# Der Einfluss von Arzneistoffen auf aquatische Invertebraten

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# INHALTSVERZEICHNIS

**Kapitel 5**

Response of benthic macroinvertebrates to micropollutants released by sewage treatment plants.

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**Kapitel 6**

# ALLGEMEINE ZUSAMMENFASSUNG

Seit einigen Jahren ist bekannt, dass Arzneimittel, die vorwiegend über den Abwasserpfad in die Gewässer eingetragen werden, aufgrund ihrer extremen Persistenz und hohen biologischen Aktivität ökotoxikologische Effekte auf Nicht-Zielorganismen hervorrufen können. Allerdings sind die Langzeiteffekte vieler Arzneimittel sowie die Auswirkungen von Stoffgemischen, insbesondere in umweltrelevanten Konzentrationen, auf aquatische Organismen noch weitgehend unbekannt. Unter Berücksichtigung dieser Aspekte wurde im Rahmen dieser Dissertation der Einfluss von Arzneistoffen auf aquatische Invertebraten untersucht. Dabei gewonnene Erkenntnisse könnten zur Überarbeitung von standardisierten Toxizitätstestverfahren herangezogen werden und somit die Managementstrategien zum Erhalt der Umwelt verbessern.

Anhand ihres ubiquitären Vorkommens und ihres erhöhten Umweltgefährdungspotentials wurden für alle Laborversuche das Antiepileptikum Carbamazepin, das Antirheumatikum Diclofenac, das synthetische Estrogen 17α-Ethinylestradiol sowie das Antihypertonikum Metoprolol ausgewählt und in umweltrelevanten Konzentrationen als Wirkstoffgemisch, teilweise auch als Einzelstoffe getestet.

Um die chronische Toxizität der Arzneistoffe auf den Wasserfloh *Daphnia magna*, einem für ökotoxikologische Studien häufig verwendeten Modelorganismus, zu ermitteln, wurde ein Lebenszyklusexperiment durchgeführt (Kapitel 2). Dabei wurden neben den in ökotoxikologischen Standardtests üblichen Parametern auch zusätzliche life-history und morphologische Merkmale erfasst. Während die Einzelstoffe keine erkennbaren Auswirkungen auf die Organismen hatten, zeigte sich eine erhöhte Toxizität der Arzneistoffmixtur, die sich vorwiegend in einer Schädigung der Nachkommen der exponierten Daphnien äußerte. So waren die neonaten Daphnien kleiner als die Kontrolltiere und wiesen zudem ein verändertes Geschlechterverhältnis sowie morphologische Deformationen auf. Aufgrund der bedeutenden Stellung von *Daphnia* in aquatischen Habitaten können solche Veränderungen zu erheblichen ökologischen Konsequenzen führen. Da all diese Effekte jedoch in Standardtests in Folge der eingeschränkten Anzahl an zu untersuchenden Parametern übersehen worden wären, scheint eine Überarbeitung der geltenden internationalen Richtlinien dringend erforderlich.

In einem Multigenerationsexperiment wurde der Einfluss der Arzneimittel auf *Daphnia magna* über sechs Generationen hinweg betrachtet (Kapitel 3). Dabei wirkten sich die

Arzneistoffe auf life-history und morphologische Parameter in der ersten exponierten Generation aus, in den darauf folgenden Generationen kam es jedoch zu einer Akklimatisierung an die Wirkstoffe. Erst in späteren Generationen traten erneut Arzneistoffeffekte bei den Daphnien auf. Daraus lässt sich schließen, dass aquatische Organismen wahrscheinlich aufgrund zu hoher Fitnesskosten keine dauerhafte Resistenz gegenüber den in die Gewässer eingetragenen Arzneistoffen entwickeln können. Eine verstärkte Toxizität der Arzneistoffmixtur gegenüber den Einzelsubstanzen konnte in diesem Experiment nicht festgestellt werden.

In einem Langzeitexperiment wurden die Auswirkungen des Arzneistoffgemisches auf das Wachstum, die Reproduktion sowie auf die Physiologie des Bachflohkrebses *Gammarus fossarum*, einem Modelorganismus in Fließgewässern, untersucht (Kapitel 4). Es zeigte sich, dass die Arzneistoffmixtur die Häutung der Gammariden beeinflusste, wobei es zu einem unregelmäßigen Größenwachstum und einer verkürzten Zeit zwischen den Häutungen kam. Bei einer permanenten Arzneimittelexposition in natürlichen Gewässern können die Gammariden in Folge des diskontinuierlichen Größenzuwachses kleiner werden, was enorme Fitnessnachteile verursachen kann. Auf die Reproduktion und Physiologie wirkte sich das Arzneimittelgemisch jedoch nicht aus.

Des Weiteren wurde in einem großräumig angelegten Freilandexperiment die Gemeinschaft der benthischen Makroinvertebraten oberhalb und unterhalb von Kläranlageneinleitungen untersucht, um mögliche Zusammenhänge zwischen der Verteilung der Invertebraten und der eingeleiteten Schadstoffe wie beispielsweise Arzneimittel zu detektieren (Kapitel 5). Die Zusammensetzung der Makroinvertebraten sowie der Artenreichtum und die Artendiversität unterschieden sich oberhalb und unterhalb der Kläranlageneinleitungen deutlich. So war der Anteil an sensitiven Arten unterhalb der Kläranlagen verringert, während dort tolerante Taxa in höheren Dichten auftraten. Da dies jedoch nicht auf den Einfluss verschiedener Umweltparameter zurückgeführt werden konnte, scheinen mit dem Abwasser eingeleitete anthropogene Schadstoffe die Invertebratengesellschaft unterhalb der Kläranlagen-einleitungen zu beeinflussen. Durch die permanente Verschmutzung werden die Gewässer letztendlich von wenigen toleranten Taxa dominiert, was eine globale Abnahme der Diversität zur Folge haben könnte.

## GENERAL ABSTRACT

In recent years, it became evident that drugs entering surface waters primarily via sewage effluents are potentially hazardous to non-target organisms due to their high persistence and biological activity. However, little is known about the long-term effects and the impact of complex drug mixtures, especially in environmentally realistic concentrations. Considering these aspects, I assessed the influence of pharmaceuticals on aquatic invertebrates. This may lead to a revision of standardised toxicity tests and foster the improvement of environmental management strategies in future.

I selected the antiepileptic agent carbamazepine, the non-steroidal anti-inflammatory drug diclofenac, the estrogen 17α-ethinylestradiol and the β-blocker metoprolol for the laboratory experiments, since these drugs occur ubiquitary in the aquatic environment and pose potential environmental risks. Pharmaceuticals were tested in environmentally realistic concentrations as drug mixture and partly also as single substances.

To assess the chronic toxicity of the pharmaceuticals, I performed a life-cycle experiment with the waterflea *Daphnia magna*, a model organism in ecotoxicology (Chapter 2). I investigated the impact of drugs on life-history traits recommended by current international guidelines and on additional parameters not controlled for in these standardised tests. The drug mixture led to decreased body sizes in the offspring of the exposed daphnids and to an increased proportion of males and non-viable neonates, whereas the single substances did not affect the organisms. Such life history changes may alter community or ecosystem-level responses because of the key role of *Daphnia* in aquatic habitats. Moreover, given that all effects seen in this study would not have been recognized in standardised tests, a revision of the current international guidelines is urgently required.

In a multigenerational experiment, I examined the influence of the drugs on life-history and morphological parameters of *Daphnia magna* over six generations (Chapter 3). Detectable effects of the pharmaceuticals occured in the first observed generation, followed by an acclimation period and a recurrence of drug effects in later generations. Hence, I anticipate that aquatic organisms may not evolve a resistance to pharmaceuticals in natural aquatic systems, presumably due to high fitness costs. However, the mixture of pharmaceuticals did not provoke stronger effects on the exposed daphnids than the single substances.

In a long-term experiment, I investigated the impact of the drug mixture on moulting, reproduction and physiology of the amphipod *Gammarus fossarum*, a model organism in

running waters (Chapter 4). I could show that the pharmaceutical mixture caused a discontinuous increase of body length and a reduced time between successive moults because of shortened intermoult periods. Due to the permanent drug exposure in natural aquatic systems, gammarids may become smaller leading to disadvantages in the fitness of the animals. Reproduction and physiology of the gammarids were not affected by the drug mixture.

A large-scale field study was performed to investigate the impact of anthropogenic contaminants released by sewage treatment plants on the benthic macroinvertebrate assemblages (Chapter 5). The community structure, species richness and species diversity of the invertebrates varied upstream and downstream of the sewage effluents. Specifically, the abundance of sensitive taxa was reduced below the effluents, whereas tolerant taxa were more abundant. Since environmental parameters could not explain the observed distribution of the benthic macroinvertebrates, I assumed that discharged anthropogenic contaminants such as pharmaceuticals may affect the invertebrates below the sewage treatment plants. As a consequence of the increasing pollution, the aquatic ecosystems may become dominated by tolerant taxa, resulting in a global decline of biodiversity.

# Einleitung

1

**Gewässerbelastung durch Arzneistoffe**

Obwohl nur ein sehr geringer Anteil der Erdoberfläche mit Süßwasser bedeckt ist, haben aquatische Ökosysteme wie Flüsse und Seen eine essentielle Bedeutung für die Menschheit. Sie sichern nicht nur unsere Trinkwasserversorgung, sondern liefern auch einen wichtigen Beitrag zu unserer Ernährung, insbesondere in Form von Fisch. Zudem wird Süßwasser zur Bewässerung von Agrarflächen, zur Erzeugung von Wasserkraft und für industrielle Prozesse genutzt (Johnson et al. 2001, Brönmark und Hansson 2002).
Da sich der anthropogene Einfluss auf die aquatische Umwelt in den letzten Jahrzehnten aufgrund des starken Bevölkerungswachstums drastisch erhöhte, verschlechterte sich der ökologische Zustand unserer Gewässer. So stellt beispielsweise die Belastung von Flüssen und Seen mit Schadstoffen eine nicht zu vernachlässigende Gefahr für die aquatischen Ökosysteme dar. Während der Eintrag von Pestiziden und Schwermetallen in unsere Umwelt in den letzten Jahren leicht zurückgegangen ist (Brönmark und Hansson 2002), wurden in den aquatischen Systemen vermehrt Rückstände von Arzneistoffen detektiert (Ternes 1998, Heberer 2002, Kolpin et al. 2002).

Der Eintrag von Arzneimitteln (Abb. 1) erfolgt hauptsächlich über den Abwasserpfad, da meist ein erheblicher Anteil der verabreichten Stoffmenge vom Körper in unveränderter oder metabolisierter Form ausgeschieden wird. Aufgrund ihrer hohen Polarität und der damit verbundenen guten Wasserlöslichkeit, adsorbieren die Arzneimittel kaum an den Klärschlamm und können somit in den Kläranlagen bisher nur unzureichend abgebaut werden. So zeigt beispielsweise das Antiepileptikum Carbamazepin nach der Kläranlagenpassage eine Eliminierungsrate von nur 7 % (Ternes 1998). Demzufolge wird ein Großteil der Arzneistoffe mit dem gereinigten Abwasser in die Oberflächengewässer eingeleitet. Hinzu kommt, dass abgelaufene Medikamente oftmals nicht sachgerecht über die Kanalisation beseitigt werden. Aber auch die Entsorgung über den Hausmüll kann zu einem Eintrag in die Umwelt führen, da auf Mülldeponien gelagerte Arzneimittel in das Grundwasser sickern können (Halling-Sørensen et al. 1998, Heberer 2002, Kolpin et al. 2002, Fent et al. 2006).

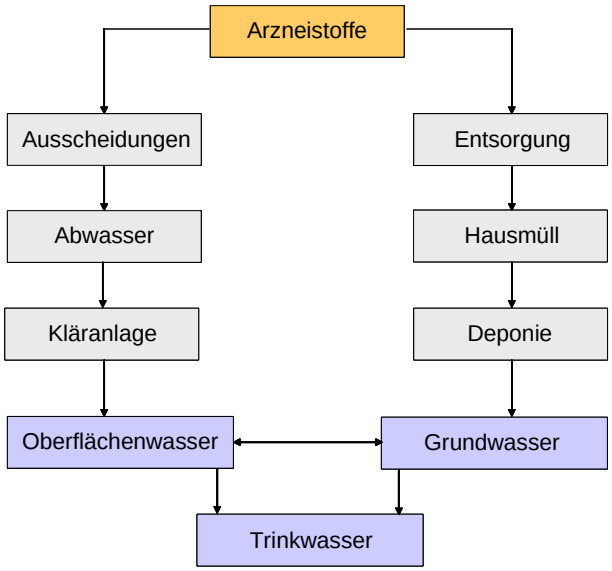


Abb. 1: Eintragswege von Arzneistoffen in die aquatische Umwelt (nach Ternes 1998)

Bislang konnten allein in Deutschland die Rückstände von etwa 120 Arzneistoffen mittels modernster Detektionsmethoden in aquatischen Systemen nachgewiesen werden (Schulte-Oehlmann et al. 2007). Obwohl die gemessenen Konzentrationen in den Oberflächengewässern nur im unteren µg/l oder ng/l Bereich liegen, können ökotoxikologische Effekte auf Nicht-Zielorganismen aufgrund der permanenten Einleitung der Stoffe sowie der extremen Persistenz und hohen biologischen Aktivität der Arzneimittel keineswegs ausgeschlossen werden (Halling-Sørensen et al. 1998, Daughton und Ternes 1999, Ferrari et al. 2003, Fent et al. 2006).

**Standardisierte Testverfahren zur Umweltrisikobewertung von Arzneistoffen**

Seit ein paar Jahren müssen die Umweltauswirkungen von Arzneimitteln vor der Zulassung der Wirkstoffe nach einer Richtlinie der EMEA (European Medicines Agency) bewertet werden (EMEA 2005). Hierbei handelt es sich um einen mehrstufigen Prozess, bei dem zunächst die erwartete Wirkstoffkonzentration in der Umwelt (PEC: predicted environmental concentration) berechnet wird. Liegt die Konzentration in den Oberflächengewässern über 0,01 µg/l, wird der Arzneistoff als potenziell umweltrelevant eingestuft und daraufhin genauer untersucht. Da jedoch hochaktive Arzneimittel wie synthetische Hormone meist in Konzentrationen unterhalb von 0.01 µg/l in Gewässern vorkommen, werden auch bei diesen Substanzen, unabhängig von ihrer erwarteten Umweltkonzentration, weitergehende

Untersuchungen durchgeführt (Christen et al. 2010). Dabei wird neben dem Abbau- und Adsorptionsverhalten des Stoffes auch dessen akute sowie chronische Toxizität auf Organismen verschiedener trophischer Ebenen wie Bakterien, Algen, Daphnien und Fische getestet. Hierzu werden standardisierte Tests nach den Richtlinien der OECD (Organisation for Economic Cooperation and Development) verwendet, um die Konzentration zu ermitteln, bei der der Arzneistoff keine Auswirkungen auf die unterschiedlichen Modellorganismen zeigt (PNEC: predicted no-effect concentration). Aus dem Verhältnis von PEC zu PNEC kann letztendlich das Umweltrisiko des untersuchten Arzneimittels berechnet werden. Liegt der Wert unterhalb von 1, wird der Stoff als ungefährlich betrachtet. Bei Werten über 1 muss jedoch von einer Umweltgefährdung ausgegangen werden, weswegen der Stoff weiteren Tests wie beispielsweise der Bioakkumulation unterzogen werden muss.

**Vorkommen und Auswirkungen von Arzneistoffen mit erhöhtem Umweltrisikopotenzial**

Zu den Humanarzneimitteln, die ein erhöhtes Risikopotenzial für die Umwelt besitzen (Rohweder 2003, LANUV-NRW 2007) und zudem sehr häufig und in erhöhten Konzentrationen in Gewässern des süddeutschen Raumes gefunden werden (Adler et al. 2001, Sengl und Schüssler 2004), gehören das Antiepileptikum Carbamazepin, das Antirheumatikum Diclofenac, das synthetische Estrogen 17α-Ethinylestradiol sowie das Antihypertonikum Metoprolol.

Carbamazepin

Carbamazepin wird nicht nur zur Epilepsiebehandlung, sondern auch als Stimmungsaufheller eingesetzt. Es wirkt als Membranstabilisator, indem es spannungsabhängige Natriumkanäle in neuronalen Membranen des Gehirns blockiert, wodurch die elektrische Entladung der Zelle verhindert wird (Mutschler et al. 2008). In Oberflächengewässern von Deutschland kommt Carbamazepin in Konzentrationen bis zu 1,1 µg/l vor und kann aufgrund der extremen Persistenz sogar mit einer Maximalkonzentration von 0,9 µg/l im Grundwasser sowie im Trinkwasser (30 ng/l) nachgewiesen werden (Ternes 1998, Sacher et al. 2001, Ternes 2001, Heberer 2002).

Lürling et al. (2006) konnten feststellen, dass sich bei einer Konzentration von 200 µg/l Carbamazepin die sexuelle Reife von *Daphnia pulex* verzögert, während die Crustaceen bei 1 µg/l Carbamazepin früher die Geschlechtsreife erlangen und mehr Nachkommen produzieren.

Des Weiteren führte eine 28-tägige Exposition von Mücken *Chironomus riparius* in mit Carbamazepin versehenem Sediment (0,16 – 100 mg/kg Trockengewicht) zu einer konzentrationsabhängigen Abnahme der Emergenz aufgrund einer Blockade bei der Verpuppung (Oetken et al. 2005).

Diclofenac

Diclofenac gehört zur Wirkstoffgruppe der nicht-steroidalen Entzündungshemmer und wird häufig als Schmerzmittel verwendet, worin die hohen Verordnungszahlen begründet liegen (Fent et al. 2006). Der Wirkstoff setzt an den Cyclooxygenasen an, durch deren Blockade die Bildung von Prostaglandinen verhindert wird. Somit werden die durch Prostaglandin vermittelten Schmerz- und Entzündungsreaktionen unterdrückt (Vane und Botting 1998). In deutschen Oberflächengewässern erreicht Diclofenac eine Maximalkonzentration von 1,2 µg/l, im Grundwasser kommt es in Konzentrationen bis 0,6 µg/l vor (Ternes 1998, Sacher et al. 2001). Laut Ternes (2001) kann Diclofenac sogar teilweise in Spuren im Trinkwasser detektiert werden.

Ferrari et al. (2003) konnten nachweisen, dass bei *Ceriodaphnia dubia* nach einer 7-tägigen Exposition in 2 mg/l Diclofenac die Reproduktion inhibiert wird. Bei Regenbogenforellen (*Oncorhynchus mykiss*) führte 5 µg/l Diclofenac nach 28 Tagen zu einer Bioakkumulation des Wirkstoffes in zahlreichen Organen sowie zu histopathologischen Veränderungen der Kiemen und Niere (Schwaiger et al. 2004). Auf cytologischer Ebene war bereits bei einer Konzentration von 1 µg/l eine Schädigung der Kiemen, Niere und Leber zu erkennen (Triebskorn et al. 2004).

17α-Ethinylestradiol

Das zur Empfängnisverhütung eingesetzte 17α-Ethinylestradiol erreicht in deutschen Oberflächengewässern eine Maximalkonzentration von 2,0 ng/l. Im Grundwasser wurden maximal 0,6 ng/l 17α-Ethinylestradiol, im Trinkwasser bis zu 0,2 ng/l EE2 gemessen (Adler *et al.* 2001).

Als hochspezialisierte Moleküle sind endokrine Substanzen bereits in geringen Konzentrationen biologisch wirksam (Fent et al. 2006) und können daher im empfindlich ausbalancierten Hormonsystem von Nicht-Zielorganismen zu Störungen der Ontogenese oder der Reproduktion führen. Beispielsweise produzieren *Daphnia magna*, die 25 Tage lang einer 17α-Ethinylestradiol-Konzentration von 100 ng/l ausgesetzt wurden, deutlich weniger Nachkommen (Goto und Hiromi 2003). Zudem erhöht 100 ng/l 17α-Ethinylestradiol bei dem

Bachflohkrebs *Gammarus pulex* die Juvenilität und ändert das Geschlechterverhältnis weiblicher zu männlicher Tiere von 1:1 auf 2:1 (Watts et al. 2002). Bei männlichen Regenbogenforellen (*Oncorhynchus mykiss*) steigt während einer dreiwöchigen Exposition in 2 ng/l 17α-Ethinylestradiol der Gehalt an Vitellogenin im Plasma an, außerdem wird das Wachstum der Hoden verhindert (Jobling et al. 1996).

Metoprolol

Metoprolol ist der am häufigsten verschriebene Wirkstoff aus der Klasse der β-Rezeptorenblocker, welcher vor allem bei der Therapie von Herzrhythmusstörungen und Bluthochdruck verwendet wird (Cleuvers 2005). Die Wirkung beruht auf der Bindung an β-adrenergen Rezeptoren des Herzens, wodurch der Einstrom von Calciumionen und somit eine Steigerung der Herzfrequenz verhindert wird (Mutschler et al. 2008). Die maximal ermittelte Konzentration von Metoprolol in Oberflächengewässern von Deutschland beträgt 2,2 µg/l (Ternes 1998). Im Grund- und Trinkwasser konnte Metoprolol bislang nicht nachgewiesen werden.

Dzialowski et al. (2006) konnten beobachten, dass Metoprolol nach 9 Tagen bei einer Konzentration unter 12 mg/l die Herzfrequenz, das Wachstum und die Fekundität von *Daphnia magna* reduziert. Ein negativer Einfluss auf die Fekundität von invertebraten und vertebraten Organismen zeigt sich auch bei strukturverwandten β-Rezeptorenblockern wie Propranolol (Hugget et al. 2002). So nahm die Reproduktion des Flohkrebses *Hyalella azteca* nach 27 Tagen bei einer Konzentration von 0,1 mg/l ab, der Japanische Reiskärpfling (*Oryzias latipes*) produzierte nach 4 Wochen bereits bei 0,5 µg/l Propranolol weniger Eier.

**Forschungsbedarf**

Umweltrelevante Arzneistoffkonzentrationen

Zwar konnten in einigen Studien bereits negative Effekte dieser Arzneimittel auf Nicht-Zielorganismen gezeigt werden, jedoch wurden dabei oftmals ziemlich hohe Stoffkonzentrationen verwendet, die nicht den in den natürlichen Systemen detektierten Konzentrationen entsprechen. Um realistische Aussagen über das Umweltverhalten der Arzneistoffe machen zu können, sollten die Substanzen in umweltrelevanten Konzentrationen getestet werden.

