of gammarids

Individuals of *G. fossarum* were sampled in Tännichtgrundbach on August 18th and 23rd, 2015. The sampling technique, transport, selection of individuals and adaptation were performed as described in Chapter 1.3.

The exposure of *G. fossarum* to a mixture of diclofenac and metoprolol started on June 25th and ended on August 4th, 2015. Over 40 days 20 individuals per replicate were exposed to 5 mixtures of diclofenac (CAS 15307-79-6) and metoprolol (CAS 56392-17-7) in 2L beakers (glassware). All treatments and the controls were run in 8 replicates to ensure a sufficient number of individuals for biomarker analysis at the end of the exposure.

Exposure conditions were identical to those in the single substance tests concerning experimental set-up, weekly medium exchange, conditioning and pre-incubation of leaf discs of *Alnus glutinosa* as food source, sampling for chemical analysis and determination of nutrients. Detailed information are presented in Chapter 1.3. The number of leaf discs of *A. glutinosa* per replicate was increased from 5 to 8 discs per week. Physico-chemical parameters were determined three times a week. Contamination of controls via the pH probe was prevented using DOSATEST paper (VWR, pH 7-10, scale 0.3 pH units; Macherey Nagel, pH 4.5-10, scale 0.5 units) for exposure concentrations. However, controls were measured using pH probe 3110 SET 2 (WTW).

For analysis of the biomarkers Hsp70 and lipid peroxides at the University of Tübingen 80 individuals of *G. fossarum* were fixed in liquid nitrogen at the beginning of the exposure and stored at -80 °C (Sanyo Ultra Low). At the end of the exposure all individuals from 4 randomly selected replicates per treatment were fixed and stored as described above. The samples were shipped on dry ice to University of Tübingen.

The endpoints mortality and juvenile/adult ratio, relative number of precopulae, relative number of egg-bearing females and juvenile length were evaluated for all 8 replicates at the end of exposure. The endpoint number of eggs per egg-bearing female was investigated only for 4 replicates due to the fixation of 4 replicates for biomarker analysis. For those analyses a stereomicroscope (Thalheim Spezial Optik) was used.

2.2.2.4 Biochemical markers

See 1.3.2.10.

2.2.2.5 Statistical Evaluation

For statistical evaluation Williams test was applied using ToxRat Software (Version 2.10, ToxRat Solutions GmbH). The confidence level for significant differences was set to $\alpha = 0.05$.

2.2.3 Results

2.2.3.1 Chemical analysis of exposure concentrations

The results of the chemical analysis of the chronic mixture toxicity test are shown for metoprolol in Table 36 and for diclofenac in Table 37.

The determined concentration of metoprolol in the control was at the beginning 0.001 mg/L and after 35 days 0.0008 mg/L. The experimental facility in the green house is an open system and other experiments with metoprolol within the project were also carried out. The decrease of the contamination with metoprolol in the control during the 35 days suggests that the low contamination was just once at the beginning of the experiment. This could explain the very low contamination close to the limit of detection. Furthermore, these concentrations detected in the control, were very low and by a factor of 15,000 lower than the LOEC that was determined in the chronic single toxicity test (48h) (Chapter 1.3).

The determined concentration at day 0 corresponds to the nominal concentration for metoprolol with 100.6% (CM1) and 66.0% (CM5). The reason for the low values of sample CM5 (day 0) was because of a break of the sample glass bottles. Due to the transfer of the frozen samples (by defrost) to new vessels a loss of diclofenac and metoprolol was assumed. This assumption is supported by the metoprolol concentration of the sample CM5 at day 7 before the medium exchange, where the determined concentration was 92.0% of nominal concentration, although nothing was added in this period to the replicas. Hence, the loss of sampling volume is the reason for the lower recovery. This explains also the calculated decline of -39.3% metoprolol after seven days in the mixture CM5.

The dissipation of metoprolol over 7 days was similar in both sampling periods (day 7 and day 35) and was approximately 10%. After the medium exchange at day 28 the detected concentration of metoprolol were 89.6% (CM1) and 92.8% (CM5) of nominal concentrations, respectively.

Table 36: Analysis of metoprolol concentrations during the 40 day exposure experiment; ME - medium exchange.

Sampling time [d]	Sample*	Nominal conc. [mg/L]	Determined conc. [mg/L]	Recovery rate [%]	Decline within 7 days [%]
0	Control **	0	0.001	-	-
0	CM1 **	3.75	3.77	100.6	-

Sampling time [d]	Sample*	Nominal conc. [mg/L]	Determined conc. [mg/L]	Recovery rate [%]	Decline within 7 days [%]
0	CM5 **	11.25	7.43	66.0	-
7 before ME	CM1	3.75	1.18	31.5	68.7
7 before ME	CM5	11.25	10.35	92.0	-39.3
28 after ME	CM1	3.75	3.36	89.6	-
28 after ME	CM5	11.25	10.44	92.8	-
35 before ME	Control	0	0.0008	-	
35 before ME	CM1	3.75	3.33	88.8	0.9
35 before ME	CM5	11.25	10.18	90.5	2.5

* Mixing ratios CM1 = ¼ - ¼, CM5 = ¾ - ¾
** short thread bottles broke after freezing the samples. Decant of the samples with glass funnel and 2 ml of Double deionised water.

At the beginning and after 35 days of the experiment, the determined concentration of diclofenac in the control was below the limit of detection (<0.0001 mg/L).The determined concentration at day 0 corresponds to the nominal concentration diclofenac with 114.7% (CM1) and 60.4% (CM5), respectively. The low recovery rate for CM5 is detailed described as above for metoprolol.

At day 7 (after ME), the recovery rate of CM1 was 82.7% and for CM5 it was 84.9% of the nominal concentrations, respectively. The decline of the diclofenac concentrations at day 35 was 60.8% (CM1) and 48.0% (CM5).

Table 37: Analysis of diclofenac concentrations during the 40 day exposure experiment; ME - medium exchange.

Sampling time [d]	Sample*	Nominal conc. [mg/L]	Determined conc. [mg/L]	Recovery rate [%]	Decline within 7 days [%]
0	Control **	0	< 0.0001	-	-
0	CM1 **	0.66	0.76	114.7	-
0	CM5 **	1.97	1.19	60.4	-
7 before ME	CM1	0.66	0.10	14.5	87.3
7 before ME	CM5	1.97	0.77	39.1	35.2
28 after ME	CM1	0.66	0.55	82.7	-
28 after ME	CM5	1.97	1.67	84.9	-
35 before ME	Control	0	< 0.0001	-	-
35 before ME	CM1	0.66	0.21	32.4	60.8
35 before ME	CM5	1.97	0.87	44.2	48.0

* Mixing ratios CM1 = ¼ - ¼, CM5 = ¾ - ¾
** short thread bottles broke after freezing the samples. Decant of the samples with glass funnel and 2 ml of distilled water.

2.2.3.2 Physico-chemical parameters

The median of pH values in the controls were in a range from 7.8 to 8.0, in the exposure concentrations pH values ranged between 7.5 and 7.6 (Table 38). Minimum and maximum values did not exceed 0.5

from the medians. Conductivity with a median of 336 µS/cm in control and medians between 334 and 336 µS/cm in exposure concentrations were very similar between all treatments. Oxygen concentration in controls had a median of 10 mg/L, in all exposure concentrations the median was 9.9 mg/L. According to this the medians of oxygen saturation were determined at 100% for control and 99% for all exposure concentrations.

Table 38: Overview of physico-chemical parameters during chronic exposure to mixtures of diclofenac (DIC) and metoprolol (MET). Median (Minimum; Maximum) are shown for every concentration.

Mixing ratio TU(DIC) – TU(MET) Parameter	Unit	Control -	CM1 ¼ – ¼	CM2 ½ – ½	CM3 ¼ - ¾	CM4 ¾ - ¼	CM5 ¾ - ¾
pH	-	7.8 (7.5-8)	7.5 (7.5-7.6)	7.5 (7.5-7.6)	7.5 (7.5-7.6)	7.5 (7.5-7.6)	7.5 (7.5-7.6)
Conductivity	µS/cm	336 (322-362)	336 (328-347)	336 (328-344)	336 (328-345)	334 (327-341)	336 (329-350)
Oxygen concentration	mg/L	10 (9.1-10.3)	9.9 (8.1*-10.3)	9.9 (9.0-10.2)	9.9 (9.0-10.2)	9.9 (9.4-10.2)	9.9 (9.0-10.3)
Oxygen saturation	%	100 (92-103)	99 (81*-103)	99 (94-102)	99 (92-102)	99 (93-102)	99 (92-103)

2.2.3.3 Determination of nitrate, ammonium and dissolved organic phosphor (SRP) concentrations

All samples for the determination of nitrate, ammonium, and SRP concentrations were taken at day 28 of the experiment.

In the control, the concentration of nitrate was 2.1 mg/L NO_3^-–N (Table 39). In the exposure ammonium concentrations ranged from 2.1 to 2.4 mg/L. The concentration of SRP in control was below the limit of detection (2 µg/L). In the exposure concentrations SPR ranged between 3.9 and 6.8 µg/L.

The ammonium concentration (NH_4-N) was lower in the control (37.6 µg/L) than in the exposure concentrations (102.8 to 266.0 µg/L). As described before a relation between metoprolol concentration and measured ammonium concentration had been observed (Chapter 1.3), possibly due to interactions between metoprolol tartrate and the substances used for the analytical method.

The ammonia concentrations (NH_3) were calculated using following equations (Emerson et al. 1975):

$$NH_3\left[\frac{mg}{l}\right] = \frac{0,94412 * NH_4 - N\left[\frac{mg}{l}\right]}{1 + 10^{pK_A - pH}} \quad (1)$$

With

$$pK_A = 0,0925 + \frac{2728,795}{t[°C] + 273,15} \quad (2)$$

For the calculation mean values of the temperature per concentration were used (14.5 – 14.6 °C). For the pH values, the geometric mean was used (7.8 in control, 7.5 in exposure concentrations).

Table 39: Concentrations of nitrate (NO_3^--N), ammonium (NH_4^+), calculated ammonia (NH_3) and SRP during exposure to the mixture of diclofenac (DIC) and metoprolol (MET).

	Mixing ratio TU (DIC) – TU (MET)	NO_3^--N [mg/L]	NH_4^+ [µg/L]	NH_3 [µg/L]	SRP [µg/L]
Control	-	2.1	37.6	0.6	1.3
CM1	¼ - ¼	2.2	108.9	0.9	5.0
CM2	½ - ½	2.1	181.7	1.4	3.9
CM3	¼ - ¾	2.4	241.1	1.9	6.8
CM4	¾ - ¼	2.1	102.8	0.8	5.5
CM5	¾ - ¾	2.2	266.1	2.1	4.3

2.2.3.4 Effects on mortality and reproduction

In two of the 8 replicates from the control treatment all individuals died during exposure. In the controls median of mortality from all replicates was 48% (Figure 69). The reason for 100% mortality in two replicates remained absolutely unclear, as the same medium was used and exposition took place in the same way for all replicates. Those two replicates were excluded from further analyses. After exclusion the median for mortality in control was 43% (Figure 70). The median for mortality in the exposure concentrations ranged between 33 and 58%. Lowest mortality occurred in the exposure concentration CM2 with ½ TU (DIC) and ½ TU (MET). Highest mortality was detected in CM4 with ¾ TU (DIC) and ¼ TU (MET).

Figure 69: Mortality of *G. fossarum* for all replicates at the end of exposure to the mixture of diclofenac and metoprolol. Medians are displayed along with 25 and 75% percentiles and minimal and maximal values. DIC – diclofenac; MET – metoprolol.

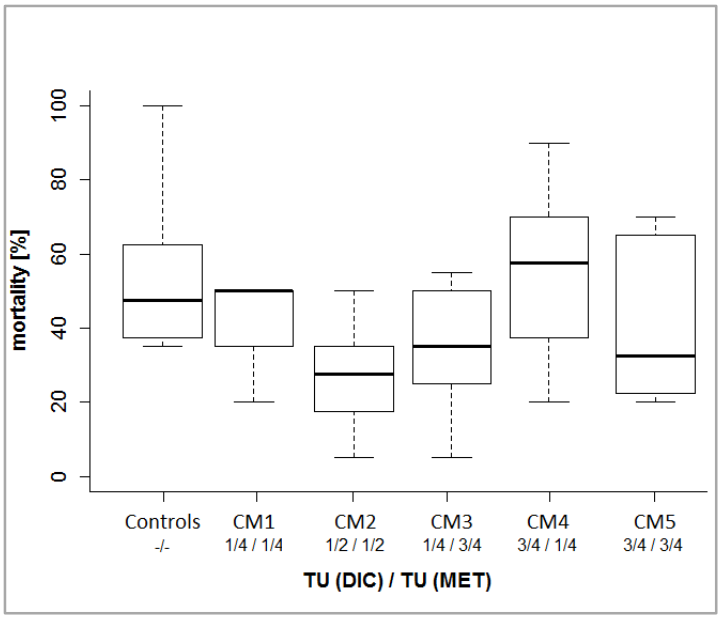
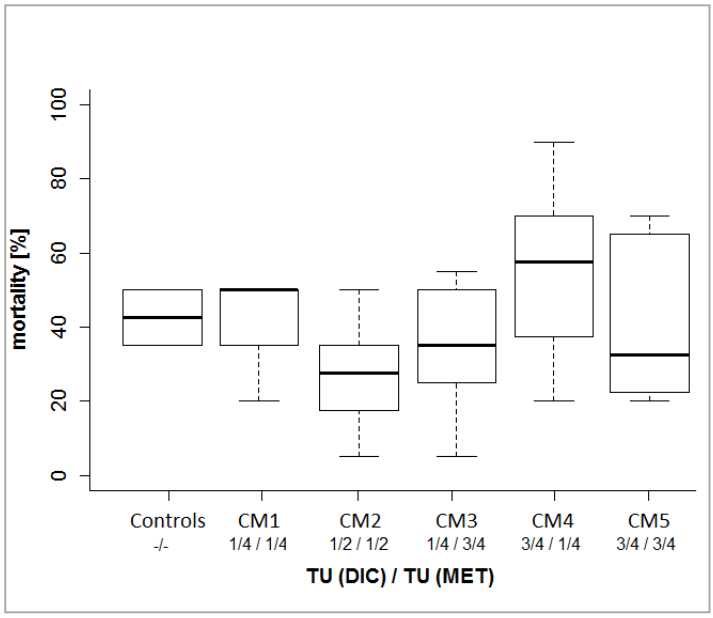
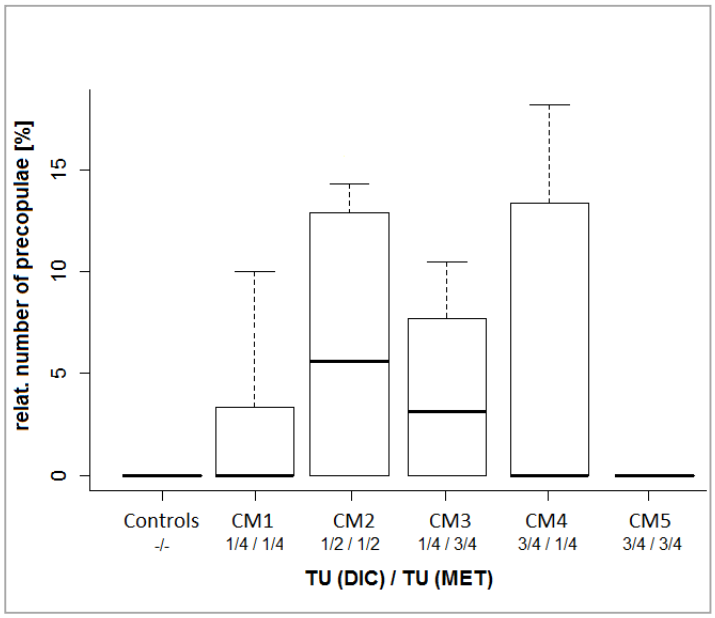


Figure 70: Mortality of *G. fossarum* after exclusion of two replicates with 100% mortality in control at the end of exposure to mixture of diclofenac and metoprolol. Medians are displayed along with 25 and 75% percentiles and minimum and maximum values. DIC – diclofenac; MET – metoprolol.



In controls no precopulae could be observed, hence the median for relative number of precopulae was 0 (Figure 71). For three mixtures (CM1, CM4, CM5) the median of relative number of precopulae was also 0. In CM3 with ¼ TU (DIC) and ¾ TU (MET) the median was 3%, minimum was 0 and maximum was 8%. Highest median was found in CM2 with ½ TU (DIC) and ½ TU (MET) at 6% with minimum at 0 and maximum at 14%.

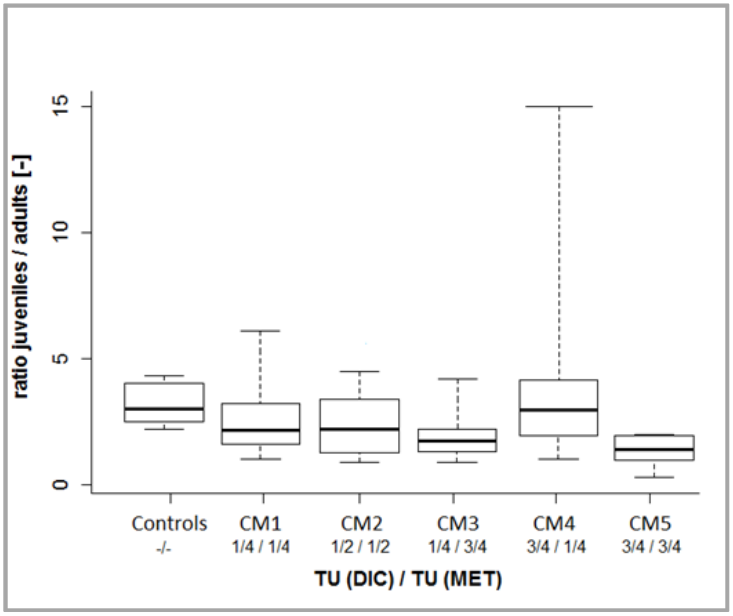
Figure 71: Relative number of precopulae of *G. fossarum* at the end of exposure to mixture of diclofenac and metoprolol. Medians are displayed along with 25 and 75% percentiles and minimum and maximum values. DIC – diclofenac; MET – metoprolol.



The ratio of juveniles to adults ranged in the controls between 2.18 to 4.3 and the median was 2.98 (Figure 72). In control and CM4 with ¾ TU (DIC) and ¼ TU (MET) highest ratios of juveniles to adults

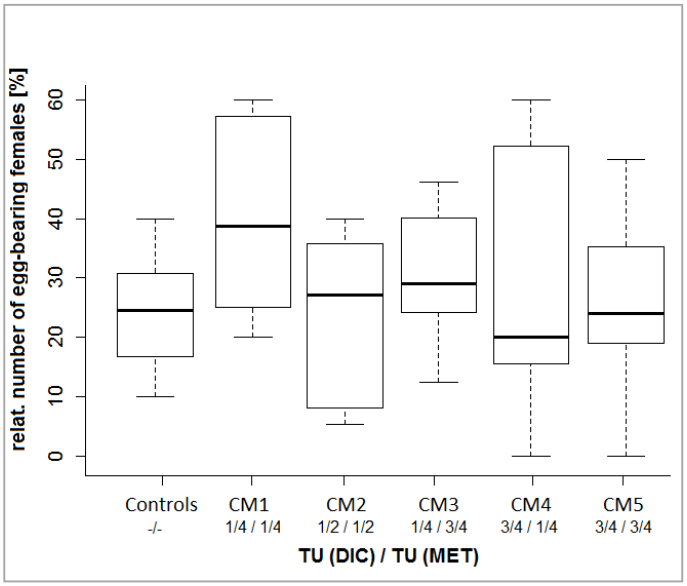
close to 3 were found. For the other mixtures the medians ranged from 1.4 (CM5) to 2.2 (CM2). The lowest value was found in concentration CM5 with ¾ TU (DIC) and ¾ TU (MET).

Figure 72: Ratio of juveniles to adults at the end of exposure to the mixture of diclofenac and metoprolol. Medians are displayed along with 25 and 75% percentiles and minimum and maximum values. DIC – diclofenac; MET – metoprolol.



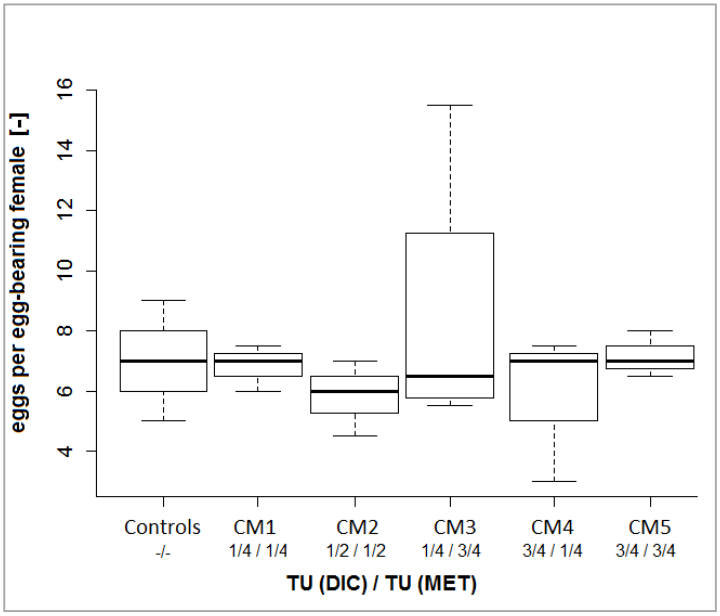
The relative number of egg-bearing females of *G. fossarum* in controls was between 10% and 40% (Figure 73), the median was at 24%. In the mixtures the medians ranged between 20% (CM4) and 39% (CM1). The highest relative number of egg-bearing females was detected in CM1 with ¼ TU (DIC) and ¼ TU (MET). The lowest relative number of egg-bearing females was found in CM4 with ¾ TU (DIC) and ¼ TU (MET).

Figure 73: Relative number of egg-bearing females of *G. fossarum* at the end of exposure to the mixture of diclofenac and metoprolol. Medians are displayed along with 25 and 75% percentiles and minimum and maximum values. DIC – diclofenac; MET – metoprolol.



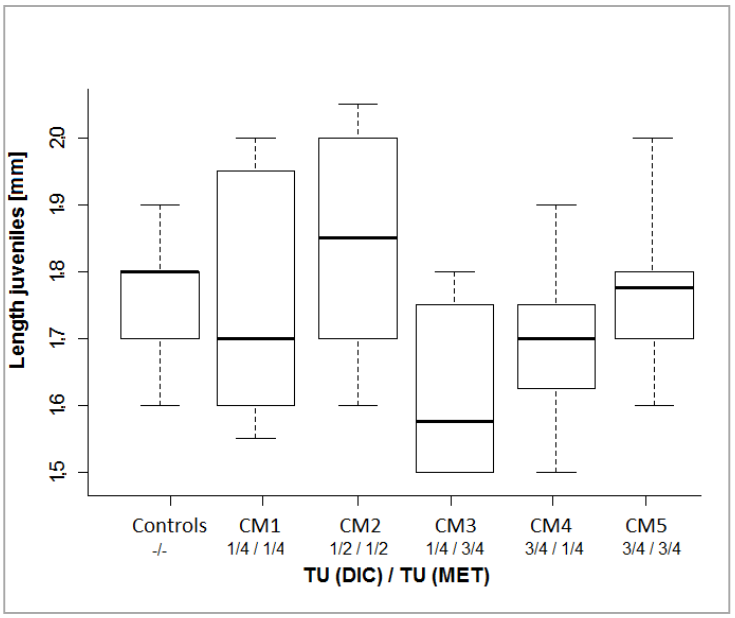
The number of eggs per egg-bearing female was calculated with four of eight replicates for each mixture concentration and three out of eight replicates for controls (Figure 74). For the number of eggs per egg-bearing female, the median in controls was 6.5, the minimum was 5.0 and the maximum was 9.0. In CM1 with ¼ TU (DIC) and ¼ TU (MET) and CM5 with ¾ TU (DIC) and ¾ TU (MET) highest median at 7.25 for the endpoint number of eggs per egg-bearing female was observed.

Figure 74: Number of eggs per egg-bearing female of *G. fossarum* at the end of exposure to the mixture of diclofenac and metoprolol. Medians are displayed along with 25 and 75% percentiles and minimum and maximum values. DIC – diclofenac; MET – metoprolol.



The length of juveniles in controls ranged between 1.6 mm and 1.9 mm with a median of 1.8 mm (Figure 75). In mixtures the medians were between 1.6 mm and 1.9 mm. Lowest median was detected in CM3 with ½ TU (DIC) and ½ TU (MET), highest was found in CM2 with ½ TU (DIC) and ½ TU (MET).

Figure 75: Length of juveniles of *G. fossarum* at the end of exposure to the mixture of diclofenac and metoprolol. Medians are displayed along with 25 and 75% percentiles and minimum and maximum values. DIC – diclofenac; MET – metoprolol.



2.2.3.5 Biochemical markers

The degree of lipid peroxidation tended to decrease with increasing toxic unit combinations (linear model, df=5/90, F=2.0240, p=0.08269), while the body mass remained unaffected (linear model, df=5/90, F=0.9253, p=0.4684). Overall, the obtained values for lipid peroxides were higher than the ones from the single substance exposure to diclofenac. Data are shown in Figure 76.

Stress proteins did show a slight tendency to decrease with increasing toxic unit mixtures. Especially the control animals showed variable levels of Hsp70. Data had to be transformed using the natural logarithm to fit the assumptions of parametric testing. However, the only significant difference could be found when comparing the experimental individuals to the t0-control (linear model, df=6/96, F=2.272, p=0.04291).

Figure 76: Lipid peroxides (top) and body mass (bottom) of *Gammarus fossarum* exposed to mixtures of diclofenac and metoprolol. Bold lines display the median values, boxes the 25% to 75% quantiles, whiskers the minimum and maximum values, open circles potential outliers. None of the treatments had an influence on the investigated responses.

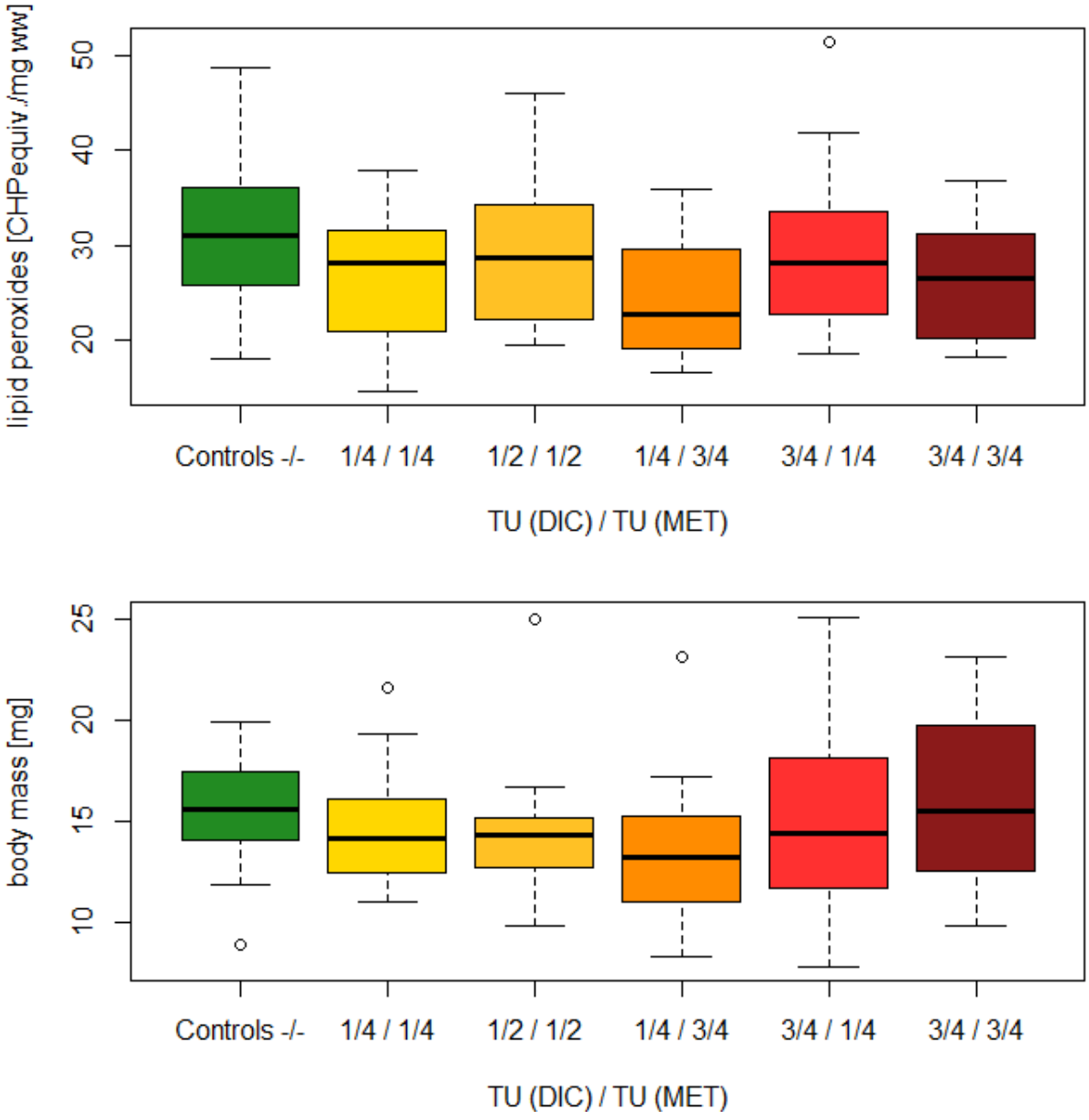
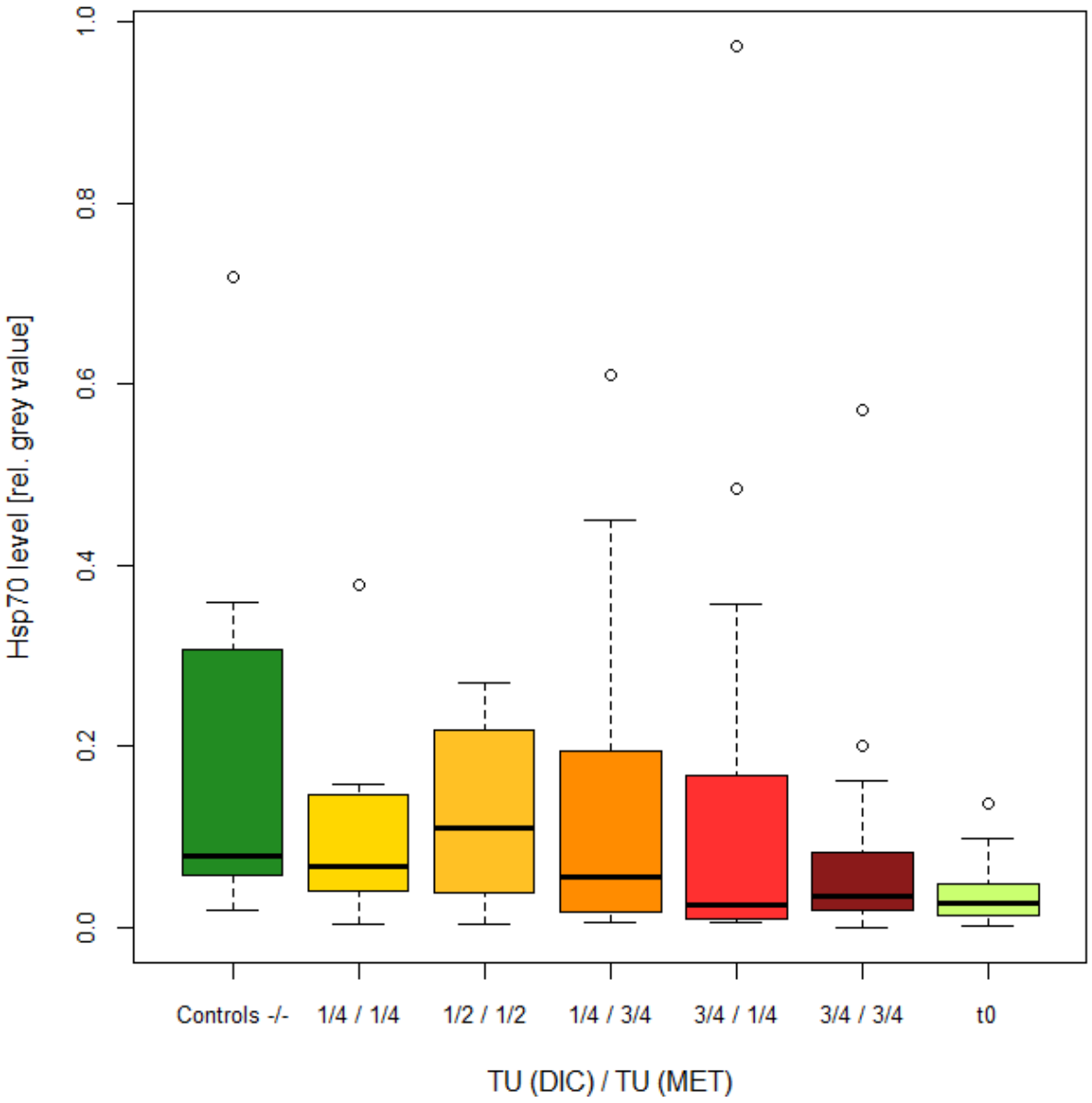


Figure 77: Stress protein levels of *G. fossarum* exposed to mixtures of Diclofenac and Metoprolol. Bold lines display the median values, boxes the 25% to 75% quantiles, whiskers the minimum and maximum values, open circles potential outliers. The only difference could be found compared to the t0-control (linear model, df=6/96, F=2.272, p=0.04291).



2.2.4 Discussion

2.2.4.1 Chemical analysis of metoprolol and diclofenac concentrations

The chemical analysis of exposure concentrations showed a higher dissipation of diclofenac compared to metoprolol. A similar process was found in the chronic toxicity tests described in Chapter 1.3.

The decline of diclofenac over 7 days was between 35 and 87% which is in the same range compared to the earlier batch experiment with a decline between 33 and 53%. The main reason is the photolytic degradation of diclofenac. The impact of light especially UV-light on the dissipation was demonstrated by experiments of P. Bartels, W. v. Tümpling, M. Schmitt -Jansen, J. Hartmann at the Centre for Environmental Research in Magdeburg. Half-live time of diclofenac, depending on season and light intensity were 2.5 - 14 h (Jansen et al.2006).

It was found that the degradation products of diclofenac are more toxic than diclofenac. However, this refers to experiments with algae. This was shown at a 48h toxicity test (endpoint inhibition of reproduction) with the green algae *Scenedesmus vacuolatus*. In future experiments, the photolytic degradation could be reduced by excluding the wavelengths from 300-340 nm (UV range) which degrade diclofenac fast (Jansen et al. 2006). This is outside of the photosynthetic active radiation of 400-700nm (Sadava et al. 2011). Thus, no adverse effects on the autotrophic part of the biofilm which is a food source for gammarids should arise. However, if the degradation products were artificially reduced it would no longer be equivalent to the natural system.

Metoprolol dissipation was much lower and decline was approximately 1% except in the samples where glass bottles were broken. This is again similar to the earlier batch experiments.

The differences in dissipation of diclofenac and metoprolol lead to a shift in the ratios of the mixture (Table 40). Thus, the ratio of the sample CM1 (day 35) had changed from 25% $LOEC_{DIC}$ - 25% $LOEC_{MET}$ to 8% $LOEC_{DIC}$ - 22% $LOEC_{MET}$.

Table 40: Nominal and measured percent of LOEC from the chemical analysis of diclofenac and metoprolol concentrations during 40 days of exposure; ME - medium exchange

Sampling time [d]	Sample*	Nominal percent of LOEC / Measured percent of LOEC	
		Diclofenac	Metoprolol
0	Control *	-	-
0	CM1 *	25/29	25/25
0	CM5 *	75/45	75/50
7 before ME	CM1	25/4	25/8
7 before ME	CM5	75/29	75/69
28 after ME	CM1	25/21	25/22
28 after ME	CM5	75/64	75/70
35 before ME	Control	-	-
35 before ME	CM1	25/8	25/22
35 before ME	CM5	75/33	75/68

* short thread bottles broke after freezing the sample. Decant of the samples with glass funnel and 2 ml of distilled water.

Since the half-live of diclofenac suggests a relatively rapid photolytic degradation it is likely that over a substantial period of the experiment, the real ratios in the mixture deviate from the nominal ratios. Therefore, it is not possible to make reliable statements about the synergistic effects based on this experimental setup.

2.2.4.2 Effects on mortality and reproduction

The median mortality in the controls was 42.5% and is comparable to mortality in earlier conducted chronic single substance tests (Chapter 1.3). However, this value excludes the replicates C6 and C8 because of their outstanding high mortality (C6 = 100%, C8 = 75%).The reason for the high mortality remains unexplained. However, as already described in chapter 1.3, mortality can be regarded as acceptable compared to literature data with mortality in controls up to 70% (Schirling et al. 2006, Dietrich et al. 2010, Oskarsson et al. 2012). Therefore, the test can be considered as valid.

Nominal concentrations of diclofenac and metoprolol in the mixtures CM2 (½TUDIC-½TUMET), CM3 (¼ TUDIC-¾ TUMET) and CM4 (¾ TUDIC - ¼ TUMET) corresponds with LOEC values from preliminary conducted single substances tests (see Chapter 1.3). Thus, significant differences to the control in mortality may occur, most likely for the mixture CM5 (¾ TUDIC-¾ TUMET). However, statistical analysis showed no significant differences between the control and exposure concentrations regarding any endpoint (Dunnett's Multiple t-Test). Therefore, an additive behaviour of diclofenac and metoprolol can be assumed rather than a synergetic.

In addition, natural fluctuations of the introduced gammarids within a mixture can affect the results. Thus, great differences regarding some endpoints were observed. For example, the mortality of CM4 (¼ TUMET-¾ TUDIC) was between 20 and 90% and of CM5 (¾TUDIC-¾ TUMET) it was between 20 and 70%.

Regarding the effect endpoint mortality the used medium could play an important role. On the one hand, the medium directly affects the organisms due to the amount of conductivity as well as indirectly through its composition of anions and cations. The used Borgmann medium (BORGMANN, 1996) contains no phosphorus compounds. Thus, an important compound for the growth of biofilm on the *Alnus glutinosa* leaf discs that serves *G. fossarum* as food was probably missing. Therefore, the growth of biofilm in the Borgmann medium is lower compared to natural environments. However, this assumption cannot be supported by the chronic single tests, because the SRP of diclofenac control with 0.78µg/L is below the SRP value of Mix control with1.27µg /l. The SRP of metoprolol-control lay with 1.71µg /L above. However, both controls of the individual substances showed a lower mortality.

However, the major reason for the missing effects is the high degradation of diclofenac (see above) resulting in lower exposure concentrations.

With regard to natural environments the importance of metabolites of the substances should be taken into account. The metabolites can reach concentrations higher than the primary form of the drug (Jansen et al. 2006). In the case of diclofenac a higher toxicity has already been demonstrated in experiments with algae. This could be a hint for an increase of the toxicity in animals e.g. Crustacea. In the present experiment, however, most of the transformation products were removed due to the weekly medium exchange. Under natural conditions, the effects of the used concentrations of substances could therefore be much higher on gammarids.

By the experiments no synergistic effects could be detected. One reason for this was the photolytic degradation of diclofenac by UV radiation. For subsequent experiments should be tried to exclude this radiation range.

2.2.4.3 Biochemical markers

None of the tested biochemical markers responded significantly to the mixtures. Body mass and lipid peroxides were on a slightly higher level than in the single substance exposure of diclofenac. Since the experiments were conducted in two different years, such differences are not surprising and are attributed to annual fluctuations and possible age differences between the individuals. The same interesting trend of decreasing amounts of lipid peroxides with increasing diclofenac concentrations, which was visible for diclofenac alone, could also be observed in the mixture. Furthermore, in this experiment there were no differences in body mass between the treatments, which could have affected the evaluation. Yet, the tendency was not significant in any of the cases. The parameters body mass and lipid peroxidation were not investigated for metoprolol in the single substance exposure, so a direct comparison is not possible in this case. This circumstance is unfortunate, since the biggest difference exists between the control and the combination of ¼ diclofenac and ¾ metoprolol. However, the differences found in lipid peroxidation seem comparably small, considering the variances. As stated before, future approaches could test pools of animals – reducing the dilution factor and therefore the variability. In our study, we wanted to produce data allowing comparability of experiments, so the procedure was unaltered.

There was a visible difference in Hsp70-level between the control and the t0-animals. This is likely due to the different age and reproductive state, so a direct comparison between these groups cannot be seen as unequivocal evidence for increased stress under laboratory conditions. In contrast to the single substance exposures, a trend of decreasing levels of stress proteins, especially at the highest combination, was observed. Considering the generally high variance, the low levels in absolute, and the lack of a concentration-dependent response it is unlikely that this trend is biologically meaningful.

In summary, the biochemical analyses did not show evidence for synergistic effects between diclofenac and metoprolol. Stress proteins remained on a low level, as observe for the single substances, and lipid peroxides showed the same decreasing trend as observed for diclofenac alone.

3 WP4 Toxicity of pharmaceuticals in mesocosms (Part III)

3.1 Studies with *Gammarus fossarum*

3.1.1 Introduction

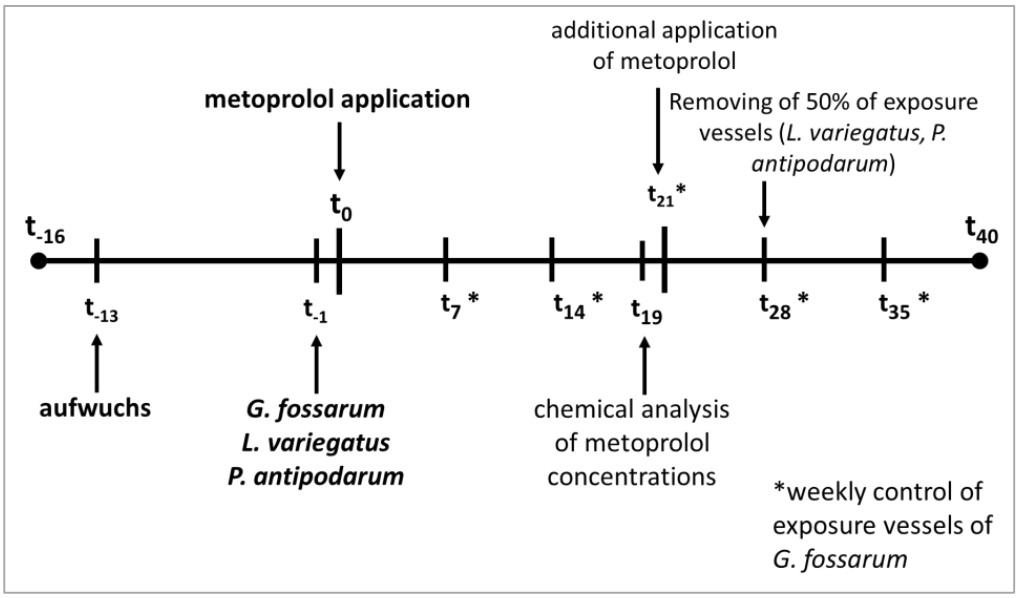
In aquatic environments various biotic and abiotic stressors have an impact on organisms, among others current velocity, inter- and intraspecific competition for food resources and habitat. Moreover, due to their ubiquitous use a tremendous amount of pharmaceuticals can be detected in surface waters (UBA, 2014). Consequently, adverse side effects on aquatic organisms occur which are not yet fully investigated. In order to study the effects of metoprolol on different aquatic organisms inhabiting lotic environments an artificial indoor stream (AIS) experiment in a greenhouse at the Institute of Hydrobiology at the Technical University of Dresden was performed (Figure 78). The results of this experiment under controlled environmental conditions provide crucial information about the impact of environmentally relevant pharmaceuticals on lotic organisms.

Figure 78: left: View on the 5 artificial indoor streams (AIS) at the Institute of Hydrobiology at the TU Dresden. right: Closed up view on complete assembly of AIS. Viewing direction in both pictures is upstream.

The 40 day exposure of *Gammarus fossarum* (Crustacea), *Lumbriculus variegatus* (Oligochaeta) *and Potamopyrgus antipodarum* (Mollusca) to metoprolol was started on June 17th, 2015 and ended on July 27th, 2015. The substance was applied as metoprolol tartrate (CAS 56392-17-7, Sigma Aldrich). The test organisms were exposed to metoprolol concentrations of 0 (control), 0.47; 1.9; 7.5; and 30 mg/L (spacing factor 4). The respective concentrations were randomly distributed to the five AIS.

The exposure experiment comprised two periods, a pre-application period for establishing aufwuchs (t_{-16}-t_{-1}) and an exposure period where the organisms were exposed to metoprolol (t_0-t_{40}) (Figure 79). Detailed information on the respective periods are provided below.

Figure 79: Schedule of the exposure experiments with metoprolol in artificial indoor streams. t_{-16}-t_{-1} pre-application period, t_0-t_{40} exposure period.



3.1.2 Materials and Methods

Nominal concentrations of metoprolol used for the chronic exposure experiment are shown in Table 41 and are derived from LOEC value from chronic test in lab scale (LOEC= 15 mg/L) (see Chapter 1.3). The given concentrations refer to the active compound of the pharmaceutical metoprolol.

Table 41: Nominal concentrations for the chronic exposure experiment to metoprolol in streams (spacing factor 4).

Concentration	control	1	2	3	4
Metoprolol [mg/L]	0	0.47	1.9	7.5	30

Stock solutions were prepared for each exposure concentration in 1 L Borgmann Medium (BORGMANN, 1996, LO4-S AND E +H) in Erlenmeyer flasks. Subsequently, for 1 minute 100 mL of the respective stock solutions were carefully and slowly poured in the respective streams at the downstream water reservoir (Jungmann et al. 2001). After a break of 2 minutes once again 100 mL of the stock solutions were poured into the stream. This procedure was repeated until 1 L of the respective stock solutions was used up.

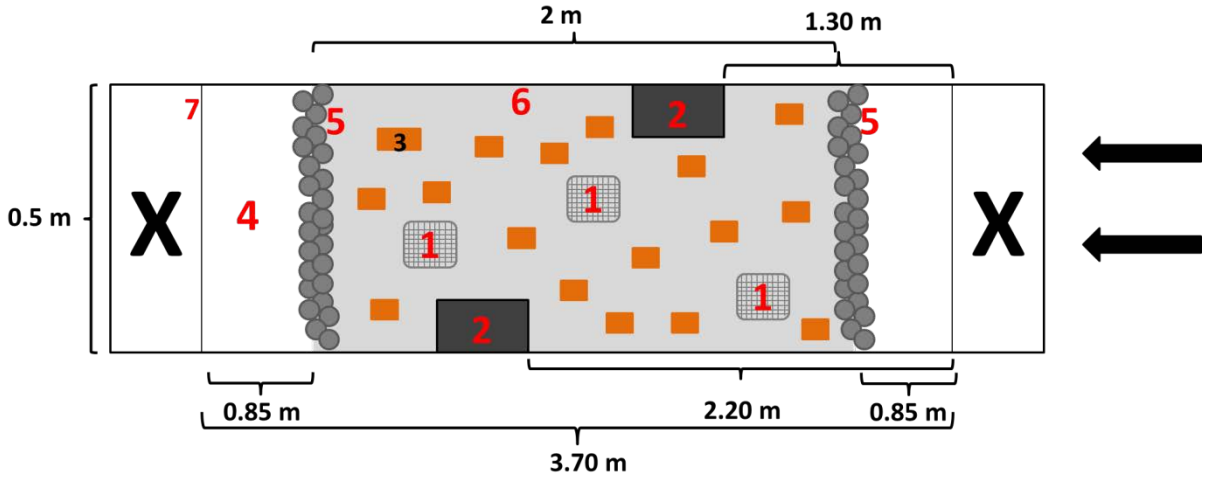
3.1.2.1 Experimental design

The AIS-system consists of 5 equal stainless steel streams in a greenhouse. Dimensions of each stream are as followed: total length of 420 cm, width of 50 cm and height of 20 cm. The used flow path of the stream has a length of 370 cm. A water reservoir exists at each end of the streams (Figure 80, X). A fine mesh sieve (mesh size: 250µm; Figure 80, #7) separates the flow path from the reservoir to prevent loss of organisms. The current velocity in the middle of the water column was adjusted to 0.15 m/s and the temperature was maintained at 15 ± 1 °C. Further technical details of the AIS-system are described by Jungmann et al. (2001).

A sketch of the complete assembly of the streams is depicted in Figure 80. In order to provide a natural habitat for the test organisms 2 m of each stream was covered with two layers of washed gravel (Figure 80, #6). The bottom layer (height: 1 cm) consisted of fine sized gravel (grain size: 0.5-1 cm) and the upper layer (height: 1 cm) consisted of median gravel (grain size: 1.5-2 cm). In addition, fist sized stones (3-5 cm) were randomly placed on the sediment. At the beginning and the end of the sediment

stretch in the respective stream a barrier of larger stones (3-5 cm) was built (Figure 80, #5). Two stainless steel boxes were placed at the longitudinal edges in the streams in order to establish areas with reduced flow (Figure 80, #2).

Figure 80: Sketch of AIS assembly (not to scale). 1: feeding cages with 5-6 conditioned leaves of *A. glutinosa*; 2: stainless steel boxes to establish areas with reduced flow; 3: 15 unglazed ceramic tiles for aufwuchs biomass analysis; 4: space for exposure vessels for the organisms(for detailed arrangement see Figure 82); 5: barrier of larger stones; 6: stream bed consisting of 2 different sized washed gravel; 7: fine mesh sieve separating flow path and water reservoirs; X: water reservoir; arrows indicate flow direction.



3.1.2.2 Pre-application period

The streams were filled with activated carbon-filtered tap water (450 L per stream) 16 days before the application of metoprolol. Additionally, micronutrients and salts were added according to BORGMANN (1996; LO4-S AND E +H). For the growth of aufwuchs in the respective streams, phosphorous (as Na_2HPO_4 * $2H_2O$; in total 80 µg/L; Merck) was added 3 times and silicate (as Na-Meta-Si-5-hydrate; in total 1 mg/L; Merck) twice according to AIS experiments of Rybicki (2014).

Furthermore, at t_{-13} aufwuchs was established in the streams in order to provide a food source for organisms. The aufwuchs was obtained from the Tännichtgrundbach (Chapter 1.3) by scraping aufwuchs with a merchantable hand brush from stones into a plastic bowl with stream water (Figure 81, left). To remove macroinvertebrates and larger particles aufwuchs suspension was filtered through a 250 µm mesh into 1 L HDPE-bottles (Figure 81, right). Subsequently, the aufwuchs suspension was transported to the laboratory, where it was pooled in a 3 L-Erlenmeyer flask. In order to determine the initial particulate organic carbon (POC) a subsample of 1 mL from the pooled aufwuchs suspension was filtered over preashed glass fibre filter (500 °C >45 min, MGF, diameter: 25 mm, Munktell) (for detailed information on the procedure see Rybicki, 2014). A total amount of 600 mL of aufwuchs suspension was then distributed in each stream with a pipette.

In order to provide an equal development of the same algae communities in all AIS a water volume of 6 L was taken out of each stream mixed together in a large plastic bowl and subsequently 6 L of water were poured back in each stream. This was done daily until application of metoprolol to streams.

Figure 81: left: stones and hand brush used for obtaining aufwuchs; right: 1 L HPLE bottle used for transport aufwuchs suspension

To analyze the aufwuchs biomass 15 unglazed ceramic tiles (6x4x1 cm LxBxH) were evenly distributed on the sediment at the top, in the center and on the bottom of the used stream bed (Figure 80, #3). Beside aufwuchs as additional food source 5-6 conditioned leaves of *A. glutinosa* were offered in three feeding cages, respectively (Figure 80, #1). Furthermore a suspension of grounded leaves of *A. glutinosa* was added to the areas with reduced current velocity of each stream (0.8 g dry mass of leaves, suspended in 20 mL Borgmann-Medium).

3.1.2.3 Exposure period

The employed test species were *Gammarus fossarum*, *Lumbriculus variegatus* and *Potamopyrgus antipodarum*.

Organisms were introduced to the AIS one day (t_{-1}) before application of metoprolol (detailed information on stock solutions see above) for adaptation to the test system. In each stream a total number of 165 gammarids, 50 individuals of *P. antipodarum* and 200 individuals of *L. variegatus* permitted to move unhampered in the streams were exposed to metoprolol.

Furthermore, six exposure vessels with 10 individuals of *L. variegatus* were introduced to every stream (for detailed information see Brust et al. 2001). The exposure vessels were composed of a glass dish (diameter 6 cm, height 3.5 cm) surrounded by a steel mesh (mesh size: 0.1 mm) filled with sediment from the AIS (height: 2cm) and were added to the AIS (Figure 82, right). As additional food source ground leaves of *A. glutinosa* in a suspension with Borgmann medium was added (0.13 g dry weight suspended in 10 mL Borgmann medium per vessel). For *P. antipodarum* six enclosures (merchantable metal egg-shaped tea infusors) with 20 individuals, respectively, were introduced to the five streams (Figure 82Figure 82, left). The enclosures were fixed to a beam with a nylon thread so that they remain in place. As food source small carrot pieces (0.5x0.5x0.5 cm, in total about 1.6 g) were added. Food was renewed if necessary.

Individuals of *G. fossarum* were sampled in Tännichtgrundbach (see Chapter 1.3) at two days (June 10th, 15th; 2015). Sampling technique, food preparation and selection of individuals for the experimental set up were done as described above (Chapter 1.3). Until transfer into the streams and exposure to metoprolol the gammarids were kept in glass aquariums (height: 30 cm, length: 40 cm, width 25 cm; along with unglazed ceramic tiles and conditioned leaves of *A. glutinosa*) filled with Borgmann

medium. Aquariums were continuously aerated (aerating pump (WISA)) over a Pasteur pipette (glassware; 3 bubbles per sec.).

Figure 82**:** Left: Six enclosures for *P. antipodarum* fixed with a nylon thread to a beam at the bottom of the respective streams; right: arrangement of three exposure vessels for *G. fossarum* and six exposure vessels for *L. variegatus* at the bottom of the respective streams.

Additionally, three exposure vessels composed of a glass petri dish (diameter: 17 cm) surrounded by a steel mesh (mesh size: 0.5 mm) were added to the AIS (Figure 83; Figure 80, #4). In each exposure vessel 20 gammarids, along with 4 unglazed ceramic tiles, 3 crushed stones (3x5 cm) and 15 conditioned leaf discs (diameter: 28 mm) of *A. glutinosa* were introduced. If necessary, further conditioned leaf discs were added to the exposure vessels.

Exposure vessels were intended to provide important information on the survival and development of the gammarids. For this purpose, once a week the total number of individuals, mortality, number of egg-bearing females and number of juveniles as well as number of precopulae was recorded for each exposure vessel. With these information, a better interpretation of the studied endpoints of the gammarids introduced directly to the stream is given.

Figure 83: Exposure vessels containing 20 individuals of *G. fossarum* along with 4 unglazed ceramic tiles, 3 crushed stones and 15 conditioned leaf discs of *A. glutinosa*

At the beginning of the experiment 80 individuals from the original population of gammarids were frozen in liquid nitrogen for analysis of stress proteins and lipid peroxides. Frozen samples were stored at -80 °C (Sanyo Ultra Low) and for analysis shipped on dry ice to the University of Tübingen.

Daily (t_0-t_{40}) at 11 o'clock drift of all organisms was recorded. For this purpose, individuals were counted and collected from the mesh sieve and the water reservoir at the outflow of each stream with a long glass pipette and then placed back in the rear part of the sediment stretch. Moreover, individuals were also counted beyond the sediment upstream. However, dead individuals were sorted out.

Furthermore, in each stream, physico-chemical parameters were measured three times a week (Monday, Wednesday, Friday) (pH: WTW pH3110, SenTix81 for control and pH paper: VWR Prolabo for exposure treatments; conductivity: WTW LF340, TetraCon325; temperature, oxygen concentration and saturation: WTW CellOx355, Oxi340i). To measure the parameters testing probes were put in the water in the middle of the respective streams.

Once a week (Wednesday), the exposure medium was sampled for chemical analysis of metoprolol concentrations (1.5 ml, Eppendorf Pipette). Samples were then deep frozen (Bosch, -22 °C) in HPLC glass bottles (1.5 ml short thread bottles, Fisher Scientific) until analysis at TZW Karlsruhe. Due to the high loss of metoprolol in the water phase an additional application of metoprolol was carried out after 21 days of the experiment based upon the chemical analysis of metoprolol concentrations at day 19. Stock solution of the respective metoprolol concentrations (Table 42) and application were conducted as at the beginning of the experiment.

Table 42: Additional application concentrations of metoprolol on day 21 of exposure.

Concentration	control	1	2	3	4
Metoprolol [mg/L]	0	0.37	1.59	6.4	11.9

Due to evaporation, the water volume of the respective streams decreased. In order to compensate the water loss, each stream was filled up weekly with purified water to the start water volume of 450 L. In addition, once a week (Wednesday), 0.5 L of stream water was sampled from each stream and filtered through a cellulose acetate filter (diameter: 0.45 µm, Sartorius Stedim Biotech) in order to analyze the

concentrations of nitrate, ammonium, and soluble reactive phosphorus (SRP). Filtration was carried out using a vacuum pump. Until further analytical processing, samples were stored in a freezer. Nitrate was analyzed using rapid test cuvettes (LCK 339, Hach Lange). Ammonium was determined according to DIN 38406-5 and SRP according to EN ISO 6878:2004 photometrically (HITACHI U-2000 Spectro-photometer).

For calculating the ammonia concentrations following equations were used (Emerson et al. 1975):

$$NH_3\left[\frac{mg}{l}\right] = \frac{0{,}94412 * NH_4 - N\left[\frac{mg}{l}\right]}{1 + 10^{pK_A - pH}} \quad (3)$$

with

$$pK_A = 0{,}0925 + \frac{2728{,}795}{t[°C] + 273{,}15} \quad (4)$$

For the temperature and the pH the values of the respective measurement dates and treatments, for NH_4^+ the medians of the values of the respective treatments were used. The temperature and pH values ranged from 14.7 to 15.5 °C and 7.9 to 8.1, respectively (for NH_4^+ values see Table 46).

At the end of the chronic exposure experiment each stream was sampled for individuals of *G. fossarum* (adults and juveniles), *L. variegatus* and *P. antipodarum* to record the required endpoints. Therefore, current velocity was reduced and feeding cages as well as the fist sized stones were removed. Then, the whole sediment was taken out gradually with a plastic shovel and transferred to plastic bowls to sort out the organisms with spring steel forceps or a Pasteur pipette (Figure 84). Detailed information about *L. variegatus* and *P. antipodarum* are provided below in this chapter.

Figure 84: Left: Removing sediment from AIS with plastic shovel into a plastic bowl. Right: Sorting out organisms with spring steel forceps.

While sorting out survived gammarids (adults and juveniles) and number of precopulae were counted in all streams. For analysis of stress proteins and lipid peroxides 40 (conc. 0 mg/L), 44 (conc. 0.47 mg/L), 50 (conc. 1.9 mg/L), 35 (conc. 7.5 mg/L) and 40 (conc. 30 mg/L) individuals of *G. fossarum* were frozen in liquid nitrogen in regular temporal intervals during sorting out. Samples were stored

at -80 °C (Sanyo Ultra Low) and shipped on dry ice to the University of Tübingen for analysis. For investigation of reproductive endpoints, 61 (conc. 0 mg/L), 60 (conc. 0.47 mg/L), 79 (conc. 1.9 mg/L), 77 (conc. 7.5 mg/L) and 65 (conc. 30 mg/L) individuals of *G. fossarum* from the respective streams were fixed in formaldehyde (Merck, 37%) in order to determine number of egg-bearing females as well as the number of eggs per egg-bearing female and the fecundity index. With a stereomicroscope (Thalheim Spezial Optik) number and size of egg-bearing females and number of eggs per egg-bearing female were determined. Moreover, number of juveniles as well as size of juveniles was recorded. In the exposure vessels the same endpoints for mortality and reproduction were determined after fixation in formaldehyde (Merck, 37%).

In the AIS experiments formal statistical testing was limited due to the lack of true replication of the artificial streams. Hence, significant differences were deduced without statistical support. However, statistic evaluation of the exposure vessels (further on referred to as enclosures) in the respective streams was conducted using the statistical software R (version 3.1.1). In order to detect significant differences in the enclosures respective data were analyzed using ANOVA. For determination of NOEC and LOEC values in the AIS Williams-test was applied, using ToxRat software (Version 2.10, ToxRat Solutions GmbH). The significance level for the above mentioned tests was set to α=0.05.

3.1.3 Results

3.1.3.1 Chemical analysis of metoprolol concentrations

At the beginning of the exposure experiment, after 19 days and after 40 days of exposure, the metoprolol concentrations in the control were below the LOD (0.002 mg/L).

At the beginning of the experiment determined concentrations in the exposure treatments were in the range of 80 to 83% of the nominal concentrations (Table 43). After 19 days, metoprolol concentrations declined by 75, 81 and 82% in the first to third lowest concentrations. However, in the highest concentration only 28% of the effective concentration dissipated from the water. In order to reach initial concentrations in the respective streams an additional application of metoprolol was carried out (see above, Table 42). After the additional application of metoprolol at day 21 the determined concentrations in the exposure treatments 0.47, 1.9, 7.5 and 30 mg/L were 94, 88, 85 and 90% of the nominal concentrations, respectively (Table 43). Thus, determined concentrations nearly correspond with respective nominal metoprolol concentrations.

At the end of the experiment, for the lowest metoprolol concentration 30% of the nominal concentration was detected. Remaining metoprolol concentrations at day 40 were highest for the nominal concentration of 30 mg/L with 63% and lowest for 1.9 (22%) and 7.5 mg/L (23%). Thus, the relative decline of metoprolol concentrations showed the same trends compared to the period after the first application until day 19. However, absolute numbers differ between day 19 and the end of the experiment.

Table 43: Analysis of metoprolol concentrations during the 40 day exposure experiment in AIS.

Sampling time [d]	Nominal concentration [mg/L]	Determined concentration [mg/L]	Recovery rate [%]	Decline corresponding to determined concentration [%]
0	0.00	0.00		
	0.47	0.39	83	
	1.90	1.50	80	
	7.50	6.10	81	
	30.00	25.00	83	
19	0.00	< 0.002		

Sampling time [d]	Nominal concentration [mg/L]	Determined concentration [mg/L]	Recovery rate [%]	Decline corresponding to determined concentration [%]
	0.47	0.096	20	75
	1.90	0.29	15	81
	7.50	1.10	15	82
	30.00	18.10	60	28
At day 21: Additional application of metoprolol in order to reach initial concentrations				
21	0.00	< 0.002		
	0.47	0.44	94	
	1.90	1.60	85	
	7.50	6.60	88	
	30.00	27.00	90	
40	0.00	< 0.002		
	0.47	0.14	30	68
	1.90	0.42	22	74
	7.50	1.70	23	74
	30.00	19.00	63	30

Determined concentrations did not remain within 80-120% of nominal and effective concentrations could be expressed relative to the geometric mean of the measured concentrations (OECD 2000). Thus, effective concentrations (EC_{geom}) were calculated by following equation:

$$EC_{geom}\left[\frac{mg}{L}\right] = \sqrt[4]{x_{t0} * x_{t19} * x_{t21} * x_{t40}} \qquad (5)$$

Where x_{t0} is the determined concentration in mg/L of the respective nominal concentrations at day 0, x_{t19} is the determined concentration in mg/L at day 19; x_{t21} at day 21 and x_{t40} at day 40 (see Table 43 for respective determined concentrations).

By means of equation (5) following effective concentrations were calculated: 0.22 (nominal 0.47 mg/L), 0.74 (nominal 1.9 mg/L), 2.95 (nominal 7.5 mg/L) and 21.95 mg/L (nominal 30 mg/L) metoprolol, respectively (Table 44).

Table 44: Comparison of nominal and effective concentrations of metoprolol of the exposure experiment in the AIS.

Metoprolol concentration [mg/L]	
nominal	effective
0.47	0.22
1.9	0.74
7.5	2.95
30	21.95

The following results refer to the respective effective concentrations and not to the nominal concentrations.

3.1.3.2 Physico-chemical parameters

In the control pH values were in the range between 7.9 and 8.5 with a median of 8.0. Measured pH values in the exposure treatments did not differ from control (Table 45). Conductivity ranged between 383 and 410 µS/cm in the control and exposures with medians between 388 and 402 µS/cm. The median values of oxygen concentrations varied between 10.1 and 10.3 mg/L in the control and exposure treatments, the lowest value was 9.9 mg/L in the control and the highest concentration and the highest value was 11 mg/L in the control. The oxygen saturation ranged between 99 and 108%, with medians between 101 and 103% in the control and exposure treatments.

Table 45: Overview of physico-chemical parameters recorded during long-term exposure with metoprolol in AIS. Medians are displayed along with minima and maxima (in parentheses) for every concentration.

