TECHNISCHE UNIVERSITÄT MÜNCHEN

Lehrstuhl für Aquatische Systembiologie

Establishing realistic test settings in aquatic ecotoxicological risk assessments

Mona Katharina Feiner

Vollständiger Abdruck der von der Fakultät Wissenschaftszentrum Weihenstephan für Ernährung, Landnutzung und Umwelt der Technischen Universität München zur Erlangung des akademischen Grades eines

Doktors der Naturwissenschaften

genehmigten Dissertation.

Vorsitzender: Prof. Dr. Johannes Kollmann

Prüfer der Dissertation:

1. Prof. Dr. Jürgen Geist
2. Prof. Dr. Christian Laforsch

Die Dissertation wurde am 23.03.2017 bei der Technischen Universität München eingereicht und durch die Fakultät Wissenschaftszentrum Weihenstephan für Ernährung, Landnutzung und Umwelt am 28.09.2017 angenommen.

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PREFACE

This thesis is intended to enhance the ecological relevance in standard aquatic ecotoxicological investigations utilized in ecotoxicological risk assessments (ERAs). For this purpose, different approaches were applied which are the investigation of substances of concern under realistic conditions, and the incorporation of ecologically relevant non-standard species. Moreover, the Miniature Circulatory System (MCS), a new exposure system for long-term investigations in riverine species, is presented.

Following a general introduction about ERAs, the current state in standard ecotoxicological tests, and deficiencies in the acquired information in prevailing test settings, the approaches of enhancing the ecological relevance in ecotoxicological investigations are presented. These three main chapters were published as distinct research papers in similar versions. In a general discussion the main findings towards more realistic exposure scenarios in view to appropriate test durations in long-term investigations and the consideration of actual circumstances at which chemicals enter the environment are recapitulated. Furthermore, the possibilities and advantages of integrating new stream-dwelling species in ecotoxicological investigations by the use of the MCS are highlighted. Finally, promising approaches for more realistic ecotoxicological investigations are identified.

SUMMARY

Ecotoxicological risk assessments (ERAs) are utilized to evaluate the potential hazards that chemical substances pose to the aquatic and terrestrial environment. For this purpose, the *predicted* or *measured environmental concentration* (PEC or MEC) of the respective substance is related to the *predicted no effect concentration* (PNEC), i.e. the threshold value of the substance below which no negative impacts on the environment are expected. In use of extrapolation techniques like assessment factors or species sensitivity distributions, PNECs are derived based on effect concentrations, which are determined using standard ecotoxicological tests. Though the first internationally accepted standard tests for aquatic compartments were already established 35 years ago, and have been under continuous revision since, there are still deficiencies in the generated information on substance toxicity concerning their ecological relevance. Thus, transferring data from the laboratory to the field is a major challenge in ecotoxicology.

The present thesis is intended to contribute to the reduction of these deficiencies in generating more realistic exposure scenarios in ecotoxicological tests. In the first step, the importance of the test duration in long-term investigations was demonstrated. The influence of the beta-blocker sotalol on the reproduction of the New Zealand mudsnail *Potamopyrgus antipodarum* occurred only after 56 d, which exceeds typical test durations in standard tests. In future ecotoxicological investigations – especially in the case of trace substances, which are continuously released into the environment – the choice of adequate test durations is essential to avoid non-detection of delayed occurring effects. Moreover, the effect was observed at concentrations that would have been rated non-hazardous to the environment based on data from current standard tests. Furthermore, the importance of investigating substances with respect to the actual form or mixture at which they enter the environment was illustrated. This was demonstrated using the example of antiscalants (Ass), which are applied in reverse osmosis (RO) systems to inhibit clogging of membranes, and which are rated as environmentally non-hazardous. Wastewater from an RO system without ASs was not acutely toxic to amphipods, whereas an increased mortality occurred when ASs were applied. Especially in these additives, which are applied to prevent membrane clogging by changing complex formation, both changes in their chemical structure as well as changes in the water matrix can result in altered performance of aquatic organisms.

Finally, the Miniature Circulatory System (MCS) was developed for the incorporation of stream-dwelling organisms in standardized long-term investigations. The applicability of the MCS for long-term investigations was exemplarily validated in an egg-to-fry bioassay with the brown trout *Salmo trutta*, and in a copper toxicity test with the amphipod *Gammarus roeseli*. Stream-dwelling organisms are currently underrepresented in ecotoxicological standard tests despite the fact, that streams and rivers are particularly threatened due to an ongoing decline of the biodiversity. The use of the MCS can greatly improve the consideration of additional data for ERAs by increasing the range of test species.

Ecotoxicological standard tests are essential to generate reproducible and comparable information on effects of chemicals in the environment. However, current test settings have to be critically discussed in terms of appropriate test durations as well as realistic application characteristics of substances, and thus the form at which they enter the environment. ERAs based on insufficient data bear the risk to underestimate the environmental hazard. However, future incorporation of stream-dwelling species as standard test organisms requires research towards the optimization of basic culturing conditions. In addition, the incorporation of site-specific ambient conditions in future ecotoxicological investigations is advisable due to relevant influences of abiotic parameters on substance effects. Moreover, local populations may be diversely affected compared to the commonly investigated “unstressed” test animals collected from unpolluted sites. Comparative investigations are needed to evaluate possible differences in sensitivities between different populations.

ZUSAMMENFASSUNG

Mittels ökotoxikologischer Risikobewertungen wird die Gefährdung der aquatischen und terrestrischen Umwelt durch chemische Substanzen bewertet. Dabei wird die vorausgesagte (PEC: *predicted environmental concentration*) bzw. gemessene (MEC: *measured environmental concentration*) Umweltkonzentration einer Substanz in Bezug zu der Substanzkonzentration gesetzt, bei der höchstwahrscheinlich keine negativen Effekte in der Umwelt zu erwarten sind (PNEC: *predicted no effect concentration*). Die PNEC wird, unter der Anwendung von Extrapolationsverfahren wie Sicherheitsfaktoren oder *species sensitivity distributions* (SSDs), anhand von Effektkonzentrationen berechnet, die mit ökotoxikologischen Standardtests bestimmt werden. Obwohl die ersten international anerkannten Standardverfahren für den aquatischen Bereich bereits vor 35 Jahren etabliert wurden, und seither unter ständiger Revision stehen, gibt es Defizite in den generierten Informationen zu den Substanztoxizitäten bezüglich ihrer ökologischen Relevanz. Dadurch ist die Übertragung der Labordaten auf die realen Gegebenheiten eine der größten Herausforderungen in der Ökotoxikologie.

Die vorliegende Arbeit soll einen Beitrag dazu leisten eben diese Defizite im aquatischen Bereich zu verringern, indem ökotoxikologische Untersuchungen mit größerem Bezug zu realen Bedingungen durchgeführt wurden. Im ersten Schritt wurde die Bedeutung der Testdauer in Langzeituntersuchungen aufgezeigt. Eine Beeinflussung der Reproduktion der Neuseeländische Zwergdeckelschnecke *Potamopyrgus antipodarum* durch den Beta-Blocker Sotalol wäre bei einer Versuchsdauer von weniger als 56 Tagen nicht aufgedeckt worden. In Standardtests wird jedoch üblicherweise eine kürzere Versuchsdauer angesetzt. In zukünftigen ökotoxikologischen Untersuchungen – insbesondere im Fall von Spurenstoffen, die kontinuierlich in die Umwelt freigesetzt werden – ist es demnach unabdingbar, die Testdauer ausreichend lang zu wählen, um etwaige verzögert auftretenden Effekte zu erfassen. Darüber hinaus trat der Effekt bei Arzneistoffkonzentrationen auf, die nach gängiger Risikobewertung mittels Standardtests als nicht umweltproblematisch einzustufen wären. Des Weiteren wurde die Notwendigkeit aufgezeigt, Chemikalien im Hinblick auf ihre tatsächliche Form und Anwendungscharakteristik, in der sie in die Umwelt gelangen, zu untersuchen. Dies wurde exemplarisch am Beispiel von Antiscalants aufgezeigt, die in Umkehrosmoseanlagen eingesetzt werden, um ein

Zusetzen der Membranen zu verhindern, und als nicht umweltgefährdend deklariert sind. Während das Abwasser dieser Anlagen ohne Antiscalants nicht toxisch auf Amphipoden wirkte, war eine erhöhte Mortalität beim Einsatz der Substanzen zu verzeichnen. Gerade diese Additive, die ein Verkalken der Membranen verhindern, indem sie in die Komplexbildung eingreifen, verändern anwendungsbedingt ihre chemischen Struktur, und können dadurch eine andere direkte Wirkung auf aquatische Organsimen vorweisen. Darüber hinaus müssen bei solchen Substanzen auch indirekte Effekte als Resultat einer veränderten Umwelt, z.B. der Wassermatrix, berücksichtigt werden. Schließlich wurde das *Miniature Circulatory System* (MCS) entwickelt. Mit diesem neuartigen Expositions-System können Fließgewässerorganismen für standardisierte Langzeituntersuchungen in Betracht gezogen werden. Die Anwendbarkeit des MCS für Langzeituntersuchungen wurde exemplarisch in einem egg-to-fry Test mit der Bachforelle *Salmo trutta* und in einem Kupfer-Toxizitätstest mit dem Amphipoden *Gammarus roeseli* validiert. Obwohl gerade Fließgewässer durch einen anhaltenden Biodiversitätsverlust stark gefährdet sind, werden Fließgewässerorganismen in ökotoxikologischen Standarduntersuchungen bislang kaum beachtet. Durch die Aufnahme dieser Organismen in künftigen Untersuchungen bietet das MCS die Möglichkeit die Datengrundlage für ökotoxikologische Risikobewertungen maßgeblich zu verbessern.

Ökotoxikologische Standardtests sind unabdingbar um reproduzierbare und vergleichbare Informationen zu Substanzwirkungen in der Umwelt zu erhalten. Diese müssen jedoch im Hinblick auf eine adäquate Testdauer sowie die Anwendung der Substanz und damit ihre chemische Struktur mit der sie in die Umwelt gelangen kritisch diskutiert werden. Bei einer unzureichenden Datengrundlage kann es mit den üblichen Extrapolationsverfahren zu einer deutlichen Unterschätzung der Umweltgefährdung kommen. Für die Aufnahme weiterer Fließgewässerorgansimen in Standardtestverfahren sind zunächst weitere Untersuchungen nötig, um die Rahmenbedingungen für Langzeituntersuchungen zu optimieren. Regionale Gegebenheiten sollten in künftige Untersuchungen einfließen, da abiotische Parameter die Wirkung von Substanzen entscheidend beeinflussen können. Darüber hinaus könnten die betroffenen Organismen andere Sensitivitäten aufweisen, als die üblicherweise verwendeten „unbelasteten“ Testorgansimen. Dies muss jedoch noch in vergleichenden Studien evaluiert werden.

1. General Introduction

1.1. Ecotoxicological risk assessments

In the course of industrialization, and thus the growth of the chemical industries, chemical substances became a part of virtually all reaches of everyday life. Beginning with chemicals that are used in raw material mining, during further processing up to the ready for use end-products. In the European Union (EU) more than 100,000 substances are currently listed in the EC inventory (European Chemicals Agency). An entrance of these substances into the aquatic environment not only occurs due to discharges of industrial wastewaters, chemical accidents like in Seveso, Baia Mare or the explosion of the Deepwater Horizon offshore oil-drilling rig (e.g. Hay 1976, Capel et al. 1988, McNutt et al. 2012) or other unintended releases. In many cases, an entrance is connected with the normal usage as in pharmaceuticals, which are excreted either unchanged or in a metabolized form, and insufficiently eliminated during sewage treatment (e.g. Kümmerer 2001, Petrie et al. 2015). Likewise, personal care products such as cosmetic products commonly end up in the sewage systems and consequently enter the aquatic environment (e.g. Kasprzyk-Hordern et al. 2008, Boxall et al. 2012, Petrie et al. 2015). In some special cases, substances are intentionally emitted into the environment. This applies e.g. to plant protection products, and road deicing salts. Though, these substances are not intended to enter the aquatic environment, they eventually reach these environmental compartments e.g. due to spray-drift, surface run-off or leaching (Carter 2000, Novotny and Stefan 2010). As a result, aquatic organisms are not only confronted with alterations in the water matrix, e.g. increased chloride concentration due to road deicing measures (e.g. Thunqvist 2004, Kaushal et al. 2005, Beggel and Geist 2015), but also with a multitude of xenobiotics, i.e. ‘a compound that is foreign to a living organism’ (IUPAC 1997). Considering the vast amounts of chemicals entering the environment, especially in the case of xenobiotics which are often designed to exhibit a specific biological or physiological effect, e.g. pharmaceuticals (Breton and Boxall 2003), negative impacts on the biocenosis are likely to occur. Thus, the evaluation of possible effects of these substances in respective systems is crucial for environmental protection purposes and associated management and mitigation measures. Using ecotoxicological risk assessments (ERAs) the potential hazards of chemicals are estimated.

The main principle of an ERA is the comparison of the p*redicted environmental concentration* (PEC; i.e. the concentration of the substance that is expected in the environment) or *measured environmental concentration* (MEC; i.e. the concentration that is actually measured in the respective environmental compartment) and the *predicted no effect concentration* (PNEC; i.e. the threshold value of the substance below which no negative impacts on the environment are expected). Thereby, the quotient PEC or MEC/PNEC is crucial in the evaluation of an ERA. The PEC is derived by model calculations. These calculations include a variety of factors which are emission, partition, degradation, and elimination (European Commission 2003). PNECs are derived from effect concentrations that are determined using standardized ecotoxicological tests. Thereby, the PNEC is calculated based on the lowest identified effect concentration, i.e. representing the most sensitive test species. Due to the limited information on substance toxicity that is derived in these tests, the application of assessment factors is stipulated, to 'predict a concentration below which an unacceptable effect will most likely not occur' (European Commission 2003). The assessment factors decrease with increasing quantity and quality (i.e. short-term or long-term tests) of the tests.

In the EU, ERAs are required nowadays for the authorization of biocides (European Union 2012), plant protection products (European Union 2009), pharmaceuticals for both human and veterinary purposes (European Communities 2001a, b), and other new industrial chemicals that are intended to be placed on the market (European Union 2006). Furthermore, along with the REACH directive, chemical substances that have been placed on the market before 1981 have to be registered and their environmental risk has to be assessed, whereat the amount of data depends on the annual production volume (European Union 2006).

1.2. Standard ecotoxicological investigations

In ERAs for freshwater systems representatives of different trophic levels are investigated, which are generally a primary producer as well as a primary and a secondary consumer (European Commission 2003). In the purpose to regulate and standardize ecotoxicological investigations that are required and applied in the course of ERAs, guidelines for the conduction of ecotoxicological single-species tests were established. Thereby, test results are comparable between countries and multiple

testing is avoided. In 1971 already the International Organization for Standardization (ISO) incorporated environmental topics in creating two new committees for air and water quality issues. Until now, 68 ISO standards for the investigation of water quality using biological methods were established, and another nine standards are currently in the developmental process. Among them are 13 guidelines for toxicity tests using freshwater organisms including three primary producers, four different invertebrate taxa and two fish species (Table 1-1). In the EU the first guidelines for the conduction of standardized ecotoxicological investigations were established in 1981 as data concerning the potential harm of substances to the environment were required in authorization processes (European Communities 1979). Those were “Alga, Growth Inhibition Test” using a green algae (OECD 1981a), *Daphnia* sp., 14-days Reproduction Test (including an Acute Immobilisation Test)” (OECD 1981b), and “Fish, Acute Toxicity Test” (OECD 1981c) established by the Organization for Economic Co-operation and Development (OECD). Since then, these guidelines have been continually revised, and additional guidelines were established. Up to now, 44 test guidelines on the effects on biotic systems were established, including 24 tests using freshwater species. These guidelines are “Accepted internationally, as standard methods for safety testing” (retrieved 16.3.17 from: www.oecd.org/env/ehs/testing/oecdguidelinesforthetestingofchemicals.htm). In 2012, the US Environmental Protection Agency (USEPA) established a series of 26 test guidelines on ecological effects assessments, including birds, terrestrial organisms (i.a. invertebrates and plants), as well as three freshwater inhabiting prime producers (USEPA 2012a). Another 23 draft and final guidelines on effect assessments in aquatic species are available including ten freshwater organisms (i.a. crustacean and fish).

Table 1-1. Available test guidelines from ISO, OECD and USEPA that incorporate freshwater species and the respective test durations and endpoints.

Trophic level	Species	Type of test	Test duration	Endpoint	Guideline
Primary producers	*Anabaena flos-aquae*		96 h	Growth (biomass)	USEPA OCSPP 850.4550 (2012d)
	Desmodesmus subspicatus	Acute/Chronic	72 h	Growth (biomass)	OECD 201 (2011a)
	Desmodesmus subspicatus/Pseudokirchneriella subcapitata	Acute/Chronic	72 h	Growth (biomass)	ISO 8692 (2012)
	Desmodesmus subspicatus/Pseudokirchneriella subcapitata	Acute/Chronic	72 h	Growth (biomass)	ISO 14442 (2006)
	Lemna sp.	Acute	7 d	Growth	OECD 221 (2006a)
	Lemna sp.	Acute	7 d	Growth	ISO 20079 (2006)
	Lemna sp.	Acute	7 d	Growth	USEPA OCSPP 850.4400 (2012b)
	Myriophyllum sp.	Acute	14 d	Growth	OECD 238 (2014a)
	Myriophyllum sp.	Acute	14 d	Growth	OECD 239 (2014b)
	Myriophyllum sp.	Acute	10 d	Growth	ISO 16191 (2013)
	Pseudokirchneriella subcapitata	Acute/Chronic	96 h	Growth (biomass)	USEPA OCSPP 850.4500 (2012c)
Primary consumers (including detritivores)	*Brachionus calyciflorus*	Chronic	48 h	Population growth	ISO 20666 (2008)
	Ceriodaphnia dubia	Chronic	7 d	Reproduction	ISO 20665 (2008)
	Chironomus sp.	Acute	48 h	Mortality	OECD 235 (2011c)
	Chironomus sp.	Acute	14 d	Mortality, growth	USEPA OPPTS 850.1790 (1996c)
	Chironomus sp.	Acute Prolonged	20-65 d	Mortality, growth, emergence	OECD 218 (2004b)

Table 1-1 (continued). Available test guidelines from ISO, OECD and USEPA that incorporate freshwater species and the respective test durations and endpoints.