Arzneistoffgemische

Ebenfalls sind bisher nur wenige Studien zu synergetischen oder antagonistischen Wirkungen von Stoffgemischen durchgeführt worden. Da jedoch die Organismen in aquatischen Systemen nicht einem Einzelstoff, sondern einer Vielzahl von Arzneimitteln zugleich ausgesetzt sind, besteht diesbezüglich ein umfangreicher Forschungsbedarf (Altenburger et al. 2004, Fent et al. 2006, Schwarzenbach et al. 2006, Borgmann et al. 2007).

Darüber hinaus konnte in aktuellen Studien bereits gezeigt werden, dass Stoffgemische bei Nicht-Zielorganismen stärkere Effekte hervorrufen als die Einzelsubstanzen in denselben Konzentrationen (Cleuvers 2003, 2004). So konnte Cleuvers (2004) feststellen, dass das Zusammenwirken verschiedener entzündungshemmender Arzneimittel bei *Daphnia magna* zu einer Abnahme der Immobilisation führte. Wirkten die Arzneistoffe in denselben Konzentrationen dagegen alleine auf die Daphnien, war kein Effekt zu beobachten. Wurde *Daphnia magna* zeitgleich in dem Antidepressivum Fluoxetin (36 µg/l) und der lipid-senkenden Clofibrinsäure (10 µg/l) exponiert, kam es zu Missbildungen des Carapaxes, der Antennen sowie der Spina, während eine Exposition in den Einzelstoffen keine Auswirkung auf die Morphologie der Daphnien hatte (Flaherty und Dodson 2005).

Langzeiteffekte von Arzneistoffen

Auch die Langzeiteffekte vieler Arzneistoffe auf aquatische Organismen sind meist noch weitgehend unbekannt. Durch den kontinuierlichen Eintrag der Stoffe in die Gewässer sind die darin lebenden Organismen jedoch permanent den Arzneimitteln ausgesetzt. Dement-sprechend ist es nötig, die Auswirkungen während einer chronischen Arzneistoffexposition auf Nicht-Zielorganismen zu untersuchen (Daughton und Ternes 1999, Fent et al. 2006).

Zwar wurden von der OECD bereits Standardverfahren entwickelt, um die chronische Toxizität von Chemikalien auf ausgewählte Modelorganismen verschiedener trophischer Ebenen zu testen. Dabei wird jedoch häufig eine Testdauer empfohlen, die das Ausmaß einer chronischen Exposition in natürlichen Systemen nur unzureichend widerspiegelt. So schreibt beispielsweise die Richtlinie 211 für den Reproduktionstest bei *Daphnia magna* eine Expositionszeit von drei Wochen vor (OECD 1998), um die chronische Toxizität einer Chemikalie auf diesen Organismus abzuschätzen. Da hierbei jedoch nicht der gesamte Lebenszyklus der Daphnien, sondern nur ein Ausschnitt davon beobachtet wird, können Effekte, die erst in späteren Lebensstadien auftreten, aufgrund der kurzen Expositionsdauer übersehen werden. Bei einer lebenslangen Exposition können hingegen auch mögliche

Langzeiteffekte eines Wirkstoffes auf die Organismen berücksichtigt werden (Segner et al. 2003).

Um potentielle multigenerationale Auswirkungen detektieren zu können, müssten die Nicht-Zielorganismen sogar über mehrere Generationen hinweg den Arzneimitteln ausgesetzt werden (Daughton und Ternes 1999, Fent et al. 2006, Marcial und Hagiwara 2007). Einige wenige Studien konnten bereits darlegen, dass die Nachfolgegenerationen von Organismen in unterschiedlicher Weise von Arzneistoffen beeinflusst werden können. So beobachteten Brennan et al. (2006), dass das Östrogen Diethylstilbestrol keinen Einfluss auf die Parentalgeneration von *Daphnia magna* hatte, während es sich toxisch auf die zweite Generation auswirkte, indem es die Fekundität verringerte. Vermutlich waren die Daphnien der zweiten Generation aufgrund der pränatalen Exposition in dem Arzneimittel bereits geschwächt und somit anfälliger für den Stoff. Im Gegensatz dazu konnten Clubbs und Brooks (2007) zeigen, dass die zweite Generation von *Daphnia magna* eine Resistenz gegenüber endokrin wirksamen Arzneistoffen entwickelte und somit hinsichtlich der Fekundität, dem Geschlechterverhältnis sowie dem Trockengewicht der Nachkommen schwächer reagierten als die Daphnien der ersten Generation.

Trotz der gegensätzlichen Resultate beider Studien wird die Notwendigkeit von Multigenerationsstudien verdeutlicht, da die Auswirkungen der Arzneimittel möglicherweise unter- oder überschätzt worden wären, hätte man die Experimente nach der ersten Generation beendet. Eine Ausweitung der Expositionsdauer über mehrere Nachfolgegenerationen könnte dabei noch detailliertere Informationen über die Langzeitwirkung der untersuchten Arzneistoffe auf aquatische Organismen liefern. Somit ließe sich die von den Arzneimitteln ausgehende Umweltgefährdung besser einschätzen.

**Ziele dieser Studie**

Im Rahmen dieser Dissertation wurde in Laborexperimenten der Einfluss der oben erwähnten Arzneimittel auf aquatische Invertebraten untersucht (Kapitel 2-4). Dabei wurden folgende Fragestellungen behandelt:

1. Wirken sich die Arzneistoffe bereits in umweltrelevanten Konzentrationen auf die Organismen aus?
2. Zeigen sich bei einer Exposition in Arzneimittelgemischen stärkere Effekte auf die Invertebraten als bei einer Exposition in den Einzelstoffen?
3. Führen die Arzneistoffe bei den Organismen zu Langzeiteffekten?

In einem Lebenszyklusexperiment (Kapitel 2) wurden die chronischen Effekte der Arzneistoffe auf *Daphnia magna* betrachtet. Aufgrund ihrer unter Normalbedingungen stattfindenden parthenogenetischen Vermehrung, die es ermöglicht, mit genetisch identischen Individuen zu arbeiten, wird *Daphnia magna* häufig als Modellorganismus für ökotoxikologische Studien verwendet (OECD 1998). Zudem besitzen Daphnien im Vergleich zu vielen anderen aquatischen Organismen eine relativ kurze Lebensspanne von wenigen Monaten. Deshalb eignen sie sich hervorragend, um eine Arzneimittelexposition vom Schlüpfen bis zum natürlichen Absterben der Individuen durchzuführen. Dabei können durch die Arzneistoffe verursachte Veränderungen bestimmter life-history und morphologischer Parameter in allen Lebensstadien erfasst werden.

Darüber hinaus wurden in einem Multigenerationsexperiment (Kapitel 3) die Effekte der Arzneistoffe auf die life-history und Morphologie von *Daphnia magna* in sechs Nachfolgegenerationen ermittelt. Da Daphnien als Hauptkonsumenten der Primärproduktion sowie als Nahrungsgrundlage vieler Fischarten eine bedeutende Rolle im Nahrungsnetz limnischer Systeme einnehmen (Tollrian und Dodson 1999), erscheint es sinnvoll, die Auswirkungen der Arzneimittel auf diese wichtigen Vertreter der aquatischen Invertebratenfauna in einer mehrere Generationen umfassenden Exposition genauer zu untersuchen.

Als wichtige Vertreter von Fließgewässern werden Gammariden unmittelbar von den in den Flüssen eingeleiteten Arzneistoffen beeinflusst. Da Gammariden sensitiv auf Schadstoffe reagieren können (Schirling et al. 2006) und zudem einen komplexen Lebenszyklus besitzen, wurden in einem Langzeitexperiment (Kapitel 4) die Auswirkungen des Arzneistoffgemisches auf das Wachstum, die Reproduktion sowie auf die Physiologie von *Gammarus fossarum* getestet. Da Gammariden als Verwerter von Detritus und essentieller Bestandteil für die Ernährung etlicher Fischarten eine bedeutende Rolle im Nahrungsnetz einnehmen (MacNeil et al. 1999, Eggers und Martens 2001), können somit mögliche Folgen für das aquatische Ökosystem ermittelt werden.

Des Weiteren wurde in einem großräumig angelegten Freilandexperiment (Kapitel 5) die Zusammensetzung der benthischen Makroinvertebraten oberhalb und unterhalb von Kläranlageneinleitungen untersucht. Dabei sollten mögliche Zusammenhänge zwischen der Verteilung der Invertebraten und der eingeleiteten Schadstoffe wie beispielsweise Arzneimittel detektiert werden.

# Beyond international guidelines – Effects of discharged pharmaceuticals on a model organism

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Nature, eingereicht

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Um die chronische Toxizität von Chemikalien auf Modelorganismen verschiedener trophischer Ebenen zu testen, wurden internationale Standardverfahren entwickelt. Dabei werden jedoch nur die Effekte von Einzelsubstanzen auf einige wenige, ausgewählte life-history Merkmale untersucht. Der Einfluss von Wirkstoffgemischen sowie die Auswirkungen auf zusätzliche Parameter werden außer Acht gelassen. Zudem wird häufig eine Testdauer empfohlen, die das Ausmaß einer chronischen Exposition in natürlichen Systemen nur unzureichend widerspiegelt.

In einem Lebenszyklusexperiment wurden die Auswirkungen eines Gemisches aus dem Antiepileptikum Carbamazepin, dem Antirheumatikum Diclofenac, dem synthetischen Estrogen 17α-Ethinylestradiol und dem Antihypertonikum Metoprolol auf den Wasserfloh *Daphnia magna* ermittelt. Zusätzlich wurden die Arzneimittel als Einzelstoffe getestet, um eine mögliche verstärkende Wirkung von Stoffgemischen zu detektieren. Bei beiden Experimenten wurden neben den in Standardtests üblichen Parametern auch zusätzliche life-history und morphologische Merkmale erfasst, wie beispielsweise die Größe der Nachkommen.

Während die Einzelstoffe keinen Einfluss auf die Größe der Nachkommen ausübten, brachten Daphnien, die in dem Arzneistoffgemisch exponiert wurden, in den anfänglichen Bruten kleinere Nachkommen hervor als die Kontrolltiere. Diese Effekte können aufgrund der wichtigen Rolle von *Daphnia* im Nahrungsnetz weitreichende Konsequenzen für das aquatische Ökosystem haben, da größenselektive Prädatoren die Gemeinschaft der Daphnien nachhaltig beeinflussen können. Zudem traten bedingt durch die Exposition in dem Wirkstoffgemisch vermehrt männliche Daphnien sowie Nachkommen mit morphologischen Deformierungen auf. Des Weiteren konnte nach 45 Tagen bei den in dem Arzneistoffgemisch exponierten Daphnien ein Anstieg in der Mortalität beobachtet werden.

All diese Effekte wären jedoch in Standardtests aufgrund der verkürzten Expositionsdauer und der eingeschränkten Anzahl an zu untersuchenden Parametern übersehen worden. Hinzu kommt, dass die Auswirkungen von Arzneimittelgemischen in standardisierten Toxizitätstests nicht berücksichtigt werden, obwohl Wirkstoffgemische stärkere Effekte hervorrufen können als die entsprechenden Einzelsubstanzen. Daher scheint eine Überarbeitung der geltenden internationalen Richtlinien dringend erforderlich, um die ökologischen Konsequenzen einer permanenten Umweltbelastung mit Arzneistoffen zukünftig besser abschätzen zu können.

# Beyond international guidelines – Effects of discharged pharmaceuticals on a model organism

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**Environmental contamination by pharmaceuticals is of global concern for both the scientific and public communities. The continuous discharge of pharmaceuticals into aquatic ecosystems and their range of biochemical activities render them potentially hazardous to the biota, even at low concentrations [1-3]. The current international guidelines recommend to use only a few selected life history traits to test the toxic effects of a single compound in model organisms [4]. As a result, the impacts of toxic substances on parameters not considered in these standardised tests are likely to be overlooked.**

**Here we show detrimental effects of pharmaceuticals, at environmental concentrations on the waterflea *Daphnia magna*, for traits not controlled in ecotoxicological tests. We found that a mixture of four human drugs led to distinct shifts in the size of newborn daphnids and to morphological abnormalities in offspring, whereas the same pharmaceuticals applied as single compounds did not affect the organisms.**

**Given that daphnids serve as a major link between primary production and higher trophic levels [5], life history changes in *Daphnia* could lead to mismatches to the predation regime. Hence, the harmful effects of chronic drug exposure may manifest gradually in organisms and populations, ultimately resulting in irreversible ecosystem changes. All the effects seen in our study would not have been recognized following the current international guidelines. This finding highlights that a revision of the guidelines is urgently required in order to assess the consequences of exposure to pharmaceutical mixtures in nature.**

In recent years, pharmaceuticals have received increased attention as a novel group of contaminants, as they have been detected in surface waters and even in drinking water resources across the world [6, 7]. Drugs are continually released into the aquatic environment via sewage treatment plants, which are often not designed to eliminate these bioactive substances effectively from the effluents [1, 6]. As pharmaceuticals were designed for human use they may elicit very different reactions in non-target organisms and consequently may lead to latent ecosystem changes. This concern has established a relatively novel field of research dealing with the sources, fate and effects of pharmaceuticals in nature [1-3, 8]. Although significant progress has been made regarding knowledge of pharmaceuticals as environmental pollutants, the ecological consequences of these contaminants, especially as complex drug mixtures, are not yet fully understood [3, 9].

The method for determining the impact of chemicals on the environment is typically a tiered task including acute and chronic toxicity tests according to current international guidelines such as the EPA (U.S. Environmental Protection Agency) and the OECD (Organisation for Economic Co-operation and Development) [10]. For testing the toxic effects of potentially hazardous substances in non-target organisms, these guidelines recommend to analyze selected life history traits during a restricted exposure time [4]. Given that the biota are exposed to a mixture of pharmaceuticals in the environment and that the life cycle of most test organisms far exceeds the standard exposure time period, it is likely that ecological consequences remain undiscovered. Furthermore, life history traits not controlled for in these standardised tests may provide more detailled insight into the impact of these toxic compounds on the test organisms and may reveal possible consequences for the environment.

We exposed *Daphnia magna*, a standard model organism in ecotoxicology, over their entire life-cycle to environmental concentrations of a mixture of the antiepileptic agent carbamazepine (CBZ), the anti-inflammatory drug diclofenac (DIC), the estrogen 17α-ethynylestradiol (EE2) and the β-blocker metoprolol (MET). These human drugs were selected for testing because they have been frequently detected in aquatic environments [6, 7, 11, 12] and are suspected of having severe effects on non-target species [13-16]. We investigated the impact of the drug mixture on life-history traits recommended by current international guidelines and on additional parameters not controlled for in these standardised tests, such as the body size of newborns.

Here we show that newborn body size was significantly reduced in initial broods when exposed to the pharmaceutical mixture (Fig. 1, Supplementary Table 1). As smaller offspring of daphnids cannot compensate for the decreased hatching size during their

development [17, 18], the reduction in size may lead to mismatches to the predation regime: On one hand, a decrease in body size may be favourable if visual oriented vertebrate predators are dominant; on the other hand, smaller animals are more vulnerable to invertebrate predators that selectively feed on small zooplankton [19]. Depending on the predation pressure on daphnids their grazing intensity on phytoplankton can vary considerably. Hence, size-selective effects may alter community or ecosystem-level responses because of the key role of *Daphnia* as primary consumers in lakes and ponds [5].

The toxic impact of the drug mixture is also shown by the percentage of nonviable offspring from daphnids faced with drugs over their entire life span: significantly more newborn daphnids with morphological abnormalities were observed in the drug mixture treatment (0.84 %) than in the control group (0.45 %; two-sample binomial test, $p = 0.039$; Fig. 2). This is in accordance with a recent study where morphological deformities in *Daphnia magna* were detected due to the toxic impact of a mixture of the pharmaceuticals clofibric acid and fluoxetine [20].

In addition, the sex ratio of the exposed organisms is usually not considered in standardised tests. Our study showed that the proportion of male offspring increased significantly in the pharmaceutical mixture treatment (0.52 %) compared to the control (0.17 %; two-sample binomial test, $p = 0.010$). The increased occurence of male daphnids in the drug mixture highlights the harmful impacts of these pharmaceuticals, as males are usually produced in response to aggravated environmental conditions [21]. As the proportion of male offspring was very low, we do not expect a severe impact on *Daphnia* populations, although we can not exclude that the effects of drug mixtures may manifest gradually on the community level.

Although significantly fewer newborn daphnids were released in the fourth (two-tailed T-test: $t_{1,35} = 6.568$, $p = 0.015$) and sixth broods only (two-tailed T-test: $t_{1,35} = 20.910$, $p < 0.001$) in the drug mixture treatment, we detected a considerable decrease (17.4 %) in the total number of offspring in the pharmaceutical mixture treatment compared to the control treatment, as daphnids exposed to the drug mixture over their lifetime showed a significant decrease in survival after 45 days of exposure (Fig. 3; one-way ANCOVA: $F_{1,33} = 4.740$, $p = 0.037$). The decreased survival in later life stages of daphnids exposed to the pharmaceutical mixture would not have been recognized when strictly following the current international guidelines which recommend an exposure period of 21 days for chronic toxicity tests with *Daphnia magna* [4].

Despite the enhanced mortality, the intrinsic rate of population growth did not differ between control animals (0.33) and the daphnids exposed to the pharmaceutical mixture (0.32). Hence,

the effects of pharmaceuticals in nature may be portrayed as negligible. However, we anticipate that the intrinsic rate of population growth may be affected in the following generation as a consequence of the reduced body size of the newborns: Smaller daphnids mature at smaller sizes [18], and in addition, the body size of females is positively correlated with the egg number [16]. Thus, the intrinsic rate of population growth is potentially decreased in the following generation due to the impact of pharmaceuticals. Since it is not required to measure the body size of newborn daphnids in standard toxicity tests, ecological consequences such as a decrease of the population growth rate and mismatches to predation regimes may be overlooked. Furthermore, under natural conditions, the mortality caused by predators will greatly enhance the fitness contributions of the first age classes.

To examine if complex drug mixtures provoke stronger effects on non-target organisms than single pharmaceuticals alone, we tested the impact of the single compounds on *Daphnia*. Since considerable effects of the drug mixture occurred predominantly during the initial broods, the single compound experiments were terminated after 21 days, according to current international guidelines [4].

The body size of newborns exposed to the single substances differed only in the fourth brood of the EE2 treatment, resulting in smaller daphnids compared to the control group (Fig. 4; nested ANOVA: $F_{4,87} = 4.063$, $p = 0.005$, post hoc: $p < 0.001$). None of the single drugs affected the number of offspring during the 21 days of exposure (two-tailed T-test: all n.s.). Our results are in accordance with recent studies which have highlighted that pharmaceutical mixtures can elicit enhanced toxicity toward invertebrate and vertebrate aquatic organisms, presumably due to synergistic effects [22-24]. As the aquatic environment is usually contaminated with a complex mixture of drugs and not only isolated compounds, it is important to study the combined effects to assess the risk of pharmaceuticals on non-target organisms in nature [3, 9, 25]. However, such combined effects of drugs are usually not considered in standard toxicity tests, which are designed to investigate the impact of single substances on organisms. It is therefore a major challenge for international authorities to further implement empirical and theoretical approaches to assess the effects of complex mixtures of pharmaceuticals into possible revisions of their risk management framework [3, 9, 25, 26]. This will foster the improvement of environmental management strategies in the future to prevent disastrous global effects that have already occurred with other pollutants.

**Methods Summary**

For the life-cycle experiment, we prepared a mixture of CBZ, DIC, EE2 and MET at environmentally relevant concentrations. Twenty newly hatched daphnids of age-synchronised *D. magna* were selected randomly for the drug mixture and the control treatment and transferred individually into glass vials containing 50 ml of test solution. Animals were fed daily with a suspension of *Scenedesmus obliquus* at a defined concentration of 2 mg C/l and were kept in a climate chamber at 20 ± 0.5 °C with a 16 h light / 8 h dark photoperiod regime.

Daphnids were controlled daily and survival of the females was recorded. For each brood the offspring per female were counted, removed from the glass vial and frozen at -80 °C. Subsequently, the body size of five randomly chosen newborn daphnids was measured with a digital image analyzing system (Cell P, Olympus, Hamburg, Germany). The body size was defined as the distance from the upper edge of the compond eye to the base of the tail spine. In addition, all preserved daphnids were examined for the percentage of nonviable newborns (with abnormal morphological deformations) and sex ratio. Nonviable and male newborns were excluded from analysis of the intrinsic rate of population growth (r), which was estimated using the Euler-Lotka equation (calculated under the assumption of zero mortality caused by predation). The experiment was terminated after 72 days, with the death of the last test animal.

For the 21-day experiment, we prepared environmentally relevant concentrations of the single compounds CBZ, DIC, EE2 and MET. The experimental design was carried out as described above with the exception that only the number of offspring and body size of newborn daphnids were analyzed.

Full methods accompany this paper.

## Methods

Test organisms

We selected a single clone of *D. magna*, originally collected from a pond in Ismaning (Germany) and cultured in the laboratory for several years. Age-synchronised females were isolated from stock cultures and maintained in artificial medium which was changed every other day. The daphnids were fed daily with a suspension of the green algae *Scenedesmus obliquus* ad libitum and were kept under constant temperature and light conditions. The experiment was started with newly hatched daphnids of the third brood from the age-synchronised mothers.

Test solutions

CBZ ((Z)-5H-dibenzo[b,f]azepine-5-carboxamide; Sigma Aldrich, Seelze, Germany), DIC (2-[(2,6-dichlorophenyl)amino]benzeneacetic acid sodium salt; Sigma Aldrich, Seelze, Germany), EE2 (17-ethynyl-13-methyl-7,8,9,11,12,13,14,15,16,17-decahydro-6H-cyclopenta[a]phenanthrene-3,17-diol; Fluka, Buchs, Switzerland) and MET ((±)-1-(isopropylamino)-3-[4-(2-methoxyethyl)phenoxy]-2-propanol tartrate salt; Sigma Aldrich, Seelze, Germany) were tested as mixture (life-cycle experiment) and as single substances (21-day experiment). Nominal test concentrations for CBZ, DIC and MET were 0.50 µg/l, 0.36 µg/l and 1.20 µg/l, respectively. These treatment levels represent the maximum concentrations found in the rivers and streams of Southern Germany [12]. The concentration of EE2 was set at 0.10 ng/l, representing the median concentration of conjugats of EE2 in surface waters of Southern and Central Germany [11].