		Metoprolol [mg/L]				
Parameter	**Unit**	**control**	**0.22**	**0.74**	**2.95**	**21.95**
pH		8.0 (7.9; 8.5)	7.9 (7.5; 8.5)	7.9 (7.5; 8.0)	7.9 (7.5; 8.0)	7.9 (7.5; 8.0)
Conductivity	µS/cm	389 (383; 393)	390 (384; 410)	388 (383; 394)	390 (385; 398)	402 (397; 405)
Temperature	°C	15.0 (14.6; 15.7)	14.9 (14,4; 15.4)	15.1 (14.4; 15.4)	15.1 (14.4; 15.5)	15.1 (14.5; 15.6)
Oxygen concentration	mg/L	10.2 (9.9; 11.0)	10.3 (10.0; 10.9)	10.3 (10.0; 10.7)	10.2 (10.0; 10.5)	10.1 (9.9; 10.5)
Oxygen saturation	%	102 (99; 110)	103 (100; 108)	102 (101; 106)	103 (99; 106)	101 (99; 104)

3.1.3.3 Nitrate, ammonium and soluble reactive phosphorus (SRP)

In the control, nitrate concentrations varied between 1.00 and 1.50 mg/L with a median of 1.20 mg/L. In the treatments with metoprolol medians ranged between 0.80 and 1.40 mg/L (Table 46).

The median ammonium concentration was lowest for the control with 26.5 µg/L and highest for the highest exposure concentration with 417.10 µg/L. The ammonium concentration increased with increasing metoprolol concentration (see Chapter 1.3). In the exposure treatments with the two lowest effective metoprolol concentrations (0.22 and 0.74 mg/L), there was only a minor increase in ammonium compared to the control. However, in comparison with exposure treatments of 0.74 and 2.95 mg/L metoprolol as well as 2.95 and 21.95 mg/L metoprolol ammonium concentrations increased by factor 3.2 and 2.7, respectively. Lowest calculated median ammonia concentration was found for control with 0.70 µg/L and a minimum of 0.12 and a maximum of 1.32 µg/L. Highest median ammonia value was detected for 21.95 mg/L metoprolol with 9.1 µg/L and a minimum of 7.76 and a maximum of 11.04 µg/L. In the two lowest exposure treatments median ammonia concentration was 1.00 µg/L. Median ammonia concentrations increased with increasing metoprolol concentration.

The median of SRP in the control was 0.90 µg/L with a minimum of 0.10 and a maximum of 1.74 µg/L. The medians in exposure treatments were in the range between 1.20 and 1.90 µg/L. Negative value indicates the limit of detection of SRP (2 µg/L).

Table 46: Concentrations of nitrate (NO_3-N), ammonium (NH_4^+), calculated ammonia (NH_3) and SRP recorded during long-term exposure with metoprolol in AIS. Medians are displayed along with minima and maxima (in parentheses) for all concentrations.

Metoprolol [mg/L]	NO_3-N [mg/L]	NH_4^+ [µg/L]	NH_3 [µg/L]	SRP [µg/L]
Control	1.2 (1.0; 1.5)	26.5 (5.4; 44.3)	0.7 (0.1; 1.3)	0.9 (0.1; 1.7)
0.22	1.1 (0.9; 1.9)	45.4 (22.8;55.9)	1.0 (0.6; 1.1)	1.5 (-0.1; 3.5)
0.74	0.8 (0.5; 2.0)	48.1 (38.9;68.0)	1.0 (0.8; 1.3)	1.2 (0.6; 3.1)
2.95	0.9 (0.8; 1.3)	156.0 (113.0; 221.9)	3.4 (2.3; 4.3)	1.4 (0.2; 2.8)
21.95	1.4 (1.1; 1.6)	417.1 (328.3; 563.6)	9.1 (7.8; 11.0)	1.9 (0.7; 3.6)

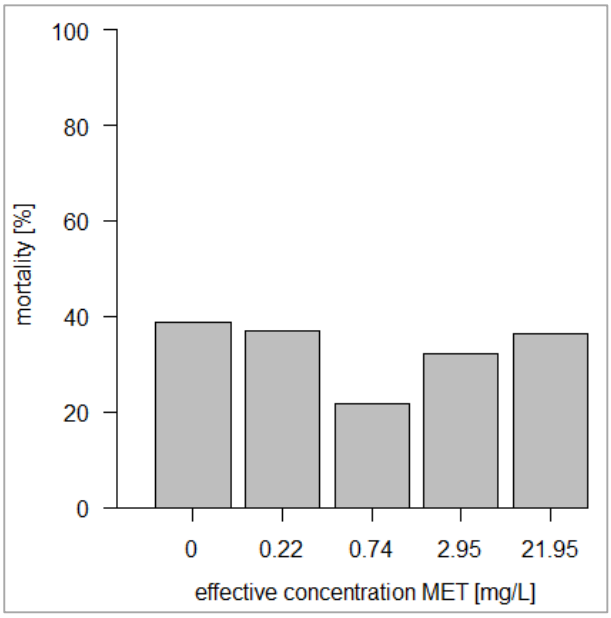
3.1.3.4 Effects on mortality and reproduction in AIS and enclosures

In the following chapter, results for the effects of metoprolol on mortality and reproduction of gammarids in the AIS and enclosures in the AIS are shown. AIS refers to the gammarids permitted to move unhampered in the streams and enclosures refer to the gammarids in the exposure vessels of the respective AIS. Due to the lack of true replication of the AIS experiments no formal statistical testing could be applied here. However, since three replicated enclosures were present in each AIS, concentration-response-relationships were detected using ANOVA if the necessary prerequisites (homogeneity of variances, normal distribution) were fulfilled.

Mortality

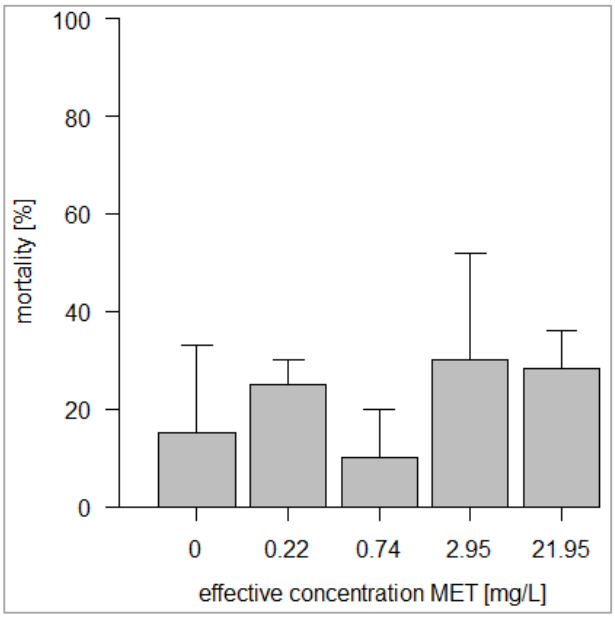
In the control treatment of the AIS, mortality reached 39% and thus showed the highest mortality in comparison to exposure treatments (Figure 85). At an effective concentration of 0.74 mg/L metoprolol mortality decreased to the lowest mortality with 22% and then increased to 36% at the highest effective metoprolol concentration (21.95 mg/L).

Figure 85: Mortality of *G. fossarum* at the end of the exposure experiment with metoprolol in AIS where 165 individuals of *G. fossarum* were introduced in each stream. n=101 for the control (0 mg/L) and n=104, n=129, n=112, n=105 for the respective increasing metoprolol concentrations.



The three enclosures of the control showed a mean mortality of 15±18% (Figure 86) which is rather low compared to the respective AIS (Figure 85). Lowest mortality (10±10%) was observed for the three enclosures in the second lowest effective concentration (0.74 mg/L) which was also detected in the respective AIS (Figure 85). In contrast to the AIS the three enclosures of the treatments with 2.95 and 21.95 mg/L metoprolol showed the highest mortality with 30±22 and 28±8%, respectively. Hence, the mortality showed neither in the AIS nor in comparison of the enclosures a concentration-response-relationship with increasing metoprolol concentration (for enclosures ANOVA, α=0.05).

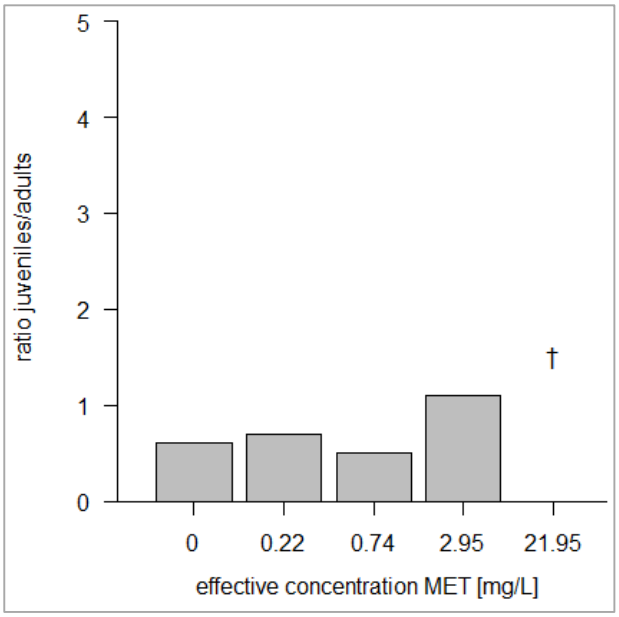
Figure 86: Mortality of *G. fossarum* in the 3 enclosures of the respective AIS at the end of the exposure experiment with metoprolol where 20 individuals of *G. fossarum* were introduced per enclosure. Means ± SD are depicted. n=51 for the control (0 mg/L) and n=45, n=54, n=42, n=43 for the respective increasing metoprolol concentrations.



Juvenile to adult ratio

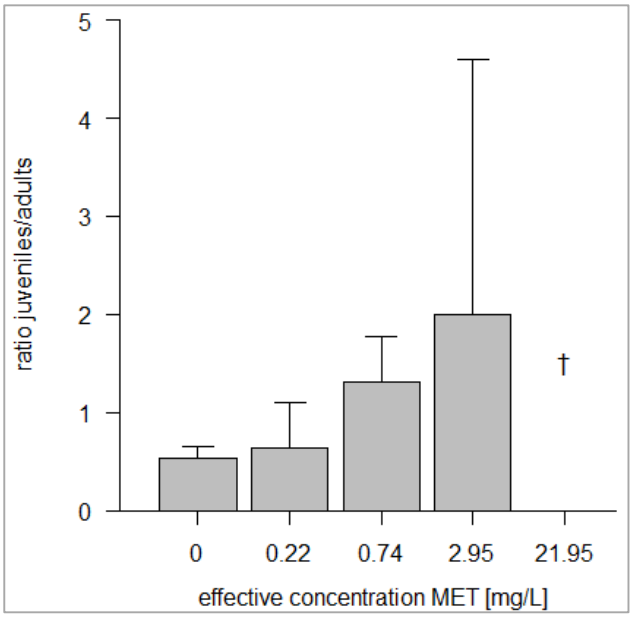
In the control of the AIS, the ratio of juveniles to adults was 0.6 (Figure 87). For exposure treatments of 0.22 and 0.74 mg/L metoprolol the quotient was found to be 0.7 and 0.5, respectively. Highest ratio of juveniles to adults was detected for the third highest metoprolol concentration (2.95 mg/L) with a quotient of 1.1. No juveniles were found in the highest exposure treatment (21.95 mg/L).

Figure 87: Ratio of juveniles to adults of *G. fossarum* at the end of the exposure experiment with metoprolol in AIS. Number of juveniles: n=60 for the control (0 mg/L) and n=73, n=68, n=120, n=0 for the respective increasing metoprolol concentrations. †: 100% mortality of juveniles.



In the three enclosures of the control the lowest ratio of juveniles to adults was found with 0.5±0.1 (Figure 88). The ratio of juveniles to adults in the enclosures increased with increasing metoprolol concentration thus showed a concentration-response relationship to metoprolol. As already detected in the respective AIS no juveniles were found in the highest concentration (21.95 mg/L). In the treatment with 2.95 mg/L metoprolol, highest ratio of juveniles to adults was found with 1.9±2.6 which is consistent with the results of the respective AIS (Figure 87).

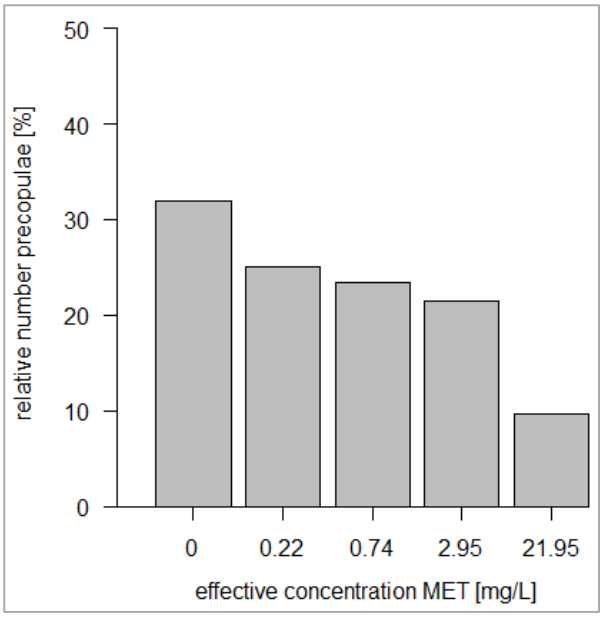
Figure 88**:** Ratio of juveniles to adults of *G. fossarum* in 3 enclosures of the respective AIS at the end of the exposure experiment with metoprolol. Means ± SD are depicted. Number of juveniles: n=27 for the control (0 mg/L) and n=28, n=70, n=90, n=0 for the respective increasing metoprolol concentrations. †: 100% mortality of juveniles.



Relative number of precopulae

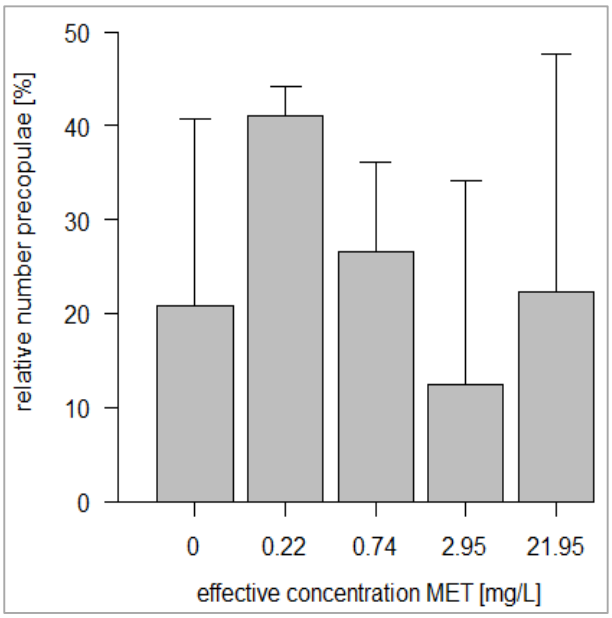
Highest relative number of precopulae was found in the control of the AIS with 32%. However, a concentration-dependent decrease of relative number of precopulae was apparent (Figure 89). For 0.22 mg/L, relative number of precopulae was found to be 25%, for 0.74 mg/L it was 23% and for 2.95 mg/L it was 21%. At an effective concentration of 21.95 mg/L metoprolol relative number of precopulae was 3-fold lower than in the control with 9%.

Figure 89: Relative number of precopulae of *G. fossarum* at the end of the exposure experiment with metoprolol in AIS. Number of precopulae pairs: n=16 for the control (0 mg/L) and n=13, n=15, n=12, n=5 for the respective increasing metoprolol concentrations.



In the three enclosures of the control, relative number of precopulae was 21±20% (Figure 90). Highest relative number of precopulae was found in the treatment 0.22 mg/L with 41±3% and lowest relative number was detected for 2.95 mg/L metoprolol with 12.5±21.7%. At effective concentrations of 0.74 and 21.95 mg/L metoprolol, relative number of precopulae was 27±10 and 22±25, respectively. In contrast to the AIS no decreasing trend regarding the relative number of precopulae with increasing metoprolol concentrations was observed. For the enclosures, ANOVA (α=0.05) showed no significant concentration-response-relationship for relative number of precopulae with increasing metoprolol concentration.

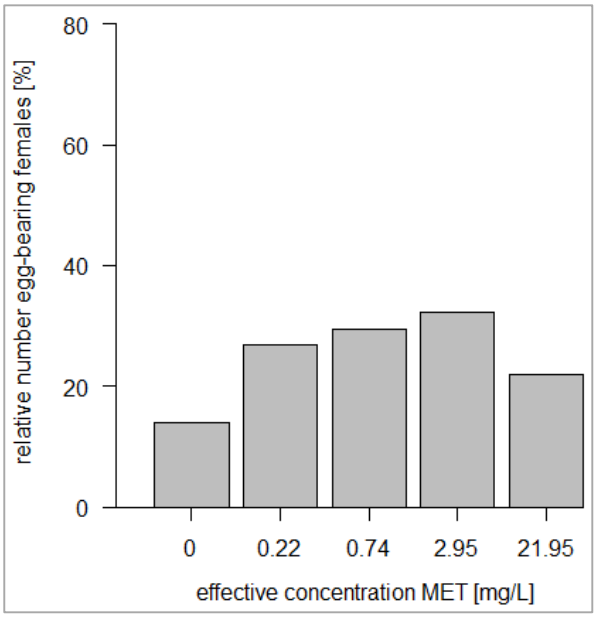
Figure 90: Relative number of precopulae of *G. fossarum* in 3 enclosures of the respective AIS at the end of the exposure experiment with metoprolol. Means ± SD are depicted. Number of precopulae pairs: n=6 for the control (0 mg/L) and n=9, n=7, n=3, n=5 for the respective increasing metoprolol concentrations.



Relative number of egg-bearing females

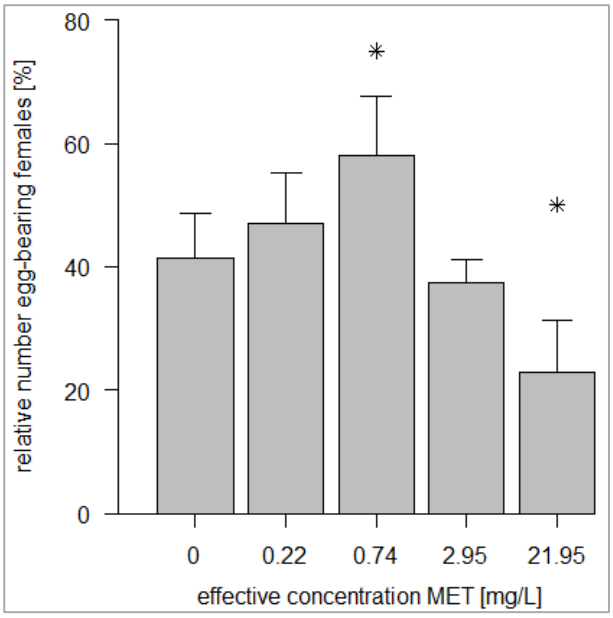
In the control of the AIS, relative number of egg-bearing females was the lowest with 14% compared to the exposure treatments (Figure 91). The relative number of egg-bearing females increased to the third highest metoprolol concentration (2.95 mg/L) with a value of 32%. For the two lowest metoprolol concentrations (0.22 and 0.74 mg/L) relative number of egg-bearing females was 27 and 30%, respectively. At a concentration of 21.95 mg/L metoprolol this number was found to be reduced to 22%.

Figure 91: Relative number of egg-bearing females of *G. fossarum* at the end of the exposure experiment with metoprolol in AIS. Number of egg-bearing females: n=14 for the control (0 mg/L) and n=28, n=38, n=36, n=23 for the respective increasing metoprolol concentrations.



As depicted in Figure 92, the relative number of egg-bearing females for the enclosures of the control was 41±7% which is 3-fold higher compared to the respective AIS (Figure 91). As shown in the results for the AIS, this number increased with increasing metoprolol concentration in the enclosures as well. However, relative number of egg-bearing females only increased to the second highest concentration (0.74 mg/L) where the highest relative number was detected with 58±10%. At 2.95 mg/L metoprolol, relative number of egg-bearing females was found to be decreased to 37±4%. Lowest relative number was observed in the highest metoprolol concentration (21.95 mg/L) with 23±8%. ANOVA (α=0.05) showed a significant increase at treatment 0.74 mg/L and a significant decrease at treatment 21.95 mg/L metoprolol.

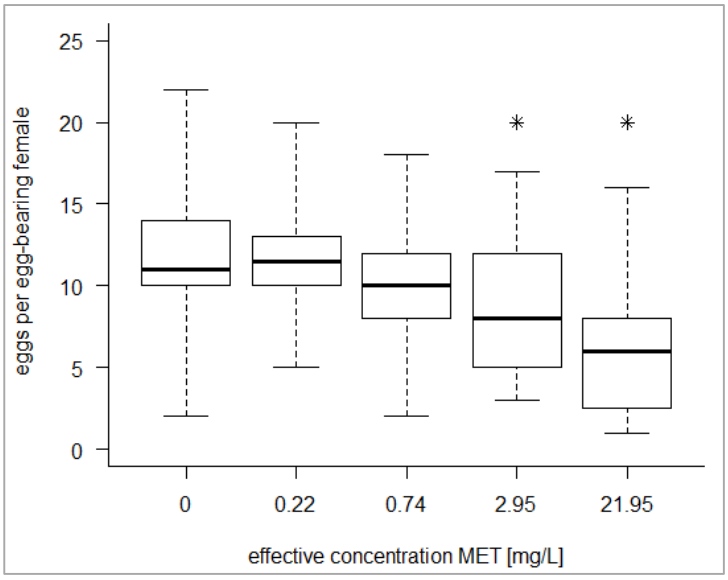
Figure 92**:** Relative number of egg-bearing females of *G. fossarum* in 3 enclosures of the respective AIS at the end of the exposure experiment with metoprolol. Means ± SD are depicted. Number of egg-bearing females: n=21 for the control (0 mg/L) and n=21, n=31, n=16, n=10 for the respective increasing metoprolol concentrations. * Significantly different from control (ANOVA, α=0.05).



Absolute number of eggs per egg-bearing female

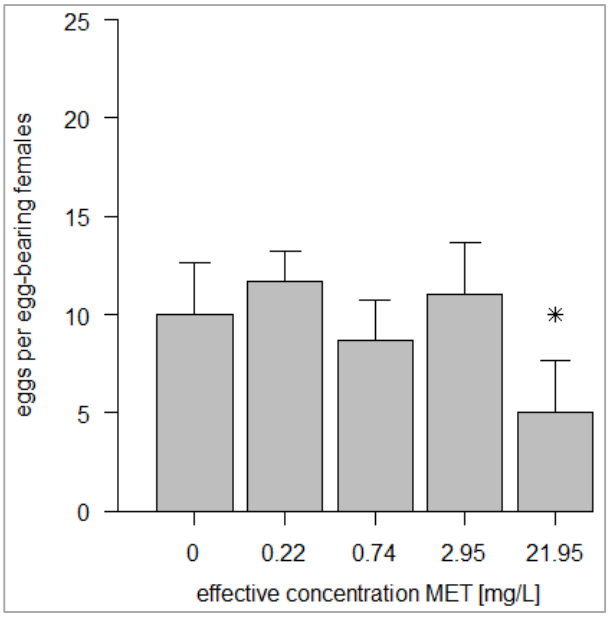
In the control of the AIS, absolute number of eggs per egg-bearing female ranged from 2 to 22, with a median of 11 (Figure 93). For 0.22 mg/L metoprolol, the median number of eggs was found to be 11.5, with a minimum of 5 and a maximum of 20. At 0.74 mg/L, the median was 10 eggs per female and at 2.95 mg/L it was 8. At the highest metoprolol concentration (21.95 mg/L), absolute number of eggs per egg-bearing female varied between 1 and 16, with a median of 6. The comparison of control and the two highest concentrations (2.95 and 21.95 mg/L) showed significant differences in number of eggs per egg-bearing females (Williams test, α=0.05). For this effect endpoint NOEC was 0.74 mg/L and LOEC was 2.95 mg/L according to Williams test (α=0.05).

Figure 93**:** Number of eggs per egg-bearing female of *G. fossarum* at the end of the exposure experiment with metoprolol in AIS. Medians are displayed along with 25 and 75% percentiles and minimum and maximum values. Total number of eggs (number of egg-bearing females): n=158 (14) for the control (0 mg/L) and n=329 (28), n=292 (38), n=255 (36), n=134 (23) for the respective increasing metoprolol concentrations. * Significantly different from control (Williams test, α=0.05).



The mean absolute number of eggs per egg-bearing female in the three enclosures of the control was 10±3 (Figure 94). The mean numbers of eggs per egg-bearing female for 0.22, 0.74 and 2.95 mg/L were in the range of the control with 12±2, 9±2 and 11±3, respectively. However, lowest mean number of eggs was detected for the highest exposure treatment (21.95 mg/L) with 5±3. For enclosures, ANOVA (α=0.05) showed a significant difference in number of eggs per egg-bearing female at the highest effective metoprolol concentration (21.95 mg/L).

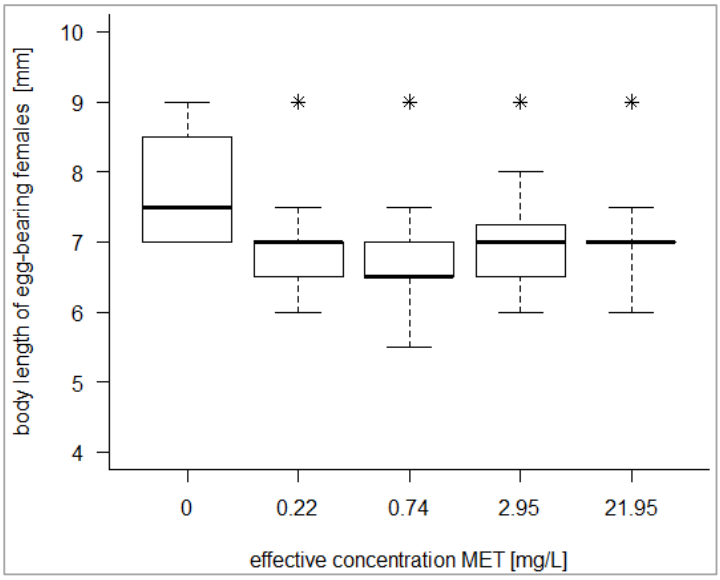
Figure 94: Number of eggs per egg-bearing female of *G. fossarum* in the 3 enclosures of the respective AIS at the end of the exposure experiment with metoprolol. Means ± SD are depicted. Total number of eggs (number of egg-bearing females): n=193 (21) for the control (0 mg/L) and n=215 (21), n=236 (31), n=156 (16), n=46 (10) for the respective increasing metoprolol concentrations. * Significantly different from control (ANOVA, α=0.05).



Body length of egg-bearing females

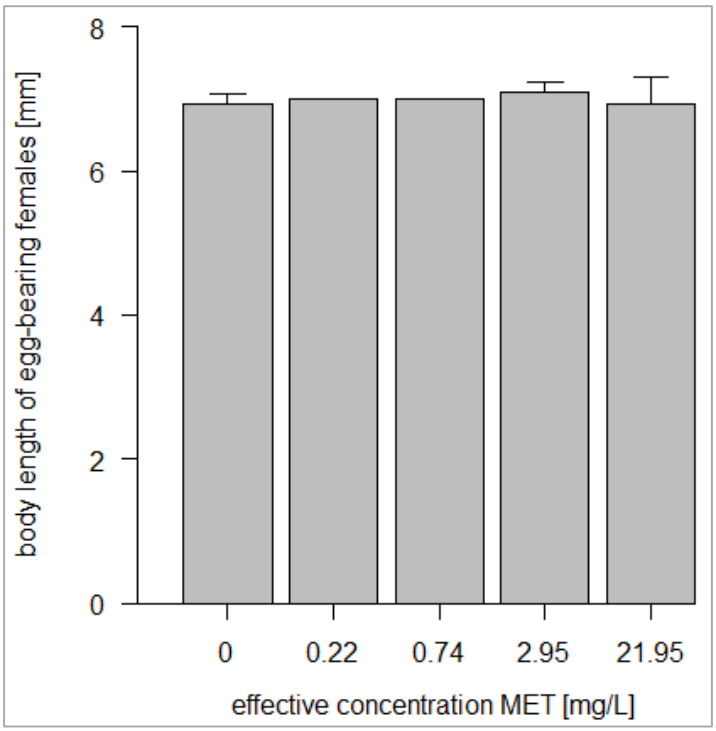
In the control of the AIS, the medium body length of egg-bearing females was 7.5 mm with a maximum of 9 and a minimum of 7 mm (Figure 95). For exposure treatments 0.22, 2.95 and 21.95 mg/L metoprolol, median body length was 7 mm with a minimum of 6 and a maximum of 7.5 and 8 mm, respectively. At 0.74 mg/L metoprolol, body length ranged between 5.5 and 7.5 mm with a median of 6.5 mm. Statistical evaluation showed significant differences of all four exposure treatments to the control (Williams test, α=0.05).

Figure 95: Body length of egg-bearing females of *G. fossarum* at the end of the exposure experiment with metoprolol in AIS. Medians are displayed along with 25 and 75% percentiles and minimum and maximum values. Number of egg-bearing females: n=14 for the control (0 mg/L) and n=28, n=37, n=35, n=22 for the respective increasing metoprolol concentrations. * Significantly different from control (Williams test, α=0.05).



For the three enclosures of the control mean body length of egg-bearing females was 6.9±0.1 mm (Figure 96). In contrast to the AIS, the mean body length in the exposure treatments did not significantly differ from control (ANOVA, α=0.05).

Figure 96: Body length of egg-bearing females of *G. fossarum* in the 3 enclosures of the respective AIS at the end of the exposure experiment with metoprolol. Means ± SD are depicted. Number of egg-bearing females: n=21 for the control (0 mg/L) and n=21, n=31, n=16, n=10 for the respective increasing metoprolol concentrations.



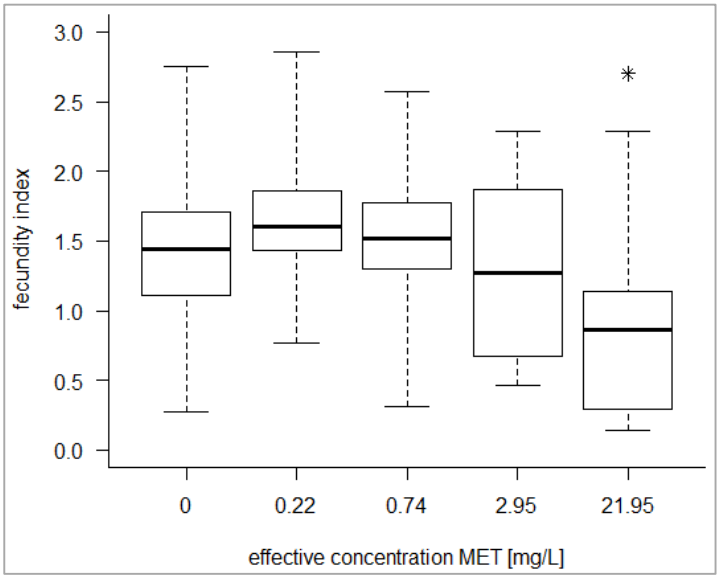
Fecundity index

To determine the fecundity in gammarids in each AIS and in the enclosures, egg-bearing females were used. Due to the fact that the fecundity is the quotient of the egg number and body length of egg-bearing females the following equation was used to calculate the fecundity index (FI) (Sundelin et al. 2008; Peschke et al. 2014):

$$\mathrm{FI} = \frac{\text{number of eggs}}{\text{female body length}} \qquad (6)$$

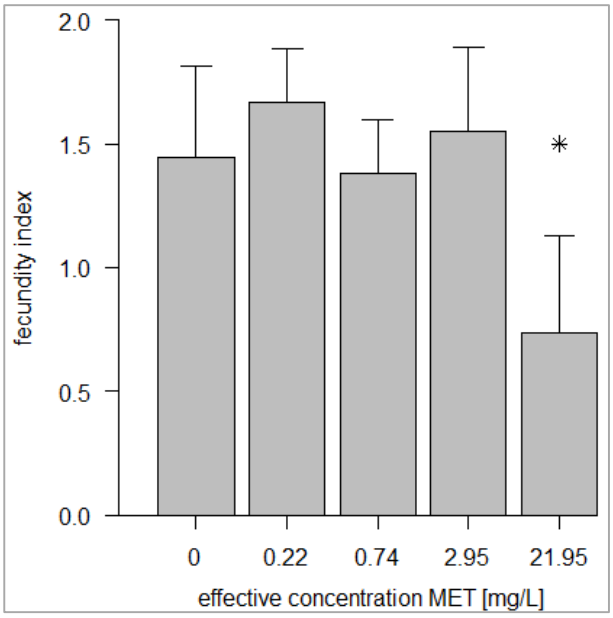
In Figure 97 the FI for the control and the exposure treatments in the AIS is depicted. Median FI in the control and in the exposure treatments 0.22, 0.74 and 2.95 mg/L were 1.4, 1.6, 1.5 and 1.3, respectively. Hence, the FIs are not statistically significant different from each other. However, the FI in the highest exposure treatment was significantly different from control with a median of 0.9 (Williams test, α=0.05).

Figure 97: Fecundity index of *G. fossarum* at the end of the exposure experiment with metoprolol in AIS. Medians are displayed along with 25 and 75% percentiles and minimum and maximum values. Total number of eggs (number of egg-bearing females): n=158 (14) for the control (0 mg/L) and n=329 (28), n=292 (38), n=255 (36), n=134 (23) for the respective increasing metoprolol concentrations. * Significantly different from control (Williams test, α=0.05).



The mean FI in the three enclosures of the control was 1.4±0.4 (Figure 98). A concentration-response trend of the FI was observed at the highest metoprolol concentration in which the FI was found to be significantly reduced (0.7±0.4) (ANOVA, α=0.05) just as in the AIS.

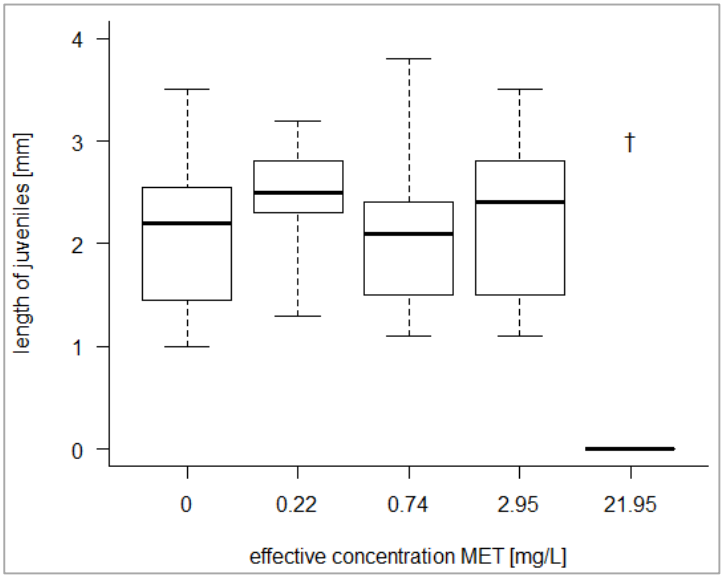
Figure 98: Fecundity index of *G. fossarum* in the 3 enclosures of the respective AIS at the end of the exposure experiment with metoprolol. Means ± SD are depicted. Total number of eggs (number of egg-bearing females): n=193 (21) for the control (0 mg/L) and n=215 (21), n=236 (31), n=156 (16), n=46 (10) for the respective increasing metoprolol concentrations. * Significantly different from control (ANOVA, α=0.05).



Juvenile body length

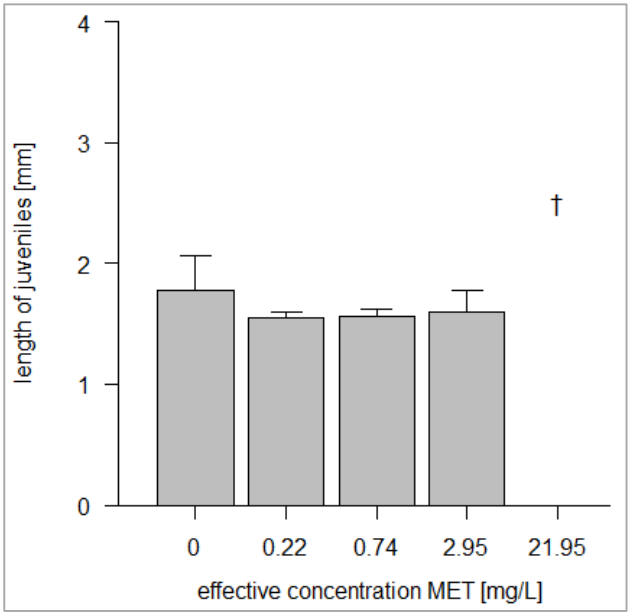
In the control of the AIS, the juvenile body length ranged between 1.0 and 3.5 mm with a median of 2.2 mm (Figure 99). The median juvenile length of the exposure treatments 0.22, 0.74 and 2.95 mg/L metoprolol were in the range of the median of the control with 2.5, 2.1 and 2.4 mm, respectively. However, no juveniles were found in the stream with the highest exposure treatment (21.95 mg/L).

Figure 99: Length of juveniles of *G. fossarum* at the end of the exposure experiment with metoprolol in AIS. Medians are displayed along with 25 and 75% percentiles and minimum and maximum values. n=60 for the control (0 mg/L) and n=73, n=68, n=120, n=0 for the respective increasing metoprolol concentrations. †: 100% mortality of juveniles.



The mean juvenile body length was 1.8±0.3 mm for the three enclosures of the control. For the exposure treatments with 0.22 and 0.74 mg/L metoprolol juvenile body length was 1.6±0.1 mm, respectively. For 2.95 mg/L it was 1.6±0.2 mm (Figure 100). The weekly monitoring of the exposure vessels showed that juveniles were present at the highest exposure treatment with metoprolol, but at day 40 no juveniles could be found. Thus, 100% mortality was detected in the highest exposure treatment. Compared to the treatments in the AIS (Figure 99) over all juvenile body length was smaller in the enclosures. For enclosures, ANOVA (α=0.05) showed no significant concentration-response-relationship of length of juveniles to metoprolol.

Figure 100: Length of juveniles of *G. fossarum* in the 3 enclosures of the respective AIS at the end of the exposure experiment with metoprolol. Means ± SD are depicted. n=27 for the control (0 mg/L) and n=28, n=70, n=90, n=0 for the respective increasing metoprolol concentrations. †: 100% mortality of juveniles.

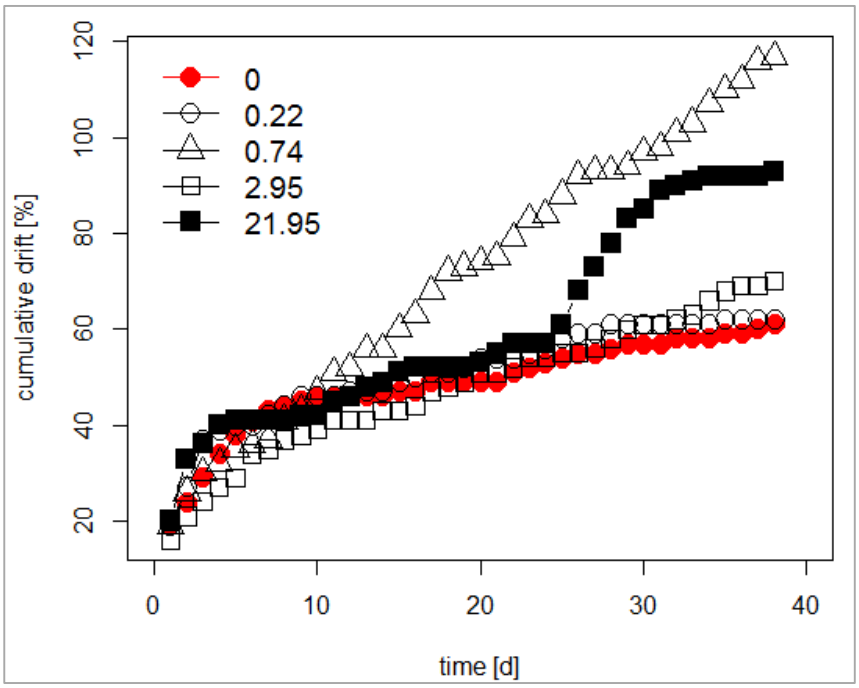


A summary of the above presented studied effect endpoints is provided in the Annexes. **Fehler! Verweisquelle konnte nicht gefunden werden.**

3.1.3.5 Drift

The cumulated drift activity of *G. fossarum* during the exposure period is depicted in Figure 101. The figure excludes the drift of day 0 because at this day gammarids were counted before metoprolol application. During the first week of exposure gammarids showed an increased drift activity in each AIS. In the control and in exposure treatment 0.22 mg/L metoprolol, drift activity was rather low after the first week and until the end of the experiment only a slight increase in drift activity was observed. Highest drift activity was detected for the exposure treatment with 0.74 mg/L metoprolol. Furthermore, there was a constant increase in drift activity until the end of the experiment in this exposure treatment. The drift activity of gammarids in the exposure treatment with 2.95 mg/L metoprolol was similar to the control. However, one week before the end of the experiment drift activity showed a slight increase in the second highest exposure treatment. The highest exposure treatment (21.95 mg/L) showed only a slight increase in drift activity after the first week, but at day 25 of the exposure experiment a strong increase in drift activity was observed. However, no other treatment showed this strong reaction in drift activity after the additional application. A clear concentration-response-relationship of the drift of gammarids to metoprolol did not become obvious.

Figure 101: Cumulative drift of *G. fossarum* during the exposure period. Cumulation started at t_1. Metoprolol treatments in mg/L (effective concentrations).



3.1.3.6 Biochemical markers

The highest degree of lipid peroxidation was measured for the "t0" individuals, which differed significantly from the other treatment groups (linear model, df=5/82, F=3.39, p=0.00781). Furthermore, the body mass was reduced for individuals from the metoprolol treated mesocosms. Yet, the only significant difference was found between the highest concentration and the control (linear model, df=5/84, F=3.168, p=0.0114). Data are shown in Figure 102.

Stress proteins were visibly reduced in all animals from the AIS compared to the original population (t0). There were no differences in relative Hsp70-level between the exposure groups (linear model, df=5/67, F=1.835, p=0.1178). Data are shown in Figure 103.

Figure 102: Lipid peroxides (top) and body mass (bottom) of *Gammarus fossarum* exposed to metoprolol in a mesocosm setup. “t0” depicts the animals at the start of the experiment. Bold lines display the median values, boxes the 25% to 75% quantiles, whiskers the minimum and maximum values, open circles potential outliers. Lipid peroxidation was higher in “t0” than in the experimental groups. Individuals from mesocosms with high metoprolol concentration were smaller than the control animals (linear model, df=5/84, F=3.168, p=0.0114).

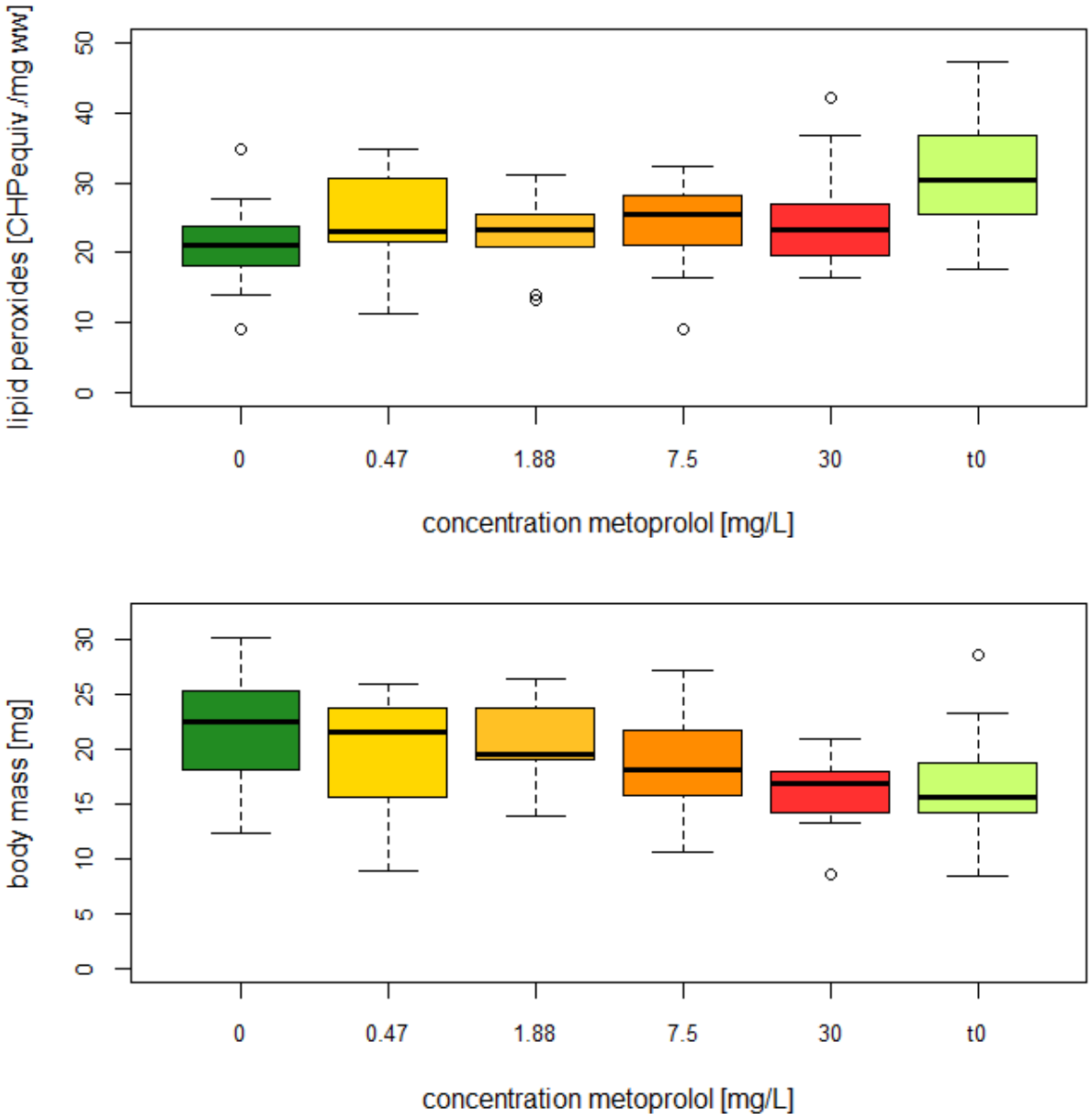
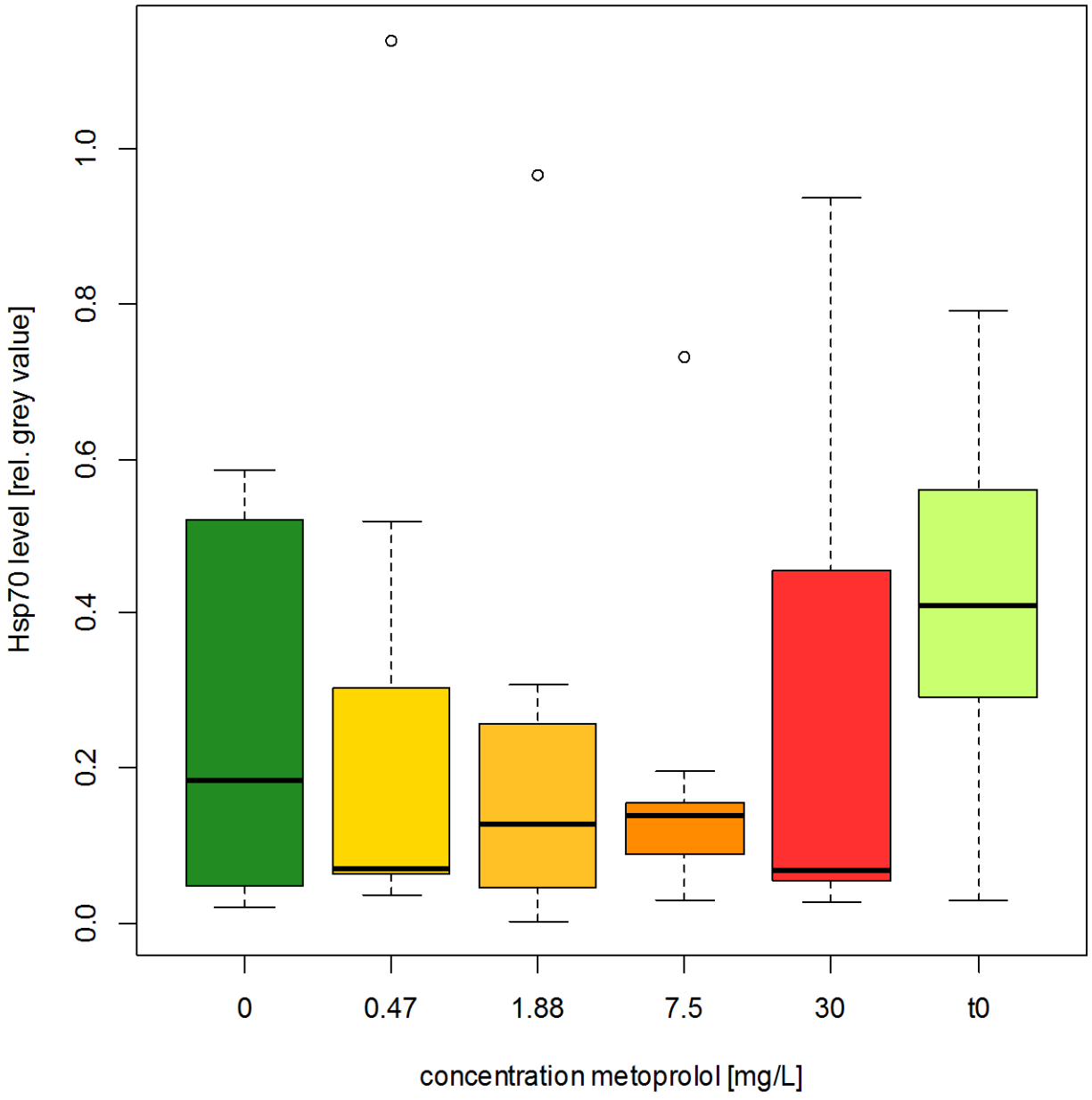


Figure 103: Stress protein levels of Gammarus fossarum exposed to metoprolol in an artificial indoor stream system. Bold lines display the median values, boxes the 25% to 75% quantiles, whiskers the minimum and maximum values, open circles potential outliers. There were no differences to be found between the experimental groups.



3.1.4 Discussion

3.1.4.1 Effects on mortality and reproduction in AIS and enclosures

In the control, the mortality of *G. fossarum* was 39% which must be regarded as acceptable compared to literature data with mortality up to 70% in the controls (Schirling et al. 2006, Dietrich et al. 2010, Oskarsson et al. 2012) which was also mentioned before in the discussion in chapter 1.3.

At the beginning of the experiment (t_0), the analytically determined metoprolol concentrations were up to 20% lower from nominal concentration which was expected due to adsorption to organic surfaces. However, the application procedure and applied concentrations are acceptable after OECD No.23 (2000). After 19 days, the dissipation from the water phase was high for the nominal concentra-

tions 0.47, 1.9 and 7.5 mg/L with 75, 81 and 82%, respectively. For the highest metoprolol concentration the dissipation from the water phase was relatively low (28%) compared to the other exposure treatments. After the additional application at day 21 of the exposure experiment the determined metoprolol concentrations were up to 15% lower compared to nominal concentrations (Table 43). At the end of the experiment (t_{40}) relative decline of metoprolol in the water phase was lower in the respective exposure treatments compared to day 19. Due to the OECD guideline No. 23 (2000) it is recommended to use calculated effective concentrations (Table 44) to describe the exposure adequately.

Regular determination of physico-chemical parameters pH, oxygen concentration, temperature and conductivity in the respective streams showed that potential effects on gammarid development by confounding factors are negligible since no critical values were detected at any time.

It was obvious that in the stream with the highest metoprolol concentration (effective: 21.95 mg/L) aufwuchs biomass was reduced compared to the other streams. It could be assumed that metoprolol at this high concentrations might alter the structure of biofilm community by negatively affecting certain bacteria and algae species in the biofilm. Bonnineau et al. (2010) assessed the acute toxicity of metoprolol on fluvial biofilms by using several biomarkers (photosynthetic efficiency, peptidase activity, live/dead ratio of bacteria, catalase activity) and found a 50% increase of bacterial mortality at the highest tested concentration of 523 µg/L metoprolol after 24h, and the determined No-Effect Concentration (NOEC) was 0.04 µg/L. However, the beta-blocker did neither significantly affect the algal photosynthetic process nor the peptidase and catalase activity. They argued that the toxicity might be caused by direct effects on the internal metabolism of the biofilm. An EC_{50} of 7.9 mg/L metoprolol for *Desmodesmus subspicatus* was found out by Cleuvers (2005) by conducting the algae growth inhibition test (Commission of the European Communities, 1993). Hence, although biofilm development was not investigated quantitatively, the observed effects could be expected when results from literature are considered.

Compared to the conducted batch experiment (see Chapter 1.3) an additional stress factor, the current velocity, was apparent in the AIS experiment. Thus, results are expected to differ from the studied effect concentrations in the batch experiment. On the other hand, a larger habitat and a different food quantity and quality in the streams can be considered as factors impacting effect strength and possibly be contradictory to the factor current velocity.

Comparing the mortality in the AIS and in the three enclosures of the respective AIS, no concentration-response-relationship was observed. In fact, at the second lowest effective concentration (0.74 mg/L) lowest mortality was found in AIS and enclosures, respectively (Figure 85, Figure 86). Hence, the additional stress factor current velocity did not enhance the effect strength of metoprolol on mortality compared to the enclosures. On the other hand the larger habitat in the AIS and consequently the higher food supply did also not show a positive effect as compensating factors.

In the AIS as well as in the enclosures of the highest exposure treatment (effective: 21.95 mg/l), no juveniles were found (Figure 87, Figure 88). It is most likely that not sufficient food was available in the stream, especially in form of fine particulate organic matter (FPOM). FPOM with bacteria and fungi living on its surfaces is a crucial food source for juveniles determining their survival and development. In the AIS, FPOM was provided among others by faeces of adult gammarids and/or by detached aufwuchs due to shearing forces of the flowing water. As mentioned above, aufwuchs biomass was lower in the AIS of the highest exposure treatment compared to the other streams. As a consequence it could be concluded that FPOM was reduced as well. Thus, a 100% mortality of juveniles could be expected in the stream with the highest metoprolol concentration. This could indicate an indirect effect of metoprolol on the survival of juveniles as well as the juvenile/adult ratio. This was also found in a 72 d experiment, in which effects of the herbicide terbutryn on *Lumbriculus variegatus* and aufwuchs were investigated in artificial indoor streams by Brust et al. (2001). They found that terbutryn inhibited the

growth of aufwuchs explained by lower detrital pool. Thus, the limited food sources inhibited the growth and reproduction of *L. variegatus* leading to an indirect effect of terbutryn on *L. variegatus*.

However, an increasing ratio of juveniles to adults with a clear concentration-response relationship was observed in the enclosures (Figure 87, Figure 88). In one of the three enclosures in the exposure treatment with effective concentration 2.95 mg/L a total number of 76 juveniles was determined whereas the other two enclosures just contained 3 and 11 juveniles, respectively. This is the reason for the high ratio of juveniles to adults in this exposure treatment. Hence, the clear concentration response relationship for the enclosures is based on the result of one solely enclosure. However, a smaller habitat with lower velocity in the enclosure might positively affect food availability for the juveniles. The FPOM pool in the enclosure is concentrated in a smaller area and additionally distribution by velocity is negligible. Hence, it can be assumed that food availability for juveniles in the enclosures is more favourable, except in the highest concentration with reduced biomass of biofilm.

With regard to the relative number of precopulae in the AIS, a negative concentration-response-relationship with increasing metoprolol concentration was observed (Figure 89). However, the difference between the control and the treatment with 2.95 mg/L metoprolol is only 10% and is therefore marginal. In comparison of the control with the highest exposure treatment the relative number of precopulae decreased by a factor of 3 and thus showed an effect of metoprolol. It might be possible that the current velocity and the larger habitat enhanced the effects of metoprolol as the chance that males find mature females to form a precopulae pair decreases with increasing size of the habitat. In addition, in the highest exposure treatment (effective: 21.95 mg/L) food supply was lower. Thus, gammarids might rather be focusing on foraging than on reproduction. Although the differences in the enclosures showed no concentration-response relationship the results support the hypothesis of habitat size and food quantity as a negative impact on precopulae formation in the AIS (Figure 90). However, the deviation in the respective enclosures is high and makes statistical analyses difficult.

In the AIS, there was an increase in relative number of egg-bearing females from the control to the exposure treatment 2.95 mg/L (Figure 91) by a factor of 2. However, a reduced number of egg-bearing females was observed in the highest concentration at 21.95 mg/L metoprolol. Hence, a concentration-response-relationship was found.

In the enclosures of the control the relative number of egg-bearing females was higher compared to the control in the AIS. Once again, it can be assumed that food availability for gammarids in the enclosures is more favourable. Furthermore in the enclosures the relative number of egg-bearing females increased and was statistically significantly higher in the treatment with 0.74 mg/L metoprolol and significantly lower at the highest exposure treatment (effective: 21.95 mg/l) (Figure 92). Hence, both treatments, AIS and enclosures, showed a similar pattern, and the significant reduction in the highest concentration can be explained by lower food availability as an indirect effect of metoprolol on biofilm development.

In the AIS number of eggs per egg-bearing female showed a concentration-response-relationship to metoprolol (Figure 94) because at the two highest exposure treatments the number of eggs per egg bearing female was significantly reduced (ANOVA, α=0.05). A significant reduction of the female body size was observed in the exposure treatments compared to the control, however, no difference was found between the treatments. The same pattern was observed in the enclosures, however, no differences in female body length between the controls and exposure treatment were detected.

As there was a significant difference in body length of egg-bearing females between the controls and exposure treatments in the AIS, however, not between the exposure treatments (Williams test, α=0.05), the reduced egg-number could be the result of the smaller size of the females. Therefore, the fecundity index (FI) was calculated (Pöckl 1993; Sundelin et al. 2008) and the egg number per female was normalised to female body length (see equation (6)). Regarding the FI, it was shown that in the

highest exposure treatment (21.95 mg/L) the fecundity was significantly lower in both treatments, enclosures and AIS. Therefore, for the highest exposure treatment, it is most likely that the reduced food source has an impact on fecundity and that the energy sources were reduced as well. Both food and energy resources are important factors for reproduction (Brust et al. 2001). Thus, in the stream with the highest metoprolol concentration reproduction was restricted by shortage of food and energy. Pöckl (1993) found that females strategically adapt to the differences in food supply by changing their reproductive effort. That means they either increase the fecundity to the detriment of egg size or increase egg size and juvenile survival by decreasing fecundity. In our experiment, the latter would apply. It can therefore be assumed that metoprolol has an indirect adverse effect on the fecundity of gammarids.

Fecundity is also influenced by e.g. strong temperature fluctuations and/or oxygen deficiency (Pöckl 1993; Sundelin et al. 2008; Peschke et al. 2014). Since no critical values for physico-chemical parameters were detected at any time (Table 45, Table 46) it can be excluded that the determined differences in number of eggs per egg-bearing female were related to these parameters.

Hence, in the enclosures a significant lower number of eggs per egg-bearing female were not due to a smaller size of the females in the highest exposure treatment. The reduced egg-number and resulting lower fecundity must therefore be a result of direct or indirect effects of metoprolol.

Summarizing, both in the AIS and the enclosures the FI was significantly lower in the highest exposure treatments (Williams test and ANOVA, α=0.05, respectively) and hence revealed a direct or indirect effect of metoprolol on reproduction of gammarids.

The length of juveniles in the exposure treatments did not significantly differ from the control, neither in the AIS nor in the enclosures (Figure 99, Figure 100). Thus, an effect of metoprolol on the length of juveniles can be excluded.

There were no signs of proteotoxic or oxidative stress potentials of metoprolol on gammarids in an artificial stream setting. The visible, yet not significant, difference in Hsp70 between exposed and t0 animals can be attributed to the different age and reproductive state of these animals. Overall, these results correspond well to the results obtained for metoprolol in the batch experiment, where alterations of stress proteins and lipid peroxides could be observed for concentrations even higher than the ones used in the AIS.

3.1.4.2 Drift

During the exposure experiment, it was investigated whether metoprolol initiates higher drift activity in *G. fossarum.* Results showed no clear concentration-response-relationship of drift to metoprolol exposure (Figure 101). During the first week of exposure gammarids showed an increased drift activity in each AIS what might indicate an acclimatization phase to the new environment. Notably, for the exposure treatment with 0.74 mg/L metoprolol highest drift activity was observed. Reasons for this remain unknown. In the highest exposure treatment (21.95 mg/L) a strong increase in drift activity was observed at day 25 of the exposure experiment. This was probably a delayed reaction on the additional application of metoprolol at day 21. However, no other treatment showed this strong reaction in drift activity after the additional application.

Organismic drift can be differentiated in 'behavioural drift' which is triggered by e.g. light intensity or predation and 'catastrophic drift' which is a result of floods and/or other physical disturbances (Waters 1965). In turn, the drift is compensated by the upstream movement of stream invertebrates in order to repopulate the depleted upper reaches (Hughes 1970, Waters 1972). Furthermore, an application of toxicants (e.g. pesticides, insecticides) can induce downstream drift of macroinvertebrates resulting in a reduction of population density of certain species and thus can alter the structure of lotic

communities (Pöckl 1993; Liess 1998; Beketov & Liess 2008). On the other hand, by drifting downstream exposed species can protect themselves from exposure and thus drift can be seen as an avoidance behaviour (Beketov & Liess 2008).

3.1.5 Comparison between batch and AIS experiment

In the following chapter, the results of the effect endpoints in the AIS experiment will be discussed with regard to the results of the respective effect endpoints in the batch experiments with metoprolol (for details to batch experiments see chapter 1.3). The tables presented here only include the median (batch experiment), absolute (AIS) and mean values (enclosures in the AIS). Analyses of metoprolol concentrations in the batch experiment showed that the determined concentrations were in good agreement with the nominal concentrations. Thus, the nominal concentrations are shown for the batch experiment. However, for the AIS experiment the effective concentrations are shown in the tables. Significant differences in the AIS were deduced without any statistical support since there was no replication of each tested metoprolol concentration.

The comparison of the three approaches batch, AIS and enclosure are undertaken to reveal the impact of two stressors, velocity and space, on the effects of metoprolol on gammarids. Batch and enclosures differ in velocity, enclosures and AIS differ in space and batch and AIS differ in velocity and space. Additionally, interactions within the organisms can be considered.

In the batch experiment, significant differences in mortality were detected at metoprolol nominal concentration of 45, 135 and 405 mg/L, respectively (Table 47) and the EC_{50} was determined with 36 mg/L with a 95% confidence interval between 17 and 67 mg/L. Hence, it was expected that significant impact on mortality would also be detected at the highest metoprolol concentrations of 22 mg/L in the AIS experiment. However, no significant impact on mortality was found in the AIS or in the enclosures. Hence, the additional stress factor current velocity in the AIS did not contribute to a higher mortality of gammarids compared to the batch experiment.

Table 47**:** Results of mortality of *G. fossarum* in batch experiment (left side) and in AIS experiment as well as in enclosures of the respective AIS (right side, grey shaded). Results are depicted without any confidence intervals for details see respective tables in the chapter results. * median values; # absolute values; + mean values. Differences are marked bold. In the batch experiment the nominal concentrations (Nom. conc.) in mg/L and in the AIS and enclosures effective concentrations (Eff. conc.) in mg/L are depicted. C – control.

	Mortality [%]			
Nom. conc.	Batch experiment *	AIS #	Enclosures +	Eff. conc.
C	35	39	15	
		37	25	0.2
		22	10	0.7
		32	30	3.0
5.0	30			
15	15			
		36	28	22.0
45	87			
135	100			
405	100			

In the batch experiment, metoprolol decreased the relative number of juveniles as determined by a lower juvenile/adult ratio. At a concentration of 45 mg/L the median was 0 and for following concentrations no juveniles were found (Table 48). For the batch experiment, a NOEC of 5 and a LOEC of 15 mg/L (nominal) metoprolol was determined. Hence, effects in the AIS and enclosures were expected. In the AIS, an increase of the J/A ratio was observed up to an effective concentration of 3 mg/L metoprolol. At 22 mg/L no juveniles were found in the AIS and in enclosures. Therefore, an increasing effect strength due to the stressor velocity was not found. However, in the batch experiments the effects were discussed as direct effects of metoprolol, and the AIS experiments revealed an important indirect effect due to shortage in food (FPOM/biofilm). According to the results, it can be expected that at a metoprolol concentration of 15 mg/L the β-blocker has a negative impact on juveniles leading to a significant decrease in J/A.

Table 48**:** Results of juveniles to adults ratio of *G. fossarum* in batch experiment (left side) and in AIS experiment as well as in enclosures of the respective AIS (right side, grey shaded). Results are depicted without any confidence intervals for details see respective tables in the chapter results. * median values; # absolute values; + mean values. Differences are marked bold. In the batch experiment the nominal concentrations (Nom. conc.) in mg/L and in the AIS and enclosures effective concentrations (Eff. conc.) in mg/L are depicted. C – control.