Trophic level	Species	Type of test	Test duration	Endpoint	Guideline
	Chironomus sp.	Acute Prolonged	20-65 d	Mortality, growth, emergence	OECD 219 (2004c)
	Chironomus sp.	Chronic	44-100 d	Time to emergence, emergence rate, sex ratio of 1st and 2nd generation	OECD 233 (2010a)
	Daphnia sp.	Acute	48 h	Immobility	OECD 202 (2004a)
	Daphnia sp.	Acute	48 h	Immobility	ISO 6341 (2012)
	Daphnia sp.	Acute	48 h	Immobility	USEPA OPPTS 850.1010 (2016a)
	Daphnia sp.	Chronic	21 d	Reproduction	OECD 211 (2012a)
	Daphnia sp.	Chronic	21 d	Reproduction	ISO 10706 (2000)
	Daphnia sp.	Chronic	21 d	Reproduction	USEPA OPPTS 850.1300 (2016d)
	Gammarus sp.	Acute	96 h	Mortality	USEPA OPPTS 850.1020 (2016b)
	Hyalella azteca	Chronic	14-28 d	Mortality, growth of juveniles	ISO 16303 (2013)
	Hyalella azteca	Acute	10-28 d	Mortality	USEPA OPPTS 850.1735 (2016f)
	Lumbriculus variegatus	Acute prolonged	28 d	Reproduction/biomass	OECD 225 (2007)
	Potamopyrgus antipodarum	Acute prolonged	28 d	Mortality, reproduction	OECD 242 (2016a)
	Lymnaea stagnalis	Acute prolonged	28 d	Mortality, reproduction	OECD 243 (2016b)

Table 1-1 (continued). Available test guidelines from ISO, OECD and USEPA that incorporate freshwater species and the respective test durations and endpoints.

Trophic level	Species	Type of test	Test duration	Endpoint	Guideline
Secondary consumers	*Danio rerio*	Acute	96 h	Mortality of adults	OECD 203 (1992)
	Danio rerio	Acute	96 h	Mortality of embryos	OECD 236 (2013b)
	Danio rerio	Acute	96 h	Mortality of adults	ISO 7346(1-3) (1996)
	Danio rerio	Acute	48 h	Mortality of embryos	ISO 15088 (2007)
	Danio rerio	Acute prolonged	Fertilized egg to end of yolk-sac stage	Mortality, development, growth, hatching rate	ISO 12890 (1999)
	Danio rerio	Chronic	30 d post-hatch	Hatching rate	OECD 210 (2013a)
	Danio rerio/Oncorhynchus mykiss/Cyprinus carpio/Oryzias latipes/Pimephales promelas	Acute prolonged	Fertilized egg to end of yolk-sac stage	Mortality, development, growth, hatching rate	OECD 212 (1998)
	Danio rerio/Pimephales promelas/Oryzias latipes	Acute prolonged	21 d	Vitellogenin, secondary sex characteristics, reproductive output	OECD 229 (2012b)
	Danio rerio/Pimephales promelas/Oryzias latipes	Acute prolonged	21 d	Vitellogenin, secondary sex characteristics	OECD 230 (2009a)
	Danio rerio/Oryzias latipes/Gasterosteus aculeatus	Chronic	60 d post-hatch	Vitellogenin, sex ratio	OECD 234 (2011b)
	Oncorhynchus mykiss	Acute prolonged	28 d	Mortality, growth	ISO 10229 (1994)
	Oncorhynchus mykiss	Acute prolonged	28 d	Growth	OECD 215 (2000)

Table 1-1 (continued). Available test guidelines from ISO, OECD and USEPA that incorporate freshwater species and the respective test durations and endpoints.

Trophic level	Species	Type of test	Test duration	Endpoint	Guideline
	Oncorhynchus mykiss/Lepomis macrochirus	Acute	96 h	Mortality of adults	USEPA OPPTS 850.1075 (2016c) USEPA OPPTS 850.1085 (1996a)
	Oncorhynchus mykiss/Pimephales promelas	Acute prolonged	60/28 d post-hatch	Mortality, hatching rate, growth, behavior	USEPA OPPTS 850.1400 (2016e)
	Oryzias latipes	Chronic	18 wk	Hatching rate, development, spawning	OECD 240 (2015a)
	Pimephales promelas	Chronic	Whole life-cycle (e.g. egg to egg)	Mortality, spawning, egg numbers, fertility, fecundity, behavior	USEPA OPPTS 850.1500 (1996b)
	Rana catesbeiana	Subchronic	30 d	Mortality, growth, behavior	USEPA OPPTS 850.1800 (1996d)
	Xenopus laevis	Acute prolonged	21 d	Mortality, morphological parameters, developmental stage	OECD 231 (2009b)
	Xenopus laevis	Chronic	Up to 115 d	Mortality, behavior, growth of larvae	OECD 241 (2015b)

1.3. Deficiencies in current test settings

As a result of the establishment of standardized ecotoxicological tests during the last 35 years, the knowledge about impacts of chemicals in the aquatic environment was greatly increased. Still there are deficiencies in the information generated in current test settings that can be attributed to the low complexity of these tests as a result of the requirements of high standardization, reproducibility and thus comparability (Breitholtz et al. 2006). Therefore, the extrapolation of laboratory ecotoxicological data to the environment, i.e. the determination of PNECs without under- or overestimation of the risks, is still a major challenge in ecotoxicology (e.g. Calow and Forbes 2003). Figure 1-1 illustrates the general procedure of an ERA and highlights the steps of PNEC determination and its deficiencies in current methodologies.

Deficiencies in information on substance toxicities are on the one hand due to the restricted number of standard test species. The common approach in ERAs is to identify PNECs based on the 'most sensitive species' in a system. Thereby, all species in a system are included and protected by the determination of threshold values for substances of concern. However, the implementation of investigating the 'most sensitive species' has to be questioned in view of the requirement to test species to be robust enough to cope with the artificial situation in the laboratories (Berger et al. 2016), but also in consideration of the scarce number of standard test species that are used in ecotoxicological investigations (Cairns and Niederlehner 1987). For instance, in the EU five freshwater invertebrate taxa (*Daphnia* sp., *Chironomus* sp., *Lumbriculus* sp., *Lymnaea stagnalis*, and *Potamopyrgus antipodarum*) are included in OECD test-guidelines (Table 1-1) which is disproportionate to 1050 invertebrate taxa that are considered in monitoring programs in Germany (Berger et al. 2016). Moreover, the determination of a single chronic *no observed effect concentration* (NOEC; i.e. the highest test concentration, at which no statistically significant difference to the control occurred), derived from one test species, is sufficient enough for the calculation of a PNEC (European Commission 2003). A PNEC can also be determined using a *species sensitivity distribution* (SSD), which is a statistical extrapolation technique of a dataset of multiple *effect concentrations* (ECx; i.e. the substance concentration, at which a defined proportion (x) of testes specimen are affected). Thereby, the PNEC is determined based on the *hazardous concentratio*n (HCp; i.e. the substance concentration, at which a defined proportion (p) of the species in a system is affected;

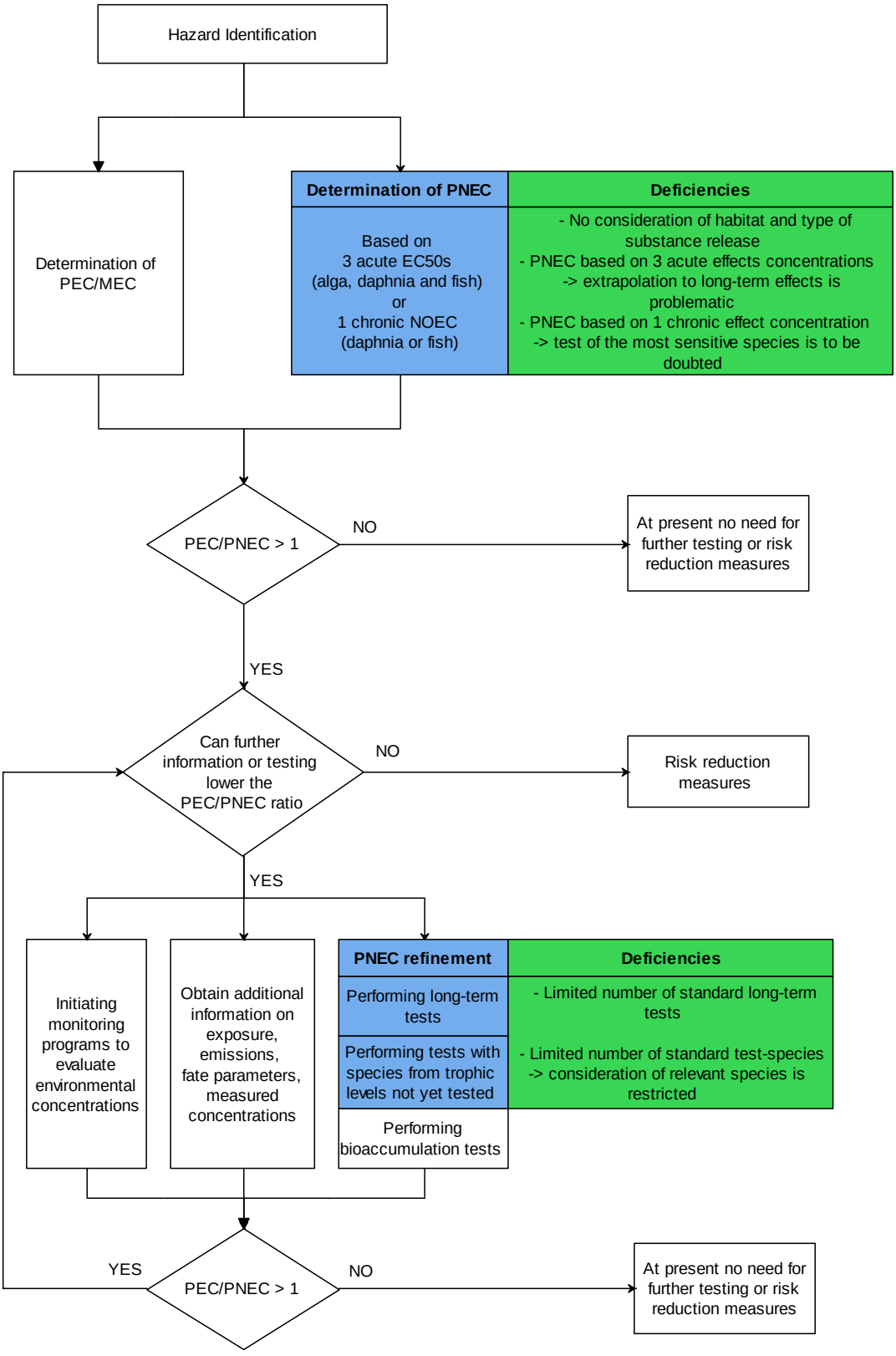

Figure 1-1. General procedure of ERAs. Blue boxes indicate steps of PNEC determination and green boxes point out deficiencies in current methodologies of PNEC determinations (adapted from European Commission 2003).

Kooijman 1987, Van Straalen and Denneman 1989, Wagner and Løkke 1991). For this purpose, ten long-term NOECs derived from representatives of eight taxonomic groups are the minimum requirements for this procedure (European Commission 2003), and optimal sample sizes range between 15 and 55 (Newman et al. 2000). Although ERAs based on the SSD approach are potentially more significant (Calow and Forbes 2003), the utilized datasets are often too small and biased, and thus not representative for the aquatic systems (Forbes and Calow 2002). This is especially true for flowing waters, as organisms originating from lotic systems are currently underrepresented in ecotoxicological investigations, although the need for information concerning riverine species is obvious. Rivers are the main receiving water bodies of chemical substances due to discharges of sewage treatment plants, which carry chemical residues from both industrial and domestic purposes (Murray et al. 2010), or unintended entry of pesticides used in agriculture (Carter 2000). The ongoing decline of the biodiversity in all freshwater systems, but especially in streams and rivers (Geist 2011), is attributed to factors like overexploitation, habitat loss and degradation, the appearance of invasive species, and also water pollution (e.g. Allan and Flecker 1993, Dudgeon et al. 2006). Among the 24 OECD test guidelines on effects on freshwater organisms, *Chironomus* sp. and *P. antipodarum* are the only invertebrate taxa from lotic systems. Similarly, in the USA 13 (draft) guidelines on ecotoxicological effects assessment in freshwater organisms are available including only three stream-dwelling species (two amphipod and one fish species). Furthermore, OECD guidelines and ISO norms on fish toxicity tests recommend the use of zebra fish *Danio rerio*, fathead minnow *Pimephales promelas*, ricefish *Oryzias latipes* or rainbow trout *Oncorhynchus mykiss* (e.g. OECD 1992 and 2013a, ISO 1994 and 1996), which are all non-native species in Europe. In addition, *D. rerio*, which is the most common fish species utilized in the EU, is not representative for lotic systems.

Further deficiencies are also due to the scarcity of information concerning chronic long-term effects as most of the standard tests are designed as acute toxicity tests (Table 1-1), and probably as a consequence of high cost and complexity of long-term exposure investigation. Short-term tests are important in effect assessments to quickly generate knowledge concerning lethal concentrations of substances of concern and if further testing is required (Hushon et al. 1979). Especially in substances with temporary entrances in the environment, like plant protection products, which mainly enter the aquatic environment via spray-drift during application (Carter 2000), short-

term tests are appropriate for the assessment of the environmental risk. In contrast, in substances that are continuously released into the environment, acute tests do not resemble realistic exposure scenarios (Eggen et al. 2004), and long-term effects might be overseen. This applies to the so-called *emerging contaminants* (ECs) which enter the aquatic environment mainly via effluents of sewage treatment plants as a result of insufficient removal (Pal et al. 2010, Petrie et al. 2015). *Emerging contaminants* are defined by the USEPA as 'chemicals without regulatory status and which impact on environment and human health are poorly understood' (Deblonde et al. 2011). Amongst others pharmaceuticals, personal care products and endocrine disrupting compounds are considered as ECs (e.g. Richardson and Ternes 2005, Pal et al. 2010, Petrie et al. 2015). Environmental concentrations of these substances are in the ng to µg/L range (Petrie et al. 2015), and thus unlikely to cause acute effects. For example, in pharmaceuticals, acute effect concentrations are several orders of magnitude higher than environmentally measured concentrations (Table 1-2). Still, chronic, sublethal effects cannot be excluded, especially as these substances were designed to exhibit a specific biological effect (Breton and Boxall 2003). Although modes of action are - at least partly - known in substances like pharmaceuticals (e.g. reviewed in Capone et al. 2007, Frishman and Saunders 2011), possible impacts – especially sublethal effects - on non-target organisms cannot be appropriately predicted. This is on the one hand due to differences in the uptake of substances in aquatic organisms, which not only occurs orally but also as a result of absorption and diffusion processes on organisms' surfaces (Fent 2007). On the other hand due to possible differences in the physiology of target and non-target organisms, and thus differences in pharmacodynamic and pharmacokinetic processes (Fent 2007). In other substances biological effects are completely unknown and are only detected by chance or in specified investigations as e.g. endocrine disrupting effects of plasticizers (e.g. Harris et al. 1997). Consequently, the environmental risks of ECs cannot be adequately assessed without precise investigations of their long-term effects on non-target organisms.

Table 1-2. Mean effect concentrations (EC_{50}) for immobility in *Daphnia magna* of the five most frequently detected pharmaceutical compounds worldwide (based on number of observations; Hughes et al. 2013) and their respective maximal *measured environmental concentration* ($MEC_{max.}$).

Compound	**EC_{50} (mg/L) *Daphnia magna***	**$MEC_{max.}$ (µg/L)**
Carbamazepine	>100[1,2]	11.56[8]
	97.8[3]	
	>13.8[4,5]	
Diclofenac	68[1]	18.74[8]
	22.43[4]	
Ibuprofen	108[1]	31.32[8]
Naproxen	174[1]	19.60[8]
	166.3[6]	
Sulfamethoxazole	189.2[2]	11.92[8]
	123.1[7]	
	>100[5]	

[1]Cleuvers (2003)
[2]Kim et al. (2007)
[3]Jos et al. (2003)
[4]Ferrari et al. (2003)
[5]Ferrari et al. (2004)
[6]Cleuvers (2004)
[7]Park and Choi (2008)
[8]Hughes et al. (2013)

Another deficiency in current standard test settings is that regional differences are not considered (Breitholtz et al. 2006), as a consequence of the requirements of high standardization and reproducibility in order to be applied on European (international) level (De Lange et al. 2010). Basic test conditions can therefore only resemble the natural circumstances of a limited proportion of ecosystems (Breitholtz et al. 2006). Thus, an adequate estimation of the environmental risk for all systems is problematic, as abiotic properties like water chemistry can strongly influence the toxicity of substances as a result of an altered bioavailability (e.g. Newman and Unger 2003). For instance, the toxicities of chloride and sulfate decrease with increasing water hardness and also depend on each other's concentration (Soucek et al. 2005, Soucek et al. 2011). Likewise, toxicities of various metal-ions are higher in soft water (Biesinger and Christensen 1972) compared with hard water (Khangarot and Ray 1989), and can also be influenced by the Mg^{2+}:Ca^{2+} ratio, which are the main ions to contribute to water hardness (Naddy et al. 2002).