Drugs were dissolved in methanol (HPLC grade) to produce solutions with a concentration of 0.1 µg/µl and then stored in the dark at -20 °C. New stock solutions were prepared every second week. For preparation of the drug mixture, 40.0 µl of the CBZ solution, 28.8 µl of the DIC solution and 96.0 µl of the MET solution were transferred to a single object slide. The EE2 stock solution was diluted to a concentration of 0.01 ng/µl and subsequently 80.0 µl of this dilution was added to the object slide which was dried under a gentle stream of nitrogen. During the nitrogen shower, only the solvent evaporates, while the drugs remain on the object slide without a loss of drug substance. The preparation of the single substances was carried out as described for the drug mixture, with the exception that the four pharmaceuticals were transferred separately to single object slides.

We placed each object slide into a 12 l glass aquarium containing 8 l of artificial medium based on ultra pure water and trace elements [27]. One aquarium filled with medium but without any pharmaceuticals served as the control treatment. The aquaria were used as reservoirs for smaller amounts of test solutions for the experiments. To avoid evaporation of test medium, we covered all aquaria with glass plates. Test solutions were renewed weekly. Every third day, we changed the test solutions in the glass vials using new medium from the corresponding reservoir aquaria. To prevent diluting the concentration of the pharmaceuticals in the vials after feeding, algae suspensions were prepared within the corresponding test solution. Additionally, before adding the algae to vials, the same amount of medium was removed from each glass vial. The abiotic parameters of temperature, dissolved oxygen, oxygen saturation, conductivity and pH were monitored once per week.

Chemical analysis

The concentrations of the pharmaceuticals were analyzed by GC-MS/MS in a previous experiment which was conducted under exactly the same conditions as described for this study [28]. The mean exposure levels differed 1.6 %, 1.4 %, 1.0 % and 2.5 % from the nominal test concentration of CBZ, DIC, EE2 and MET, respectively. For more detailed information of the chemical analysis see [28].

Statistical analysis

All statistical analysis were performed with SPSS 15.0 (SPSS inc., Chicago, IL, USA). We utilized histograms to validate that observations within treatments were normally distributed. A general linear model (one-way ANCOVA) with treatment as a fixed factor and brood number as covariate was used to compare differences in female survival. Differences in the number of offspring were compared to the control using two-tailed T-tests with treatment as a fixed factor. Two-level nested general linear models (nested ANOVAs) with treatment as a fixed factor and five replicates per treatment as a random factor were performed to compare differences in offspring body size. For the 21-day experiment, Tamhane´s post hoc tests were applied to distinguish differences between means. To compare differences in the percentage of nonviable newborn daphnids and the percentage of males, two-sample binomial tests were performed.

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## Supplementary Information

Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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## Author Contributions

C.L. and S.D. designed and performed the research, analyzed the data and wrote the manuscript. W.G. analyzed the intrinsic rate of population growth. F.P. and F.B. designed and performed the chemical analyses.

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**Figures**

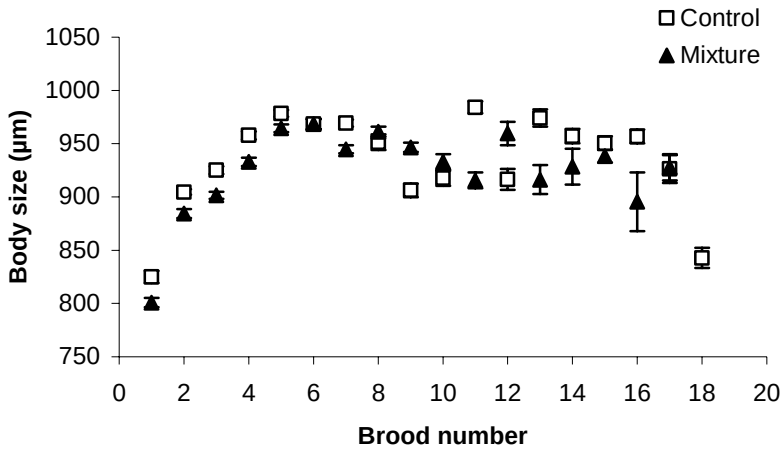
Fig. 1: Body size of newborn *D. magna* (mean ± s.e.m.) exposed to the pharmaceutical mixture at environmentally relevant concentrations over their entire lifetime.

Fig. 2: Birth deformities (development of two heads; right side) in a newborn offspring of *D. magna* exposed to environmental concentrations of a drug mixture in comparison to a healthy newborn animal (left side).

Fig. 3: Survival of *D. magna* exposed to the pharmaceutical mixture at environmentally relevant concentrations over their entire lifetime.

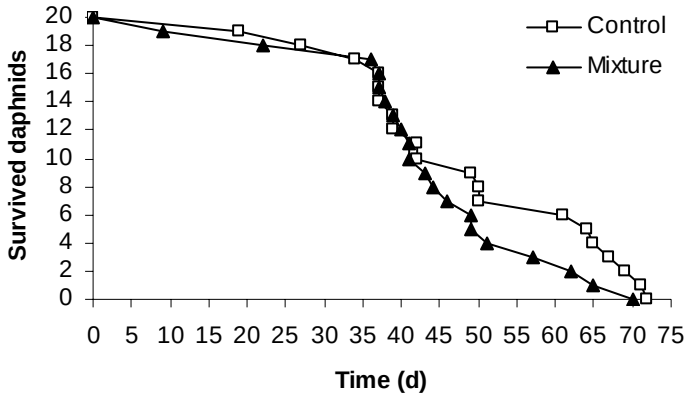
Fig. 4: Body size of newborn *D. magna* (mean + s.e.m.; $90 \leq n \leq 100$) exposed to single substances CBZ, DIC, MET and EE2 at environmentally relevant concentrations for 21 days.

1)

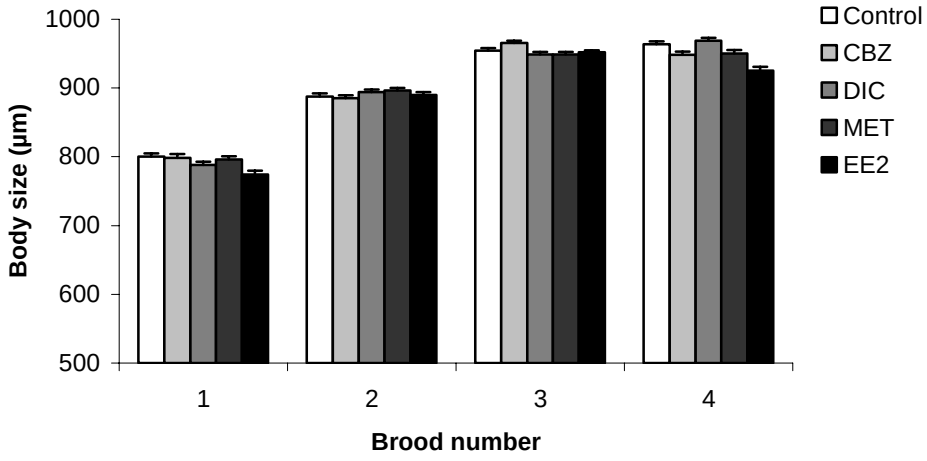

Control
Mixture
Body size (µm)
1050
1000
950
900
850
800
750
0
2
4
6
8
10
12
14
16
18
20
Brood number

2)

3)


Survived daphnids
Time (d)
Control
Mixture
0
2
4
6
8
10
12
14
16
18
20
0 5 10 15 20 25 30 35 40 45 50 55 60 65 70 75

4)


Body size (µm)
Brood number
Control
CBZ
DIC
MET
EE2
500
600
700
800
900
1000
1
2
3
4

**Supplementary Information**

Supplementary Table 1: Statistical analyses of newborn body size for *D. magna* exposed to the pharmaceutical mixture at environmentally relevant concentrations over their entire lifetime.

| brood | control | | mixture | | nested ANOVA | |
|---|---|---|---|---|---|---|
| number | mean ± s.e.m. | n | mean ± s.e.m. | n | F | p |
| 1 | 824.99 ± 5.80 | 90 | 800.82 ± 4.23 | 94 | $F_{1,35} = 3.057$ | $p = 0.089$ |
| 2 | 904.56 ± 2.58 | 100 | 884.50 ± 4.20 | 93 | $F_{1,37} = 7.508$ | **$p = 0.009$** |
| 3 | 925.12 ± 3.64 | 95 | 901.47 ± 3.30 | 95 | $F_{1,36} = 7.613$ | **$p = 0.009$** |
| 4 | 957.86 ± 3.75 | 95 | 932.98 ± 4.02 | 90 | $F_{1,35} = 9.449$ | **$p = 0.004$** |
| 5 | 978.33 ± 3.15 | 95 | 964.48 ± 3.61 | 91 | $F_{1,37} = 3.048$ | $p = 0.089$ |
| 6 | 968.43 ± 4.89 | 95 | 968.30 ± 4.69 | 90 | $F_{1,35} < 0.001$ | $p = 0.991$ |
| 7 | 969.31 ± 3.33 | 90 | 944.82 ± 3.84 | 90 | $F_{1,34} = 6.830$ | **$p = 0.013$** |
| 8 | 951.43 ± 7.42 | 85 | 961.31 ± 4.61 | 90 | $F_{1,33} = 0.307$ | $p = 0.583$ |
| 9 | 906.20 ± 6.51 | 80 | 946.62 ± 4.23 | 80 | $F_{1,30} = 6.208$ | **$p = 0.018$** |
| 10 | 917.65 ± 7.03 | 58 | 933.03 ± 7.21 | 54 | $F_{1,21} = 0.876$ | $p = 0.360$ |
| 11 | 984.11 ± 5.62 | 50 | 915.59 ± 7.43 | 45 | $F_{1,17} = 13.282$ | **$p = 0.002$** |
| 12 | 916.48 ± 9.93 | 48 | 959.55 ± 10.88 | 32 | $F_{1,15} = 0.840$ | $p = 0.374$ |
| 13 | 974.01 ± 8.10 | 35 | 916.35 ± 13.70 | 24 | $F_{1,10} = 3.504$ | $p = 0.091$ |
| 14 | 957.08 ± 6.79 | 35 | 928.51 ± 16.84 | 15 | $F_{1,8} = 0.853$ | $p = 0.383$ |
| 15 | 950.52 ± 6.55 | 32 | 938.47 ± 6.17 | 12 | $F_{1,9} = 0.864$ | $p = 0.378$ |
| 16 | 956.78 ± 6.48 | 31 | 895.39 ± 27.59 | 10 | $F_{1,6} = 1.898$ | $p = 0.217$ |
| 17 | 926.27 ± 13.07 | 15 | 927.90 ± 12.33 | 5 | $F_{1,2} = 0.001$ | $p = 0.983$ |
| 18 | 842.83 | 5 | | | | |

# Single and combined toxicity of pharmaceuticals at environmentally relevant concentrations in *Daphnia magna* – A multigenerational study

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Bedingt durch die kontinuierliche Freisetzung von Arzneimitteln in die aquatische Umwelt sind die darin lebenden Organismen permanent diesen Stoffen ausgesetzt. Daher wurde ein Multigenerationsexperiment mit der Cladocere *Daphnia magna* durchgeführt, um die chronische Toxizität des Antiepileptikums Carbamazepin, des Antirheumatikums Diclofenac, des synthetischen Estrogens 17α-Ethinylestradiol sowie des Antihypertonikums Metoprolol zu ermitteln. Diese Arzneistoffe wurden anhand ihres ubiquitären Vorkommens und ihres erhöhten Umweltgefährdungspotentials als Testsubstanzen ausgewählt. Dabei wurden die Substanzen sowohl als Einzelstoffe als auch als Wirkstoffgemisch getestet, da Arzneimittel in den Gewässern meist als komplexe Stoffgemische vorliegen. Zudem wird einer Mixtur aus Schadstoffen eine höhere Toxizität zugeschrieben als den Einzelsubstanzen. Um möglichst naturnahe Bedingungen zu simulieren, wurden ausschließlich umweltrelevante Arzneimittelkonzentrationen verwendet. Die Effekte der Arzneistoffe wurden in einer sechs Nachfolgegenerationen umfassenden Exposition auf life-history und morphologische Parameter der Daphnien untersucht.

Generell wirkten sich die Arzneimittel bei allen untersuchten Parametern in der ersten exponierten Generation aus, in den darauf folgenden Generationen kam es jedoch zu einer Akklimatisierung an die Wirkstoffe. Erst in späteren Generationen traten erneut Arzneistoffeffekte bei den Daphnien auf. So waren die Organismen, die in Metoprolol exponiert wurden, in den Generationen F0, F3 und F4 zum Zeitpunkt der ersten Reproduktion kleiner und produzierten zudem in den Generationen F0 und F4 weniger Nachkommen. Dieselben Auswirkungen zeigten mit 17α-Ethinylestradiol behandelte Daphnien, mit der Ausnahme, dass die Körpergröße zum Zeitpunkt der ersten Reproduktion nur in den Generationen F0 und F4 verringert war. Diclofenac verzögerte das Alter bei der ersten Reproduktion in den Generationen F0 und F2. Des Weiteren war die Körpergröße der neonaten Daphnien in Folge der Exposition mit Diclofenac in den Generationen F1 und F5 erhöht. Bei den Organismen, die mit Carbamazepin behandelt wurden, konnte nur in der F0 Generation eine Verzögerung bei der ersten Reproduktion beobachtet werden. Durch die Exposition in dem Arzneimittelgemisch verkürzte sich zum einen das Alter der ersten Reproduktion der Daphnien in den Generationen F0 und F2, zum anderen verlängerte sich die Körpergröße zum Zeitpunkt der ersten Reproduktion in den Generationen F0 und F3. Zudem war die Körpergröße der neonaten Daphnien in der F1 Generation erhöht, in der F5 Generation jedoch reduziert.

In diesem Multigenerationsexperiment konnte bei *Daphnia magna* keine verstärkte Toxizität der Arzneistoffmixtur gegenüber den Einzelsubstanzen festgestellt werden. Allerdings zeigte

sich, dass sich weder das Arzneimittelgemisch noch die Einzelstoffe kontinuierlich auf die Nachfolgegenerationen der Daphnien auswirkten. Diese Ergebnisse verdeutlichen die Notwendigkeit von Multigenerationsstudien, da die Effekte von Arzneistoffen auf die untersuchten Parameter bei einer kürzeren Expositionsdauer unter- oder überschätzt werden könnten. Darüber hinaus liegt die Vermutung nahe, dass aquatische Organismen wahrscheinlich aufgrund zu hoher Fitnesskosten keine dauerhafte Resistenz gegenüber den in die Gewässer eingetragenen Arzneistoffen entwickeln können.

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# Single and combined toxicity of pharmaceuticals at environmentally relevant concentrations in *Daphnia magna* – A multigenerational study

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ABSTRACT

Pharmaceuticals are continuously discharged into the environment, resulting in the chronic exposure of aquatic organisms to these compounds. In this multigenerational study, we examined the influence of four pharmaceuticals (carbamazepine (CBZ), diclofenac (DIC), 17α-ethinylestradiol (EE2) and metoprolol (MET)) as single substances and as a drug mixture at environmentally relevant concentrations on life-history and morphological parameters over six generations of the cladoceran *Daphnia magna*.

Detectable effects of the used pharmaceuticals occured in the first observed generation, followed by an acclimation period and a recurrence of drug effects in later generations: daphnids exposed to MET were affected by the pharmaceutical, resulting in a decreased body length at first reproduction in the generations F0, F3 and F4 and in a reduced number of offspring in the generations F0 and F4. Similar effects were observed in daphnids exposed to EE2. DIC delayed age at first reproduction in the F0 and F2 generations and increased the body length of neonates in the generations F1 and F5. Daphnids exposed to CBZ showed a delay in the age at first reproduction for the F0 generation only. The drug mixture reduced the age at first reproduction of daphnids in the F0 and F2 generation and increased the body length at first reproduction in the generations F0 and F3.

Neither the single drugs nor the pharmaceutical mixture influenced the successive generations of *D. magna* in a steady way. Thus, multigenerational studies are necessary to prevent insufficient assessments on the impact of pharmaceuticals in aquatic ecosystems.



## 1. Introduction

Due to anthropogenic activity, large amounts of pollutants such as pesticides, heavy metals and organic contaminants are released into the environment. Pharmaceuticals are an additional group of chemicals, which are discharged into aquatic systems (Ternes, 1998; Heberer, 2002; Kolpin et al., 2002). These substances enter sewage treatment plants predominantly after consumption by humans and subsequent excretion or directly, as many unused drugs are flushed down toilets (Halling-Sørensen et al., 1998; Heberer, 2002). Since sewage treatment plants do not remove pharmaceuticals effectively (Ternes, 1998), unchanged parent compounds or metabolites are permanently released via effluents into the aquatic environment. In addition, drugs enter aquatic systems as run-off from sewage sludge, which is dispersed on fields as manure (Halling-Sørensen et al., 1998; Heberer, 2002).

The antiepileptic agent carbamazepine (CBZ), the non-steroidal anti-inflammatory drug diclofenac (DIC), the estrogen 17α-ethinylestradiol (EE2) and the β-blocker metoprolol (MET) are drugs that are found commonly and in increasing quantities in rivers and streams throughout Southern Germany (Adler et al., 2001; Sengl and Schüssler, 2004). CBZ has been detected in German surface waters at a maximum concentration of 1.1 $\mu g\,L^{-1}$, and DIC occurs at concentrations up to 1.2 $\mu g\,L^{-1}$ (Ternes, 1998). The estrogenic compound EE2 was reported to reach 2.0 $ng\,L^{-1}$ in surface waters of Germany (Adler et al., 2001), and MET has been found with a maximum concentration of 2.2 $\mu g\,L^{-1}$ (Ternes, 1998).

Although these pharmaceuticals occur in the aquatic environment at low concentrations, in the nanogram-per-liter or microgram-per-liter range, they were assessed to pose potential environmental risks (Rohweder, 2003), due to either high persistence or biological activity (Kreuzinger et al., 2004; Fent et al., 2006). The toxic effects of the single substances studied here (CBZ, DIC, EE2 and MET) toward daphnids have been established in recent studies. Exposition of *Daphnia pulex* to 200 $\mu g\,L^{-1}$ CBZ resulted in delayed maturation, whereas animals exposed to 1 $\mu g\,L^{-1}$

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CBZ matured sligthly earlier and produced more offspring than the control group (Lürling et al., 2006). A concentration of 40 mg $L^{-1}$ DIC induced significantly elevated levels of the stress protein hsp70 in *Daphnia magna* after an exposure time of 48 h (Haap et al., 2008). Goto and Hiromi (2003) observed a reduced number of offspring in *D. magna* exposed to EE2 for 25 d at concentrations of 100 and 500 ng $L^{-1}$. Moreover, *D. magna* exposed to the β-blocker MET for 9 d showed decreased growth, fecundity and heart rate at concentrations ranging from 3.1 to 25 mg $L^{-1}$ (Dzialowski et al., 2006).

However, the majority of published data is based on far higher concentrations than those detected in freshwater systems. Therefore, studies considering the implications of drugs on aquatic organisms at environmentally realistic concentrations are required (Dietrich et al., in press).

In addition, our knowledge regarding the influence of pharmaceutical mixtures on aquatic organisms remains limited. As the aquatic environment is usually contaminated with a mixture of drugs and not only single compounds, it is important to study possible combined effects of these compounds (Fent et al., 2006; Schwarzenbach et al., 2006; Borgmann et al., 2007). Recent studies have highlighted that pharmaceutical mixtures can elicit different effects than those predicted from single compounds. For instance, Cleuvers (2003) showed that a mixture of the antiepileptic drug CBZ and the lipid lowering agent clofibrinic acid had stronger effects on *D. magna* during immobilization tests than the single substances at the same concentrations. In addition, in acute *Daphnia* and algal tests, Cleuvers (2004) detected considerable mixture toxicity of different anti-inflammatory drugs, even at concentrations for which the single compounds showed no effects.

A complete understanding of the effects of these chemicals on natural systems remains elusive, as most studies consider only the short-term effects of drug mixtures, as especially acute toxicity tests with very short exposure times are conducted. Given the continuous entry of pharmaceuticals into aquatic environments, it is necessary to perform chronic studies to observe the long-term effects of drugs on non-target organisms (Daughton and Ternes, 1999; Fent et al., 2006). Full life-cycle studies may detect sub lethal and late-life effects which are missed in short-term tests (Segner et al., 2003). Furthermore, to elucidate possible multigenerational effects, it has been suggested to extend the exposure time of pharmaceuticals on aquatic organisms to several generations (Daughton and Ternes, 1999; Fent et al., 2006; Marcial and Hagiwara, 2007). By assessing the effects of environmental estrogens on *D. magna* over two generations, Brennan et al. (2006) detected enhanced toxicity in the second generation resulting in decreased fecundity and increased mortality. In contrast, Clubbs and Brooks (2007) found a less sensitive reaction of second generation *D. magna* exposed to endocrine-active pharmaceuticals, most likely resulting from daphnids developing resistance to these contaminants. These contradictory studies highlight the importance of multigenerational tests, as effects could be under- or overestimated if experiments are terminated after one generation. Testing more than two successive generations may provide further and more detailed informations about the possible effects of drugs on non-target organisms.

In the present study, we performed a multigenerational study over six generations using the cladoceran *D. magna*. We investigated the effects of CBZ, DIC, EE2 and MET as single compounds and as a drug mixture on life-history and morphological parameters of *Daphnia*. To simulate natural conditions as closely as possible, we used environmentally relevant drug concentrations. Our hypotheses were: (1) the effects of pharmaceuticals differ between generations, resulting in either an enhanced toxicity or resistance in subsequent generations (2) the pharmaceutical mixture induces stronger effects on daphnids than the single drugs alone.

## 2. Materials and methods

### 2.1. Test solutions

Environmentally relevant concentrations of CBZ ((Z)-5H-dibenzo[b,f]azepine-5-carboxamide; Sigma Aldrich, Seelze, Germany), DIC (2-[(2,6-dichlorophenyl)amino]benzeneacetic acid sodium salt; Sigma Aldrich, Seelze, Germany), EE2 (17-ethynyl-13-methyl-7,8,9,11,12,13,14,15,16,17-decahydro-6H-cyclopenta[a]phenanthrene-3,17-diol; Fluka, Buchs, Switzerland) and MET ((±)-1-(isopropylamino)-3-[4-(2-methoxyethyl)phenoxy]-2-propanol tartrate salt; Sigma Aldrich, Seelze, Germany) were tested as single substances and as a drug mixture. Nominal test concentrations for CBZ, DIC and MET were 0.50 μg $L^{-1}$, 0.36 μg $L^{-1}$ and 1.20 μg $L^{-1}$, respectively. These treatment levels represent the maximum concentrations found in the rivers and streams of Southern Germany (Sengl and Schüssler, 2004). The concentration of EE2 was set at 0.10 ng $L^{-1}$, representing the median concentration of conjugates of EE2 in the surface waters of Southern and Central Germany (Adler et al., 2001). Since endocrine compounds such as EE2 possess a very high biological activity, they may affect non-target organisms already at very low concentrations (Fent et al., 2006). Therefore, we used only the median concentration for EE2 to avoid that EE2 may mask the impact of the other pharmaceuticals in the drug mixture. The latter was a combination of the single substances at the same concentrations.