	J/A			
Nom. conc.	Batch experiment *	AIS #	Enclosures +	Eff. conc.
C	1.0	0.6	0.5	
		0.7	0.6	0.2
		0.5	1.3	0.7
		1.1	1.9	3.0
5.0	0.6			
15	0.7			
		0	0	22.0
45	0			
135	0			
405	0			

In the batch experiment with metoprolol, relative number of precopulae significantly decreased at nominal concentration 45 mg/L. Hence, it could be expected that no adverse effects on precopulae in the AIS experiment will be observed since highest effective metoprolol concentration was 22 mg/L. However, relative number of precopulae showed a concentration-response-relationship to metoprolol in the AIS (Table 49). On the contrary, no concentration-dependent decrease in relative number of precopulae was detected in the enclosures. Thus, regarding the AIS the stress factor current velocity and the larger habitat could be taken into account as stressors; both enhance the adverse effect of metoprolol on forming precopulae pairs.

Table 49: Results of relative number of precopulae of *G. fossarum* in batch experiment (left side) and in AIS experiment as well as in enclosures of the respective AIS (right side, grey shaded).Results are depicted without any confidence intervals for details see respective tables in the chapter results. * median values; # absolute values; + mean values. Differences are marked bold. In the batch experiment the nominal concentrations (Nom. conc.) in mg/L and in the AIS and enclosures effective concentrations (Eff. conc.) in mg/L are depicted. C – control.

	Precopulae [%]			
Nom. conc.	Batch experiment *	AIS #	Enclosures +	Eff. conc.
C	3.9	32	21	
		25	41	0.2
		23	27	0.7
		21	12.5	3.0
5.0	11.03			
15	5.9			
		9	22	22.0
45	0			
135	0			
405	0			

In the batch experiment, a NOEC of 15 mg/L and a LOEC of 45 mg/L was determined for the endpoint relative number of egg-bearing females. Hence, effects in the AIS and enclosures were expected at least in the highest concentration. These were found in the enclosures, with a significant increase in egg-bearing females at 0.7 mg/L and a significant reduction was observed at the highest effective concentration of 22 mg/L (ANOVA, α=0.05) (Table 50). In the AIS, a similar tendency was found, however, in the control the relative number of egg-bearing females was lower compared to the enclosures. Therefore, there was no decrease in the highest concentration compared to the control.

The stressor current velocity did not enhance the effects of metoprolol on number of egg-bearing females since in the AIS and enclosures relative number of egg-bearing females showed effects at metoprolol concentrations similar to the batch experiments, respectively.

Table 50: Results of relative number of egg-bearing females of *G. fossarum* in batch experiment (left side) and in AIS experiment as well as in enclosures of the respective AIS (right side, grey shaded). Results are depicted without any confidence intervals for details see respective tables in the chapter results. * median values; # absolute values; + mean values. Differences are marked bold. In the batch experiment the nominal concentrations (Nom. conc.) in mg/L and in the AIS and enclosures effective concentrations (Eff. conc.) in mg/L are depicted. C – control.

	Egg-bearing females [%]			
Nom. conc.	Batch experiment *	AIS #	Enclosures +	Eff. conc.
C	12.2	14.0	41.5	
		27.0	47.0	0.2
		30.0	58.0	0.7

Nom. conc.	Egg-bearing females [%]			Eff. conc.
	Batch experiment *	AIS #	Enclosures +	
		32.0	37.0	3.0
5.0	25.6			
15.0	18.8			
		22.0	23.0	22.0
45.0	0.0			
135.0	0.0			
405.0	0.0			

In the batch experiment, for the endpoint number of eggs per egg-bearing female a NOEC of 5 mg/L and a LOEC of 15mg/L were determined. Hence, it could be expected that a significant decrease in number of eggs per egg-bearing females in the AIS and enclosures will occur at the highest metoprolol concentration of 22 mg/L. This was confirmed by the results of the AIS and enclosures (Table 51). In fact, for the AIS a LOEC of 3 mg/L metoprolol was determined. The significant lower number of eggs per egg-bearing female in the highest exposure treatment is due to the shortage of food and energy in the stream which are important factors for the reproduction of gammarids (Brust et al. 2001). Additionally, velocity and space have an impact on effect strength of metoprolol, as effective concentrations decreased to NOEC values of 0.7 mg/L in the AIS.

Table 51: Results of number of eggs per egg-bearing females of *G. fossarum* in batch experiment (left side) and in AIS experiment as well as in enclosures of the respective AIS (right side, grey shaded). Results are depicted without any confidence intervals for details see respective tables in the chapter results. * median values; # absolute values; + mean values. Differences are marked bold. In the batch experiment the nominal concentrations (Nom. conc.) in mg/L and in the AIS and enclosures effective concentrations (Eff. conc.) in mg/L are depicted. C – control.

Nom. conc.	Eggs per egg-bearing females [%]			Eff. conc.
	Batch experiment *	AIS #	Enclosures +	
C	6	11	10	
		11.5	12	0.2
		10	9	0.7
		8	11	3.0
5.0	5.3			
15	3.7			
		6	5	22.0
45	0			
135	0			
405	0			

Summarising, the results of the AIS experiment showed a similar trend regarding the effects of metoprolol on mortality and reproductive endpoints of gammarids as for the single substance batch experiments in chapter 1.3. However, lower metoprolol concentrations were applied in the AIS experiment compared to the batch experiments because additional factors such as current velocity and a

larger habitat impacting the fitness of gammarids were present. In general, the current velocity and space did not really enhance the effects of metoprolol on the effect endpoints. Except for the relative number of precopulae where the chance that males find mature females to form a precopulae pair decreased with increasing size of the habitat. Regarding the relative number of eggs the decreasing trend in the highest exposure treatment was rather an effect of the reduced food and energy source, both important factors for reproduction (Brust et al. 2001), than of the velocity or larger habitat, respectively. This could also be the reason for the survival of juveniles. A 100% mortality of juveniles was found in the highest exposure treatment which was mostly due to the shortage of food in form of FPOM which is a crucial food source for juveniles. Thus, an indirect effect of metoprolol on some effect endpoints is indicated according to the results. Although lower metoprolol concentrations were applied in the AIS experiment compared to the batch experiments the effect concentration remains clearly above the effect concentration in fish (this report).

3.2 Studies with *Potamopyrgus antipodarum*

3.2.1 Materials and Methods

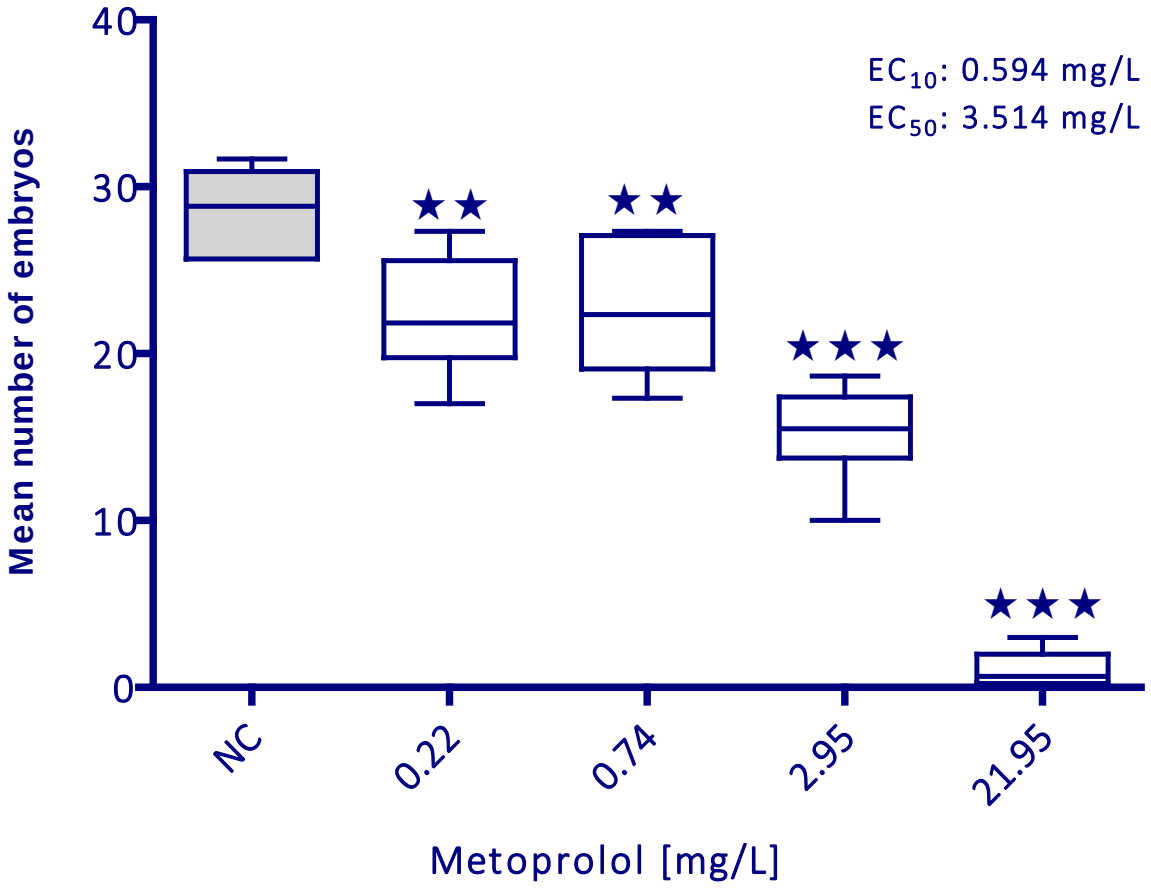
See 3.1.2

3.2.2 Results and Discussion

3.2.2.1 Flow channel experiment (28d)

At the beginning of the experiment, 20 snails were placed in each enclosure. After 28 days in the treatment groups between 17 (2.95 mg/L) and 20 (control) living snails were found (data not shown). Hence, in the concentrations range up to 22 mg/L metoprolol had no effect on mortality. In contrast, for the endpoint reproduction a notable concentration-response relationship was observed (Figure 104). The number of embryos decreased with increasing metoprolol concentrations significantly. While in the control a mean of 28.56 embryos per female was counted, reproduction had been reduced significantly in the lowest concentration to an average of 22.28 per female. In the highest concentration offspring decreased to 1.06 embryos per female. Consequently, the NOEC is <0.22 mg/L and the EC_{10} amounts to 0.594 mg/L (0.140/1.11 mg/L, lower and upper confidence interval).

Figure 104: *Potamopyrgus antipodarum* reproduction in an artificial indoor stream. Mean embryo numbers of caged snails after a 28d-exposure to metoprolol (left). Measured concentrations, NC=negative control, one-way-ANOVA, **p<0.01, ***p<0.001, n=18, logistic non-linear regression model, EC_{10}: 0.594 mg/L (0.140/1.11 mg/L), EC_{50}: 3.514 mg/L (2.17/4.89 mg/L).

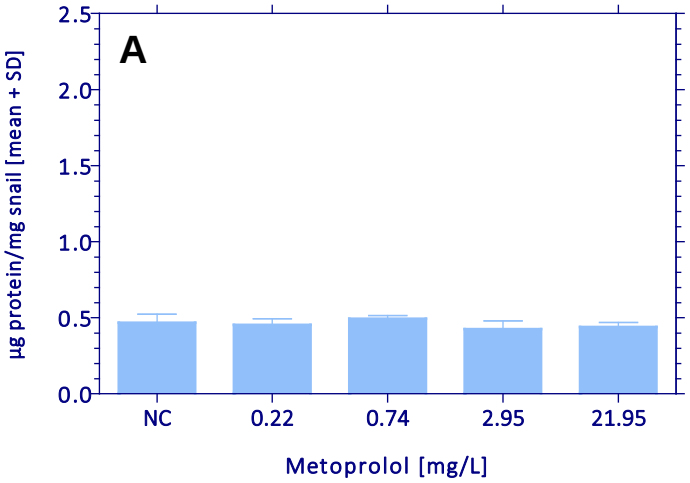


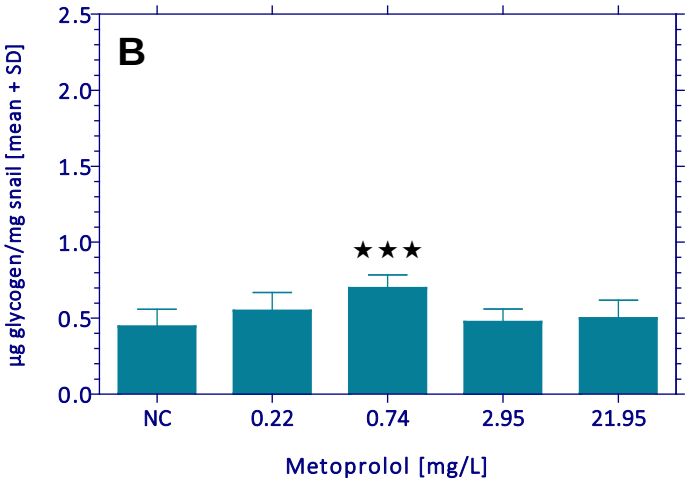
Biomarker (Energy status)

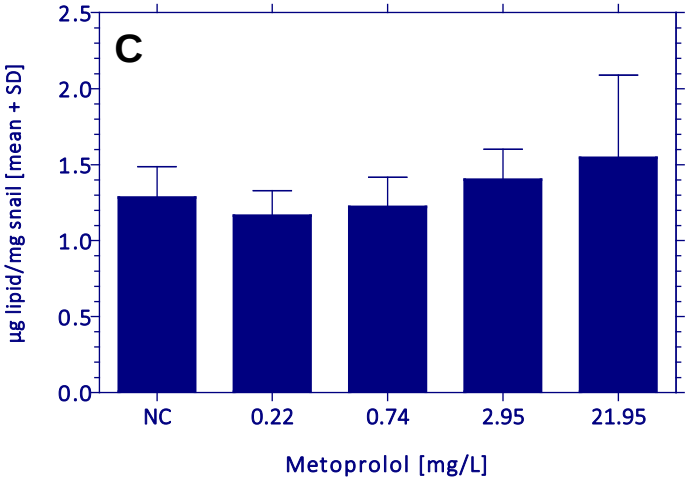
In Figure 105, the energy status of the snails is shown after exposure to different metoprolol concentrations. The protein contents were very similar in all treatment groups and reached values between 0.425 µg/mg (2.95 mg/L) and 0.494 µg/mg (0.74 mg/L) per snail. Total protein levels were comparable to those measured at day 28 in the batch experiment (see 1.4.3.1). However, the metoprolol treatment resulted not in a concentration-dependent toxicity as indicated by Figure 105 A. The glycogen content of treatment 0.74 mg/L showed a significant increase compared to the control (one-way ANOVA, Dunnett's test) (Figure 105 B), higher beta-blocker concentrations only reached the control level (Figure 105 B). The lipid content of the snails indicated a slight increase with increasing metoprolol concentration (Figure 105 C). As already mentioned (see chapter 1.4), the lipid level seems to be coupled to the reproduction of the gastropods.

Since neither the protein nor the glycogen or lipid content of metoprolol treated *P. antipodarum* displayed significant differences compared to the energy status of the control snails, the assessment of these biomarkers seems to be less meaningful compared to reproductive endpoints.

Figure 105: 28-day exposure of caged *Potamopyrgus antipodarum* in AIS. Mean energy status (protein (A), glycogen (B) and lipid content (C)) in *P. antipodarum* after 28 days of exposure to metoprolol. Measured concentrations, NC=negative control, one-way-ANOVA, *p<0.05, N = 6.



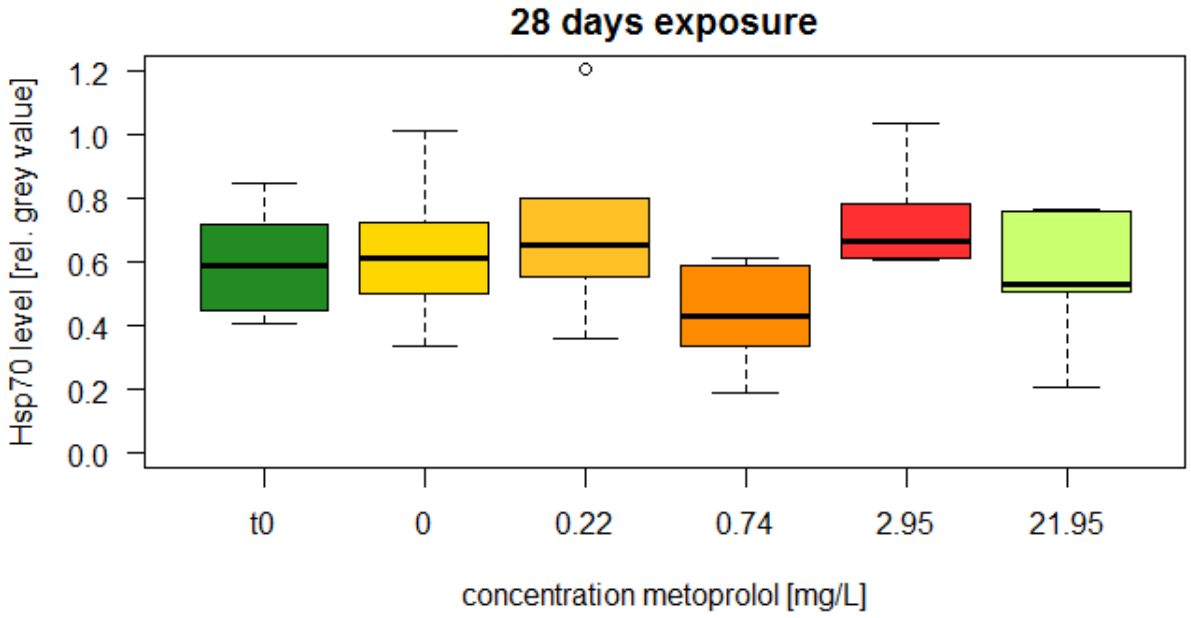




Biomarker (Hsp70 Analysis)

The level of the stress protein Hsp70 was not affected by metoprolol in an AIS setup (linear model, df=5/36, F=1.819,p=0.1339). Data are shown in Figure 106. This is an interesting finding in comparison to the batch experiment, were a clear reaction of the Hsp70-system was found.

Figure 106: Stress protein levels in *Potamopyrgus antipodarum* exposed to metoprolol in an artificial indoor stream for 28 days. "t0" depicts the animals at the start of the experiment. Bold lines display the median values, boxes the 25% to 75% quantiles, whiskers the minimum and maximum values, open circles potential outliers. There were no differences between the treatment groups.



Comparison between the batch and AIS experiment (28 day)

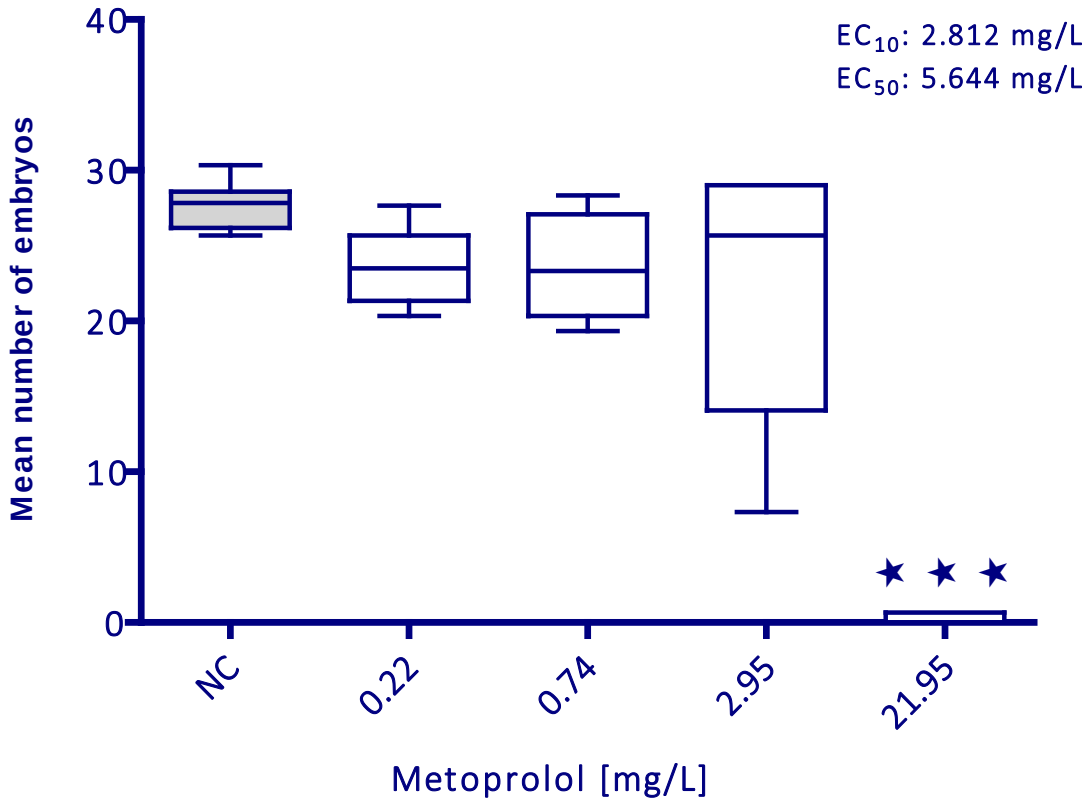
In the batch experiment in the laboratory we calculated an EC_{10} value of 2.922 mg/L when snails were exposed to the beta-blocker for 28 days. In contrast, toxicity in the AIS was higher by a factor of 5 (EC_{10} value of 0.594 mg/L). The higher toxicity in the AIS could not be explained by physico-chemical parameters. The average values of water temperature, pH and oxygen content were comparable (cf. Table 33 and Table 45). Only the conductivity was in AIS twice as high as in the batch experiment (389 µS/cm and 799 µS/cm, respectively). *P. antipodarum* appears to be highly tolerant towards different conductivity, as they can successfully reproduce both at 400 µS/cm (Herbst et al. 2008) and at 800 µS/cm (OECD 2016).

However, it is more likely that the permanent flow velocity in the channel functioned as an additional stressor leading to an increase of toxicity. Sieratowicz et al (2013) found also an increased toxicity in *P. antipodarum* when snails have been exposed to a second stressor (in this case they used cadmium). For the Hsp70 content we determined a NOEC of 1 mg/L (batch experiment), while after the exposure of snails in the AIS Hsp70 content, we found no difference between metoprolol concentrations and the control. The relative values were in general higher than in the batch exposure, indicating more stressful conditions in the flow-through system. Further external stressors may mask the proteotoxic effect of metoprolol in this kind of test setup. As already mentioned, the energy status of the snails in the batch and the AIS was comparable.

Flow channel experiment (40d)

As shown in Figure 107, there is also a concentration-effect relationship even after 40 days. The control produced slightly fewer embryos per female (27.67) compared to day 28 (28.56), while the offspring of the treatment groups 0.22 to 2.95 mg/L was quite similar (23.61; 23.61 and 22.17 embryos per female). In the highest concentration offspring collapsed significantly, here only 0.22 embryos per female were counted. The resulting EC_{10} value was 2.812 mg/L (0.055/n.d. mg/L lower/upper 95% Cl) and was therefore higher compared to the EC_{10} value calculated for a 28 day exposure. Since the confidence intervals were very wide and overlap with those of the 28-day value, it can be assumed that there is actually no difference in response between these two exposure periods.

Figure 107: *Potamopyrgus antipodarum* reproduction in an artificial indoor stream. Mean embryo numbers of caged snails after a 40d-exposure to metoprolol. Measured concentrations, one-way-ANOVA, ***p<0.001, n=18), 3-parameters logistic non-linear regression model, EC_{10}: 2.84 mg/L (0.055/n.d.), EC_{50}: 5.644 mg/L (0.551/10.693 mg/L).



Biomarker (Energy status)

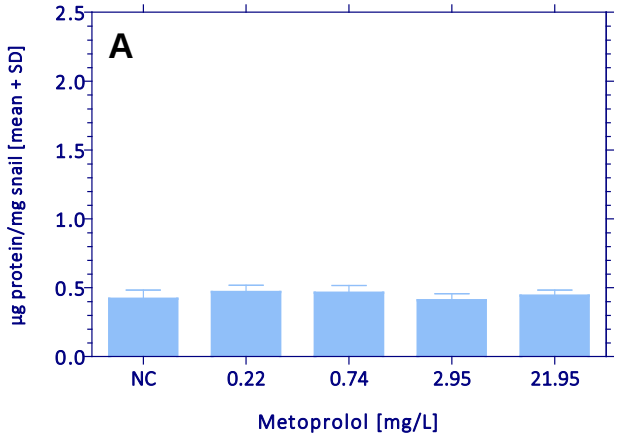
After 40 days, the protein content of the gastropods was almost the same in all treatment groups and overall very comparable to that the animals showed after 28 days (Figure 105 and Figure 108). The same applies to the glycogen content, which is also very similar in all treatment groups (Figure 63B). In contrast to the 28 day-exposure we did not found in any of the treatment groups significant differences to the negative control after 40 days. The lipid content of the snails was increased in the second highest treatment (2.95 mg/L) significantly compared with the control. In the highest concentration the lipid content decreased.

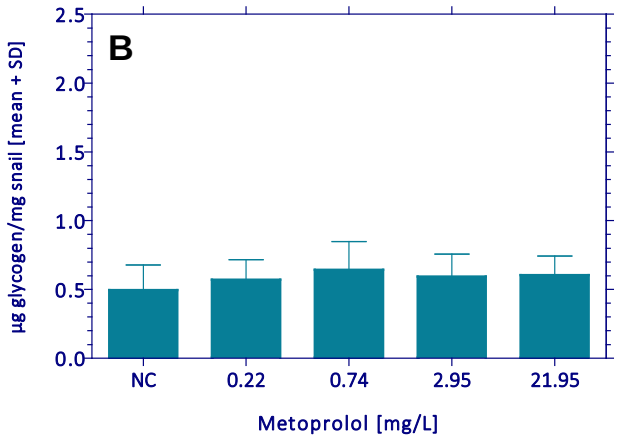
After 40 days, *P. antipodarum* produced significantly fewer embryos in the highest concentration (Figure 107), and in addition lower lipid content decreased (1.379 µg lipid/mg snail) compared to 28-days exposure (1.546 ug lipid/mg snail). Possibly, 40 day-exposure to metoprolol required energy for the

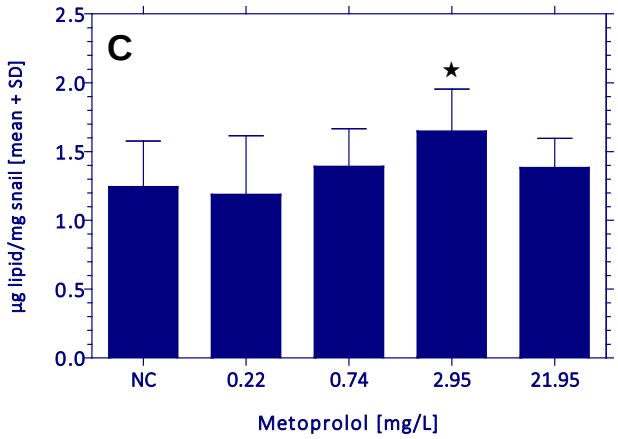
preservation of vital functions and detoxification processes, so *P. antipodarum* had mobilized fat stores.

Overall, the EC_{10} value for the reproductive endpoint was in the same range as the values for any biomarker, so that it is not recommended to measure energy status additionally.

Figure 108: 40-day exposure of caged *Potamopyrgus antipodarum* in AIS. Energy status (protein (A), glycogen (B) and lipid content (C)) in *P. antipodarum* after 40 days of exposure to metoprolol. Measured concentrations, one-way-ANOVA, *p<0.05, N = 6.



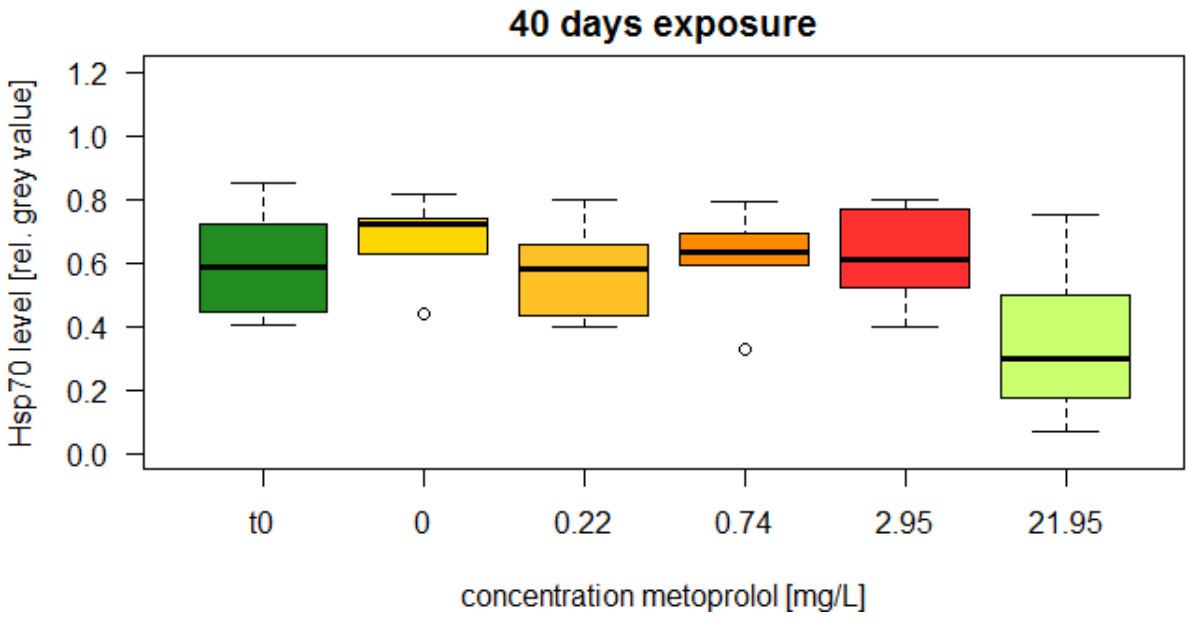




Biomarker (Hsp70 Analysis)

After an exposure time of 40 days, the stress protein level was significantly reduced at the highest teste metoprolol concentration of 21.95 mg/L (linear model, df=5/36, F=2.906, p=0.02645). This finding contrasts with the results of the batch experiment, where metoprolol led to an increase in stress protein level. The elongated exposure time, 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 was overwhelmed after a longer time – resulting in strongly reduced levels of Hsp70 at the highest test concentration.

Figure 109: Stress protein levels in *P. antipodarum* exposed to metoprolol in an artificial indoor stream for 40 days. "t0" depicts the animals at the start of the experiment. Bold lines display the median values, boxes the 25 % to 75 % quantiles, whiskers the minimum and maximum values, open circles potential outliers. The relative Hsp70 level was significantly reduced at the highest tested concentration (linear model, df=5/36, F=2.906, p=0.02645).



3.2.2.2 Free living snails in the AIS at day 40

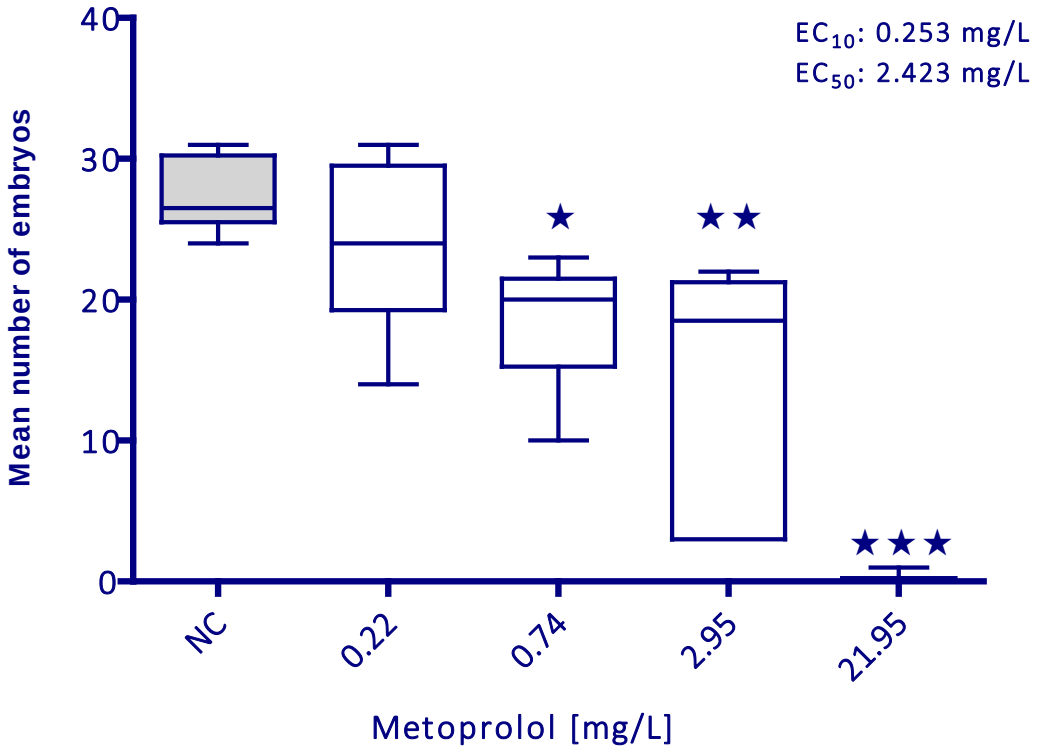
50 snails were inserted free in each flow channel at the start of the experiment. As shown in Table 52 between 22 (0.74 mg/L) and 29 (control) of them were counted at day 40 (however, it is uncertain where the other individuals had remained). On basis of the found individuals, no effect of metoprolol on the endpoint mortality was shown. In contrast, for the endpoint reproduction a significant concentration-response relationship was observed, decreasing gradually with increasing beta-blocker concentration (Figure 110). While the mean offspring of free living control snails in the AIS amounted to 27.33, females from the highest metoprolol concentration only produced embryos on an average of 0.167. Hence, we calculated a NOEC of 0.22 mg/L (one-way ANOVA, Dunnett's test) and an EC_{10} value of 0.253 mg/L (0.017/0.676 mg/L) (logistic non-linear regression model).

Moreover, it is notable, that the variability of offspring increased with increasing beta-blocker concentrations. Especially in the second highest concentration there are obviously individuals having many embryos and those which produce virtually no offspring.

Table 52: Number of snails found in the flow channels at day 40.

Metoprolol [mg/L]	*Potamopyrgus antipodarum* (individuals per flow channel)
0.00	29
0.22	23
0.74	22
2.95	27
21.95	25 (+2 dead)

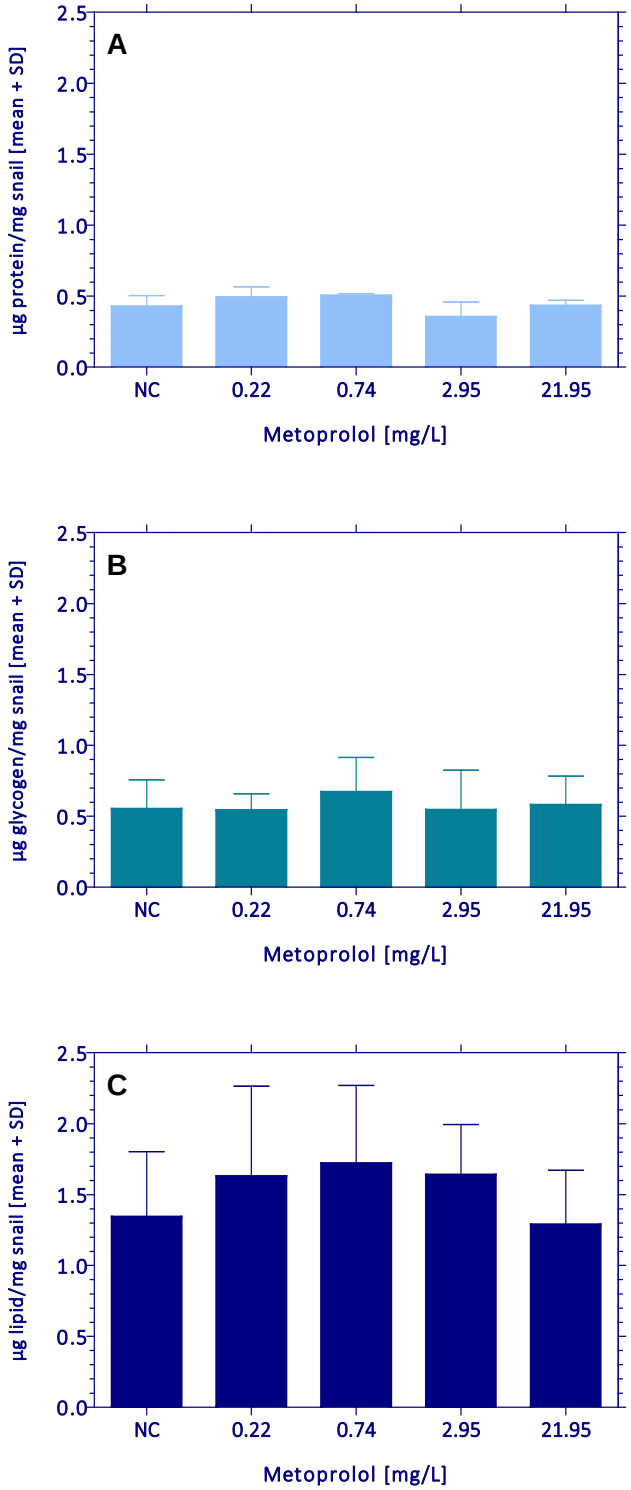
Figure 110: *Potamopyrgus antipodarum* reproduction in an artificial indoor stream (snails were free exposed in each channel). Mean embryo numbers after a 40d-exposure to metoprolol. Measured concentrations, one-way-ANOVA, *p<0.05, **p<0.01, ***p<0.001, n=6, logistic non-linear regression model, EC_{10}: 0.253 mg/L (0.017/0.676 mg/L), EC_{50}: 2.423 mg/L (0.780/4.157 mg/L).



Biomarker (Energy status)

While the protein and glycogen content over all treatment groups exhibited no differences to the negative control (Figure 111 A, B), the lipid content showed an inverted U-shaped concentration-effect relationship (Figure 111 C). Here, the lipid levels increased up to an average of 0.505 µg lipid/mg snail (treatment 0.74 mg/L) and decreased in higher metoprolol concentrations. Even in the case of the lipid content, we calculated no significant differences to the negative control. However, it is noticeable that the energy reserves (in particular the lipid content) of the free-living animals were higher than in the caged snails.

Figure 111: 40-day exposure of free living *Potamopyrgus antipodarum* in AIS. Energy status (protein (A), glycogen (B) and lipid content (C)) in *P. antipodarum* after 40 days of exposure to metoprolol. Measured concentrations, one-way-ANOVA, *p<0.05, N = 6.



Since the number of pools obtained for the free living animals was too low, no reliable evaluation could be performed for the stress protein level of these individuals.

3.2.2.3 Comparison between caged and free living snails in the AIS experiment

Compared to the caged snails, the beta-blocker toxicity is eleven times higher when the animals were free living in the AIS. The main reason for this could be that *P. antipodarum* was exposed to the water flow in the AIS constantly (a competition for resources (food, space) is less likely), so that current was acting as an additional stressor. On the other hand in the enclosures the current was significantly reduced, so that metoprolol showed only in higher concentrations adverse effects on the endpoint reproduction. In addition, the free-living gastropods had a higher lipid content compared to the caged snails. Possibly, the free living snails in the AIS were investing relatively more energy into maintenance of their endogenous functions and less in the production of offspring than the caged gastropods.

3.3 Studies with *Lumbriculus variegatus*

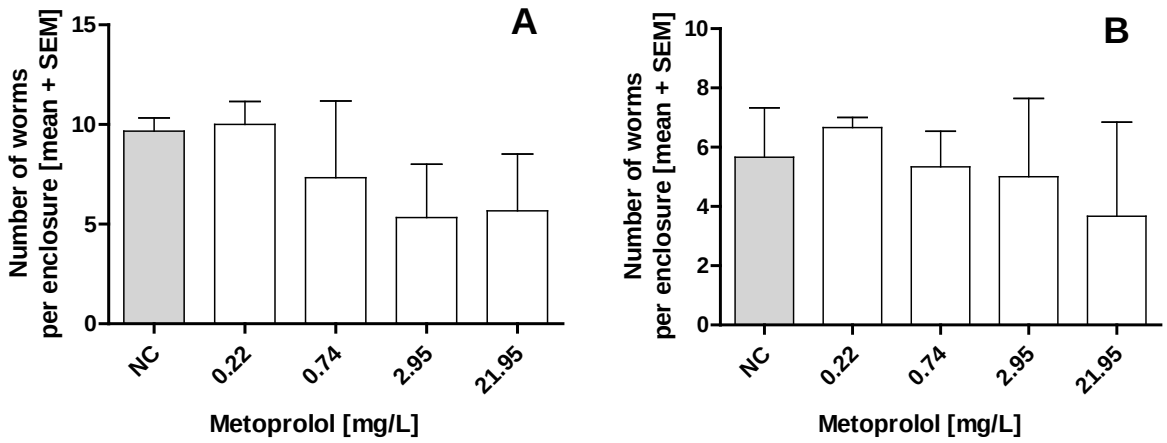
3.3.1 Materials and Methods

See chapter 3.1.2

3.3.2 Results and Discussion

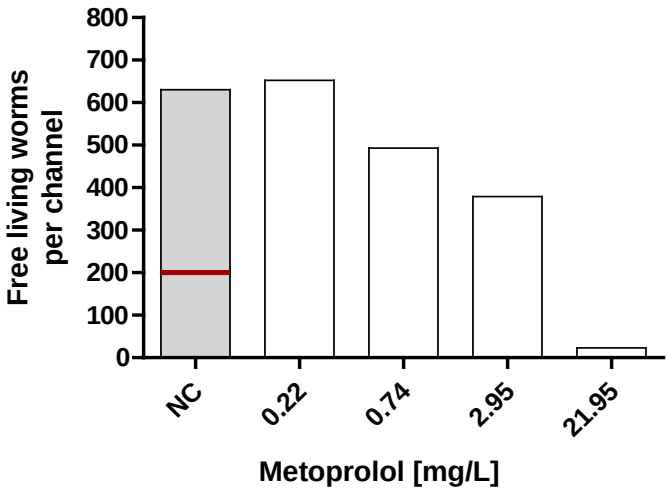
At day 0, six enclosures (containing 10 worms each) per flow channel were introduced. As shown in Figure 112 A, in the control an average of 9.67 worms was counted at day 28. At day 40, the average number of *L. variegatus* in the control decreased to 5.67 (Figure 112 B). Consequently, no reproduction had occurred in the control. It seems highly probable that some of the caged worms have escaped throughout the steel mesh (mesh size: 0.1 mm) of the exposure vessels. Due to that fact, we could not determine any ECx-values for both exposure times.

Figure 112: Number of *Lumbriculus variegatus* in an artificial indoor stream at day 28 (A) and day 40 (B). At day 0 worms were caged in enclosures (10 worms/enclosure; 6 enclosures/treatment) and exposed to different concentrations of the beta-blocker metoprolol.



As mentioned earlier, at the start of the experiment, 200 worms were inserted free into each flow channel. At day 40, we observed a clear concentration-response relationship (Figure 113). The number of worms decreased with increasing metoprolol concentrations. While in the control we counted a total of 630 *L. variegatus* at day 40 (worms have reproduced by a factor of 3.15), in the highest treatment only 23 worms were found. Due to these data we calculated an EC_{10} value of 0.569 mg/L (0.171/1.892 mg/L) for the endpoint reproduction (probit analysis, 3-parameters logistic non-linear regression model).

Figure 113: Total number of free living *L. variegatus* per artificial indoor stream (worms were exposed free in each flow channel over 40 days to the beta-blocker metoprolol). At the start of the experiment 200 worms were inserted in each indoor stream (red line).



	EC10	EC50
Value [mg/L]	0.569	3.381
lower 95%-cl	0.171	1.865
upper 95%-cl	1.892	6.129

4 WP6 Sediment toxicity: single substances and mixtures

4.1 Introduction

In Germany, about 2,300 different active pharmaceutical ingredients are approved for use in humans, whereas approximately half of these active ingredients are potentially environmentally relevant (Ebert et al., 2014). These mainly include antibiotics, hormone preparations, analgesics and antihypertensives. The latter as well as analgesics are active compounds with high annual consumption. One of the most important representatives of non-steroidal anti-inflammatory drugs (NSAID) is diclofenac with an annual consumption rate of 84.4 t in Germany. The most widely used antihypertensive drug is metoprolol with a consumption rate of 157 t/a (IMS Health AG, 2012).

Both pharmaceuticals are eliminated poorly in conventional sewage treatment plants (STP) and find their way into the aquatic environment (Ebert et al., 2014). Fatta-Kassinos et al. (2011) reported high concentrations of diclofenac (5.51 µg/L) and metoprolol (9.59 µg/L) in the final effluent of a STP located in Cyprus. In surface waters mean metoprolol concentrations of 0.310 µg/L (Kunkel & Radke, 2012) and 0.404 µg/L were measured in the water body and 33 µg/kg (Ramil et al., 2010) in the sediment. For diclofenac, average concentrations in surface water were 0.09 µg/L (Camacho-Muñoz et al., 2010) and 0.65 µg/L (Kunkel & Radke, 2012). In marine sediments, diclofenac concentrations of 1.06 µg/kg were reported (Beretta et al., 2014) and in freshwater sediments maximum diclofenac concentrations of 52.1 µg/kg dw were detected (Camacho-Muñoz et al., 2013).

The aim of the present work package was to evaluate adverse effects of diclofenac and metoprolol on the endobenthic oligochaete *Lumbriculus variegatus*. For this purpose, 28-days sediment toxicity tests were performed according to OECD 225 (OECD, 2007). Since for both drugs no data are available concerning the effects on sediment organisms, preliminary tests (range-finder) followed by final tests were conducted over a wide concentration range. Due to the results of the range-finder we have conducted a high-concentration experiment (20 mg/kg dw to 320 mg/kg dw) to determine effects on the terminal endpoints reproduction and biomass. Additionally, we performed 28-days sediment toxicity tests using low concentrations of each drug (0.039 mg/kg dw to 10 mg/kg dw) to identify effects on bio-markers (Hsp70 level, inhibition of cyclooxygenase).

The quantification of the stress protein Hsp70 is an established method for the determination of proteotoxic stress. As a chaperone, Hsp70 plays a major role in folding and re-folding proteins. Organisms experiencing protein damage react by increasing the production of Hsp70 to compensate for this damage. However, the protective system can be overwhelmed if protein damage is too severe – resulting in a decreased Hsp-level, even below the constitutive level, under highly stressful conditions. The protein family is highly conserved and can be found in a wide variety of taxa.

Lipid peroxidation is one major contributor to the loss of cell function (Hermes-Lima, 1995), since the peroxidation of cell membranes exerts strong negative effects on their integrity and function. The generation of lipid peroxides is also linked to the COX I and II system, which is targeted by non-steroidal anti-inflammatory drugs (NSAIDs). Therefore, we hypothesize that the exposure to NSAIDs, like diclofenac, can result in changed lipid peroxidation; either by directly interfering with the COX I and II system, which should result in decreased levels of lipid peroxides, or by indirect effects increasing the level of oxidative stress resulting in higher lipid peroxide levels. Lipid peroxides can be quantified by the FOX assay (ferrous oxidation of xylenol orange). This test is based on a colour change resulting from the reaction of Fe3+, which is generated through the reduction of Fe2+ by peroxided lipids, with xylenol orange.

The quantification of the protein Hsp70 and lipid peroxides in *L. variegatus* has, to the best of our knowledge, never been conducted before. Therefore, the aim of our examination was to investigate

whether biomarker analysis of stress proteins and lipid peroxides in *L. variegatus* can be used as means to gain further knowledge on the toxicity of pharmaceuticals from samples deriving from OECD 225 guideline testing.

4.2 Toxicity tests

4.2.1 Materials and Methods

4.2.1.1 Test Substances

The β-blocker metoprolol is used to treat high blood pressure, to prevent angina and to improve survival after a heart attack. Metoprolol (CAS 56392-17-7) was obtained from Sigma Aldrich (Taufkirchen, Germany). Relevant physical and chemical properties are as follows: chemical purity > 98%; molecular formula $(C_{15}H_{25}NO_3)_2C_4H_6O_6$; molecular weight 684.81 g/M). Kujawa-Roeleveld et al. (2008) reported a log K_{OW} of 1.90.

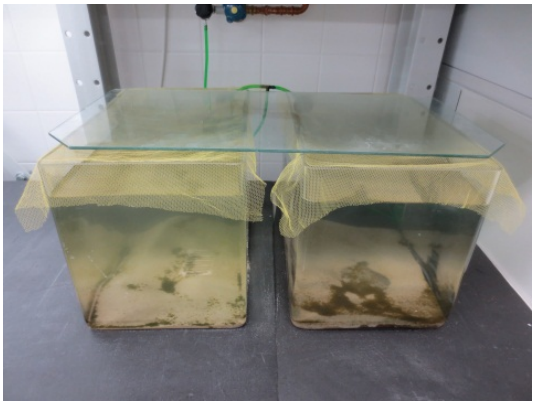
Diclofenac is a NSAID acting as cyclooxygenase inhibitor. Diclofenac (CAS 15307-79-6) was also obtained from Sigma Aldrich (Taufkirchen, Germany). Relevant physical and chemical properties are: molecular formula $C_{14}H_{10}Cl_2NNaO_2$; molecular weight 318.13 g/M; log K_{OW} 0.70 (Knappe et al., 2007)

4.2.1.2 Test Organism

L. variegatus (Figure 114) has been used as a standard organism (OECD 225) in sediment toxicity tests (Phipps et al., 1993; Oetken et al., 2005; Nentwig, 2007; Galluba et al., 2012). The oligochaetes come from our in-house culture and were kept in continuously aerated tap water in 10-L glass aquaria at 20 °C ± 1 °C under a 16 hours light to 8 hours dark photoperiod. For the breeding culture, artificial sediment with a mean particle size of 175 µm was used. Worms were fed once a week with TetraMin®. The reproduction of this species is mainly asexual. By self-fragmentation the posterior regenerates a new head within 10 to 14 days, whereas the anterior part generates a new tail. Organisms from the stock culture were cut in the middle of their body to use worms of the same developmental and physiologic status (so-called synchronization). After 14 days, the posterior fragments were grown to complete worms, which then were used in the experiment.

Figure 114: *Lumbriculus variegatus* (A) and in-house culture (B).

A B



4.2.1.3 28-Days Sediment Tests with *L. variegatus*

Preliminary tests (range-finder)

In a first step, preliminary tests (range-finders) were performed to determine the concentrations for the final toxicity tests with the β-blocker metoprolol and the NSAID diclofenac. For that purpose,

worms were synchronized as previously described. The artificial sediment was prepared according to Table 52. Peat and stinging nettle were treated at the beginning with the appropriate volume of reconstituted water (OECD, 2007) and this mixture was put for one day on a shaker (Society for Laboratory Technology (GFL) 3017, Großburgwedel). Thereafter, the other ingredients kaolin and cellulose were added, and then shaken for another day. The pH value had to be adjusted at 6.5 - 7.5 using calcium carbonate (VWR, Darmstadt, Germany). Both pharmaceuticals were tested using a negative control, a solvent control (methanol) and 14 concentrations with one replicate each. The concentrations ranged from 0.12 to 1000 mg/kg dry weight (spacing factor 2).

First, each 250 mL beaker was filled with 10 g of quartz sand. After that, each drug was solved in 50 mL methanol (Merck, Darmstadt) to produce a concentrated stock solution. The quartz sand was spiked with an appropriate amount of diclofenac or 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 sand as well as 20 mL of the sediment components that was taken from the shaker. Finally, the whole sediment was mixed homogenously. Each beaker was filled carefully with 150 mL reconstituted water (OECD, 2007). After spiking, the test system was incubated for 2 days under test conditions to ensure equilibration of the test substance between water and sediment. After this equilibration period, the exposure started with insertion of the test organisms (day 0). Experiments were terminated on day 28.

The experiments were conducted in a controlled-climate room at 20 °C ± 1 °C under a cycle of 16 hours light to 8 hours dark (light intensity 500 to 1000 lux). For the preliminary tests, 30 synchronized worms were randomly placed into each test beaker. A glass Pasteur pipette was fixed with the plastic cap 0.5 cm above the sediment layer for gentle aeration. At the end of the test, the worms were removed from the sediment, and the number of worms and their biomass (dry weight) were recorded as end points.

Table 53: Composition of the sediment per glass beaker (50.5 g)

Ingredients	Amount	Properties
Quartz sand (*Baumit*, Bad Hindelang)	37.5 g (= 74.2%)	Grain size: ≤ 2 mm
Kaolin (*Merck*, Darmstadt)	10 g (= 19.8%)	-
Peat (*Floragard*, Oldenburg)	2.5 g (= 5.0%)	Particle size ≤ 0.5 mm
Stinging nettle (*Ceasar&Loretz*, Hilden)	0.25 g (= 0.5%)	Particle size ≤ 0.5 mm
Cellulose (*Sigma-Aldrich*, Steinheim)	0.25 g (= 0.5%)	-
Demineralized water	15 - 25 mL	-

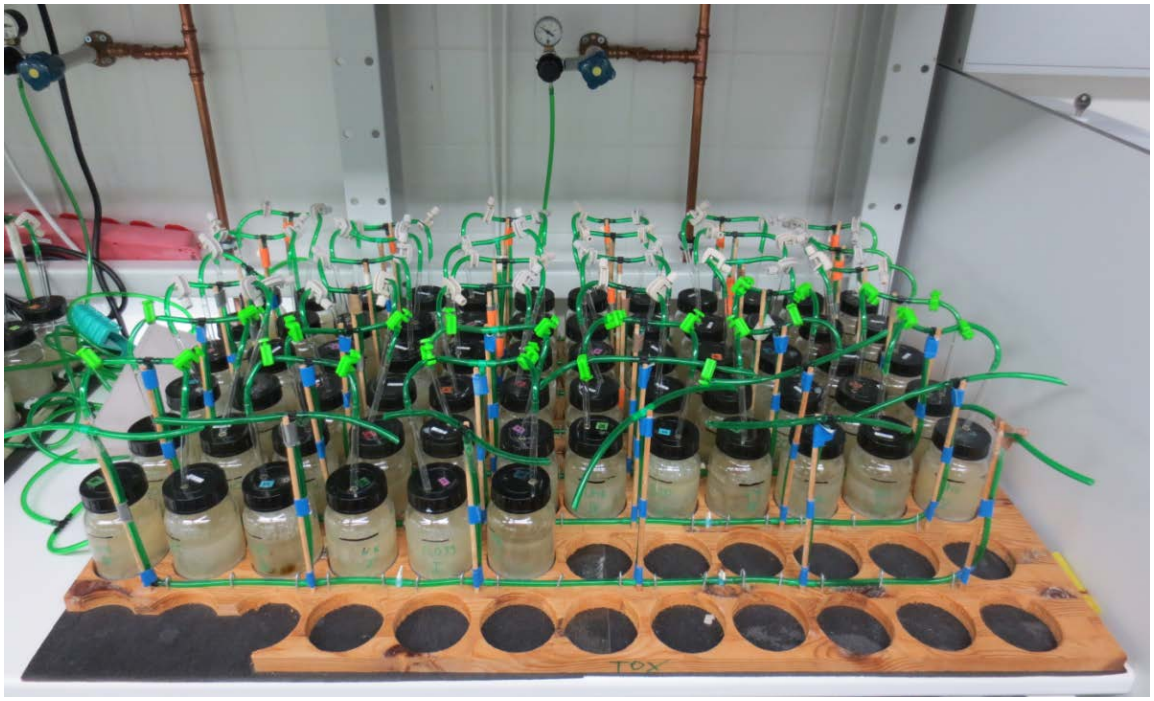
Due to experimental restrictions, the content of the organic carbon could not be determined.

Final tests

Two kinds of 28-days sediment toxicity tests were conducted: (1) Tests using high sublethal concentrations of the respective pharmaceuticals (high concentration tests), which were conducted according to OECD 225 and (2) tests using low concentrations for biomarker studies (low concentration tests). The test design is shown in Figure 115. For the high concentration tests, the concentrations 20, 40, 80, 160 and 320 mg/kg dw were investigated. In these tests, effects on reproduction and biomass were studied. Biomarker responses were not measured. According to the OECD guideline 225, tests included a water control, a solvent control and five concentrations of the respective pharmaceuticals in four replicates each (OECD 2007). For the low concentration tests, the concentrations used were 0.039, 0.078, 0.156, 0.312, 0.62, 1.25, 2.5, 5 and 10 mg/kg dw. For these concentrations, effects on biomarkers, but not on the endpoints reproduction and biomass (which are addressed in the OECD

guideline 225) were expected. Also for these tests, water and solvent controls were included. The final tests were conducted in 250 mL beakers containing 2 cm of spiked artificial sediment (composition, see Table 53), 150 mL of reconstituted water and aerated with compressed air (2 bubbles/s). The spiking procedure was the same as described for the preliminary tests. For the mixture toxicity test both diclofenac and metoprolol were combined in a 1:1 mass ratio, whereas the same concentrations were used as in the experiment with the single compounds. As recommended in the 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 temperatures (20 °C ± 1 °C) and at a light / dark period of 16 to 8 hours with a light intensity of 500 to 1000 lux. Under these conditions, the sediment was aged for two days to ensure equilibrium. Before starting, water parameters (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 beginning (day 0) and end (day 28) of the test using the Ammonium test (Merck Darmstadt). At the start of the experiment, 10 synchronized worms were added to each beaker.

Figure 115: 28 d sediment toxicity test with the endobenthic oligochaete *Lumbriculus variegatus*.



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).

4.2.1.4 Statistics

Statistical analysis was performed using *GraphPad Prism* (GraphPad® Software, Version 5.00, San Diego, USA) and *ToxRat* software (ToxRat® Solutions, Version 2.10.05, Aachen, Germany). Control and solvent control were pooled when no statistically significant differences were found (t-test), otherwise treatments were compared to the solvent control. ECx-values were computed using probit analysis (dose-response fitting: normal sigmoid, maximum-likelihood-regression). No observed effect concentration (NOEC) and lowest observed effect concentration (LOEC) values were determined by analyses of variance (one-way) followed by Dunnett's multiple t-test procedure or Williams´ multiple sequential t-test procedure as post-hoc tests.

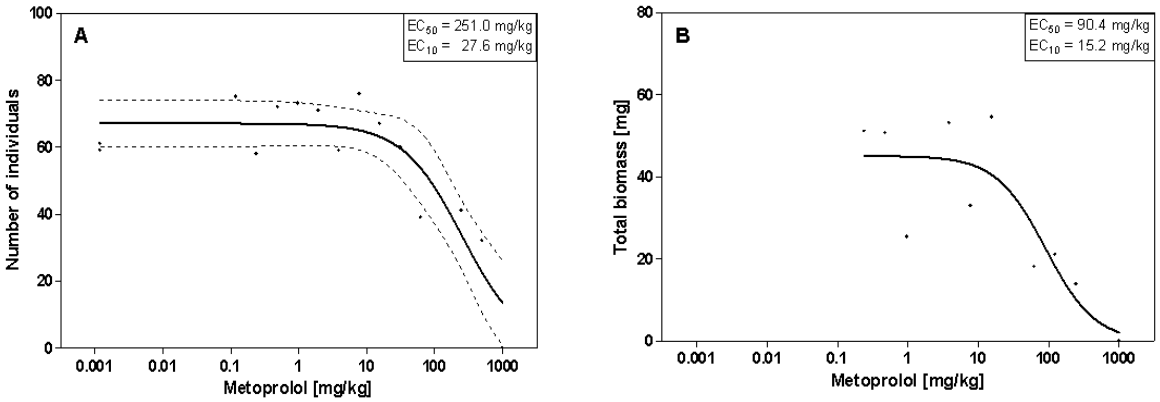
4.2.2 Results

Since only three concentrations (0.039, 20 and 320 mg/kg) were quantified analytically (see chapter 4.4), the nominal concentrations are given in the following. In addition, the NOEC, LOEC and ECx were calculated approximately on real concentrations. This was done under the conservative assumption, that we found diclofenac in the sediment at the start of the experiments with a maximum of 62.5% and metoprolol with 79.7%.

Preliminary tests (range finding)

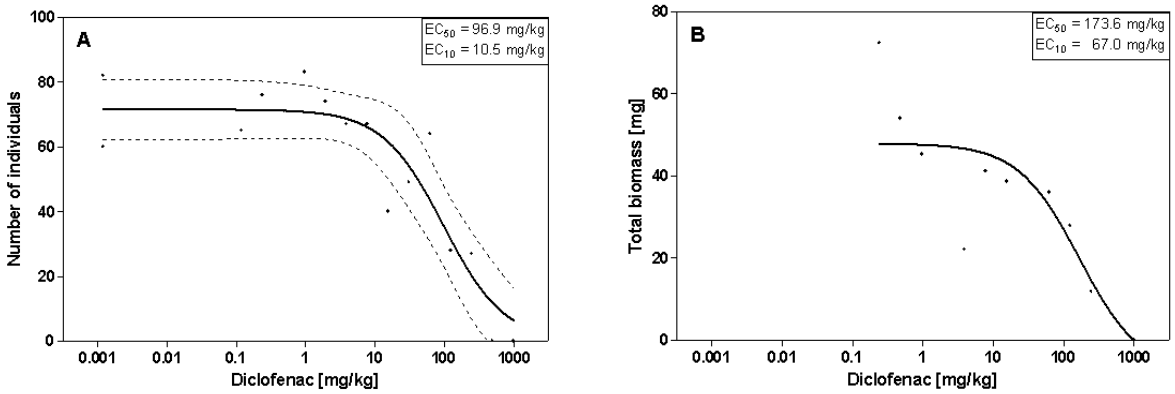
In the solvent control, a mean number of 59.5 individuals (untreated control 71.5 individuals) were found. Thus, the reproductive factors were 1.97 to 2.73. The pH of the overlaying water was between 6 and 9, the oxygen content was always greater than 30%. Therefore, the test fulfilled all validity criteria according to OECD guideline 225. Both reproduction and biomass of *L. variegatus* decreased with increasing concentrations of metoprolol in the sediment (Figure 116). Using non-linear regression for the endpoint reproduction an EC_{50} (28d) of 251 mg/kg dry weight (confidence interval from 127 to 495 mg/kg dry weight) was calculated (Figure 116 A) (the real concentration was approximately 200 mg/kg dw).. The corresponding EC_{10} (28d) was 27.6 mg/kg dry weight (confidence interval 5.08 to 150 mg/kg dry weight) (the real concentration was approximately 22 mg/kg dw). For the total biomass EC_{50} and EC_{10} values of 90.4 mg/kg dry weight (confidence interval 20.8 – 393 mg/kg dry weight) (the real concentration was approximately 72 mg/kg dw) and 15.2 mg/kg dry weight (confidence interval 0.287 to 806 mg/kg dry weight) (the real concentration was approximately 12.1 mg/kg dw) were determined (Figure 116 B).

Figure 116: Preliminary 28-days sediment toxicity test with the oligochaete *Lumbriculus variegatus* and metoprolol. Number of individuals (A) and total biomass of *L. variegatus* (B). (At the start of the experiment 30 synchronized worms were added to each beaker, probit analysis, n = 1 replicate per treatment group).



In Figure 117, the reproduction and the biomass of *L. variegatus* are shown after 28 days of *diclofenac* exposure. The number of individuals decreased with increasing concentration of diclofenac (Fig. 4A). For the endpoint reproduction an EC_{50} of 96.9 mg/ kg dw (confidence interval 43.8 to 214 mg/kg dw) (the real concentration was approximately 60.6 mg/kg dw) and an EC_{10} value of 10.5 mg/kg dw (confidence interval 1.58 to 70.0 mg/kg dw) (the real concentration was approximately 6.56 mg/kg dw). were found. Figure 117B indicates an EC_{50} of 174 mg/kg dw (confidence interval 73.1 to 413 mg/kg dw) (the real concentration was approximately 108.8 mg/kg dw) and an EC_{10} of 67.0 mg/kg dw (confidence interval from 8.59 to 522 mg/kg dw) (the real concentration was approximately 41.9 mg/kg dw) for the endpoint biomass.