1.4. Objectives

The aim of this thesis was to increase the ecological relevance in ecotoxicological investigations in order to enhance the assignability of laboratory-derived knowledge on substance toxicity to real conditions in ERAs. For this purpose, three different approaches were utilized. First, chronic, sublethal effects of a pharmaceutical were investigated in a realistic long-term exposure using measured environmental concentrations. Second, actual conditions under which chemicals enter the environment were considered by the investigation of wastewater instead of the single substance. Third, ecologically relevant non-standard test species were incorporated in the investigations, which required the development of a novel exposure system.

In the first part, a proposed new standard test with the New Zealand mudsnail *Potamopyrgus antipodarum* (OECD 2010b) for the investigation of chronic, long-term effects was utilized to investigate the impacts of environmentally occurring concentrations of the beta-blocker sotalol. In addition, the potential to predict sublethal effects based on known pharmacodynamic properties of pharmaceuticals in humans was assessed.

In the second part, an ERA of an intended wastewater (concentrate) discharge of a reverse osmosis system into a nearby stream was conducted in consideration of the real circumstances. Therefore, both water and test organisms *Gammarus pulex* and *Gammarus roeseli* were collected at the specific site and used in acute single-species tests. In addition, concentrates containing commonly applied scale-building inhibitors (antiscalants; AS) were compared to concentrate without AS. These additives are rated not harmful to the environment, but had only solely been tested as single substance.

In the third part, the Miniature Circulatory System (MCS) was developed for the long-term investigation of the concentrate in an egg-to-fry bioassay with the brown trout *Salmo trutta*. The MCS was also evaluated for its adaptability to long-term investigations with other stream-dwelling species using the example of the amphipod *G. roeseli*.

2. SUBLETHAL EFFECTS OF THE BETA-BLOCKER SOTALOL AT ENVIRONMENTALLY RELEVANT CONCENTRATIONS ON THE NEW ZEALAND MUDSNAIL *POTAMOPYRGUS ANTIPODARUM*

A similar version of this chapter was published as:

Feiner M, Laforsch C, Letzel T, and Geist J (2014). Sublethal effects of the beta-blocker sotalol at environmentally relevant concentrations on the New Zealand mudsnail *Potamopyrgus antipodarum*. *Environmental Toxicology and Chemistry* 33, 2510-2515.

2.1. Abstract

Monitoring sublethal effects of pharmaceuticals on non-target species in aquatic environments has become an important topic in ecotoxicology, yet there are few studies concerning the effects of beta-blockers on aquatic organisms. We investigated the effects of the beta-blocker sotalol (SOT) at three environmentally relevant concentrations on life-history traits of the New Zealand mudsnail *Potamopyrgus antipodarum*. Based on the pharmacodynamic properties of SOT, we hypothesized reduced numbers of embryos in the brood pouches, decelerated growth of adult snails, and smaller size of neonates, but no effect on mortality rates of adults. Contrary to our expectations, the total number of embryos was significantly higher after 56 days of exposure at nominal concentrations of 0.05 and 1.0 µg/L by 107 and 73%, respectively. No differences in embryo numbers were observed at earlier time-points. Therefore, the mode of action seems to be an extension of the reproductive period rather than an increase of the embryo production. Furthermore, our results indicate a hormetic dose-response-relationship, as no effects were observed at the highest test concentration (6.5 µg/L). Mortality, growth of adult snails and neonate sizes were not affected by the beta-blocker. Given the strong influence on reproduction, the effects of sublethal concentrations of SOT and other beta-blockers deserve better consideration in ERAs.

2.2. Introduction

The occurrence of pharmaceuticals in the aquatic environment has become an important issue for ecotoxicologists and water managers alike (Connon et al. 2012). Numerous studies reported the detection of drug residues in surface waters worldwide (e.g. Halling-Sørensen et al. 1998, Kolpin et al. 2002). In the context of the targets set by the European Water Framework Directive, there is increasing interest in understanding and mitigating the effects of these compounds (Geist 2014, European Union 2013). Beside an improper disposal of unused or expired medications, excretion by humans via sewage is considered as the major source of pharmaceuticals entering the environment (Kümmerer 2001). Up to 95% of the active ingredients are excreted in an unchanged or metabolized form (Salomon 2007). Due to their stability against enzymes or acids, in order to reach their specific sites of action (Halling-Sørensen et al. 1998), sewage treatment plants are not able to completely eliminate these compounds. Removal rates vary between 0 and 100% depending on the agent and the wastewater treatment process (e.g. Miège et al. 2009, Gabet-Giraud 2010).

Among frequently detected pharmaceuticals in effluents of sewage treatments plants, receiving rivers, and even the groundwater are beta-blockers (e.g. Sacher et al. 2001, Ternes 1998, Vieno et al. 2006), which belong to the substance class of sympatholytics. These agents are primarily prescribed to treat cardiovascular diseases like hypertension, and act as antagonists to beta-adrenoceptors of the vertebrate adrenergic hormone system (Frishman and Saunders 2011). Besides the smooth muscles of the heart, beta-adrenoceptors are located in a variety of different tissues in vertebrates (Ahlquist 1948). The three known subtypes differ in their structure and function (Strosberg 1993, Bylund et al. 1994), and therefore are involved in several physiological processes in vertebrates (Massarsky et al. 2011). Their activation by the neurotransmitters epinephrine and norepinephrine leads, for instance, to an increase of the intracellular calcium concentration during the heart muscle contraction or initiates the glycogenolysis. In contrast to vertebrates, invertebrate species lack the adrenergic system and have the octopaminergic system instead. Due to the structural homology of the two hormone systems, potential points of action for beta-blockers also exist in aquatic invertebrates (Massarsky et al. 2011, Stefano et al. 1978), especially molluscs, as the transmitter norepinephrine was detected in this phylum (e.g. Stefano et al. 1978, Croll et al. 1999).

One of the most common beta-blockers detected in surface waters in Germany is sotalol (SOT) (Rohweder 2003). It acts as a non-selective beta-receptor antagonist (Antonaccio and Gomoll 1990) and therefore binds to all types of beta-receptors. Ecotoxicological investigations into the biological effects of this substance are scarce, despite its frequent appearance in all types of surface waters (e.g. Sacher et al. 2001) and its classification as potentially harmful to the environment (LANUV 2007). Moreover, SOT is only slightly biodegraded and hydrolyzed (Letzel 2007) and not prone to direct photolysis (Piram et al. 2008). These factors are likely to contribute to a high persistence of this pharmaceutical in the aquatic environment. Though, SOT does not seem to be acutely toxic to invertebrates. The 48 h acute *Daphnia magna*-Immobilisation-Test revealed a LC_{50}-value (*lethal concentration*$_x$; i.e. the concentration, at which x% of the test animals die) of more than 300 mg/L (Hernando et al. 2004), which exceeds environmental concentrations by several orders of magnitude (Rohweder 2003). Due to a lack of information on the chronic effects of this chemical at low exposure concentrations, we focused on possible sublethal effects at environmentally relevant concentrations on a non-target aquatic invertebrate.

Besides the standard aquatic ecotoxicological test organisms like *Daphnia spp.*, which inhabit standing water bodies, there is a tendency in ecotoxicology to also develop and include new standard tests with organisms originating from running waters (e.g. Duft et al. 2007, Gerhardt 2011). Among them is the New Zealand mudsnail *Potamopyrgus antipodarum* (Gastropoda: Hydrobiidae). *Potamopyrgus antipodarum* is an invasive and well-established species in many countries worldwide (DAISIE 2009), making knowledge on its sensitivity to pharmaceuticals in comparison to native species particularly important. Due to its parthenogenetic reproduction, ubiquitous distribution, sensitivity to endocrine disrupting chemicals (Duft et al. 2007), and easy cultivation in the laboratory *P. antipodarum* is a suitable organism for single-species tests.

We investigated the effects of a long-term (56 d) exposure of *P. antipodarum* to SOT on growth, reproduction, offspring size, and mortality, using environmentally relevant concentrations. The synthetic estrogen 17α-ethinylestradiol with known endocrine effects was used as a positive control. Due to the pharmacodynamic properties of the beta-blocker, we hypothesized that increasing concentrations of SOT would result in reduced growth and reproduction of the adult snails, and smaller sizes of the neonates. In contrast, we expected no direct effect on the mortality of adult snails.

2.3. Material and Methods

2.3.1. Pharmaceutical preparation and chemical fate

The pharmaceutical substances 17α-ethinylestradiol (EE2) and sotalol-hydrochloride (SOT) were purchased from Sigma-Aldrich. Test concentrations resembled reported concentrations of SOT in surface waters (Rohweder 2003) (Table 2-1), whereat Levels 1 and 2 represent the median and the maximum values, respectively, found in rivers, and level 3 corresponds to the highest concentration in effluents of sewage treatment plants. EE2 at a concentration of 25 ng/L served as a positive control (OECD 2010b). Stock solutions of the pharmaceuticals were prepared by dissolving the chemicals in double-distilled water using a magnetic stirrer one day prior to use. For the application of the pharmaceuticals, the stock solutions were diluted with double-distilled water to working solutions with nominal SOT concentrations of 0.03, 0.60 and 3.90 mg/L. Thus, 1 ml of the working solutions had to be applied to the corresponding beaker to generate the appropriate SOT concentrations for the treatments SOT1, SOT2, and SOT3, respectively.

Table 2-1. Concentrations of sotalol measured in surface waters (Rohweder 2003) and test concentrations used in this study (µg/L).

Level	Environmental concentration	Test concentration
1	0.049	0.050
2	0.950	1.000
3	6.500	6.500

Due to the high detection limit of SOT (1.67 mg/L), the actual test concentrations could not be verified and presentation of results thus refers to nominal concentrations. With regard to the low test concentrations, a preconcentration of test medium still would have resulted in concentrations below the detection limit. An additional test was conducted to examine the fate of the beta-blocker under simulated conditions. For the test, a stock solution was prepared by dissolving 22.52 mg SOT in 100 ml of the artificial medium used for snail culture and in the effects assessment test (see *Test organisms and test setup*). Twelve 100 ml glass beakers were filled with 92.45 ml medium and 7.55 ml stock solution each to gain test solutions with a SOT concentration of 16.99 mg/L. Immediately, the concentration of SOT was measured in

three beakers. The remaining ones were stored in a climate chamber, which was set to 16 ± 1 °C with a day-night rhythm of 16:8 hours. Additionally, the beakers were slightly aerated with a Pasteur pipette connected to an air pump, but not covered, to resemble test conditions. No food and snails were added, because SOT is not likely to adsorb to organic compounds (Maurer et al. 2007), and the purpose of this test was to investigate the general fate of the beta-blocker in aqueous solutions. Furthermore, with the test concentration used, a realistic test condition concerning the proportionality between SOT, food, and snails could not be realized. After two, four, and seven days SOT concentrations were determined in three beakers, respectively. For this purpose, test solutions were filtered through 0.22 µm PVDF-filters for the analyses of SOT concentrations. HPLC was applied for the separation of the substances (Poroshell 120-EC-C18, 3.0x50 mm, 2.7 µm, Agilent Technologies Deutschland GmbH). Eluent A and B consisted of 90/10 and 10/90 acetonitrile/ammonium acetate (10mM), respectively. The run was performed at 0.5 ml/min. Gradient of the mobile phase was 0% B (minutes 0-2), 50% B (minutes 6-9), and 0% B (minutes 10-15). Following spectroscopic measurements were performed at 220, 280, 237, and 228 nm using an UV-detector (1260 Infinity Diode Array Detector, Agilent Technologies Deutschland GmbH). The pharmaceutical fate during the test was analyzed both with the actually measured concentrations and in consideration of the water evaporation, which occurred due to the aeration. For the latter, the daily evaporation rate was determined based on the volumes of the test solutions after seven days, and hence, volumes of the test solutions were calculated for each measuring time-point. The SOT compound of each beaker was calculated using the analyzed SOT concentrations. Concentrations were assessed using the initial volume of 100 ml. We used EE2 as a positive control to ensure sensitivity of the test snails to endocrine-disrupting chemicals, which was verified in the present study.

2.3.2. Test organisms and test setup

The *Potamopyrgus antipodarum* snails for the test were derived from our own laboratory breeding stock. This culture was started with snails that were collected in the river Moosach near Freising, Germany, which is uninfluenced by sewage treatment effluents. The animals were cultured in aerated 12 L glass aquaria filled with artificial medium based on distilled water (0.3 g synthetic sea salt, 0.18 g $NaHCO_3$ and 0.1 ml

saturated $CaCO_3$ solution per liter), which was also used in the test. The medium was prepared in a 45 L aquarium and aerated at least 24 h prior to use. Once a week, half of the culturing medium was renewed. The snails were fed with finely ground flaked fish food (Tetra GmbH) *ad libitum* three times a week. Animal densities were about 100 snails per liter.

The exposure test was performed on the basis of the OECD Series on Testing and Assessment No. 121: Detailed review paper (DRP) on molluscs life-cycle toxicity testing (OECD 2010b). At the beginning of the test, the shell height of the snails was measured with a digital caliper (to the nearest tenth mm) and 20 animals per replicate were randomly assigned to 920 mL glass beakers filled with 600 ml of the artificial medium containing the different SOT or EE2 treatments. Control beakers did not contain any pharmaceutical compound. For each treatment, eleven replicates were used. Due to mortality, replicate numbers decreased towards test terminations, but were always at least eight. To ensure the inclusion of reproductively active snails, only specimens with a shell height between 3.7 and 4.3 mm were used. The beakers were aerated as described above. The test system was semi-static with a renewal of the test medium three times a week by transferring the animals into new beakers containing new test solutions and food *ad libitum* (approximately 0.25 mg per animal and day). Because of the frequent renewal of the test medium, water evaporation was considered to be low, and thus beakers were not covered. With regular measurements of the oxygen saturation, pH and temperature, the validity of the test was conducted. Both culture and test set-up were stored in a climate chamber at 16 ± 1 °C and a day-night rhythm of 16:8 h.

2.3.3. Growth, reproduction, mortality, and offspring size

On each of the exposure days 14, 28, 42, and 56, five adult snails were randomly sampled from each beaker. Subsequently, their shell heights, shell widths (both to the nearest tenth mm), and the wet weights (to the nearest tenth mg) were determined, and the snails were transferred into a 2.5% (w/v) $MgCl_2$-solution for narcotization. After 45-60 min, the number of embryos (subdivided into those with and without shell) in the brood pouch of each snail was counted under a stereomicroscope. In addition, unfertilized or undeveloped eggs, i.e. egg capsules without an embryonic structure,

were separately recorded. Beakers were checked for dead snails during the renewal of the test medium. Once a week during the exposure period from day 14 to day 56 five newly hatched snails were randomly removed from the beakers and immediately fixed in 70% (v/v) EtOH for subsequent size measurements, which were conducted using a stereomicroscope and the digital imaging software Cell*D (Olympus). As the shells of neonate *P. antipodarum* are rather plane, the size was defined as the shell area of the top-view. Therefore the snails were set up in the same position with the apex on the upper side and the aperture perpendicular to the bottom. Additionally, as a standard size parameter of gastropods, the shell width was determined. Measurements were up to the nearest µm² and µm, respectively. In order to avoid pseudo-replication, means of all collected data (i.e. size parameters of the neonate and adult snails, and number of embryos) were calculated per beaker and measuring day for further analyses. In accordance to the DRP (OECD 2010b) all snails without reproduction, i.e. no embryos in the brood pouch, were excluded.

2.3.4. Statistical analyses

Normal distribution and homoscedasticity of the data were analyzed with Kolmogorov-Smirnov-tests and Levene's-tests, respectively. Pharmaceutical fate under test conditions was tested for changes over time with a simple linear regression. Adult snails were checked for differences in the number of embryos, size, and mortality between treatments with one-way ANOVAs (analyses of variance) and following Dunnett's post-hoc-tests. One-way ANOVAs were also used to check for differences between treatments in neonate sizes. Changes over time in offspring sizes within treatments were tested using a simple linear regression. All statistical analyses were performed with the software IBM SPSS Statistics 20 (IBM Corporation).

2.4. Results

2.4.1. Chemical fate of SOT

The chemical analyses revealed a slight increase of the SOT concentration during seven days by three percent, if the reduction in the water volume due to evaporation was not considered (linear regression: $y = 0{,}0814x + 18{,}026$, $r^2 = 0.561$, $f_{(1,10)} = 12.769$, $p = 0.005$, Table 2-2). After correcting data by considering the daily evaporation rate (1.57 ml per day), a significant decrease of the SOT concentration within seven days by eight percent (linear regression: $y = -0{,}2119x + 18{,}056$, $r^2 = 0.916$, $f_{(1,10)} = 109.72$, $p < 0.001$) was observed. Irrespective of the determination method, the SOT concentrations remained within the range of ± 20% of the nominal concentration throughout the test.

Table 2-2. Mean sotalol concentrations (± standard deviation) at test initiation, after 48, 96, and 168 h.