Drugs were dissolved in methanol (HPLC grade) to produce solutions with a concentration of 0.1 μg $\mu L^{-1}$ and then stored in the dark at −20 °C. New stock solutions were prepared every second week. For preparations of single substances, 40.0 μL of the CBZ solution, 28.8 μL of the DIC solution and 96.0 μL of the MET solution were transferred to a single object slide and dried under a gentle stream of nitrogen. The EE2 stock solution was diluted to a concentration of 0.01 ng $\mu L^{-1}$ and subsequently 80.0 μL of this dilution was added to an object slide which was similarly dried under a gentle stream of nitrogen. In the case of the drug mixture, preparation was carried out as described for single substances, with the exception that all four pharmaceuticals were transferred to a single object slide.

We placed each object slide into a 12 L glass aquarium containing 8 L of artificial medium based on ultra pure water and trace elements (Laforsch et al., 2006). One aquarium filled with medium but without any pharmaceuticals served as the control treatment. The aquaria were used as reservoirs for smaller amounts of test solutions for the following experiment. To avoid evaporation of test medium, we covered all aquaria with glass plates. Test solutions were renewed weekly. The abiotic parameters of temperature, dissolved oxygen, oxygen saturation, conductivity and pH were monitored once per week.

### 2.2. Chemical analysis

During the experiment, concentrations of the pharmaceuticals were measured weekly in the drug mixture treatment. Nominal exposure concentrations were analyzed by GC–MS/MS, using a Varian triple quadrupole 1200 coupled with a Varian 3800 gas chromatograph equipped with CTCs CombiPAL (Palo Alto, CA, USA). Varian VF-5 ms capillary columns (Middelburg, The Netherlands) of 30 m × 0.25 mm × 0.25 μm with 10 m guard columns were employed throughout the study.

A 1 L sample of the drug mixture test solution was run through RGL-60 membrane filters (Schleicher & Schüll, Dassel, Germany) with a 1 μm pore size. After filtration, 800 g of water were acidified with phosphoric acid to a pH of 3.0 and triphenylmethane was added as an internal standard. A Varian Bondelut Plexa PCX cartridge (Middelburg, The Netherlands) was conditioned with

3 mL methanol and 3 mL water. The aqueous sample was then passed through the cartridge at a rate of 5 mL $h^{-1}$ and subsequently dried under a gentle stream of nitrogen for 15 min. The absorbed drugs were eluted with 3 mL of 10% diethylamine in methanol.

Dichlorodiphenyltrichloroethane was added as an internal standard and the solvent was then evaporated until dry under a gentle stream of nitrogen. The residue was finally redissolved in 900 μL of 20% 1-chlorbutane in acetonitril. For GC–MS/MS determination, the samples were derivatized using N-trimethylsilylimidazol and N-methyl-N-(trimethylsilyl)trifluoroacetamide for 1 h at 60 °C.

### 2.3. Test organisms

*D. magna is* a common invertebrate model organism and keystone species in freshwater systems, playing a central role as an algal grazer and an important food source for fish. We selected a single clone of *D. magna*, originally collected from a pond in Ismaning (Germany) and cultured in the laboratory for several years. Age-synchronised females were isolated from the stock culture and maintained in artificial medium which was changed every other day. The daphnids were fed daily with a suspension of the green algae *Scenedesmus obliquus* ad libitum and were kept in a temperature chamber at 20 ± 0.5 °C with a 16 h light/8 h dark photoperiod regime. All experiments were performed under the same constant temperature and light conditions.

### 2.4. Multigeneration experiment

Twenty newly hatched neonates (F0) of the third brood from the age-synchronised mothers were selected randomly for each pharmaceutical treatment and transferred individually into glass vials containing 50 mL of test solution. The control treatment was started with 100 neonate daphnids. Test solutions in the glass vials were changed every third day using new medium from the corresponding reservoir aquaria. Animals were fed daily with a suspension of *S. obliquus* at a defined concentration of 2 mg C $L^{-1}$. To prevent diluting the concentration of pharmaceuticals in the vials, algae suspensions were prepared within the corresponding test solutions. Additionally, before adding algae to vials, the same amount of medium was removed from each glass vial.

F0 generation daphnids were controlled twice per day at a defined time. Age at first reproduction was recorded, and the body length of egg-carrying females was measured with a digital image analyzing system (Cell P, Olympus, Hamburg, Germany). The body length was defined as the distance from the upper edge of the compound eye to the base of the tail spine. After hatching of offspring, females of the F0 generation were removed from the glass vials. All newly hatched neonates from each female were counted and subsequently, a single neonate per clutch was randomly selected as the F1 generation to continue the experiment. The remaining neonate daphnids were removed and frozen at −80 °C for determination of body length and sex ratio. Five (if available) randomly

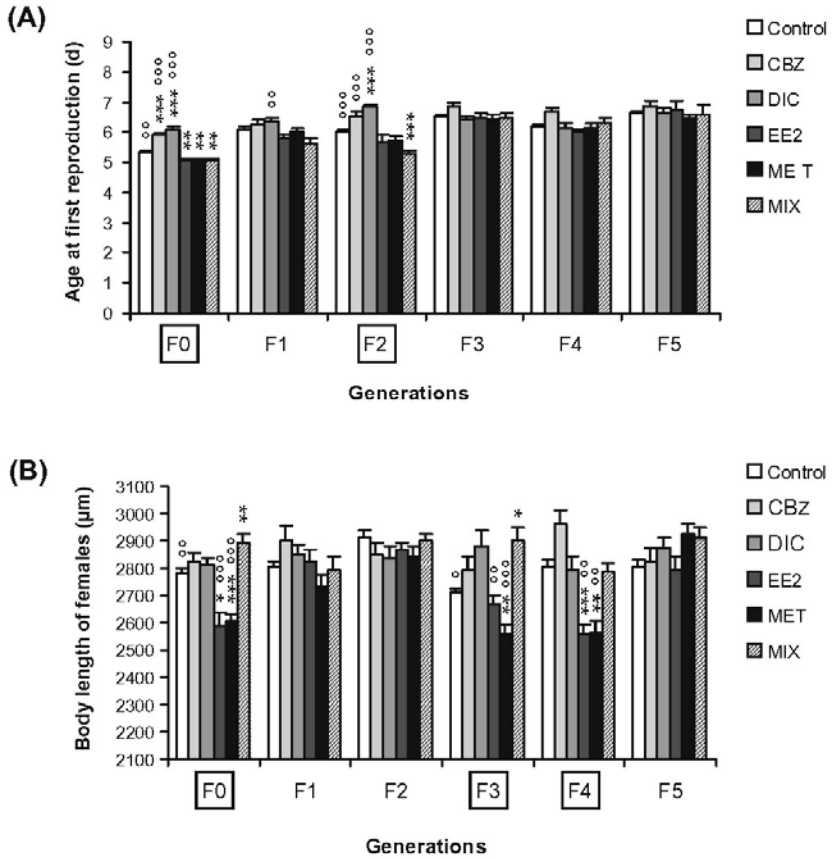


**Fig. 1.** (A) Age at first reproduction (mean + SE) and (B) body length of females (mean + SE) of *D. magna* exposed to pharmaceuticals (single substances and drug mixture) for six generations. Asterisks indicate statistically significant differences from the control treatment ($^{*}p \leq 0.050$; $^{**}p \leq 0.010$; $^{***}p < 0.001$). Open circles indicate statistically significant differences from the drug mixture ($^{\circ}p \leq 0.050$; $^{\circ\circ}p \leq 0.010$; $^{\circ\circ\circ}p < 0.001$). Frames indicate generations with one or more significant differences from the control.

chosen neonate daphnids per female were measured according to the method described above. Neonates that had already moulted were excluded from the analysis. Daphnids of subsequent generations were treated as in the F0 generation. Dead animals were counted, but not replaced in any treatment. The experiment was terminated after the neonates (F6) of the generation F5 had been hatched.

### 2.5. Statistical analysis

All statistical analyses were performed with SPSS 15.0 (SPSS Inc., Chicago, IL, USA). We utilized histograms to validate that observations within treatments were normally distributed. Differences in age at first reproduction, body length of females and number of offspring were compared to the control and to the drug mixture using one-way ANOVAs. Nested ANOVAs were performed to compare differences in body length of offspring to the control and to the pharmaceutical mixture. Tamhane's post hoc tests were applied to distinguish differences between means.

## 3. Results

### 3.1. Chemical analysis

During the experiment, we measured the following pharmaceutical concentrations in the drug mixture treatment: mean exposure level of CBZ: $0.492 \pm 0.007\ \mu g\ L^{-1}$ (98.4% of the nominal test concentration of $0.50\ \mu g\ L^{-1}$); mean exposure level of DIC: $0.355 \pm 0.007\ \mu g\ L^{-1}$ (98.6% of the nominal test concentration of $0.36\ \mu g\ L^{-1}$); mean exposure level of EE2: $0.101 \pm 0.003\ ng\ L^{-1}$ (101% of the nominal test concentration of $0.10\ ng\ L^{-1}$); mean exposure level of MET: $1.170 \pm 0.019\ \mu g\ L^{-1}$ (97.5% of the nominal test concentration of $1.20\ \mu g\ L^{-1}$).

### 3.2. Multigeneration experiment

Daphnids exposed to EE2, MET and to the drug mixture matured significantly faster than control animals in the F0 generation (Fig. 1A, Table S1 in Supplementary material). In contrast, age at first reproduction of daphnids in the CBZ and DIC treatments was significantly delayed compared to the control and the pharmaceutical mixture. In the F1 generation, we observed a significant increase in the age at first reproduction in the DIC treatment in comparison to the drug mixture. Animals exposed to the pharmaceutical mixture showed a significant decrease in the age at first reproduction in the F2 generation compared to the control, whereas daphnids of the DIC treatment matured significantly slower than the control treatment. A significantly slower maturation was also detected in the CBZ and DIC treatments compared to the pharmaceutical mixture. Age at first reproduction was not affected by drugs in generations F3, F4 and F5.

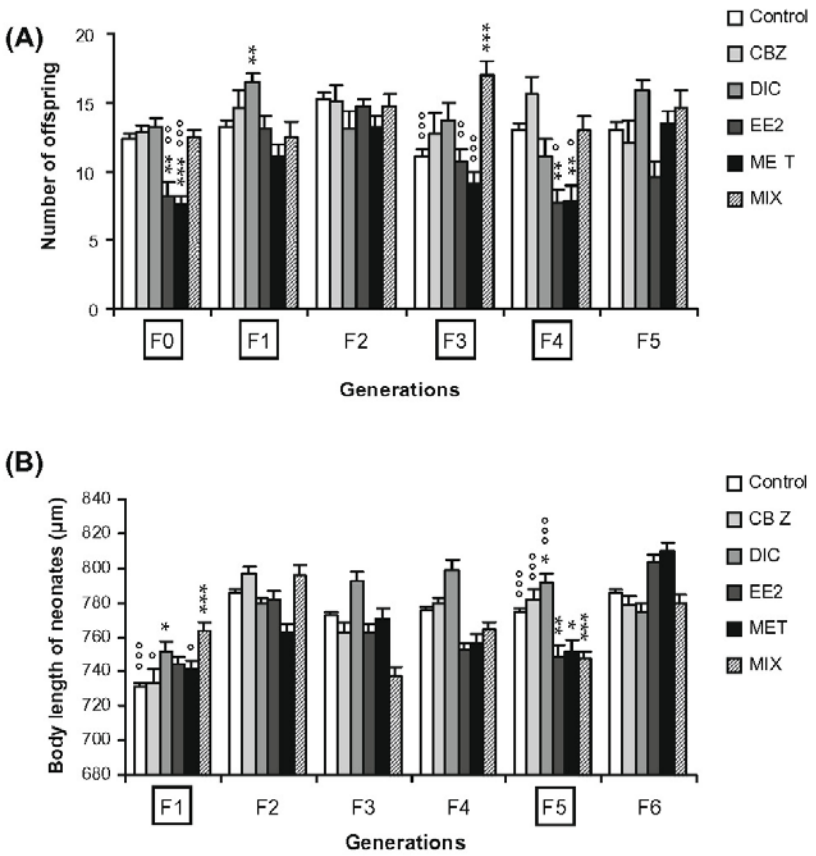


**Fig. 2.** (A) Number of offspring (mean + SE) and (B) body length of neonates (mean + SE) of *D. magna* exposed to pharmaceuticals (single substances and drug mixture) for six generations. Asterisks indicate statistically significant differences from the control treatment ($^{*}p \leq 0.050$; $^{**}p \leq 0.010$; $^{***}p < 0.001$). Open circles indicate statistically significant differences from the drug mixture ($^{\circ}p \leq 0.050$; $^{\circ\circ}p \leq 0.010$; $^{\circ\circ\circ}p < 0.001$). Frames indicate generations with one or more significant differences from the control.

Body length of females at first reproduction was significantly reduced in the F0 generation for the treatments EE2 and MET compared to the control and to the pharmaceutical mixture (Fig. 1B, Table S1 in Supplementary material). In contrast, daphnids exposed to the drug mixture were significantly larger than control animals. The same pattern was observed in the F3 generation except that the body length of females in the EE2 treatment did not differ from the control. In the F4 generation, daphnids of the treatments EE2 and MET were significantly smaller than the controls and daphnids exposed to the pharmaceutical mixture. No differences in body length at first reproduction were observed in generations F1, F2 and F5.

The number of offspring per female was significantly reduced in the F0 generation for the treatments EE2 and MET as compared to the control treatment and to the drug mixture (Fig. 2A, Table S2 in Supplementary material). In the F1 generation, we counted significantly more neonates in the DIC treatment than in the control. A significant increase in the number of offspring was also observed in daphnids exposed to the pharmaceutical mixture in the F3 generation. Additionally, compared to the drug mixture we detected a significantly decreased number of offspring in the treatments EE2 and MET. Significantly less neonates were also counted in the F4 generation for the EE2 and MET treatments in comparison to the control and the pharmaceutical mixture. There were no effects of pharmaceuticals on the number of offspring in the F2 and F5 generations.

Body length of neonates was only affected in generations F1 and F5 (Fig. 2B, Table S2 in Supplementary material). In generation F1, daphnids exposed to DIC and to the drug mixture were significantly larger than control animals. In addition, body length of neonates in the CBZ and MET treatments was significantly reduced as compared to the pharmaceutical mixture. In generation F5, we observed a significant decrease in body length for the treatments EE2 and MET and in the drug mixture treatment in comparison with the control. In contrast, daphnids exposed to DIC were significantly larger than control animals. Body length of neonates in the treatments CBZ and DIC was also significantly increased compared to the pharmaceutical mixture treatment.

No male offspring were produced in the control group or the pharmaceutical treatments in any generations. The mortality rates of control animals and exposed daphnids are presented in Table S3 (Supplementary material) for each generation.

## 4. Discussion

### 4.1. Multigenerational effects

In the multigenerational study, we examined the toxicity of pharmaceuticals at environmentally relevant concentrations in *D. magna* over six generations. Concerning the first investigated generation, considerable drug effects occurred over all observed life-history and morphological parameters of the exposed daphnids. In the following generation, we could not detect the same impact of drugs as in the initial generation. Moreover, with the exception of the number of offspring, none of the other three measured parameters were affected by any pharmaceutical or by the drug mixture. A potential explanation for this pattern is the development of resistance. Klerks and Weis (1987) postulated two possible reasons why organisms exhibit resistance to a pollutant. They may acquire tolerance via physiological acclimation, during exposure to sub lethal concentrations of a toxic substance at a prior period of their life span. Alternatively, populations may evolve genetically based resistance due to natural selection. We can exclude the latter in our experiment; thus we suppose that physiological acclimation is the probable mechanism for resistance to the pharmaceuticals in our study.

As our experiment was started with neonates from unexposed mothers, a pharmaceutical exposure during developmental stages as oogenesis and embryogenesis was lacking in the first investigated generation. However, we assume that a prenatal exposure to drugs is necessary for daphnids to acclimate to these toxic substances. This may explain why the animals of the first investigated generation exhibited no resistance toward the pharmaceuticals, whereas the successive generations could develop drug resistance. This is in accordance with Tanaka and Nakanishi (2002) who suggested that parental exposure during developmental stages of *D. galeata* may be responsible for the decreased effects of the endocrine disruptor p-nonylphenol (50 $\mu g\,L^{-1}$) on the second generation.

Development of resistance toward toxic compounds has also been shown by Clubbs and Brooks (2007), who observed that second generation *D. magna* can acclimate to endocrine-active drugs, leading to diminished sub lethal responses in fecundity of females and in sex ratio and dry weight of neonates.

In contrast, some multigenerational studies have found no acclimation, but enhanced effects of contaminants in later generations of aquatic invertebrates. For instance, male amphipods (*Hyalella azteca*) exposed to 0.1 and 0.32 $\mu g\,L^{-1}$ EE2 developed significantly smaller second gnathopods in the second generation than in the parental generation, and a slightly increased occurence of females was noted in later generations (Vandenbergh et al., 2003). Moreover, Brennan et al. (2006) showed that 0.2–0.5 $mg\,L^{-1}$ of the estrogen diethylstilbestrol reduced the number of offspring in the second generation of *D. magna*, whereas no effects were observed in the parental generation. They supposed that the offspring of the first generation were weakened, leading to a higher susceptibility to the toxic effects of the estrogenic compounds in the second generation.

Resistance or enhanced toxicity in aquatic organisms depends presumably on the mode of action of the toxic compounds. In addition, we assume that the various taxa of non-target organisms possess different susceptibilities to toxic substances, resulting in contrary effects. Furthermore, it is likely that some aquatic invertebrates cannot develop resistance toward the permanent exposure to toxic substances over generations probably due to high energetic costs.

Moreover, several studies have demonstrated that acclimation to toxic compounds is often associated with elevated fitness costs. For instance, Kwok et al. (2009) showed that copper resistant copepods (*Tigriopus japonicus*) had a significantly lower intrinsic population growth rate, presumably since acclimation to a toxic compound demands energetic costs for defense, detoxification and repair mechanisms. The recurrence of drug effects, observed in our experiment in later generations for all life-history and morphological parameters, may indicate that the daphnids cannot maintain costly resistance to pharmaceuticals over several generations.

Xie and Klerks (2004) postulated that high fitness costs may restrict the evolution of resistance to pollutants. Although non-target organisms can presumably acclimate to toxic compounds for a short time, they may not evolve resistance to these substances in natural aquatic systems. Therefore, we assume that detrimental effects of pharmaceuticals in aquatic ecosystems cannot be excluded.

### 4.2. Single and combined drug effects

In this study, we analysed the toxic effects of CBZ, DIC, EE2 and MET on *D. magna* as single compounds and compared them with the influence of a drug mixture of all compounds. Daphnids exposed to the β-blocker MET were in some generations significantly smaller at first reproduction and released significantly less neonates than

the control animals. This is in accordance with a study by Dzialowski et al. (2006) who found decreased growth and reduced fecundity in *D. magna* after subchronic exposure to MET at concentrations in the mg $L^{-1}$ range. Moreover, exposure of the cladoceran *Ceriodaphnia dubia* and the amphipod *H. azteca* to the β-blocker propranolol caused a decrease in the production of neonates at concentrations above 250 μg $L^{-1}$ and 100 μg $L^{-1}$, respectively (Huggett et al., 2002). The decreased number of offspring in our study is likely a consequence of the reduced size at first reproduction, as body length of female daphnids and the number of eggs per clutch are positively correlated (Lürling et al., 2006). Although a small clutch size is often compensated by larger offspring (Ebert, 1993), we could not observe an increase in body length of neonate daphnids exposed to MET. Instead, the body length of neonates in the MET treatment was similar or even shorter than the size of the control animals, presumably due to the toxicity of this pharmaceutical.

The estrogen EE2 caused almost the same effects on exposed daphnids as MET, resulting in a reduced body size at first reproduction and a decreased number of offspring in some generations. Several studies showed similar effects for endocrine compounds toward aquatic invertebrates. For example, Ladewig et al. (2006) detected an influence of endocrine substances on the body size of *Gammarus fossarum*, resulting in smaller animals downstream from sewage treatment plant effluents. In addition, Goto and Hiromi (2003) showed in chronic toxicity tests that the number of offspring in *D. magna* was reduced by EE2 at 100 and 500 ng $L^{-1}$.

The non-steroidal anti-inflammatory drug DIC had a slightly stimulatory effect on the body length of neonates, resulting partly in an significant increase in body size. However, maturation was negatively affected by DIC which led to a delayed age at first reproduction in some generations. An exposition of *D. magna* to 40 mg $L^{-1}$ of the non-steroidal anti-inflammatory drug ibuprofen also caused a significant delay in the age at first reproduction (Heckmann et al., 2007). Furthermore, Ferrari et al. (2003) showed that 2 mg $L^{-1}$ of DIC inhibited the reproduction of *C. dubia* after 7 d of exposure.

We could not detect any effects of the antiepileptic agent CBZ on the observed life-history and morphological parameters of *D. magna* which occurred in more than one generation. This is in accordance with Lürling et al. (2006) who did not observe any effects of CBZ (0.1–200 μg $L^{-1}$) on the size at first reproduction of D. *pulex* and on the body length of neonates.

Comparing the influence of the drug mixture with the impact of the single compounds CBZ, DIC, EE2 and MET, it seems that the pharmaceutical mixture did not provoke stronger effects on the exposed daphnids than the single drugs. This is in disagreement with Cleuvers (2003, 2004) who detected an enhanced toxicity of drugs toward daphnids when pharmaceuticals were applied as a mixture. In addition, our results are in contrast to a study by Schnell et al. (2009) who showed that the toxic effects of a mixture of pharmaceuticals and personal care products from different therapeutic classes on liver cells of rainbow trouts (*Oncorhynchus mykiss*) were greater than predicted due to synergistic effects of the substances.