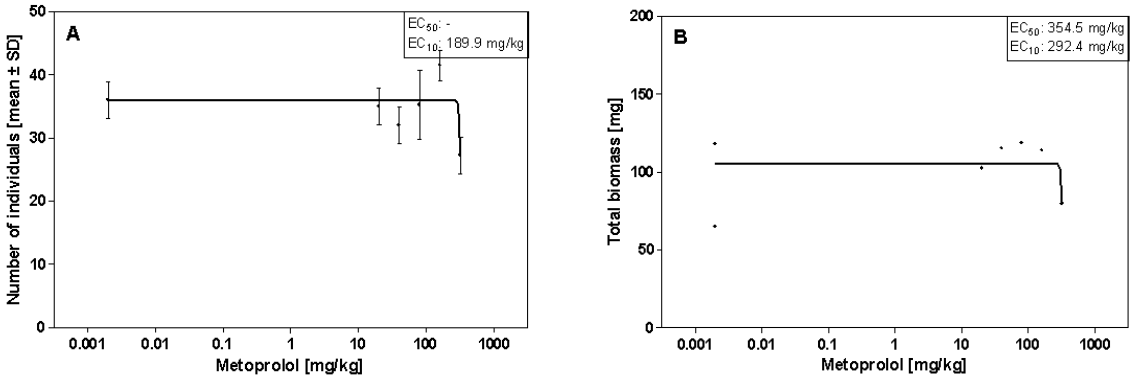
Figure 117: Preliminary 28-days sediment toxicity test with the oligochaete *Lumbriculus variegatus* and diclofenac. Number of individuals (A) and total biomass of *L. variegatus* (B) per beaker. (At the start of the experiment 30 synchronized worms were added to each beaker, probit analysis, n = 1 replicate per treatment group, nominal concentration).



Final tests

The aim of the final test was to assess the effective range for effects of metoprolol and diclofenac on *Lumbriculus variegatus*. For this purpose, both the effects of the single compound as well as of the binary mixture were tested. All definitive tests fulfil the validity criteria according to the guideline 225 (OECD, 2007). As recommended in the guideline 225 pentachlorophenol (30 mg/kg) was used as a positive control (PC) in the mixture experiment only. In the PC treatments, we found no individuals at the end of the test (Day 28).

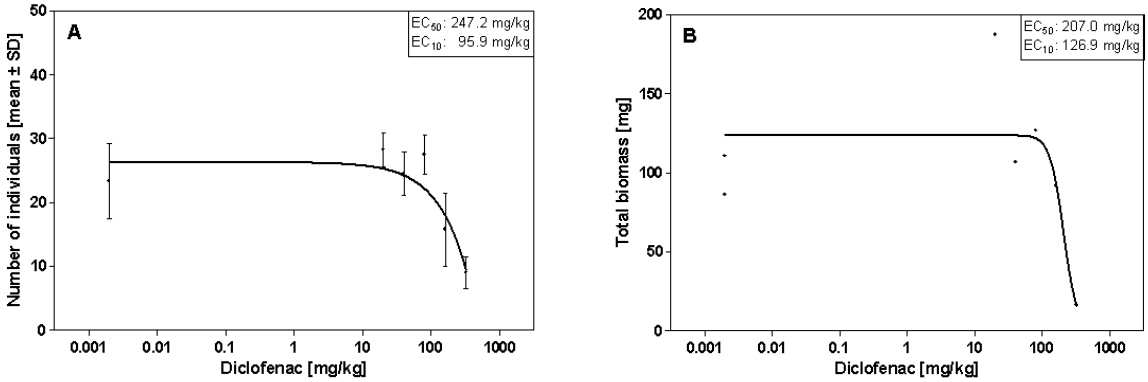
Figure 118: Definitive 28-days sediment toxicity test with the oligochaete *Lumbriculus variegatus* and metoprolol according to the OECD guideline 225. Mean number of individuals (± SD, n = 4 replicates, probit analysis, nominal concentrations) (Figure 118A) and total biomass of *L. variegatus* (mean, data were pooled from 4 replicates, probit analysis) (B,). At the start of the experiment 10 synchronized worms were added to each beaker.



In Figure 119, the effects of metoprolol on the endpoints reproduction (Figure 119A) and biomass (Figure 119B) are shown. The number of individuals decreased slightly with increasing β-blocker concentration, however in the highest concentration (320 mg/kg dw) a maximal reduction of reproduction of 24.3% (compared to the solvent control) was observed. Thus only an EC_{10} value (189.9mg/kg dw) was computable. For the endpoint reproduction NOEC and LOEC values were 160 mg/kg dw and 320 mg/mg dw, respectively (Dunnett´s test and Williams´ test). Also the total biomass decreased with increasing metoprolol concentration. The EC_{10} and EC_{50} values were 292.4 mg/kg dw and 354.5 mg/kg dw, respectively. Since the highest test concentration was 320 mg/kg, these values can only be used

with reservations. In general, confidence intervals were not calculable (very wide). A positive control was only included in the mixture experiments, not in the single compound tests.

Figure 119: Definitive 28-days sediment toxicity test with the oligochaete *Lumbriculus variegatus* and diclofenac according to the OECD guideline 225. Mean number of individuals (± SD, n = 4 replicates, probit analysis, nominal concentrations) (Figure 119A) and total biomass of *L. variegatus* (mean, data were pooled from 4 replicates, probit analysis) (Figure 119B,). At the start of the experiment 10 synchronized worms were added to each beaker.



The NSAID diclofenac showed pronounced effects on reproduction and biomass of *L. variegatus* (Figure 119). For the endpoint reproduction a significant concentration-effect relationship was observed, whereas the number of individuals decreased with increasing diclofenac in the sediment (Figure 119A). While the EC_{10} was calculated with 95.9 mg/kg (confidence interval 37.9 to 134 mg/kg dw), the EC_{50} was 247.2 mg/kg (confidence interval 198 to 335 mg/kg dw). The deviation of these results from data of the range finding tests might possibly be due to the higher density of animals in the preliminary tests resulting in an increased toxicity of diclofenac. The corresponding NOEC and LOEC values were 80 mg/kg dw and 160 mg/kg dw, respectively (Dunnett test and Williams´ test). As shown in Figure 119B, also the total biomass decreased with increasing concentrations of diclofenac. Here, an EC_{10} of 126.9 mg/kg dw (confidence interval 27.8 to 570 mg/kg dw) as well as an EC_{50} of 207.0 mg/kg (confidence interval 82.2 to 521 mg/kg dw) were determined.

Figure 120: Definitive 28-days sediment toxicity test with the oligochaete *Lumbriculus variegatus* and mixture of metoprolol and diclofenac according to the OECD guideline 225. Reproduction compared to the solvent control of both the single compounds and the mixture. n = 4, probit analysis

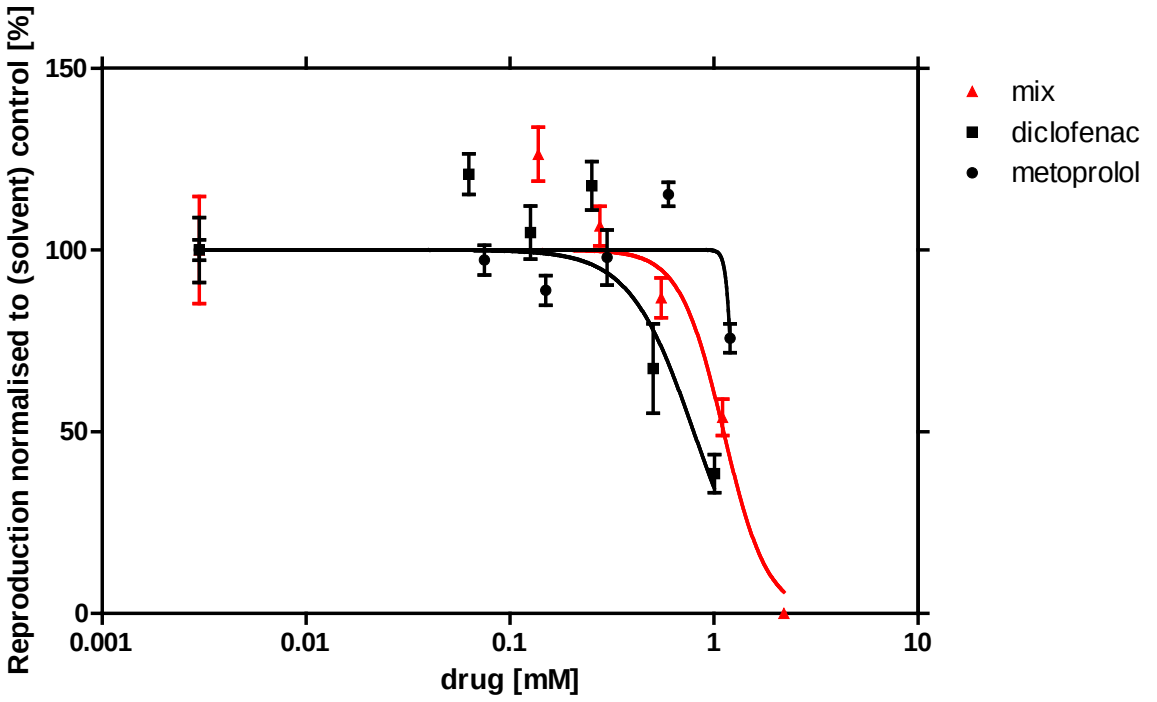


Figure 120 shows the reproduction of *L. variegatus* which were exposed over 28 days to a mixture (1:1) of metoprolol and diclofenac. The number of individuals decreased significantly with increasing concentrations of the two drugs. Calculated EC_{10} and EC_{50} values were 0.466 and 1.11 mM, respectively. The corresponding NOEC and LOEC values were 0.551 and 1.10 mM, respectively (Dunnett's test and Williams´ test). The total biomass decreased with increasing concentrations of metoprolol and diclofenac in the sediment. Here, we calculated an EC_{10} of 1.16 mM and an EC_{50} of 1.26 mM. Table 2 summarizes the effect concentrations (and 95% confidence limits) and the NOEC/LOEC values for both the single compounds and the mixture in the 28-days sediment toxicity test with *L. variegatus* (OECD, 2007).

Table 54: 28-days sediment toxicity test with *L. variegatus* (OECD 225) and both pharmaceuticals. Effect concentrations (upper/lower 95% confidence limits) and NOEC/LOEC-values for the endpoint reproduction (all values in mM) in the final test.

Substance	EC_{10}	EC_{50}	NOEC	LOEC
Diclofenac	0.302 (0.119/0.421)	0.777 (0.620/1.05)	0.252	0.503
Metoprolol	0.710 (n.d./n.d.))	n.d.	0.598	1.200
Mixture (1:1)	0.466 (0.269/0.807)	1.110 (0.928/1.34)	0.551	1.100
Mixture (referred to diclofenac)	0.213	0.509		

n.d.: not determinable

4.2.3 Discussion

All experiments conducted in the present work package lasted for 28 days. Since in the literature no effect data for sediment organisms were found, preliminary tests (range-finders) were performed with the single compounds diclofenac and metoprolol. Based on the results of these preliminary tests a wide range of concentrations (0.039 mg/kg dw to 320 mg/kg dw) was covered in the final tests. In the low concentration range (0.039 mg/kg dw to 10 mg/kg dw) we expected mainly proteotoxicity (Hsp 70) and specific effects of the NSAID, such as an inhibition of cyclooxygenases. In contrast, in the high concentration range (20 mg/kg to 320 mg/kg) we focused on effects on the endpoints reproduction and biomass, which provide data for regulatory ecotoxicology. The results of the preliminary tests have also led to renounce toxicity tests with effluent from wastewater treatment plants, as the drug concentrations in the effluent (e.g. 1-2 µg diclofenac/L) were too low to expect effects on terminal endpoints.

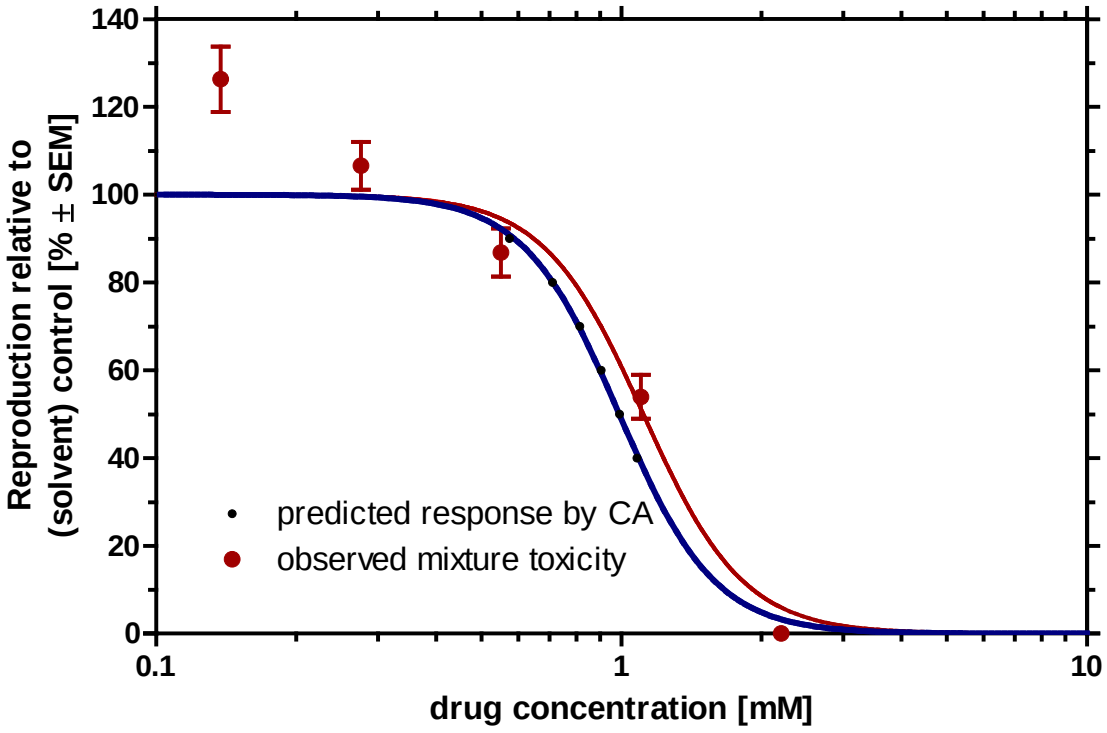
The high concentration experiments were performed according to OECD guideline 225 (OECD, 2007). As already mentioned, all tests complied with the validity criteria of the guideline. However, the results from the preliminary tests could not be confirmed by the final tests. For example, in the pre-test with diclofenac, we calculated an EC_{50} reproduction value of 96.9 mg/kg dw. In the final test (high concentration experiment), however, this value increased up to 247 mg/kg by factor 2.5. This has probably been caused by the use of 30 individuals per beaker in the preliminary test, while in the final test only 10 individuals per beaker were used, according to OECD (2007). Possibly, the higher density of animals resulted in an increased toxicity of diclofenac.

In the test with the binary mixture, high ECx values and thus low ecotoxicity were determined. As shown in Figure 120 for the endpoints reproduction, the action of both drugs follows the model of concentration addition (CA). However, toxicity is increasing only slightly due to the marginal effect of metoprolol. When we refer to the amount of diclofenac in the binary mixture, it becomes obvious that the toxicity increases compared to the toxicity of diclofenac as single compound (Table 54).

Diclofenac and metoprolol enter the aquatic environment mainly via municipal waste water discharge. For example, Camacho-Muñoz et al. (2014) found a mean of 570 ng diclofenac/L in STP effluent. As already mentioned in the introduction, the average environmental concentrations of metoprolol in German streams are 404 ng/L in the water phase and 33 µg/kg dw in sediments (Ramil et al., 2010). In Spanish rivers mean diclofenac concentrations of 90 ng/L in the water column and highest concentrations of 52.1 µg/kg dw in the sediment were measured (Camacho-Muñoz et al., 2010; 2013).

Obviously, the calculated effect concentrations for the endpoints reproduction and biomass are above the environmental concentrations. Even if an appropriate assessment factor is used, the resulting PEC/PNEC ratio would be well below 1. For example, the NOEC for diclofenac in the final test was determined as to be 95.9 mg/kg dw. The use of an assessment factor of 100 (EMEA, 2006) results in a predicted environmental concentration (PNEC) of 950 µg/kg dw. As stated above, a measured environmental concentration (MEC) of 52.1 µg/kg dw was found. Hence, the MEC/PNEC ratio is 0.055 for the compartment sediment.

Figure 121: Observed and predicted mixture toxicity of diclofenac and metoprolol observed in the 28-days sediment toxicity test using *Lumbriculus variegatus* (CA = concentration addition model, probit analysis). In the low concentration experiment no effects on the apical endpoints were observed neither for diclofenac nor metoprolol. The biomarkers (Hsp 70 and inhibition of lipid peroxidation) also showed no significant effects compared with the control.



4.3 Biomarker analyses: stress protein level and degree of lipid peroxidation

4.3.1 Materials and Methods

4.3.1.1 Stress protein quantification

Per concentration, 15 individuals of *L. variegatus* were used *in toto* for stress protein quantification. Samples were stored at -80 °C until processing. Due to their small size and low protein content, individuals had to be pooled for the analyses. Each pool consisted of three individuals. The frozen samples were mechanically homogenized on ice in 40 µL extraction medium (consisting of 96% concentrated extraction buffer (80 mM potassium acetate, 5 mM magnesium acetate, 20 mM Hepes in bidestilled water, adjusted to a pH of 7,5 and 4% protease inhibitor). Homogenized samples were centrifuged at 4 °C and 20000 rcf for 10 min. 5 µL of the supernatant were used for protein quantification. The remaining supernatant was diluted with SDS buffer (20% glycerin, 3% sodium dodecyl sulfate, 0.3% β-mercaptoethanol, 10 mM Tris pH 7 and 0.005% bromophenol blue in bidestilled water) in a proportion of 2 parts supernatant to 1 part SDS buffer, and boiled at 95-100 °C for 5 min. Both mixtures were stored at -20 °C until further usage.

To quantify the protein content according to Bradford (Bradford, 1976), 5 µL of supernatant were diluted with 245 µL of 1:10 extraction buffer. 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 straight calibration line. Each sample was tested in triplicates. 20 µL of the samples´ supernatant mixture were mixed with 250 µL of Bradford

mixture (0.001% Coomassie brilliant blue G-250, 4.75% ethanol, 8.5% phosphoric acid in bidestilled water) and the extinction at 595 nm was measured using an automated microplate reader.

For the electrophoresis, polyacrylamide minigels (12% acrylamide, 0.12% bisacrylamide) were loaded with the sample/SDS mixtures. Constant protein weights (60 µg per sample) were applied to the gels, following the results of the Bradford analysis. To allow for a comparison between different gels, a standard in duplicate was added to every gel. Gel chambers were placed in E-buffer + SDS (0.19 M glycin, 25 mM $Tris_{base}$ and 0.1% SDS). Gels were run for 15 min at a voltage of 80 V, followed by 1 h at 120 V to separate the proteins.

Protein transfer from the gel to a nitrocellulose membrane was performed via semi-dry Western blot. The membrane was fitted to the gel, encased in Whatman-papers soaked in transfer buffer (0.095% $Tris_{HCl}$, 6.34% $Tris_{base}$, 3.63% glycine and 0.046% SDS in bidestilled water, adjusted to a pH of 9) and placed in a blotting chamber. For each gel used in the blotting, an amperage of 90 mA was applied, with the voltage not exceeding 10 V. The blotting process was done over a time period of 2 hours.

After the blotting process, the filter was blocked in blocking solution (TBS (0.88% sodium chloride, 0.635% Tris-HCl and 0.118% Tris-base in bidestilled water, adjusted to a pH of 7.5) with horse serum in a mixture of 1:2) for one hour. 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 (1 mM 4-chloro-1-naphtol, 6% methanol, 0.015 % hydrogen peroxide in 30 mM Tris pH 8.5) until the protein bands were visibly stained. The reaction was stopped by transferring the filter into bidestilled 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 E.A.S.Y. Win 32 (Herolab, Wiesloch, Germany). To assure comparability, each sample was related to an Hsp70 standard.

4.3.1.2 Quantification of lipid peroxides

The FOX assay procedure was conducted according to a modified version of the protocol proposed by Hermes-Lima et al. (1995). 15 individuals of *L. variegatus* per treatment were used *in toto* for the lipid peroxide quantification. Samples were stored at -80 °C until further processing. Due to their small size the samples had to be pooled for further analysis. Each pool consisted of three individuals. The samples were weighed, diluted in a ratio of 1:10 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 bidestilled water. Each sample was tested in triplicates, 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 48 hours at room temperature. The absorbance at 580 nm was measured using an automated microplate reader. After the first measurement, 1 µL of 1 mM cumene hydroperoxide solution was added to each well. After another incubation period of 1 hour, 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 ($CHP_{equiv.}$/mg wet weight) were calculated using the following equation:

$$\frac{A580nm}{A580nmCHP} * volumeCHP * \frac{total\ volume}{sample\ volume} * dilution\ factor = \frac{A580nm}{A580nmCHP} * 1 * \frac{200}{20} * 10$$

4.3.1.3 Statistical analysis

Statistical analysis was conducted using SAS JMP 11.0. Data were checked for normal distribution using the Pearson-D'Agostino Omnibus K2 test. If necessary, data were transformed using square root or 3rd root transformations to fit the 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 post hoc test) was used to compare the means between the different treatment groups. If no homogeneity of variance was given, a Welch-ANOVA was used instead. In order to check the data for concentration-effect relationships , a correlation analysis was conducted using Kendall's Tau test. The significance level was set to α=0.05 for all tests.

4.3.2 Results

4.3.2.1 Diclofenac – Stress protein Hsp70

Data showed normal distribution after transformation using the 3rd root (Pearson-D'Agostino-Omnibus, n=30, p=0.1599), homogeneity of variance was given (Levene, *df*=5,24, F=1.2216, p=0.3295).

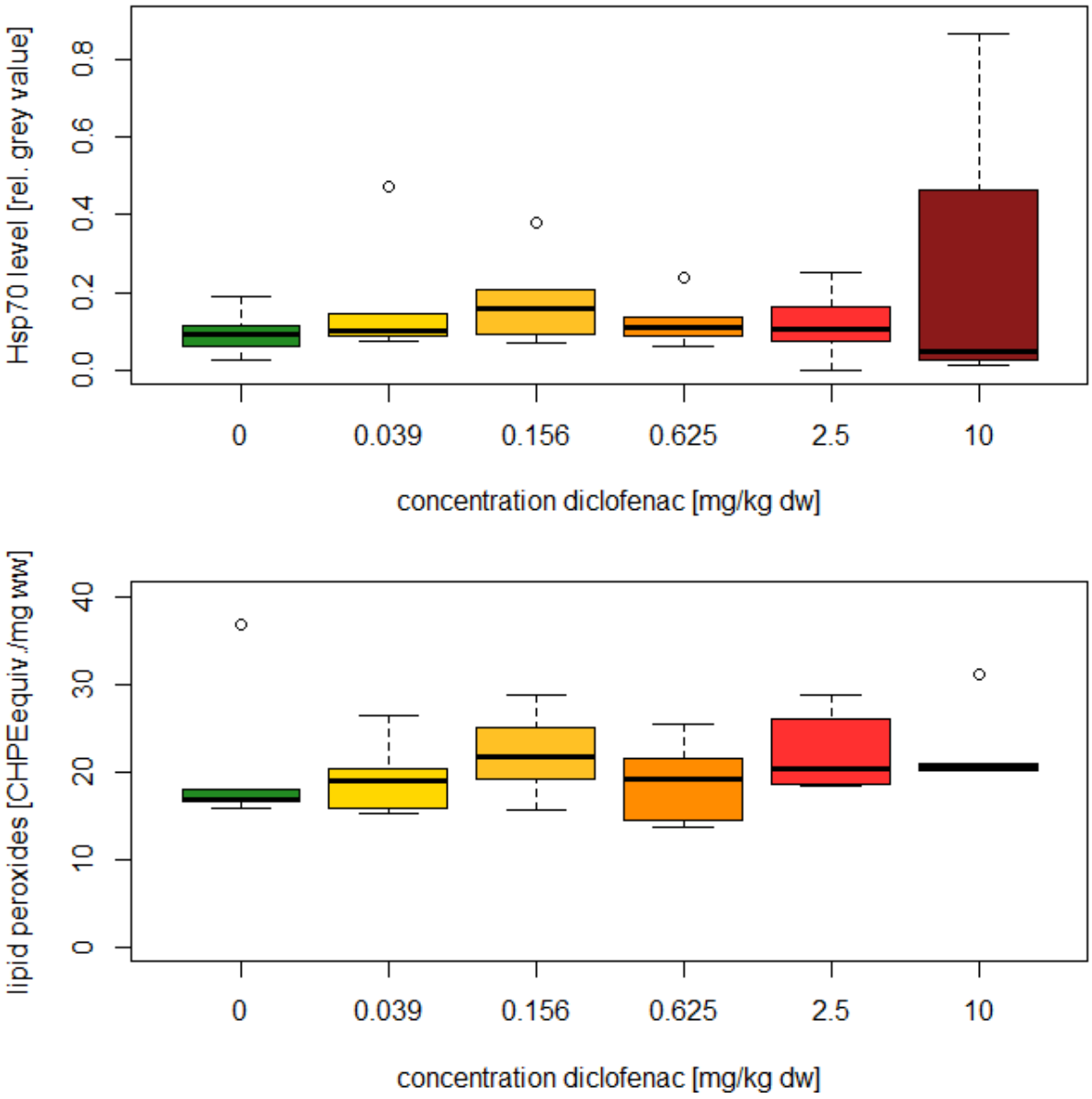
The stress protein level of *L. variegatus* exposed to diclofenac did not change with increasing concentration (ANOVA, *df*=5.24, F=0.5211, p=0,7579). However, it was striking that individuals exposed to higher concentrations (particularly 10 mg/kg dw) seemed to show a higher variability (Figure 122).

4.3.2.2 Diclofenac – Lipid peroxidation

Data showed normal distribution after transformation using the square root (Pearson-D'Agostino-Omnibus, n=30, p=0.1292), homogeneity of variance was given (Levene, *df*=5.24, F=0.5062, p=0.7687).

The degree of lipid peroxidation did not show significant differences between the investigated concentrations of diclofenac (ANOVA, *df*=5.24, F=0.4661, p=0.7975) (Figure 122).

Figure 122: Stress protein Hsp70 (top) and lipid peroxide (bottom) levels of *Lumbriculus variegatus* exposed to diclofenac. Bold lines display the median values, boxes the 25% to 75% quantiles, whiskers the minimum and maximum values, circles potential outliers. There were no significant differences to be seen between the treatment groups.



4.3.2.3 Metoprolol – Stress protein Hsp70

Data showed normal distribution after transformation using the 3rd root (Pearson-D'Agostino-Omnibus, n=26, p=0.2364), homogeneity of variance was given (Levene, *df*=5, 20, F=0.5410, p=0.7431).

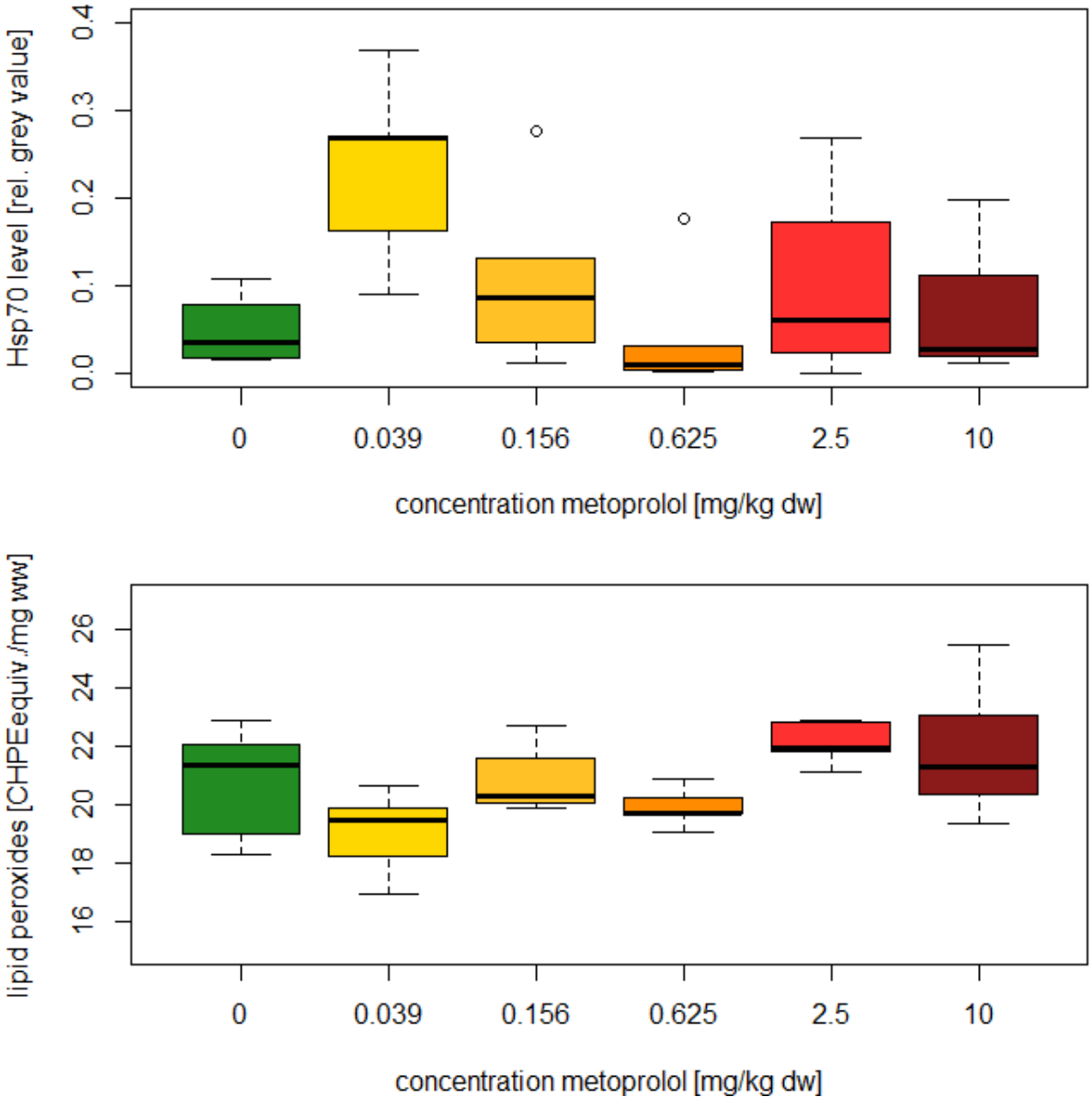
The stress protein level of *L. variegatus* exposed to metoprolol was conspicuously, but most probably due to the low sample size (which will be discussed later on) not significantly (ANOVA, df=5, 20, F=2.2054, p=0.0942), increased in the group treated with the lowest concentration of 0.039 mg/kg (Figure 123). The other treatment groups showed Hsp70 levels comparable to the control.

4.3.2.4 Metoprolol – lipid peroxidation

Data showed normal distribution after transformation using the square root (Pearson-D'Agostino-Omnibus, n=30, p=0.6766), homogeneity of variance was not given (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, τ=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 of any treatment group compared to the control (Figure 123).

Figure 123: Stress protein Hsp70 (top) and lipid peroxide (bottom) levels of *Lumbriculus variegatus* exposed to metoprolol. Bold lines display the median values, boxes the 25% to 75% quantiles, whiskers the minimum and maximum values, circles potential outliers. The stress protein level was slightly, but not significantly, increased at the lowest tested concentration. The level of lipid peroxides slightly increased with rising metoprolol concentrations (Kendall's Tau, n=30, τ=0.2847, p=0.0371).



4.3.3 Discussion

The necessity of pooling individuals for Hsp70 quantification and the FOX-Assay can be regarded as a drawback in terms of reducing the sample size in these analyses. It should be kept in mind that the lack of significances in some of our results may be due to the low number of independent data points.

4.3.3.1 Diclofenac - Stress protein Hsp70

In general, the stress protein levels were low, symbolized by a weak staining of the blotted protein bands. Nevertheless, a comparison on this low level remains possible.

The slight increase of the relative Hsp70 level in the lower diclofenac concentrations 0.039 and 0.156 mg/kg, followed by a decreased level at higher concentrations corresponds to the kinetics of a typical Hsp70 response curve. However, the effects were not significant and could also be due to natural variability of the tested organisms. The increased variability of the data at the highest examined concentration (10 mg/kg) may be regarded as an indicator of increased overall stress in these treatments. In summary, our results do not support the hypothesis of diclofenac exhibiting a proteotoxic effect in *L. variegatus*.

4.3.3.2 Diclofenac – lipid peroxidation

The samples used for the FOX-Assay had to be diluted in a ratio of 1:10, even if the samples were pooled. As a consequence, the incubation time had to be increased to 48 h and the measured extinction values were comparably low. Since the dilution factor directly affects the calculation of CHP equivalents, a further increased dilution could have intensified measurement inaccuracies.

The degree of lipid peroxidation in *L. variegatus* treated with diclofenac did not differ significantly between the tested concentrations. However, the high mean in the control is mainly due to a single data point. Lipid peroxidation was slightly increased in the two highest concentrations 2.5 and 10 mg/kg.

Previous studies with diclofenac in *Cyprinus carpio* showed on the one side decreased levels of oxidative stress biomarkers (TBARS) at concentrations of 0.03 mg/L (Stepanova et al., 2013), but increased levels at 7.098 mg/L (Islas-Flores et al., 2013). The mollusc *Mytilus galloprovincialis* showed increased levels of antioxidant enzymes at concentrations of as low as 0.25 µg/L diclofenac and ibuprofen (Gonzalez-Rey & Bebianno, 2011). However, none of those studies applied the FOX assay as a measure of oxidative stress. The experiments on gammarids and trout performed in the course of the running project will give us further insights into the sensitivity of the FOX-assay method

, and also allow us to compare sensitivities between species.

4.3.3.3 Metoprolol – Stress protein Hsp70

The stress protein level was conspicuously increased by the lowest tested concentration, 0.039 mg/kg. This can be seen as an indicator of the organisms' reaction to proteotoxic stress. The lower stress protein values obtained for concentrations of 0.156 mg/kg or higher may indicate capacity overload of this system. However, due to the lack of significance this remains speculative, and further tests, covering the concentration range of 0 to 0.156 mg/kg more in detail, would be needed.

4.3.3.4 Metoprolol – Lipid peroxidation

The analysis revealed a weak positive correlation between the exposure to metoprolol and the degree of lipid peroxidation. The organisms seem to experience increased oxidative stress in high metoprolol concentrations. However, since the comparison of the means did not show a significant difference between the highest concentration and the control, a LOEC cannot be calculated. Further tests, investigating the potential effect of metoprolol on the oxidative stress level, are advised. Other pharmaceuticals, like anticonvulsants, antibiotics, and lipid regulators can change levels of oxidative stress in a variety of different organisms (Schmidt et al., 2011; Zhang et al., 2012; Nie et al., 2013; Yang et al., 2013)

in concentrations as low as 1 µg/L. However, any effect of beta blockers on the oxidative stress level has never been reported before.

4.4 Chemical analyses

4.4.1 Materials and Methods

Water and sediment samples were analyzed by high performance liquid chromatography (HPLC, series 1290, Agilent, Waldbronn, Germany) coupled to tandem mass spectrometry (Tandem-MS, API 5500, AB Sciex, Foster City, USA) in the MRM-Mode (MRM: multiple reaction monitoring) with a positive ionization by electrospray-interface. The optimized interface parameters are shown in table 3. The analytical techniques used for solid samples were similar to those used for water samples but required a sample preparation which efficiently removed co-extracted matrix compounds. By combining appropriate extraction (solid phase) and enrichment techniques (Strata-X, 200 mg, Phenomenex, Aschaffenburg) during sample pre-treatment, a high selectivity and sensitivity was achieved.

Table 55: Optimized interface parameters

parameter	Unit
collision gas	medium
curtain gas	30 psi
ion source gas 1	60 psi
ion source gas 2	80 psi
ion spray voltage	5.500 V
ionisation mode	positive
temperature	450 °C

For each substance, two suitable values for mass transfer were selected with highest intensity possible. Furthermore, the optimal adjustments for the MS were selected. In table 4, details for the MS/MS-parameters parameters are summarized.

Table 56: MS/MS-parameters for diclofenac und metoprolol

analyte	precursor ion	production	DP [a] (V)	CE [b] (eV)	CXP [c] (V)
Diclofenac	296.0	213.8	26	37	4
	296.0	249.8	26	19	6
Metoprolol	268.1	116.2	46	27	0
	268.	56.2	46	43	0

[a] DP = declustering potential in volt, [b] CE = collision energy in electron volt, [c] CXP = cell exit potential in volt

4.4.2 Results

The results for the real 1concentrations of diclofenac and metoprolol in sediment and overlaying water in the tests with isolated substances and the substance mixture are summarized in table 5 and 6.

At day 0, the real concentrations of diclofenac were approximately half of the nominal concentrations in the sediment. After 28 days, the real concentration represents less than 30% of the nominal concentration, however, the concentration in the overlaying water increased. In the following figures, therefore measured concentrations are shown.

Also for metoprolol, the concentrations in sediment decreased from day 0 to day 28. For both substances, similar trends became obvious in the mixture experiment.

Table 57: Measured concentrations of diclofenac and metoprolol in sediment and overlaying water at the start (day 0) and the end (day 28) of the final tests. (SC = solvent control, n.m. = not measured).

Drug	Nominal concentration (mg/kg dw)	Measured concentrations			
		Day 0		Day 28	
		Sediment (mg/kg dw)	Overlaying water (mg/L)	Sediment (mg/kg dw)	Overlaying water (mg/L)
Diclofenac	SC	0.0077	< 0.00005	n.m.	0.0015
	0.039	0.0112	< 0.00005	< 0.001	0.0031
	20	10.9	n.m.	5.7	n.m.
	320	200	45	105	69.2
Metoprolol	SC	< 0.001	0.00005	n.m.	< 0.00005
	0.039	0.0068	0.00055	< 0.001	0.0003
	20	12.5	n.m.	3.1	n.m.
	320	255	7.4	156	11

Table 58: Measured concentrations of the mixture experiment in sediment and overlaying water at the start (day 0) and the end (day 28) of the final tests. (SC = solvent control, n.m. = not measured)

Drug	Nominal concentration (mg/kg dw)	Measured concentrations			
		Day 0		Day 28	
		Sediment (mg/kg dw)	Overlaying water (mg/L)	Sediment (mg/kg dw)	Overlaying water (mg/L)
Diclofenac	SC	< 0.0010	< 0.00005	n.m.	0.0093
	0.039	0.0185	0.0032	0.0104	0.0055
	20	7.4	n.m.	5.3	n.m.
	320	118	58	77	62
Metoprolol	SC	0.0173	0.00079	n.m.	0.0014
	0.039	0.009	0.0013	0.0082	0.003
	20	9	n.m.	7.7	n.m.
	320	150	28	141	19

4.5 Conclusions

For reproduction and biomass, the calculated effect concentrations summarized in table 2 are far above the environmental concentrations. Even if an appropriate safety factor is used, the resulting PEC/PNEC ratio is below 1, thus indicating negligible risk of the two pharmaceuticals diclofenac and metoprolol (even when applied as a 1:1 mixture) for sediment-dwelling organisms represented by the oligochaete *Lumbriculus variegatus.*

To the best of our knowledge, biomarkers have been applied to this species for the very first time. Despite some drawbacks with respect to the necessity to pool individuals in order to receive the critical protein content for analysis, we succeeded to analyze stress proteins and lipid peroxides in the exposed worms.

The tests on *L. variegatus* showed that it is generally possible to transfer existing methods for the quantification of stress proteins and lipid peroxides to this test organism. However, the investigations also revealed several difficulties, which are mainly due to the small size of these oligochaetes. In order to gain sample sizes that allow a reliable statistical analysis, a much higher number of individuals would be necessary. It remains to test whether this is feasible.

Nevertheless, the first results already revealed apparent, albeit not always statistically significant, influence of the tested pharmaceuticals. While diclofenac showed only weak effects on the tested parameters, metoprolol exposure resulted in some interesting findings. Correlation analyses revealed the degree of lipid peroxidation to be significantly correlated with the concentration of metoprolol; A visibly increased stress protein level without significance became obvious for the lowest tested concentration of metoprolol. However, due to the low sample size and the generally low absolute values, these results should be substantiated by further analyses before drawing final conclusions.

Whereas diclofenac showed only weak effects on the tested parameters, biomarkers indicated tendencies towards elevated stress in *L. variegatus* when exposed to metoprolol at concentrations of 0.039 mg/kg and higher.

4.6 Appendix

Raw data for toxicity tests

Preliminary tests

Table 59: Preliminary 28-days sediment toxicity test with *Lumbriculus variegatus* and diclofenac and metoprolol. Number of individuals per beaker at day 28 (30 individuals per beaker were inserted at day 0). C = control, SC = solvent control

Concentration	Metoprolol Number of individuals	Diclofenac Number of individuals
C	61	82
SC	59	60
0.12	75	65
0.24	58	76
0.49	72	71
0.98	73	83
1.95	71	74
3.91	59	67
7.81	76	67
15.63	67	40
31.25	60	49
62.5	39	64
125	45	28
250	41	27

Concentration	Metoprolol Number of individuals	Diclofenac Number of individuals
500	32	0
1000	0	0

Table 60: Preliminary 28-days sediment toxicity test with *Lumbriculus variegatus* and diclofenac or metoprolol. Total biomass per beaker at day 28 (30 individuals per beaker were inserted at day 0).

Concentration	Diclofenac Total biomass [mg]	Metoprolol Total biomass [mg]
0.24	72.4	51.1
0.48	54.1	50.7
0.98	45.3	25.4
3.91	22.1	53.1
7.81	41.3	33.0
15.63	38.7	54.6
62.50	36.1	18.2
125.00	28.0	21.1
250.00	11.9	13.9
1000.00	0.0	0.0

Final tests

Table 61: Definitive 28-days sediment toxicity test with *Lumbriculus variegatus* and metoprolol. Number of individuals of individuals per beaker at day 28 (10 individuals per beaker were inserted at day 0), light grey = low concentration range, dark grey = high concentration range) C = control, SC = solvent control

Concentration	Replicate Number of individuals			
	I	II	III	IV
C	37	36	41	31
SC	37	37	35	34
0.039	32	31	36	
0.078	37	24		
0.156	25	38	32	
0.312	33	39		
0.62	36	34	40	
1.25	28	34		
2.5	37	35	47	
5	38	32		
10	42	39	38	
20	37	38	32	33
40	35	33	28	32

Concentration	Replicate Number of individuals			
	I	II	III	IV
80	37	28	35	41
160	44	40	43	39
320	25	31	25	28

Table 62: Final 28-days sediment toxicity test with *Lumbriculus variegatus* and diclofenac. Number of individuals of individuals per beaker at day 28 (10 individuals per beaker were inserted at day 0), light grey = low concentration range, dark grey = high concentration range) C = control, SC = solvent control

Concentration	Replicate Number of individuals			
	I	II	III	IV
C	24	29	13	31
SC	20	22	28	20
0.039	28	31	30	
0.078	13	32		
0.156	10	10	18	
0.312	29	33		
0.62	27	14	23	
1.25	27	6		
2.5	21	17	24	
5	35	30		
10	28	12	28	
20	26	26	31	30
40	29	21	25	23
80	26	24	31	29
160	13	20	21	9
320	9	9	6	12

Table 63: Definitive 28-days sediment toxicity test with *Lumbriculus variegatus* and mixture of diclofenac/metoprolol. Number of individuals of individuals per beaker at day 28 (10 individuals per beaker were inserted at day 0, PC = positive control (30 mg/kg pentachlorophenol), light grey = low concentration range, dark grey = high concentration range) C = control, SC = solvent control, PC = positive control

Concentration	Replicate Number of individuals			
	I	II	III	IV
C	28	28	29	27
SC	11	20	24	21
0.039	24	28	27	
0.078	28	30		
0.156	20	19	23	

Concentration	Replicate Number of individuals			
	I	II	III	IV
0.312	30	28		
0.62	16	20	19	
1.25	25	29		
2.5	27	26	22	
5	27	25		
10	20	22	18	
20	28	22	22	24
40	20	20	23	18
80	17	14	19	16
160	13	9	10	9
320	0	0	0	0
PC	0	0	0	0

Table 64: Definitive 28-days sediment toxicity test with *Lumbriculus variegatus* and mixture of diclofenac/metoprolol. Total biomass (all replicates per treatment were pooled) at day 28 (10 individuals per beaker were inserted at day 0, PC = positive control (30 mg/kg pentachlorophenol), light grey = low concentration range, dark grey = high concentration range) C = control, SC = solvent control, PC = positive control

Concentration	Metoprolol Total biomass [mg]	Diclofenac Total biomass [mg]	Mixture Total biomass [mg]
C	118	111	80.8
SC	64.9	86.3	31.8
0.039	40.9	58.5	103
0.078	39.3	24.1	39.7
0.156	34.8	11.2	37.0
0.312	72.3	45.1	33.9
0.62	60.4	14.7	39.4
1.25	40.7	56.1	39.1
2.5	126	38.0	22.9
5	47.2	89.4	30.9
10	50.7	59.1	24.6
20	103	187	89.8
40	115	107	67.2
80	119	127	69.4
160	114	91.8	66.3
320	79.4	16.5	0
PC	*	*	0

*positive control was considered in the mixture experiment only.

B. Raw data for biomarker studies

Table 65: Relative Hsp70-Level of *L. variegatus* exposed to diclofenac. Empty cells indicate that there was no measureable staining of the protein band. LMK = solvent control

Sample ID	Substance	Concentration [mg/kg dw]	Hsp-Level [Rel. Grey value]
DF LMK 1-3	Diclofenac	LMK	0.0938
DF LMK 4-6	Diclofenac	LMK	0.0627
DF LMK 7-9	Diclofenac	LMK	0.0245
DF LMK 10-12	Diclofenac	LMK	0.1139
DF LMK 13-15	Diclofenac	LMK	0.1887
DF 0,03 1-3	Diclofenac	0.039	0.0872
DF 0,03 4-6	Diclofenac	0.039	0.0745
DF 0,03 7-9	Diclofenac	0.039	0.1015
DF 0,03 10-12	Diclofenac	0.039	0.1450
DF 0,03 13-15	Diclofenac	0.039	0.4740
DF 0,15 1-3	Diclofenac	0.156	0.0687
DF 0,15 4-6	Diclofenac	0.156	0.1600
DF 0,15 7-9	Diclofenac	0.156	0.2081
DF 0,15 10-12	Diclofenac	0.156	0.3814
DF 0,15 13-15	Diclofenac	0.156	0.0937
DF 0,6 10-12	Diclofenac	0.625	0.0888
DF 0,6 1-3	Diclofenac	0.626	0.0634
DF 0,6 13-15	Diclofenac	0.627	0.2389
DF 0,6 4-6	Diclofenac	0.628	0.1096
DF 0,6 7-9	Diclofenac	0.629	0.1356
DF 2,5 1-3	Diclofenac	2.5	0.0015
DF 2,5 4-6	Diclofenac	2.5	0.2500
DF 2-5 7-9	Diclofenac	2.5	0.1058
DF 2,5 10-12	Diclofenac	2.5	0.1618
DF 2,5 13-15	Diclofenac	2.5	0.0742
DF 10 1-3	Diclofenac	10	0.0361
DF 10 4-6	Diclofenac	10	0.0591
DF 10 7-9	Diclofenac	10	0.0147
DF 10 10-12	Diclofenac	10	
DF 10 13-15	Diclofenac	10	0.8651

Table 66: Relative Hsp70-Level of *L. variegatus* exposed to metoprolol. Empty cells indicate that there was no measureable staining of the protein band. LMK = solvent control

Sample ID	Substance	Concentration [mg/kg dw]	Hsp-Level [Rel. Grey value]
MP LMK 1-3	Metoprolol	LMK	0.1078
MP LMK 4-6	Metoprolol	LMK	
MP LMK 7-9	Metoprolol	LMK	0.0212
MP LMK 10-12	Metoprolol	LMK	0.0151
MP LMK 13-15	Metoprolol	LMK	0.0481
MP 0,03 1-3	Metoprolol	0.039	0.2683
MP 0,03 4-6	Metoprolol	0.039	0.2713
MP 0,03 7-9	Metoprolol	0.039	0.1624
MP 0,03 10-12	Metoprolol	0.039	0.0898
MP 0,03 13-15	Metoprolol	0.039	0.3685
MP 0,15 1-3	Metoprolol	0.156	0.0859
MP 0,15 4-6	Metoprolol	0.156	0.0128
MP 0,15 7-9	Metoprolol	0.156	0.1314
MP 0,15 10-12	Metoprolol	0.156	0.2757
MP 0,15 13-15	Metoprolol	0.156	0.0357
MP 0,6 1-3	Metoprolol	0.625	0.0023
MP 0,6 4-6	Metoprolol	0.626	0.0035
MP 0,6 7-9	Metoprolol	0.627	0.0310
MP 0,6 10-12	Metoprolol	0.628	0.1763
MP 0,6 13-15	Metoprolol	0.629	0.0105
MP 2,5 1-3	Metoprolol	2.5	0.0002
MP 2,5 4-6	Metoprolol	2.5	0.0756
MP 2,5 7-9	Metoprolol	2.5	0.2686
MP 2,5 10-12	Metoprolol	2.5	0.0473
MP 2,5 13-15	Metoprolol	2.5	
MP 10 1-3	Metoprolol	10	0.0284
MP 10 4-6	Metoprolol	10	0.0114
MP 10 7-9	Metoprolol	10	0.1972
MP 10 10-12	Metoprolol	10	
MP 10 13-15	Metoprolol	10	

Table 67: Level of lipid peroxidation (in cumene hydroperoxide equivalents per mg wet weight) of *L. variegatus* exposed to diclofenac. LMK = solvent control

Substance	Concentration [mg/kg dw]	ID	Sample mass [g]	Lipid peroxidation [CHPequiva-lents/mg ww]
Diclofenac	LMK	16-18	0.0173	36.6791
Diclofenac	LMK	19-21	0.0108	16.7215
Diclofenac	LMK	22-24	0.0178	15.7107
Diclofenac	LMK	25-27	0.0226	16.6131
Diclofenac	LMK	28-30	0.0162	17.8645
Diclofenac	0.039	16-18	0.0185	26.4732
Diclofenac	0.039	19-21	0.0315	20.2111
Diclofenac	0.039	22-24	0.0165	19.0043
Diclofenac	0.039	25-27	0.0144	15.2311
Diclofenac	0.039	28-30	0.0143	15.8915
Diclofenac	0.156	16-18	0.0174	25.1039
Diclofenac	0.156	19-21	0.0171	21.7321
Diclofenac	0.156	22-24	0.0138	28.7774
Diclofenac	0.156	25-27	0.0162	15.5776
Diclofenac	0.156	28-30	0.0184	19.1478
Diclofenac	0.625	16-18	0.0200	21.4327
Diclofenac	0.626	19-21	0.0210	25.4391
Diclofenac	0.627	22-24	0.0151	19.1476
Diclofenac	0.628	25-27	0.0197	14.4526
Diclofenac	0.629	28-30	0.0168	13.5886
Diclofenac	2.500	16-18	0.0108	28.7004
Diclofenac	2.500	19-21	0.0087	26.0328
Diclofenac	2.500	22-24	0.0175	18.4504
Diclofenac	2.500	25-27	0.0245	18.2857
Diclofenac	2.500	28-30	0.0232	20.2654
Diclofenac	10.000	16-18	0.0107	20.9553
Diclofenac	10.000	19-21	0.0099	31.1813
Diclofenac	10.000	22-24	0.0092	20.0511
Diclofenac	10.000	25-27	0.0145	20.5711
Diclofenac	10.000	28-30	0.0108	20.0827

Table 68: Level of lipid peroxidation (in cumene hydroperoxide equivalents per mg wet weight) of *L. variegatus* exposed to metoprolol. LMK = solvent control

Substance	Concentration [mg/kg dw]	ID	Sample mass [g]	Lipid peroxidation [CHPequiva-lents/mg ww]
Metoprolol	LMK	16-18	0.0194	18.9920
Metoprolol	LMK	19-21	0.0217	18.2840
Metoprolol	LMK	22-24	0.0358	22.0172
Metoprolol	LMK	25-27	0.036	22.8320
Metoprolol	LMK	28-30	0.042	21.3315
Metoprolol	0.039	16-18	0.0111	16.8919
Metoprolol	0.039	19-21	0.0188	19.4359
Metoprolol	0.039	22-24	0.0186	20.6096
Metoprolol	0.039	25-27	0.0162	18.1818
Metoprolol	0.039	28-30	0.0234	19.8424
Metoprolol	0.156	16-18	0.0204	20.0511
Metoprolol	0.156	19-21	0.0152	19.8680
Metoprolol	0.156	22-24	0.0147	22.6634
Metoprolol	0.156	25-27	0.0186	21.5833
Metoprolol	0.156	28-30	0.0185	20.2693
Metoprolol	0.625	16-18	0.0214	19.6532
Metoprolol	0.626	19-21	0.0152	20.2346
Metoprolol	0.627	22-24	0.0161	20.8648
Metoprolol	0.628	25-27	0.0178	19.0045
Metoprolol	0.629	28-30	0.0169	19.6875
Metoprolol	2.5	16-18	0.0265	21.0922
Metoprolol	2.5	19-21	0.0149	21.9381
Metoprolol	2.5	22-24	0.0155	22.8144
Metoprolol	2.5	25-27	0.0184	22.8346
Metoprolol	2.5	28-30	0.0138	21.8236
Metoprolol	10	16-18	0.017	21.2610
Metoprolol	10	19-21	0.0196	20.3448
Metoprolol	10	22-24	0.0217	23.0334
Metoprolol	10	25-27	0.0272	25.4735
Metoprolol	10	28-30	0.0191	19.3443

5 WP 5 Toxicity of pharmaceuticals in the field (WWTP exposure) (Part II)

5.1 Studies with juvenile trout

5.1.1 Introduction

Since the envisaged *in vitro*-assays shall also be applicable for chemical mixtures and complex matrices, validation with respect to effects under field conditions is necessary. We therefore investigated the influence of a wastewater treatment plant effluent - a highly complex mixture of different chemicals in low concentrations – on fish development.

5.1.2 Materials and Methods

Freshly fertilized eggs of brown trout (origin: Forellenzucht Lohmühle, Am Lohmühlebach 85, 72275 Alpirsbach-Ehlenbogen) were exposed to the effluent of the wastewater treatment plant Eriskirch (Gmünd 2, 88097 Eriskirch) in a continuous flow-through system (Figure 124). The effluent water was directed into a 250 L glass aquarium for aeration and temperature adjustment, and subsequently passed into the exposure aquarium. In the exposure aquarium, six steel cages made of perforated metal plate were placed to keep the embryos. In each steel cage, 50 eggs were exposed, thus, an overall number of 300 eggs were used per treatment. As a control, a set up at the "ISF – Institut für Seenforschung" (Argenweg 50/1, 88085 Langenargen) was established, where an aquarium was supplied with water directly taken from the Lake of Constance. Eggs developed in darkness until the stadium of eye pigmentation was reached. Temperature and oxygen content were checked regularly. An additional experiment was conducted in the laboratory where both, controls and effluent-treatments were kept under identical conditions in a climate chamber.

Figure 124: Exposure design of brown trout eggs/larvae directly at the wastewater treatment plant and control station at the Lake of Constance. Eggs were placed in metal sieves (left) within a flow-through system. At the wastewater treatment plant, a second aquarium connected the effluent and exposure aquarium to adjust the temperature and oxygen content (right).

The lab experiment started on December 15th 2014 and followed the instructions already described in 1.2.2: Eggs were exposed in 200 mL petri dishes that either contained cooled, aerated effluent, or aerated artificial water as a control. The whole experiment was performed in a thermo-constant chamber

in which temperature was adjusted to 7 °C. Eggs were kept in darkness until eye pigmentation was visible (Figure 125). The recorded parameters match those described in 1.2.2.

Figure 125: Exposure design of brown trout eggs/larvae to wastewater treatment plant effluents in the lab. Eggs were kept in petri dishes (left) and developed in complete darkness (right) until the stadium of eye pigmentation.

5.1.3 Results

Chemical analyses on effluent samples at selected time points revealed concentrations of 1.44 µg/L diclofenac and 1.39 µg/L metoprolol in the effluent and concentrations below the detection limit at the control station.

Overall, it became obvious that the eggs exposed at the wastewater treatment plant developed faster than those exposed to the water of Lake of Constance. Eggs exposed in the lab developed even slower – here, the hatching was only beginning at the end of February 2015. At that time, all surviving embryos in the field were already hatched. Sampling took place on March 31st at the wastewater treatment plant and April 14th 2015 at the control station, the lab exposures ended on May 13th 2015. At the wastewater treatment plant, one of the six replicates was lost due to a leak in the steel cage that allowed the larvae to escape. This cage was excluded from the evaluation of the relevant parameters.

The heart beat rate of the larvae was generally higher than in the lab experiments with metoprolol and diclofenac, and the lab experiments with wastewater effluents. There was neither a visible difference between the effluent exposure and the control at the Lake of Constance, nor between the lab exposure and the lab control (Figure 127).

In the lab experiment, hatching began around 70 days post fertilization and was finished 84 days post fertilization. The embryos of the WWTP treatment hatched slightly faster than in the control. There were no differences in mortality (Figure 128), nor in the time of eye pigmentation.

Figure 126: Hatching rate (relative to the total number of exposed individuals) of brown trout exposed to wastewater treatment plant effluent in the lab. Hatching started 70 dpf and was finished 84 dpf. There were no clear differences between control and effluent.

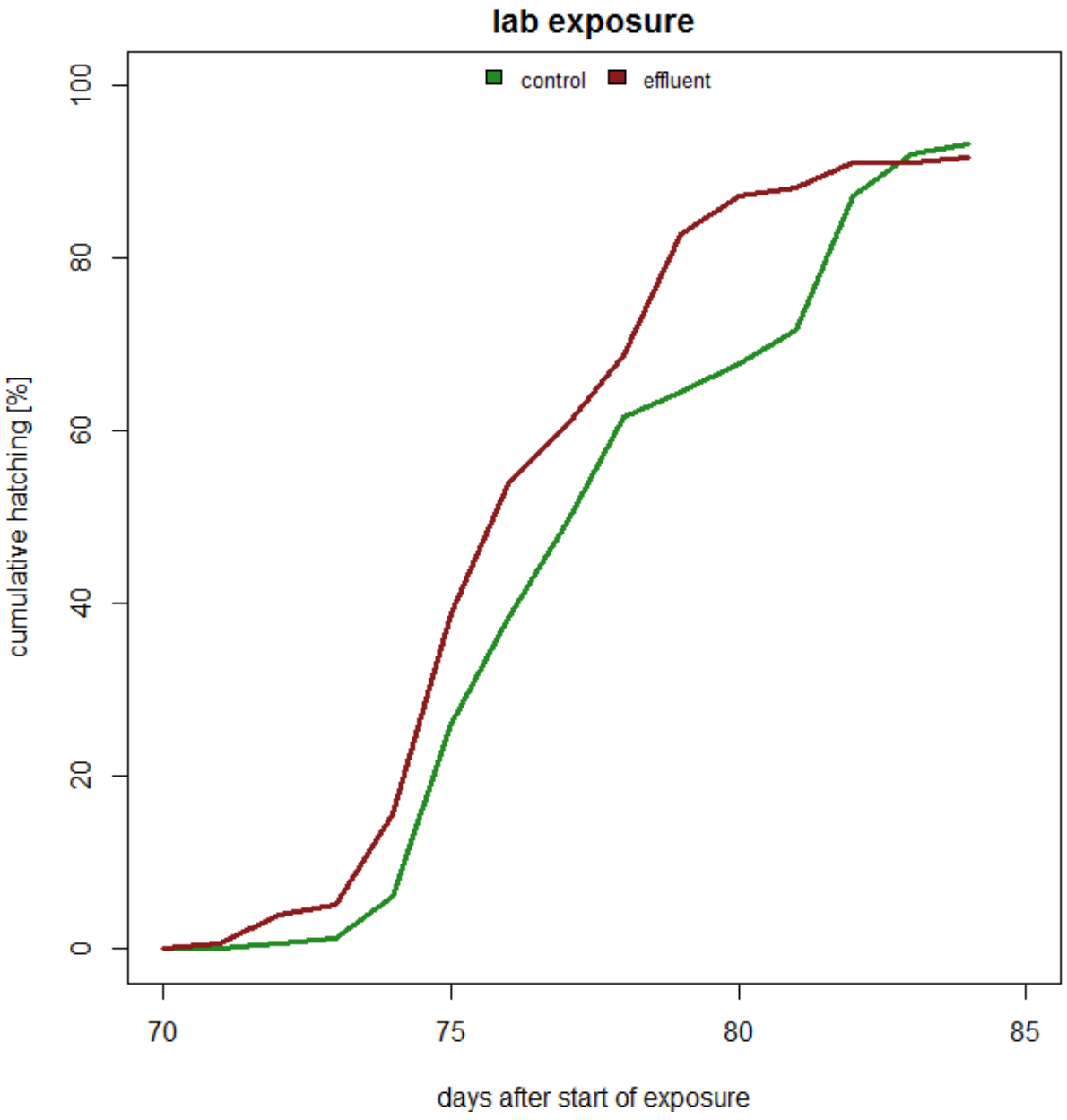


Figure 127: Heart beat rate one week post hatch of brown trout exposed to wastewater treatment plant effluents in field stations (top) and lab (bottom). Bold lines display the median values, boxes the 25% to 75% quantiles, whiskers the minimum and maximum values, circles potential outliers. In the field, the heart beat rate is generally higher and shows greater variability. There are no differences between effluent and control.

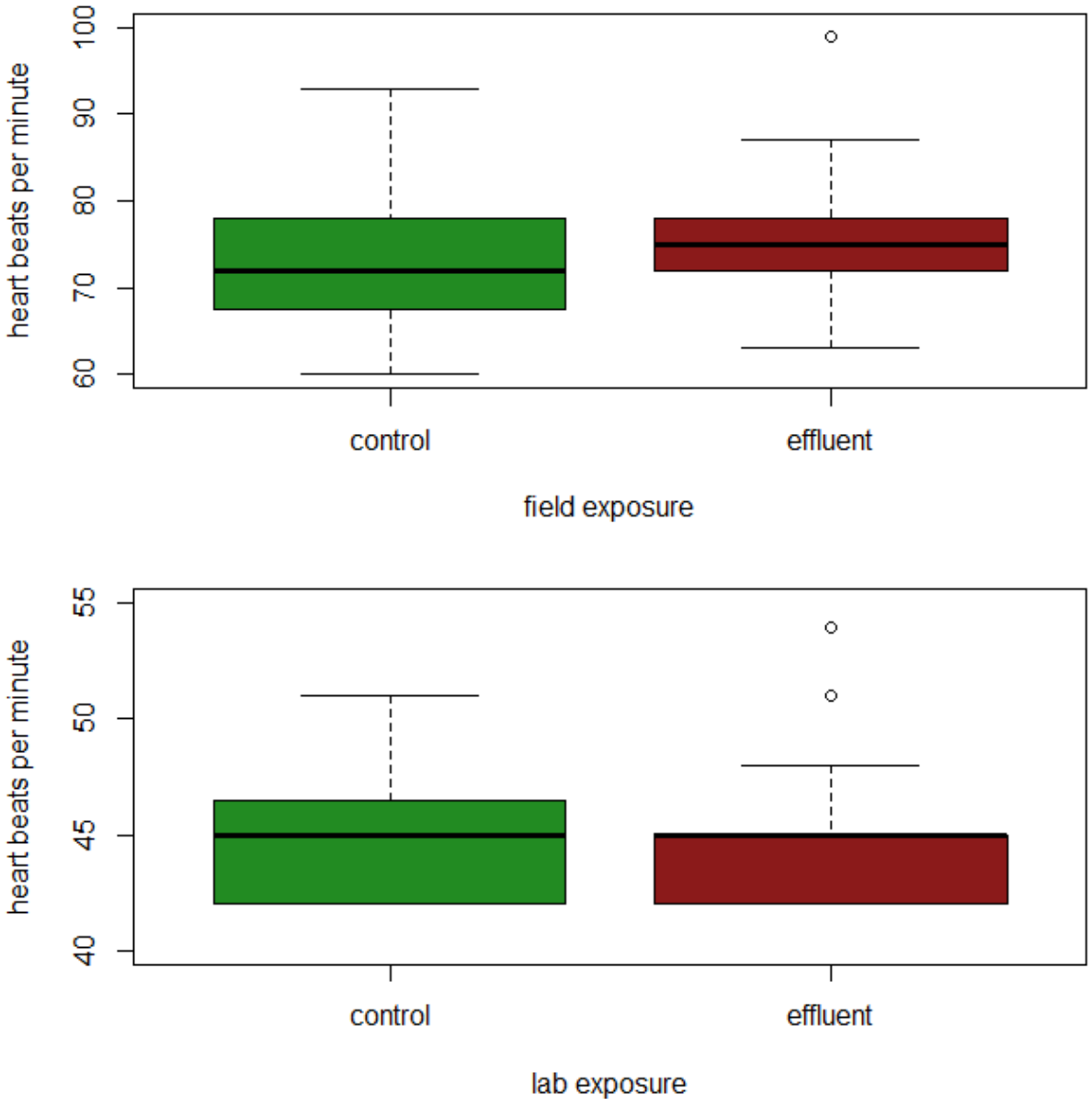


Figure 128: Cumulative mortality during the exposure of brown trout larvae to wastewater treatment plant effluents directly at the station (top) or in a controlled lab setting (bottom). The larvae in the field developed faster, so their exposure was terminated at an earlier point. There were no clear differences between the effluent and control.