Time Point (h)	Analyzed c (mg/L)	Percentage of nominal c	Calculated c (mg/L)	Percentage of nominal c
0	18.12 ± 0.15	107	18.12 ± 0.15	107
48	18.12 ± 0.22	107	17.57 ± 0.22	103
96	18.25 ± 0.29	107	17.15 ± 0.27	101
168	18.67 ± 0.09	110	16.62 ± 0.08	98

c = concentration

2.4.2. Reproduction, growth, and mortality

The snails exposed to SOT at the concentrations 0.05 µg/L (SOT1), 1 µg/L (SOT2), and to EE2 (25 ng/L) had a significantly higher total number of embryos in their brood pouches than the untreated animals after 56 days (one-way ANOVA: $f_{(4,37)} = 5.128$, $p = 0.002$, Dunnett (> control) $p = 0.002$ (SOT1), $p = 0.03$ (SOT2), and $p = 0.001$ (EE2), Figure 2-1). Embryo numbers exceeded the control-level by 107, 73, and 112%, respectively. The same result was observed considering only unshelled embryos (one-way ANOVA: $f_{(4,37)} = 4.835$, $p = 0.003$, Dunnett (> control) $p = 0.005$ (SOT1), $p = 0.038$ (SOT2), and $p < 0.001$ (EE2)). Numbers of unshelled embryos were 294 (SOT1), 215 (SOT2), and 385% (EE2) higher than in the control. A similar pattern occurred

concerning the number of shelled embryos, where only the difference between treatment SOT1 and the control was significant (one-way ANOVA: $f_{(4,37)} = 2.802$, $p = 0.040$, Dunnett (> control) $p = 0.017$). This pattern became already apparent after 42 days in the number of unshelled embryos. Though the differences were not significant, a clear trend towards an increased number was noticeable in treatments SOT1, SOT2, and EE2 (one-way ANOVA: $f_{(4,48)} = 2.332$, $p = 0.069$, Figure 2-1C). No differences were observed after 14 and 28 days (Figure 2-1A and B). Animals exposed to 6.5 µg/L SOT (SOT3) were not different to the control throughout the entire testing period. Undeveloped eggs were found at a frequency of 11% in the brood pouches of snails in all treatments, without differences between treatments.

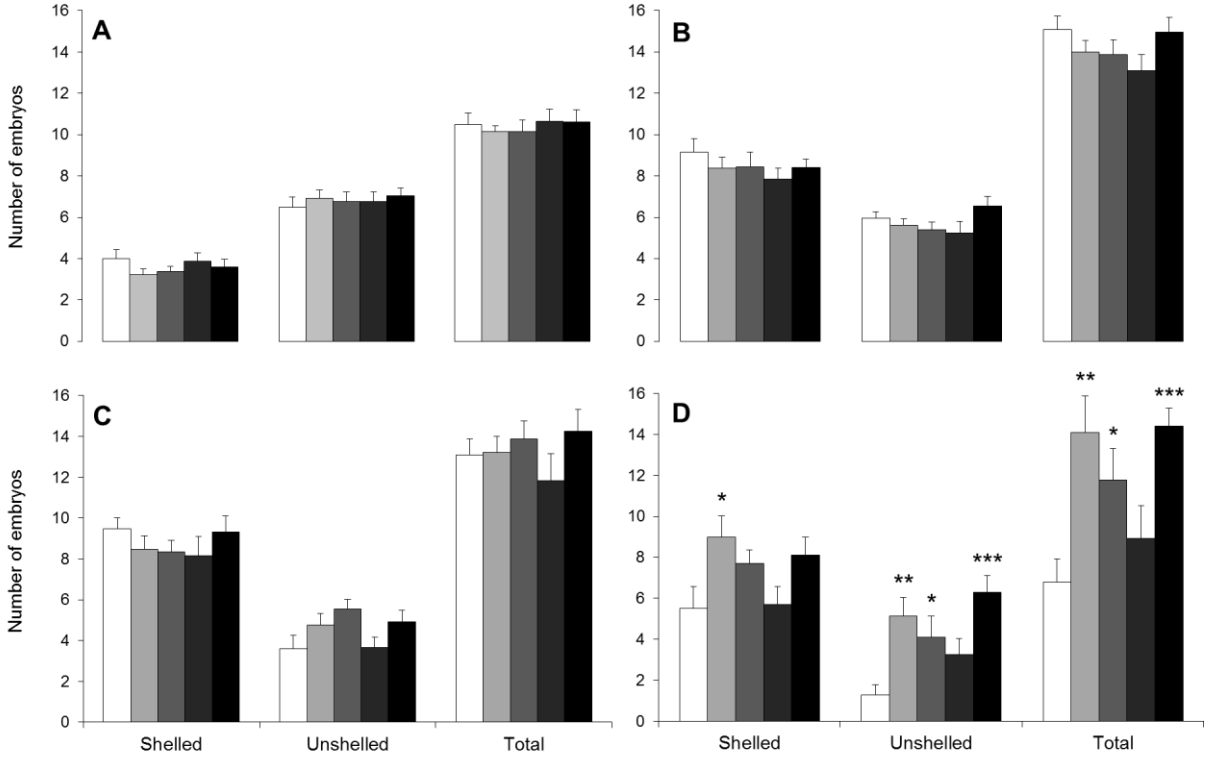

Figure 2-1. Number of shelled and unshelled embryos and total number after (A) 14, (B) 28, (C) 42, and (D) 56 days at different sotalol and 17α-ethinylestradiol exposures. ☐ Control, ▒ 0.05 µg/L sotalol, ▒ 1.0 µg/L sotalol, ■ 6.5 µg/L sotalol und ■ 25.0 ng/L 17α-ethinylestradiol (mean ± SE). Asterisks mark significant differences to the control: * p ≤ 0.05, ** p ≤ 0.01, *** p ≤ 0.001.

Shell-height and wet-weight of adult snails did not differ between treatments at any sampling time point. In all treatments, mean shell-height increased from 3.9 to 4.0 mm between day 0 and day 56. The wet-weight varied considerably throughout the entire

test period between 5.8 and 6.3 mg and was thus a weak indicator of snail performance.

Mean mortality rates were between 6% (EE2) and 14% (control) at test termination, and thus complied with the validity criteria of control mortality less than 20%. In line with our hypothesis, no differences in mortality rates were observed between treatments and the control (one-way ANOVA: $f_{(4,48)} = 0.725$, $p = 0.579$).

2.4.3. Offspring size

Measurements of the neonate sizes as top-view shell area were not significantly different between treatments at any time point except for significantly smaller offspring in SOT3 after 14 days (one-way ANOVA: $f_{(4,15)} = 5.084$, $p = 0.009$, Dunnett (two sided) $p = 0.006$).

The simple linear regression revealed a slight, but significant and continuous decrease of the neonate sizes based on the top-view shell area over time in all SOT treatments and the control (Figure 2-2, Table 2-3). In contrast, measurements of shell widths did not follow this trend.

Table 2-3. Results of the simple linear regression for neonate sizes as top-view shell area over time.

Treatment	***r²***	***df***	***f***	***p***
Control	0.108	1,56	6.749	0.012
SOT1	0.073	1,63	4.984	0.029
SOT2	0.161	1,64	12.319	0.001
SOT3	0.095	1,64	6.739	0.012

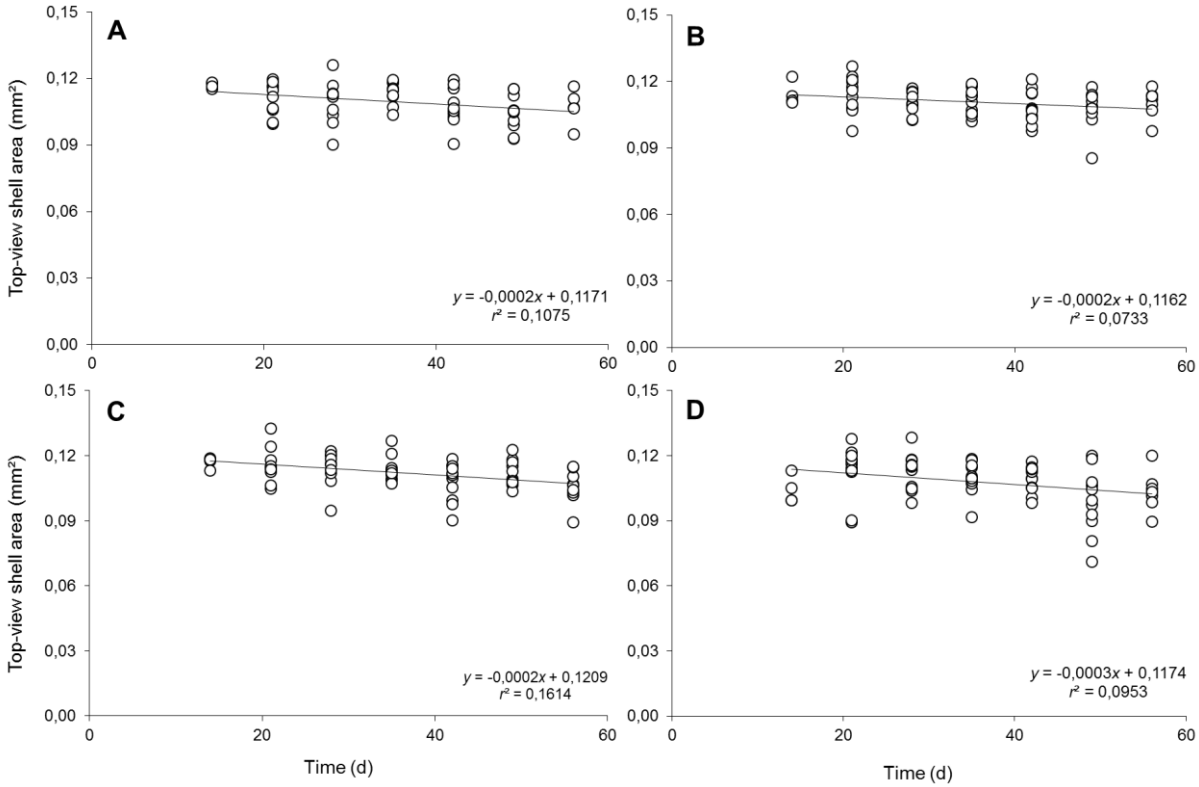

Figure 2-2. Neonate sizes of *P. antipodarum* as top-view shell area during the exposure test with the beta-blocker sotalol (A) in the control, (B) at 0.05 µg/L, (C) at 1 µg/L, and (D) at 6.5 µg/L.

2.5. Discussion

The results of the present study indicate that environmentally observed concentrations of the beta-blocker SOT can significantly influence reproduction in the aquatic snail *P. antipodarum*. This is, to our knowledge, the first detection of a low-dose-effect of this single substance. Up to now, the lowest effect concentration observed was 0.5 mg/L in the 21 days *Daphnia magna* reproduction test (Maurer et al. 2007) which exceeds our results by a factor of 1000. In contrast to our expectations of a reduced reproduction, female snails of *P. antipodarum* exposed to the agent showed a significantly increased reproductive output at the environmentally relevant SOT concentrations of 0.05 and 1 µg/L after eight weeks compared to the untreated snails, which can mostly be explained by prolongation of the reproductive period. However, no effect occurred at the highest test concentration of 6.5 µg/L. This is in concordance with Stanley et al. (2006), who observed an increased reproduction in *D. magna* exposed to the beta-blocker propranolol at the lowest test concentrations, but no effect at higher exposure levels. Moreover, Dietrich et al. (2010) observed changes in the reproduction in *D. magna* due to an exposure to the beta-blocker metoprolol at the environmentally occurring concentration of 1.2 µg/L, though the effects were antithetic to our results with reduced offspring numbers. Likewise, Huggett et al. (2002) demonstrated a diminished reproduction in *Hyalella azteca* and *Ceriodaphnia dubia* caused by propranolol. However, this impact only occurred at rather high test concentrations of 0.1 and 0.25 mg/L, respectively. In consistence with our results, Gust et al. (2009) noticed a similar effect of the antidepressant fluoxetine in *P. antipodarum*, with a stimulation of the reproduction at low concentrations, no effect at mid concentrations, and reduced reproduction at high concentrations. According to Duft et al. (2007), New Zealand mudsnails reproduce throughout the year, but with fluctuations in the number of offspring. This applies to the reproductive behavior of the control snails in our study with highest numbers of embryos after 28 and 42 days and a reduced number at the end of the test. In contrast, the total number of embryos in treatments SOT1 and SOT2 remained at a constant high level towards test termination. According to our results, the underlying mechanism is obviously an extension of the highly reproductive period rather than an increase of the embryo production at any specific time point. This may have severe consequences for the affected species, both in terms of possible positive and negative effects. Considering allochthonous species,

an extension of the reproductive period might accelerate their reproductive output, resulting in an increased invasive potential of the species. In contrast, an artificially modified reproduction behavior and/or timing of reproduction might be disadvantageous for autochthonous species. Juveniles born during an extended reproduction period are more likely to be born outside the periods with optimal environmental conditions (Duft et al. 2007). This may be either due to seasonal changes, or in consequence of increased intraspecific competition, or predation. Moreover, the size measurements of neonate snails in our study revealed a continuous decline in offspring sizes over time. Though, only for the sizes based on the top-view shell area, but not for the standard size parameter shell width. This trend might be in consequence of the depletion of energy storage compounds such as glycogen and lipids, which are mobilized during the reproductive cycle in *P. antipodarum* (Gust et al. 2011). Thus, the fitness of late-born neonates might be lower. Furthermore, adult specimens presumably are negatively affected as well. As a result of the increased energy demand along with an enlarged population size, a shift in their foraging behavior, e.g. into daytimes when predation risk is higher (Levri and Lively 1996), might occur. Moreover, an increased reproduction caused by an anthropogenic introduced chemical might inhibit self-regulating mechanisms like density-dependent fecundity in an ecosystem.

The beta-blocker SOT proved to be stable in aqueous solutions for at least seven days. The actually analysed concentrations showed a slight, significant increase of the SOT concentration over seven days, which is due to the water evaporation. Taking it into account, a significant decrease of the concentration during the test period was identified. This indicates a degradation of SOT in aqueous solutions. However, the concentration remained within the ± 20% range of the nominal concentration. Hence, a renewal of the test-medium in weekly intervals is sufficient for constant SOT concentrations in further investigations.

Our results demonstrate the challenges in the risk assessment of pharmaceuticals in aquatic environments. In particular, the strong and previously undescribed effects of SOT at environmentally relevant concentrations suggest that beta-blockers deserve better consideration in ERAs. Although the modes of action of these substances are – at least in some cases – well investigated, the pharmacodynamic properties of an agent in humans cannot readily be transferred to non-target animal species, especially

invertebrates. Furthermore, the results indicate a hormetic dose-response relationship of SOT to the reproduction of *P. antipodarum* by the induction of a positive effect, and the effect occurring only at the lowest test concentrations. Although we did not demonstrate negative impacts on reproduction caused by high SOT concentrations, attributable to the specific test concentration choice, decreased reproduction was previously observed in *D. magna* exposed to 0.5 g/L SOT (Schüssler and Sengl 2004). Moreover, the present study emphasizes the importance of appropriate test durations, especially for the investigation of sublethal effects. Although Macken et al. (2012), for example, demonstrated that significant differences in the reproduction can occur after 28 d, the impact of SOT would not have been observed within this time period. Therefore, our results support the recommended test duration of 56 d for the establishment of a standard chronic ecotoxicological test with the New Zealand mudsnail *P. antipodarum*.

3. INCREASED RO CONCENTRATE TOXICITY FOLLOWING APPLICATION OF ANTISCALANTS – ACUTE TOXICITY TESTS WITH THE AMPHIPODS *GAMMARUS PULEX* AND *GAMMARUS ROESELI*

A similar version of this chapter was published as:

Feiner M, Beggel S, Jaeger N, and Geist J (2015). Increased RO concentrate toxicity following application of antiscalants – Acute toxicity tests with the amphipods *Gammarus pulex* and *Gammarus roeseli*. *Environmental Pollution* 197, 309-312.

3.1. Abstract

In reverse osmosis (RO), a frequently used technology in water desalination processes, wastewater (concentrate) is generated containing the retained solutes as well as so-called antiscalants (ASs), i.e. chemical substances that are commonly applied to prevent membrane-blocking. In this study, an ERA of a possible discharge of concentrate into a small stream was conducted. The acute toxicity of two concentrates containing two different ASs and of concentrate without AS to the amphipods *Gammarus pulex* and *Gammarus roeseli* was studied. Mortality of gammarids exposed to the concentrate without AS was not different to the control, whereas concentrates including ASs caused mortality rates up to 100% at the highest test concentrations after 168 h. Resulting EC_{50}-values were 36.2–39.4% (v/v) after 96 h and 26.6–58.0% (v/v) after 168 h. These results suggest that the ecotoxicological relevance of ASs is greater than currently assumed.

3.2. Introduction

The provision of potable domestic water in high quality is a great challenge in many regions worldwide (e.g. Geist 2014). Membrane-based technologies are thereby most widely applied for raw water purification, with reverse osmosis (RO) being used in 80% of the desalination plants worldwide (Fritzmann et al. 2007, Greenlee et al. 2009). Especially in arid and semi-arid regions, the use of desalination of seawater is necessary for the provision of drinking water. Also, desalination of groundwater can become necessary, even in regions without water scarcity, due to elevated amounts of undesired ions. Quality requirements of water for human consumption are defined by legislation in the European Union and elsewhere (European Communities 1998, USEPA 2009). In addition to microbiological parameters, threshold values for chemical (e.g. heavy metals, salts, or pesticides) and indicator parameters (e.g. taste, smell, and conductivity) are regulated. Regional variations in aquifer geologies can thereby imply the application of purification technologies.

This can exemplarily be studied in shell-limestone dominated areas such as lower Frankonia in Germany. The groundwater used as local drinking water supply of the city of Würzburg has a high hardness and exceeds national quality criteria in sulfate-concentration of 250 mg/L (Lipp 2010). Therefore, desalination by RO is planned to be applied. However, the efficiency of an RO system is mainly limited by membrane-blocking due to precipitation of inorganic solutes like carbonate and sulfate scales (Rautenbach and Melin 2003). In order to inhibit scale formation, so-called antiscalants (ASs; i.e. substances that bind to different types of solutes) are applied. The exact compositions of ASs are trade secrets, but principle components are phosphonates, polycarboxylates or polyacrylic acid (e.g. Darton 2000, Genesys® International 2007b and Toray Membrane Europe 2010b).