However, the results of our study indicate that the drug mixture affected *D. magna* partly in a contrary way to single substances. For example, daphnids exposed to the pharmaceutical mixture were in some generations significantly larger at first reproduction than control animals, although the single substances caused either no effect or even reduced the body length. In addition, the drug mixture influenced the successive generations of exposed daphnids partly in a quite inconsistent way. For instance, the body length of neonates was either unchanged by the pharmaceutical mixture or significantly increased or decreased compared to the control. As the influence of the pharmaceutical mixture was very inconsistent in our study, it was not possible to draw conclusions from the effects of the single compounds CBZ, DIC, EE2 and MET in comparison to their mixture toxicity. This is supported by Flaherty and Dodson (2005) who showed that the toxicity of drug mixtures is unpredictable and complex compared to effects of single pharmaceuticals. Therefore, it is very difficult to assess the risk of pharmaceuticals on non-target organisms in natural aquatic systems, where animals are permanently exposed to complex drug mixtures.

## 5. Conclusions

Our results demonstrate inconsistent effects of pharmaceuticals on *D. magna*. In particular, drugs acting in combination can lead to impairments that are not predicted by the response to single substances alone. Further research is needed to extend our knowledge of the influence of pharmaceuticals and especially of complex drug mixtures on non-target organisms. In addition, more multigenerational studies are urgently needed, as potential effects can be missed in single-generation experiments. Our study shows that drug effects can recur after a short acclimation period in later generations of *D. magna*. Hence, we anticipate that aquatic organisms may not evolve a resistance to pharmaceuticals in natural aquatic systems, presumably due to high fitness costs. We thus cannot exclude harmful effects of pharmaceuticals in aquatic ecosystems.

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## Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.chemosphere.2009.12.069.

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# Effects of a pharmaceutical mixture at environmentally relevant concentrations on the amphipod *Gammarus fossarum*

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Aufgrund des kontinuierlichen Arzneimitteleintrags in die Gewässer sind aquatische Organismen permanent komplexen Arzneistoffgemischen ausgesetzt. So treten das Antiepileptikum Carbamazepin, das Antirheumatikum Diclofenac, das synthetische Estrogen 17α-Ethinylestradiol sowie das Antihypertonikum Metoprolol häufig in Kombination in Gewässern des süddeutschen Raumes auf.

In einem Laborexperiment wurden die Auswirkungen dieses Arzneistoffgemisches sowohl in einer umweltrelevanten als auch in einer artifiziell erhöhten Konzentration auf den Bachflohkrebs *Gammarus fossarum* untersucht. Da Gammariden sensitiv auf Schadstoffe reagieren können, wurden sie bereits bei Toxizitätstest mit Schwermetallen und endokrinen Substanzen eingesetzt. Jedoch gibt es bislang kaum Studien zum Einfluss von Arzneistoffgemischen auf Gammariden. Daher wurden Langzeitexperimente durchgeführt, bei denen die Auswirkungen des Wirkstoffgemisches auf die Häutung, die Reproduktion sowie auf den Gehalt des Energiespeicherproduktes Glykogen von *Gammarus fossarum* getestet wurden. Da die Häutung und Reproduktion von Gammariden von einem hohen metabolischen Umsatz begleitet sind, könnten diese sensitiven Lebenszyklusstadien anfällig für Schadstoffe sein. Des Weiteren kann es durch die Arzneimittelexposition zu einem veränderten Energiehaushalt kommen.

Es zeigte sich, dass die Arzneistoffe in der umweltrelevanten wie auch in der erhöhten Konzentration die Häutung der Gammariden beeinflussten: Während in der Kontrollgruppe nach jeder Häutung ein kontinuierlicher Größenzuwachs verzeichnet werden konnte, kam es durch die Arzneistoffbehandlung zu einem unregelmäßigen Größenwachstum. Zudem war die Zeit zwischen den Häutungen in beiden Arzneistofftreatments im Vergleich zur Kontrolle verkürzt. Auf die Reproduktion wirkte sich das Arzneimittelgemisch jedoch nicht aus. Ebenfalls konnte kein Einfluss der Arzneistoffe auf den Glykogengehalt ermittelt werden.

Aufgrund der gezeigten Auswirkungen auf die Häutung kann bei einer permanenten Arzneimittelexposition in natürlichen Gewässern eine Beeinträchtigung der gesamten Gammaridenpopulation nicht ausgeschlossen werden. So können die Organismen in Folge des durch die Arzneimittel verursachten diskontinuierlichen Größenzuwachses kleiner werden, was zu erheblichen Fitnessnachteilen und einer verringerten Überlebenswahrscheinlichkeit führen kann. Da Gammariden ein essentieller Bestandteil für die Ernährung etlicher Fischarten sind und somit eine wichtige Rolle im Nahrungsnetz spielen, könnte es durch die permanente Belastung der Umwelt mit Arzneimitteln zu schwerwiegenden Folgen für das aquatische Ökosystem kommen.

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# Effects of a pharmaceutical mixture at environmentally relevant concentrations on the amphipod *Gammarus fossarum*

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**Abstract.** The continuous discharge of pharmaceuticals into the environment results in the chronic exposure of aquatic organisms to complex drug mixtures. We examined the influence of a mixture of pharmaceuticals (carbamazepine (CBZ), diclofenac (DIC), metoprolol (MET) and 17α-ethinylestradiol (EE2)) at environmentally relevant ('env') and artificially high ('high') concentrations on *Gammarus fossarum*. Different sublethal responses such as moulting, reproduction and the content of the energy-storage component glycogen were analysed. The drug mixture influenced the moulting behaviour of gammarids at both the 'env' and 'high' concentration levels, leading to a discontinuous increase of body length in successive moults, compared with the constant increase of body length in the control treatment. Moreover, the time between successive moults of animals exposed to the 'env' and 'high' pharmaceutical concentrations was decreased because of shortened intermoult periods. We observed no significant impact of the pharmaceuticals on reproduction. In addition, the content of glycogen was not significantly affected by the drug mixture. Permanent exposure of *G. fossarum* to a wider range of pharmaceuticals in natural aquatic systems may influence moulting behaviour and accompanied life-history parameters, followed by severe ecological consequences as gammarids play an important role in many freshwater ecosystems of the northern hemisphere.

**Additional keywords:** amphipoda, life-history traits, micropollutants.

## Introduction

In recent years, an increasing number of pharmaceuticals have been detected in aquatic systems across the world (e.g. Ternes 1998; Heberer 2002; Kolpin *et al.* 2002). Pharmaceuticals are bioactive and often extremely persistent (Halling-Sørensen *et al.* 1998; Fent *et al.* 2006); thus, they are potentially hazardous to aquatic organisms, even at low concentrations (Daughton and Ternes 1999; Ferrari *et al.* 2003). Drugs enter surface waters primarily via sewage effluents after consumption by humans and subsequent excretion (Halling-Sørensen *et al.* 1998; Kreuzinger *et al.* 2004; Fent *et al.* 2006). Incomplete removal of many pharmaceuticals by sewage treatment plants (Ternes 1998) leads to a permanent discharge of metabolites and parent compounds at low concentrations into the aquatic environment.

Among the pharmaceuticals that are found commonly and in increased quantities in rivers and streams of Germany are the anti-epileptic agent carbamazepine (CBZ), the non-steroidal anti-inflammatory drug diclofenac (DIC), the β-blocker metoprolol (MET) and the synthetic oestrogen 17α-ethinylestradiol (EE2) (Adler *et al.* 2001; Sengl and Schüssler 2004). CBZ is one of the most persistent compounds in aquatic ecosystems, having an elimination rate from sewage treatment plants below 10% (Kreuzinger *et al.* 2004), and has been detected in German surface waters with a maximum concentration of 1.1 $\mu g L^{-1}$ (Ternes 1998; Heberer 2002). The anti-inflammatory agent DIC occurs in German rivers and streams at concentrations up to 1.2 $\mu g L^{-1}$ (Ternes 1998). MET was reported to reach 2.2 $\mu g L^{-1}$ in surface waters of Germany (Ternes 1998) and the estrogenic compound EE2 has been found with a maximum concentration of 2 $ng L^{-1}$ (Adler *et al.* 2001).

All these drugs pose potential environmental risks (Rohweder 2003). The toxic effects of such single substances towards aquatic vertebrates and invertebrates have been documented; however, these were frequently documented at much higher concentrations than measured in the field. For instance, exposure of the midge *Chironomus riparius* to sediments spiked with the anti-epileptic agent CBZ (0.16–100 $mg kg^{-1}$ dry weight) for 28 days resulted in a concentration-dependent decrease of emergence because of a blockade of pupation (Oetken *et al.* 2005). Schwaiger *et al.* (2004) and Triebskorn *et al.* (2004) exposed rainbow trout (*Oncorhynchus mykiss*) for 28 days to DIC (1–500 $\mu g L^{-1}$), resulting in histopathological and cytological

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alterations of various organs and in a concentration-related bioaccumulation of DIC in all investigated tissues. The freshwater cladoceran *Daphnia magna* exposed to the β-blocker MET for 9 days showed decreased growth, fecundity and heart rate at concentrations ranging from 3.1 to 25 mg $L^{-1}$ (Dzialowski *et al.* 2006). Although the concentrations of the oestrogen EE2 detected in aquatic systems are very low, EE2 may still affect non-target organisms because of its high biological activity (Fent *et al.* 2006). For example, *D. magna* exposed for 25 days to EE2 at concentrations of 100 and 500 ng $L^{-1}$ showed a reduced number of offspring (Goto and Hiromi 2003).

Since organisms are exposed not only to isolated pharmaceuticals in the aquatic environment, but to complex mixtures of drugs, it is important to study the combined effects (e.g. Altenburger *et al.* 2004; Fent *et al.* 2006; Schwarzenbach *et al.* 2006). Cleuvers (2003, 2004) highlighted in recent studies with *Daphnia* that pharmaceutical mixtures can elicit stronger effects than the single compounds at the same concentrations.

However, most studies on the mixture toxicity of pharmaceuticals have been performed with relatively short-lived organisms such as daphnids (Christensen *et al.* 2007; Henry and Black 2007). To determine the impact of chemical compounds in aquatic ecosystems, more than one invertebrate taxon should be used (McCahon and Pascoe 1988*a*). Despite the difficulty of maintaining field-collected organisms in the laboratory, it is important that long-lived freshwater invertebrates with complex life histories, such as gammarids, are assessed for testing the mixture toxicity of pharmaceuticals.

Gammarids are among the most common invertebrates found in European streams. They feed predominantly on detritus (Eggers and Martens 2001) and are an important prey item for many species of fish (MacNeil *et al.* 1999). Therefore, they play a key role in the food chain of freshwater ecosystems. Because gammarids are sensitive to a wide range of pollutants, they are often used in toxicity tests, predominantly for testing toxic effects of heavy metals and endocrine-disrupting chemicals (McCahon and Pascoe 1988*b*; Schirling *et al.* 2006). However, studies concerning the chronic influence of drug mixtures on gammarids are scarce.

In the present study, *Gammarus fossarum* was used as a model organism for stream invertebrates (Ladewig *et al.* 2006) to test a mixture of CBZ, DIC, MET and EE2. These drugs often occur in combination in German surface waters and their environmental fate renders them potentially hazardous to the aquatic biota. Pharmaceuticals were examined at environmentally relevant concentrations to simulate natural conditions as closely as possible and at artificially high concentrations to test for any potential effects. To assess the impact of the drug mixture on *G. fossarum*, we performed an analysis on different sublethal responses, including moulting and reproduction. Because the latter are accompanied by considerable metabolic stress (Buikema and Benfield 1979; McCahon and Pascoe 1988*b*, 1988*c*), we hypothesised that these very sensitive life-cycle stages are susceptible to a mixture of pharmaceuticals, potentially resulting in an abnormal moulting behaviour and a reduced fecundity. An analysis of the glycogen content, a major energy-storage component in animals, may provide insights into the metabolic energy management and the ecological fitness (Winkelmann and Koop 2007) of *G. fossarum* exposed to a mixture of drugs. We anticipated that a pharmaceutical mixture may diminish the stored-energy component glycogen.

## Materials and methods

### Test organism

*Gammarus fossarum* was collected by kick-sampling from the Schlierbach, an unpolluted mountain creek situated near Hinterwössen in south-eastern Germany (47°42′N, 12°27′E). Gammarids were transported to the laboratory immediately after collection and cultured in 30-L plastic buckets for 2 days before the experiments. The buckets contained 10 L of stream water that was continually diluted with artificial medium based on ultra pure water and trace elements (Laforsch *et al.* 2006) to allow for a steady acclimatisation to laboratory conditions. A sufficient oxygen supply was ensured by an airstone. Gammarids were fed with dried nettles (*Urtica dioica*) and flaked fish food which is suggested as a surrogate food for shredding species (OECD 2004). Additionally, dried leaves from beeches (*Fagus sylvatica*) representing the natural riverine vegetation of the sampling site were added as food and shelter for the animals. Gammarids were kept in a temperature-controlled room at 12 ± 1°C with a 12 h light/12 h dark photoperiod regime. All experiments were performed under the same constant conditions.

### Chemical compounds

A mixture of CBZ ((Z)-5H-dibenzo[b,f]azepine-5-carboxamide; Sigma Aldrich, Seelze, Germany), DIC (2-[(2,6-dichlorophenyl) amino]benzeneacetic acid sodium salt; Sigma Aldrich, Seelze, Germany), MET (1(±)1-(Isopropylamino)-3-[p-(β-methoxyethyl)phenoxy]-2-propanol tartrate salt; Sigma Aldrich, Seelze, Germany) and EE2 (17-ethynyl-13-methyl-7,8,9,11,12,13,14, 15,16,17-decahydro-6H-cyclopenta[a]phenanthrene-3,17-diol; Fluka, Buchs, Switzerland) was prepared in environmentally relevant ('env') and artificially high ('high') concentrations. Test concentrations for the treatment 'env' were 0.50 μg $L^{-1}$, 0.36 μg $L^{-1}$ and 1.20 μg $L^{-1}$ for CBZ, DIC and MET, respectively. These treatment levels represent the maximal concentrations found in rivers and streams of southern Germany (Sengl and Schüssler 2004). The concentration of EE2 was set at 0.10 ng $L^{-1}$ representing the median concentration of forms of EE2 found in surface waters of southern and central Germany (Adler *et al.* 2001). Concentrations for the treatment 'high' were 1 μg $L^{-1}$ for EE2 and 1 mg $L^{-1}$ for the other three pharmaceuticals.

Drugs were dissolved in methanol (HPLC grade) to produce solutions with a concentration of 0.1 μg $\mu L^{-1}$ which were stored in the dark at −20°C. New stock solutions were prepared every second week. For preparation of the treatment 'env', 40.0 μL of the CBZ solution, 28.8 μL of the DIC solution and 96.0 μL of the MET solution were transferred to an object slide. The EE2 stock solution was diluted to a concentration of 0.01 ng $\mu L^{-1}$ for the preparation of the slides. A volume of 80 μL of the latter was added to the object slide with the other three chemicals, and the mixture was subsequently dried under a gentle stream of nitrogen. By using a nitrogen shower, only the solvent evaporates, whereas the drugs remain on the object slide without a decrease in concentration. Hence, the dissolution of the pharmaceuticals can be provided without the side effect of introducing organic solvents into the biological system.

For the preparation of the treatment 'high', 8.0 mg CBZ, 8.6 mg DIC sodium salt and 10.3 mg MET tartrate salt were weighed into a 2-mL reaction tube. Because only a very low concentration of EE2 was required, it was not added to the reaction tube. Instead, 80 μL of EE2 stock solution was separately transferred to an object slide and dried under a nitrogen stream.

If possible, pharmaceuticals were used in ionic form to enhance their dissolution in the test media. Chemical analyses by GC–MS/MS, which were conducted in preliminary experiments to test for the dissolution of the pharmaceuticals, showed that the initial drug concentrations were reached after 1 day. However, we cannot exclude that the pharmaceuticals are partially decomposed over the entire exposure period.

### *Moulting behaviour*

At the beginning of the experiment, body lengths of the gammarids were measured with a digital image analysing system, using polygon length determination (Cell P, Olympus, Hamburg, Germany). The body length was defined as the distance from the base of the first antennae along the dorsal side of the body to the edge of the third urosome segment.

For each treatment, 30 juvenile gammarids ranging 3.5–7.0 mm were selected randomly and transferred individually into a 30-mL glass vial, which was closed with gauze (500-μm mesh size). Three randomly chosen vials were put into each of 30 12-L glass aquaria containing 8 L of artificial medium. A single object slide of the treatment 'env' was randomly placed into each of 10 aquaria. The same procedure was carried out for treatment 'high', except that the object slide as well as the dry chemical mixture were added. The 10 remaining aquaria without any pharmaceuticals served as the control treatment. Test solutions were exchanged every second week. All aquaria were aerated with an airstone that was placed directly in front of the glass vials facing the side closed by the gauze; this was done to ensure an exchange of the test solution and a sufficient supply of oxygen. To avoid evaporation, the aquaria were covered with glass plates. One flake of fish food as well as one dried leaf of nettle (*U. dioica*) and one dried leaf of beech (*F. sylvatica*) were added to each glass vial. The fish food and the nettle were renewed once a week, whereas the leaf of beech was not exchanged during the experiment because gammarids feed additionally on the microflora of bacteria and fungi developing on the surface of decaying leaves (Kaushik and Hynes 1971; Sutcliffe *et al.* 1981). Because only beech leaves previously submerged in the Schlierbach were collected, recolonisation with bacteria and fungi was provided by resting stages on these leaves.

The gammarids were examined individually every second day to determine the effects of the mixture of CBZ, DIC, MET and EE2 on mortality and moulting behaviour. When a *Gammarus* moulted, the exuvium was removed from the glass vial and the body length of the *Gammarus* was measured. Additionally, the time between successive moults was noted. The experiment was terminated after 100 days.

### *Reproduction*

Precopula pairs were separated from the stock culture and maintained in a 30-L plastic bucket containing 15 L of artificial medium and food. Immediately after copulation, egg-carrying females were isolated and transferred individually into a 250-mL glass vial closed with gauze (500-μm mesh size). Thirteen egg-carrying females were randomly assigned to each treatment group. The experimental design was carried out as described above, with the exception that a single glass vial was placed into an aquarium and the gammarids were checked once a day.

The time for egg development was recorded, which was defined as the period from copulation to the release of neonate gammarids out of the brood pouch. The degree of degeneration of the brood of egg-carrying females was noted and the number of offspring per female counted. Additionally, the body length of three (if available) randomly chosen neonate gammarids per female was measured according to the method described above. The experiment was terminated after all females had released their offspring.

### *The energy-storage component glycogen*

Juvenile *G. fossarum* were selected randomly from the stock culture. Thirty gammarids were transferred randomly into each of nine 12-L glass aquaria filled with 8 L of artificial medium. The object slides of the treatment 'env' were placed randomly into three of the nine aquaria. For the treatment 'high', the object slides plus the dry chemical mixture were added to three additional aquaria. Three remaining aquaria without any pharmaceuticals served as the control treatment. Test solutions were exchanged every second week. Gammarids were fed with flaked fish food and dried nettles (*U. dioica*) *ad libitum*. Additionally, about 15 dried leaves of beeches (*F. sylvatica*) were added as food and shelter for the animals. With the exception of the beech leaves, which were not replaced during the experiment, food was renewed twice per week.

After 4, 8 and 12 weeks, three gammarids were selected randomly from each aquarium. However, sampling of precopula pairs or egg-carrying females was avoided, because these animals probably have other glycogen contents owing to the costs of reproduction. Gammarids were transferred individually into 1.5-mL screw-cap micro tubes and fixed with liquid nitrogen for further analyses of their glycogen. Enzymatic assays according to the method of Bergmeyer (1985) and Winkelmann and Koop (2007) were applied for the determination of the glycogen content of individual gammarids. Because a marginal weight of the homogenate of fixed animals can cause considerable measuring inaccuracies, samples of ≤1 mg were excluded from the analysis.

### *Statistical analysis*

All statistical analyses were performed with SPSS 15.0 (SPSS Inc., Chicago, IL, USA). If there was more than one individual per aquarium, we pooled the gammarids in each aquarium and calculated average values to avoid pseudoreplication. Differences in the body-length increase and time between successive moults were compared with the control by using simple linear regressions and two-tailed *t*-tests. Mortality rate was $\sqrt{}$arcsin-transformed and a one-way ANOVA was performed to compare differences between the treatments. Differences in the time for egg development and numbers of offspring were compared with the control by using one-way ANOVAs. To compare differences in the body length of offspring, a two-level nested ANOVA

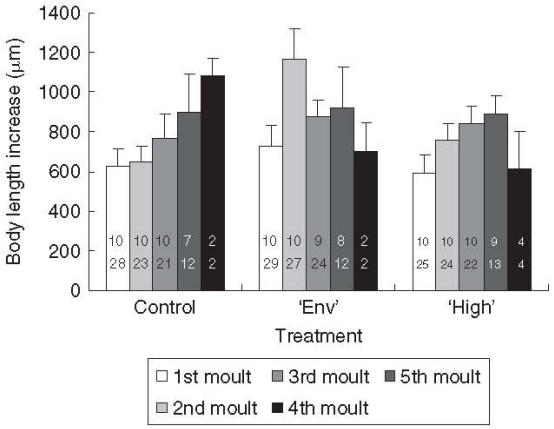


**Fig. 1.** Body-length increase (mean + s.e.) of *Gammarus fossarum* exposed to a mixture of CBZ, DIC, MET and EE2 at environmentally relevant ('env') and artificially high ('high') concentrations for 100 days. The upper value in each bar indicates the number of aquaria (i.e. the number of replicates), the lower value indicates the total number of gammarids. See text for definition of CBZ, DIC, MET and EE2.

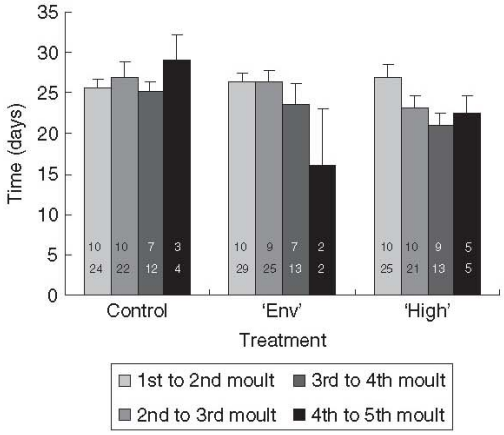


**Fig. 2.** Time between successive moults (mean + s.e.) of *Gammarus fossarum* exposed to a mixture of CBZ, DIC, MET and EE2 at environmentally relevant ('env') and artificially high ('high') concentrations for 100 days. The upper value in each bar indicates the number of aquaria (i.e. the number of replicates), the lower value indicates the total number of gammarids. See text for definition of CBZ, DIC, MET and EE2.

with treatment as a fixed factor and 13 replicates per treatment as a random factor was performed. The glycogen content was compared with the control by using a two-way ANOVA, with treatment and time as fixed factors. Histograms were used to validate that observations within treatments were normally distributed. Homogeneity of variance was checked by means of residual analysis for the linear regressions and with Levene's test for the ANOVAs. As the variances of the observations were not always homogenous, Tamhane's post hoc tests were utilised to distinguish differences between means.