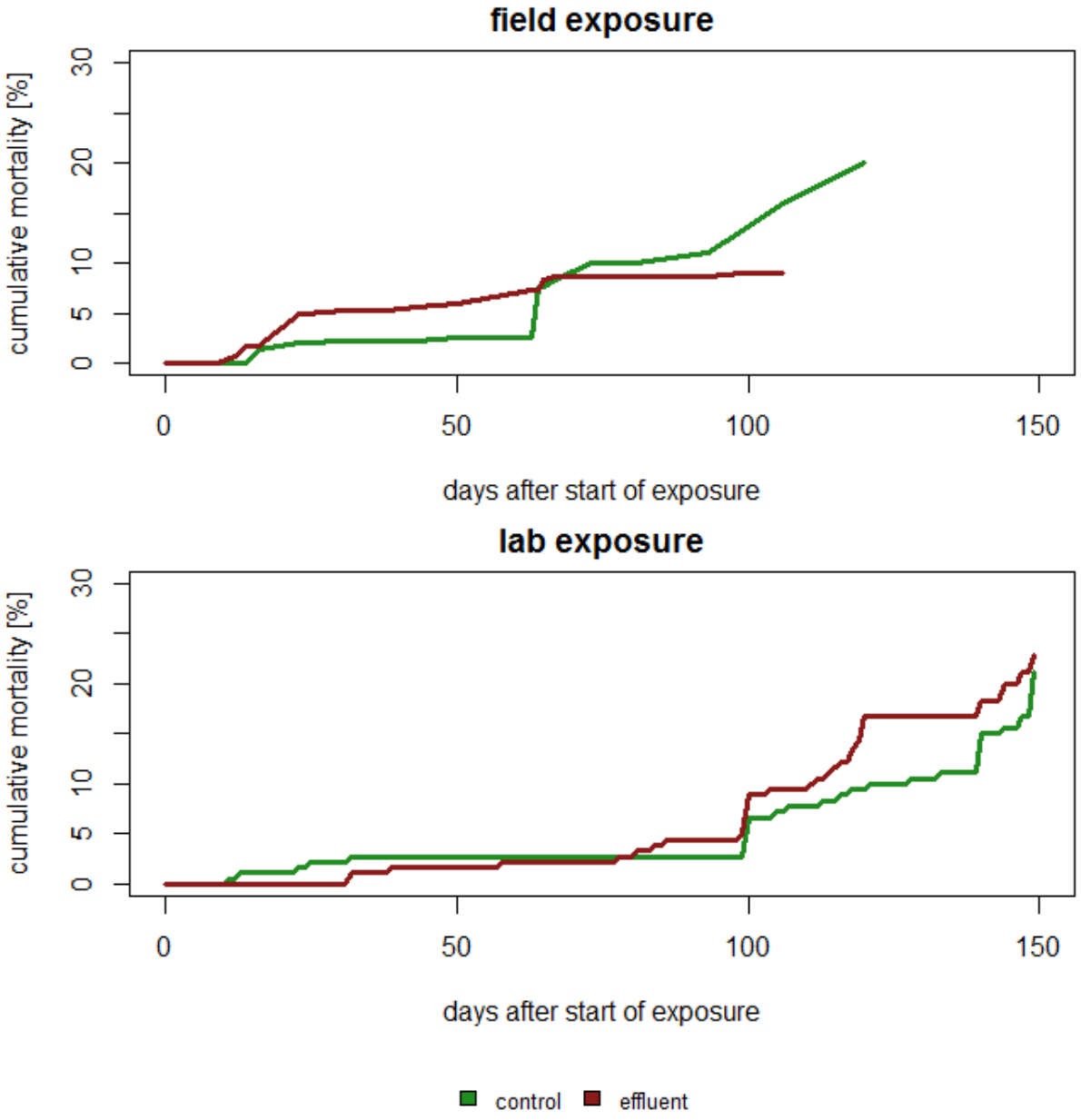
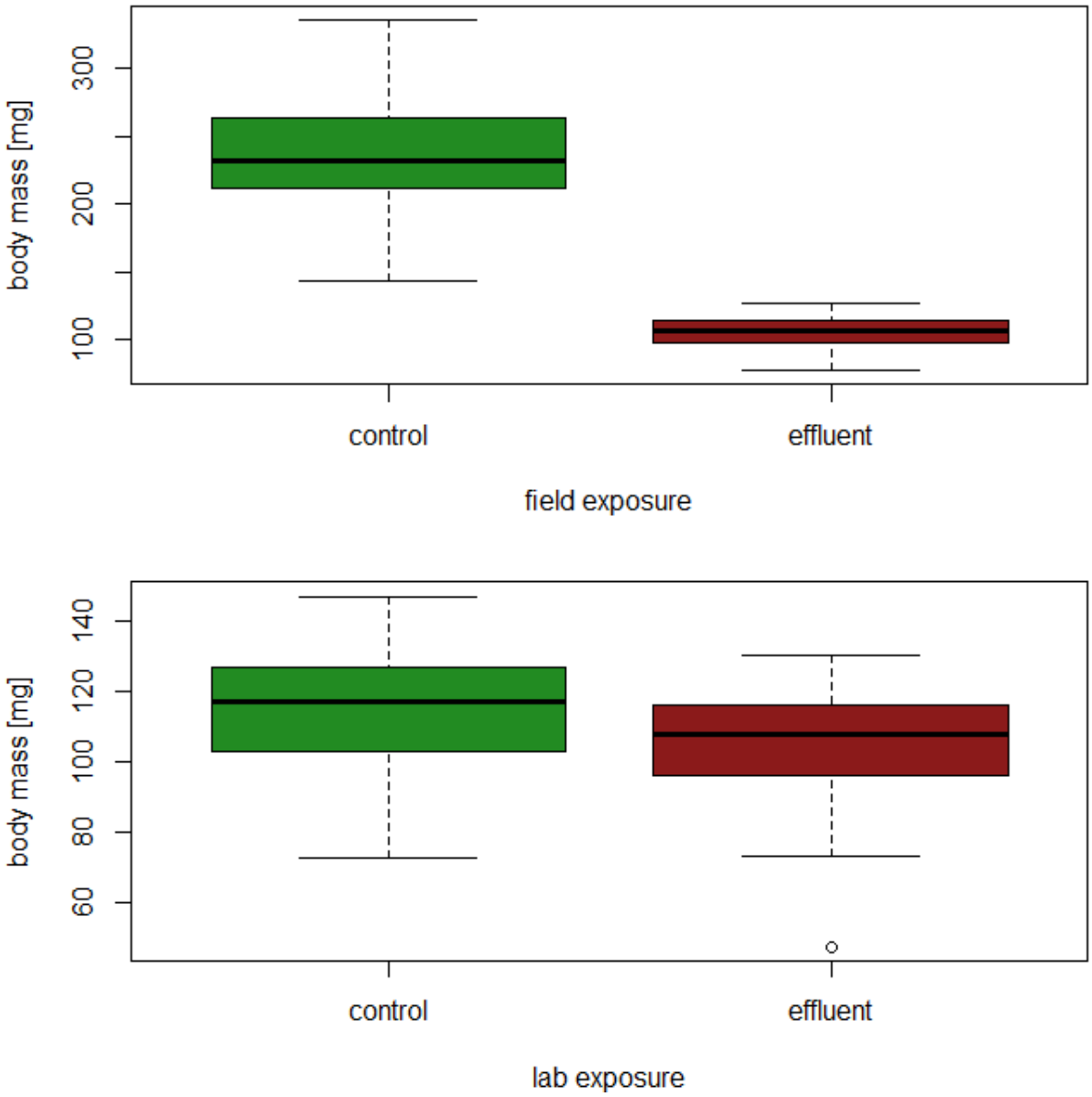


Figure 129: Body mass of brown trout larvae exposed to wastewater treatment plant effluents at sampling time. Bold lines display the median values, boxes the 25% to 75% quantiles, whiskers the minimum and maximum values, circles potential outliers. In the field exposure (top), samples were taken at different time points, so the difference is not due to the effluent itself. In the lab exposure (bottom), effluent treatment had a negative effect on embryo mass (linear model, df=1/138, F=17.42, p<0.0001).



Considering the mass of the larvae at sampling time, samples from effluent treatments were smaller than control samples. A statistical evaluation was only meaningful for the lab experiment, since the field exposure and field control developed with different speed and were sampled at different time points. Control larvae averaged at 115.5 mg and effluent larvae at 105.5 mg (linear model, df=1/138, F=17.42, p<0.0001), the test vessel had no noticeable influence (Figure 129).

Samples from the wastewater exposure showed a mean of 5.0 CHP-equiv./mg ww, while the control larvae from Lake of Constance displayed a lower mean of 4.34 CHP-equiv./mg ww. This slightly increased level of lipid peroxides proved to be significant (linear model, df=1/141, F=6.827, p=0.0995), while the test vessel had no influence. For the lab exposure, an opposite effect was visible. With a mean

of 3.79 CHP-equiv./mg ww, compared to 4.67 CHP-equiv./mg ww, the samples from the effluent had a lower degree of lipid peroxides than the control (linear model, df=1/138, F=42.714, p=0.0018). Neither the test vessel nor the sample mass had an influence on this parameter. Data are illustrated in Figure 130.

Overall, the larvae were in a good histological state. There were no histological differences to be found between the control and the effluent-exposed larvae. Most individuals were in a control state, few showed mild to moderate reactions. Observed peculiarities were vacuolisations 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. In two cases, necrosis of single cells was observed. The findings are illustrated in Figure 131.

Figure 130: Lipid peroxides in brown trout larvae exposed to wastewater treatment plant effluents directly at the station (top) or the lab (bottom). Bold lines display the median values, boxes the 25% to 75% quantiles, whiskers the minimum and maximum values, circles potential outliers. In the field, the heart beat rate is generally higher and shows greater variability. In the field, effluent treatment increased the level of lipid peroxides while in the lab, larvae from effluents showed a slightly reduced level of lipid peroxides.

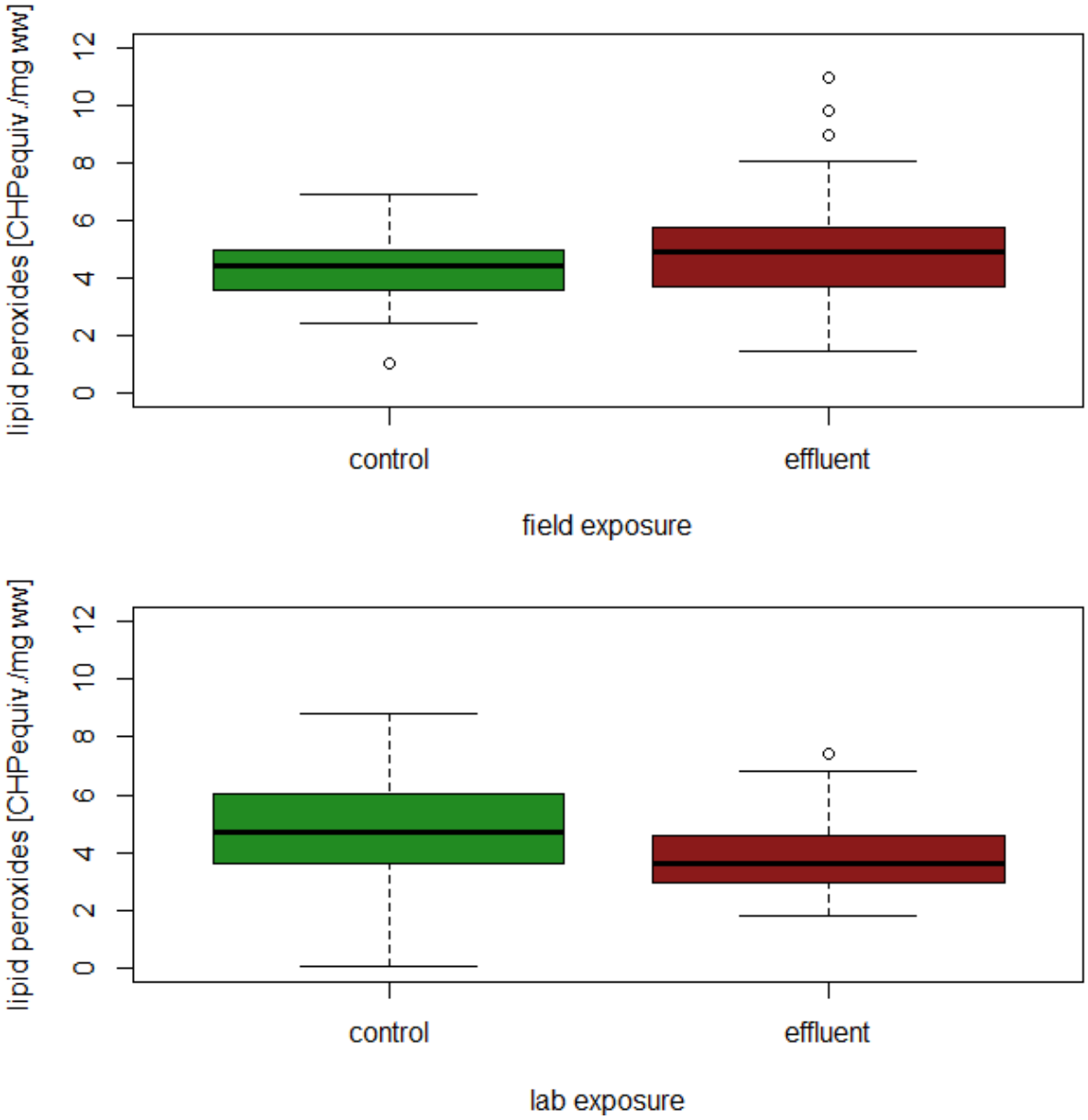
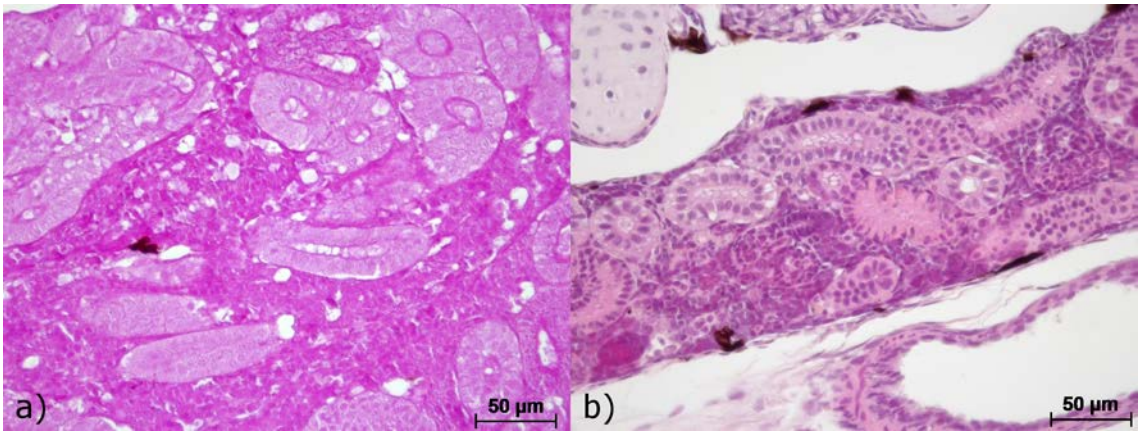


Figure 131: Histological sections of brown trout embryos exposed to wastewater treatment plant effluent (field experiment): a) kidney, control (AB-PAS): reactions in hematopoietic tissue. B) kidney, effluent (HE): vacuolization in tubular cells.



5.1.4 Discussion

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 pharmaceutical exposure and the wastewater exposure in the lab. While hatching in the pharmaceutical exposure was finished after 70 days, it took 84 days for the wastewater exposure. These experiments took place in separate thermoconstant chambers – whose mean temperatures differed by ca. 1 °C. This further illustrates the strong effect of even slight temperature differences on trout development.

The apparent difference in mortality between the field effluent and control during the last days of exposure is difficult to evaluate. At this time, the effluent was already sampled, but the control animals had not reached the necessary life stage.

In general, a direct comparison of field effluent and field control is problematic, due to a variety of possible confounding factors. Besides the chemical stress through remaining pollutants in the water, the temperature differences between both treatments could play a major role. The lab experiment can be viewed as more reliable. Here, the concentration of chemical pollutants was the only difference between the treatments, while temperature, light and other environmental factors were the same. This allows us to draw a clearer conclusion on the effects of wastewater on trout embryos.

The most interesting effect of the effluent exposure 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, we 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.

Regarding the results on lipid peroxides, there are two possible explanations for the reduced level in the effluent samples. On the one side, certain substances in wastewater (e.g. vitamins, pharmaceuticals) 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 hand, the vast diversity of different trace substances in the effluent could induce the overall oxidative stress response of the organism. In contrast, organisms raised in pristine water containing no more than the essential electrolytes, have less reason for this induction. Increased levels of antioxidant enzymes would reduce the effects of oxidative stress, 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, this hypothesis is

plausible. Future studies could investigate this more closely by having a look on the activity of antioxidative enzymes like catalase, ascorbate-peroxidase or superoxide dismutase.

5.2 Studies with gammarids

5.2.1 Toxicity tests

5.2.1.1 Materials and Methods

As an example for toxicity of pharmaceuticals in the field gammarids were exposed to a wastewater treatment plant (WWTP) effluent. Effects of a variety of pharmaceuticals and other micropollutants in the WWTP effluent on reproductive endpoints in *G. fossarum* were investigated. As a control set-up gammarids were exposed to natural stream water in Tännichtgrundbach, further on referred to as stream.

Individuals of *G. fossarum* for the exposure at the wastewater treatment plant (WWTP Eriskirch) were sampled in Tännichtgrundbach (Chapter 1.3) at two days (April 15th, 16th; 2015). Sampling technique, culture conditions, food preparation and selection of individuals for the experimental set-up were done as described above (Chapter 1.3). Transport of the gammarids to Tübingen (journey time: 8hrs) and further on to Eriskirch took place in plastic vessels along with conditioned leaves (see above, Chapter 1.3) filled with Borgmann medium (particle free and active carbon filtered tap water with additives LO4-S and E + H as described by Borgmann (1996)) which was continuously aerated (mobile aeration unit, Marina Germany). The vessels were placed in a cool box together with cooling elements. For analysis of stress proteins and lipid peroxides at the University of Tübingen 100 individuals from the original population of gammarids were frozen in liquid nitrogen at the beginning of the experiment in Tübingen. Subsequently, the vessels with the gammarids were stored in a refrigerator overnight (15 °C) and transport continued next day to the WWTP in Eriskirch.

The exposure of *G. fossarum* to WWTP effluent was started on April 21st, 2015 and ended 40 days later on June 2nd, 2015. A number of 11 glass tubes (diameter: 3.5 cm, length: 25 cm) with 20 gammarids, respectively, were exposed to the WWTP effluent in a continuous flow-through system (for detailed information see Chapter 5.1 (see above, Chapter 1.3) from *A. glutinosa* (10 per tube) were added and the tubes were then closed with nets (mesh size: 0.5 mm) on each side. Finally, the tubes were placed in the exposure aquarium (Figure 132).

Figure 132: Arrangement of 11 glass tubes, each containing 20 individuals of *G. fossarum* and 10 conditioned leaves of *A. glutinosa* in the exposure aquarium in the WWTP Eriskirch.

Control of the glass tubes took place biweekly (M. Weyhmüller (Biologiebüro Weyhmüller) - responsible for the setup and maintenance of the flow-through systems at Eriskirch and Langenargen) and if necessary, conditioned leaves consumed by the gammarids were replaced by newly conditioned leaves. A multi parameter sensor (Hach/Hydrolab MS5) measured continuously pH, conductivity as well as temperature, oxygen concentration and saturation during the exposure experiment in WWTP effluent.

The multi parameter sensor was temporary turned off due to voltage peaks in the power supply system. Hence, data for physico-chemical parameters in WWTP Eriskirch are just provided for a period between the beginning of the experiment (April 21st, 2015) to April 24th, 2015, and then again between May 7th, 2015, to one week before the end of the experiment (May 24th, 2015). Nevertheless, according to M. Weyhmüller the installed aeration equipment in the flow-through system worked properly. However, at some point errors occurred when measuring oxygen concentration and saturation identified by not reasonable high values. This is probably due to the fact that the galvanic sensor provides values that are too high when higher amounts of iron ions are present in the effluent because of phosphate precipitation in the WWTP. It can be assumed that oxygen saturation values above 105% are not valid (according to M. Weyhmüller). For this reason, oxygen saturation values higher than 105% and the respective oxygen concentrations were excluded, i.e. from a total of 2065 measured values only 805 values were used for calculation of median, minimum and maximum of oxygen saturation and concentration in WWTP effluent.

The exposure of *G. fossarum* in a natural stream started on April 29th, 2015 and ended 40 days later on June 8th, 2015. The control set-up has been established at the Tännichtgrundbach, where boxes made of stainless steel were placed in the stream (Figure 133, for detailed information of stainless steel boxes see Jungmann et al., 2004) including 5 and 6 glass tubes, respectively. Each end and the top of the metal boxes are provided with a metal grid to enable stream water to flow through. Identically equal to exposure in WWTP effluent the glass tubes contained 10 conditioned leaves of alder and 20 individuals of *G. fossarum* which were sampled and selected for respective size-class as described above (Chapter 1.3).

At the beginning of the control experiment in the stream 85 individuals from the original population were frozen in liquid nitrogen for the stress protein and lipid peroxides quantification conducted by the University of Tuebingen. Glass tubes were controlled weekly and consumed alder leaves were replaced by newly conditioned leaves if necessary. The physico-chemical parameters pH (WTW pH3110, SenTix81), conductivity (WTW LF340, TetraCon325) as well as temperature, oxygen concentration and saturation (WTW CellOx355, Oxi340i) were measured at day 22 of the exposure and at the end of the experiment.

Figure 133**:** Top: Stainless steel boxes closed with metal grids; bottom: stainless steel boxes in the stream containing 5 and 6 glass tubes respectively as a control set-up, glass tubes including 20 individuals of *G. fossarum* and 10 conditioned leaves of *A. glutinosa*.

At the end of both exposure experiments mortality and precopulae was recorded in all glass tubes (11). Then, 100 individuals (WWTP effluent) and 70 individuals (stream), respectively, from 5 randomly chosen glass tubes were frozen in liquid nitrogen for analysis of stress proteins and lipid peroxides carried out by the University of Tübingen. Samples were stored at -80 °C (Sanyo Ultra Low) and shipped on dry ice to the University of Tübingen. From 6 randomly chosen glass tubes 112 individuals (WWTP effluent) and 52 individuals of *G. fossarum* (stream), respectively, were fixed in formaldehyde (Merck, 37%) in order to determine number of egg-bearing females as well as the number of eggs per egg-bearing female. With a stereomicroscope (Thalheim Spezial Optik) number of egg-bearing females and number of eggs per egg-bearing female were determined.

For the evaluation of concentrations of pharmaceuticals in biota, conducted by TZW Karlsruhe, 40 gammarids from the 2 remaining glass tubes of WWTP effluent were fixed in formaldehyde. In the control set-up in the stream 40 of the 52 gammarids used for reproduction analysis were then also used to evaluate the concentrations of pharmaceuticals in biota. After determining their fresh weight gammarids were freeze-dried for 24 h (Alpha 1-2, Fa. Christ). Dry weight was determined and after grinding (mortar and pestle) samples were sent to TZW Karlsruhe. Data are shown in Chapter 6.3.

Statistical analysis was conducted using the statistical software R (version 3.1.1). Data were checked for normal distribution using Shapiro-Wilks test as well as for homogeneity of variance using Fligner

Killeen-Test. Significant differences were determined using Kruskal-Wallis-test, respectively. The significance level was set to α=0.05.

5.2.1.2 Results

Physico-chemical parameters

During exposure of gammarids to WWTP effluent, measured pH values ranged between 5.0 and 7.1 with a median of 6.1 (Table 69). In the stream measured pH values were 6.7 at day 22 of the exposure and 7.6 at the end of the control experiment. The median values for conductivity ranged between 580 and 1659 µS/cm in WWTP effluent. Conductivity values in the stream were 731 and 732 µS/cm at both days. In the WWTP effluent, the median value for oxygen concentration was 9.04 mg/L, the lowest measured value was 7.24 mg/L and the highest 10.26 mg/L. The measured values of oxygen concentrations in the stream were 12 mg/L and 13.2 mg/L. The oxygen saturation in WWTP effluent ranged between 77% and 105% with a median of 93%. The measured oxygen saturation values in the stream were 86 and 92%.

Table 69: Overview of physico-chemical parameters recorded during long-term exposure in WWTP effluent and control set-up stream. Medians are displayed along with minima and maxima (in parentheses) for WWTP Eriskirch.

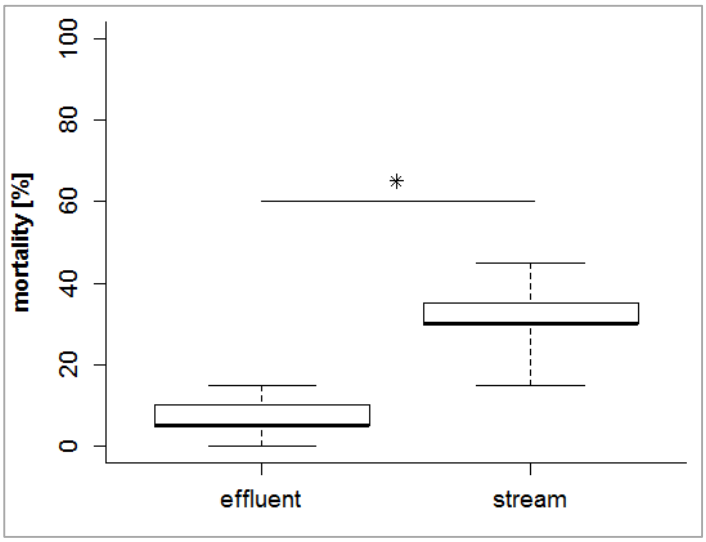
Parameter	Unit	WWTP effluent Median (min; max)	Stream	
			20.05.2015	08.06.2015
pH		6.1 (5.0; 7.1)	6.7	7.6
Conductivity	µS/cm	1053 (580; 1659)	731.0	732.0
Temperature	°C	14.35 (12.7; 16.5)	12.0	13.2
Oxygen concentration	mg/L	9.04 (7.2; 10.2)	9.2	9.6
Oxygen saturation	%	93 (77; 105)	86.0	92.0

Effects on mortality and reproduction

In the WWTP effluent the median of mortality was 5% with a minimum of 0 and a maximum of 15% (Figure 134). The mortality in the stream was significantly higher (Kruskal-Wallis-test, α=0.05) with a median of 30%, minimum of 15 and a maximum of 45%.

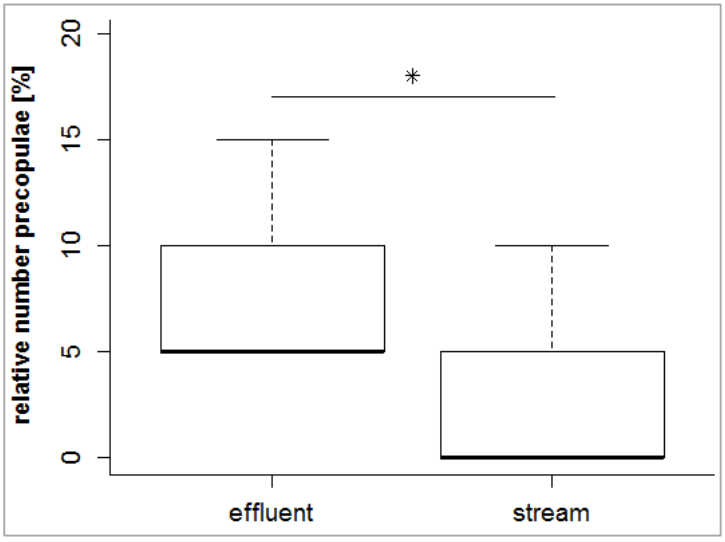
First control of the glass tubes in the stream took place one week after the start of exposition. In 2 of 11 glass tubes only 1 and 2 of 20 gammarids, respectively, were found. It can be assumed that these glass tubes were not completely tight so the gammarids were able to escape. Hence, these 2 glass tubes from the stream were excluded from analysis. For this reason 2 other glass tubes from the stream were used for both reproduction analysis and evaluation of concentrations of pharmaceuticals in biota.

Figure 134: Mortality of *G. fossarum* after exposure to WWTP effluent (n= 205) and stream (n=121). Medians are displayed along with 25 and 75% percentiles and minimum and maximum values. * Significant differences between sampling sites (Kruskal-Wallis-test, α=0.05).



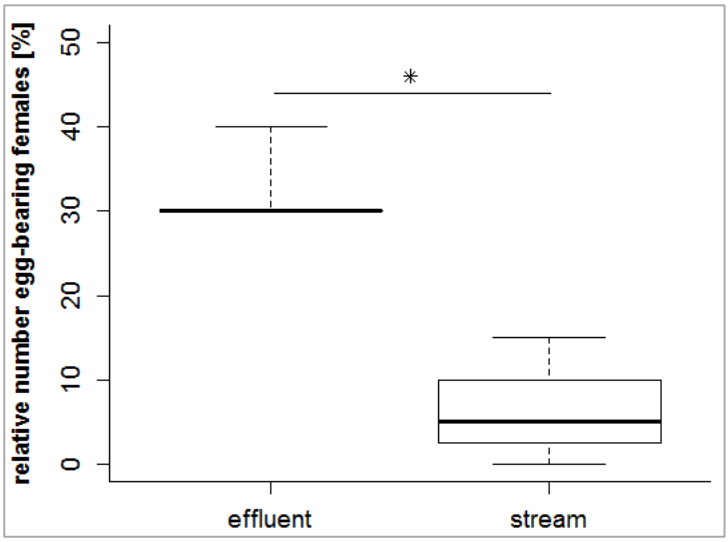
The median of the relative number of precopulae in WWTP effluent was 5%, as well as the minimum, and the maximum was 15% (Figure 135). The median of relative number of precopulae in the stream was 0% as well as the minimum, the maximum was 10%, which was significantly different compared to WWTP effluent (Kruskal-Wallis-test, α=0.05).

Figure 135: Relative number of precopulae of *G. fossarum* after exposure to WWTP effluent (n = 76) and stream (n = 52), respectively. Medians are displayed along with 25 and 75% percentiles and minimum and maximum values. * Significant differences between sampling sites (Kruskal-Wallis-test, α=0.05).



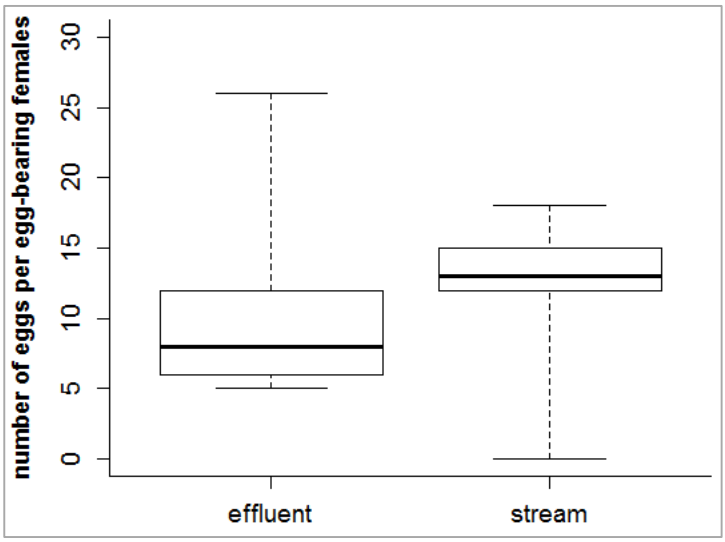
The median of the relative number of egg-bearing females in WWTP effluent was found to be 30% (Figure 136). Minimum value was also 30% and maximum was 40%, as in all glass tubes from WWTP effluent 6 egg-bearing females were found, except for one tube where 8 egg-bearing females were found. In the stream, the relative number of egg-bearing females ranged between 0 and 15%, with a median of 5%. The comparison of WWTP effluent and stream showed significant differences in relative number of egg-bearing females (Kruskal-Wallis-test, α=0.05).

Figure 136: Relative number of egg-bearing females of *G. fossarum* after exposure to WWTP effluent (n = 76) and stream (n = 52), respectively. Medians are displayed along with 25 and 75% percentiles and minimum and maximum values. * Significant differences between sampling sites (Kruskal-Wallis-test, α=0.05).



The median of the absolute number of eggs per egg-bearing female in WWTP effluent was 8 with a minimum of 5 and a maximum of 26 (Figure 137). In the stream, the median was 13 with a minimum of 0 and a maximum of 18. Statistical evaluation regarding the number of eggs per egg-bearing female was not possible due to the poor distribution of the data at both sampling sites. Nevertheless, a tendency towards fewer eggs in the WWTP effluent is indicated.

Figure 137: Number of eggs per egg-bearing female of *G. fossarum* after exposure to WWTP effluent (n= 24) and stream (n = 5). Medians are displayed along with 25 and 75% percentiles and minimum and maximum values.



Due to the fact that juveniles were able to escape through the fine mesh sieve at each side of the glass tubes no precise indications could be provided about the total number of juveniles in the respective glass tubes.

5.2.1.3 Discussion

Mortality in the WWTP effluent (median 5%) was significantly lower compared to the control set-up in the stream (Kruskal-Wallis-test, α=0.05). The flow-through system in the WWTP was continuously aerated and a high biomass of bacteria in the WWTP effluent provided a high quantity of food by establishing aufwuchs. Although data of oxygen concentration and oxygen saturation are missing to some extend and certain measured values were not plausible, a sufficient oxygen supply can be supposed as aeration system worked continuously.

In the control set-up in the stream the median mortality of gammarids was 30% which must be regarded as low compared to literature data with mortality up to 70% in controls (Schirling et al. 2006, Dietrich et al. 2010, Oskarsson et al. 2012) which was also mentioned before in the discussion in chapter 2.3.3. In spring-time 2015, significantly less precipitation and more sunshine hours compared to the long-time means in the Free State of Saxony resulted in very low water levels (DWD, 2015). Due to the low water level during the control experiment in the stream the metal boxes were barely flowed through and therefore oxygen supply provided by fresh water was possibly not sufficient. Furthermore, heterotrophic processes in the glass tubes may have caused additional oxygen consumption leading to lower oxygen concentrations.

Due to the relatively high mortality in the stream fewer gammarids were available for precopulae formation and hence the probability to find a partner decreases. Thus, in the stream relative number of precopulae and relative number of egg-bearing females were significantly lower compared to the WWTP effluent (Kruskal-Wallis-test, α=0.05). In addition, a strong influence of oxygen concentrations on egg-bearing females has to be considered since in the stream relative number of egg-bearing females was significantly lower compared to WWTP effluent (Kruskal-Wallis-test, α=0.05). Furthermore, a brown-red color was detected at some of the eggs in egg-bearing females in the stream. This may indicate that the eggs were either unfertilized or ceased in development due to oxygen deficiency or increased water temperature (Sundelin et al. 2008). On the basis of measured water parameters in the stream (Table 69), however, it can be excluded that the determined egg aberration was related to the water temperature.

Nevertheless, a lower number of eggs per egg-bearing female is indicated in WWTP effluent in contrast to the stream. Probably, the mixture of several chemicals contained in the WWTP effluent has an adverse impact on the fecundity of gammarids resulting in a lower number of eggs per egg-bearing female (Peschke et al. 2014). Moreover, fecundity is related to the size and weight of females (Sundelin et al. 2008). Hence, it could be possible that the females in WWTP effluent showed a smaller size than in the stream and consequently bearing fewer eggs.

Results of the studied endpoints of both experiments in WWTP effluent and stream are summarized in Table 70. The endpoints mortality, relative number of egg-bearing females and number of eggs per egg-bearing females showed significant differences between WWTP effluent and stream. Opposing trends could be observed regarding effects on reproduction. Relative number of egg-bearing females was significantly higher, however number of eggs per egg-bearing female was lower in WWTP effluent compared to the stream. To assess these effects for the population dynamics a population model could be applied.

Table 70: Results of studied endpoints on *G. fossarum* after exposure to WWTP effluent and stream. Calculated values of median along with percentiles (25% - 75%) are depicted. Significant differences are marked bold.

study site	Mortality [%]	Precopulae [%]	egg-bearing females [%]	number of eggs per egg-bearing female
effluent	5 (5-10)	5 (5-10)	30 (30-30)	8 (6-11)

study site	Mortality [%]	Precopulae [%]	egg-bearing females [%]	number of eggs per egg-bearing female
stream	30 (30-35)	0 (0-5)	5 (3.75-7.5)	13 (12-14.75)

Concentrations of diclofenac (1.3 µg/L) and metoprolol (1.4 µg/L) in the WWTP effluent are far from exposure concentrations in single substance laboratory experiments (see above Chapter 1.3) and resulting LOEC values. Therefore, diclofenac and metoprolol are not primarily responsible for the detected effects in WWTP effluent. It is more likely that the detected effects on gammarid reproduction are caused by the complex interaction between the various pharmaceuticals and micropollutants present in the WWTP effluent. On the contrary in the stream abiotic factors, e.g. oxygen concentration, are the crucial factors determining the survival and reproduction of gammarids rather than chemical substances present in the water phase, e.g. diclofenac (0.025 µg/L) and metoprolol (below the limit of detection).

Summarising beside chemical exposure at the different study sites differences in oxygen concentration and food quality respectively aufwuchs quantity were found. A solely adverse effect of WWTP effluent on gammarid development compared to the stream and single substance exposure was not detected. However, it has to be taken into account that the presented results only apply to the studied sample sites and the observed effects cannot be generalized to other WWTPs and streams. Therefore, more WWTP effluents and natural streams should be included into the investigations regarding adverse effects of wastewater effluents on reproductive endpoints of aquatic organisms. Ladewig et al. (2006) investigated the population dynamics and structure of gammarids upstream and downstream of a sewage treatment plant (STP) in two streams in Germany (Körsch and Lockwitzbach). They found out that downstream the STP the proportion of breeding females was decreased. However, gammarids reached their highest abundances at the downstream sampling site at the Körsch. Additionally, Peschke et al. (2014) showed an adverse effect in the health of gammarids (e.g. decreased fecundity, increased hsp70 levels) downstream a WWTP (Langwiese, Baden-Wurttemberg, Germany).

5.2.2 Biochemical markers

5.2.2.1 Materials and Methods

See 1.3.2.10.

5.2.2.2 Results and discussion

Data on lipid peroxides had to be log-transformed, data on body mass fourth-root transformed to meet the assumptions of parametric testing. Lipid peroxides were of a comparable level in effluent-exposed gammarids and the control taken at the same time point (t40). Values obtained for the control at the start of the experiment (t0) were higher, but did not differ significantly (linear model, df=2/91, F=2.651, p=0.07604). Body mass showed great differences between all treatment groups (linear model, df=2/91, F=25.39, p<0.0001). Control individuals sampled after 40 days were heavier than at the beginning (ANOVA + Tukey-Kramer, p=0.0003), and individuals exposed in the effluent had an even bigger body mass than t 40 (ANOVA + Tukey-Kramer, p=0.0107) and t0 (ANOVA + Tukey-Kramer, p<0.0001). Data are shown in Figure 138.

Data on stress proteins had to be square root-transformed to fit the assumptions of parametric testing. There was an overall significant difference between the treatments (linear model, df=2/98, F=3.845, p=0.02469), which was driven by the lower Hsp70 level in “t0” animals compared to the control. There were no differences between the control and the effluent-exposed animals. Data are shown in Figure 139.

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. 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, our results are not surprising.

Figure 138: Lipid peroxidation (top) and body mass (bottom) of *Gammarus fossarum* exposed to wastewater treatment plant effluent for 40 days. "t0" depicts control animals sampled at the beginning of the experiment, "t40" those samples after 40 days. Bold lines display the median values, boxes the 25% to 75% quantiles, whiskers the minimum and maximum values, circles potential outliers. There were no differences in the level of lipid peroxides, but individuals exposed to the effluent were considerably heavier than control animals (linear model, df=2/91, F=25.39, p<0.0001).

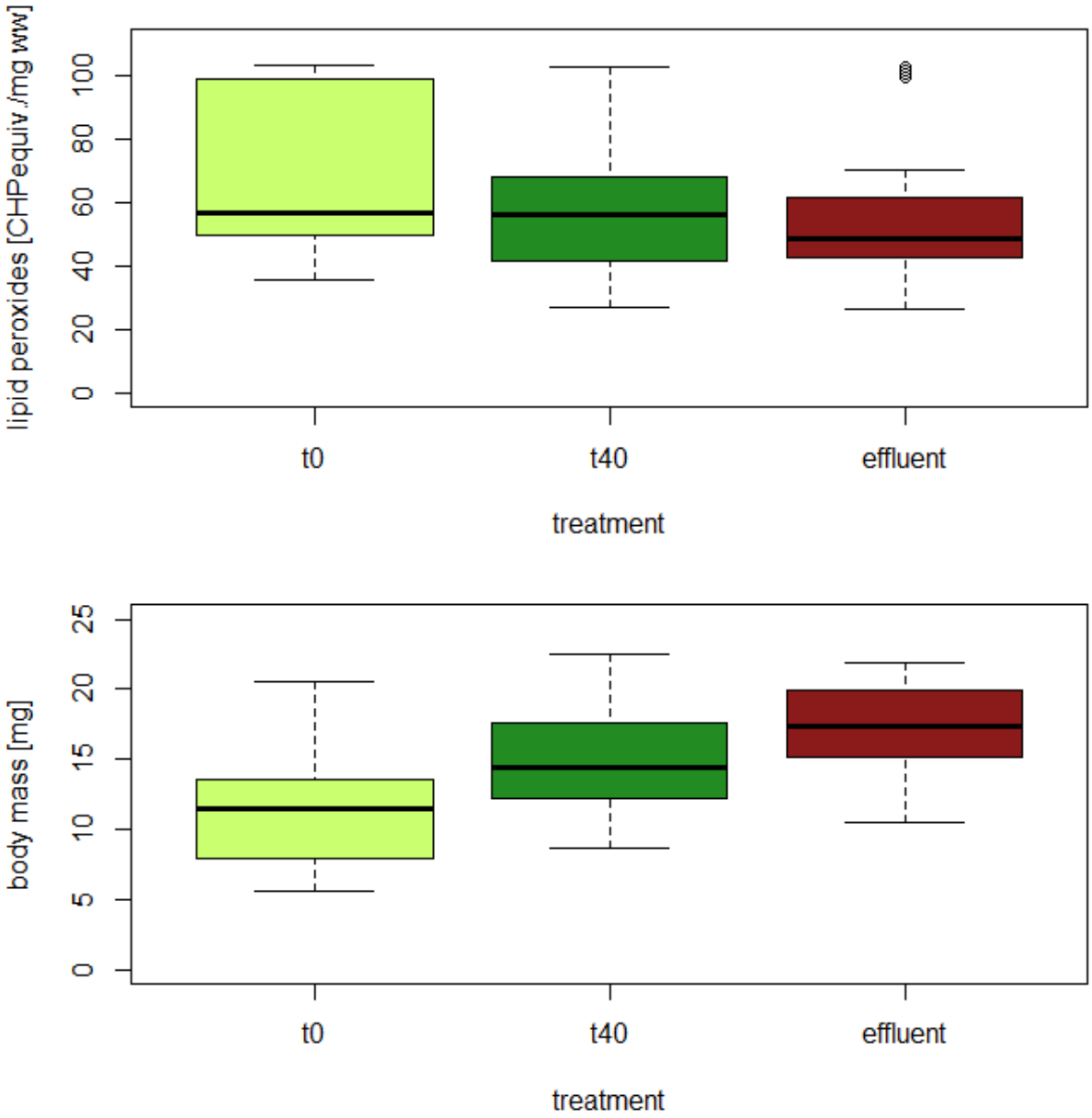
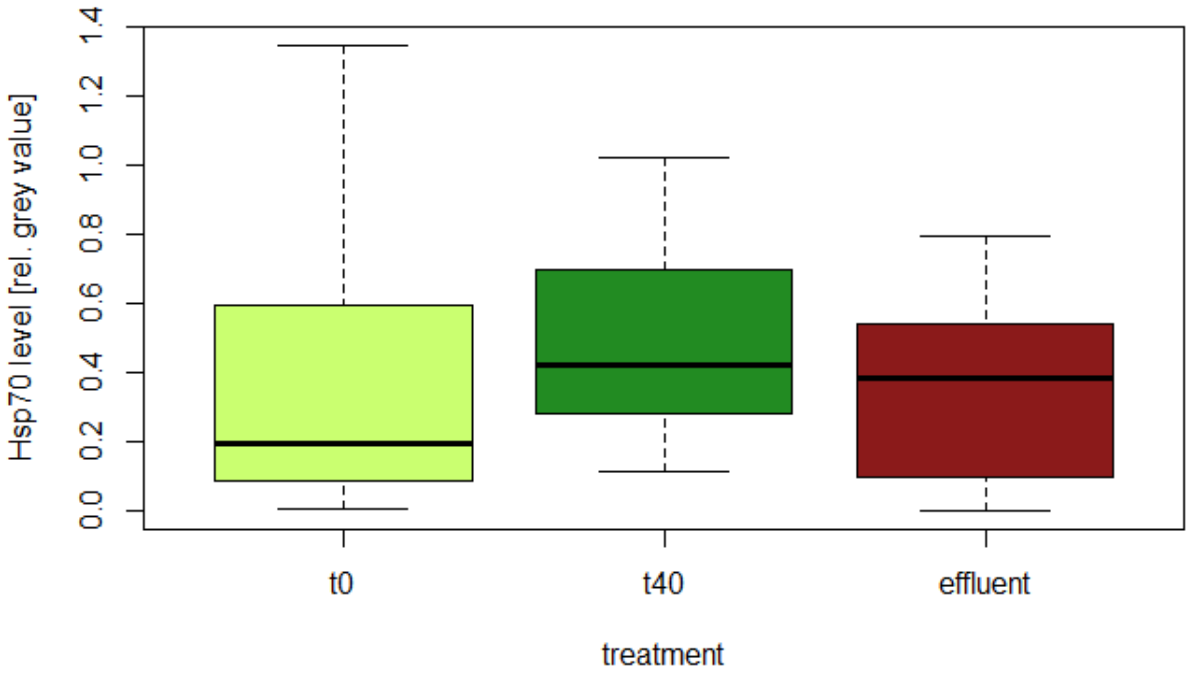


Figure 139: Relative Hsp70-level of *Gammarus fossarum* exposed to wastewater treatment plant effluent for 40 days. “t0” depicts control animals sampled at the beginning of the experiment, “t40” those samples after 40 days. Bold lines display the median values, boxes the 25% to 75% quantiles, whiskers the minimum and maximum values, circles potential outliers. The only difference was found between t0 and the control, none between effluent and control.



6 WP7A (Part II), WP7B (Part III) Chemical analyses

6.1 General aspects

For aquatic laboratory-based tests with fish, gammarids and for sediment toxicity tests the concentrations of the respective stock solutions as well as the test concentrations were checked by chemical analyses.

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 analytes were pre-concentrated with a polymeric sorbent material (Strata-X, 200 mg, Phenomenex, Aschaffenburg, Germany). Afterwards, the sorbent was dried under a gentle stream of nitrogen and eluted with 8 mL methanol and 2 mL acetonitrile. Analytes of interest were analyzed with 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 parameters are displayed in Table 71.

Table 71: Optimized interface parameters for the analyses of diclofenac and metoprolol

parameter	setting
collision gas	medium
curtain gas	30 psi
ion source gas 1	60 psi
ion source gas 2	80 psi
ion spray voltage	5,500 V
ionisation mode	positive
temperature	450 °C

For the target analytes the two mass transitions with the highest intensities were chosen for optimizing the MS/MS parameters. Table 72 summarizes the mass transitions and the optimized conditions for the fragmentation of diclofenac and metoprolol.

Table 72: MS/MS parameters for diclofenac and metoprolol

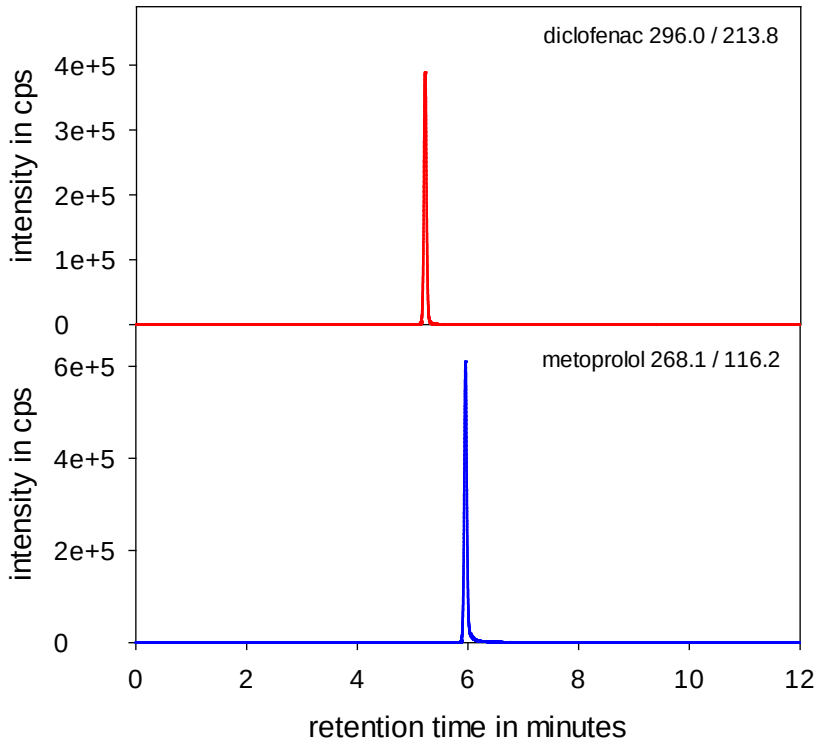
compound	precursor ion	product ion	DP [a] (V)	CE [b] (eV)	CXP [c] (V)
diclofenac	296.0	213.8	26	37	4
	296.0	249.8	26	19	6
metoprolol	268.1	116.2	46	27	0
	268.1	56.2	46	43	0

[a] DP = declustering potential in volt, [b] CE = collision energy in electron volt, [c] CXP = cell exit potential in volt

After injection of the SPE eluate or direct injection of the aqueous sample, chromatographic separation was achieved with an Ultra Aromax column (150 x 2.1 mm, 3 µm, Restek, Bad Homburg, Germany). A gradient system with a flow rate of 220 µL/min was used. Eluents were (A) ultrapure water + 5 mM ammonium formate and (B) methanol / acetonitrile (⅓ / ⅔, v/v) + 5 mM ammonium formate. Chromatographic separation started with 20% eluent B, which is increased to 100% within 5 min, held for 6 min before it was returned within 1 min to the initial conditions. The first minute of the chromatographic run was directed to the solvent waste to prevent the interface of the mass spectrometer from any avoidable contamination of the eluting salt burden. The method allows for a satisfactory retention

of the analytes and their base line separation (Figure 140). For quantitative analyses, the isotopically labelled standards diclofenac-d_4 and metoprolol-d_7 were used for the correction of signal enhancement or suppression caused by matrix components.

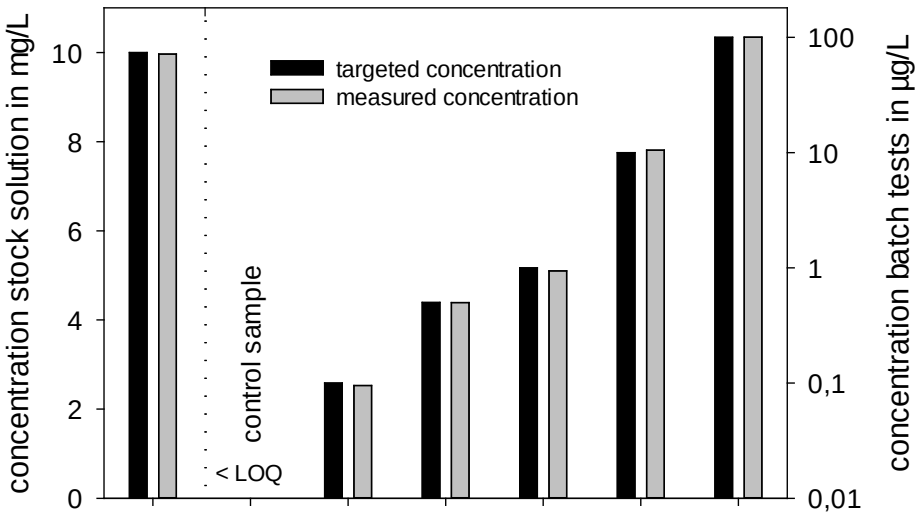
Figure 140: Extracted chromatograms for the most intensive mass transitions of diclofenac and metoprolol.



6.2 Chemical analyses for WP3A1: Studies with juvenile trout

At the kick-off meeting, Umweltbundesamt and the project partners agreed to check the concentrations of the aqueous phase of the biotests at the beginning, the end and at an intermediate date. It was decided to pool aqueous samples of aquaria with the same test concentration (n=3). At the beginning of the biotests, all concentration levels, the control sample and the stock solution were analyzed. Additionally, three test concentrations and the control sample were analyzed prior to the first water change and at the end of the tests in order to gain maximum information by a limited number of analyses. In biomarker studies with juvenile trout the stock solution of diclofenac and the initial test concentrations differed to a maximum of 6% from the target concentration and thus, were within the analytical uncertainty (Figure 141).

Figure 141: Target and measured concentrations of diclofenac at the beginning of the biotests with juvenile trout (WP3, Test 1).



6.3 Chemical analyses for WP3A2: Tests with gammarids

6.3.1 Chemical analyses of test concentrations for acute toxicity tests

Prior to the laboratory-based experiments with gammarids (40 d, four test concentrations and control sample), pre-tests for the evaluation of their sensitivity were performed for two pharmaceuticals with three different test concentrations. Deviations between targeted (nominal) and measured (real) concentrations at the beginning of the test were between 0% und 15%. After 48 h, for two diclofenac batches 76% and 77% of the expected concentrations were measured. In the other batch tests, between 88% and 99% of the initial concentrations were found (Figure 142). Based on these results, a good correlation between targeted and measured concentrations could be expected.

Figure 142: Targeted (nominal) concentrations compared to measured concentrations at the beginning of the acute toxicity test with gammarids and after 48 h for metoprolol (left) and diclofenac (right).

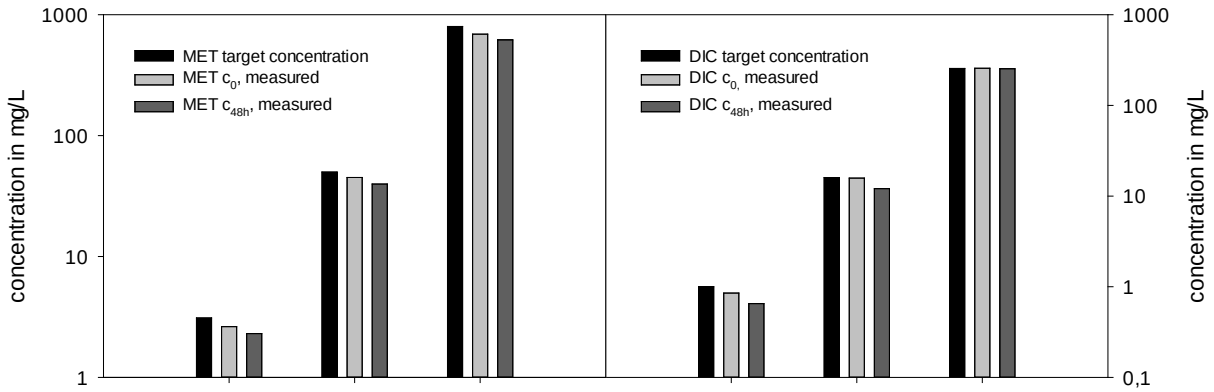


Table 73: Measured concentrations of pharmaceuticals in the exposure medium at beginning of experiment and after 48 h

Pharmaceutical	Sampling time [h]	nominal conc. [mg/L]	measured conc. [mg/L]	recovery [%]	decline [%]
diclofenac	0	5	4.6	91	
	48	5	3.6		22
metoprolol	0	45	44.6	99	
	48	45	42.6		4

6.3.2 Chemical analyses of test concentrations for chronic toxicity tests

Measured concentrations of the pharmaceuticals were determined in the experiments with nominal concentrations of 1.48 and 4.44 mg/L. In these experiments, effects were visible in gammarids. A total of 81% of the nominal concentrations were detected in the two treatments at the beginning of the experiment (Table 74). Concentrations decreased throughout the first week of exposure by 33 and 41%, respectively. After 28 days, 65 and 72% of nominal concentrations were detected after exchange of 80% of the medium. This decline was caused by the weekly exchange of medium, where one fifth of old medium, presumably with decreasing concentrations of the pharmaceuticals, remained in the vessels. This effect could have been avoided by an exchange of the entire medium. However, this procedure is not recommended in order to avoid handling stress for the test organisms. At the beginning of exposure, the concentration of diclofenac was below 0.001 mg/L in the controls. After 35 days, a concentration of 0.003 mg/L was determined in the controls.

Between days 28 and 35 of exposure, concentrations decreased by 47 and 53%, respectively. The relative decline was higher at lower exposure concentration (1.48 mg/L) in both time frames investigated. In this second period of investigation (day 28 to day 35) a 12% and 15% larger decline of diclofenac concentration was observed than in the first period (day 1 to day 7).

Table 74: Analysis of concentrations during 40 days of exposure to diclofenac; ME medium exchange

sampling time [d]	nominal conc. [mg/l]	determined conc. [mg/L]	recovery rate [%]	decline per 7 days [%]
0	0.00	< 0.001	-	
0	1.48	1.200	81	
0	4.44	3.600	81	
7 before ME	1.48	0.710	48	41
7 after ME	4.44	2.400	54	33
28 after ME	1.48	0.970	65	
28 after ME	4.44	3.200	72	
35 before ME	0.00	0.003		
35 before ME	1.48	0.460	31	53
35 before ME	4.44	1.700	38	47

6.3.3 Sorption of diclofenac and metoprolol onto leaves of Alnus *glutinosa*

During the tests, gammarids were fed with leaf disks of *Alnus glutinosa*, which were incubated in the respective concentrations of pharmaceuticals already two days prior to the start of the test. In order to

assess the sorption of the target compounds onto the leaf material during the exposure of the gammarids, batches containing leaf disks were spiked with diclofenac (5 mg/L) and metoprolol (45 mg/L), respectively. The aqueous phase of the batches (n = 3) was sampled at the beginning and after 48 h. The leaf disks were separated and extracted after 48 h and the results were compared with non-spiked batches, which also contained leaf material.

During the test duration of 48 h, diclofenac concentrations decreased to a similar degree (21%) in the aqueous phase as observed in the acute toxicity tests without leaf disks. For metoprolol similar concentrations at the beginning and after 48 h were measured.

When taking into account the measured concentrations at time t_0 and the water volume of the batch tests (50 mL), an absolute amount of 0.23 mg diclofenac and 2.23 mg metoprolol were spiked into the tests. Converted to the mass of the freeze dried leaves 2.6 ± 1.1 µg diclofenac und 43 ±7.2 µg metoprolol could be extracted, which account for a percentage of about 1% and 2%, respectively. The results corroborate the low sorption tendency of the two pharmaceuticals onto leaf material. Exemplarily the incubation test was run at medium concentrations of the pharmaceuticals. Sorption processes from the aqueous phase to leaf surfaces were considered negligible, being as low as 1 -2% of the initial pharmaceutical concentration.

6.3.4 Concentrations of pharmaceuticals in the water from the original habitat of the gammarids

Tännichtgrundbach water was sampled at two dates and analysed for a total number of 25 selected anthropogenic organic compounds. From these compounds, only diclofenac and gabapentin were detected for both sampling dates Table 75 Diclofenac was present at concentrations of 17 and 33 ng/L. Gabapentin was determined at concentrations of 25 and 28 ng/L. All other pharmaceuticals traced in the screening campaigns were not found in concentrations above their limit of quantification.

Table 75: Results of selected micropollutants for two sampling campaigns at the Tännichtgrundbach, concentrations in µg/L.

Parameter	creek sample 31.03.2014	creek sample 15.04.2014
N-Acetyl-Sulfamethoxazole	< 0.010	< 0.010
Diatrizoic acid	< 0.010	< 0.010
Benzotriazole	< 0.010	< 0.010
Bezafibrate	< 0.010	< 0.010
Carbamazepine	< 0.010	< 0.010
Carbendazim	< 0.010	< 0.010
Cetirizine	< 0.010	< 0.010
Clarithromycin	< 0.010	< 0.010
Diclofenac	0.033	0.017
Diuron	< 0.010	< 0.010
Gabapentin	0.028	0.025
Hydrochlorothiazide	< 0.010	< 0.010
Ibuprofen	< 0.010	< 0.010
Iohexol	< 0.010	< 0.010
Iomeprol	< 0.010	< 0.010
Iopamidol	< 0.010	< 0.010

Parameter	creek sample 31.03.2014	creek sample 15.04.2014
Iopromide	< 0.010	< 0.010
Isoproturon	< 0.010	< 0.010
Lamotrigine	< 0.010	< 0.010
Metoprolol	< 0.010	< 0.010
N-Acetyl-4-aminoantipyrine	< 0.010	< 0.010
N-Formyl-4-aminoantipyrine	< 0.010	< 0.010
Oxazepam	< 0.010	< 0.010
Paracetamol	< 0.010	< 0.010
Sotalol	< 0.010	< 0.010

6.4 Chemical analyses for WP3A3: Tests with *Lumbriculus variegatus*

The analytical techniques used for solid samples were similar to those used for water samples but required a sample preparation which efficiently removed co-extracted matrix compounds. By combining appropriate extraction (solid phase) and enrichment techniques (Strata-X, 200 mg, Phenomenex, Aschaffenburg) during sample pre-treatment, a high selectivity and sensitivity could be achieved

The results for the measured concentrations of diclofenac and metoprolol in sediment and supernatant in the tests with isolated substances and the substance mixture are summarized in Table 76 and Table 77.

At day 0, the measured concentrations of diclofenac were approximately half of the nominal concentrations in the sediment. After 28 days, the measured concentration represents less than 30% of the nominal concentration, however, the concentration in the supernatant increased.

Table 76: Measured concentrations of diclofenac and metoprolol in sediment and overlaying water at the start (day 0) and the end (day 28) of the final tests. (SC = solvent control, n. m. = not measured).

drug	Nominal concentration	Measured concentration			
	(mg/kg dw)	Sediment (mg/kg dw)	Supernatant (mg/L)	Sediment (mg/kg dw)	Supernatant (mg/L)
		day 0		day 28	
diclofenac	SC	0.0077	< 0.00005	n. m.	0.0015
	0.039	0.0112	< 0.00005	< 0.001	0.0031
	20	10.9	n. m.	5.7	n. m.
	320	200	45	105	69.2
metoprolol	SC	< 0.001	0.00005	n. m.	< 0.00005
	0.039	0.0068	0.00055	< 0.001	0.0003
	20	12.5	n. m.	3.1	n. m.
	320	255	7.4	156	11

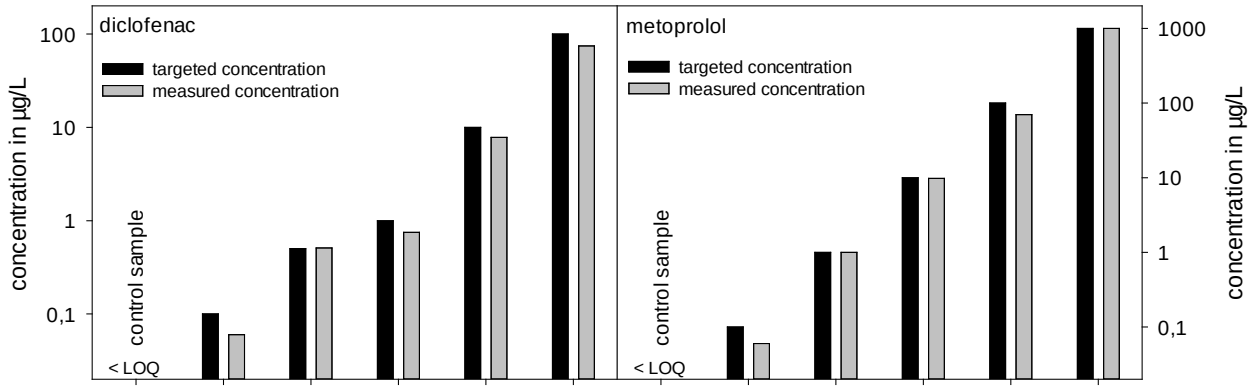
Table 77: Measured concentrations of the mixture experiment in sediment and overlaying water at the start (day 0) and the end (day 28) of the final tests. (SC = solvent control, n.m. = not measured)

drug	Nominal concentration (mg/kg dw)	Measured concentration			
		Sediment (mg/kg dw)	Supernatant (mg/L)	Sediment (mg/kg dw)	Supernatant (mg/L)
		day 0		day 28	
diclofenac	SC	< 0.001	< 0.00005	n. m.	0.0093
	0.039	0.0185	0.0032	0.0104	0.0055
	20	7.4	n. m.	5.3	n. m.
	320	118	58	77	62
metoprolol	SC	0.0173	0.00079	n. m.	0.0014
	0.039	0.009	0.0013	0.0082	0.003
	20	9	n. m.	7.7	n. m.
	320	150	28	141	19

For WP3A, “toxicity of pharmaceuticals in surface water with single substances”, the initial concentrations of the tests with brown trout embryos were analyzed.