During the RO process, deionized water (permeate) and wastewater (concentrate) are produced, with the latter containing the retained solutes and the AS (Fritzmann et al. 2007) making its disposal an important issue, especially in inland RO applications (Greenlee et al. 2009). One option is to discharge concentrate directly into surface waters, which requires a governmental permission. Minimum requirements – concerning chemical and biological parameters – for wastewater to be discharged via natural waters are regulated in the wastewater ordinance in Germany (AbwV 1997). Beyond these specifications, the principles of the federal water act have to be met

(WHG 2009), which include prohibition of water body degradation and the retention of a high environmental protection level in order to meet the aims of the Water Framework Directive (European Union 2000, Geist 2014). Whilst the impacts of seawater desalination and concentrate disposal in coastal regions have already been studied (e.g. Miri and Chouikhi 2005, Del-Pilar-Ruso et al. 2008), there is a lack in ecotoxicological effect assessments of concentrate discharge into freshwater ecosystems.

Therefore, an ERA of the possible discharge of the concentrate into the nearby stream Kürnach was conducted. Acute toxicity tests with the two amphipods *Gammarus pulex* and *Gammarus roeseli* were conducted, which are naturally the most abundant benthic species in such streams, including the Kürnach (Tombek 2012). The effects of concentrate containing one of two different ASs, which were considered for usage, were tested in seven different concentrations including PECs. For comparison, the effects of concentrate without any AS were investigated.

3.3. Material and Methods

3.3.1. Test organisms

Gammarus pulex and *G. roeseli* were collected by kick-sampling at least one week prior to the beginning of each test in the stream Kürnach (Würzburg, Germany), in June and July 2013. Immediately after collection, the gammarids were transferred into buckets filled with water from the sampling site and previously conditioned alder leaves (*Alnus glutinosa*) to provide feed. After transportation to the facilities of the Aquatic Systems Biology Unit at Technische Universität München, gammarids were sorted by species and separately maintained in aerated boxes filled with water from the sampling site. Gammarids were fed *ad libitum* with conditioned alder leaves which also served as shelter (Welton and Clarke 1980). The cultures were stored in a climate chamber at 13±1 °C and a day-night rhythm of 16:8 h.

3.3.2. Antiscalants, concentrate, and test-medium

Concentrates from an RO system containing one of the two ASs FreeFlow8 (Genesys® International, Middlewich, UK; FF8) and RPI-2100 (Toray Membrane Europe, Münchenstein, Switzerland; RPI) were tested in this investigation. For comparison, concentrate without AS (PURE) was tested following the same exposure protocol. Concentrates FF8 and PURE were derived from a pilot station which was run by the Technologiezentrum Wasser Karlsruhe (TZW) using the actual groundwater to be treated. The chemical composition of the concentrate is displayed in Table 3-1. Concentrate RPI was prepared by adding 15 mg of the AS to one liter PURE, which represents the maximal concentration of an AS in the concentrate (Lipp, P., personal communication). Seven concentrations per concentrate were tested, with the lowest four concentrations representing PECs at various outputs of the RO system, and various possible discharge scenarios of the Kürnach stream: 5, 13 (mean PEC), 24, 33 (“worst-case” PEC), 50, 75 and 100% (v/v). Control treatments did not contain concentrate. Water from the sampling site was used in the test as medium. Test-solutions were prepared by mixing concentrates and water in the appropriate ratios.

Table 3-1. Mean concentrations of main solutes in reverse osmosis concentrate derived from the pilot station (± standard deviation). The station was run with feed at a rate of 40 L/s and an antiscalant metering of 1.5 g/m³. The recovery rate was 80%.

Solute	Concentration (mg/L)
Ca^{2+}	1201 ± 80
Cl^{-}	407 ± 64
K^{+}	14.9 ± 2.1
Mg^{2+}	289 ± 15
Na^{2+}	232 ± 46
NH_4^{+}	0.05 ± 0.03
NO_2^{-}	0.03 ± 0.03
NO_3^{-}	118 ± 10
SO_4^{-}	2562 ± 242

3.3.3. Acute toxicity test

The acute toxicity tests were performed according to the USEPA Ecological Effects Test Guideline OPPTS 850.1020 Gammarid Acute Toxicity Test (1996e). The tests were conducted in a climate chamber under the same conditions as the culturing. Glass beakers (1 L) were filled with one liter of the test-solution and 20 green-colored glass-nuggets (d = 15 mm) as shelter. Ten gammarids were transferred into each beaker using five replicates per treatment for *G. pulex*. Due to the lower abundance of *G. roeseli* at the sampling site, the number of replicates was three, four and five for the tests with PURE, RPI, and FF8, respectively. However, minimum requirements concerning the number of test animals (20) and replicates (2) were met in all tests (USEPA 1996e). Beakers were randomly distributed in the climate chamber. Gammarids were not fed during the exposure. Beakers were checked every 24 h, and exuviae and dead animals were removed. Mortality rates were determined after 96 and 168 h. Gammarids were considered dead in the absence of a locomotion reaction following mechanical stimulation. Physico-chemical parameters were measured at the beginning of the test, after 96 and 168 h in all beakers of the control and the highest test concentration. Conductivity (control: 885–934 µS/cm; 100% (v/v) concentrate: 5250–5890 µS/cm), pH (7.3–8.2), and temperature (12.6–13.6 °C) were stable during the tests. However, O_2-saturation continuously declined (Table 3-2), but survival of gammarids was presumably not affected due to their ability to acclimate to low-oxygen conditions (Vobis 1973, Toman und Dall 1998) and as evident from the high survival rates in several treatments in this study.

Table 3-2. Range of mean O_2-saturation (%) in the acute toxicity tests with the three different concentrates at the beginning, and after 96 and 168 h. Measurements were conducted in the control treatment and the highest test concentration.

Treatment	**0h**	**96 h**	**168 h**
control	92–95	82–88	70–86
33% (v/v) concentrate[a]			28-58
50% (v/v) concentrate[a]		51–71	
100% (v/v) concentrate	90–93	77–86	74–76

[a] Additional measurements in tests with FF8 due to 100% mortality in the highest treatments at this time points.

3.3.4. Statistical analyses

Analyses were conducted using the software SciDAVis 0.2.4 and SPSS 20 (IBM Corporation, NY, USA). Data were checked for normal distribution using Kolmogorov-Smirnov tests, and Levene's tests were applied to check for homoscedasticity. If appropriate, EC_{50}-values were determined with probit-analyses. Additionally, NOECs were assessed with one-way ANOVAs or Kruskall-Wallis-tests followed by Dunnett's post-hoc tests and Mann-Whitney-U tests, respectively. Afterwards, the Holm-Bonferroni method was applied to correct for multiple testing.

3.4. Results and Discussion

The two *Gammarus* species were similarly affected by FF8. The NOEC was 24% (v/v) for both species after 96 and 168 h (Mann-Whitney-U: *G. pulex*: $p = 0.421$ (96 and 168 h); *G. roeseli*: $p = 0.345$ (96 h), $p = 0.155$ (168 h; Figure 3-1 A and B). In contrast, EC_{50}-values decreased with increasing exposition duration (Table 3-3). For both species, mortality rate of the control was less than 10% after 168 h.

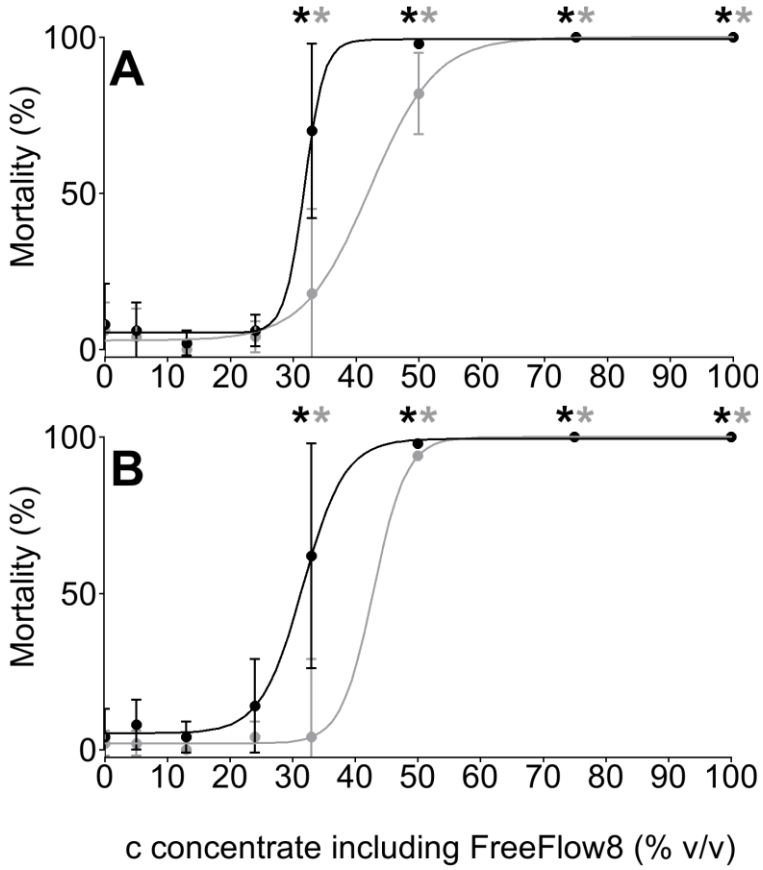

Figure 3-1. Mean mortality (± standard deviation) of *G. pulex* (A) and *G. roeseli* (B) exposed to FF8 after 96 (grey) and 168 h (black). Asterisks indicate significant differences to the control. Note: mortality rates did not increase in the concentration range up to 24% (v/v) representing PECs.

Concentrate RPI differently affected the two *Gammarus* species with *G. pulex* being the more sensitive species. For this species, the determined NOEC was 33% at both measuring time points (Kruskal-Wallis: $p < 0.001$ (96 and 168 h), Mann-Whitney-U: $p = 0.075$ (96 h), $p = 0.016$ (168 h) not significant according to Holm-Bonferroni method, Figure 3-2 A). The EC_{50}-value decreased between the two time points (Table

3-3). Mortality of *G. pulex* in the control did not exceed 10% after 96 h, and was less than 20% after 168 h.

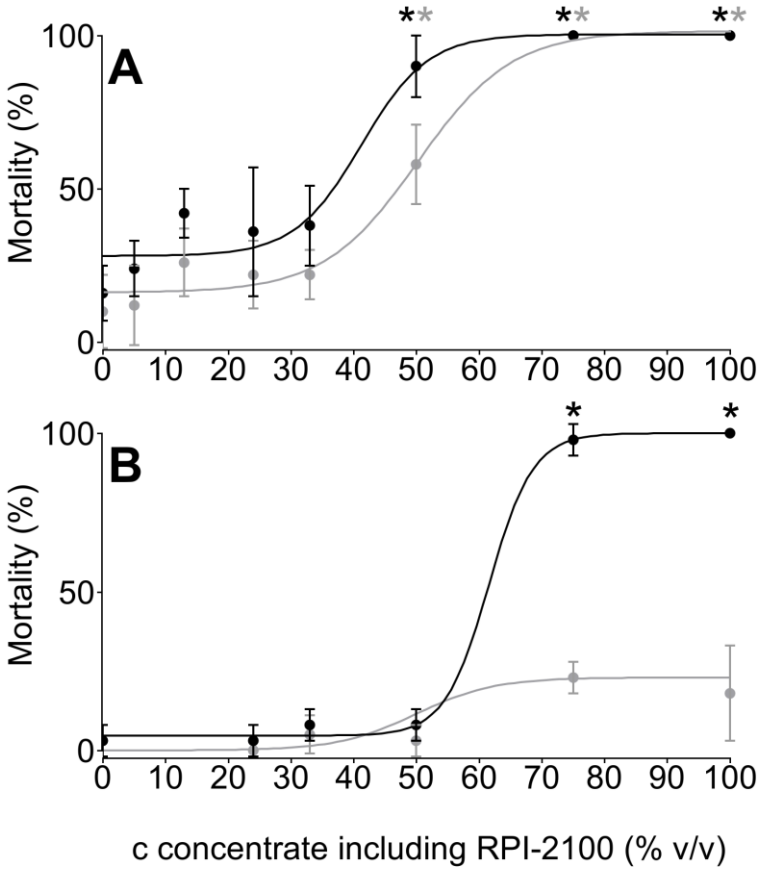

Figure 3-2. Mean mortality (± standard deviation) of *G. pulex* (A) and *G. roeseli* (B) exposed to RPI after 96 (grey) and 168 h (black). Asterisks indicate significant differences to the control. Note: mortality rates did not increase in the concentration range up to 33% (v/v) representing PECs.

In contrast, no significantly increased mortality was observed in *G. roeseli* in any test concentration after 96 h (Kruskal-Wallis: $p = 0.017$, Mann-Whitney-U: $p = 0.015$ (75%), not significant according to Holm-Bonferroni method, Figure 3-2 B). Though, mortality increased up to 100% in the two highest test concentrations until test termination (Kruskal-Wallis: $p = 0.002$, Mann-Whitney-U: $p = 0.015$ (75 and 100%)). No other test concentrations affected the survival of the gammarids. Due to the high survival, the EC_{50}-value was only determinable after 168 h (Table 3-3). Mean mortality rate of the control group remained below 10% throughout the test.

Table 3-3. EC_{50}-values determined by probit-analyses in the acute toxicity tests with FF8 and RPI, respectively, for *G. pulex* and *G. roeseli* after 96 and 168 h.

Test species	**Test concentrate**	**Time point (h)**	**EC_{50} (% (v/v))**	**95% confidence interval**
G. pulex	FF8	96	38.2	32.2–46.4
		168	28.6	23.4–35.7
	RPI	96	39.4	34.6–45.0
		168	26.6	22.7–30.6
G. roeseli	FF8	96	36.2	-
		168	29.3	25.1–34.5
	RPI	96	-	-
		168	58.0	-

Mean mortality of both gammarid species exposed to PURE was not increased compared to the control in any test concentration after 96 h (*G. pulex*: one-way ANOVA: $f_{(7,31)} = 1.391$, $p = 0.244$; *G. roeseli*: Kruskal-Wallis: $p = 0.161$, Figure 3-3 A and B). Though, mortality of *G. roeseli* was higher than in the controls by trend at concentrations above 24% (v/v). This tendency was also evident after 168 h, but no significant differences were observed (Kruskal-Wallis: $p = 0.024$, results of pairwise Mann-Whitney-U comparisons not significant according to Holm-Bonferroni method). However, mortality rate in the control exceeded the accepted value of 10% after 168 h. In contrast, mortality rates in *G. pulex* remained at low levels of about 10% in all treatments throughout the test with the exception of test concentration 75% after 168 h (one-way ANOVA: $f_{(7,31)} = 2.539$, $p = 0.035$, Dunnett-T (> control): $p = 0.008$).

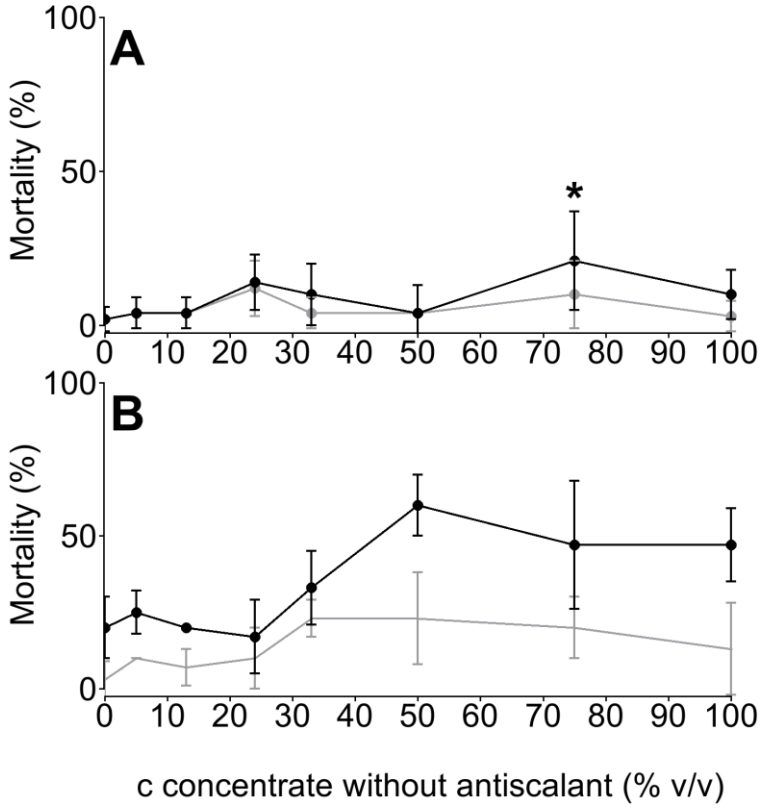

Figure 3-3. Mean mortality (± standard deviation) of *G. pulex* (A) and *G. roeseli* (B) exposed to PURE after 96 (grey) and 168 h (black). Asterisks indicate significant differences to the control.