## Results

### *Moulting behaviour*

The body length of gammarids from the control treatment showed a continuous increase from the first to the fifth moult (Fig. 1; simple linear regression: $F_{1,37} = 4.918$, $P = 0.033$). In contrast, the increase in body length of *G. fossarum* exposed to the 'env' and 'high' treatments of drug concentrations was discontinuous with an irregular pattern (simple linear regressions: 'env': $F_{1,37} = 0.011$, $P = 0.916$, 'high': $F_{1,41} = 1.881$, $P = 0.178$). However, there was no significant difference in the gradient of body-length increase between either the control and the 'env' treatment ($t_{77} = 1.307$, $P = 0.195$) or the control and the 'high' treatment ($t_{81} = 0.983$, $P = 0.329$).

The time between successive moults did not change in the control treatment (Fig. 2; simple linear regression: $F_{1,28} = 0.405$, $P = 0.530$). However, the intermoult period of *G. fossarum* exposed to the 'env' pharmaceutical concentrations was shortened starting from the third moult (simple linear regression: $F_{1,26} = 5.107$, $P = 0.032$). There was a significant difference in the time between successive moults for the control and the 'env' treatment ($t_{57} = 2.159$, $P = 0.035$). In addition, gammarids exposed to the 'high' drug concentrations showed a shortened intermoult period starting from the second moult (simple linear regression: $F_{1,32} = 5.820$, $P = 0.022$), resulting in a significant difference in the time between successive moults for the control and the 'high' treatment ($t_{63} = 2.080$, $P = 0.042$).

During the 100-day exposure period, 12 gammarids (40.0%) from the control treatment died, with a marked increase in mortality observed after 30 days. The mortality of *G. fossarum* exposed to the mixture of drugs was approximately the same at both concentrations; in the 'env' treatment there were six dead gammarids (20.0%) and in the 'high' treatment there were seven dead gammarids (23.3%) at the end of this experiment. Mortality rate did not differ significantly between the treatments (one-way ANOVA: $F_{2,27} = 1.730$, $P = 0.196$).

### *Reproduction*

During the experiment, four egg-carrying females (30.8%) exposed to the 'high' treatment levels and one egg-carrying female (7.7%) exposed to the 'env' mixture of pharmaceuticals lost their broods. A degeneration of the brood also occurred in two egg-carrying females (15.4%) from the control.

Compared with the control treatment, the time for egg development was decreased slightly at the 'env' and 'high' drug concentrations, but there was no significant difference in the time for egg development between the gammarids from the control treatment and those exposed to the pharmaceuticals (Table 1; one-way ANOVA: $F_{2,29} = 0.907$, $P = 0.415$).

The mixture of pharmaceuticals showed no significant impact on the number of offspring (Table 1; one-way ANOVA: $F_{2,29} = 0.006$, $P = 0.994$).

The body length of the neonate gammarids was not affected significantly by the drugs, but the offspring of *G. fossarum* exposed to the 'high' pharmaceutical concentrations were marginally smaller than the gammarids from the control (Table 1; nested ANOVA: $F_{2,30} = 0.477$, $P = 0.626$).

**Table 1. Reproduction parameters (mean ± s.e.) of *Gammarus fossarum* exposed to a mixture of CBZ, DIC, MET and EE2 at environmentally relevant ('env') and artificially high ('high') concentrations**
See text for definition of CBZ, DIC, MET and EE2

| Parameter | Control | | 'Env' | | 'High' | |
|---|---|---|---|---|---|---|
| | Mean ± s.e. | *n* | Mean ± s.e. | *n* | Mean ± s.e. | *n* |
| Time for egg development (days) | 43.82 ± 1.08 | 11 | 41.92 ± 1.32 | 12 | 41.56 ± 1.41 | 9 |
| Number of offspring | 10.73 ± 1.78 | 11 | 10.50 ± 1.91 | 12 | 10.78 ± 2.13 | 9 |
| Body length of offspring (μm) | 2301.52 ± 18.87 | 33 | 2298.28 ± 15.41 | 33 | 2272.15 ± 26.08 | 25 |

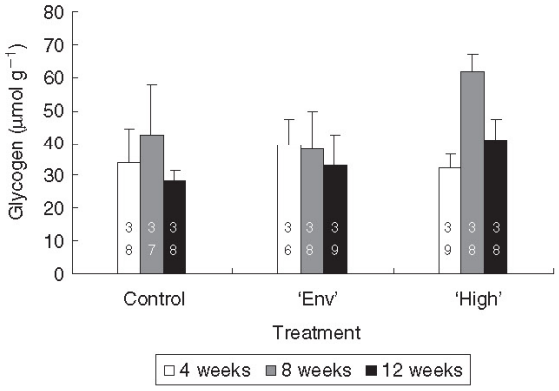


**Fig. 3.** Glycogen content (mean + s.e.) of *Gammarus fossarum* after 4, 8 and 12 weeks of exposure to a mixture of CBZ, DIC, MET and EE2 at environmentally relevant ('env') and artificially high ('high') concentrations. The upper value in each bar indicates the number of aquaria (i.e. the number of replicates), the lower value indicates the total number of gammarids. See text for definition of CBZ, DIC, MET and EE2.

### *The energy storage component glycogen*

The glycogen content in *G. fossarum* from the control group was nearly constant over the entire exposure period (Fig. 3). Furthermore, gammarids exposed to the 'env' treatment had glycogen concentrations of almost the same level. In contrast, animals at the 'high' drug concentrations showed a slight increase in glycogen after 8 weeks of exposure, whereas the glycogen content after 4 and 12 weeks was comparable. However, because of the high variances within treatments, there were no significant differences in glycogen content between gammarids exposed to the pharmaceuticals and the control group (two-way ANOVA: $F_{2,18} = 1.088$, $P = 0.358$). In addition, no time effect (two-way ANOVA: $F_{2,18} = 2.086$, $P = 0.153$) and no interaction between time and treatment (two-way ANOVA: $F_{4,18} = 0.808$, $P = 0.536$) were detected.

## Discussion

### *Chemical compounds*

Depending on their chemical structure, pharmaceuticals are subject to chemical decomposition. Moreover, in our experiments, the drugs used may form hydrophobic interactions with the silicon sealings of the aquaria, other parts of the experimental setup or with the biofilms that form over the course of the experiments. Hence, we cannot exclude that the drug concentrations in our study were partially decreased, although preliminary experiments showed that the drug mixture was completely dissolved in the test media at the beginning of the experiments. Thus, it may be speculated that the impact of the pharmaceutical mixture on *G. fossarum* is slightly underestimated in our study.

### *Moulting behaviour*

Our results show that the growth of animals exposed to the 'env' and 'high' pharmaceutical concentrations followed an irregular pattern, whereas the control group exhibited a continuous increase in body length (Fig. 1). Hence, the drug mixture apparently influenced the moulting behaviour of *G. fossarum*, resulting in abnormal growth patterns. This tendency of exposed animals to have a decreased growth indicates that gammarids may become smaller if they are chronically exposed to such chemical compounds. This is in accordance with Ladewig *et al.* (2006) who observed populations of *G. fossarum* upstream and downstream from effluents of sewage treatment plants. They found smaller animals at the downstream site, presumably as a result of elevated concentrations of endocrine substances.

Smaller gammarids are considered to have a decreased chance of survival, because they suffer intense cannibalism and intraguild predation by larger animals (MacNeil *et al.* 2008), which in turn could have consequences at the community level. In addition, a decrease in the body length could lead to reduced fitness because the body size of female gammarids and the number of eggs per clutch are positively correlated (Hynes 1955; Sheader 1983). There is a similar disadvantage for males in mating owing to male–male competition, because larger males are more successful in entering into precopula (Elwood *et al.* 1987) and in defending females against take-overs by other males (Ward 1983). Furthermore, Greenwood and Adams (1984) suggested that male gammarids showing a decreased body size can swim in precopula at lower current speeds than larger males, emphasising that smaller animals are inferior competitors.

According to Pöckl (1992), the mean interval between moults in *G. fossarum* is linearly related to the moult number and exponentially related to age. We did not find this pattern in any of our treatments, possibly because animals were observed only for a limited period of time and not from birth to sexual maturity. Instead, the intermoult period of gammarids exposed to the 'env' and 'high' drug concentrations was shortened (Fig. 2). Because we selected organisms in a similar size class, which were randomly distributed into the different treatments, the intermoult interval should not be influenced by the age and hence, the body

size of the gammarids. Pöckl (1992), additionally, argued that the intermoult intervals of *G. fossarum* varied considerably with the water temperature, resulting in shorter intermoult periods with increasing water temperatures. In our experiment, gammarids were kept in a temperature-controlled room; therefore, we can exclude a possible influence of changing water temperatures. Thus, it can be assumed that the shortened time between successive moults is due to the pharmaceutical mixture.

An effect on moulting evoked by estrogenic environmental contaminants has already been shown for the cladoceran *D. magna*. Yet, this resulted in elongated intermoult periods and reduced moulting frequencies (Baldwin *et al.* 1995; Zou and Fingerman 1997). Moulting in arthropods is mainly regulated by ecdysteroid hormones (Chang *et al.* 1993). Zou and Fingerman (1997) argued that the moulting process could be slowed by oestrogen chemicals acting as antagonists of ecdysteroids, by binding to and blocking the ecdysteroid receptor. Therefore, it can be anticipated that the endocrine compound EE2 used in our study may influence the moulting process of *G. fossarum* in the same way. However, because time between moultings of gammarids exposed to the 'env' and 'high' drug mixture was decreased in our experiment, in contrast to the studies of Baldwin *et al.* (1995) and Zou and Fingerman (1997), it may be speculated that the mode of action of the single compounds CBZ, DIC and MET differs from that of the endocrine chemical EE2. Furthermore, a potential explanation for the shortened intermoult interval could also be that a mixture of pharmaceuticals can lead to effects that are not expected from exposure to single compounds (Cleuvers 2003, 2004). As moulting is an essential physiological process for growth and reproduction of crustaceans, any disruption of this complex mechanism can affect whole populations of gammarids.

The mortality did not differ significantly among the treatments. However, variable mortality rates among the treatments may be explained by an altered food quality, because drugs potentially improved the development of the microflora of bacteria and fungi on the surface of the beech leaves, which is an essential food constituent for gammarids (Kaushik and Hynes 1971; Sutcliffe *et al.* 1981). This is supported by Lawrence *et al.* (2007), who observed a significant increase in bacterial biomass and biofilm thickness at 100 $\mu g\,L^{-1}$ DIC. Moreover, CBZ, ibuprofen and furosemide also had a positive effect on fungi (Lawrence *et al.* 2005).

Another possible pathway for the detected effects regarding the moulting behaviour in our experiment might be that the drug mixture caused a shift in feeding activity. Blockwell *et al.* (1998) detected reduced feeding activity in *G. pulex* following a 96-h exposure to 12.1 $\mu g\,L^{-1}$ of copper, which may result in growth inhibition and impaired reproduction.

### *Reproduction*

Female amphipods practice active brood care, involving ventilation of the brood pouch through increased pleopod beating and cycling of eggs, as well as selective ejection of non-viable eggs from the brood pouch (Dick *et al.* 1998). A release of eggs or offspring from the brood pouch was shown for *G. pulex* in response to exposure to the insecticide esfenvalerate (Cold and Forbes 2004). Because an increase in egg volume during development can be partially explained by an uptake of the surrounding liquid (Sheader 1996), toxic compounds in this liquid may lead to egg deformities and thus a premature release of embryos. Although a slight increase in brood loss at the 'high' drug concentration was observed, our results do not indicate that the mixture of CBZ, DIC, MET and EE2 induced aberration of egg development in *G. fossarum*.

Concerning the time for egg development, both the 'env' and 'high' pharmaceutical treatment showed a tendency towards a slightly quicker time of development, although not significantly (Table 1). Schirling *et al.* (2006) observed an accelerated maturation of oocytes in females of *G. fossarum* exposed to the endocrine compound bisphenol A. Stimulatory effects on the maturation and reproduction caused by CBZ and acetylsalicyl acid have also been demonstrated in daphnids at environmentally realistic concentrations (Marques *et al.* 2004; Lürling *et al.* 2006).

The drug mixture tested did not affect the number of offspring (Table 1). This is in accordance with Borgmann *et al.* (2007), who observed no influence of a mixture of seven pharmaceuticals on the number of neonates produced by the amphipod *Hyalella azteca*. However, *Daphnia pulex* exposed to 1 $\mu g\,L^{-1}$ CBZ produced a higher number of offspring (Lürling *et al.* 2006), whereas EE2 (100 and 500 $ng\,L^{-1}$) as well as MET (6.15 $mg\,L^{-1}$) caused a decrease in the number of offspring in *D. magna* (Goto and Hiromi 2003; Dzialowski *et al.* 2006). Nevertheless, if female gammarids are exposed to the pharmaceuticals before ovulation, an effect on the number of offspring cannot be excluded.

The body length of neonate gammarids exposed to the 'high' pharmaceutical concentrations was marginally smaller than the size of control animals, although this result was not significant (Table 1). Because pharmaceuticals in the surrounding liquid are taken up by eggs during development (Sheader 1996), the drug mixture may impair the growth of embryos resulting in a decreased hatching size. Buikema and Benfield (1979) proposed that juvenile and, hence, smaller invertebrates are more sensitive to toxic compounds than older and, thus, larger animals; the former have a greater surface-to-volume ratio and consequently a higher capacity for exchange with the pollutant. However, if gammarids are affected only at the artificially high drug concentrations, the impact of the pharmaceuticals may be negligible in the field.

### *The energy-storage component glycogen*

In our experiment, the content of glycogen was not altered by exposure of the gammarids to the drug mixture (Fig. 3). This is in accordance with a study of Schill and Köhler (2004); even though Schill and Köhler (2004) detected a correlation between various polluted stream sites and levels of stress proteins at *G. fossarum*, the energy-storage compounds of the gammarids were similar at all study sites and independent of the pollution rate. Moreover, stress caused by predation from benthivorous fish showed no influence on the concentration of energy-storage components in *G. pulex* and the mayfly *Rhithrogena semicolorata* (Winkelmann *et al.* 2007).

## Conclusions

Neither the content of glycogen nor reproduction of *G. fossarum* was significantly influenced by the mixture of CBZ, DIC, MET

and EE2. Nevertheless, the partially lower drug concentrations caused by the experimental setup may mask possible effects in our study.

Whether the impacts of pharmaceuticals on aquatic environments are severe or negligible is still under discussion and in need of more detailed analyses. However, in our study, pharmaceuticals affected the moulting behaviour during an exposure period of 100 days. Hence, it can be speculated that chronic exposure to drugs in the surface waters of urban areas may lead to impairments for entire populations of gammarids. Because gammarids play a key role in the food chain, representing an important prey item for a large number of fish species, harmful consequences for aquatic ecosystems may be expected.

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# Response of benthic macroinvertebrates to micropollutants released by sewage treatment plants

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Obwohl sich die Reinigungsleistung unserer Kläranlagen ständig verbessert, belastet das eingeleitete Abwasser dennoch die Gewässer, da etliche Substanzen im herkömmlichen Reinigungsverfahren bisher nicht oder nur unvollständig eliminiert werden können. So gelangen beispielsweise anthropogene Schadstoffe wie Schwermetalle, Arzneistoffe und Nanopartikel über die Kläranlagenpassage in die Gewässer. Da viele dieser Stoffe äußerst persistent sind, akkumulieren sie in der aquatischen Umwelt. Dabei lagern sich die meisten Substanzen in den Sedimenten ab, wodurch es zu einer dauerhaften Exposition von benthischen Makroinvertebraten gegenüber diesen Schadstoffen kommt.

In dieser großräumig angelegten Freilandstudie wurde der Einfluss von acht Kläranlagen auf die Invertebratengemeinschaft bayerischer Fließgewässer untersucht. Dabei wurden in zwei Probenahmeserien jeweils oberhalb und unterhalb der Kläranlageneinleitungen die Zusammensetzung der Makroinvertebratengesellschaft sowie deren Artenreichtum und Artendiversität aufgenommen. Begleitend wurden zahlreiche abiotische und biotische Parameter gemessen, um einen möglichen Zusammenhang mit der Verteilung der Invertebraten zu detektieren.

Oberhalb und unterhalb der Kläranlageneinleitungen unterschied sich die Zusammensetzung der Makroinvertebraten deutlich. Während sensitive Arten wie *Rhithrogena sp.* unterhalb der Kläranlagen in geringeren Dichten auftraten, nahm der Anteil an toleranten Taxa wie Oligochaeten unterhalb der Einleitungen zu. Entgegen unserer Erwartungen war sowohl der Artenreichtum als auch die Artendiversität unterhalb der Kläranlageneinleitungen erhöht. Vermutlich siedelten sich aufgrund des erhöhten Nährstoffgehalts unterhalb der Kläranlage mehr tolerante Taxa an, als dass dort sensitive Arten verschwanden. Diese lokale Zunahme der Diversität spiegelt jedoch nicht die globale Situation wider. Durch die permanente Verschmutzung der Gewässer werden letztendlich etliche sensitive Arten aussterben, was eine Dominanz von wenigen toleranten Taxa und somit eine globale Abnahme der Diversität zur Folge haben könnte.

Da in dieser Studie kein Zusammenhang zwischen den gemessenen abiotischen und biotischen Parametern und der Verteilung der benthischen Makroinvertebraten bestand, scheinen mit dem Abwasser eingeleitete anthropogene Schadstoffe die Invertebraten-gesellschaft unterhalb der Kläranlageneinleitungen zu beeinflussen. Eine Veränderung der Artengemeinschaft kann sich jedoch nachteilig auf das aquatische Ökosystem auswirken, da Makroinvertebraten eine wichtige Stelle in der Nahrungskette einnehmen. Deshalb muss der Eintrag von umweltgefährdenden Substanzen zukünftig weitestgehend reduziert werden.

# Response of benthic macroinvertebrates to micropollutants released by sewage treatment plants

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**Abstract**

Aquatic ecosystems have been severely affected by the continuous discharge of wastewater into the environment. In this large-scale field study, we investigated the impact of eight sewage treatment plants (STPs) on benthic macroinvertebrate assemblages. We examined community structure, taxon richness and diversity of the invertebrates upstream and downstream of the STP effluents in spring and summertime. Additionally, several abiotic (e.g. nutrients and stream habitat parameters) and biotic measurements (e.g. algal biomass) were taken at each sampling site to detect correlations with the distribution of the invertebrates.

We found that community structure differed between the sampling sites. Specifically, the abundance of sensitive taxa (*Rhithrogena sp.*) was reduced below the effluents, whereas tolerant taxa (oligochaetes) were more abundant. In addition, we observed a higher taxon richness and diversity downstream of the STPs.

None of the measured abiotic or biotic parameters explained the observed distribution of benthic macroinvertebrates. Therefore, we assume that anthropogenic contaminants, which cannot be completely removed from wastewater, may affect benthic macroinvertebrates below the STPs. As changes to invertebrate assemblages may lead to severe ecological consequences, it should be a major objective to further reduce the discharge of environmentally hazardous micropollutants into aquatic ecosystems.

**Keywords**

Benthic macroinvertebrates, communtiy structure, diversity, micropollutants, sewage treatment plant

**Abbreviations**

CBZ: carbamazepine

EPT: Ephemeroptera, Plecoptera and Trichoptera

STP: sewage treatment plant

## 1. Introduction

Although freshwater systems occupy less than 1 % of the earth´s surface, they possess considerable global value by supplying drinking water and providing food, especially fish, which represents an essential part of the human diet in many regions of the world (Johnson et al., 2001). However, aquatic ecosystems have been severely affected by anthropogenic activities resulting in habitat fragmentation and pollution.

Before the first sewage treatment plants (STPs) were constructed at the end of the 19$^{th}$ century, common practice was to directly discharge wastewater into rivers and streams without cleaning (Wiesmann et al., 2007). Starting at the end of the 1970s, different purification methods were developed to eliminate phosphorus and nitrogen from sewage leading to a reduced nutrient flow into the aquatic ecosystems (Krug, 1993; Obenaus and Kraft, 2004). However, despite continually improved cleaning technologies, STPs are generally not designed to remove all contaminants completely from wastewater (Paul and Meyer, 2001). Particularly micropollutants such as heavy metals, organochlorines, pharmaceuticals and engineered nanoparticles pass through STPs without a complete elimination (Ternes, 1998; Paul and Meyer, 2001; Brönmark and Hansson, 2002; Limbach et al., 2008). Thus, Sumpter (2009) proposed that almost all rivers in densely populated areas are contaminated with a complex mixture of chemicals discharged by STP effluents.

Due to the permanent release and high persistence of many pollutants (Halling-Sørensen et al., 1998; Brönmark and Hansson, 2002), they can accumulate in the aquatic environment. Especially sediments act as a sink for contaminants, thus benthic macroinvertebrates are exposed to higher concentrations of pollutants than free-swimming fauna (Holthaus et al., 2002). Therefore, the species diversity and population size of benthic macroinvertebrates are often used to indicate the water quality in freshwater systems (Bacey and Spurlock, 2006). Particularly invertebrates of the orders Ephemeroptera, Plecoptera and Trichoptera (EPT) possess a high sensitivity to environmental impacts (Rosenberg and Resh, 1993; Thorne and Williams, 1997), whereas chironomids and oligochaetes are considered as pollution-tolerant taxa (Crawford et al., 1992; Bacey and Spurlock, 2006).

As sewage effluents are the primary source for water quality degradation (Paul and Meyer, 2001), the stream community may differ upstream and downstream of STPs. Ortiz et al. (2005) found a lower diversity of benthic macroinvertebrates below a STP effluent, with decreased numbers of sensitive EPT taxa and an increased abundance of resistant taxa such as chironomids and oligochaetes. However, EPT taxa are considered as important prey for a

large number of fish species (Meissner and Muotka, 2006; Gregory et al., 2007). Thus, changes to the invertebrate community caused by pollutants may lead to harmful consequences for aquatic systems because many benthic macroinvertebrate taxa play a key role in the food web.