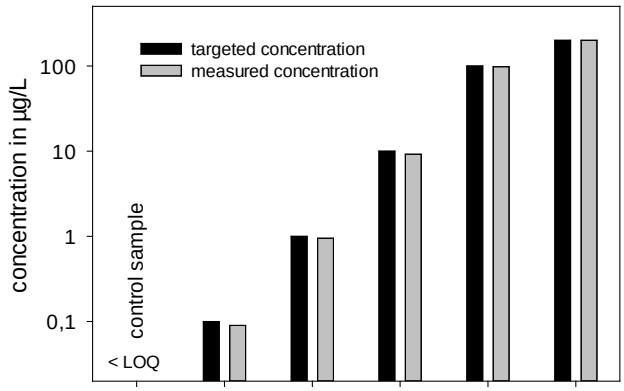
One control and five concentrations of diclofenac (0.1, 0.5, 1, 10 and 100 µg/L) and five concentrations of metoprolol (0.1, 1, 10, 100 and 1,000 µg/L) were used. Experiments were set up in triplicates, but for chemical analyses aliquots of replicates with the same concentration were pooled. The results for both target analytes were heterogeneous (Figure 143). In half of the samples targeted and measured concentrations matched perfectly. In other samples deviations were between 20% and 30%, in the samples with the lowest concentration of 0.1 µg/L only 60% of the intended concentration was detected.

Figure 143: Targeted concentrations of diclofenac (left) and metoprolol (right) compared to measured concentrations at the beginning of single substance testing for brown trout embryos.



Due to high mortality during the exposure of juvenile brown trout to diclofenac in 2014, the test was repeated. The initial concentrations were checked by chemical analysis and all were in good agreement with targeted concentrations (Figure 144). The highest deviation was 10% for the lowest concentration of 0.1 µg/L.

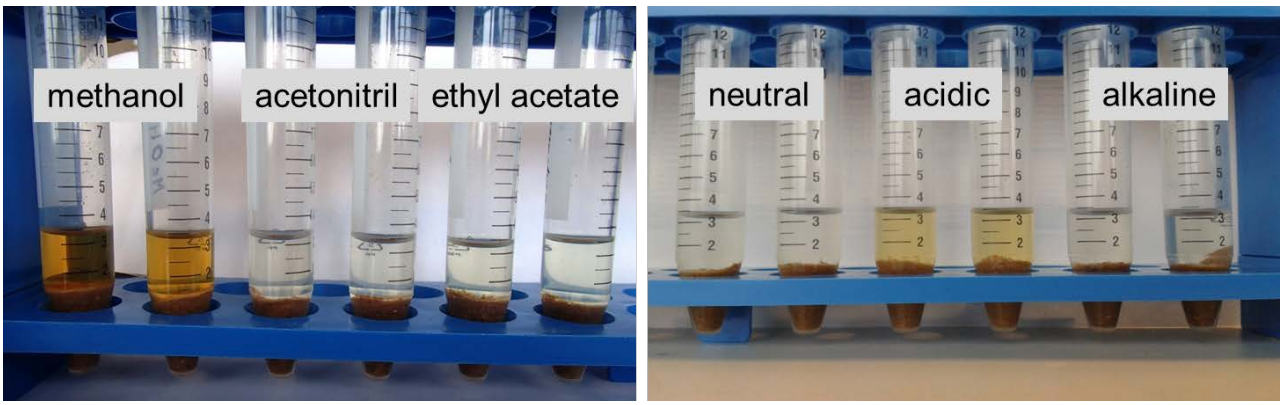
Figure 144: Targeted concentrations of diclofenac compared to measured concentrations at the beginning of single substance testing for juvenile brown trout (test repetition, July 2015).



Prior to the analysis of fish, several parameters for optimum extraction like organic solvents, pH of extraction and injection volume were tested to assess the effect of co-extracted matrix. As matrix turtle food containing complete freeze-dried fish and crustaceans was used. The matrix was ground in a blender to obtain a fine powder for extraction experiments. 0.5 g of 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 resolved with 500 µL of methanol and 2 mL of ultra-pure water. 1 mL was transferred to a 1.5 mL snap-cap tube suitable for micro-centrifugation. After 15 min of centrifugation an aliquot of 200 µL was transferred into a HPLC-vial and diluted with 800 µL of ultra-pure water. Target analytes were spiked at the end of this procedure. To assess matrix suppression, peak areas of these samples with external standards were compared.

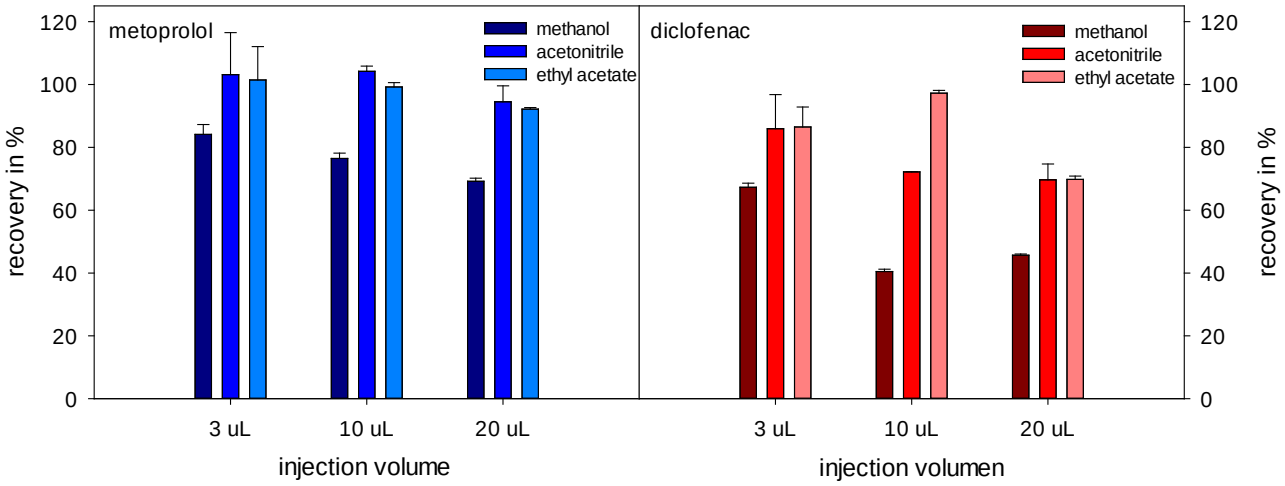
As it can be seen from Figure 145 the extraction solvent as well as the extraction pH had a distinct impact on the extracted matrix components. Methanol extraction resulted in the most colored extract and produced a very fine precipitant, which may result in problems in subsequent clean-up steps. Acetonitrile 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 (data not shown).

Figure 145: Extracts obtained after ultra-sonic extraction of 0.5 g matrix and after centrifugation: with different organic solvents (left) and at different pH values in methanol (right).



In most cases not the extraction efficiency, but the co-extracted matrix components limit signal intensities during MS/MS detection. For the extracts from the preliminary experiments a considerable amount of matrix is still co-extracted and present in the final sample although two dilution steps were applied. This was proven by the injection of different sample volumes in the LC-MS/MS system (Figure 146). When increasing the injection volume the recoveries of the target analytes metoprolol and diclofenac decreased. The phenomenon of matrix effects, resulting in signal suppression or (less frequently) in signal enhancement was already intensively addressed in other studies. Scheurer et al. (2010) reported low absolute recoveries when analyzing beta-blocker drugs in different sewage sludges. Reduction of the injection volume and dilution of the extracts improved the absolute recoveries in some cases by a factor of two. Hernando et al. (2004) even reported a complete elimination after dilution, but as a consequence also a decreased sensitivity. Therefore, sample volumes, injection volumes and dilution of the final extract is always a compromise between reducing negative matrix effects and the method sensitivity. In any case, when dealing with difficult matrices like biota samples acceptable relative recoveries can only be achieved when using isotopically labelled internal standards to compensate for matrix effects.

Figure 146: Recoveries for metoprolol and diclofenac obtained for different organic solvents and injection volumes. Extracts were spiked after extraction procedure, which is described above.



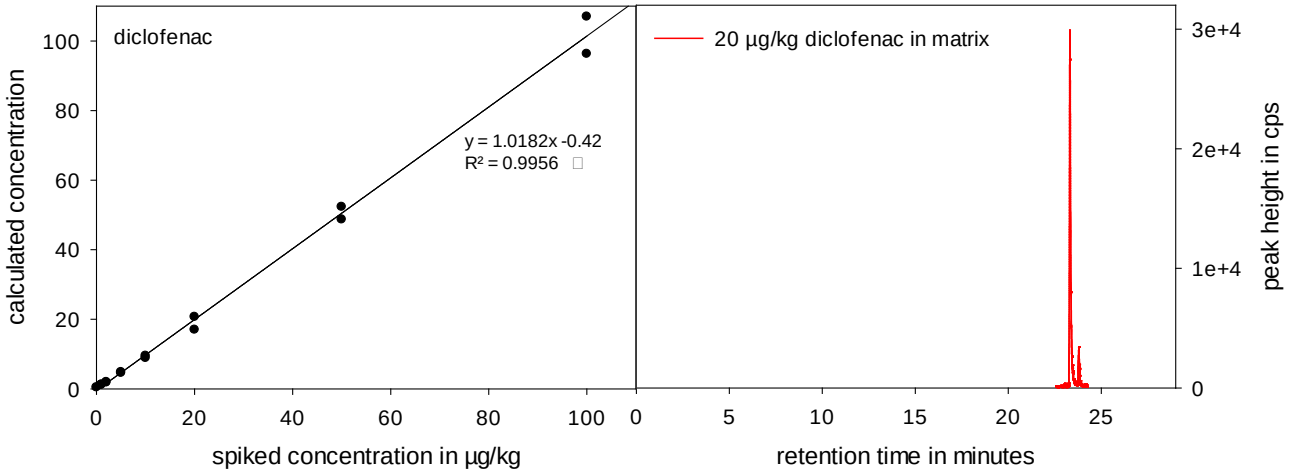
For the analysis of biota samples the same analytical instrumentation as described for water samples, but different LC buffers and another LC gradient program were used due to co-eluting matrix components. Eluents were (A) ultrapure water + 5 mM ammonium formate + 0.05% formic acid and (B) methanol / 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.

For the extraction of trout samples from WP 3 and 5, the procedure described above was applied with the exception that the dry residue was resolved with 200 µL of methanol and 0.8 mL of ultra-pure water, which was directly injected into the LC-MS/MS system after micro-centrifugation. This was done to obtain a more concentrated sample in order to achieve a lower limit of quantification. A matrix matched calibration with turtle food was prepared for quantification (Figure 147). For the analysis of juvenile trout, filets of test fish were available for analyses as organs like liver were needed for biomarker studies. Therefore, samples may underestimate diclofenac concentrations in fish, as muscle

tissue comparably accumulates less organic micropollutants than organs with a higher fat content. 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. By this means, a good linear calibration curve with a regression coefficient of 0.996 was achieved.

The limit of quantification 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 analytical limit of quantification, minimum bio-concentration factors (BCF) between 50 L/kg and 0.025 L/kg can be proven for the different test concentration (0.1 µg/L to 200 µg/L).

Figure 147: Left: Calibration curve of diclofenac spiked in matrix and resulting linear equation and regression coefficient (every concentration was processed in duplicates). Right: Signal of 20 µg/kg diclofenac in matrix.



In the control samples and the two lowest test concentrations of 0.1 µg/L and 1µg/L no diclofenac was detected in the fish in concentrations above the limit of quantification. 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. In the spiked control sample, the expected concentration was found proving the applicability of the method also for trout samples.

Table 78 Concentrations of diclofenac in pooled trout samples.

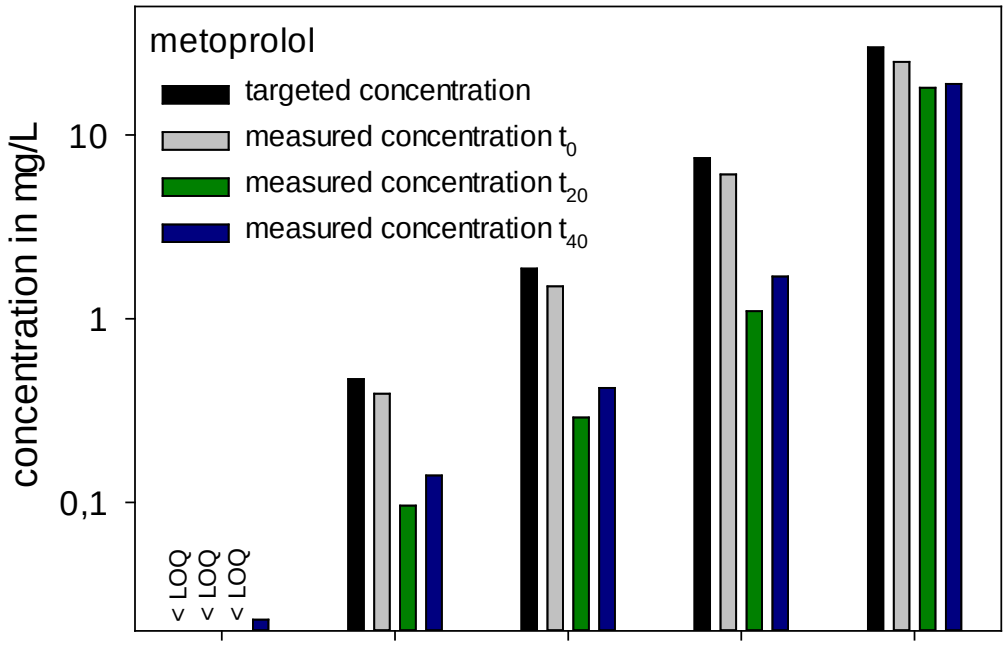
Parameter	measured concentration in µg/kg
control sample, pool 1	< 5
control sample, pool 2	< 5
test concentration 0.1 µg/L, pool 1	< 5
test concentration 0.1 µg/L, pool 2	< 5
test concentration 1 µg/L, pool 1	< 5
test concentration 1 µg/L, pool 2	< 5
test concentration 10 µg/L, pool 1	11.1
test concentration 10 µg/L, pool 2	9.2
test concentration 100 µg/L, pool 1	99

Parameter	measured concentration in µg/kg
test concentration 100 µg/L, pool 2	70
test concentration 200 µg/L, pool 1	202
test concentration 200 µg/L, pool 2	137
control sample, pool 1 spiked with 20 µg/kg	20.3

6.5 Chemical analyses for WP4: Mesocosms

Based on the results of WP3, metoprolol was chosen to be tested in mesocosm experiments in WP4. During the test duration of 40 d, the different concentration levels (0.47; 1.88, 7.5 and 30 mg/L) at t0, t20 and t40 were controlled (Figure 148). The deviations at the starting date of the experiment were between 17% and 20%. After 20 d, concentrations had decreased up to 15% of the targeted levels, except at the highest concentration of 30 mg/L (60%). The mesocosms were spiked again to re-establish the intended levels and checked after 40 d at the end of the test. The decrease in concentration during the second half of the test was similar to the first. Again a high decrease in mesocosms with the three lowest concentrations was observed, whereas the highest concentration decreased only by 37%.

Figure 148: Targeted concentrations of metoprolol compared to measured concentrations at the beginning of mesocosm studies as well as after day 20 and day 40.

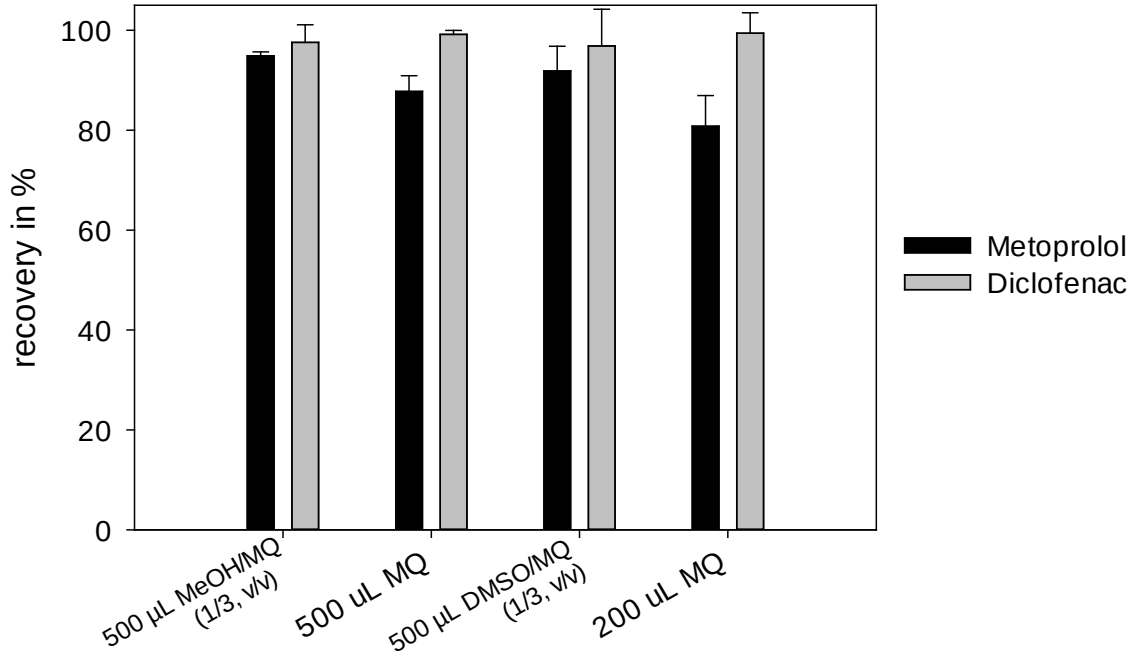


6.6 Chemical analyses for WP8-10: Development, testing and validation of *in vitro*-assays

At the beginning of the project a limit of detection (LOD) for metoprolol in the low nM/L range in the cell culture assays was assumed. For testing and validation of the assays a LOD of 100 nM/L metoprolol (26.7 µg/L) was aimed at. Since concentrations in the aquatic environment are much lower (e. g. in wastewater treatment plant effluents about 1 µg/L and streams influenced by wastewater about 0.1 µg/L) environmental samples had to be enriched. A common technique for the extraction of polar compounds from aqueous samples is solid-phase extraction (SPE). The eluate deriving from SPE is evaporated to dryness to enable a solvent exchange. To reconstitute the dry residue, water mixed with

an organic solvent is used in many cases. As most organic solvents cannot be used in cell culture assays it was tested if water or dimethyl sulfoxide can be used for reconstitution. For this, 9 mL methanol, which represented the SPE eluate, were spiked and evaporated to dryness under a gentle stream of nitrogen. Afterwards, analytes were reconstituted with water or water/solvent mixtures, analyzed and peak areas were compared with a directly injected standard. Figure 149 shows, that for metoprolol a better recovery can be achieved when organic solvents are used. However, for metoprolol and diclofenac recoveries are satisfactory when exclusively using water. At the same time the results show, that it is important to use a sufficient amount of liquid to enable properly rinsing of the inner surface of the vial.

Figure 149: Recovery in% of metoprolol and diclofenac after reconstitution of a dry residue with different solvents. MeOH = methanol, MQ = ultra-pure water, DMSO = dimethyl sulfoxide.



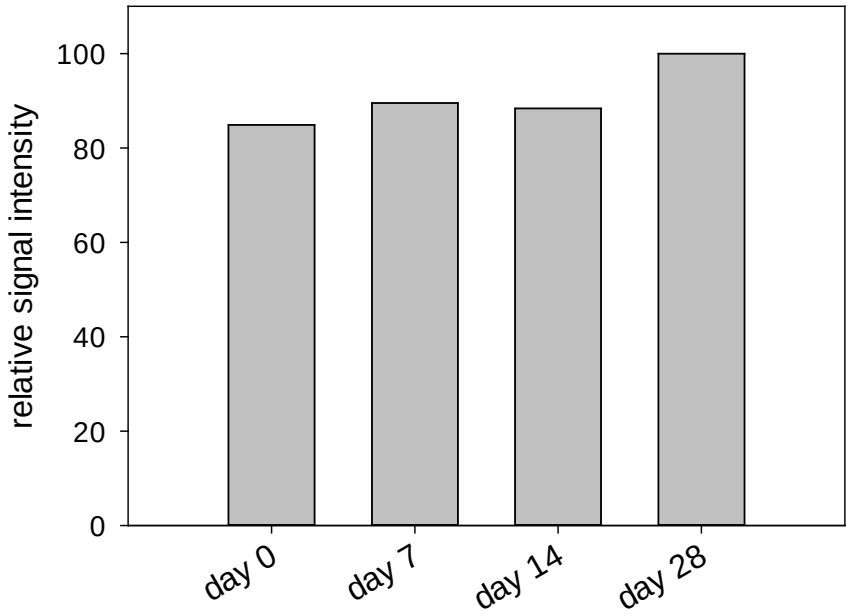
The pre-concentration with SPE is not exclusively selective for diclofenac and metoprolol but leads to the enrichment of undesired matrix components at the same time. Therefore, the objective of further pre-tests was to assess the robustness of the cell culture assays against those unknown compounds present in environmental samples. Water of a small creek was analyzed to ensure the absence of metoprolol and other beta-blocker compounds (LOQ = 10 ng/L). After confirmation, six SPE replicates were prepared and the dry residues were reconstituted and applied in the bioassays. The results have been used as a part of the validation process and are described in chapter 7.

6.6.1 Stability of beta-blockers in environmental samples enriched by SPE

After the development of the cell culture assays, wastewater treatment effluent and surface water samples were enriched for validation purposes and testing of environmental samples. It is crucial to ensure the stability of the analytes when a time offset between SPE and the application in the bioassays exists. For this reason, surface water samples were spiked with metoprolol and pre-concentrated with SPE (n = 8). All eluates were evaporated to dryness. The dry residues of two samples were reconstituted immediately. All other samples were deep-frozen and reconstituted after seven, 14 and 28 d. By this means, knowledge should be gained if the immediate and complete sample preparation or freezing of the dry residues is advantageous for conservation. Figure 150 shows that samples can be

enriched as soon as possible after sampling and the resulting dry residues can be deep-frozen and reconstituted just before analyses. For sample preparation this procedure was adapted for the rest of the project.

Figure 150: Relative signal intensity in% of metoprolol of samples which were reconstituted immediately or which were deep-frozen for one, two and four weeks, respectively. All samples were measured together four weeks after SPE.



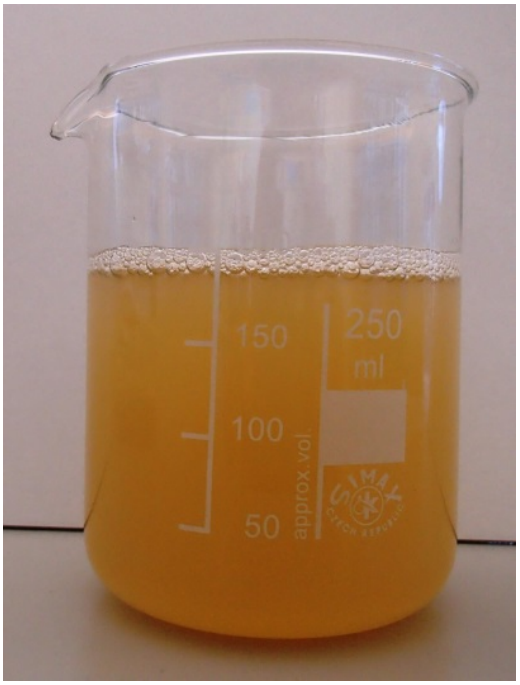
During the further course of the project, several series of samples were provided for validation purposes and sensitivity testing of the bioassays in WP 10.

In a first series replicates of surface water (n=5) and wastewater treatment plant effluent (n=2) were enriched by SPE. One sample of each matrix was spiked with a defined concentration of metoprolol. Spiking was conducted after SPE extraction to exclude any losses of metoprolol occurring during enrichment. Spiking amount was 500 nM to provide a concentration of about 134 µg/L metoprolol in the final extract. Surface water was sampled in Frauenalb at the upper course of the Alb, a small creek in the Northern Black Forest, to provide an original surface water matrix without any burden of metoprolol. By this means a comparison of spiked and non-spiked surface water was possible and thus conclusions about potential matrix effects in the bioassays can be made. The treated wastewater had a concentration of 0.94 µg/L metoprolol, which is in the range typically found in domestic wastewater after activated sludge treatment. Taking into account an enrichment factor of 400 and an average absolute recovery of metoprolol by SPE of 85% the concentration in the non-spiked wastewater extract was about 320 µg/L and in the spiked one 454 µg/L.

To assess matrix effects of treated wastewater it was necessary to create an artificial wastewater matrix in a second series of samples as metoprolol is ubiquitarily present in wastewater treatment effluents. Artificial wastewater was prepared according to DIN 38412/26 (1995) “German standard method for the examination of water, wastewater and sludge; surfactant biodegradation and elimination test for simulation of municipal wastewater treatment plants” (Figure 151). Besides inorganic salts the artificial wastewater contains an organic burden comprising peptone, urea and meat extract.

Artificial wastewater and potable water from Karlsruhe were solid-phase extracted and provided non-spiked as well as spiked with 500 nM and 1,000 nM metoprolol.

Figure 151: Stock solution A according to German DIN standard 38412/26 containing inorganic salts, peptone, urea and meat extract for the preparation of artificial wastewater.



In a third series treated wastewater from the municipal treatment plant in Karlsruhe was sampled and analyzed for metoprolol with LC-MS/MS. SPE was used to pre-concentrate the sample undiluted (n=2) and with a dilution factor of 5 (n=3) to further asses the influence of the sample matrix on bioassays. Furthermore, SPE extracts of diluted wastewater and with two spiking levels as well as native wastewater were provided for direct testing in the beta-blocker bioassay. The concentration of metoprolol in the effluent was 1.1 µg/L and the concentrations in the respective sample extracts could be calculated based on SPE enrichment factors, absolute recovery, dilution level and spiking amount. The treated wastewater was also enriched with a different SPE protocol to determine the concentration of diclofenac and to provide pre-concentrated native and diluted samples for the NSAID bioassays. Diclofenac was measured with a concentration of 2 µg/L in the effluent sample. Taking into account an enrichment factor of 500 and an absolute recovery of 100% a concentration of 1 mg/L diclofenac was provided in the extract of the undiluted sample.

After testing of these three series in WP10, it was reported that effects in drinking water samples after SPE were observed in the bioassays. For this reason, further samples were prepared for validation to exclude effects deriving from the water matrix, the vessels or the SPE material itself. Drinking water and ultra-pure water were enriched according to the SPE protocol for diclofenac to assess any differences arising from the water matrix. Furthermore, drinking water was filled in two different plastic vessels and glass vials used in laboratory routine. The samples were stored for several days prior to shipping at ambient temperature and light to allow leaching of unknown compounds. The effect of the SPE sorbent material was also determined: pristine SPE cartridges were compared with cartridges conditioned with methanol and water. 50 mL drinking water were extracted and collected in glass vials. By this means it was possible to assess negative effects of the sorbent material itself and the importance of conditioning. Additionally, wastewater treatment plant effluent was provided in this series of samples. Two replicates each were extracted undiluted, diluted with drinking water and diluted with ultra-pure water. The effluent used for this extraction had concentration of 1 µg/L diclofenac.

During the course of the development and validation of the beta-blocker bioassay, higher activity in the original water samples than in the SPE extracts were detected. It was assumed, that the reason are compounds that show activity in the bioassays but are not retained by the SPE sorbent material. As a

consequence these compounds would break through the sorbent and still be present in the SPE-extracted water sample. Therefore, the SPE flow-through as well as the eluates of a new series of wastewater samples were provided for analyses by the bioassays. Samples from different stages of a wastewater treatment plant in the Southwest of Germany were collected. This treatment plant applies powdered activated carbon as an additional treatment step for the biologically treated water. As a consequence, the samples also provided important information if the bioassays developed in WP 10 are able to monitor and control upgrading measures in wastewater treatment plants. Besides the target compounds metoprolol and diclofenac, further beta-blocker compounds, NSAIDs, and other pharmaceuticals were quantified in these samples in order to identify compounds which may contribute to the activities measured in the bioassays. Results for bioassays are displayed in chapter 7.

As it can be seen from the data in Table 79 the usage of powdered activated carbon is an effective measure to reduce micro-pollutant concentrations in biologically treated wastewater and to improve effluent quality. The beta-blocker compound with the highest concentration in the conventional effluent was metoprolol with 0.76 µg/L. Metoprolol concentration was reduced by 96% by the application of activated carbon. Other beta-blocker found in the effluent of the activated sludge treatment were atenolol, bisoprolol and Sotalol, but all of them were reduced to concentrations below the limit of quantification in the following treatment step. Diclofenac was quantified with 2.5 µg/L in wastewater after biological treatment and with 0.38 µg/L after treatment with activated carbon (reduction: 85%) and thus was one of the micropollutants with the highest concentration among the compounds analyzed in these samples. Besides diclofenac, other NSAIDs like ibuprofen or naproxen were present in the both wastewater samples.

Table 79: Results of selected micropollutants in wastewater after activated sludge treatment and additional treatment with powdered activated carbon, values in µg/L

parameter	effluent of activated sludge treatment	effluent after powdered activated carbon
atenolol	0.24	0.03
betaxolol	< LOQ	< LOQ
bisoprolol	0.34	< LOQ
clenbuterol	< LOQ	0.06
cyclophosphamide	< LOQ	< LOQ
aminophenazone	< LOQ	< LOQ
ifosfamide	< LOQ	< LOQ
metoprolol	**0.76**	**0.03**
phenazone	0.17	0.06
pindolol	< LOQ	< LOQ
propranolol	< LOQ	< LOQ
propyphenazone	< LOQ	< LOQ
salbutamol	< LOQ	< LOQ
simvastatin	< LOQ	< LOQ
sotalol	0.39	0.04
terbutaline	< LOQ	< LOQ
4-AAA	3.2	0.54
4-FAA	2.3	1.2

parameter	effluent of activated sludge treatment	effluent after powdered activated carbon
bezafibrate	0.54	0.10
carbamazepine	0.77	0.06
clofibric acid	< LOQ	< LOQ
diazepam	< LOQ	< LOQ
diclofenac	**2.5**	**0.38**
etofibrate	< LOQ	< LOQ
fenofibrat	< LOQ	< LOQ
fenofibric acid	0.22	0.02
fenoprofen	< LOQ	< LOQ
gemfibrozil	0.06	< LOQ
ibuprofen	1.6	0.13
indometacin	< LOQ	< LOQ
ketoprofen	< LOQ	0.01
naproxen	0.64	0.11
pentoxifylline	< LOQ	< LOQ
phenacetin	< LOQ	< LOQ
paracetamol (acetaminophen)	1.5	0.16

To further elucidate the character of compounds which break through the cartridge material and cause activity in the bioassays, two sample series from the wastewater treatment plant in Karlsruhe were run. In both samples water treated by SPE (flow-through of cartridges) and the dry residues deriving from SPE standard operating procedures of NSAIDs and beta-blockers were provided for WP 10. Additionally, the wastewater was SPE extracted by weak and strong anion and cation exchanger SPE materials, to elucidate if unknown target compounds can be assigned to molecules preferably extracted by those materials.

For both samples from the WWTP in Karlsruhe selected pharmaceutical compounds and some of their metabolites were analyzed. Several beta-blocker compounds were detected in the WWTP effluent with metoprolol being again the representative with the highest concentrations (1.2 µg/L). Besides metoprolol atenolol, sotalol, bisoprolol, and propranolol were incompletely removed by biological wastewater treatment and were present in concentrations between < LOQ and 0.4 µg/L in the effluent (Table 80). Values of the two sampling campaigns were comparable and similar to those displayed in Table 80. In the second effluent sample from March 2016 atenolol acid was additionally analyzed and quantified with 0.54 µg/L. Atenolol acid is an important metabolite of metoprolol and atenolol and proofed to be more stable than its precursor atenolol in laboratory-based biodegradation tests (Kern et al., 2010, Barbieri et al., 2012). The analytical standard was provided for further testing in WP 10.

Diclofenac was measured with a concentration of 2.2 µg/L in the effluent of the WWTP in Karlsruhe, proving again the rather recalcitrant behaviour of this compound during wastewater treatment and the necessity of additional treatment steps to further reduce its effluent concentrations.

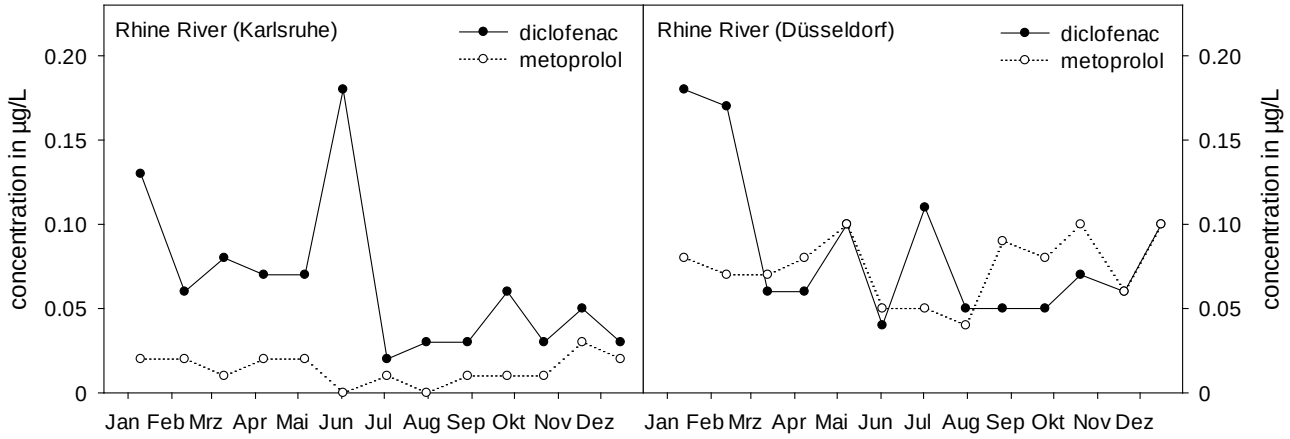
Table 80: Results of selected micropollutants in effluent from the wastewater treatment plant in Karlsruhe, Germany from two sampling campaigns. Samples were taken in February and March 2016, values in µg/L. n. a. = not analyzed

parameter	February 2016	March 2016
atenolol	< LOQ	0.07
betaxolol	< LOQ	< LOQ
bisoprolol	0.28	0.4
clenbuterol	< LOQ	< LOQ
cyclophosphamide	< LOQ	< LOQ
aminophenazone	< LOQ	< LOQ
ifosfamide	< LOQ	< LOQ
metoprolol	**1.2**	**1.2**
phenazone	< LOQ	0.05
pindolol	< LOQ	< LOQ
propranolol	< LOQ	0.05
propyphenazone	< LOQ	< LOQ
salbutamol	< LOQ	< LOQ
simvastatin	< LOQ	< LOQ
sotalol	0.10	0.14
terbutaline	< LOQ	< LOQ
atenolol acid	n. a.	0.54
4-AAA	2.3	2.4
4-FAA	0.95	1.2
bezafibrate	n. a.	1.1
carbamazepine	n. a.	0.71
clofibric acid	n. a.	< LOQ
diazepam	n. a.	< LOQ
diclofenac	**n. a.**	**2.2**
etofibrate	n. a.	< LOQ
fenofibrat	n. a.	< LOQ
fenofibric acid	n. a.	0.14
fenoprofen	n. a.	< LOQ
gemfibrozil	n. a.	< LOQ
ibuprofen	n. a.	1.1
indometacin	n. a.	< LOQ
ketoprofen	n. a.	< LOQ
naproxen	n. a.	1.4
paracetamol (acetaminophen)	n. a.	< LOQ
pentoxifylline	n. a.	< LOQ

parameter	February 2016	March 2016
phenacetin	n. a.	< LOQ

The incomplete removal of diclofenac and metoprolol during conventional wastewater treatment results in their presence in surface waters receiving the discharge of wastewater treatment plants. In Figure 152 the concentrations of both compounds in the Rhine River at the monitoring stations Karlsruhe and Düsseldorf are exemplarily displayed. Both compounds are detected in comparable concentrations of about 0.1 µg/L. The higher concentration level in the upper Rhine at Düsseldorf can be explained by an increasing percentage of wastewater in the course of the river. However, the dilution of wastewater in the Rhine is comparably high and in smaller rivers and creeks with a higher percentage of wastewater considerably higher concentrations of both compounds can be expected. For example in the Glems, a small creek in the Southwest of Germany, mean annual concentrations of 0.91 µg/L diclofenac and 0.75 µg/L metoprolol were reported (LUBW, 2014), which are concentrations similar to those in most wastewater treatment plant effluents.

Figure 152: Concentrations of diclofenac and metoprolol in the Rhine River at Karlsruhe (Rhine km 359.3) and Düsseldorf (Rhine km 732.1) in 2014 for comparison with WWTP effluent values (AWBR, 2014 and ARW, 2014).



7 WP 8-10 Development, testing and Validation of *in vitro*-test systems

The aim was the development of mode of action (MOA) based *in vitro*-assays for environmental monitoring of beta-blocker and NSAIDs.

Mode of action-based specific biomonitoring of pharmaceuticals is made possible by sensor cell lines. Such sensor cell lines can be categorized as either reporter or biosensor cell lines. In reporter cell lines, an analyte induces the expression of a reporter protein enabling an enzymatic or optical assay. The reporter is produced 12-24 hours after incubation of the cell line with the analyte. In contrast to that, biosensor cell lines generate signals directly. In such assays, commonly used fluorescence signals can be measured immediately after incubation of the cells with the analyte. Biosensor cell lines express recombinant sensor proteins that act as nanoswitches. Such nanoswitches contain a sensing unit that senses a change of the analyte concentration and a reporting unit to indicate the sensing unit's state. Upon binding of the analyte, the protein undergoes a change in structure and, in turn, changes its fluorescence characteristics which could be measured by live cell imaging.

For the development of *in vitro*- test systems, recombinant cell lines were constructed for the sensing of beta-blocker and NSAIDs. For the biomonitoring of beta-blockers, three concepts were followed in parallel.

7.1 WP8-WP9 Development and testing of *in vitro*-test systems (Part II)

A *beta-blocker CRE reporter cell line* was developed containing the reporter enhanced green fluorescent protein (EGFP) under the control of a cAMP response element. cAMP synthesis results in induction of EGFP which can be measured by fluorescence activated cell sorting (FACS).

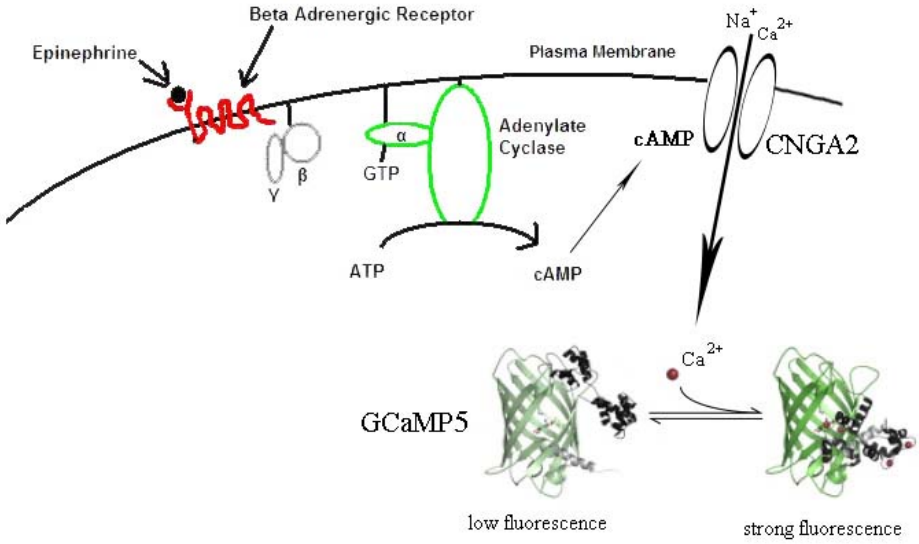
In the *beta-blocker GCaMP5 biosensor cell line*, cAMP generated by the ß-1 adrenergic receptor leads to Ca^{2+} influx into the cell due to activation of the cyclic nucleotide-gated ion channel CNGA2. An increase in intracellular Ca2+ concentration is monitored by the fluorescent Ca2+ nanoswitch GCaMP5 (Figure 153).

For the development of the *beta-blocker CEPAC biosensor cell line*, a FRET sensor containing the exchange protein directly activated by cAMP (EPAC) was used. cAMP generated by the ß-1 adrenergic receptor binds to the regulatory domain of EPAC which results in a decrease of FRET intensity (Figure 154)

The development concept for a *NSAID biosensor cell line* was to sense changes of the intracellular redox potential. Such redox potential changes are influenced by the generation of reactive oxygen species (ROS). The glutathione redox couple, being the most important determinant of the intracellular redox environment, is altered by the generation of intracellular ROS. The glutathione redox balance can be sensed by redox sensitive green fluorescent protein (roGFP). Alterations in the ratio of oxidized (GSSH) and reduced glutathione (GSH) influence the ratio of reduced and oxidized roGFP and results in changes of roGFP emission ratio (Figure 155). The metabolizing of arachidonic acid by cyclooxygenase leads to the generation of lipid peroxides. After incubation with arachidonic acid, a cell line overexpressing cyclooxygenase will generate significant intracellular amounts of ROS.

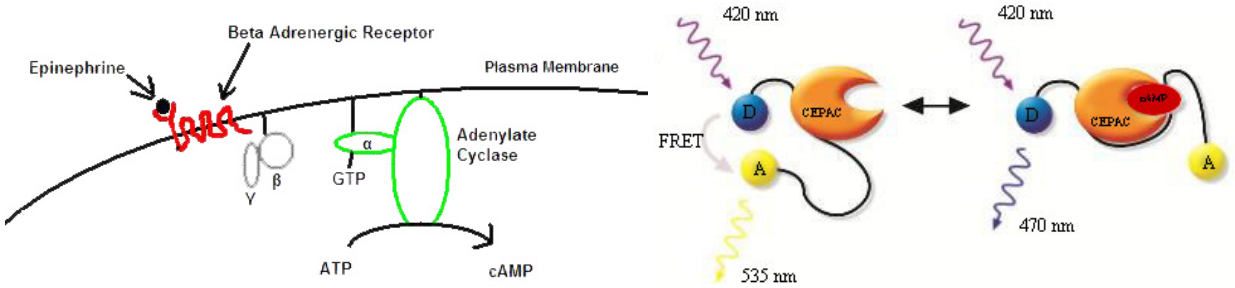
For the development of the NSAID biosensor cell lines, CHO cells were developed that express cyclooxygenase 1 (Cox-1) together with roGFPs.

Figure 153: Sensor mechanism of beta-blocker GCaMP5 biosensor cell line



After binding of the agonist, cAMP generation leads to Ca2+ channel opening. Ca2+ ion influx is monitored by an increase of Ca2+ dependent GCaMP5 fluorescence.

Figure 154: Sensor mechanism of beta-blocker CEPAC biosensor cell line



After binding of the agonist, cAMP is formed and binds to the regulatory domain of the indicator CEPAC. The distance between the donor (D) and acceptor (A) fluorophore increases after cAMP-binding, resulting in a decrease of FRET intensity.

7.1.1 Materials and Methods

Reagents were purchased from Sigma Aldrich unless specified otherwise.

Cell culture

The following cell lines were used in this study, Cho-K1 (DSMZ, ACC-110), HELA (DSMZ, ACC-57), T47D (DSMZ, ACC-739). Cells were cultivated in an incubator under 5% CO2 atmosphere and at 37°C using the appropriate media, supplemented with 10% FCS if not otherwise specified. CHO-K1 cells were grown in F12 medium, HELA cells were grown in DMEM medium and T4D cells were grown in RPMI medium. All media and serum was purchased from Sigma Aldrich.

DNA constructs

Open reading frames were synthesised by Geneart. Sequences were triplet optimized for expression in CHO-K1 cells. Genes were cloned in vectors Ga_G418-TBS-TSF3, Ga-TBS-Zeo, Ga-TBS-TSF3-IRES-Hyg, STZ-AG, STZ-CMV-CRE (STZ Angew. Biol. Chem., Mannheim).

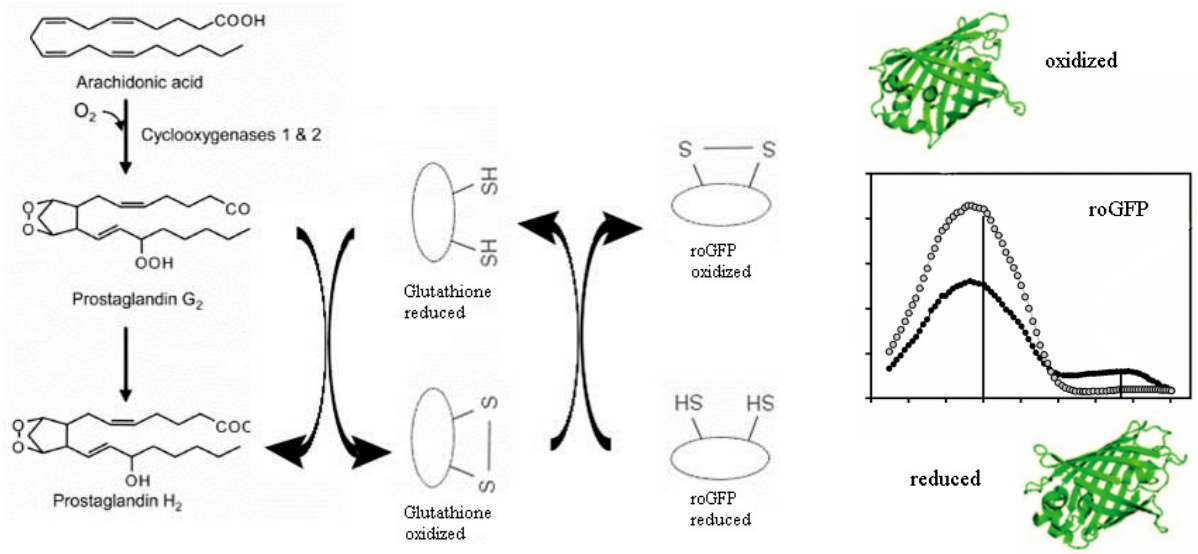
Open reading frames of the following protein sequences were used:

EGFP (GenBank: BAP87017), EGFP-d2 (d2EGFP, Clonetech), ß1-adrenergig receptor (NCBI: NP_00675), CNG-A2 (NCBI: NP_037060), GCaMP5 (GenBank: ADJ53338, delta 1-10, T292L, R293P, D370Y), ro-GFP (GenBank AAF06117, GFP C48S, S147C, Q204C), ro-GFP2 (GeneBank AAF6117, C48S, S147C, Q204C, S65T), Grx-1 (NCBI NP_001230588), ro-GFP3 (STZ Angew. Biol. Chem), CEPAC (Saloni-kidis, P.S. et al. (2011))

Cell line transfection and generation of stable cell lines

CHO cells were transfected using FUGENE-HD (Promega). All transfections were conducted according to the respective manufacturer's instructions. In order to generate stable transfected cell lines, cells were transfected at a confluence of 75% in 12-well plates. After 48 h of expression, cells were selected and expanded under 0.6 mg/ml G418-sulfate (Invivogen) or 0.6 mg/ml Hygromycin (Invivogen). In-duction of expression follows withdrawing of Doxycyclin (normally 10 ng/ml) from the medium.

Figure 155: Sensing mechanism of roGFP



Microscopic analysis

Image acquisition was conducted with a Zeiss Axiovert 200 Fluorescence microscope (Axiovision soft-ware) and an Axiocam camera. In general, microscopical images were taken with a 10x magnification lens. Quantitative measurements were conducted under constant exposure times. Images were back-ground corrected. Image analysis was conducted using the ImageJ software.

Fluorescence microplate reader measurements

Fluorescence measurements were done with the CLARIOstar plate reader (BMG Labtech). Clariostar instrument setting for the Grx-roGFP3 assay: Ex: 395-15 and Ex. 485-15; for the CEPAC assay: Ex. 420-10; Em. 470-30 and Em. 535-30

Beta-blocker assay (CEPAC biosensor cell line)

Cells were seeded 16-24h before assay in 96-wells in Ham's F12 medium (10% FCS). Induction of ex-pression followed the withdrawal of doxycycline. Fluorescence measurements were done with the

CLARIOstar plate reader (BMG Labtech). Clariostar instrument settings for the CEPAC assay: Ex. 420-10; Em. 470-30 and Em. 535-30; plate mode or well mode.

After pre-incubation with the antagonist metoprolol (5min) measurement of light emission intensity ratio at 470/535nm (excitation wavelength 420nm) was taken prior to the addition of the ß-1 adrenergic receptor agonist isoproterenol. Then, isoproterenol (0,5nM) in assay buffer was added and measurement of the fluorescence intensity ratio continued for further 23-70min in plate mode or 80-180s/well in well mode.

NSAID assay (Grx-roGFP3/Cox-1 biosensor cell line)

Cells were seeded 24h before assay in 96-wells in Ham's F12 medium (10% FCS). Induction of expression followed the withdrawal of doxycycline. Cells were pre-incubated with 100µl Diclofenac in assay buffer in the appropriate concentrations for 5min prior to start of assay. Three measurements of light emission intensity ratio at 528nm (excitation wavelengths 395/485nm) were taken prior to the addition of arachidonic acid. After 6s, to each well, 40µl of arachidonic acid in assay buffer were added to yield the concentrations of diclofenac given below and a final concentration of 50-150µM arachidonic acid. Assay buffer contained 0.1nM DTT per 50µM arachidonic acid in order to optimally protect arachidonic acid from auto-oxidation without diminishing the Cox-1 dependent oxidative Grx-roGFP3 signal. Measurement for each well continued for further 80s. Fluorescence measurements were done with the CLARIOstar plate reader (BMG Labtech). Clariostar instrument setting for the Grx-roGFP3 assay: Ex: 395-15 and Ex. 485-15; Em. 528-20; well mode.

7.1.2 Results

7.1.2.1 Beta-blocker CRE reporter cell line

The genes enhanced green fluorescent protein (EGFP) and the mutant EGFP-d2 were used in reporter constructs. The EGFP-d2 mutant contains a c-terminal PEST sequence from the mouse ornithine decarboxylase gene resulting in a reduced half-life. As a consequence EGFP-d2 protein does not accumulate inside the cell during cultivation. After transfection of CHO-K1, HELA and T47D cells resistant cell clones could only be isolated with T47D and CHO cells. Expression of the reporter controlled by the CMV CRE minimal promotor was induced by forskolin which is an unspecific activator of cellular adenylate cyclases.

cAMP dependent induction of reporter gene expression in T47D (Figure 156) and CHO cells (Figure 157) were characterized by FACS. The intracellular cAMP concentration results in cAMP binding to the cellular cAMP response element binding protein (CREB). This leads to induction of the reporter gene EGFP at a low concentration. Due to a high stability of the EGFP reporter and a concomitant protein accumulation the fluorescence is clearly measured without additional cAMP generation. In contrast the EGFP-d2 protein does not accumulate, resulting in lower fluorescence intensity (Figure 156, Figure 157). Best results were obtained with CHO cells. After forskolin dependent cAMP synthesis CHO cells showed an increase in fluorescence by a factor of two, 24h after forskolin incubation (Figure 157)

Figure 156: Forskolin dependent reportergen induction in T47D cells

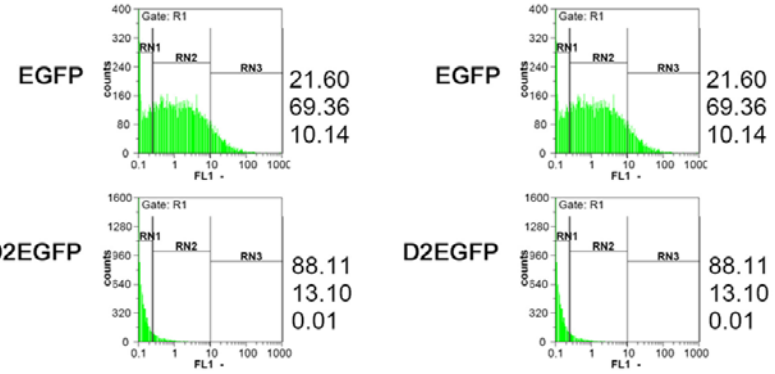
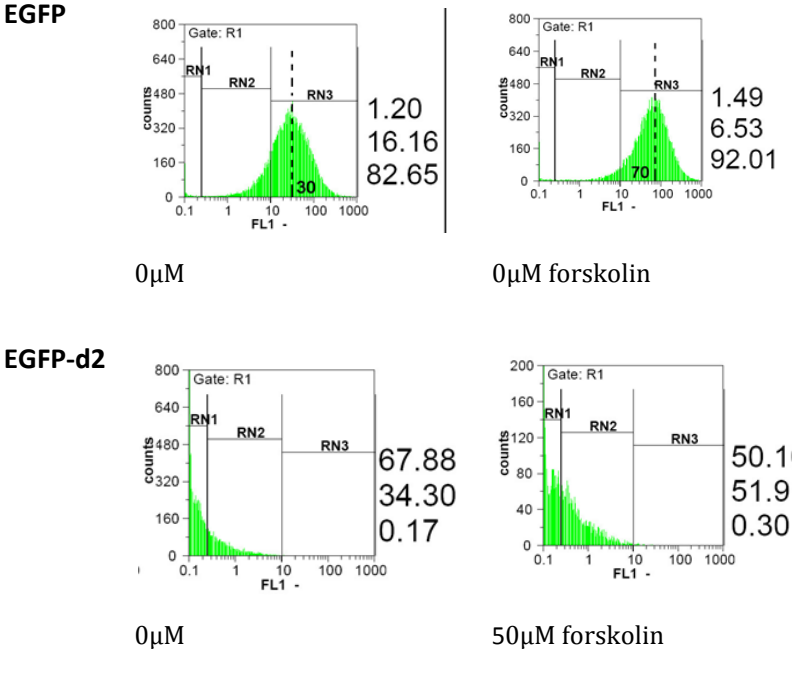


Figure 157: Forskolin dependent reportergen induction in CHO cells



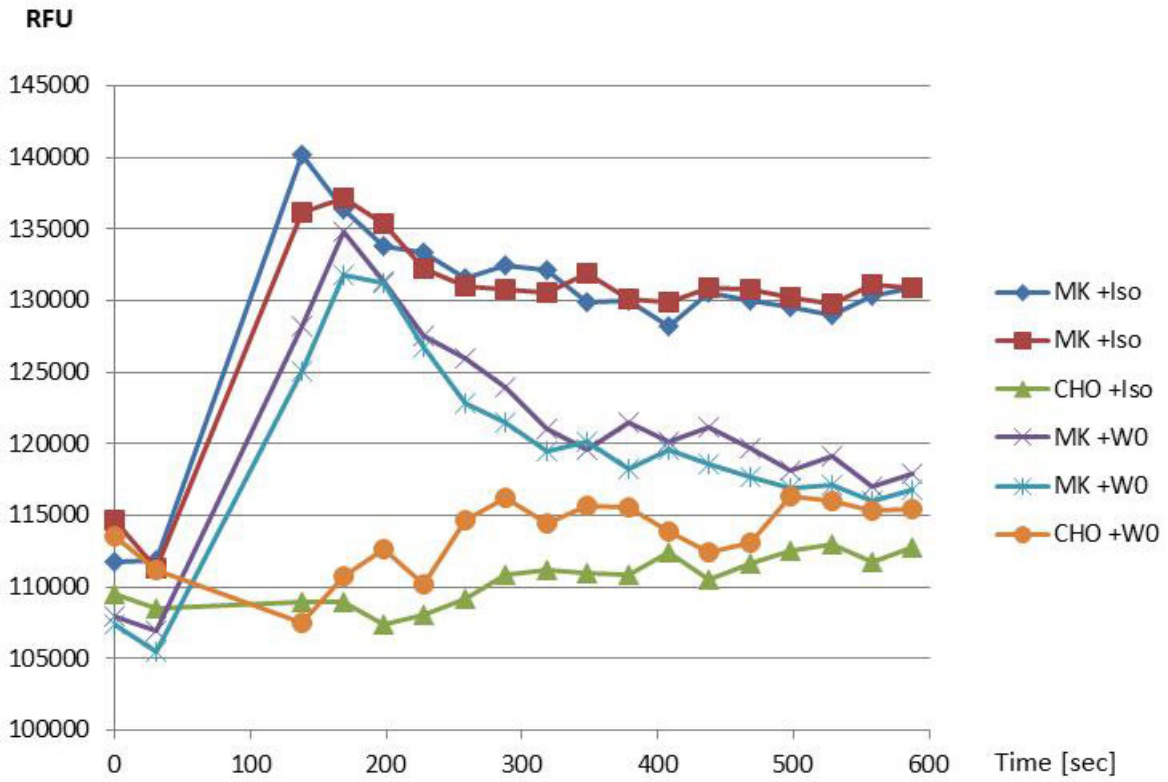
7.1.2.2 Beta-blocker GCaMP5 biosensor cell line

The genes ß-1 adrenergic receptor, CNGA2 and GCaMP5 were cloned in appropriate STZ vectors. In a first step CHO-K1 cells were transfected with a plasmid coding for inducible expression of the cAMP dependent calcium ion channel CNGA2. After G418 selection, subcloning and screening a selected CNGA2 expressing single clone was transfected with a vector containing the calcium sensor GCaMP5 and the ß-1 adrenergic receptor gene regulated by an inducible dox-off promotor. Hygromycin resistant cells were subcloned and screened. After simultaneous induction of the CNGA2 calcium channel, the ß1-adrenergic receptor and GCaMP5 cells were incubated with 1µM isoproterenol, an agonist of the ß-1 adrenergic receptor. Only cells expressing all three proteins should show a fluorescence increase after addition of isoproterenol.

Cells stably transfected with GCaMP5, CNGA2 and the ß-1 adrenergic receptor showed an increase in fluorescence after gene induction and addition of isoproterenol (Figure 158). However, adding of di-

methylsulfoxide containing buffer without isoproterenol resulted in a Ca^{2+} dependent fluorescence increase too. In contrast to the isoproterenol induced signal the buffer induced signal was only transient (Figure 158). Cells transfected with GCaMP5 only were used as a control. Cells expressing GCaMP5 only did not show any increase in Ca^{2+} dependent fluorescence after isoproterenol addition. Hygromycin resistant cell lines (MK) were incubated with (Iso) or without 1µM isoproterenol (W0). As control CHO cells expressing the GCaMP5 only (CHO) were used.

Figure 158: Isoproterenolassay with cells expressing GCaMP5, CNGA2 and ß1-adrenergic receptor

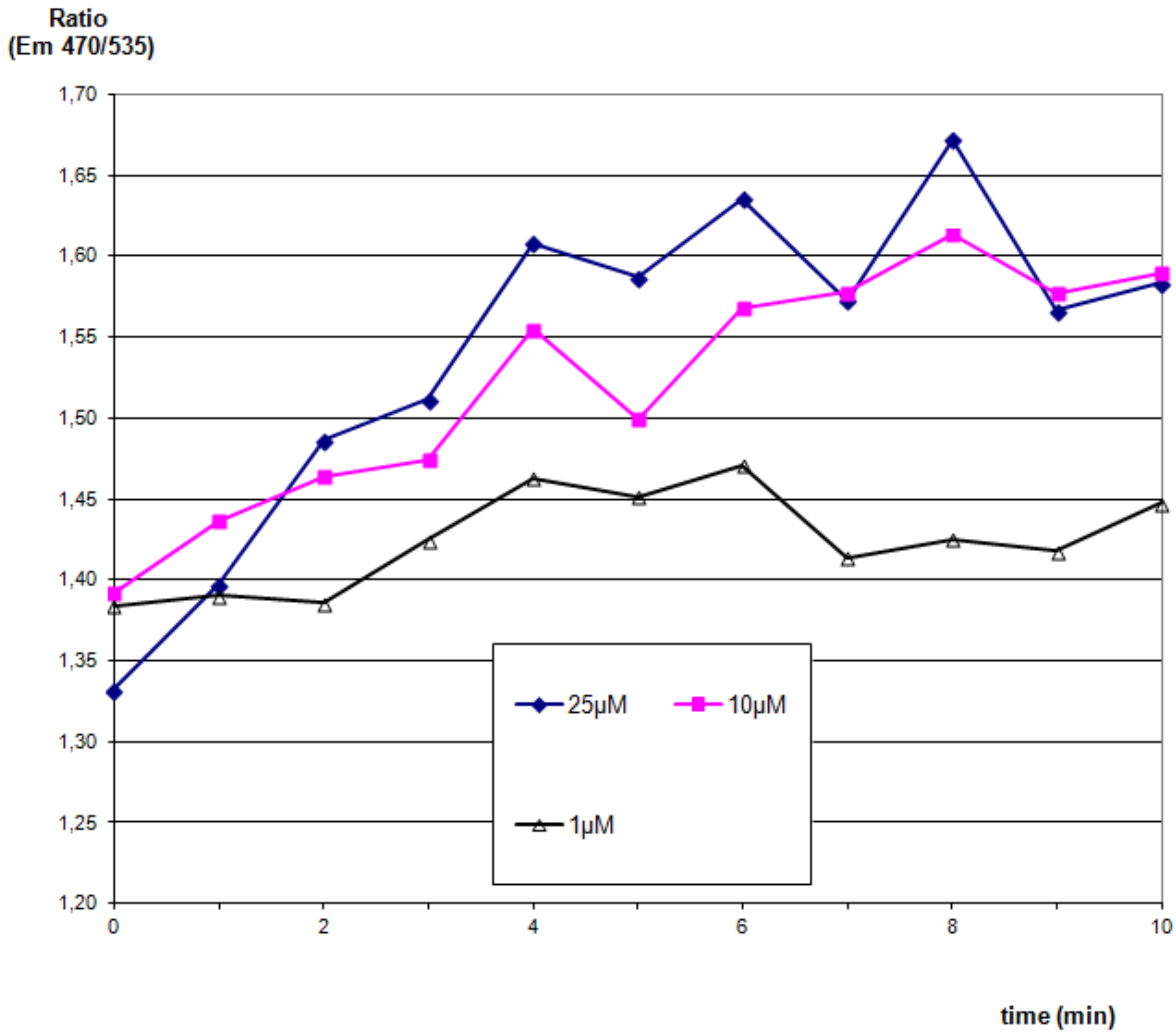


Cells stably transfected with GCaMP5, CNGA2 and the ß-1 adrenergic receptor (MK) showed an increase in fluorescence after gene induction and addition of isoproterenol (Iso). Control cells (CHO) showed no signal increase.

7.1.2.3 Beta-blocker CEPAC biosensor cell line and assay

The CEPAC (Cerulean-Citrine fusion with exchange protein directly activated by cAMP) gene was cloned in a STZ expression vector for inducible expression. The regulatory domain of the CEPAC sensor binds cAMP. Due to a conformational change the distance between donor and acceptor fluorophore increases after cAMP binding, resulting in a decrease of FRET intensity (Figure 154). CEPAC expression in G418 resistant cells was induced with doxycycline and cells were then incubated with different amounts of forskolin, an unspecific activator of cellular adenylate cyclases. The cAMP dependent FRET signal efficiency was determined by measuring the emission ratio E470/E535 after excitation at 420 nm. Higher forskolin concentrations resulted in higher cAMP concentration and higher ratios due to lower FRET efficiencies (Figure 159)

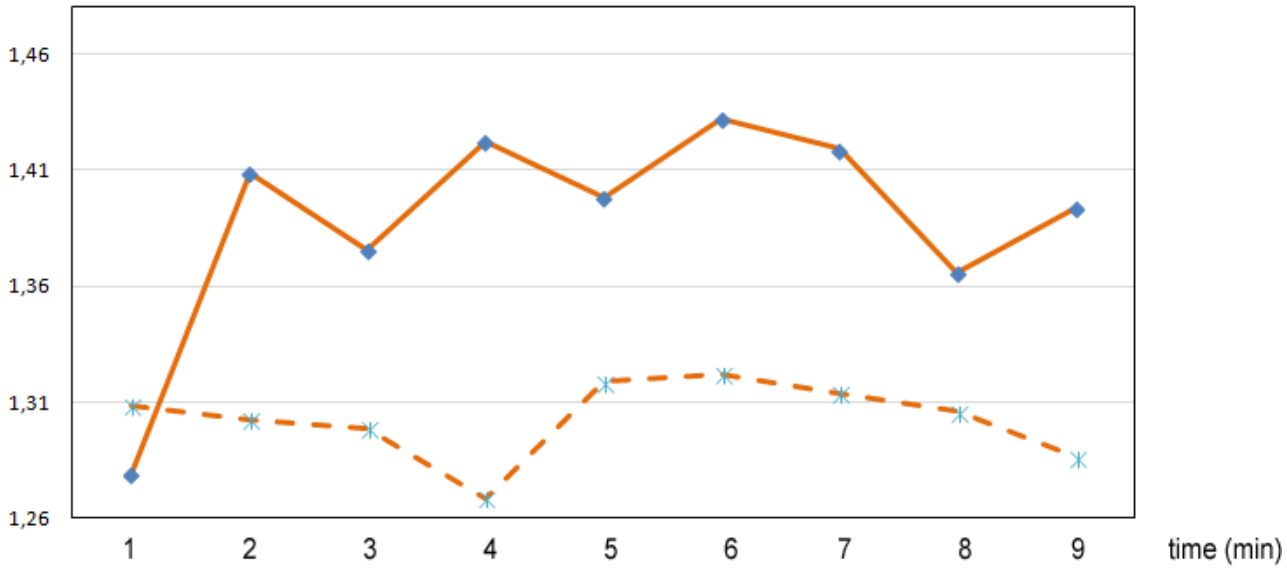
Figure 159: Forskolin dependent FRET in CEPAC sensor expressing cells



After CEPAC induction with doxycycline G418 resistant cells were incubated with 1µM, 10µM and 25µM forskolin. The cAMP dependent FRET signal efficiency was determined by measuring the emission ratio E470/E535 after excitation at 420 nm.