Based on these results, the originally intended discharge of concentrate containing an AS is ecologically problematic at the intended quantities. Our results indicate toxic synergistic interdependencies between concentrate derived from RO and ASs. In both concentrates containing an AS, dose-dependent increased mortality rates up to 100% were, at least after 168 h, evident. These results are likely of great environmental relevance since the two amphipod species studied were the dominant invertebrate fauna of this and many other streams, with great importance for food web functioning. In contrast, the concentrate without AS did not impair survival in both gammarid species. This result contradicts the expectation that the AS formulations should not have any adverse effect, since they officially do not contain environmentally hazardous ingredients according to the Council Directive 67/548/EEC (Genesys® International 2007a and Toray Membrane Europe 2010a). Moreover, the ecotoxicological data report EC_{50}-values of > 1,100 mg/L (*O. mykiss*, 96 h) and > 1,040 mg/L (*D. magna*, 48 h) for FreeFlow8, and > 500 mg/L (*Leuciscus idus*, 96 h) for RPI-2100, greatly exceeding the concentrations of AS in our exposures. In our study, the determined

EC_{50}-values correspond to 4–8.7 mg/L AS. According to Soucek and Kennedy (2005) and Elphick et al. (2011), sulfate and chloride toxicity decreases with increasing water hardness. Hence, with the ASs binding to water hardening compounds, toxicity of these solutes in the concentrate might be increased. However, the application of an AS is inevitable for an economic use of a RO plant (Rautenbach and Melin 2003). With the "pure" concentrate being much less toxic, a treatment of the concentrate in order to remove the AS should be applied, if the concentrate is to be discharged into surface waters. In conclusion, the results of this study suggest that the effects of AS require more careful consideration in ERA and regulation.

4. Miniature circulatory System (MCS): A New Exposure System For Ecotoxicological Effect Assessments In Riverine Organisms

A similar version of this chapter was published as:

Feiner M, Beggel S, and Geist J (2016). Miniature Circulatory System (MCS): A new exposure system for ecotoxicological effects assessments in riverine organisms. *Environmental Toxicology and Chemistry* 35, 2827-2833.

4.1. Abstract

Long-term effect assessments in ecotoxicological investigations are important, yet there is a lack of suitable exposure systems for these experiments that can be used for riverine species. We developed a cost-efficient Miniature Circulatory System (MCS), which was evaluated for its applicability in long-term exposures in two stream-dwelling species: brown trout (*S. trutta*) and an amphipod (*G. roeseli*). In an egg-to-fry exposure of *S. trutta*, the toxicity of two RO concentrates was exemplarily investigated. Control hatching rate of yolk sac fry was 75 ± 7%, and thus complies with the OECD validity criterion (≥ 66%). RO concentrates did not impair the hatching rate in any tested concentration. In *G. roeseli*, mortality rates remained below 20% during a 21 d cultivation, fulfilling the common validity criterion in ecotoxicological tests. Mortality was significantly lower when the species was fed with conditioned alder leaves instead of an artificial shrimp food. Finally, a toxicity test on *G. roeseli*, using copper as test substance, revealed LC_{50} values of 156 µg/L after 96 h and 99 µg/L after 264 h, which is in line with literature findings using other accepted exposure units. In conclusion, the MCS provides a novel and cost-efficient exposure system for long-term investigations on riverine species that may also be applicable for other species of fishes and macroinvertebrates.

4.2. Introduction

The assessment of possible adverse impacts of anthropogenic introduced chemical substances in the environment is one of the main objectives in ecotoxicology. Legislative regulations require the assessment of the environmental risk emanating from the test-substances in terrestrial and aquatic systems, especially for chemical registration processes (European Union 2006) or marketing authorization of pharmaceuticals (European Communities 2001b). Thus, several biological test procedures have been developed, and guidelines (e.g. OECD, USEPA) were established for the investigation of acute and chronic effects on model-species of different trophic levels. In this process, the development of ecotoxicological tests is subject to trade-offs between cost, logistics, ecological relevance, reproducibility, and sensitivity (Breitholtz et al. 2006).

ERAs for aquatic systems in the European Union include investigations of acute and chronic effects with representatives of green algae, crustaceans, and fish (European Commission 2003). Despite this attempt for taxonomic representativeness, the common standard test organisms, used in ecotoxicological tests, are mostly from standing waters (e.g. *Daphnia* sp. and *D. rerio*). The use of these species has advantages such as simple, and thus cost-efficient cultivation under laboratory and test conditions. However, the extrapolation and generalization of laboratory results to the environmental situation becomes difficult, if test species from stream ecosystems are excluded. This is especially critical as organisms from diverse habitats might have fairly different and even inversed sensitivities to substances of concern (Deanovic et al. 2013). Along with the fact that there are species which are more sensitive than the standard test species (Barbour et al. 1996), as well as the likely chance of divergences in sensitivities between habitats, the current practice in identifying the 'most sensitive species' in a system needs to be questioned in view of the scarce number of standard toxicity tests considering riverine species. The application of assessment factors is a common approach to address the problem of the uncertainty of the data sets examined (Kooijman 1987). However, they are not based on scientific reasoning (Chapman et al. 1998) implicating the possibility of an underestimation of the environmental risks. Hence, there is an urgent demand for new methods in ecotoxicology in order to realistically estimate the hazards of chemical substances in freshwater ecosystems, especially as an ongoing decline in biodiversity is observed in all freshwater systems

(Dudgeon et al. 2006), out of which river ecosystems are probably most strongly affected (Geist 2011). Along with the overexploitation, severe transformations of hydromorphological parameters, and the introduction and spread of non-native species in rivers and streams, water pollution is one of the most important hazards to lotic freshwater systems (Geist 2011). Consequently, ERAs also need to consider typical riverine species that have important ecosystem functions. In particular, the presence or absence of keystone species in headwater areas is of great importance for stream reaches further downstream. These headwater habitats are typically inhabited by amphipods and salmonids. Amphipods such as *G. fossarum, G. pulex,* and *G. roeseli* are shredder organisms that are particularly important in terms of nutrient turnover, breakdown of organic matter (Anderson and Sedell 1979), and as fish prey in the aquatic food web (Maitland 1966, Andersen et al. 1992, Suter and Cormier 2014). Salmonids, such as species of the genus *Oncorhynchus* in North America and of the genus *Salmo* in Europe, are important due to their regulatory function as predators and habitat engineers (Geist et al. 2009). Moreover, they are also host species of endangered freshwater mussels (Taeubert et al. 2010). An approach for the inclusion of these species of concern in ERAs is the use of model ecosystems, which resemble natural water bodies, either lentic or lotic, and thus give the opportunity to increase the number of species in a test, including non-standard test organisms. These test systems allow a more environmentally realistic effect assessment, but are only required in higher tier assessments for certain substance classes (Breitholtz et al. 2006) and implicate disadvantages like reduced reproducibility (Connon et al. 2012) and high cost. Lab-scale test systems for the inclusion of stream-dwelling organisms in ecotoxicological investigations are very limited.

For several years, ecotoxicologists have been interested in using representatives of the amphipoda suborder gammaridea as an alternative in single-species tests with crustaceans (McCahon and Pascoe 1988, Gerhardt 2011). As a result of their ubiquitous distribution, high abundance, and continuous availability throughout the year, these species qualify as representative standard test organisms concerning flowing waters and are considered ecologically relevant test organisms (OECD 2005). Specific guidelines on amphipods as test species are already used by the USEPA, including *H. azteca* (USEPA 2000, USEPA 2002) and *Gammarus* spp. (USEPA 1996e), and a variety of studies on short-term effects assessments of different test substances on these species have been published (e.g. Phipps et al. 1995, Feiner et

al. 2015). However, investigations on long-term effects are scarce possibly due to difficulties in the cultivation of amphipods (Kunz et al. 2010), and a lack of standardized exposure systems.

Due to the trend in ecotoxicology to reduce experiments using (vertebrate) animals, as defined by law (at which all embryonal stages as well as yolk sac fry of fish are not considered vertebrates and thus excluded) (European Union 2010, Rufli and Springer 2011), acute fish toxicity tests with adult *D. rerio* (ISO 1996) were replaced by the acute toxicity test using eggs of the same species (ISO 2007) in wastewater surveys. However, a major point of criticism refers to the use of the rather robust warm water species *D. rerio* in assessing ecotoxicological impacts on cold water habitats that are inhabited by salmonid species. An OECD guideline on fish toxicity including both embryonic and sac-fry stages was established (OECD 1998), which does not replace the chronic fish early-life stage test, but is applicable for long-term effect assessments in slowly developing species of the ecologically relevant salmonid fish family. Due to the high water quality requirements of salmonid eggs, the breeding is linked to high investments, e.g. concerning the space needed for flow-through systems, thus often limiting the number of experimental units. In addition, the use of flow-through systems requires a great amount of water, which has to be treated before disposal in many cases driving up costs. However, the investigation of possible impacts on fish resulting from anthropogenic introduced substances in aquatic systems is inalienable, especially in the light of the European Water Framework Directive (Geist 2014). Consequently, salmonid egg exposure systems have already been established for bioindication in streams (Pander et al. 2009, Pander and Geist 2010), but these systems are not ideal for ecotoxicological tests in the laboratory.

The aim of the present study was to establish and validate a novel exposure system that is applicable in ecotoxicological investigations using riverine organisms. Therefore, the MCS was developed and its suitability for long-term investigations was exemplarily tested using an egg-to-fry exposure of the salmonid species *S. trutta* and an exposure of adult amphipods (*G. roeseli*). In the first experiment, a possible discharge of wastewater (concentrate), generated during drinking-water purification by RO, into a nearby stream was investigated. The main issue in raw water treatment resulting in this discharge product is the required reduction of sulfate in drinking water, which locally exceeds the maximum permissible value in water intended for human

consumption in the EU. The local fauna is dominated by the amphipods *G. pulex* and *G. roeseli*, as well as the brown trout *S. trutta* (Tombek 2012) providing an additional argument for the choice of the two test species in our study. Following acute toxicity tests with the two *Gammarus* species (Feiner et al. 2015), a chronic early-life stage test with *S. trutta* was conducted in the present study. Using the MCS, possible effects of the concentrate at different concentrations on the embryo development and yolk sac fry in *S. trutta* were assessed. In the second experiment, the feasibility of a long-term cultivation of the amphipod *G. roeseli* was evaluated. In order to optimize the culturing conditions, two different food types and light intensities, respectively, were taken into account. In both experiments, the suitability of the MCS was evaluated considering common validity criteria in standard ecotoxicological test guidelines. Finally, a toxicity test with *G. roeseli* was conducted to validate the suitability of the MCS for ecotoxicological investigations. Copper as Cu^{2+} was used as test substance, as data on copper toxicity to different *Gammarus* species with variable test durations already exist (e.g. Sroda and Cossu-Leguille 2011, Schmidlin et al. 2015) allowing a qualitative assessment of the results obtained by the novel exposure system by comparing the LC_{50} values.

4.3. Material and Methods

4.3.1. Miniature Circulatory System (MCS)

The MCS consists of two borosilicate glass beakers (1 and 5 L) placed in a plastic tray (l: 310 mm, w: 270 mm, h: 60 mm) with the small beaker being placed on a rack (h: 100 mm) made of stainless steel EN 1.4301. The beakers are positioned in a way that the water runs from the 5 L beaker into the 1 L beaker and alongside the 5 L beaker into the plastic tray (Figure 1). A water pump (flow rate: 150 L/h, Eheim compact 300, Eheim GmbH & Co. KG, Germany) inside the tray pumps the water via a silicone hose (ø: 10 mm, l: 500 mm) and a glass tube (ø: 10 mm, l: 200 mm) back into the 5 L beaker. The glass tube leads through the middle of an inset (ø: 140 mm, h: 50 mm) with an exchangeable perforated plate as base (both made of stainless steel EN 1.4301). Thus, a continuous water flow solely through the base-plate is generated in the inset. The base is covered with gauze (mesh size: 250 µm) from below to avoid the escape of small organisms. The system has a total water holding capacity of 9 L. A partial water renewal can easily be conducted by exchanging the small beaker with a new one containing new water. All parts made of glass and stainless steel as well as the plastic trays were thoroughly rinsed with deionized water before use. All other parts made of plastic were placed in deionized water for 24 h before use.

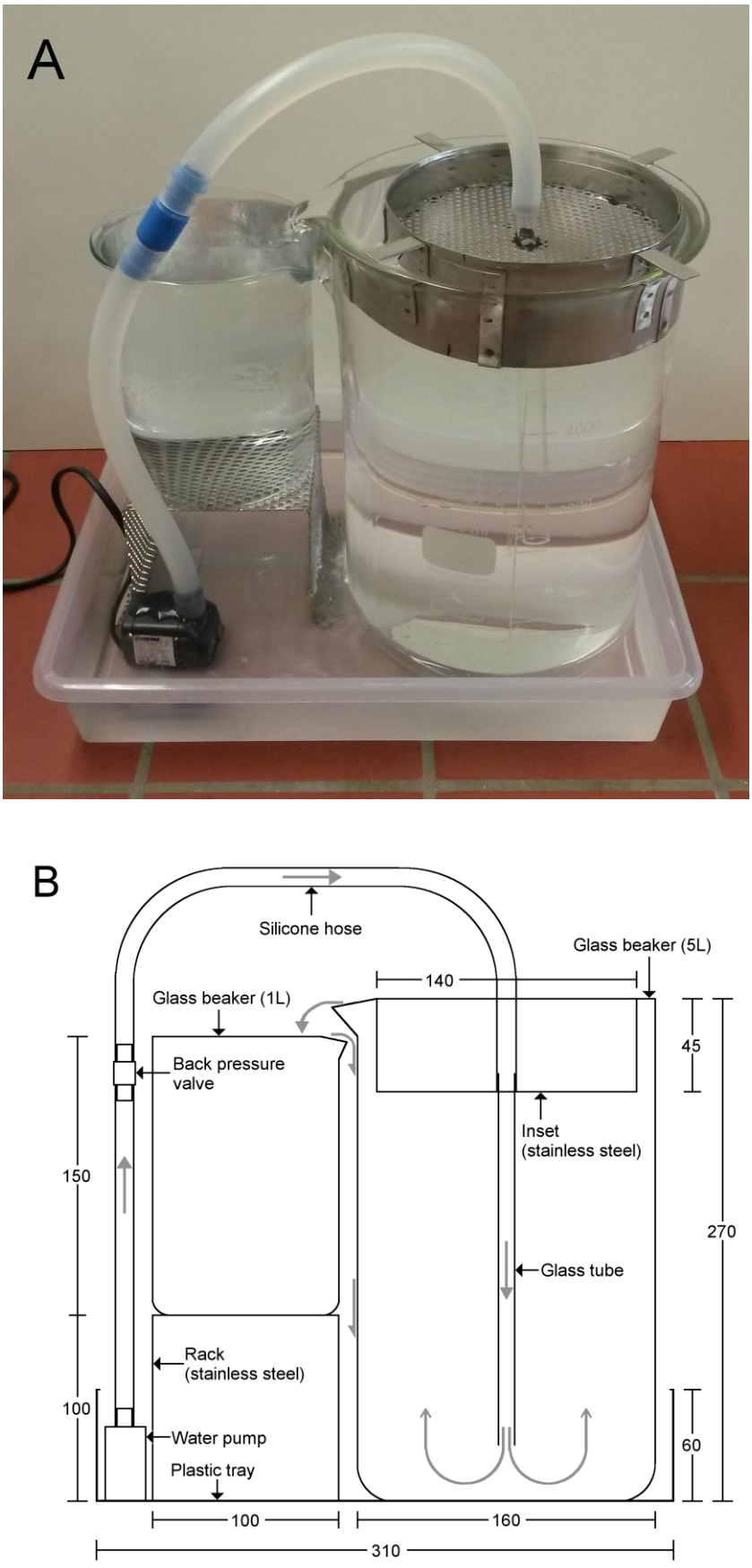

Figure 4-1. Photograph (A) and construction schematic (B) of the MCS. Grey arrows indicate the direction of flow. Indications of size are in mm.

4.3.2. Reverse osmosis concentrate

RO concentrates including 15 mg/L of the AS FreeFlow8 (Genesys® International; FF8) and without AS (PURE) were used in the salmonid egg-to-fry development test. Both RO concentrates were derived from a pilot plant. Concentrates are characterized by high amounts of sulphate (> 2500 mg/L) and a very high water hardness (> 230 dGH). For detailed information on source and chemical characterization of the concentrates see Chapter 3. Corresponding to previously conducted acute toxicity tests with the amphipods *G. pulex* and *G. roeseli* (Feiner et al. 2015), the test concentrations were 5, 10 and 24% (v/v) for FF8 and 5, 10, 24, 50, 75 and 100% (v/v) for PURE with the highest test concentrations representing NOECs. RO concentrates were mixed with water from the stream Kürnach (Würzburg, Germany) into which the effluent was to be released to generate the chosen test concentrations under a realistic exposure scenario. Water without RO concentrate from the stream Kürnach and the river Moosach (Freising, Germany), which corresponds to the water from the fish hatchery where *S. trutta* were held, were used as control treatments with the latter being used as a reference for egg development.

4.3.3. Salmonid egg development

MCSs were placed in a dark climate chamber which was set to 7 ± 1 °C. For each treatment, three systems were filled with the corresponding test water. Eggs of *S. trutta* (mixed eggs from four females fertilized with sperm from four males) were derived from a fish hatchery (Landesfischzuchtanstalt Mauka, Germany). Immediately after fertilization they were transported to the facilities of the Aquatic Systems Biology Unit. Each MCS was loaded with 50 eggs. Additionally, a batch of 3000 eggs was reared under common salmonid hatchery conditions (i.e. upflow incubation tray in a groundwater supplied flow-through system) as reference for egg quality and comparison with the MCSs. MCSs were checked daily and dead eggs and larvae, and egg membranes were removed to avoid development of mold. The same treatment was also performed for the upflow incubation trays. One liter of the test medium was renewed twice a week and evaporated water was substituted by deionized water once a week. Physicochemical parameters temperature, pH, oxygen saturation, dissolved oxygen, and conductivity were measured once after beginning of hatch to avoid disturbances of embryo development. The hatching rate was determined based on the

number of successfully hatched yolk sac larvae divided by the initial total number of eggs. The test was terminated 14 days post-hatch.