In our large-scale field study, we examined the assemblages of benthic macroinvertebrates upstream and downstream of eight STP effluents. We assumed that not only the increased nutrient contents, but also anthropogenic contaminants such as micropollutants, which are continuously discharged into the rivers, may influence the distribution of invertebrates. Hence, we hypothesized that the density of sensitive taxa, such as EPT, is reduced below STPs compared to the less polluted upstream sites, whereas tolerant taxa, such as chironomids and oligochaets, may sustain the contamination evoked by the effluents. Furthermore, we anticipated a decreased taxon richness and diversity downstream of the STPs.

## 2. Materials and Methods

### 2.1 Study sites

The study was carried out in six pre-alpine streams in Southern Germany, which are assigned to similar stream types according to the European Water Framework Directive (Meier et al., 2006). As study sites we selected stream sections within eight towns with sewage treatment plants (STPs) discharging their effluents into one of the six streams. Study sites were labelled as follows (town/stream): Garmisch/Loisach (GL), Grassau/Tiroler Achen (GTA), Lenggries/Isar (LI), Mittenwald/Isar (MI), Oberammergau/Ammer (OA), Peißenberg/Ammer (PA), Piding/Saalach (PS) and Traunstein/Traun (TT). We investigated each study site approximately 100 m above the STP and 50-300 m below the effluent. The downstream sites were determined depending on the complete mixture of the treated water and the stream water which was verified using a conductivity meter.

### 2.2 Biological sampling

Samples were taken in two test series in May and July 2007. To investigate the benthic macroinvertebrate community, five Surber samples (25 cm x 25 cm, 200 µm mesh size) were collected within riffles of each upstream and downstream sampling site. The Surber sampler was placed randomly on the stream bed and the sediment was disturbed by an iron fork at a sampling depth of 10 cm for three minutes. Invertebrate samples were preserved in 70 % ethanol in the field. We used a plancton divider to subsample rich samplings to one half, one fourth or one eighth depending on the number of invertebrates. If there were less than 250 organisms in the subsample, we also analyzed the other partition. Macroinvertebrates were sorted, identified at least to the family level and counted using a stereomicroscope (Wild M3Z, Heerbrugg, Switzerland, magnification 6.5 - 40 x). Afterwards, invertebrate numbers were converted to individuals per m².

### 2.3 Environmental parameters

After removing the invertebrates, benthos samples were dried at 90 °C to constant mass and subsequently ashed at 550 °C for examining of the particulate organic matter (POM; APHA, 1998).

To determine algal biomass a single stone was randomly chosen from each Surber sampling area before disturbing the sediment. All macroinvertebrates on this stone were carefully washed off into the sampler. We scraped the entire surface of each stone with a toothbrush

and fixed the algal sample immediatly with formaldehyde (final concentration 4 %). Algal biomass of cool and dark stored samples were analyzed fluorometrically (GAT TD 700, Bremerhaven-Lehe, Germany) as chlorophyll *a* (Chl *a* ). To convert algal biomass to values per cm² of stone surface area, we wrapped each stone in aluminium foil of known weight per unit area and weighed the foil (Townsend et al., 1997).

For each Surber site we measured water depth and near-bed stream velocity with a flow meter (Hoentzsch, Waiblingen, Germany). We also estimated the substratum composition defined as the average particle widths of the first- and second-most common grain size classes inside each Surber sample. These size classes were identified using a modified Wentworth scale with a half-phi scale (Harrelson et al., 1994).

Upstream and downstream of each study site we additionally measured the following physical parameters: oxygen content (WTW Oxi 340, Weilheim, Germany), pH (WTW pH 330, Weilheim, Germany) and conductivity (WTW LF 196, Weilheim, Germany).

For the chemical parameters we collected one liter of stream water close to each Surber site. Subsequently, the five water samples from each upstream or downstream range were mixed in a bucket. We filled 125 ml of the mixed stream water into a plastic bottle and stored it on ice for further water chemistry analyses. The content of total phosphor (TP) was measured spectrophotometrically (Shimadzu UV-1700, Shanghai, China) at 880 nm after acid peroxydisulfate digestion of the water samples (DIN EN 1189). We determined chloride ($Cl^-$), nitrate ($NO_3^-$) and sulfate ($SO_4^{2-}$) from stream water with a ion chromatography system (Dionex DX 100, Idstein, Germany).

To test for the occurence of micropollutants in the stream water, we filled 1 l of the mixed water samples from each upstream and downstream location in glass bottles. Additionally, we took a water sample directly at the STP effluent of each study site. The water samples were stored in the dark at -20° C for subsequent analyses using GC-MS/MS. For a detailed description of the chemical analysis see Dietrich et al. (2010). As Clara et al. (2004) suggested the antiepileptic agent carbamazepine (CBZ) as a suitable wastewater marker, we focused only on the concentrations of this substance.

2.4 Data analysis

To evaluate the impact of the STPs, we considered the community structure, the taxon richness and diversity of the benthic macroinvertebrates upstream and downstream of the effluents. The invertebrate data sets were analysed with a non-parametric multivariate analysis of variance based on permutations (PERMANOVA; Anderson, 2001). We used a

three-level design with sampling sites nested in study sites, and samples (five replicates per sampling site) as a random factor. Invertebrate abundances were fourth root transformed and standardized to each samples´ total. PERMANOVAs on community data were conducted with 9999 permutations on Bray-Curtis similarities. Additional PERMANOVAs on taxon richness and diversity (Shannon index) were calculated on Euclidean distances of untransformed data.

To assess the influence of the measured environmental parameters on the distribution of the invertebrates, we included them as covariables in the PERMANOVAs. As we assume that the physical and chemical parameters vary slightly among the five samples of each sampling site, we added a minute random variation in the range of 0 to 0.001 to each measured parameter. Data which deviated considerably from normal distribution were fourth root (May and July: POM, Chl *a*) or log (x + 1) (May and July: TP; May: $Cl^-$) transformed. Conductivity values from the May data set were excluded due to missing values from technical defects in the measuring instrument.

We checked which parameters contributed most to the difference between both sampling sites using the similarity percent method (SIMPER) based on Bray-Curtis similarities. To evaluate habitat differences between sampling sites, PERMANOVAs on parameters that explained ≥ 1 % of the difference between the upstream and downstream sites were conducted with 9999 permutations on Bray-Curtis similarities. The same parameters were included as covariables in the PERMANOVA tests on community data to determine possible interactions with the sampling sites.

For significant differences in community structure at the upstream and downstream sites, SIMPER tests based on Bray-Curtis similarities were used on the community data to ascertain which taxa were predominantly responsible for the different invertebrate compositions. For taxa which explained ≥ 5 % of the difference between the upstream and downstream sites, the means of each sampling site were calculated. The proportions of these means were compared between sampling sites across all study sites using non-parametric tests for two dependant samples (Wilcoxon signed rank tests). The same tests were applied to examine differences in taxon richness and diversity.

All statistical analyses were conducted with PRIMER 6 (PRIMER-E Ltd, Lutton, Ivybridge, United Kingdom) and SPSS 15.0 (SPSS inc., Chicago, IL, USA).

## 3. Results

### 3.1 Community structure

The community structure differed significantly between the upstream and downstream sites in May and July (PERMANOVAs, $p < 0.001$). Including parameters that explained ≥ 1 % of the difference between the upstream and downstream sites (Tab. 1), the community structure of both the May and July samplings remained significantly different between the sampling sites (PERMANOVAs, $p < 0.001$). Moreover, none of the measured environmental parameters influenced the composition of the benthic macroinvertebrates upstream and downstream of the STPs significantly (May: PERMANOVA, all parameters $p \geq 0.270$; July: PERMANOVA, all parameters $p \geq 0.078$), although the habitat was significantly different between the sampling sites in May and July (May: PERMANOVA, $p = 0.011$; July: PERMANOVA, $p < 0.001$). Furthermore, we did not find any significant interaction between the parameters and sampling site (May: PERMANOVA, all interactions $p \geq 0.086$; July: PERMANOVA, all interactions $p \geq 0.257$).

Taxa which contributed most to the different compositions of benthic macroinvertebrates are listed in Tab. 2. In the May sampling, the relative percentage of *Rhithrogena sp.* was significantly reduced at the downstream sites compared to the upstream sites (Wilcoxon signed rank tests, $p = 0.036$, $n = 8$; Fig. 1 a). In contrast, the proportion of oligochaetes increased significantly below the STP effluents (Wilcoxon signed rank tests, $p = 0.012$, $n = 8$; Fig. 1 b). At the July sampling, the relative percentage of Hydracarinae decreased significantly at the downstream sites in comparison to the sampling sites above the STPs (Wilcoxon signed rank tests, $p = 0.025$, $n = 8$; Fig. 1 c).

### 3.2 Taxon richness

Due to an increase in taxa below the STPs (Wilcoxon signed rank tests, $p = 0.036$, $n = 8$; Fig. 2) in the May sampling, taxon richness differed significantly between the upstream and downstream sites (PERMANOVA, $p = 0.022$). In contrast, taxon richness of the July sampling showed no differences between the sampling sites (PERMANOVA, $p = 0.101$). Including environmental parameters into the model, we did not detect differences between the sampling sites (May: PERMANOVA, $p = 0.402$; July: PERMANOVA, $p = 0.467$). There was no significant influence of the measured parameters on the taxon richness upstream and downstream of the STPs (May: PERMANOVA, all parameters $p \geq 0.225$; July: PERMANOVA, all parameters $p \geq 0.225$). Furthermore, we did not observe any significant

interactions of the parameters and the sampling sites (May: PERMANOVA, all interactions $p \geq 0.236$; July: PERMANOVA, all interactions $p \geq 0.663$).

3.3 Shannon diversity

The Shannon diversity index showed no significant differences between the upstream and downstream sites (PERMANOVA, $p = 0.259$) in the May sampling. In the July sampling, the Shannon diversity index was significantly different between the sampling sites (PERMANOVA, $p < 0.001$) with an increased diversity downstream of all sudy sites except at OA; however, this was not significant (Wilcoxon signed rank tests, $p = 0.161$, $n = 8$; Fig. 3). Including the environmental parameters into the model, we found no significant differences between the upstream and downstream sites (May: PERMANOVA, $p = 0.065$; July: PERMANOVA, $p = 0.071$). Moreover, none of the measured parameters influenced significantly the Shannon diversity upstream and downstream of the STPs (May: PERMANOVA, all parameters $p \geq 0.220$; July: PERMANOVA, all parameters $p \geq 0.300$). However, in the May sampling, there were significant interactions between sampling sites and substratum composition (PERMANOVA, $p = 0.012$), as well as sampling sites and oxygen content (PERMANOVA, $p = 0.040$; all other interactions $p \geq 0.103$). We did not find any significant interactions of the parameters with the sampling sites in the July sampling (PERMANOVA, all interactions $p \geq 0.327$).

3.4 Content of CBZ

We could detect CBZ in all water samples of the STP effluents in the May and July samplings (Tab. 3). However, the concentrations of CBZ in the stream water of the upstream and downstream sampling sites were very low and especially in the May sampling not measurable with the exception of TT where the content of CBZ reached 301 ng/l below the STP. In the July sampling we measured the following CBZ concentrations: 158 ng/l at GTA downstream, 116 ng/l at MI upstream, 356 ng/l at MI downstream, 190 ng/l at PS upstream and 131 ng/l at TT downstream.

## 4. Discussion

Our large-scale field study demonstrates that assemblages of benthic macroinvertebrates are impacted by STPs leading to different community structures upstream and downstream of effluents. As hypothesized, the mean proportion of pollution-tolerant oligochaetes was higher downstream of the STPs compared to upstream sites (Tab. 2, Fig. 1 b). This is consistent with the findings of Marcogliese et al. (2009), who observed a higher density of oligochaetes below STP effluents resulting from organic enrichment of the sediments. In addition, Lin and Yo (2008) found a positive correlation between the total abundance of oligochaetes and the aquatic pollution measured as River Pollution Index.

Although chironomids can occur in severely contaminated areas (Bacey and Spurlock, 2006), their mean proportion in our study was only at the July sampling slightly higher at the downstream site than at the upstream site (Tab. 2). However, we can not exclude that the composition of the chironomid assemblage varied on taxon level below the STP effluents leading to a community dominated by more pollution-tolerant chironomid taxa (Gresens et al., 2007).

According to a study by Stryjecki (2002), who detected a negative impact of water pollution on the water mite fauna in Poland, the proportion of Hydracarinae decreased significantly at the downstream sites of the STP effluents (Tab. 2, Fig. 1 c). Moreover, as expected, the mean proportion of several sensitive EPT taxa was reduced below the STPs compared to the less polluted upstream sites (Tab. 2, Fig. 1 a). A decline in the abundances of EPT due to degradation in the quality of the habitat was observed in several studies. For instance, Gresens et al. (2007) found a lower EPT density in urban versus rural reaches of streams, presumably because of the chemical impacts of urban stormwater runoff. Furthermore, Hepp and Santos (2009) demonstrated that the density of EPT taxa was negatively correlated with parameters indicating environmental disturbances, such as increased nutrient concentrations, higher conductivity and turbidity or lower oxygen content.

All of these studies found a correlation between the assemblages of benthic macroinvertebrates and factors demonstrating aquatic pollution caused by human activity. Although we detected physical and chemical habitat differences between the upstream and downstream sites (e.g. higher nutrient contents below the STPs) in our large-scale field survey, none of the measured abiotic and biotic parameters explained the difference between the community structure upstream and downstream of the effluents. Additionally, we did not observe any significant interactions with the measured parameters and community structure.

However, this could be due to minute, not detectable effects or to inconsistent effects between streams. Moreover, because of the complex nature of streams, field experiments are influenced by various parameters and it is not possible to measure each factor and to determine all possible interactions. Although we tried to consider all factors that are known to be crucial for the distribution of the benthic macroinvertebrates (e.g. Ulfstrand, 1967; Hearnden and Pearson, 1991; Holomuzki and Messier, 1993), some parameters may be neglected. For instance, micropollutants like heavy metals, organochlorines, pharmaceuticals and engineered nanoparticles which are incompletely removed from wastewater may affect the benthic macroinvertebrates. Ladewig et al. (2006) found higher abundances of *Gammarus fossarum* downstream of effluents. However, there was a reduced proportion of breeding females and juvenile gammarids, presumably as a result of elevated concentrations of endocrine substances below the effluents. Investigating the benthic community structure of the Llobregat River Basin in Spain, Munoz et al. (2009) detected a potential causal relation between the concentrations of a few pharmaceuticals and the abundance and biomass of some macroinvertebrates: Sites with higher concentrations of anti-inflammatories and β-blockers showed an increased abundance and biomass of chironomids and oligochaetes. Furthermore, the community structure of macroinvertebrates in streams polluted with heavy metals differed significantly from the control sites, resulting in reduced animal densities (Doi et al., 2007). Although the concentrations of the wastewater marker CBZ were often not measurable in the stream water of our study sites, we detected CBZ ubiquitary in each effluent, thus we conclude that the downstream sites are more polluted with anthropogenic contaminants than the upstream sites. This may presumably contribute to the different community structures upstream and downstream of the STPs.

In contradiction to our hypothesis, taxon richness and diversity was increased downstream of the STPs at least at one of the test series. That is in contrast to Ortiz et al. (2005), who detected a reduced taxon richness and diversity of macroinvertebrates below a STP effluent. Also many other studies found negative correlations between the biodiversity of invertebrates and disturbances associated with urbanization or other kinds of human impacts (e.g. Roy et al., 2003; Urban et al., 2006; Hepp and Santos, 2009). However, Pinder and Farr (1987) and Thorne and Williams (1997) documented that the macroinvertebrate richness and diversity can increase with slight pollution. This could be explained by the intermediate disturbance hypothesis (Connell, 1978), which proposes that diversity is greatest at an intermediate level of disturbance.

We assume that caused by the nutrient enrichment below the STP effluents more tolerant macroinvertebrate species colonized the downstream sites, whereas sensitive taxa disappeared. This pattern may explain the observed local increase in biodiversity. However, in the progress of the increasing pollution of freshwater habitats it might be expected that sensitive macroinvertebrate species will get extinct over a wide area, since they cannot adapt to the adverse environmental conditions. As a consequence, the aquatic ecosystems may become dominated by tolerant taxa, resulting in a global decline of biodiversity. A study by the invasion ecologists McKinney and Lockwood (1999) showed that many endemic species declined due to human disturbances and were replaced by fewer widespread species, which are successful in the altered environment. Biotic homogenization with reduced biodiversity of the ecosystems is the consequence.

## 5. Conclusion

This large-scale field study showed that the structure and function of aquatic ecosystems are impaired by STPs. Benthic macroinvertebrates play a key role in aquatic food webs as important prey items for a large number of fish species, thus changes to the invertebrate assemblages may lead to severe ecological consequences. Although we do not know the exact reasons for the altered distribution of invertebrates, we assume that micropollutants which cannot be completely removed from the wastewater may affect the benthic macroinvertebrates below the effluents. Consequently, more effort should be invested to further reduce the amounts of environmentally hazardous contaminants which are discharged into rivers by STPs to prevent harmful consequences for aquatic ecosystems.

## Acknowledgments

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**Tables**

Table 1: Parameters which explained ≥ 1 % of the difference between the upstream and downstream sites at the a) May and b) July samplings.

1 a)

| Parameters | Contribution to the difference (%) |
|---|---|
| Substratum composition (mm) | 41.91 |
| Water depth (cm) | 28.88 |
| $SO_4^{2-}$ (mg/l) | 21.13 |
| $Cl^-$ (mg/l) | 2.09 |
| TP (µg/l) | 1.96 |
| Oxygen content (mg/l) | 1.75 |

1 b)

| Parameters | Contribution to the difference (%) |
|---|---|
| Conductivity (µS/cm) | 44.18 |
| Substratum composition (mm) | 33.49 |
| Water depth (cm) | 11.10 |
| $SO_4^{2-}$ (mg/l) | 5.61 |
| $Cl^-$ (mg/l) | 3.49 |

Table 2: Taxa which contributed ≥ 1 % to the different compositions of the benthic macroinvertebrates upstream and downstream of the STPs at the a) May and b) July samplings.

2 a)

| Taxa | Contribution to the difference (%) | Mean proportion upstream | Mean proportion downstream |
|---|---|---|---|
| Oligochaetae | 20.02 | 11.63 | 23.27 |
| Chironomidae | 19.52 | 32.43 | 29.56 |
| Leuctridae | 14.98 | 16.30 | 15.10 |
| *Rhithrogena sp.* | 14.90 | 18.22 | 11.36 |
| Chloroperlidae | 7.67 | 4.64 | 5.81 |
| Baetidae | 4.55 | 5.40 | 4.50 |
| Simuliidae | 2.61 | 2.14 | 1.16 |
| *Protonemura sp.* | 2.37 | 2.08 | 0.47 |
| Hydracarinae | 2.26 | 1.22 | 1.48 |
| *Dicranota sp.* | 1.64 | 1.59 | 1.29 |
| Nemouridae | 1.58 | 0.45 | 1.49 |
| Tanypodinae | 1.49 | 0.77 | 1.06 |

2 b)

| Taxa | Contribution to the difference (%) | Mean proportion upstream | Mean proportion downstream |
|---|---|---|---|
| Baetidae | 16.56 | 23.97 | 20.32 |
| Chironomidae | 14.69 | 18.52 | 20.04 |
| Oligochaetae | 13.72 | 12.96 | 13.16 |
| *Rhithrogena sp.* | 13.38 | 15.23 | 12.88 |
| Simuliidae | 7.72 | 5.34 | 7.84 |
| Leuctridae | 7.22 | 6.32 | 7.56 |
| Chloroperlidae | 7.05 | 3.98 | 5.92 |
| Hydracarinae | 6.28 | 5.59 | 3.58 |
| *Protonemura sp.* | 1.10 | 0.80 | 0.68 |
| Nemouridae | 1.02 | 0.65 | 0.82 |

Table 3: Concentrations of carbamazepine (CBZ) in the STP effluents of all study sites at the May and July samplings.

| Study site | Concentration of CBZ (ng/l) at the May sampling | Concentration of CBZ (ng/l) at the July sampling |
|---|---|---|
| GL | 505 | 1564 |
| GTA | 2894 | 271 |
| LI | 390 | 1113 |
| MI | 688 | 1518 |
| OA | 43 | 141 |
| PA | 92 | 77 |
| PS | 1609 | 896 |
| TT | 2942 | 585 |

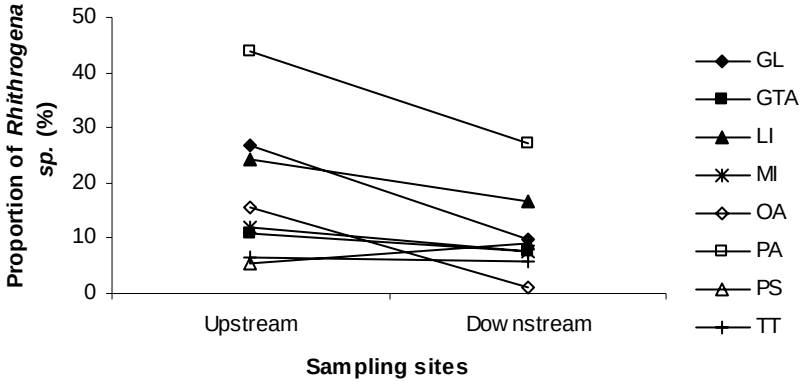
**Figures**

Fig. 1: Proportion of a) *Rhithrogena sp.* b) Oligochaetae and c) Hydracarinae upstream and downstream of each STP in May (a, b) and July (c) 2007.

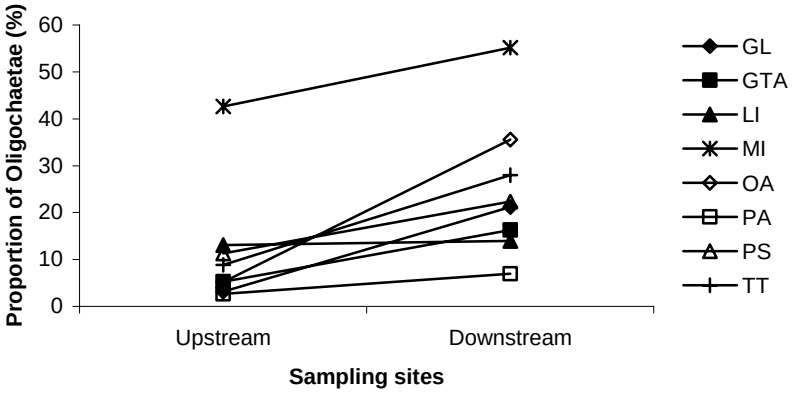
Fig. 2: Taxon richness upstream and downstream of each STP in May 2007.