G418 resistant CEPAC cells were sub-cloned and screened for maximal inducible CEPAC expression. A selected single clone was then transfected with an inducible vector coding for the ß-1 adrenergic receptor. After selection, resistant cells were screened in an isoproterenol assay. In this assay CEPAC and ß-1 adrenergic receptor were simultaneously induced by doxycycline. After adding of the agonist isoproterenol The cAMP dependent FRET signal efficiency was determined by measuring the emission ratio E470/E535 after excitation at 420 nm. Cells showed a ratio increase after isoproterenol incubation (Figure 160). The emission ratio change could be inhibited by pre-incubation with the beta-blocker metoprolol. Buffer containing DMSO showed no influence on the emission ratio.

Figure 160: Isoproterenolassay with cells expressing CEPAC and ß-1 adrenergic receptor



After induction of ß1-adrenergic receptor and CEPAC expression 0µM (broken line) or 1µM isoproterenol (solid line) was added. Excitation wavelength: 420 nm, emission wavelength: 470 nm and 535 nm.

Cell lines expressing CEPAC and ß-1 adrenergic receptor upon induction, were isolated via selection with hygromycin and G418, subcloned and 60 single clones were screened using the beta-blocker assay described in methods. Addition of the ß-1 adrenergic receptor antagonist metoprolol diminished the FRET signal due to inhibition of the ß-1 adrenergic receptor-induced cAMP synthesis stimulated by the agonist isoproterenol. Single clones were screened for maximal signal intensity and ß-1 adrenergic receptor-dependent sensitivity. Here, the concentration of metoprolol required to reduce the FRET signal by 50% (IC_{50}) was determined. IC_{50} concentrations were measured by dose response curves, which were fitted using the program "sigma plot". The single clones CEPAC ß1-adr #27 and subclone #27.2.1 showed maximal signal intensity and the highest sensitivity having an IC_{50} around 15nM (Figure 161 and Figure 162)

7.1.2.4 NSAID biosensor cell line

For the development of NSAID biosensor cell lines, CHO cells were developed that express Cox-1 together with roGFPs.

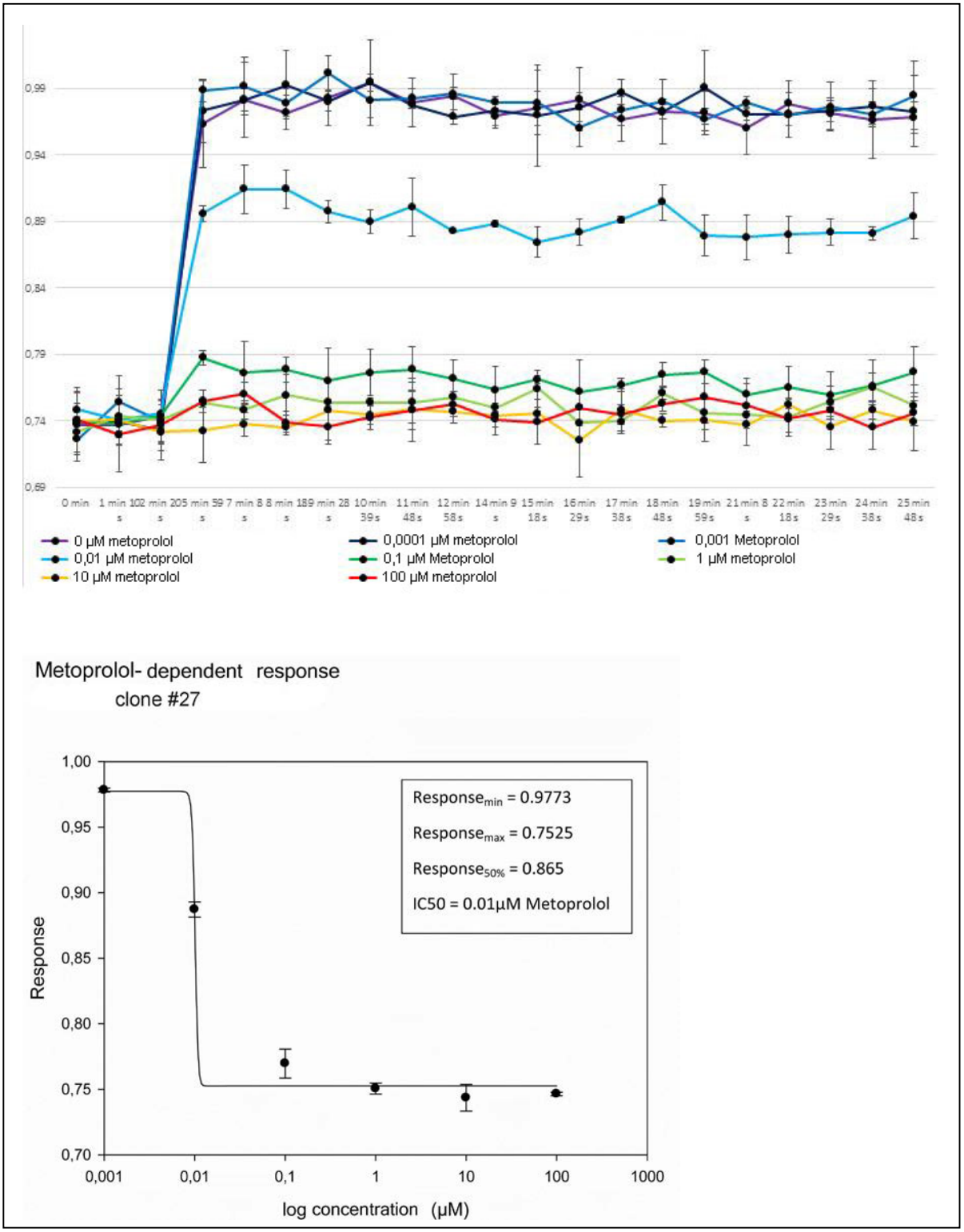
RoGFP1 and roGFP2, genes were cloned in a STZ expression vector for continuous expression. After selection, resistant cells were characterized by fluorescence analysis. Transfected cell lines showed only weak signals which were measurable using spatially-resolved fluorescence microscope measurements only. Although roGFP2 showed higher fluorescence intensity than roGFP1, addition of H_2O_2 resulted in a very weak signal change. A roGFP mutant with higher fluorescence intensity and better signal-to-noise ratio is a prerequisite for the development of a cell based microtiter plate assay which could be used in a higher throughput format. Because of the weak signal and a poor signal-to-noise ratio it was decided to check a new mutation with improved signal intensity.

We fused human glutaredoxin-1 to the new roGFP3 because it was recently published that the Grx-roGFP2 fusion protein allows dynamic live cell imaging of intracellular glutathione redox potential (Gutscheret al., 2008). Transfection and selection of cells with a construct for continuous Grx-roGFP3 expression resulted in cells expressing Grx-roGFP3. These cells showed improved fluorescence signal intensity compared to Grx-roGFP2 expressing cells and were therefore used in all further experiments. Oxidative stress induced by adding 50µM H_2O_2 into the buffer resulted in a decrease of fluorescence

emission intensity at 485nm and an increase at 395nm (Figure 163). Therefore intracellular glutathione redox potential was measured by calculating the emission ratio E395/E485. The thiol-disulfide exchange over Grx-roGFP3 enabled dynamic and rapid detection of changes of GSH/GSSH poise in cells stably expressing Grx-roGFP3. We treated cells with 50 µM H_2O_2, followed 45 seconds later by exposure to 500 µM dithiothreitol (DTT). Addition of H_2O_2 to the medium led to an immediate and strong oxidative response in Grx-roGFP3 expressing cells (Figure 164). The sensor remained in the oxidized state until DTT induced its reduction.

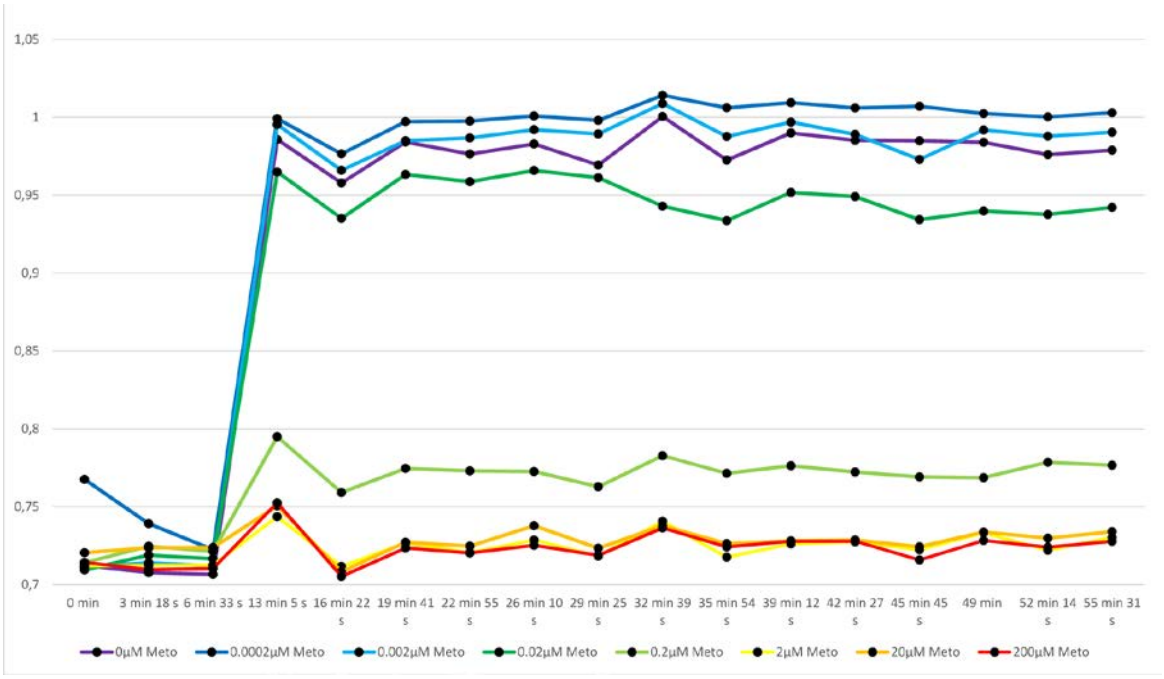
A screened single Grx-roGFP3 expressing clone was then transfected with an inducible vector coding for the ß-1 adrenergic receptor and Cox-1. Hygromycin resistant cells expressing Cox-1 and ß-1 adrenergic receptor after induction were incubated with 100µM arachidonic acid or 50µM H_2O_2. A positive shift of the emission ratio (395/485) indicated an increase of oxidized Grx-roGFP3 over reduced Grx-roGFP3 (Figure 165). Addition of arachidonic acid resulted in a ratio increase only with Cox-1 expressing cells (Figure 165A). Cells expressing Grx-roGFP3 without Cox-1 showed only an increase of the signal after incubation with H_2O_2 (Figure 165B). Resistant mixed cell clones were sub-cloned and single clones were screened by the redox sensor assay (Figure 165).

Figure 161: Beta-blocker assay clone #27



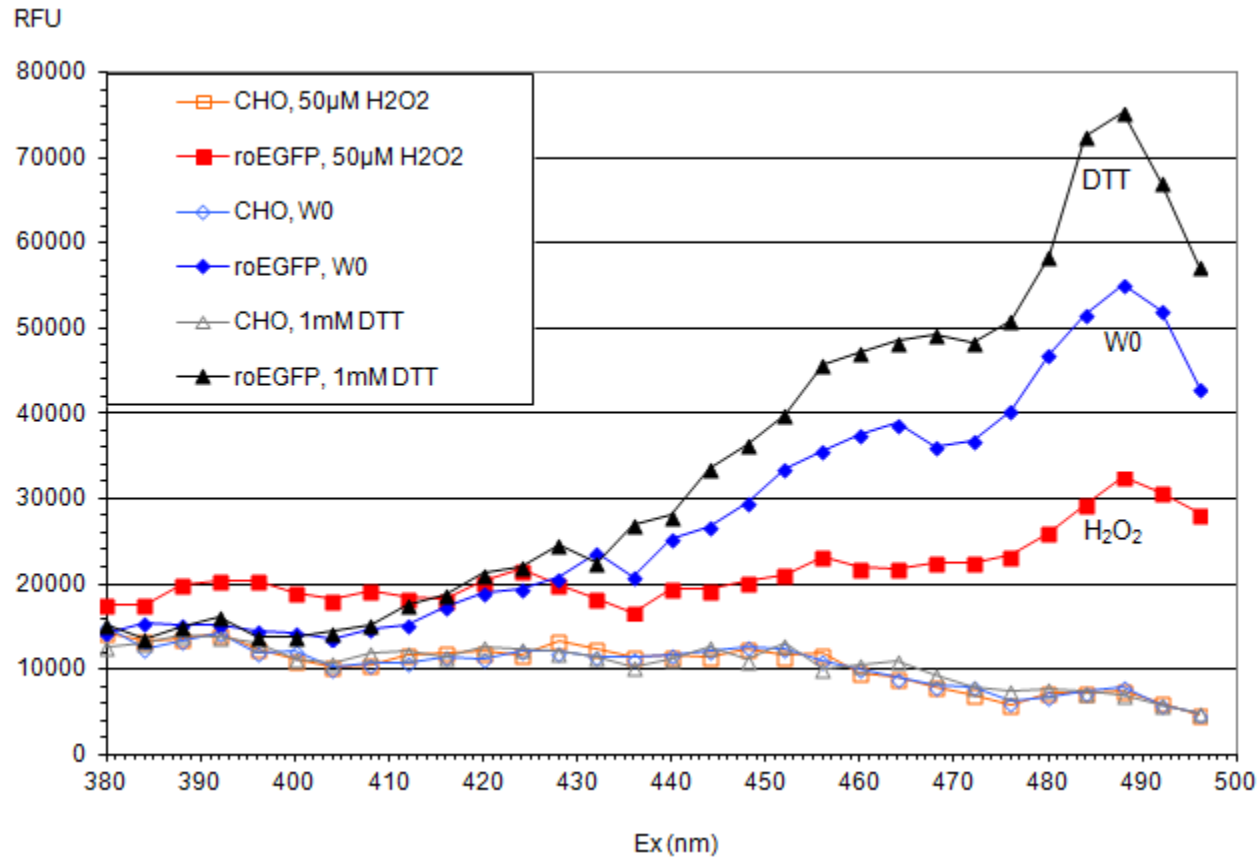
Isoproterenol assay with cell clone #27 expressing CEPAC and ß-1 adrenergic receptor. Analysis in plate mode.

Figure 162: Beta-blocker assay clone #27.2.1



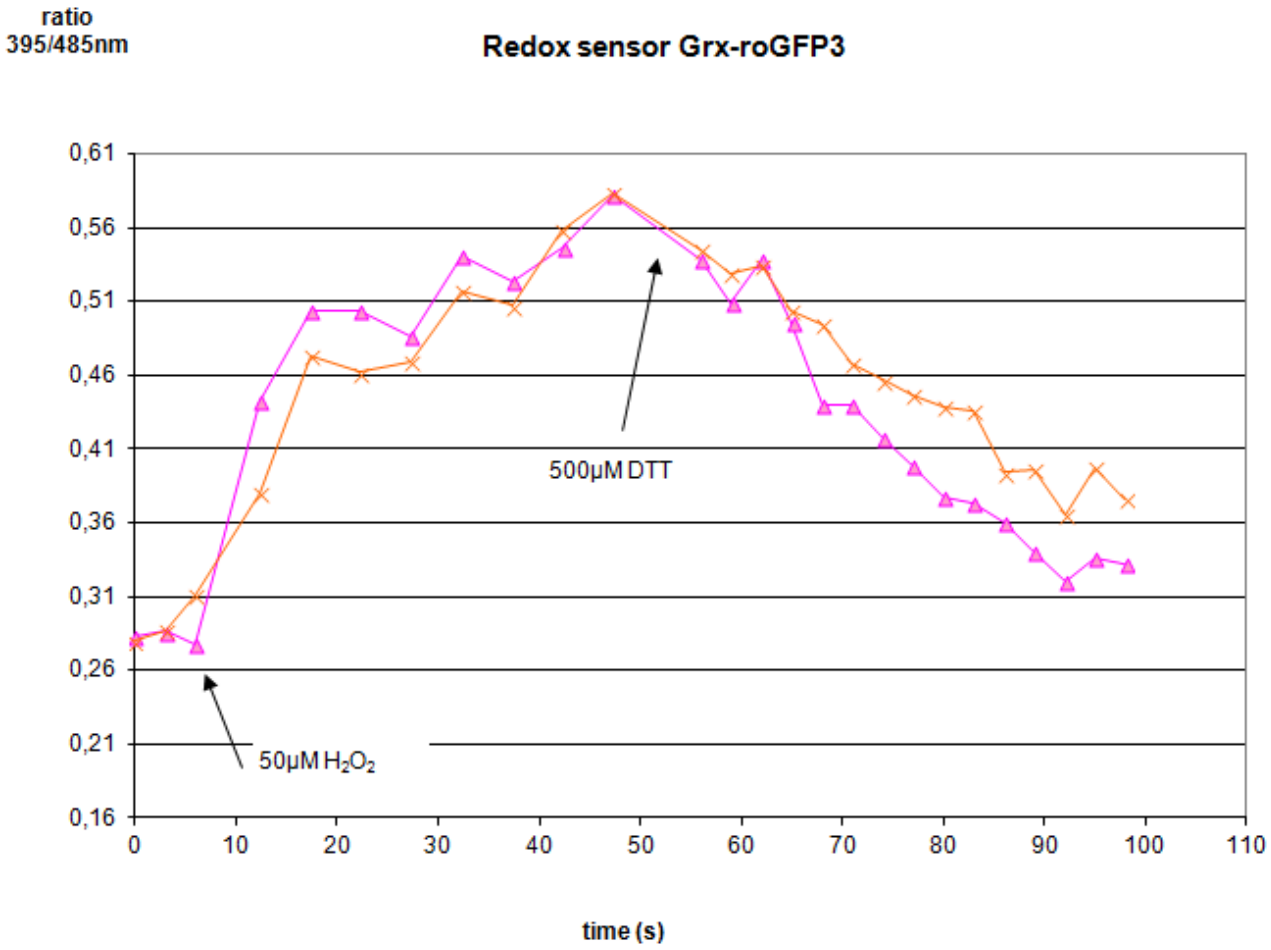
Isoproterenol assay with cell clone #27.2.1 expressing CEPAC and ß-1 adrenergic receptor. Analysis in well mode. Half maximal inhibitory concentration of metoprolol was around 20nM.

Figure 163: Spectral scan of Grx-roGFP3 expressing cells



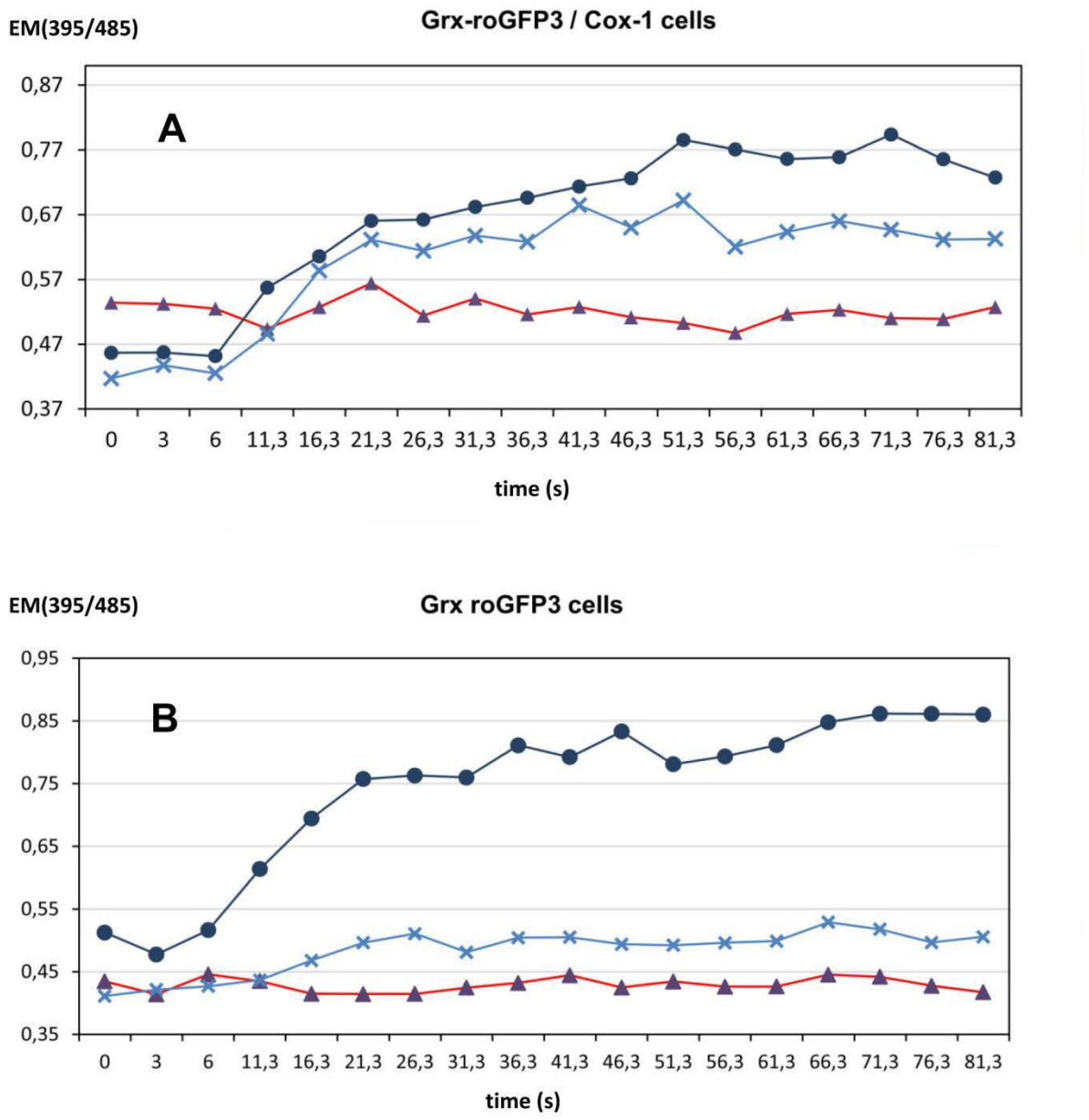
Excitation wavelength was scanned from 380-500 nm. Fluorescence intensity was measured in relative fluorescence units (RFU) at the emission wavelength 528 nm. Grx-roGFP3 expressing cells were incubated with 50µM H2O2 (red), 1mM DTT (black) or normal buffer (W0).

Figure 164: Live imaging of rapid intracellular redox changes



CHO cells expressing Grx-roGFP3 were excited at 395 and 485 nm and the ratio of emissions at 528 nm was calculated. After 6s cells were treated with 50µM H2O2 followed by DTT addition 50s later.

Figure 165: Redox sensor assay with Grx-roGFP3 expressing cells



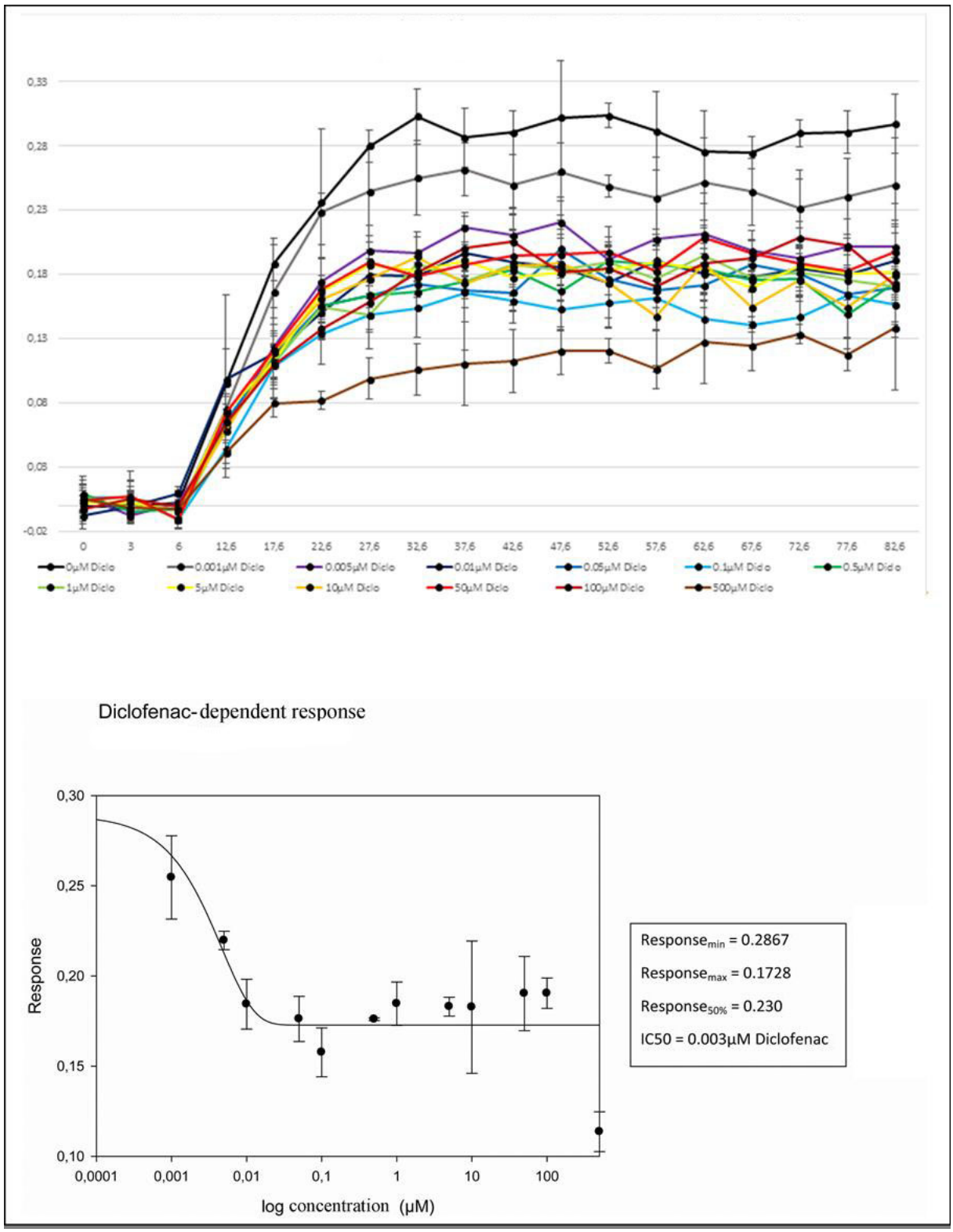
CHO cells expressing Grx-roGFP3(**B**) or Grx-roGFP3as well as Cox-1 (**A**) were incubated with 50µM H_2O_2 (●), 100µM arachidonic acid (X) or buffer (▲). Cells were excited at 395 and 485 nm and the ratio of emissions at 528 nm were calculated.

NSAID assay (Grx-roGFP3/Cox-1 biosensor cell line)

Cell lines expressing Grx-roGFP3 and Cox-1 upon induction were subcloned and 80 single clones were screened using the established NSAID assay described in methods. Cyclooxygenase-catalyzed arachidonic acid oxidation resulted in the oxidation of Grx-roGFP3 and a concomitant change of the fluorescence properties of the redox sensor. Oxidation of the redox sensor resulted in an increase of the 528nm emission intensity (E485) after excitation at 485nm and a decrease of the 528nm emission intensity (E395) after excitation at 395nm. The increase of the ratio E395/E485 due to oxidation of arachidonic acid was reduced by the Cox-1 inhibitor diclofenac. Single clones were screened for maximal signal intensity and Cox-1 dependent sensitivity. Concentration of diclofenac required to reduce the signal ratio by 50% (IC_{50}) was determined. IC_{50} concentrations were measured by dose response

curves, which were fitted using the program “sigma plot”. The single clone roGFP3/Cox-1 #1.2.14 showed maximal signal intensity and the highest sensitivity having an IC_{50} of 3-1.6nM (Figure 166).

Figure 166: NSAID assay



Grx-roGFP3 as well as Cox-1 were induced in CHO cells which were incubated with different concentrations of diclofenac (Diclo). After addition of 100µM arachidonic acid ells were excited at 395 and 485 nm and the ratio of emissions at 528 nm were calculated. Half maximal signal reduction (IC50) was seen around 3 nm diclofenac.

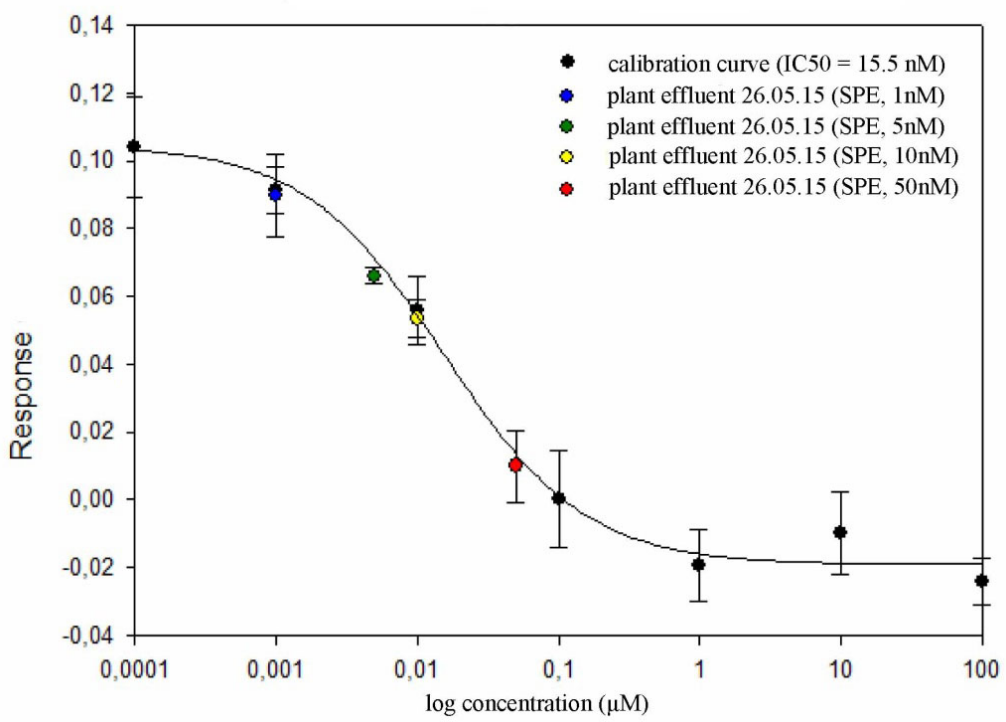
7.2 WP10A Validation of *in vitro*-test systems (Part II)

The standard analysis for the determination of diclofenac or metoprolol in municipal wastewater samples is liquid chromatography along with mass spectrometry detection (LC-MS). Due to the complex composition of the samples it is necessary to apply an extraction technique for the sample preparation before LC-MS analysis. Solid phase extraction (SPE) is the preferred sample pre-treatment technique.

7.2.1 Determination of beta-blocker activity using wastewater samples after SPE enrichment

For the extraction of metoprolol from aqueous wastewater samples solid phase extraction (SPE) was used by the project partner TZW. The eluate deriving from SPE was blown down to dryness to enable a solvent exchange. The dry residue was reconstituted with water and diluted directly in the beta-blocker assay buffer. Beta-blocker activity was measured using the beta-blocker CEPAC biosensor cell line assay. Metoprolol containing wastewater samples enriched by solid phase extraction were diluted in assay buffer and beta-blocker activity was determined in the beta-blocker assay. Dose response curve was fitted using the program sigma plot and was used for calculation of metoprolol concentration in the wastewater sample (Figure 167). Beta-Blocker activity was calculated in metoprolol units (nm metoprolol). Metoprolol concentration of the wastewater SPE samples were determined in parallel using established LC-MS methods by TZW. Measured beta-blocker activities (response) of SPE enriched wastewater plant effluents (26.05.15) were plotted in the diagram at metoprolol concentrations determined by LC-MS analysis. The beta-blocker activity calculated in the dose response curve correlated with the determination according to mass spectrometry (Figure 167).

Figure 167: Determination of beta-blocker activity in wastewater samples enriched by SPE



Calibration curve was calculated with metoprolol standards diluted in assay buffer (black circles). Measured beta-blocker activities (response) of SPE enriched wastewater samples were plotted in the diagram at metoprolol concentrations determined by LC-MS analysis. Measured beta-blocker activities (coloured points) were comparable to Metoprolol concentration determined by MS analysis.

7.2.2 Determination of NSAID activity using wastewater samples after SPE enrichment

7.2.2.1 Effects of SPE protocol on NSAID activity determination by NSAID assay

SPE was performed with wastewater samples which were undiluted, diluted with drinking or distilled water. After removal of organic solvent SPE residues were re-dissolved in distilled water and buffer concentrate was added resulting in ionic strength of assay buffer.

Dose response curve was generated with diclofenac standard, fitted using the program sigma plot and used for calculation of NSAID activities which were measured in nm or µg/L diclofenac.

Table 81 NSAID *in vitro*-assay. Effects of SPE protocol on NSAID activity determination

Wastewater plant effluent (20.7.15)	Diclofenac assay concentration [nM] (MS calculation]	NSAID activity assay concentration [nM diclofenac] (NSAID assay)	NSAID activity plant effluent [nM diclofenac] (NSAID assay)	NSAID activity plant effluent [µg/L diclofenac] (NSAID assay)
undiluted	0.5nM 1 nM 5nM 10nM	0.0nM 0.0nM 4.89nM 8,86nM	0.0nM 0.0nM 6,6nM 6,0nM	0.0µg/L 0.0µg/L 1.95µg/L 1.78µg/L
Diluted with drinking water	0.5nM 1 nM 5nM 10nM	0.5nM 1 nM 5nM 10nM	3.9nM 5.9nM 8.3nM 5.4nM	1.15µg/L 1.75µg/L 2.46µg/L 1.60µg/L
Diluted with distilled water	0.5nM 1 nM 5nM 10nM	0.0nM 0.0nM 4.0nM 5.6nM	0.0nM 0.0nM 5,4nM 3.8nM	0.0µg/L 0.0µg/L 1.60µg/L 1.13µg/L

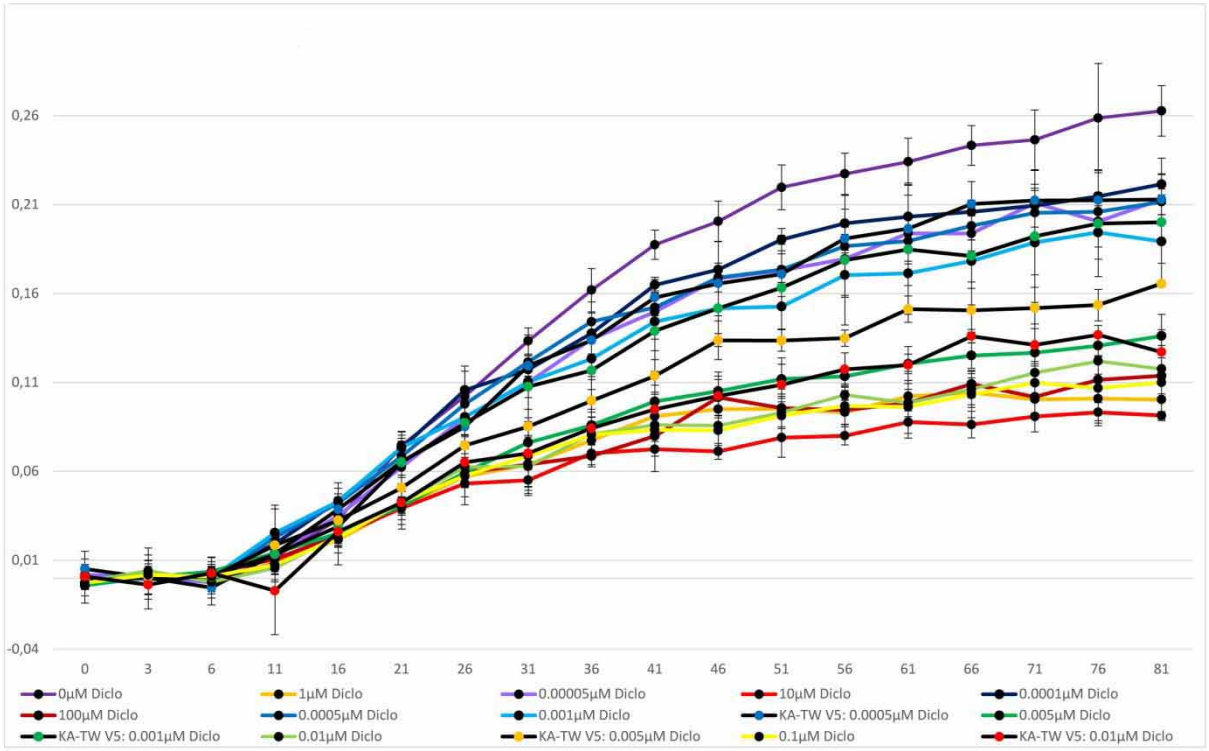
Wastewater sample from treatment plant was diluted before SPE enrichment by a factor of 5. LC-MS analysis yielded a diclofenac concentration in the undiluted treatment plant effluent of 6.75 nM (2µg/L).

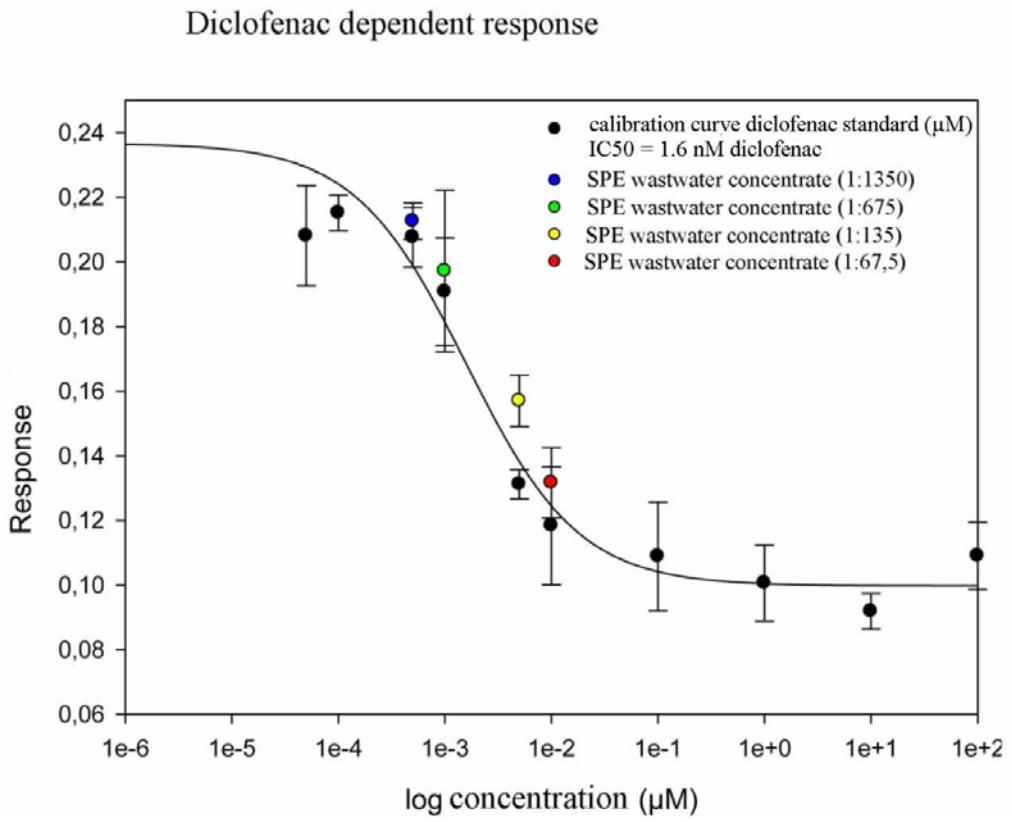
Best NSAID assay test performance was observed using wastewater diluted with drinking water before SPE enrichment. Undiluted wastewater sample or diluted sample with distilled water resulted in failure of *in vitro* activity measurement of low diclofenac concentrations (Table 81)

7.2.2.2 NSAID activity in SPE enriched wastewater treatment plant effluent

A diclofenac containing wastewater sample, enriched by solid phase extraction, was diluted in assay buffer and NSAID activity was determined in the NSAID assay. NSAID activity measured is given as "nM diclofenac" or "µg/L diclofenac" values The NSAID activity calculated using the NSAID dose response curve was comparable according to the to mass spectrometry analysis (Figure 168).

Figure 168: Determination of NSAID activity in wastewater samples enriched by SPE





Measured NSAID activities (response, coloured points) were plotted in the diagram at diclofenac concentrations determined by LC-MS analysis of the respective SPE enriched samples.

7.3 WP10B Further Validation of *in vitro*-test systems (Part III)

Solid phase extraction (SPE), the preferred sample pre-treatment technique for analysis for the determination of diclofenac or metoprolol in municipal wastewater samples, has to be optimized for each particular chemical to be analyzed. Moreover, retention of the substance of interest during SPE has to be verified by the addition of internal standards in the form of special isotopic derivatives. Cell based *in vitro*-assays would represent a major advantage over standard LC-MS analysis, if activities of pharmaceutical chemicals were measured directly in wastewater samples without the need for SPE enrichment.

During first experiments beta-blocker and NSAID activities of municipal wastewater samples could be detected without SPE enrichment. During this work package measurements were taken using native wastewater samples. Measurement data of SPE enriched wastewater samples were compared with corresponding data of non SPE enriched samples and mass spectrometry analysis, respectively.

7.3.1 Determination of beta-blocker activity using wastewater samples without SPE enrichment

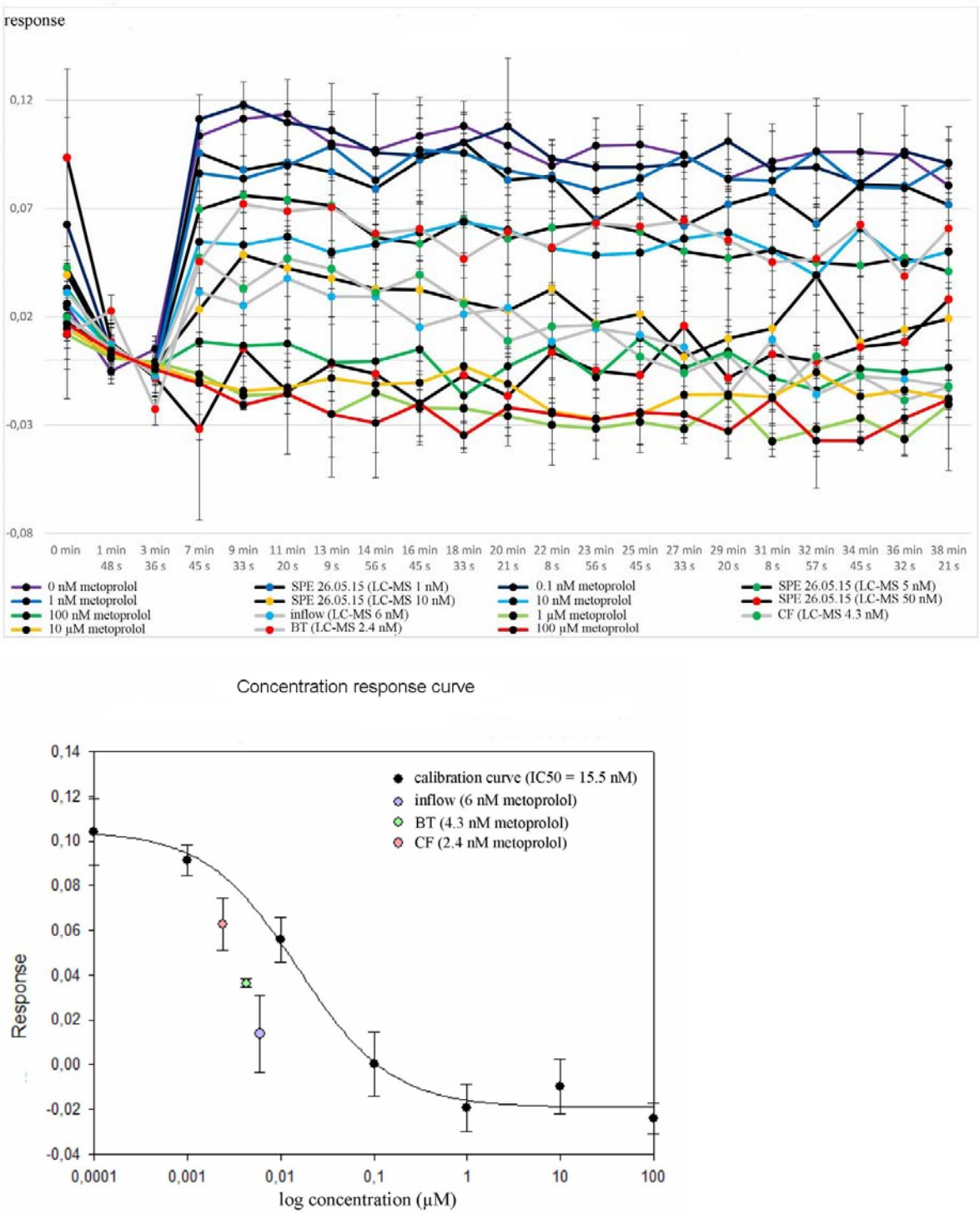
Wastewater samples were collected from a municipal treatment plant. Samples were collected at the inlet of the treatment plant (inflow), after the biological treatment (BT) and after the activated carbon filter (CF). Buffer concentrate was added resulting in wastewater samples with ionic strength of assay buffer and beta-blocker activity was characterized by measuring the 470/535 ratio after adding the agonist isoproterenol (Figure 169). Beta-blocker activity was measured with ß1-adrenoceptor/CEPAC clone #27.2.1 and is given as “nM metoprolol” or “µg/L metoprolol” values. Measured beta-blocker activities of wastewater samples were plotted in the dose response diagram at metoprolol concentrations determined by LC-MS analysis of the respective SPE enriched samples. In Table 82 beta-blocker activities were listed together with the corresponding metoprolol concentrations determined by LC-MS. Beta-blocker activities differed significantly from metoprolol concentrations determined by LC-MS of corresponding samples.

Table 82 Beta-blocker assay. Beta-blocker activity of samples not enriched by SPE

Wastewater plant effluent (03.08.15)	Metoprolol Assay concentration [nM] (LC-MS)	ß-blocker activity Assay concentration [nM metoprolol] (ß-blocker assay)	ß-blocker activity Effluent [nM metoprolol] (ß-blocker assay)	ß-blocker activity Effluent [µg/L metoprolol] (ß-blocker assay)
inflow	6.0 nM	49 nM	73.5 nM	19.6 µg/L
BT	4.3 nM	20 nM	29.8 nM	8.0 µg/L
CF	2.4 nM	7.2 nM	10.8 nM	2.9 µg/L

Wastewater samples from treatment plant were measured directly without SPE enrichment. Beta-blocker activity (ß-blocker activity) was determined by beta-blocker assay. Samples were collected at the inlet of the treatment plant (inflow), after the biological treatment (BT) and after the activated carbon filter (CF). Metoprolol concentration of corresponding samples determined by LC-MS after SPE was: 9 nM/2.4 µg/L (inflow), 6.4 nM/1.7 µg/L (BT) and 3.6 nM/0.9 µg/L (CF).

Figure 169: Determination of beta-blocker activity in wastewater samples not enriched by SPE



Wastewater samples from treatment plant (03.08.15) were measured directly without SPE enrichment. Beta-blocker activities of inflow and of effluent after the biological treatment (BT) and after activated carbon filter (CF) were measured during the assay. Measured beta-blocker activities (response, colored points) were plotted in the diagram at metoprolol concentrations determined by LC-MS analysis of the respective SPE enriched samples

7.3.2 Determination of NSAID activity using wastewater samples without SPE enrichment

Wastewater samples were collected from a municipal treatment plant. Samples were collected at the inlet of the treatment plant (inflow), after the biological treatment (BT) and after the activated carbon filter (CF). Buffer concentrate was added resulting in wastewater samples with ionic strength of assay

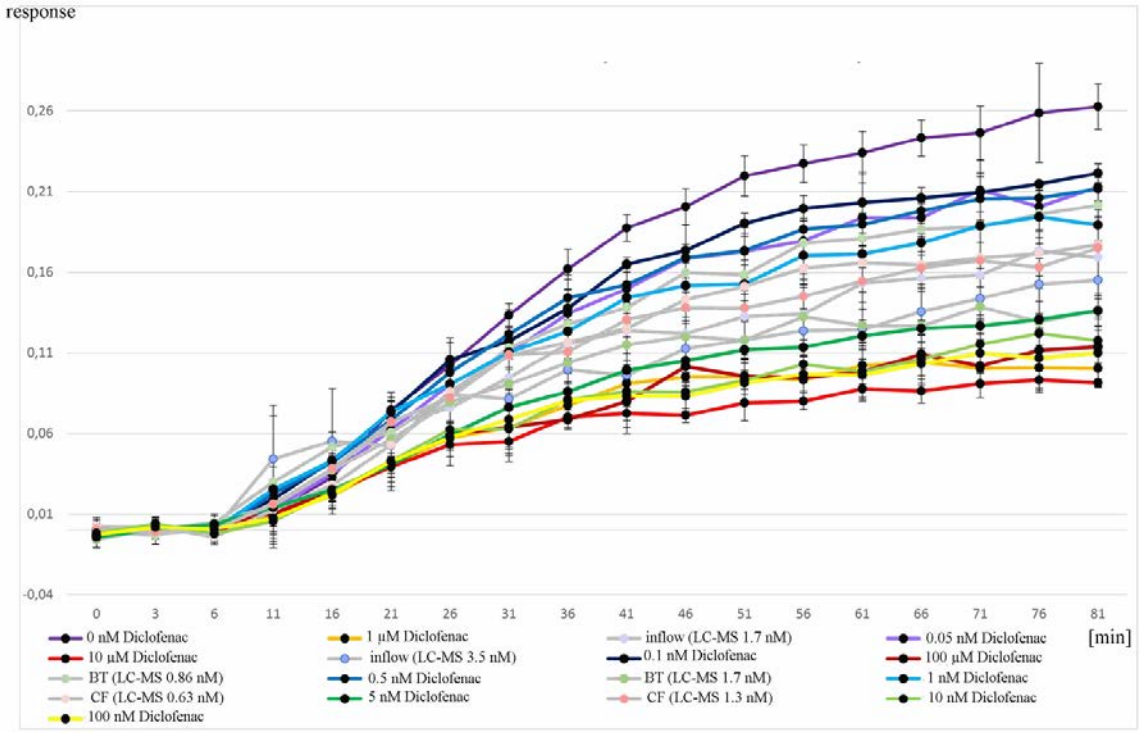
buffer. Dose response curve with diclofenac standards was fitted using the program sigma plot and used for calculation of NSAID activities. NSAID activity was measured with Cox-1/roGFP3 clone #1.33 and is given as "nM diclofenac" or "µg/L diclofenac" values. Wastewater dependent response was characterized by measuring the change of ratio (395/485) after addition of arachidonic acid. Measured NSAID activities of wastewater samples were plotted in the dose response diagram at diclofenac concentrations determined by LC-MS analysis of respective SPE enriched samples (Figure 170). In Table 83 NSAID activities were listed together with the corresponding diclofenac concentrations determined by LC-MS. NSAID activities measured in the NSAID assay of wastewater samples not enriched by SPE were in the concentration range measured by LC-MS determination of corresponding SPE enriched samples.

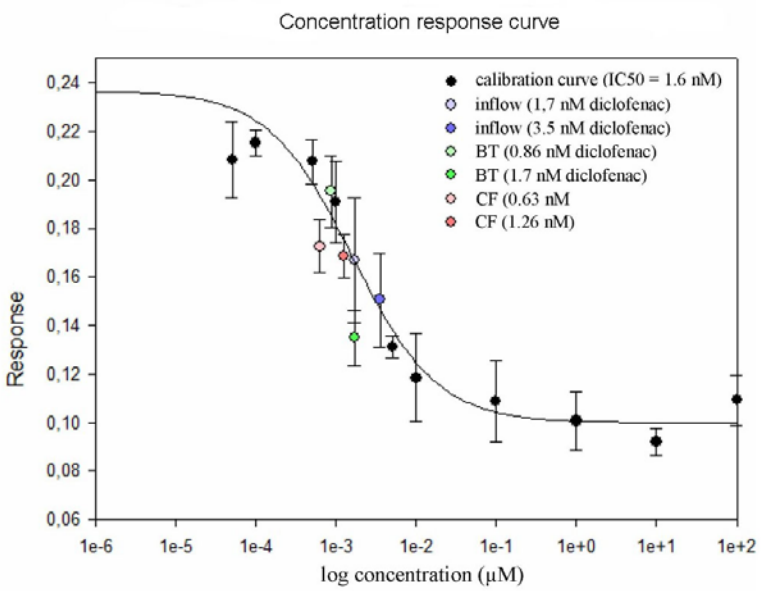
Table 83 NSAID assay. NSAID activity of samples not enriched by SPE

Wastewater plant effluent (03.08.15)	Diclofenac Assay concentration [nM] (LC-MS)	NSAID activity Assay concentration [nM diclofenac] (NSAID assay)	NSAID activity Effluent [nM diclofenac] (NSAID assay)	NSAID activity Effluent [µg/L diclofenac] NSAID assay)
Inflow	1.7 nM 3.5 nM	1.7 nM 3.1 nM	6.1 nM 5.4 nM	1.8 µg/L 1.6 µg/L
BT	0.68 nM 1.7 nM	0.6 nM 5.8 nM	2.1 nM 10.2 nM	0.6 µg/L 3.0 µg/L
CF	0.63 nM 1.26 nM	1.4 nM 1.6 nM	4.9 nM 2.8 nM	1.5 µg/L 0.8 µg/L

Wastewater samples from treatment plant were measured directly without SPE enrichment. For two different sample concentrations NSAID activity was determined by NSAID assay. Samples were collected at the inlet of the treatment plant (inflow), after the biological treatment (BT) and after the activated carbon filter (CF). Diclofenac concentration of corresponding samples determined by LC-MS after SPE was: 6.1 nM/1.8 µg/L (inflow), 3.0 nM/0.9 µg/L (BT) and 2.2nM/0.65µg/L (CF).

Figure 170: NSAID assay. Wastewater samples not enriched by SPE





Wastewater sample from treatment plant (03.08.15) were measured directly without SPE enrichment. Samples were collected at the inlet of the treatment plant (inflow), after the biological treatment (BT) and after the activated carbon filter (CF). Measured NSAID activities (coloured points) were plotted in the diagram at diclofenac concentrations determined by LC-MS analysis of the respective SPE enriched samples.

7.3.3 *In vitro*-assays in comparison with standard LC-MS/MS analysis

The project partner TZW Karlsruhe sampled and analyzed wastewater treated by an activated sludge process prior to its characterization in the *in vitro*-assays. From the measurement of cellular responses in the developed NSAID and ß-blocker *in vitro*-assays, cox-1 inhibitory or antagonistic ß-1 adrenoceptor effects of the wastewater extracts were determined as equivalents (EQ). Cox-1 inhibitory activity was measured in diclofenac equivalent quantity (DicloEQ) in µg/L diclofenac and antagonistic ß-1

adrenoceptor activity was measured in metoprolol equivalent (MetoEQ) in µg/L metoprolol. All measured EQs exceeded the respective amount of diclofenac and metoprolol concentration determined by LC-MS/MS.

Samples from the wastewater treatment plant (WWTP) in Karlsruhe from two sampling campaigns (February 2016 and March 2016) were analyzed. The determined EQs exceeded the concentration of diclofenac and metoprolol determined by LC-MS/MS (Table 80). In Table 84 EQs of samples extracted by solid-phase extraction (SPE) are listed together with the respective concentration of diclofenac and metoprolol determined by LC-MS/MS.

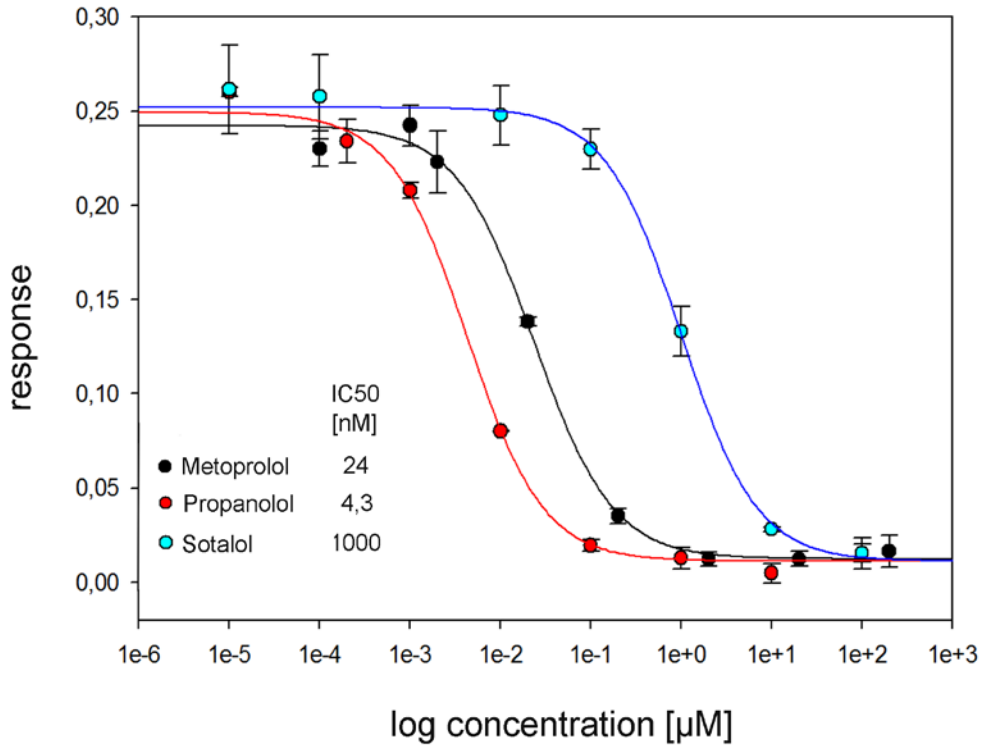
Table 84: Diclofenac and metoprolol equivalent quantities of wastewater treatment plant samples enriched by SPE

Wastewater plant effluent	Diclofenac [µg/L] LC-MS/MS	Metoprolol [µg/L] LC-MS/MS	DicloEQ [µg/L] NSAID assay	MetoEQ [µg/L] ß-blocker assay
February 2016	n.a.	1.2	n.a	3.2±1.7
March 2016	2.2	1.2	3.5±1.0	4.2±0.9

Wastewater plant effluent after activated sludge treatment was sampled from two different days. Diclofenac and metoprolol concentrations were measured by chemical analysis (LC-MS/MS) and EQs were determined by the respective *in vitro*-assays. Values represent the mean ± SEM (n=4-9).

The MetoEQs of the WWTP samples February 2016 and March 2016 differed although the metoprolol concentration determined by chemical analysis was identical. Here, the question rises whether the WWTP March sample contains additional ß-blocker activities compared to the WWTP February sample resulting in a higher MetoEQ. For the evaluation of the MetoEQ of both WWTP samples, first, an extensive chemical analysis of selected pharmaceutical compounds and some of their known metabolites was performed by the project partner TZW Karlsruhe. Besides metoprolol, the ß-blocker substances atenolol, sotalol, bisoprolol, and propranolol were present in concentrations up to 0.4 µg/L in the effluent due to incomplete removal by biological wastewater treatment (Table 80). Concentration-response curves of antagonistic activity were obtained from the ß-blocker *in vitro*-assay. In Figure 171 ß-blocker response curves of the ß-blocker metoprolol, propanolol and sotalol are shown. Table 85 lists IC_{50} values of metoprolol, atenolol, sotalol, bisoprolol, propranolol and the major metoprolol-metabolite atenolol acid, as determined by the *in vitro*-ß-blocker assay. Respective MetoEQ factors of tested compounds were calculated based on the IC_{50} values measured by the ß-blocker assay and are listed in Table 85. Bisoprolol and propranolol showed a higher and sotalol and atenolol a lower antagonistic activity than metoprolol.

Figure 171: ß-Blocker concentration-response curves



Concentration-response curves of the ß-blocker substances metoprolol, propranolol and sotalol. IC_{50} values were calculated by fitting of the concentration response curve (sigmoidal fitting, 4 parameter, SigmaPlot). Values represent the mean ± SEM (n=3).

Table 85: IC_{50} of ß-1 adrenoceptor antagonists

Antagonist	IC_{50} [µg/L]	IC_{50} [nM]	Factor MetoEQ
Atenolol	13.7	51.6±2	0.63
Bisoprolol	2.9	8.8±2.6	3.70
Metoprolol	8.7	32.6±14	1,0
Propanolol	1.9	6.5±3	5.02
Sotalol	277.7	1019±30	0.03
Atenolol acid	114 000	$426x10^3$±$241x10^3$	$8,78x10^{-6}$

Values represent the mean ± SEM of 3-4 different measurements. Propanolol and bisoprolol showed a higher antagonistic activity in the ß-blocker *in vitro*-assay than metoprolol. For the calculation of MetoEQs a respective factor was calculated for all measured compounds.

With the calculated MetoEQ factors, it is possible to estimate the antagonistic ß-1 adrenoceptor activities of the WWTP effluent samples. In Table 86 the MetoEQs were estimated under the assumption that the antagonist compounds show an additive activity only. Although the value of the antagonist metoprolol of the two sampling campaigns was comparable, the total MetoEQ differed due to a higher concentration of the antagonists atenolol, bisoprolol and propranolol in the WWTP effluent of March 2016 compared to the WWTP effluent of February 2016.

Table 86: ß-1 adrenoceptor antagonist equivalent quantities of wastewater treatment plant samples based on chemical analysis

Wastewater plant effluent	ß-blocker	concentration [µg/L] / [nM] LC-MS/MS	factor MetoEQ	MetoEQ [µg/L]
February 2016	atenolol	<LOQ	0.63	0
	bisoprolol	0.28 / 0.86	3.7	0.85
	metoprolol	1.2 / 4.49	1.0	1.2
	propanolol	<LOQ	5.02	0
	sotalol	0.1 / 0.37	0.03	0
	atenolol acid	n.a.	$8{,}78 \times 10^{-6}$	0
			total MetoEQ	2.06
March 2016	atenolol	0.07 / 0.26	0.63	0.04
	bisoprolol	0.4 / 1.23	3.7	1.22
	metoprolol	1.2 / 4.49	1.0	1.2
	propanolol	0.05 / 0.17	5.02	0.23
	sotalol	0.14 / 0.51	0.03	0
	atenolol acid	0.54 / 2.02	$8{,}78 \times 10^{-6}$	0
			total MetoEQ	2.69

Wastewater plant effluents of two sampling campaigns after activated sludge treatment were analyzed for selected ß-blocker compounds and the metoprolol metabolite atenolol acid by chemical analysis. ß-1 adrenoceptor antagonist equivalent quantities (MetoEQs) were estimated under the assumption that the antagonist compounds show an additive activity only.

The MetoEQs measured by the ß-blocker *in vitro*-assay (Table 84) exceeded the MetoEQs calculated from chemically analyzed ß-blocker concentrations (Table 86) by a factor of 1.5 (WWTP February and WWTP March 2016). This additional ß1-adrenoceptor antagonistic activity measured by the ß-blocker *in vitro*-assay indicates that further ß-blocker compounds are present in the WWTP effluent, which were not analyzed by the chemical analysis.

As well as, in the ß-blocker case, the DicloEQs measured by the NSAID *in vitro*-assay (3.5 µg/L diclofenac, Table 84) for the WWTP effluent March 2016 exceeded the diclofenac concentration determined by chemical analysis (2.2 µg/L diclofenac, Table 84). This indicates that beside diclofenac there are additional active NSAID compounds present in the WWTP effluent. The IC_{50} of the metabolite 4-hydroxy-diclofenac was determined in the diclofenac *in vitro*-assay to amount 10 µM.