4.3.4. *Gammarus roeseli* test condition improvement

Specimen of *G. roeseli* were collected in the river Moosach (Freising, Germany) one week prior to the beginning of the test and immediately transported to the facilities of the Aquatic Systems Biology Unit. Both animal cultivation as well as the test condition improvement study were conducted in a climate chamber which was set to 10 ± 1 °C and a day:night rhythm of 16:8 h. Gammarids were cultivated in aerated aquaria filled with water from the sampling site. This natural water was also used in the test, which is an accepted method in standard test guidelines (USEPA 1996e, OECD 2004a and c). Previously conditioned black alder leaves (*A. glutinosa*) were provided *ad libitum* as food. Insets of the MCSs contained 20 green glass-nuggets (ø: 15 mm), which served as artificial substrate. Fifteen adult gammarids were transferred into each of 28 systems. To test for a possible influence of food type, three discs of alder leaves (40x40 mm, conditioned for one week) or shrimp food (one pellet per animal; shrimps natural, sera GmbH, Germany) was provided as food in each of 14 systems. Food items were renewed twice a week. To account for potential effects of light intensity on gammarid survival, half of the systems of the two different nutrition treatments were placed on shelves, which were covered from all sides with sheets, and the remaining were placed on uncovered shelves. Once a week evaporated water was substituted by deionized water, and 1 L of the water was renewed twice a week. Physicochemical parameters temperature, pH, oxygen saturation, dissolved oxygen, and conductivity were measured twice a week. The test ended after 21 days.

4.3.5. *Gammarus roeseli* copper toxicity test

Specimen of *G. roeseli* were collected and cultivated as described in section *Gammarus roeseli* test condition improvement. The test set-up was modified in that way that food (three discs of conditioned alder leaves) was exchanged once a week which was considered sufficient since leaf-material was still left after seven days. Four concentrations of Cu^{2+} were tested: 50, 100, 150, and 200 µg/L. Copper was applied as a solution based on deionized water. For this purpose, 392.9 mg $CuSO_4*5H_2O$

(Carl-Roth GmbH + Co. KG) were dissolved in deionized water to obtain a stock solution with a Cu^{2+} concentration of 100 mg/L. Each test concentration as well as the control without Cu^{2+} was replicated five times, and 15 adult gammarids were transferred into each MCS. Shelves were not covered, as no other works were done in the climate chamber at the same time. MCSs were checked for mortality every two to three days to minimize handling stress, and dead gammarids as well as exuviae were removed. Two times a week, 1 L of the test solution was substituted by new water followed by an application of the appropriate amount of the Cu^{2+} stock solution. Evaporated water was substituted by deionized water once a week. Copper concentration in the MCSs was measured every third day using the Spectroquant® Copper Test (Merck KGaA) and photoLab® S12 photometer (WTW). When the Cu^{2+} concentration was > 20% below the nominal concentration, the appropriate amount of the stock solution was added to restore the test concentrations. Physicochemical parameters temperature, pH, oxygen saturation, dissolved oxygen, and conductivity were measured twice a week. Additionally, 100 ml water samples were collected at the beginning of the test and subsequently every third day and stored at -20 °C before chemical analyses (anions: F^-, Cl^-, NO_2^-, Br^-, NO_3^-, PO_4^{3-}, and SO_4^{2-}, cations: Li^+, Na^+, NH_4^+, K^+, Mg^{2+}, and Ca^{2+}), which were conducted using ion chromatography (DIONEX ICS 1100, Thermo Scientific). For anion analysis an AS-22 column was used with a mixture of 4.5 mM Na_2CO_3 and 1.4 mM $NaHCO_3$ as eluent. Cations were separated using a CS-12 column and 20 mM CH_4O_3S as eluent. Flow rate was 0.3 mL/min. Only water samples from control treatment were analyzed, as the copper ions would have damaged the columns. The test ended after 21 days.

4.3.6. Statistical analyses

Statistical analyses were conducted using the software IBM SPSS 20. Kolmogorov-Smirnov tests and Levene's tests were used to test the data for normal distribution and homoscedasticity, respectively. Hatching success and survival of salmonid larvae were compared between treatments using a one-way ANOVA and the Kruskal-Wallis test, respectively. Effects of nutrition and light intensity interaction on the survival of *G. roeseli* were analyzed with a two-way ANOVA. Probit-analyses were used to determine LC_{50} values for Cu^{2+} toxicity after 96 h and 264 h. Differences in mortality per day (96 h, 264 h, and 21 d) between copper treatments and the control were tested

using Krukal-Wallis tests followed by pairwise Mann-Whitney-U tests with Bonferroni correction. Changes in ion concentrations between test beginning (day 0) and end (day 21) were checked using Wilcoxon signed-rank tests.

4.4. Results

4.4.1. Miniature circulatory system performance

MCSs properly worked for up to 67 days in the salmonid egg exposure experiment, when yolk-sac larvae were 14 days post-hatch, and the test was terminated. However, in four out of the 33 systems, a technical failure of the pump caused the leakage of the systems, resulting in a decrease in the number of replicates in four treatments. Consequently, a back pressure valve was inserted in the silicone hose which eliminated this problem. Water pumps caused an average increase of the water temperature by approximately 1.5 °C compared to the ambient temperature in the climate chamber of 7 and 10 °C in the *S. trutta* and *G. roeseli* experiment, respectively (Table 4-1). Thus, the MCSs should be started one day prior to the beginning of a test in a constant ambient environment, to generate constant values. Daily evaporation rate was 70 ± 12 ml (mean ± standard deviation), which can easily be substituted by deionized water at regular intervals.

Table 4-1. Physicochemical parameters in the MCS in the experiments with *S. trutta* and *G. roeseli*. Numbers are overall range (conductivity in *S. trutta* experiment) and overall means ± standard deviations.

Experiment	pH	Conductivity (µS/cm)	O_2-saturation (%)	Dissolved O_2 (mg/L)	Temperature (°C)
S. trutta	8.0 ± 0.3	922-5200[a]	94 ± 5	8.6 ± 0.4	8.6 ± 0.3
G. roeseli test condition	8.2 ± 0.3	1030 ± 51	95 ± 3	9.9 ± 1.1	11.5 ± 0.4
G. roeseli Cu^{2+} toxicity	8.4 ± 0.1	987 ± 21	92 ± 1	9.6 ± 0.1	11.5 ± 0.3

[a] High variations in conductivity between treatments are due to the high conductivity of the concentrate (5200 µS/cm). Within treatment variations were < 10%.

4.4.2. Salmonid egg development

Hatching rates in all concentrate-treatments and the controls were between 74 ± 6% (PURE 100%) and 85 ± 1% (PURE 5%; mean ± standard deviation, Figure 4-2). Thus, control hatching rate complied with the requirements of at least 66% of the Fish, Early-Life Stage Toxicity Test (OECD 2013a). There were no differences between treatments (one-way ANOVA: $f_{(1,18)} = 0.727$, $p = 0.691$). Egg developmental time was 419 ± 19 degree days (dd; overall mean ± standard deviation). Mean survival of yolk-sac larvae

was 100% in both control treatments and 97% to 100% in the concentrate treatments without statistically significant differences between treatments and the control (Kruskal-Wallis: $p = 0.508$). Hatching rate in the flow-through reference was 80%. Physicochemical parameters are displayed in Table 4-1.

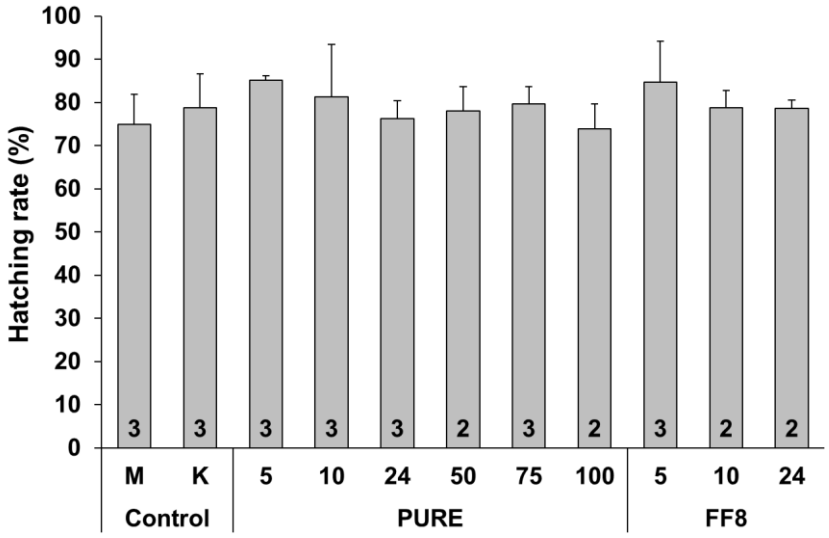

Figure 4-2. Mean hatching rate (± standard deviation) of *S. trutta* yolk-sac larvae exposed to RO concentrates PURE and FF8 (numbers indicate volume fractions of the concentrate) and in the control (M: water from river Moosach; K: water from stream Kürnach). Numbers on bars indicate number of replicates.

4.4.3. *Gammarus roeseli* test condition improvement

Survival of *G. roeseli* was significantly influenced by nutrition with higher survival rates of more than 90% in *A. glutinosa* treatments after 21 d (two-way ANOVA: $f_{(1,24)} = 4.6$, $p = 0.042$, Figure 4-3A). Differences in light intensity were marginal (5 and 10 lux) and did not influence survival of gammarids (two-way ANOVA: $f_{(1,24)} = 2.684$, $p = 0.114$). However, in both nutrition-treatments, gammarid survival was higher in covered systems by trend (Figure 4-3B). No interaction of the two tested factors was observed (two-way ANOVA: $f_{(1,24)} = 0.141$, $p = 0.710$, Figure 4-3). Physicochemical parameters were stable throughout the test (Table 4-1).

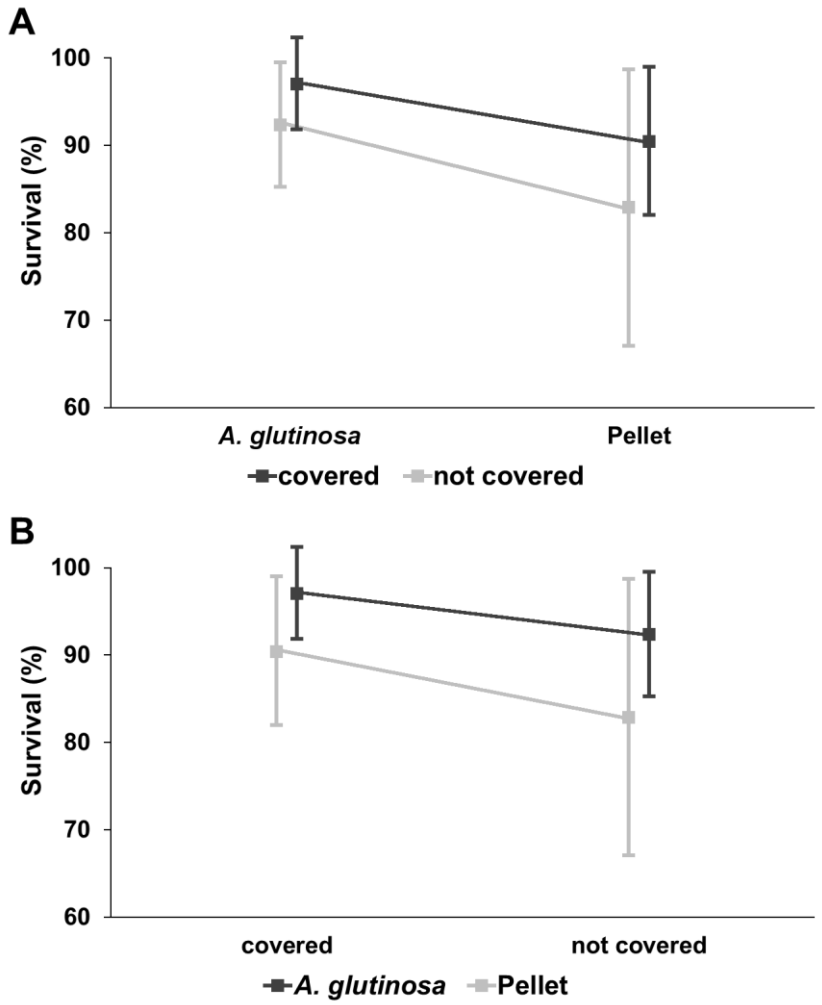

Figure 4-3. Mean survival (± standard deviation) of *G. roeseli* after 21 days in the MCSs as a function of (A) food type (conditioned leaves of *A. glutinosa* and artificial shrimp food pellets, shrimps natural, sera GmbH, Germany) and (B) covering of test systems. Nutrition significantly influenced the survival of gammarids. As evident from the near-parallel lines, no interaction of the two tested factors was observed.

4.4.4. *Gammarus roeseli* copper toxicity test

Copper analyses confirmed constant Cu^{2+} concentrations (i.e. less than 20% deviation from nominal concentrations) during the test (Table 4-2). Thus, results are presented in relation to nominal concentrations in the treatments.

Table 4-2. Concentration (c; µg/L) of Cu^{2+} ions in the 21 d *G. roeseli* toxicity test. Concentrations were measured eight times in all copper treatments during the test. Given are overall means ± standard deviations.

Nominal c	**50**	**100**	**150**	**200**
Measured c	47 ± 11	97 ± 7	143 ± 10	190 ± 8
Relative Deviation	6 %	3 %	5 %	5 %

In the Cu^{2+} toxicity test, LC_{50} values of 156 µg/L (96 h) and 99 µg/L (264 h) were determined. Gammarid mortality was significantly higher in copper treatments 100, 150, and 200 µg/L at each time point (Kruskal-Wallis: $p < 0.001$, Mann-Whitney-U: $p = 0.032$ (Figure 4-4); results are the same for the three treatments at the time points 96 h, 264 h, and 21 d). No differences in mortality between control and the lowest copper treatment (50 µg/L) were observed (Mann-Whitney-U: $p = 0.128$ (96 h), $p = 1$ (264 h), $p = 0.064$ (21 d)). From day 0 to day 21, a significant increase of chloride, sulfate and sodium-ions was observed, whereas calcium concentration significantly decreased (Wilcoxon signed-rank test: $p = 0.043$ (Cl^-, SO_4^{2-}, Na^+, and Ca^{2+}; Table 4-3). For nitrate, potassium and magnesium-ions no significant differences between days 0 and 21 were observed (Wilcoxon signed-rank test: $p = 0.223$ (NO_3^-), $p = 0.068$ (K^+), and $p = 0.498$ (Mg^{2+})). Changes in ion concentrations generally occurred during the first week and remained stable afterwards (Table 4-3). Anions F^-, NO_2^-, Br^-, and PO_4^{3-} as well as cations Li^+ and NH_4^+ were below detection limits. Physicochemical parameters were stable throughout the test (Table 4-1).

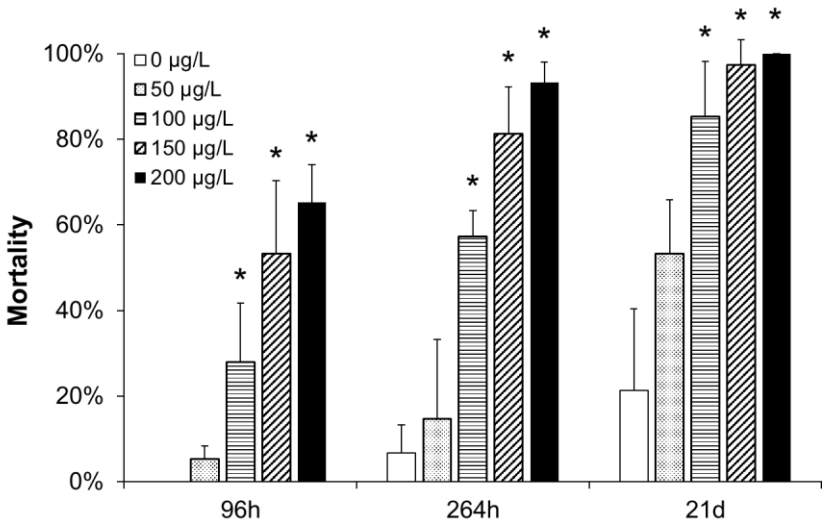

Figure 4-4. Mean mortality rates (± standard deviation) of *G. roeseli* exposed to 0, 50, 100, 150, and 200 µg/L Cu^{2+} after 96 h, 264 h, and 21 d. Asterisks indicate significant differences to the control (0 µg/L Cu^{2+}).

Table 4-3. Concentrations (mg/L) of anions and cations on days 0, 6, 13, and 21 in control treatments in the Cu^{2+} toxicity test with *G. roeseli*. Given are means ± standard deviations.

Day of test	**0**	**6**	**13**	**21**
Cl^-	117 ± 8	138 ± 6	139 ± 1	139 ± 2
NO_3^-	9.0 ± 0.8	9.5 ± 1.0	8.6 ± 1.3	7.3 ± 2.9
SO_4^{2-}	52 ± 3	65 ± 5	64 ± 3	68 ± 9
Ca^{2+}	48 ± 6	40 ± 1	34 ± 8	35 ± 4
K^+	7.0 ± 0.6	7.9 ± 0.3	7.0 ± 0.1	6.3 ± 0.2
Mg^{2+}	32 ± 3	34 ± 2	32 ± 1	31 ± 1
Na^+	82 ± 8	93 ± 5	89 ± 1	91 ± 3

4.5. Discussion

This present study presents a novel laboratory exposure system, to be used for investigations on organisms originating from flowing waters. The applicability of the MCS was positively validated in the long-term cultivation of *G. roeseli*, as well as in two ecotoxicological experiments with *S. trutta* eggs and adult *G. roeseli*, representing two ecologically relevant test organisms for stream and river ecosystems.

We demonstrated the suitability of the MCS for a successful salmonid egg development to meet the validity criterion concerning the hatching success (≥ 66%) of the OECD guidelines on chemical toxicity assessment with early-life stages of fish (OECD 1998, OECD 2013a). Control hatching rate in the MCSs complied with the hatching rate in the classical hatchery-based flow-through reference, indicating that the new system yields the same hatching rates. The system enables the assessment of impacts on the whole embryonal and early larval development of fish species (egg-to-fry) with little requirements concerning space, equipment, and amount of work. It provides the possibility to easily investigate long-term effects on sensitive life-stages using slowly developing fish species like salmonids. It is thus possible to obtain information about chronic effect concentrations regarding fish species, as requested in ERAs (European Commission 2003), while avoiding the use of older life-stages.

Neither of the two RO concentrates affected the hatching rate and the survival of yolk-sac larvae of *S. trutta* at any test concentration. This result supports our previous findings of the undiluted RO concentrate without AS not being toxic to aquatic organisms (Chapter 3). Furthermore, the concentrate including the AS did not impair the development of brown trout early life-stages even at the highest test concentration representing the short-term NOEC. This indicates the requirement of a minimum dilution by a factor of four, and thus the reduction of the AS concentration to 3.75 mg/L, to eliminate the concentrate toxicity.

In addition to the suitability for salmonid egg tests, the MCS also provides the possibility of a long-term culturing of aquatic invertebrates like *Gammarus* spp. for chronic toxicity effect assessments. In the test condition improvement study, gammarid mortality was below 20% in all treatments at test termination after 21 days which is a common validity criterion in ecotoxicological investigations (OECD 2006b). Accepted values for control mortality are even higher in tests with longer test durations (OECD 2004c, OECD 2013a). The present Cu^{2+} toxicity test revealed an LC_{50} value of 156 µg/L after 96 h

which is higher than the LC_{50} values ranging between 22 µg/L and 53 µg/L in the study of Sroda and Cossu-Leguille (2011). The finding of a lower sensitivity of gammarids to Cu^{2+} exposed in the MCS compared to the unspecified exposure systems used by Sroda and Cossu-Leguille (2011) could be explained by the better holding conditions in the novel MCS. However, in the present study, gammarids were fed during the test, which can obviously influence copper toxicity as demonstrated for *Gammarus fossarum* by Schmidlin et al. (2015). In their study, the determined LC_{50} value were 239 µg/L after 96 h when gammarids were not fed, and 304 µg/L after 264 h when gammarids were fed during the test. Hence, the determined LC_{50} values for 96 h and 264 h in the present study seem reasonable in consideration of both a lower tolerance to copper of *G. roeseli* compared to *G. fossarum* (LC_{50}(*G. roeseli*) < LC_{50}(*G. fossarum*)), and an increased tolerance to copper of gammarids when food is available (LC_{50}(+food) > LC_{50}(-food)). However, control mortality was slightly over 20% after 21 days, which can be attributed to a high mortality rate (47%) in one MCS of the control.

Although gammarids feed on a variety of food types (MacNeil et al. 1997), the positive effect of providing conditioned alder leaves for *G. roeseli* as food was clearly evident with mortality rates below 10% in the test condition improvement study. These observations are in line with previous studies, which demonstrated the importance of microbial and especially fungal colonization of leaf litter in the diet of gammarids (Bärlocher and Kendrick 1973, Sutcliffe et al. 1981). Though the use of conditioned leaves involves disadvantages like reduced standardization and an increased amount of work, this natural food source should be considered in investigations with gammarids. Furthermore, we observed the tendency to a lower survival rate in uncovered test systems. As gammarids are negatively phototactic (Bethel and Holmes 1973, Bakker et al. 1997), higher light intensities may increase stress levels in turn resulting in higher mortality rates. Differences in light intensity were negligible in our study and not likely to affect gammarid survival. However, we observed flight behavior of gammarids when approaching the cultures (i.e. simultaneous swimming of a multitude of gammarids away from the approaching person) indicating an influence of the surrounding activities. We therefore recommend the covering of test systems in future investigations with *Gammarus* spp. to minimize disturbance by experimenters in the surrounding of the test systems at the same time.

In conclusion, long-term, chronic exposures of organisms to low contaminant concentrations are still regarded as a major challenge to be evaluated in ecotoxicology (Eggen et al. 2004). The MCSs provide a novel and promising exposure system for ecotoxicological tests in stream-dwelling species such as salmonids and gammarids, which are currently underrepresented in such studies. The same exposure system can also be used in other species (fishes, macroinvertebrates) as well as for other purposes such as testing of gene-environment interaction or standardized ecologically relevant experiments.

5. General Discussion

In this thesis, different measures to enhance the ecological relevance of ecotoxicological investigations were evaluated. As evident from the above case studies, the need to incorporate more realistic exposure scenarios in ERAs was clearly demonstrated. This is on the one hand the choice of appropriate test durations in chronic effect assessments, as demonstrated in the *P. antipodarum* reproduction assay with the effects only occurring after 56 days (Chapter 2). The study illustrates the necessity for sufficiently long test durations to adequately investigate chronic, sublethal effects particularly in (trace-) substances that are continuously released into the environment. Furthermore, in ecotoxicological tests with specific chemical substances, especially additives like ASs, it is essential to investigate the substances with regard to the circumstances such as they enter the environment, i.e. in the context of their application, as effects might be seriously modified. This was demonstrated in the RO concentrate investigation with toxic effects occurring in concentrates containing ASs (at concentrations far below their respective single-substance EC_{50}) reflecting the real condition at which ASs might enter the aquatic environment, but not in concentrate without AS (Chapter 3). Finally, the Miniature Circulatory System was developed as long-term exposure system for future incorporation of current non-standard stream-dwelling test species (Chapter 4). The system was successfully applied in an egg-to-fry test with *S. trutta* as well as in a 21 d toxicity test with *G. roeseli*, and common validity criteria regarding hatching rate and survival in control treatments were met.

5.1. Appropriate test durations in long-term investigations

The presented study on effects of environmentally relevant concentrations of the beta-blocker sotalol on the reproduction of *P. antipodarum* clearly demonstrated the need for long-term investigations and appropriate test durations in effect assessments (Chapter 2). As in the example of sotalol, an influence on the reproduction of *P. antipodarum* occurred at the lowest test concentrations, but not until the 56th day of exposure. This is especially important as in the meanwhile established OECD guideline on the *P. antipodarum* reproduction test from July 2016 (OECD 2016a), the test duration was shortened to 28 days compared to the Detailed review paper on molluscs life-cycle toxicity testing (OECD 2010b), which was used in the present study. Thus, in the case of sotalol, the influence on the reproduction of *P. antipodarum* would have been overseen with a shorter test duration.

Investigations of long-term effects at low substance concentration must occupy a higher position in future ERAs. Due to environmental protection managements, like the application of recycling processes or improvements in wastewater treatment technologies, the chemical pollution in (freshwater) ecosystems decreases, but still a multitude of substances at low concentrations is present (Eggen et al. 2004, Daughton and Ternes 1999, Deblonde et al. 2011). Thus, the organisms in receiving waters are continuously exposed to these substances at low concentrations, and acute, short-term effect assessments do not adequately reflect the actual circumstances (Eggen et al. 2004). Moreover, short-term investigations of those trace-substances are inappropriate as extrapolating from acute to chronic toxicity might not be possible in view of different modes of action in acute lethal and chronic sublethal effects (Eggen et al. 2004, Fent 2007), and high variabilities in substances' modes of action even within chemical classes (Calow and Forbes 2003). The latter can be exemplified on the class of beta-blockers, which bind either selectively or non-selectively to different types of beta-adrenergic receptors (β1, β2 and β3) and act as antagonist, but can also stimulate the receptors (reviewed in Frishman and Saunders 2011). The application of assessment factors is the usual approach to address this problem of extrapolating short-term test results to real conditions (Kooijman 1987). However, assessment factors are not based on scientific knowledge but chosen rather arbitrarily (Chapman et al. 1998). On the example of the beta-blocker sotalol, the present study demonstrated the possibility of assessment factors being insufficient to adequately determine PNECs. Based on the chronic *D. magna* 21 d reproduction test

(NOEC = 0.1 mg/L, Hernando et al. 2004), the derived PNEC is 1 µg/L. However, in the present study, effects on the reproduction of *P. antipodarum* were observed at a concentration equal to the PNEC and even at a concentration twenty times lower than the PNEC (0.05 µg/L; Chapter 2). Moreover, a sufficiently long test duration in the effects assessments of substances at low concentrations in invertebrates is crucial, as delayed appearing impacts are likely to occur. Invertebrates have low Cytochrome P450 contents, which are amongst the most important enzyme classes in detoxification processes (Snyder 2000). Thus, metabolism- and excretion-rates are restricted, and contaminants accumulate in the organism (Fent 2007). This even more applies to poikilothermic species that inhabit cold water habitats, as physiological processes depend on the ambient temperature (Heugens et al. 2001).

5.2. Conditions of substances entering the environment

In the case study on RO concentrate toxicity, synergistic interdependencies between concentrate and AS were illustrated (Chapter 3). RO concentrates without an AS turned out not to be toxic to the amphipods *G. pulex* and *G. roeseli*. Likewise, ASs are rated as not being harmful to the environment, as they officially do not contain environmentally hazardous ingredients (Genesys® International 2007a and Toray Membrane Europe 2010a). However, the present study revealed toxic effects of concentrates that contained ASs, which might have been overseen in an assessment considering the "single substances" (i.e. AS and concentrate) individually. AS concentrations in the concentrates at the determined EC_{50} values in the present study were at least 84 times lower than respective EC_{50} values of the ASs tested individually. Multiple stressors can generate interdependent effects (additive, synergistic or antagonistic; Crain et al. 2008) resulting in different impacts of substance mixtures compared to the single substances (e.g. Pape-Lindstrom and Lydy 1997, Silva et al. 2002, Eggen et al. 2004). Furthermore, abiotic parameters like pH and the chemical composition of the water can influence substance toxicity (e.g. Bryant et al. 1985, Naddy et al. 2002). However, chemical substances are commonly evaluated regardless of their application and actual form, in which they enter the environment. Especially in substances like additives, which are applied to exhibit a specific effect, e.g. ASs to prevent clogging of RO membranes by interacting with scale forming components in the water (e.g. Darton 2000), both the chemical structure of the substances itself as well as the chemical composition of the water is altered (Ang et al. 2016). Resulting impacts on the biota due to direct effects might be different in comparison to the raw material of the substance in consequence of a changed molecule structure, and thus a possible shift in the mode of action. On the other hand, organisms could be indirectly affected as a result of stress due to an altered environment (Fischer et al. 2013). As in the example of ASs, these substances not only interact and bind to scale forming ions like Ca^{2+} (Darton 2000), but also form complexes with other metal-ions like Fe^{2+} and Al^{3+} (Dalvi et al. 1999, Shih et al. 2006). Thus, ASs might also influence the bioavailability of respective ions, which is especially significant in a lowered bioavailability of essential trace elements. As a consequence, the affected organisms might be forced to operate near or over the edges of their ecological niche (Van Straalen 2003). Though organisms are able to cope with the altered condition, at least for some time (Van Straalen 2003), they might be more

sensitive to further unfavorable conditions as a result of the compensating processes, and thus, higher energetic costs (Fischer et al. 2013). These indirect effects cannot adequately be investigated in the use of standardized artificial freshwater, which only resembles water parameters for a limited proportion of aquatic systems and disregards regional differences. Investigating substances at their original quality might be inappropriate, if their application involves changes both in the substance's chemical structure and the water chemistry.

5.3. New exposure systems for non-standard stream-dwelling species

The investigation of substance effects on stream-dwelling organisms is neglected in current standard ecotoxicological tests as evident from the small amount of species considered in test protocols from ISO, OECD, and USEPA (Table 1-1). Only five stream-dwelling species are included in 29% of the guidelines, with two thirds of these only dealing with effects on the two species *Chironomus* sp. and *O. mykiss*. In order to enhance the percentage of riverine species in standard investigations, the Miniature Circulatory System (MCS) was developed and successfully applied in an egg-to-fry investigation with the brown trout *S. trutta* and also validated for further aquatic organisms on the example of the amphipod *G. roeseli* (Chapter 4). The MCS is a cost effective, small-scale, semi-static system which generates a continuous water flow in the exposure chamber making it a promising tool for future long-term investigations using non-standard stream-dwelling organisms. The underrepresentation of these species in ecotoxicological investigations is possibly due to difficulties in long-term cultivation (e.g. Kunz et al. 2010). The MCS is capable to generate appropriate basic conditions for long-term maintenance of these species in terms of constant physicochemical water properties like pH and high dissolved oxygen content (Table 4-1). As evident from the high hatching rate (up to 85%) of larvae of *S. trutta*, the MCS is actually suitable for species with high requirements to water quality for a successful development. Moreover, the case study on copper toxicity to *G. roeseli* revealed short-term and prolonged EC_{50} values for gammarid mortality, which are in line with further studies using other accepted exposure units (Sroda and Cossu-Leguille 2011, Schmidlin et al. 2015), validating the usability of the MCS in ecotoxicological investigations. Thus, the MCS can contribute to improve data sets used in ERAs by integrating test species that are representative for both the aquatic systems of concern, especially flowing waters, and for the major and ecologically most important proportion of the respective taxonomic group (Breitholtz et al. 2006). This is especially important in view of the utilization of SSDs, which are rated to generate a more stable basis for ERAs compared to the assessment factor approach, provided that data are representative in terms of species and endpoints (Calow and Forbes 2003). Though the MCS was successfully validated for macroinvertebrates, it is not suitable for microorganisms, which are able to escape through the gauze. Also, the use of the system for organisms that inhabit the hyporheic interstitial, i.e. the functioning of the MCS, when the exposure chamber is loaded with natural sediment, is not investigated

yet. Further exposure systems are needed to include these species. In conclusion, the MSC is a promising exposure system for the investigation of a variety of species, both fish and macroinvertebrates, and endpoints including behavioral and physiological responses as well as the identification of underlying molecular mechanisms. Thereby, it can significantly contribute to decrease the problem of biased data-sets used in SSDs (Forbes and Calow 2002), which are the result of both limited data available and common standard test species that are used due to historical establishment (Breitholtz et al. 2006).

6. OUTLOOK

Standardized ecotoxicological tests are essential in generating reproducible and comparable information on substance toxicity. However, current standards should be critically discussed and improved in view of the incorporation of more realistic test settings. Exposure scenarios should be adapted to the test substances with regard to (1) the type of their release, i.e. temporary or continuous, and to (2) their application and chemical structure at which they enter the environment (Chapter 2 and 3). Thus, test duration and form at which the test substance is applied have to be appropriately chosen.

The utilization of assessment factors for the determination of PNECs must be critically discussed, as they might not be sufficient for the protection of the environment despite the common opinion of being a conservative approach. Species Sensitivity Distributions are capable of more accurately predicting the environmental hazard provided that the underlying data set is representative. Therefore, the incorporation of further test species in ERAs should be promoted in consideration of species from flowing waters to expand knowledge on chemical effects in general, but particularly in rivers, which are severely threatened by an ongoing decline in biodiversity. The MSC creates the opportunity to develop new standard tests on relevant test species including both macroinvertebrates and fish (Chapter 4). However, further investigations on optimizing the basic conditions for novel test species are crucial. At the same time, local differences should be taken into account in view to differences in abiotic parameters, which can strongly influence substance toxicities. Moreover, the common approach of using specimen from unpolluted sites for ecotoxicological tests disregards the possibility of a differing sensitivity of the local population of a test species, which can be either lower or higher compared to unstressed organisms. Especially in locally limited emissions, the use of relevant organisms from the affected system as well as the water might be a measure to more precisely assess the environmental risk. Comparative investigations are needed to verify sensitivity differences amongst different populations.

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8. List Of Publications Included In This Thesis

Feiner M, Laforsch C, Letzel T, and Geist J (2014). Sublethal effects of the beta-blocker sotalol at environmentally relevant concentrations on the New Zealand mudsnail *Potamopyrgus antipodarum*. *Environmental Toxicology and Chemistry* 33, 2510-2515.

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9. ACKNOWLEDGEMENT

First of all I want to thank my supervisor and head of the Aquatic Systems Biology Unit, Prof. Dr. Jürgen Geist, for his continuous support and valuable advices, and for giving me the freedom to realize this work in my own way.

I also want to thank the committee, Prof. Dr. Jürgen Geist, Prof. Dr. Christian Laforsch, and Prof. Dr. Johannes Kollmann, for the evaluation of this thesis.

I am grateful for the scholarship of the UniversitätBayern e.V. I want to thank the Trinkwassergewinnung Würzburg GmbH for financial support, and namely Dr. Nadine Jäger and Alfred Lanfervoß for the good collaboration.

I want to thank Dr. Thomas Ternes and Andrea Boltner from the Chair of Urban Water Systems Engineering for the chemical analyses of sotalol.

I also want to thank the Landesfischzuchtanstalt Mauka for providing salmonid eggs.

Thank you to all my present and former colleagues Dr. Sebastian Beggel, Dr. Ursula Dawo, Christine Seidel, Jörg Steinhilber, Dr. Katharina Sternecker, and Dr. Katharina Stöckl for your help, support and all the good times.

Finally, I want to thank my parents Helga and Erich and my sister Nina for their enduring support in every way.