A more detailed chemical analysis determined compounds with cyclooxygenase-1 inhibitory activity. The compounds ibuprofen (1.1 µg/L) and naproxen (1.4 µg/L) were among the unspecific cyclooxygenase inhibitors with the highest concentration analyzed in the WTTP effluent (Table 80). Although the specific inhibitory activity of these compounds was not measured by the NSAID *in vitro*-assay, it seems plausible that in the WWTP effluent the DicloEQ must be higher than the diclofenac concentration.

This result indicates that pharmaceuticals with similar mode of action in wastewater effluent might have additive and/or synergistic effects. This emphasizes the necessity for *in vitro* assays to detect the total activity of pharmaceutical mixtures being present in WWTP effluents.

7.3.4 Determination of metoprolol and diclofenac activities in municipal wastewater treatment plant effluent without SPE extraction

During the ß-blocker *in vitro*-assay development wastewater samples were tested in the in *vitro-assays* directly without SPE enrichment. Thereby higher ß-1 adrenoceptor antagonist activity in the original water samples than in the SPE extracts were detected. It was assumed, that compounds that show activity in the bioassays are not retained by the SPE sorbent material. As a consequence these compounds would break-through the sorbent and would still be present in the SPE-extracted water sample.

Therefore, the SPE flow-through as well as wastewater extracted by weak (WAX) and strong (MAX) anion and weak (X-CW) and strong (MCX) cation exchanger SPE materials was prepared by the partner TZW and was analyzed by the *in vitro*-assays. The used anion or cation exchanger could not enrich the complete metoprolol antagonistic equivalent activity found in the breakthrough of the standard SPE (Table 87).

Table 87: ß-1 adrenoceptor antagonist equivalent quantities of WWTP samples (February 2016) measured by the ß-blocker *in vitro*-assay

Wastewater plant effluent native MetoEQ µg/L	SPE MetoEQ µg/L	WAX extract MetoEQ µg/L	MAX extract MetoEQ µg/L	X-CW MetoEQ µg/L	MCX MetoEQ µg/L
4.1±2.0	3.2±1.7 (extract)	0.23±0.21	0.23±0.27	0.08±0.04	0.0
	2.8±1.1 (breakthrough)				

SPE column breakthrough was fractionated by weak (WAX) and strong (MAX) anion and weak (X-CW) and strong (MCX) cation exchanger SPE materials. Metoprolol antagonistic equivalent activity was found in the breakthrough of the standard SPE column. Values represent the mean ± SEM of 3-4 different measurements.

In native WWPT effluent (March 2016) DicloEQ determined by the diclofenac *in vitro*-assay was in the range of 1.8 µg/L compared to the SPE enriched respective sample which was determined to be 3.5 µg/L.

7.3.5 Summary

All concepts for the development of beta-blocker sensing cell lines were successful. 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.

Several published roGFP redox sensors, which were tested in CHO cells after stable transfection, showed only 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 micro plate 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 require only short incubation times of sensor cells with analyte solutions. Consequently there is no need for working under sterile conditions during measurement which is 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 seems that beta-blocker activities of wastewater samples are significantly higher than metoprolol concentrations measured by LC-MS of corresponding SPE enriched samples. This does not seem to be the case for NSAID activities. 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.

8 Part IV: Studies with *Daphnia magna*

8.1 Introduction

The objective of the work package was to evaluate toxic effects of diclofenac and metoprolol on the reproductive output of the plankton free water species *Daphnia magna*. This Crustacean species is one of the most common standard test organisms in regulatory assessment for e.g. pharmaceuticals or plant protection products.

Three 21 days toxicity tests according to OECD TG 211 (OECD, 2012) were performed with both single substances and a mixture of diclofenac and metoprolol. The semi-static exposure of parent females started with animals aged less than 24 h and test substances added to water at a range of concentrations. 1.9 – 50 mg diclofenac/L and 0.1 – 25 mg metoprolol/L were investigated as test concentrations related to free acid and free base, respectively.

Stress protein Hsp70 quantification was used as a well established method to determine any proteotoxic effects of the test substances; for details see chapter 8.3. This protein group can be found in a wide range of taxonomic groups as it is phylogenetic highly conserved. The increase of the Hsp70 level of exposed animals, compared to control individuals, indicates a protein damage caused by chemical stress. However, also a decreased Hsp70 level might be the result of a stressful condition.

8.2 Toxicity tests

8.2.1 Materials and Methods

8.2.1.1 Test substances

Diclofenac sodium salt (CAS: 15307-79-6, batch BCBK6371V) and metoprolol tartrate (CAS: 56392-17-7, batch BCBH0239V) were obtained from Sigma Aldrich (Taufkirchen). The chemical purity of both test substances was ≥98%. For more details of physical/chemical properties please see e.g. chapter 4.2.1.1.

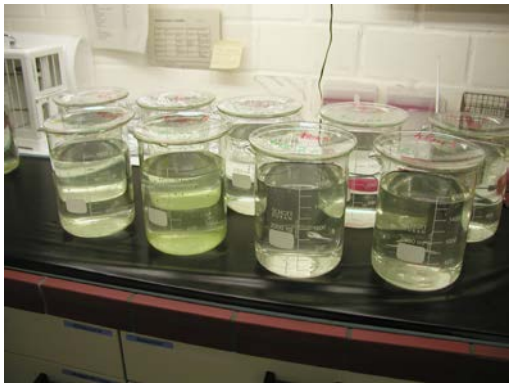
8.2.1.2 Test organism and culture

The parent females dreived from an in-house culture (UBA, Berlin Marienfelde). They were kept in 2 L glass beakers with 1.6 L aerated Elendt M4-medium for three weeks. In the following, the pre-adults were isolated and kept individually under the same conditions in smaller glass vessels for another three weeks. As food an algae suspension of *Desmodesmus subspicatus* was administered according to OECD TG 211 (OECD, 2012), corresponding to 0.1 – 0.2 mg TOC per animal and day. The environmental conditions were adjusted as follows: temperature 18 – 22°C; light regimen 16 h light / 8 h dark; illumination 15-30 µmol m^{-2} s^{-1}.

Figure 172: *Daphnia magna* (A) and in-house culture of daphnids at UBA, Berlin Marienfelde (B)

A

B



8.2.1.3 Test design and endpoints

The exposure test was performed according to OECD TG 211 and GLP specification (OECD, 2012). For the single substance tests with diclofenac and metoprolol, ten replicates with one animal each (<24 h old) per test concentration (five test concentrations overall, see Table 88) were used in the 21 day semi-static exposure design. For the test with the mixture of both test substances twelve replicates were used. 150 mL glass beakers were used as exposure vessels covered with glass lids to prevent evaporation. Every two or three days the test solutions based on Elendt M4-medium were renewed. The pH value, oxygen content and temperature were measured in both old and new test solutions. Additionally, the temperature in the climate test unit was measured continuously by data logging. A food suspension of *D. subspicatus* was administered daily, corresponding to TOC of 0.1 – 0.2 mg TOC per animal and day. The following endpoints were evaluated: mortality of parent females, number of offspring (total and per parent), and time to first hatch. Replicates with dead parent animals were excluded from statistical evaluation. Additionally, the biomarker Hsp70 was measured to evaluate proteotoxic effects.

Table 88: Test concentrations of diclofenac and metoprolol in single substance toxicity tests with *D. magna*

Identification	Concentration [mg/L, nominal]		Metoprolol [mg/L, nominal]	
	Diclofenac sodium	Diclofenac	Metoprolol tartrate	Metoprolol
Control	–	–	–	–
Concentration 1	2.0	1.9	0.128	0.1
Concentration 2	6.7	6.25	0.641	0.5
Concentration 3	13.4	12.5	3.20	2.5
Concentration 4	26.9	25.0	12.81	10.0
Concentration 5	53.7	50.0	32.03	25.0

8.2.1.4 Mixture test concentrations

The aim was to investigate the mixture toxicity of diclofenac and metoprolol in *D. magna* and to compare it with the results of the single substance toxicity test. According to the several mode of actions of pharmaceuticals a mixture may act additive, synergistic or antagonistic. To enable the comparison all exposure conditions and endpoints were identical in all toxicity tests with *D. magna* (cf. 8.2.1.3).

The mixture concentrations were determined using the concept of the toxic unit (TU). Hereby, one TU was defined as the lowest observed effect concentration (LOEC) derived from single substance tests with *D. magna*. The LOEC for diclofenac was determined with 6.25 mg/L, for metoprolol with2.5 mg/L for most relevant endpoint number of offspring (cf. 8.2.2). Table 89 summarizes the ratios and concentrations tested in the mixture toxicity test with *D. magna*.

Table 89: Mixture ratios and test concentrations of diclofenac (DIC) and metoprolol (MET) in toxicity test with *D. magna*; toxic unit (TU) DIC = 6.25 mg/L, MET = 2.5 mg/L

Identification	Mixture ratio of TU		Mixture concentration [mg/L, nominal]		Mixture sum [mg/L, nominal]
	TU (DIC)	TU (MET)	DIC	MET	
Control	0	0	–	–	–

Identification	Mixture ratio of TU		Mixture concentration [mg/L, nominal]		Mixture sum [mg/L, nominal]
	TU (DIC)	TU (MET)	DIC	MET	
CM1	¼	¼	1.56	0.625	2.19
CM2	¼	¾	1.56	1.875	3.44
CM3	½	½	3.125	1.25	4.38
CM4	¾	¼	4.68	0.625	5.31
CM5	¾	¾	4.68	1.875	6.56

8.2.1.5 Chemical analyses

At the beginning and on days 3, 6, 8, 10, 13, 15, 17, 20, and 21 of the test 500 mL exposure medium and 500 mL control medium were sampled - pooled from the replicate vessels. The glass bottles with the medium samples were stored at 5 – 10°C in the dark not for longer than one week (diclofenac) or at 2 – 4°C (metoprolol). To start the analytical procedure 20 mL from each sample were filtered via glass fibre filter. Subsequently, an aliquot of the filtrate was blended with 1 mL ammonium acetate solution and 0.5 mL acetonitrile (diclofenac samples). Metoprolol samples were treated with a PS-DVB SPE column for enrichment. HPLC-UV/VIS was used for analytical verification of the test compounds.

8.2.1.6 Statistics

Statistical analysis was performed with ToxRat software (ToxRat 2.10.06 and 3.1.0, Aachen, Germany). Due to the properties of both test substances no solvent needed to be used and, therefore, all results could be compared to the control directly. ECx-values were computed using probit analysis and linear maximum likelihood regression as concentration-response fitting. No observed and lowest observed effect concentration (NOEC, LOEC) values were determined by test on normal distribution (Shapiro-Wilk) and analyses of variance (Levene). If normal distribution and variance homogeneity requirements were fulfilled the Williams´ multiple sequential t-test procedure as parametric post-hoc test was used ($\alpha = 0.05$, one-sided). Alternative approaches different to that are descripted in the biological effects chapter below.

8.2.2 Results and discussion

8.2.2.1 Physico-chemical parameters

The physico-chemical parameters in all tests were determined in biologically acceptable ranges and in accordance with the OECD TG 211 (OECD, 2012). The measurement details are summarized in Table 90 below.

Table 90: Water parameters measured in test vessels of the exposure tests with diclofenac, metoprolol and the mixture of both test substances.

Test	Parameter		
	Oxygen [mg/L]	Water temperature [°C]	pH
Diclofenac	8.0 - 10.1	21.2 - 23.7*	7.3 - 9.0
Metoprolol	8.2 - 10.9	20.4 - 22.6	6.3 - 9.2*
Mixture	8.5 - 10.0	21.3 - 23.1*	7.4 - 8.8

* outside the given range in guideline; but slightly higher value is considered not relevant for validity and acceptability of the test procedure.

8.2.2.2 Analytical verification of test concentrations

The test concentrations of diclofenac, metoprolol and the mixture of both were analytically verified using HPLC-DAD (UV/VIS). For detection of diclofenac a C18-HD column was used with 0.1M ammonium acetate and 0.2M ammonium acetate – acetonitrile mixture as eluent solutions. A C18 aqua column was used with mixtures of water and acetonitrile added by 0.1M phosphoric acid as well as 0.01M sodium 1-octanesulfonate. The values of the limit of detection and limit of quantification for diclofenac are 4 µg/L and 8 µg/L and for metoprolol 10µg/L and 40 µg/L, respectively. The analytical recovery of the diclofenac samples from single substance test varied between 94% and 99%, and of the metoprolol samples between 91% and 101%. For the mixture experiment the recovery contained 93%-98% for both compounds. The recovery of all tests exceeded 80% and therefore, in all three experiments the test concentrations for effect value calculation were based on a nominal level.

8.2.2.3 Biological effects

In all exposure tests with daphnids the following endpoints were investigated: parent mortality, number of living offspring (total and per parent), number of dead offspring, time to first hatch and abnormal behaviour of the parent animals.

8.2.2.4 Biological effects of diclofenac

As mortality parameter the immobility of the parent animals was determined and contained in control and lower test concentrations up to 20%, while in the both highest test concentrations mortality of 60% and 70% occurred, respectively. Nevertheless, in consequence of the data no statistical significance was obtained (exact Fisher t-test with Bonferroni), but an EC_{50} value of 25.2 mg/L and an EC_{10} value of 3.6 mg/L were calculated. As most relevant reproduction parameter the cumulative number of offspring per living parent animal (survivor) was evaluated. In the concentration of 6.25 mg/L and higher statistically significant effects were observed, resulting in a NOEC of 1.9 mg/L. Based on a concentration response and using the Probit method an EC_{50} value of 15.0 mg/L and an EC_{10} value of 3.2 mg/L were calculated. The most sensitive endpoint was time to first hatch. The multiple Welsh-t test (Bonferroni-Holm) was used to determine NOEC and LOEC values. As already in the lowest test concentration significant effects occurred a NOEC value of <1.9 mg/L was deviated. For summarised endpoints and (no-) effect values, see Table 91 below. With 20% immobility and an number of 86 as mean offspring number per survivor in the control the diclofenac exposure test was considered valid and acceptable according to OECD TG 211 (OECD, 2012).

8.2.2.5 Biological effects of metoprolol

A concentration response was observed for the endpoint immobility; starting with 20% effects in the lowest test concentration 0.1 mg/L up to 80% effects in the highest test concentration 25 mg/L. Due to significant effects in the both highest concentrations a NOEC value of 2.5 mg/L was inferred. Furthermore, an EC_{50} value of 2.0 mg/L was calculated. The EC_{10} was not applicable as 20% effects occurred already in the lowest test concentration and a 10%-value needed to be extrapolated. The evaluation of the cumulative number of offspring per survivor resulted in a NOEC of 0.5 mg/L despite the fact of no clear concentration response in the two lowest test concentrations. Using the Probit method and a maximum-likelihood regression an EC_{50} value of 2.9 mg/L and an EC_{10} value 0.47 mg/L were calculated. Also in the metoprolol exposure test the time to first hatch represented the most sensitive endpoint. All test concentrations showed significant effects and, therefore, a NOEC value of <0.1 mg/L had to be inferred. For summarised endpoints and (no-) effect values, see Table 91 below. With 10% immobility and a mean number of 72offspring per survivor in the control the metoprolol exposure test was considered valid and acceptable according to OECD TG 211 (OECD, 2012).

8.2.2.6 Biological effects of the mixture of diclofenac and metoprolol

In the mixture test a control replicate had to be excluded due to accidental death (handling) of the parent. No clear concentration response for the endpoint immobility was observed. 16.7%-25% effects occurred in the most test concentrations. However, the trend showed increased immobility (58.3%) in highest test concentration. No statistical significant effects were observed and a NOEC value of >6.56 mg/L as sum concentration was deduced (exact Fisher t-test with Bonferroni). Using the Probit method an EC_{50} value of 8.25 mg/L and an EC_{10} value of 2.13 mg/L were calculated. It has to be stated that both values are outside the tested concentration range. In the lowest test concentration already 17% effects occurred. On the other side, based on the fitted curve a 50% effect value of 9.32 mg/L was calculated, although in the highest test concentration 58.3% effects occurred. A NOEC value of 5.31 mg/L was deduced for the endpoint cumulative number of offspring per survivor. Only the living offspring was included for evaluation of this endpoint. With the exception of the intermediate concentration of 4.38 mg/L a clear concentration response was observed. Using the Probit method EC_{50} values of 6.50 mg/L and an EC_{10} of 5.04 mg/L were calculated. In all test concentrations a delay in time to first hatch was observed but without clear concentration response. Nevertheless, also in the mixture test this endpoint showed the most sensitive reaction with a NOEC value of 2.19 mg/L caused by significant effects in all other test concentrations. For summarised endpoints and (no-) effect values, see Table 91 below. With 18.2% immobility and a number of 98 as mean offspring number per survivor in the control the mixture test was considered valid and acceptable according to OECD TG 211 (OECD, 2012).

Table 91: 21 days toxicity test with *D. magna* (OECD 211); endpoints and effect concentrations (mg/L); values in brackets: lower and upper 95%-confidence limit

Endpoint	Diclofenac	Metoprolol	Mixture *
Cumulative offspring per survivor	NOEC: 1.9 EC_{10}: 3.2 (0.75-5.60) EC_{50}: 15 (10.3-21.6)	NOEC: 0.5 EC_{10}: 0.47 (n.d.-1.6) EC_{50}: 2.9 (n.d.-72.2)	NOEC: 5.31 EC_{10}: 5.04 (0.53-5.71) EC_{50}: 6.50 (5.77-11.4)
Time to first hatch	NOEC: <1.9 EC_{10}: n.d. EC_{50}: n.d.	NOEC: <0.1 EC_{10}: n.d. EC_{50}: n.d.	NOEC: 2.19 EC_{10}: n.d. EC_{50}: n.d.
Immobility	NOEC: ≥50 EC_{10}: 3.6 (0.28-7.74) EC_{50}: 25.2 (13.8-84.5)	NOEC: 2.5 EC_{10}: n.d. EC_{50}: 2.0 (0.31-10.9)	NOEC: ≥6.56 EC_{10}: 2.13 (n.d.)** EC_{50}: 8.25 (n.d.)**

*: mixture concentrations stated as sum concentration of diclofenac and metoprolol proportions; **: extrapolated values outside the tested concentration range; n.d.: not determined

To assess the real and the expected effect potency of the mixture, model deviation ratios (MDR) of the equipotent mixtures (each ¼, ½ and ¾ of TU) of both compounds were compared: MDR = predicted EC_{10} / determined EC_{10}. No clear concentration response of the endpoint cumulative offspring per survivor was observed between equipotent mixtures: ¼ DIC + ¼ MET: 8.1% effects; ½ DIC + ½ MET: 0.3% effects [sic]; ¾ DIC + ¾ MET: 52.2% effects. Nevertheless, ECx and NOEC/LOEC values (see Table 91) were calculated from the mixture test with five rising mixture sum concentrations as stated in Table 89. The determined EC_{10} values of the endpoint cumulative offspring per survivor were used as relevant endpoint reaction and to calculate the MDR.

Diclofenac EC_{10} = 3.2 mg/L

Metoprolol EC_{10} = 0.47 mg/L

Mixture of diclofenac and metoprolol EC_{10}: 5.04 mg/L

For the equipotent mixtures ¼, ½ and ¾ MDRs of 0.918, 1.835 and 2.75 were calculated, respectively. These values resulted in ratio corrected MDRs of 0.73 each. Consequently, in the reproduction toxicity test with *D. magna* exposed to diclofenac and metoprolol no synergistic properties of the mixed substances were estimated and no increased toxicity of the mixture was observed.

8.3 Biomarker analyses

8.3.1 Material and methods

8.3.1.1 Origin of samples

A first test was done to assure the general applicability of the standard method: This included testing the protein content of samples prepared from different pool sizes (2, 4, 6 and 8 individuals) to determine the minimum pool size. Furthermore, we tested whether the daphnid Hsp70 can be stained and quantified with the applied antibody.

The diclofenac treatment included only 21 samples that were sent to Tübingen. Five treatments plus control were included in the analysis, each with a replicate size of two to five. For metoprolol, only 18 samples were available – with especially low replicate numbers of one or two pools in the two highest concentrations. For the mixtures, 28 samples were obtained – the highest mixture treatment yielded only three pools, of which one could not be analysed due to the low protein content.

8.3.1.2 Sample extraction

Stress protein quantification was done using pools of two daphnids at a time. Samples were stored at -80 °C until processing. The frozen samples were mechanically homogenized on ice in an appropriate volume (40 to 60 µL) of extraction medium (consisting of 96% concentrated extraction buffer (80 mM potassium acetate, 5 mM magnesium acetate, 20 mM Hepes in bidestilled water, adjusted to a pH of 7,5 and 4% protease inhibitor). Homogenized samples were centrifuged at 4 °C and 20000 rcf for 10 min. 5 µL of the supernatant were used for protein quantification. The remaining supernatant was diluted with SDS buffer (20% glycerin, 3% sodium dodecyl sulfate, 0.3% β-mercaptoethanol, 10 mM Tris pH 7 and 0.005% bromophenol blue in bidestilled water) in a proportion of 2 parts supernatant to 1 part SDS buffer, and boiled at 95-100 °C for 5 min. Both mixtures were stored at -20 °C until further usage.

8.3.1.3 Quantification of protein content

To quantify the protein content according to Bradford (Bradford, 1976), 5 µL of supernatant were diluted with 245 µL of 1:10 extraction buffer. 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 straight calibration line. Each sample was tested in triplicates. 20 µL of the samples´ supernatant mixture were mixed with 250 µL of Bradford mixture (0.001% Coomassie brilliant blue G-250, 4.75% ethanol, 8.5% phosphoric acid in bidestilled water) and the extinction at 595 nm was measured using an automated microplate reader (Bio-Tek Instruments, Winooski VT, USA).

8.3.1.4 Quantification of relative Hsp70 level

For the electrophoresis, polyacrylamide minigels (12% acrylamide, 0.12% bisacrylamide) were loaded with the sample/SDS mixtures. Constant protein weights (40 µg per sample) were applied to the gels, following the results of the Bradford analysis. To allow for a comparison between different gels, a standard in duplicate was added to every gel. Gel chambers were placed in E-buffer + SDS (0.19 M glycin, 25 mM $Tris_{base}$ and 0.1% SDS). Gels were run for 15 min at a voltage of 80 V, followed by 1 h at 120 V to separate the proteins.

Protein transfer from the gel to a nitrocellulose membrane was performed via semi-dry Western blot. The membrane was fitted to the gel, encased in Whatman-papers soaked in transfer buffer (0.095% $Tris_{HCl}$, 6.34% $Tris_{base}$, 3.63% glycine and 0.046% SDS in bidestilled water, adjusted to a pH of 9) and

placed in a blotting chamber. For each gel used in the blotting, an amperage of 90 mA was applied, with the voltage not exceeding 10 V. The blotting process was done over a time period of 2 hours.

After the blotting process, the filter was blocked in blocking solution (TBS (0.88% sodium chloride, 0.635% Tris-HCl and 0.118% Tris-base in bidestilled water, adjusted to a pH of 7.5) with horse serum in a mixture of 1:2) for one hour. 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 (1 mM 4-chloro-1-naphtol, 6 % methanol, 0.015 % hydrogen peroxide in 30 mM Tris pH 8.5) 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 an Hsp70 standard (prepared from full body homogenate of *Salmo trutta* f. *fario*).

8.3.2 Results

8.3.2.1 Protein quantification in samples

The first test showed that the protein content of a pool consisting of two individual daphnids is already sufficient for the described analysis. Therefore, all further tests could be performed on samples of this pool size.

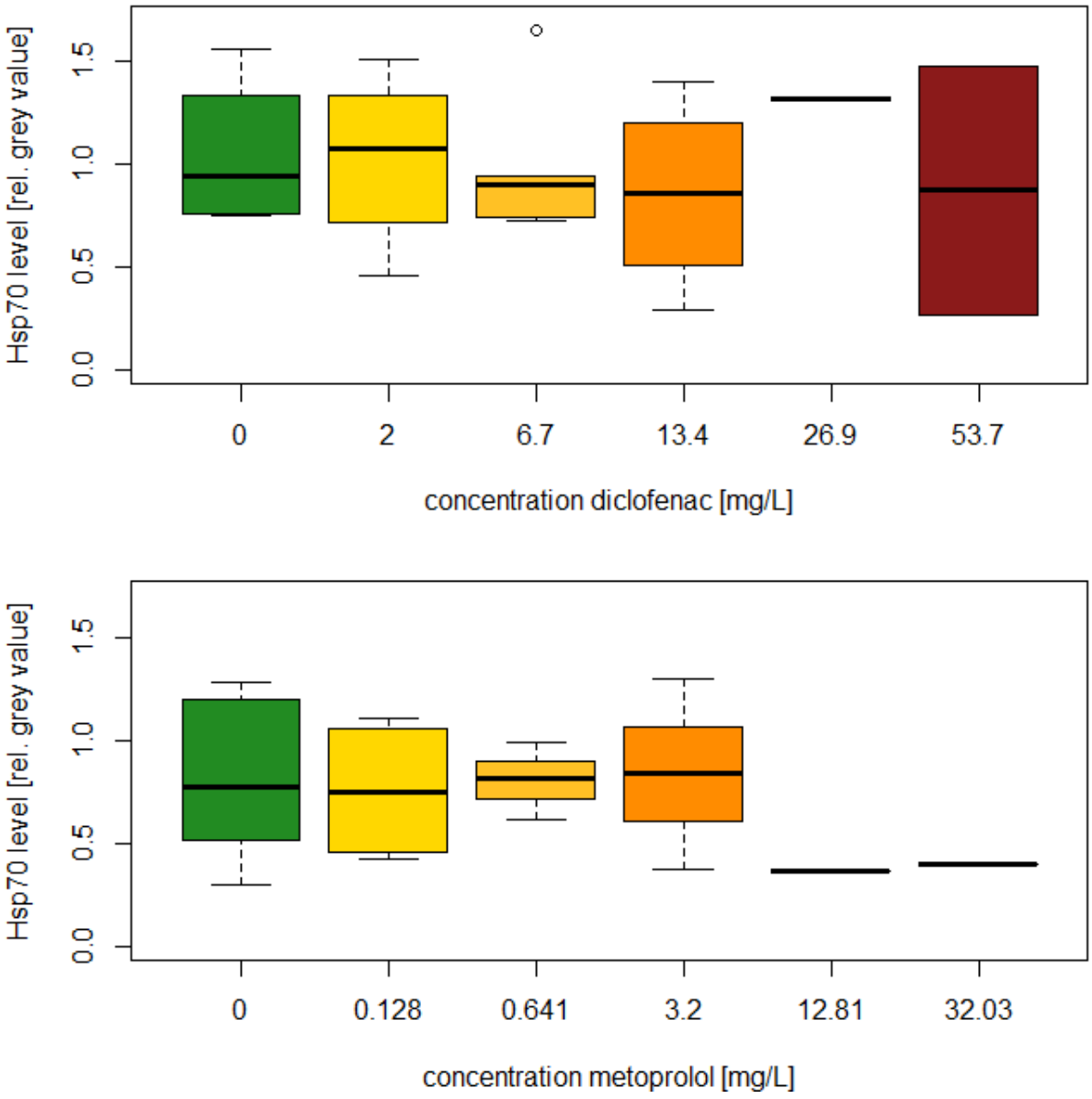
The samples of the diclofenac treatment showed an even higher protein content of the homogenate. So even the individual samples tested in the 6,7 and 53,7 mg/L treatments yielded a sufficient protein content for further processing.

8.3.2.2 Single substances

Samples treated with low diclofenac concentrations did not show a difference in their Hsp70 level compared to the control treatment (linear model, df=5/15, F=0.3356, p=0.8835). The two highest concentrations had a strongly reduced sample size and resulted in highly variable values, rendering a comparison with the control very difficult.

Likewise, metoprolol did not have an effect on stress proteins levels in any tested concentration (linear model, df=5/11, F=0.4475, p=0.8067). For the two highest concentrations, only one pool was available for each treatment. Results are shown in Figure 173.

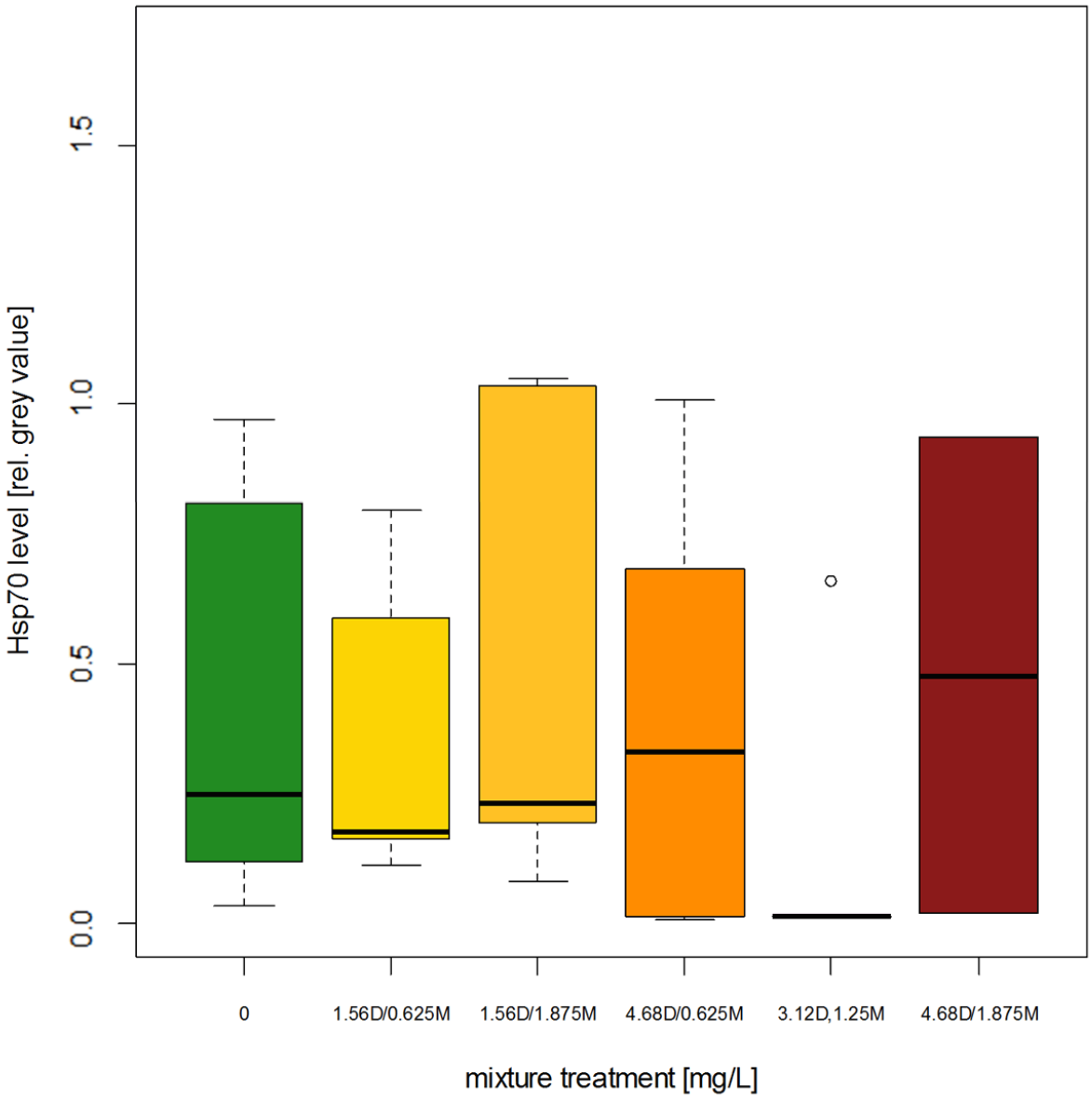
Figure 173: Stress protein Hsp70 level of *Daphnia magna* exposed to diclofenac (top) and metoprolol (bottom). Bold lines display the median values, boxes the 25% to 75% quantiles, whiskers the minimum and maximum values, circles potential outliers. None of the treatment influenced the level of Hsp70. At the highest test concentrations, the sample size was reduced due to the high mortality in these treatments.



8.3.2.3 Mixtures

The stress protein levels of daphnids exposed to mixtures were in general much lower than those seen for the single substances. Especially the mixture of 3.12 mg/L diclofenac and 1.25 mg/L metoprolol showed low levels that were hardly quantifiable. Yet, there were no statistically significant differences to be found (linear model, df=5/21, F=1.5, p=0.2323). Data are shown in Figure 174.

Figure 174: Stress protein Hsp70 level of *Daphnia magna* exposed to binary mixtures of diclofenac (D) and metoprolol (M). Bold lines display the median values, boxes the 25% to 75% quantiles, whiskers the minimum and maximum values, circles potential outliers. None of the treatments influenced the level of Hsp70. At the highest test concentration, the sample size was reduced due to mortality in this treatment.



8.3.3 Discussion

The obtained results for the diclofenac exposure indicate no proteotoxic effects of diclofenac concentrations as high as 13.4 mg/L. In no case, the results were significant. However, the situation in higher concentrations opens room for speculations. The second highest concentration yielded high Hsp70 levels, which can be seen as a sign of increased proteotoxic stress. The two highly variable levels obtained for the highest concentration led to suggest a beginning breakdown of the Hsp70 protection system. While one sample still has a high level of stress proteins, the other already lacks the resources to do so. This corresponds to the high mortality observed in those treatments. Unfortunately, the mortality also resulted in the drastically reduced sample size, which complicates further interpretations. Future approaches would need a greatly increased sample size for the evaluation of biochemical markers, to produce more reliable results. Other studies on daphnids found a LOEC of 30 mg/L for the induction of Hsp70 (Haap et al. 2008).

The evaluation of the metoprolol exposure experiment suffered from the same problems. Low to medium concentrations did not induce proteotoxic stress. High concentrations drastically decreased the sample size, so a reliable evaluation is hardly possible.

In mixtures, there was the peculiar finding of very low values at ½ TU diclofenac and ½ TU metoprolol. However, it is unlikely that this difference is biologically meaningful. On the one side, the difference was not statistically significant, which is not surprising considering the high variance and comparably low sample size. On the other side, there were no clear signs of proteotoxic action on daphnids even at much higher test concentrations of the single substance. Furthermore, the effect was not present at the combination of ¾ TU diclofenac and ¾ TU metoprolol and there were no preceding increases in Hsp70 at lower combinations. Therefore, it is likely that the observed effect was merely due to normal biological variation.

Overall, we could also observe a high variation in the basic Hsp70-level between the three experiments. This is probably due to potential external factors like age, reproductive state, clone, season or previous keeping conditions. There were always several months of time between the experiments, which makes slight differences in external factors highly likely.

9 Annexes

Annex 1: Overview on effect data of Crustacea for diclofenac.

Pharmaceutical	Acute/ chron.	Endpoint	Organism	Duration	T [°C]	NOEC [mg/L]	LOEC [µg/L]	EC50 [µg/L]	EC100 [µg/L]	Author
Diclofenac	acute	Mortality	*Daphnia magna*	48	18-22			60.70		Lee, 2011
	acute	Mortality	*Daphnia magna*	48				67.00		Quinn, 2011
	acute	?	*Daphnia magna*	24		18.0				Hanisch, 2002
	acute	Mortality	*Daphnia magna*	48	20.0			22.43		Ferrari, 2003
	acute	Mortality	*Daphnia magna*	48				34.00		CPSE, 1999
	acute	Mobility	*Daphnia magna*	48	20.0			68.00		Cleuvers, 2003
	acute	Mortality	*Daphnia magna*	24				69.05		CPSE, 1999
	acute	?	*Daphnia magna*	24				56.00	100	Hanisch, 2002
	acute	Mortality	*Ceriodaphnia dubia*	48	25.0			22.7		Ferrari, 2003
	acute	Mortality	*Moina macrocopa*	48	18-22			1426.		Lee, 2011
	chronic	Reproduction	*Daphnia magna*	504	25.0	10.0				Han, 2006
	chronic	Mortality	*Daphnia magna*	504	25.0				40	Han, 2006
	chronic	Reproduction	*Moina macrocopa*	504	18-22	16.7	50			Lee, 2011
	chronic	Reproduction	*Ceriodaphnia dubia*	168	25.0	1.0	2			Ferrari, 2003
	chronic	Reproduction	*Daphnia magna*	504	18-22	8.3	25			Lee, 2011
	chronic	Mortality	*Daphnia magna*	504		10.0	32			Quinn, 2011
	chronic	Reproduction	*Daphnia magna*	multigen	20.0		0.00036			Dietrich, 2010
	subacute	physiological responses	*Gammarus spp.*	1344	10.5	1.0				Oskarsson, 2012

Annex 2: Overview on effect data of Crustacea for metoprolol.

Pharmaceutical	Acute/ chron.	Endpoint	Organism	Duration	T [°C]	NOEC [mg/L]	LOEC [µg/L]	EC50 [µg/L]	EC100 [µg/L]	Author
Metoprolol	acute	Mortality	*Daphnia magna*	48	20.0			100		Cleuvers, 2003
	acute	Mortality	*Daphnia magna*	48	20.0			438		Cleuvers, 2003
	acute	heart rate	*Daphnia magna*	0.5	25.0		32.0			Dzialowski, 2006
	acute	Mortality	*Ceriodaphnia dubia*	48	25.0			45.3		Fraysse, 2005
	acute	Mortality	*Daphnia magna*	48	20.0			200		Hernando, 2004
	acute	Mortality	*Ceriodaphnia dubia*	48	25.0			8.8		Huggett, 2002
	acute	Mortality	*Daphnia magna*	48	25.0			63.9		Huggett, 2002
	acute	Mortality	*Hyalella azteca*		25.0			>0100	100	Huggett, 2002
	acute	Mortality	*Daphnia magna*	48	20.0			76.2		Villegas-Navarro, 2003
	chronic	Reproduction	*Daphnia magna*		20.0		0.0012		40	Dietrich et al. 2010
	chronic	fecundity (9d, 2 generations)	*Daphnia magna*		25.0	3.1	6.2			Dzialowski, 2006
	chronic	growth (dry body mass, 9d)	*Daphnia magna*		25.0		3.1			Dzialowski, 2006
	chronic	heart rate (9d)	*Daphnia magna*		25.0		3.1			Dzialowski, 2006

Annex 3: Physico-chemical parameters of Borgmann medium and stock solution before realization of the acute *G. fossarum* tests with metoprolol and diclofenac.

Pharmaceutical	Medium	PH	Lf [µS/cm]	O_2 [mg/l]/[%]	T [°C]
Metoprolol	Borgmann medium	7.91	701	8.1 / 91	20.8
	Stock solution	7.76	895	8.3 / 95	21.0
Diclofenac	Borgmann medium	7.82			
	Stock solution	7.94			

Annex 4: Physico-chemical parameters after conduction of the acute *G. fossarum* test with diclofenac (48 h).

Parameter	Unit	Controls	1 mg/L	4 mg/L	16 mg/L	64 mg/L	256 mg/L
pH	-	7.14	7.20	7.26	7.32	7.30	7.21
Conductivity	µS/cm	630.00	482.00	490.00	495.00	533.00	632.00
Oxygen	mg/L	6.80	6.90	7.00	7.00	6.60	6.30
Oxygen saturation, T=21 °C	%	81.00	81.00	84.00	84.00	78.00	76.00

Annex 5: Physico-chemical parameters after conduction of the acute *G. fossarum* test with metoprolol (144 h).

Parameter	Unit	Controls	1 mg/L	4 mg/L	16 mg/L	64 mg/L	256 mg/L
pH	-	7.87	7.88	7.97	7.94	8.01	7.94
Conductivity	µS/cm	810.00	728.00	729.00	739.00	786.00	967.00
Oxygen	mg/L	7.10	7.00	7.20	7.40	7.30	4.90
Oxygen saturation, T=21 °C	%	83.00	80.00	81.00	85.00	81.00	54.00

Annex 6: Results (medians along with percentiles (25% - 75%)) obtained for studied endpoints in *G. fossarum* after exposure to diclofenac.

Diclofenac [mg/L]	Num. adults	Num. juveniles	Num. precopula	Num. e.b. females	Mortality [%]	J/A	Precopula [%]	E.b. females [%]	Eggs
0.00	58	71	2	12	28 (21-34)	1.0 (0.7-1.5)	3.3 (0-7)	25 (18-28)	3.4 (3-4)
0.26	58	59	3	14	28 (23-33)	1.1 (0.8-1.2)	6.9 (5-7)	26 (23-27)	5.6 (5-6)
0.79	52	64	1	15	38 (33-40)	1.2 (1.0-1.6)	0 (0-2)	30 (26-33)	4.1 (3-5)
2.62	58	29	4	10	28 (25-30)	0.5 (0.3-0.7)	7.1 (0-14)	17 (14-20)	5.3 (3-7)
8.00	62	4	3	3	25 (18-30)	0 (0-0.1)	0 (0-4)	3.6 (0-8)	3.8 (4-4)
24.10	40	0	2	1	50 (48-53)	0	5 (0-11)	0	1 (1-1)

Annex 7: Results obtained for studied endpoints in *G. fossarum* after exposure to metoprolol. Medians along with percentiles (25% - 75%) are depicted. J/A – ratio of juveniles to adults.

Metoprolol [mg/L]	Num. adults	Num. juve-niles	Num. preco -pula	Num. fe-males	Mortality [%]	J/A	Preco-pula [%]	E.b. fe-males [%]	Eggs
0.00	55	70	2	10	35 (30-36)	1.0 (0,8-1,5)	3.90 (0-8)	12.2 (6-23)	6.0 (6-7)
5.00	57	45	6	14	30 (24-35)	0.6 (0,5-0,9)	11.03(5-17)	25.6 (17-33)	5.3 (5-6)
15.00	61	37	4	9	15 (15-24)	0.7 (0,4-0,8)	5.90 (4-7)	18.8 (13-21)	3.7 (3-5)
45.00	13	3	0	0	87 (81-90)	0	0	0	0
135.00	0	0	0	0	100	0	0	0	0
405.00	0	0	0	0	100	0	0	0	0

Annex 8**:** Results obtained for studied endpoints in *G. fossarum* at the end (t_{40}) of the exposure experiments with metoprolol in AIS. J/A – ratio of juveniles to adults. Medians along with percentiles (25-75%) are depicted for number of eggs.

Metoprolol [mg/L]	Number adults	Number juveniles	Number of eggs	Mortality [%]	Precopu-lae [%]	Egg-bearing females [%]	J/A
control	101	60	11 (10-13.5)	39	32	14	0.6
0.47	104	73	12 (10-13)	37	25	27	0.7
1.9	129	68	10 (8-12)	22	23	30	0.5
7.5	112	120	8 (5-12)	32	21	32	1.1
30	105	0	6 (2.5-8)	36	9	22	0

Annex 9**:** Results obtained for studied endpoints in *G. fossarum* in the 3 enclosures of the respective AIS at the end (t_{40}) of the exposure experiments with metoprolol. J/A – ratio of juveniles to adults. Means ± SD are depicted for mortality, relative number of precopulae as well as egg-bearing females, J/A ratio, and number of eggs.

Metoprolol [mg/L]	Number Adults	Number juveniles	Number of eggs	Mortality [%]	Precopulae [%]	Egg-bearing females [%]	J/A
control	51	27	10.0±2.7	15±18	20.7±20.0	41.5±7.0	0.5±0.1
0.47	45	28	11.7±1.5	25.0±5.0	41.0±3.1	47.0±8.0	0.6±0.5
1.9	54	70	8.7±2.1	10.0±10.0	26.6±9.5	58.0±10.0	1.3±0.5
7.5	42	90	11.0±2.7	30.0±21.8	12.5±21.7	37.0±4.0	1.9±2.6
30	43	0	5±2.7	28.3±7.6	22.2±25.5	23.0±8.5	0

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11 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.

ARW, 2014.. Jahresbericht der Arbeitsgemeinschaft Rhein Wasserwerke e. V. (ARW).

AWBR, 2014. Jahresbericht der Arbeitsgemeinschaft der Wasserwerke Bodensee-Rhein (AWBR).

Aydin, E., and I. Talinli. 2013. Analysis, occurrence and fate of commonly used pharmaceuticals and hormones in the Buyukcekmece Watershed, Turkey. Chemosphere 90:2004-2012.

Barbieri, M., Licha, T., Nödler, K., Carrera, J., Ayora, C., Sanchez-Vila, X., 2012. Fate of b-blockers in aquifer material under nitrate reducing conditions: batch experiments. Chemosphere 89 (11), 1272-1277.

Beketov, M. A., Liess, M. (2008): Potential of 11 pesticides to initiate downstream drift of stream macroinvertebrates. Archives of Environmental Contamination and Toxicology, 55:247–253.

Beretta M., Britto V., Tavares T.M., da Silva S.M.T., Pletsch A.L. (2014): Occurrence of pharmaceutical and personal care products (PPCPs) in marine sediments in the Todos os Santos Bay and the north coast of Salvador, Bahia, Brazil. Journal of Soils and Sediments 14: 1278-1286.

Bonnineau, C., Guasch, H., Proia, L., Ricart, M., Geiszinger, A., Romaní, A. M., Sabater, S. (2010): Fluvial biofilms: A pertinent tool to assess beta-blockers toxicity. Aquatic Toxicology 96: 225–233.

Borgmann, U. (1996). Systematic analysis of aqueous ion requirements of Hyalella azteca: a standard artificial medium including the essential bromide ion. Archives of Environmental Contamination and Toxicology 30:356-363.

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.

Bradford, M.M. (1976): A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248-254.

Brust, K., Licht, O., Hultsch, V., Jungmann, D. and R. Nagel. 2001. Effects of terbutryn on aufwuchs and Lumbriculus variegatus in artificial indoor streams. Environmental toxicology and chemistry 20:2000-2007.

Camacho-Muñoz D., Martín J., Santos J. L., Aparicio I., Alonso E. (2010): Occurrence, temporal evolution and risk assessment of pharmaceutically active compounds in Doñana Park (Spain). Journal of Hazardous Materials 183: 602-608.

Camacho-Muñoz D., Martín J., Santos J. L., Aparicio I., Alonso E. (2013): Distribution and risk assessment of pharmaceutical compounds in river sediments from Doñana Park. Water Air Soil Pollution 224: 1665

Camacho-Muñoz D., Martín J., Santos J.L., Aparicio I., Alonso E. (2014): Concentration evolution of pharmaceutically active compounds in raw urban and industrial wastewater. Chemosphere 111: 70-79.

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.

Deutscher Wetterdienst, Pressemitteilung vom 29.05.2015: „Deutschlandwetter im Frühling 2015“. http://dwd.de

DIN 38412-26:1994-05. German standard methods for the examination of water, waste water and sludge; bio-assays (group L); surfactant biodegradation and elimination test for simulation of municipal waste water treatment plants (L 26)

Clauss T. M., Dove A. D. M., Arnold J. E. (2008): Hematologic disorders of fish. *Veterinary Clinics of North America: Exotic Animal Practice* **11**(3):445-462.

Cleuvers, M. (2003). Aquatic ecotoxicity of pharmaceuticals including the assessment of combination effects. Toxicology Letters 142: 185-/194.

Cleuvers, M. (2005): Initial risk assessment for three beta-blockers found in the aquatic environment. Chemosphere 59 (2), 199–205.

Commission of the European Communities (1993): Methods for determination of ecotoxicity; Annex V, C.3, Algal inhibition test. L 383A, 179-186. EEC Directive 92/69/EEC.

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

Dietrich, S., S. Dammel, F. Ploessl, F. Bracher, and C. Laforsch. 2010a. Effects of a pharmaceutical mixture at environmentally relevant concentrations on the amphipod Gammarus fossarum. Marine and Freshwater Research 61:196-203.

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

Duft M, Schulte-Oehlmann U, Tillmann M, Markert M, Oehlmann J (2003): Toxicity of triphenyltin and tributyltin to the freshwater mudsnail *Potamopyrgus antipodarum* in a new sediment biotest. Environmental Toxicology and Chemistry 22: 145–152.

Dzialowski, E., Turner, P., Brooks, B. (2006). Physiological and reproductive effects of beta adrenergic receptor antagonists in Daphnia magna. Arch. Environ. Contam. Toxicol. 50: 503–510.

Ebert I., Amato R., Hein A., Konradi S. (2014): Arzneimittel in der Umwelt - vermeiden, reduzieren, überwachen. Herausgeber: Umweltbundesamt, Fachgebiet IV 2.2 Arzneimittel, Wasch- und Reinigungsmittel, Dessau-Roßlau.

EC. 2003. Technical Guidance Document on Risk Assessment. European Commission Joint Research Centre.

EMEA (2006): Guideline on the environmental risk assessment of medicinal products for human use (EMEA/CHMP/SWP/4447/00). European Medicines Agency Pre-Authorization Evaluation of Medicines for Human Use. London

Emerson, K; Russo, RC; Lund, RE et al. (1975). Aqueous Ammonia Equilibrium Calculations: Effect of pH and Temperature, Journal of the Fisheries Research Board of Canada 32 (12): 2379-2383.

Fatta-Kassinos D., Hapeshi E., Achilleos A., Meric S., Gros M., Petrovic M., Barcelo D. (2011): Existence of pharmaceutical compounds in tertiary treated urban wastewater that is utilized for reuse applications. Water Resources Management 25: 1183-1193.

Ferrari, B, Paxe, N., Giudice, R., Pollio,A., Garric,J. (2003). Ecotoxicological impact of pharmaceuticals foam in treated wastewaters: study of carbamazepine, clofibric acid, and diclofenac. Ecotoxicology and Environmental Safety 55: 359–370.

Fraysse, B., Garric, J. (2005). Prediction and experimental validations of acute toxicity of b-blockers in Ceriodaphnia dubia. Environmental Toxicology and Chemistry 24 (10): 2470–2476.

Fretter V, Graham A. (1994): British Prosobranch Molluscs: Their Functional Anatomy and Ecology. Ray Society, London, UK

Galluba S., Oetken M., Oehlmann J. (2012): Comprehensive sediment toxicity assessment of Hessian surface waters using *Lumbriculus variegatus* and *Chironomus riparius*. Journal of Environmental Science and Health, Part A 47: 507-521.

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.

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

Gust M, Mouthon J, Queau H, Dussart C, Buronfosse T, Garric J (2011): Natural variability and response interpretation of fecundity, vertebrate-like sex-steroid levels and energy status in the New Zealand mudsnail *Potamopyrgus antipodarum* (Gray). General and Comparative Endocrinology 172: 243–250.

Gutscher, M. et al. (2008), Real-time imaging of the intracellular glutathione redox potential, Nature Methods, Vol. 5, No. 6, 553–559, June 2008

Haap, T., Triebskorn, R. and Kohler, H.R. (2008) Acute effects of diclofenac and DMSO to *Daphnia magna*: Immobilisation and hsp70-induction. Chemosphere 73(3), 353-359.

Hallare, A.V., Kohler, 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.

Han, G., Hur, H., Kim, S. (2006). Ecotoxicological risk of pharmaceuticals from wastewater treatment plants in Korea: occurrence and toxicity to Daphnia magna. Environmental Toxicology and Chemistry 25 (1): 265–271.

Hanisch, B., Abbas, B., Kratz, W., (2002). Ökotoxikologische Bewertung von Humanarzneimitteln in aquatischen Ökosystemen. Landesumweltamt Brandenburg, Potsdam, Germany.

Hansch, C., Leo, A., D. Hoekman. Exploring QSAR - Hydrophobic, Electronic, and Steric Constants. Washington, DC: American Chemical Society. 1995., p. 137

Herbst, DB, Bogan MT, Lusardi RA (2008): Low specific conductivity limits growth and survival of the New Zealand mud snail from the upper Owens River, California. North American Naturalist 68(3), pp. 324–333.

Hermes-Lima, M., Willmore, W.G., Storey K.B. (1995). Quantification of lipid peroxidation in tissue extracts based on Fe(III)xylenol orange complex formation. Free Rad. Biol. Med. 19: 271-280.

Hernando, M., Petrovic, M., Fernandez-Alba, R. Barcelo, D. (2004). Analysis by liquid chromatography–electrospray ionization tandem mass spectrometry and acute toxicity evaluation for b-blockers and lipid-regulating agents in wastewater sample. Journal of Chromatography A, 1046: 133–140.

Hoeger, B., Kollner, 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.

Huggett, D., Brooks, B., Peterson, B., Foran,C., Schlenk, D. (2002). Toxicity of selected beta adrenergic receptor-blocking pharmaceuticals (b-Blockers) on aquatic organisms. Arch. Environ. Contam. Toxicol. 43: 229–235.

Hughes, D. A. (1970): Some factors affecting drift and upstream movements of gammarus pulex.Ecology, Volume 51, Issue 2, 301-305.

Hurlbert, S.H. (1984). Pseudoreplication and the Design of Ecological Field Experiments Ecological Monographs 54 (2) 187-211

IMS Health AG (2012). MIDAS Database. Frankfurt/Main, Germany. In: http://www.umweltbundesamt.de/sites/default/files/medien/377/dokumente/compilation-pharmaceuticalsintheenvironment_uba.pdf

Islas-Flores H., Gomez-Olivan L.M., Galar-Martinez M., Colin-Cruz A., Neri-Cruz N., 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.

Jansen et al. (2006). Phytotoxicity assessment of diclofenac and its phototransformation products. Springer-Verlag.

Jobling S, Casey D, Rodgers-Gray T, Oehlmann J, Schulte-Oehlmann U, Pawlowski S, Braunbeck T, Turner AP, Tyler CR (2003). Comparative responses of molluscs and fish to environmental estrogens and an estrogenic effluent. Aquatic Toxicology 65: 205–220.

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.

Jungmann, D., Brust, K., Licht, O., Mahlmann, J., Schmidt, J., and Nagel, R. (2001): Artificial indoor streams as a method to investigate the impact of chemicals on lotic communities. Environmental Science and Pollution Research, 8(1):49–55.

Jungmann, D., Ladewig, V, Ludwichkowski, K.U., Petzsch, P., Nagel, L.R. (2004). Intersexuality in Gammarus fossarum Koch – A common inducible phenomenon? Archiv für Hydrobiologie 159:511-529.

Kern, S., Baumgartner, R., Helbling, D.E., Hollender, J., Singer, H., Loos, M.J., Schwarzenbach, R.P., Fenner, K., 2010. A tiered procedure for assessing the formation of biotransformation products of pharmaceuticals and biocides during activated sludge treatment. J. Environ. Monit. 12 (11), 2100-2111.

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

Killeen, J. R., H. A. McLay, and I. A. Johnston. 1999b. Temperature and neuromuscular development in embryos of the trout (Salmo trutta L.). Comp. Biochem. Physiol. Part A: Mol. & Integr. Physiol. 122: 53-64.

Knappe D.R.U., Rossner A., Snyder S.A. (2007): Alternative Adsorbents for the Removal of Polar Organic Contaminants. Amer Water Works Assn, 90p.

Kujawa-Roeleveld K., Schuman E., Grotenhuis T., Kragić D., Mels A., Zeeman G. (2008): Biodegradability of human pharmaceutically active compounds (PhAC) in biological systems treating source separated wastewater streams. Third SWITCH Scientific Meeting Belo Horizonte, Brazil.

Kunkel D., Radke M. (2012): Fate of pharmaceuticals in rivers: Deriving a benchmark dataset at favorable attenuation conditions. Water Research 46: 5551-5565.

Ladewig, V., D. Jungmann, H.-R. Köhler, O. Licht, K. U. Ludwichowsk, M. Schirling, R. Triebskorn, and R. Nagel. 2006. Effects of Bisphenol A on Gammarus fossarum and Lumbriculus variegatus in artificial indoor streams. Toxicological and Environmental Chemistry 88:649-664.

Ladewig, V., Jungmann, D., Köhler, H.-R., Schirling, M., Triebskorn, R., Nagel, R. (2006): Population structure and dynamics of Gammarus fossarum (Amphipoda) upstream and downstream from effluents of sewage treatment plants. Arch. Environ. Contam. Toxicol. 50, 370–383.

Lamoureux, E. M. and B. J. Brownawell. 1999. Chemical and biological availability of sediment-sorbed hydrophobic organic contaminants. Environmental toxicology and chemistry 18:1733-1741.

Lee, J., K. Ji, et al. (2011). Chronic exposure to diclofenac on two freshwater cladocerans and Japanese medaka. Ecotoxicology and Environmental Safety 74(5): 1216-1225.

LfW (2004) Ökotoxikologische Auswirkungen von Arzneimitteln. Langzeitwirkungen bei Fischen.

Liess, M. (1998): Insektizide in Fließgewässern – Kopplung von Freilanderfassung und Testsystemen. Z. Umweltchem. Ökotox. 10 (1): 31-36.

Liu, Q.-T. and H. E. Williams. 2007. Kinetics and degradation products for direct photolysis of beta-blockers in water. Environmental Science & Technology 41:803-810.

LUBW, 2014. Spurenstoffinventar der Fließgewässer in Baden-Württemberg - Ergebnisse der Beprobung von Fließgewässern und Kläranlagen 2012/2013. Hrsg: Ministerium für Umwelt, Klima und Energiewirtschaft Baden-Württemberg Stuttgart; LUBW Landesanstalt für Umwelt, Messungen und Naturschutz Baden-Württemberg; Karlsruhe, ISBN 978-3-88251-379-0

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.

Nentwig G (2007): Effects of pharmaceuticals on aquatic invertebrates. Part II. The antidepressant drug fluoxetine. Archives of Environmental Contamination and Toxicology 52: 163-170.

Nie X.P., Liu B.Y., Yu H.J., Liu W.Q., Yang Y.F. (2013): Toxic effects of erythromycin, ciprofloxacin and sulfamethoxazole exposure to the antioxidant system in *Pseudokirchneriella subcapitata*. Environmental Pollution 172: 23-32.

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.

OECD-23. (2000). Guidance document on aquatic toxicity testing of difficult substances and mixtures. OECD Series on Testing and Assessment 23. 23, Paris.

OECD (2007): Sediment-water Lumbriculus toxicity test using spiked sediment (Guideline 225). OECD, Paris.

OECD (2015). OECD Draft guideline for the testing of chemicals. *Potamopyrgus antipodarum* reproduction test. Organisation for Economic Co-operation and Development, 2015, Paris, France.

OECD (2016): *Potamopyrgus antipodarum* Reproduction Test. OECD draft Guideline for the testing of chemicals. Paris.

Oetken M., Nentwig G., Löffler D., Ternes T., Oehlmann J. (2005): Effects of pharmaceuticals on aquatic invertebrates. Part I. The antiepileptic drug carbamazepine. Archives of Environmental Contamination and Toxicology 49: 353-361.

Organisation for economic co-operation and development (OECD) (Hrsg.) (2000): OECD Series on Testing and Assessment No. 23. Guidance Document on Aquatic Toxicity Testing of Difficult Substances and Mixtures. ENV/JM/MONO(2000)6

Oskarsson, H., A.-K. E. Wikland, et al. (2012). Effect studies of human pharmaceuticals on Fucus vesiculosus and Gammarus spp. Marine Environmental Research 74: 1-8.

Peschke, K., Geburzi, J., Köhler, H.-R., Wurm, K., Triebskorn, R. (2014). Invertebrates as indicators for chemical stress in sewage-influenced stream systems: Toxic and endocrine effects in gammarids and reactions at the community level in two tributaries of Lake Constance, Schussen and Argen. Ecotoxicology and Environmental Safety 106: 115–125.

Phipps G.L., Ankley G.T., Benoit D.A., Mattson V.R. (1993): Use of the aquatic oligochaete *Lumbriculus variegatus* for assessing the toxicity and bioaccumulation of sediment-associated contaminants. Environmental Toxicology and Chemistry 12: 269-279.

Pöckl, M. (1993): Reproductive potential and lifetime potential fecundity of the freshwater amphipods *Gammarus fossarum* and *G. roeseli* in Austrian streams and rivers. Freshwater Biology 30, 73-91.

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). TheScientificWorldJournal 2014, 645737.

Quinn, B., W. Schmidt, et al. (2011). Effects of the pharmaceuticals gemfibrozil and diclofenac on biomarker expression in the zebra mussel (Dreissena polymorpha) and their comparison with standardised toxicity tests. Chemosphere 84(5): 657-663.

Radke, M. and M. P. Maier. 2014. Lessons learned from water/sediment-testing of pharmaceuticals. Water Research 55:63-73.

Ramil M., El Aref T., Fink G., Scheurer M., Ternes T.A. (2010): Fate of beta blockers in aquatic-sediment systems: sorption and biotransformation. Environmental Science and Technology 44: 962-970.

Rybicki, M. (2014): Effects of Single and Combined Pesticide Exposure on the Grazer–Aufwuchs–Interaction – Micro- and Mesocosm Experiments. Dissertation. TU Dresden, Fakultät Umweltwissenschaften.

Sadava et al. (2011). Purves Biologie, 9, Spektrum Verlag, Heidelberg.

Salonikidis, P.S. et al. (2011), An Ion-insensitive cAMP Biosensor for Long Term Quantitative Ratiometric Fluorescence Resonance Energy Transfer (FRET) Measurements under Variable Physiological Conditions, JBC, Vol. 286, No. 26, 23419–23431, July 2011

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., Ternes, T.A., 2010. The challenge of analyzing beta-blocker drugs in sludge and wastewater. Anal Bioanal Chem ;396(2),845-56.

Schirling, M., Jungmann, D., Ladewig, V., Ludichowsky, K-U., Nagel, R., Köhler, H-R., Triebskorn, R. (2006). Bisphenol A in Artificial Indoor Streams: II. Stress Response and Gonad Histology in Gammarus fossarum (amphipoda), Ecotoxicology, 15, 143–156.

Schmidt W., O'Rourke K., Hernan R., 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: 1389-1395.

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.

Sieratowicz A, Schulte-Oehlmann U, Wigh A, Oehlmann J (2013): Effects of test media on reproduction in *Potamopyrgus antipodarum* and of pre-exposure population densities on sensitivity to cadmium in a reproduction test. J. Environ. Sci. Health A 48, 481-488.

Steinbach C., Burkina V., Fedorova G., Grabicova K., Stara A., Velisek J., Zlabek V., Schmidt-Posthaus H., Grabic R., 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.

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

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

Sundelin, B., Eriksson Wiklund, A.-K., Ford, A. T. (2008). Biological effects of contaminants: the use of embryo aberrations in amphipod crustaceans for measuring effects of environmental stressors. ICES techniques in marine environmental sciences. No 41. 21 pp.

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

Triebskorn, R., Casper, H., Heyd, A., Eikemper, R., Kohler, HR., 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 68:151-166.

Triebskorn, R-, Schwarz,S., Köhler, H-R., Jungmann, D., Berg, K., Frey, M., Oehlmann. J, Oetken, M. 2014. From theory to reality – Evaluation of suitable organisms and test systems for the biomonitoring of pharmaceuticals. Part I: Literature review. Federal Environmantal Agency, Germany.

Umweltbundesamt (UBA) (1996): Ammoniak im Wasser – Ableitung einer Formel zur Berechnung Ammoniak in wässrigen Lösungen. UBA-BE-076. Wien.

Umweltbundesamt (UBA) (2014): Pharmaceuticals in the environment – the global perspective - Occurrence, effects, and potential cooperative action under SAICM. IWW Rheinisch-Westfaelisches Institut für Wasser.

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 Handel E (1965): Microseparation of Glycogen, Sugars, and Lipids. Analytical Biochemistry 11(2): 266–271.

Van Handel E (1985): Rapid determination of glycogen and sugar in mosquitoes. Journal of the American Mosquito Control Association 1: 299-304.

Velisek J., Sudova E., Machova J., Svobodova Z. (2010): Effects of sub-chronic exposure to terbutryn in common carp (*Cyprinus carpio* L.). *Ecotoxicology and Environmental Safety* **73**(3):384-390.

Villegas-Navarro, A., Rosas-L, E., Reyes, J. (2003). The heart of Daphnia magna: effects of four cardioactive drugs. Comparative Biochemistry and Physiology Part C 136: 127–134.

Völker C, Gräf T, Schneider I, Oetken M, Oehlmann J (2014). Combined effects of silver nanoparticles and 17α-ethinylestradiol on the freshwater mudsnail *Potamopyrgus antipodarum*. Environmental Science and Pollution Research: 10661-10670.

Wang, J. and P.R. Gardinali 2012. Analysis of selected pharmaceuticals in fish and the fresh water bodies directly affected by reclaimed water using liquid chromatography-tandem mass spectrometry. Analytical and Bioanalytical Chemistry 404:2711-2720

Waters, T. F. (1965): Interpretation of invertebrate drift in streams. Scientific Journal Series, Minnesota, Paper No. 5333.

Waters, T. F. (1972): The drift of stream insects. Annual Review of Entomology 17, 253-272.

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.

Winkelmann, C., Petzoldt, T., Koop, J., Matthaei, C., Benndorf, J. (2008). Benthivorous fish reduce stream invertebrate drift in a large-scale field experiment. Aquatic Ecology 42 (3): 483-493.

Yang W.W., Tang Z.P., Zhou F.Q., Zhang W.H., Song L.R. (2013): Toxicity studies of tetracycline on *Microcystis aeruginosa* and *Selenastrum capricornutum*. Environmental Toxicology and Pharmacology 35: 320-324.

Zhang W., Zhang M., Lin K.F., Sun W.F., Xiong B., Guo M.J., Cui X.H., Fu R.B. (2012): Eco-toxicological effect of carbamazepine on *Scenedesmus obliquus* and *Chlorella pyrenoidosa*. Environmental Toxicology and Pharmacology 33: 344-352.