Fig. 3: Shannon diversity index upstream and downstream of each STP in July 2007.

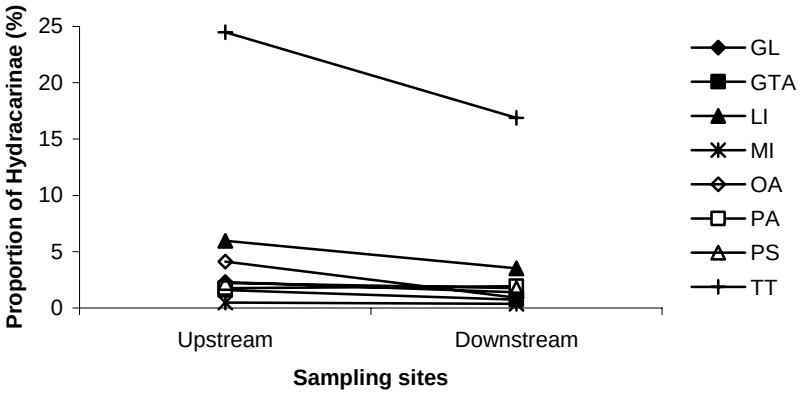
1 a)


Proportion of Rhithrogena sp. (%)
50
40
30
20
10
0
Upstream
Dow nstream
Sampling sites
GL
GTA
LI
MI
OA
PA
PS
TT

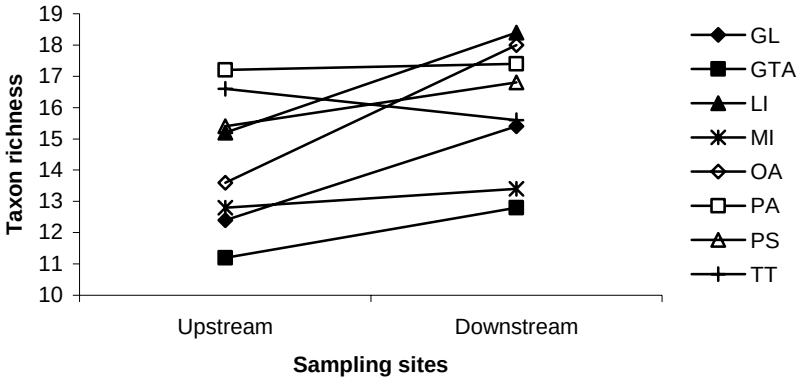
1 b)


Proportion of Oligochaetae (%)
60
50
40
30
20
10
0
Upstream
Downstream
Sampling sites
GL
GTA
LI
MI
OA
PA
PS
TT

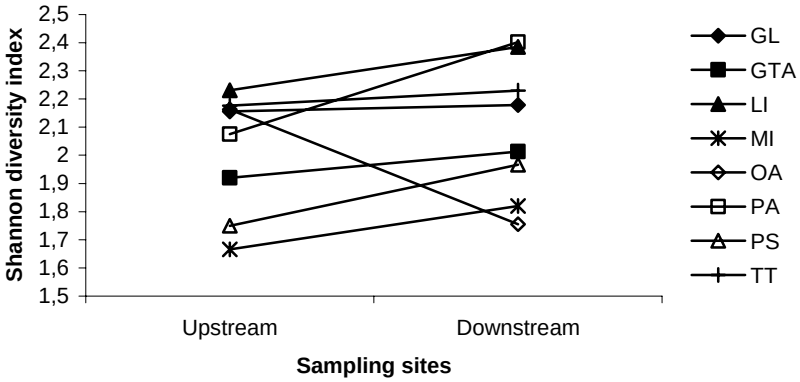
1 c)


Proportion of Hydracarinae (%)
25
20
15
10
5
0
Upstream
Downstream
Sampling sites
GL
GTA
LI
MI
OA
PA
PS
TT

2)


Taxon richness
19
18
17
16
15
14
13
12
11
10
Upstream
Downstream
Sampling sites
GL
GTA
LI
MI
OA
PA
PS
TT

3)


Shannon diversity index
2,5
2,4
2,3
2,2
2,1
2
1,9
1,8
1,7
1,6
1,5
Upstream
Downstream
Sampling sites
GL
GTA
LI
MI
OA
PA
PS
TT

# Diskussion

6

Im Rahmen dieser Dissertation wurde der Einfluss von Arzneistoffen auf aquatische Invertebraten untersucht. Dabei wurden die Arzneistoffe in umweltrelevanten Konzentrationen als Arzneimittelgemisch, teilweise auch als Einzelstoffe getestet. Da zudem die Langzeiteffekte vieler Arzneimittel weitgehend unbekannt sind, wurden ausschließlich Langzeitstudien durchgeführt.

**Notwendigkeit von Langzeitstudien**

Meine Ergebnisse verdeutlichen die Notwendigkeit von Langzeitstudien, da die Arzneistoffe bei allen im Labor durchgeführten Experimenten Langzeiteffekte verursachten. So traten bei den Experimenten mit *Daphnia magna* Effekte auf (Kapitel 2), die bei einer kürzeren Expositionsdauer nicht berücksichtigt worden wären. Wäre beispielsweise das Lebenszyklusexperiment nach 21 Tagen beendet worden, so wie es in Standardtoxizitätstests üblich ist (OECD 1998), hätte man bei den in dem Arzneistoffgemisch exponierten Daphnien die erst in den späteren Lebensstadien auftretende erhöhte Mortalität übersehen.
Ebenso hätte eine vorzeitige Beendigung des Multigenerationsexperimentes (Kapitel 3) aufgrund der Diskontinuität der durch die Arzneimittel bedingten Effekte zu falschen Rückschlüssen bezüglich der Langzeitwirkung dieser Substanzen geführt. Denn wäre das Experiment nach der ersten Generation beendet worden, wären die Auswirkungen der Arzneistoffe möglicherweise überschätzt worden, da die Vermutung nahe liegt, dass alle nachfolgenden Generationen auf die selben Weise von den Arzneimitteln beeinträchtigt werden. Bei Beendigung des Experiments nach der zweiten Generation, wären die Arzneistoffeffekte vermutlich unterschätzt worden, da man von einer vollständigen Akklimatisierung an die Substanzen ausgegangen wäre. Dementsprechend lässt sich die von den Arzneimitteln ausgehende Umweltgefährdung erst durch eine mehrere Generationen umfassende Studie genauer einschätzen.
Auch bei *Gammarus fossarum*, die der Arzneimittelmixtur in umweltrelevanten Konzentrationen ausgesetzt waren, konnte erst ab der dritten Häutung eine Verkürzung der Zeiträume zwischen den Häutungen beobachtet werden (Kapitel 4).

**Notwendigkeit von Studien zur Mischtoxikologie**

Weitere Studien zur Mischtoxikologie scheinen unabdingbar, da die Effekte von Arzneistoffgemischen oftmals unvorhersehbar sind und sehr verschiedenartig ausfallen können (Flaherty und Dodson 2005). So konnten auch im Rahmen dieser Dissertation für das gleiche Arzneimittelgemisch unterschiedliche Auswirkungen auf denselben Klon von *Daphnia magna* ermittelt werden. Während sich im Lebenszyklusexperiment verstärkte Effekte des Arzneistoffgemisches zeigten (Kapitel 2), konnte im Multigenerationsexperiment entgegen aktueller Studien (Cleuvers 2003, 2004, Schnell et al. 2009) keine gesteigerte Toxizität der Arzneistoffmixtur gegenüber den Einzelsubstanzen festgestellt werden (Kapitel 3). Möglicherweise können minimale Schwankungen bei der Laborhaltung, beispielsweise eine leicht veränderte Qualität der Futteralgen, zu solch unterschiedlichen Ergebnissen führen.

**Ökologische Konsequenzen umweltrelevanter Arzneistoffkonzentrationen**

Da bei unseren Experimenten umweltrelevante Arzneistoffkonzentrationen verwendet wurden, lassen sich anhand der in den Laborstudien beobachteten Auswirkungen auf die Testorganismen mögliche Konsequenzen für das komplette Ökosystem ableiten. So können beim Lebenszyklusexperiment (Kapitel 2) die sublethalen Auswirkungen zu Veränderungen innerhalb der Daphnienpopulation führen, die wiederum die verschiedenen trophischen Ebenen beeinflussen können. Beispielsweise kann sich aufgrund der geringeren Körpergröße der neonaten Daphnien der Prädationsdruck durch größenselektive Räuber wandeln. Während optisch orientierte vertebrate Prädatoren großes Zooplankton bevorzugen, jagen invertebrate Prädatoren vornehmlich kleine Zooplankter. Abhängig vom Prädationsdruck auf die Daphnien kann sich deren Konsumintensität auf das Phytoplankton verändern. Aufgrund der wichtigen Rolle von Daphnien im Nahrungsnetz von Seen und Teichen können schädliche Auswirkungen für das aquatische Ökosystem nicht ausgeschlossen werden.
Das Multigenerationsexperiment (Kapitel 3) zeigt, dass sich die Daphnien zwar kurzzeitig an die schädliche Wirkung der Arzneimittel anpassen können, jedoch lässt sich aus dem erneuten Auftreten der Arzneistoffeffekte schließen, dass aquatische Organismen wahrscheinlich aufgrund zu hoher Fitnesskosten keine dauerhafte Resistenz gegenüber den in die Gewässer eingetragenen Arzneistoffen entwickeln können (Xie und Klerks 2004).

Weiterhin kann bei einer permanenten Arzneimittelexposition in natürlichen Gewässern eine Beeinträchtigung der gesamten Gammaridenpopulation nicht ausgeschlossen werden (Kapitel 4), da die Organismen in Folge des durch die Arzneimittel verursachten diskontinuierlichen Größenzuwachses kleiner werden können. Dies kann wiederum zu erheblichen Fitness-nachteilen und einer verringerten Überlebenswahrscheinlichkeit führen. Da Gammariden wichtige Vertreter der Fließgewässerfauna darstellen und ein essentieller Bestandteil für die Ernährung etlicher Fischarten sind (MacNeil et al. 1999), kann es durch die permanente Belastung der Umwelt mit Arzneimitteln zu schwerwiegenden Folgen für das aquatische Ökosystem kommen.

**Umweltrelevanz der Laborstudien**

Da bei meinen Laborexperimenten möglichst naturnahe Voraussetzungen geschaffen wurden, indem die Arzneimittel über einen langen Expositionszeitraum hinweg in umweltrelevanten Konzentrationen und als Stoffgemische getestet wurden, könnten die gewonnenen Erkenntnisse zur Überarbeitung von standardisierten Toxizitätstestverfahren herangezogen werden und somit die Managementstrategien zur Erhalt der Umwelt verbessern. Jedoch muss berücksichtigt werden, dass Laborbedingungen nie vollständig die Beschaffenheit natürlicher Systeme widerspiegeln. So kann sich die Wirkung eines Arzneistoffgemisches in Abhängigkeit von der Zusammensetzung der Substanzen komplett ändern (Flaherty und Dodson 2005). Obwohl die vier ausgewählten Arzneimittel zu den am häufigsten in der Umwelt auftretenden Wirkstoffen gehören und zudem ein erhöhtes Risikopotenzial besitzen (Adler et al. 2001, Rohweder 2003, Sengl und Schüssler 2004), sind die meisten Gewässer zusätzlich mit anderen Stoffen kontaminiert. Je nach Komplexität der Mixtur kann es dadurch zu den verschiedenartigsten Wechselwirkungen zwischen den Arzneimitteln kommen.
Zudem findet man in Gewässern häufig Metabolite von Arzneistoffen, da viele Substanzen vom Körper umgewandelt und in veränderter Form wieder ausgeschieden werden. Miao und Metcalfe (2003) konnten in den Abflüssen von Kläranlagen fünf Metabolite des Anti-epileptikums Carbamazepin detektieren, wobei die Konzentration von trans-10,11-Dihydroxy-10,11-Dihydrocarbamazepin im Oberflächenwasser dreimal so hoch war wie die von Carbamazepin. Wie ich in zusätzlich durchgeführten Experimenten belegen konnte, besitzen diese beiden Substanzen allerdings eine sehr ähnliche Wirkweise: Sowohl Carbamazepin als auch trans-10,11-Dihydroxy-10,11-Dihydrocarbamazepin steigern die

Produktion von Nachkommen bei *Daphnia magna* (Abb. 2). Weiterhin konnte ich zeigen, dass sich auch Diclofenac und dessen Metabolit 1-(2,6-Dichlorphenyl)-1,3-dihydro-2H-indol-2-on sehr ähnlich auf die Morphologie und Life-history von *Daphnia magna* auswirkten. Jedoch können sich die Effekte von Metaboliten auch deutlich von den Auswirkungen des Reinstoffes unterscheiden (Marques et al. 2004). Dadurch wird es schwierig, Vorhersagen über das Umweltverhalten von Arzneistoffmetaboliten zu treffen, die ausschließlich auf Tests mit den zugehörigen Reinstoffen beruhen.

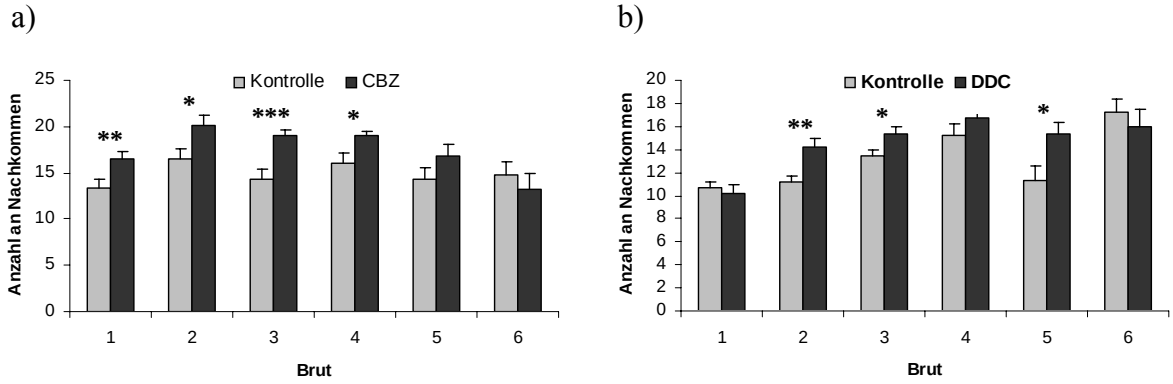



Abb. 2: Anzahl an produzierten Nachkommen (Mw + Sf; 20 ≥ n ≥ 11) pro Brut bei *Daphnia magna* (T-Test; * p ≤ 0,050; ** p ≤ 0,010; *** p < 0,001)
a) Exposition in 0,5 µg/l Carbamazepin (CBZ)
b) Exposition in 0,5 µg/l trans-10,11-Dihydroxy-10,11-Dihydrocarbamazepin (DDC)

Darüber hinaus reagieren aquatische Organismen verschieden sensitiv und somit sehr individuell auf die Schadstoffe. Beispielsweise konnten Oetken et al. (2005) unterschiedliche Auswirkungen des Wirkstoffs Carbamazepin auf aquatische Invertebraten verschiedener taxonomischer Gruppen beobachten: Weder auf den Oligochaet *Lumbriculus variegatus* noch auf die Schnecke *Potamopyrgus antipodarum* konnten Arzneimitteleffekte festgestellt werden. Bei der Mücke *Chironomus riparius* kam es jedoch bedingt durch Carbamazepin zu einer konzentrationsabhängigen Abnahme der Emergenz aufgrund einer Blockade bei der Verpuppung. In weiteren Versuchen konnte ich zeigen, dass die Häutung bei der Eintagsfliegenlarve *Baetis rhodani* im Gegensatz zu dem Amphipoden *Gammarus fossarum* nicht von dem Wirkstoffgemisch aus Carbamazepin, Diclofenac, 17α-Ethinylestradiol und Metoprolol beeinflusst wird. Darüber hinaus habe ich nachgewiesen, dass es selbst innerhalb derselben Gattung zu unterschiedlichen Reaktionen auf eine Arzneistoffexposition kommen kann. So waren Daphnien der Art *Daphnia cucullata*, die dem Wirkstoff trans-10,11-Dihydroxy-10,11-Dihydrocarbamazepin ausgesetzt waren, im Primiparstadium größer als die

Kontrolltiere, während Daphnien der Art *Daphnia magna*, die in der selben Substanz exponiert wurden, deutlich kleiner waren als die Kontrolle (Abb.3). Dementsprechend ist es möglich, dass einige Organismen in natürlichen aquatischen Systemen keinerlei Beeinträchtigung durch eingeleitete Arzneistoffe erlangen, wohingegen es bei anderen zu erheblichen Schädigungen kommen kann.

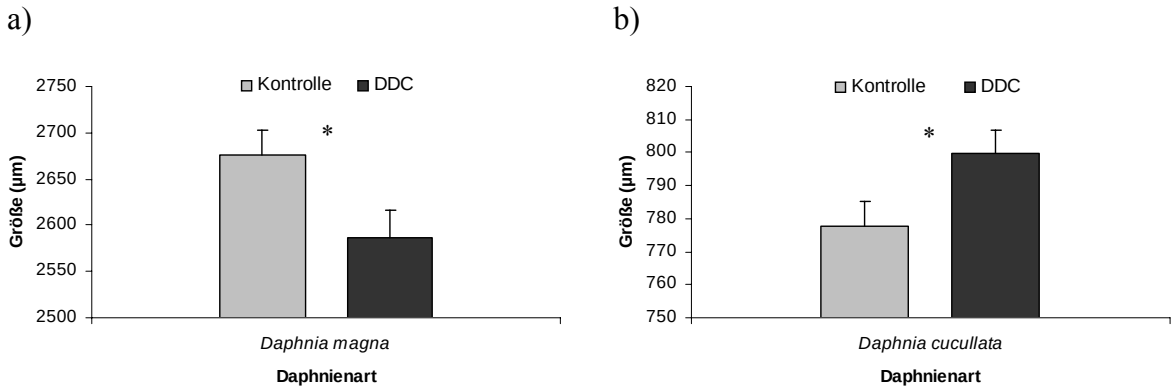



Abb. 3: Größe im Primiparstadium von verschiedenen Daphnienarten bei einer Exposition in 0,5 µg/l DDC (Mw + Sf; n = 20; T-Test; * $p \leq 0{,}050$)
a) *Daphnia magna*
b) *Daphnia cucullata*

Außer Acht gelassen wurde bei unseren Laborexperimenten auch der Einfluss multipler Stressoren (Heugens et al. 2001, Sih et al. 2004, Coors und De Meester 2008), obwohl die Organismen in der aquatischen Umwelt mehreren Stressoren zugleich ausgesetzt sind. So müssen sie abiotische Stressfaktoren wie beispielsweise Temperaturschwankungen und suboptimale Sauerstoffkonzentrationen tolerieren und biotischem Stress wie Nahrungslimitation, Konkurrenz und Prädation standhalten. Relyea und Mills (2001) konnten feststellen, dass sich das Pestizid Carbaryl sowie Räuberkairomone des Molches *Ambystoma maculatum* synergetisch auf Kaulquappen des grauen Baumfrosches (*Hyla versicolor*) auswirkten. Durch die Anwesenheit der Räuberkairomone erhöhte sich die lethale Wirkung des Pestizids enorm. Ein ähnliches Ergebnis konnte ich in einem zusätzlich durchgeführten Experiment mit *Daphnia magna* erzielen: Der Einfluss des Arzneistoffgemisches gekoppelt mit Kairomonen des Räubers *Triops cancriformis* verursachte bei den Daphnien eine erhöhte Mortalität (Abb. 4). Hinsichtlich morphologischer und life-history Parameter ergaben sich bei den Daphnien jedoch keine verstärkten Effekte.

Weiterhin kann sich die Toxizität eines Arzneistoffes mit der Qualität des Futters verändern (Hansen et al. 2008). Im Gegensatz dazu konnte ich in einem weiteren Versuch nachweisen,

dass die Futterquantität keinen negativen Einfluss auf *Daphnia magna* ausübte, die zugleich in der Arzneimittelmixtur exponiert wurden. Allerdings gilt *Daphnia magna* als sehr tolerante Art, da sie aufgrund ihrer Körpergröße und dem damit verbundenen kleineren Oberflächen zu Volumen Verhältnis eine geringere Menge an toxischen Substanzen aufnehmen können als kleinere Arten (Lilius et al. 1995). Somit wäre es vorstellbar, dass sich bei sensitiveren Daphnienarten hinsichtlich der Futterquantität synergetische Effekte zeigen.

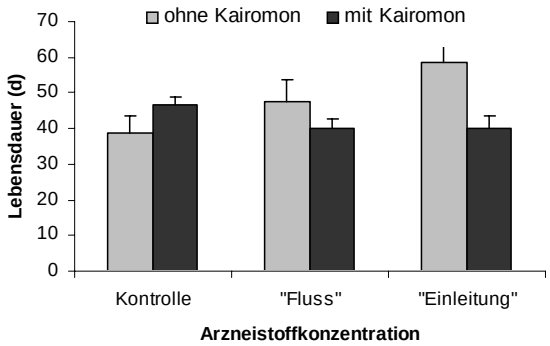



Abb. 4: Lebensdauer von *Daphnia magna* in An- und Abwesenheit von Kairomonen des Räubers *Triops cancriformis* bei verschiedenen umweltrelevanten Konzentrationen des Arzneistoffgemisches aus Carbamazepin, Diclofenac, 17α-Ethinylestradiol und Metoprolol.
(Mw + Sf; $15 \geq n \geq 12$; Zwei-Wege ANOVA; $p = 0{,}008$)
„Fluss“: Maximalkonzentrationen bayerischer Fließgewässer
„Einleitung“ Maximalkonzentrationen bayerischer Kläranlagenabläufe

In zukünftigen Laborstudien könnten die oben erwähnten Aspekte noch intensiver berücksichtigt und integriert werden. Somit ließen sich noch detailliertere Erkenntnisse über den Einfluss von Arzneistoffen auf aquatische Invertebraten gewinnen. Da sich jedoch kaum alle Aspekte in Laborstudien vereinen lassen, können nur Vermutungen über mögliche Konsequenzen für das aquatische Ökosystem angestellt werden, aber keine Aussagen über das tatsächliche Umweltverhalten der getesteten Arzneistoffe getroffen werden.

**Relevanz von Freilandstudien**

Um die von den freigesetzten Arzneistoffen ausgehende Umweltgefährdung zu ermitteln, sind daher Freilandstudien unabdingbar. Hierbei werden sämtliche abiotischen und biotischen Parameter berücksichtigt, allerdings ist es oftmals schwierig, aufgrund der Vielzahl an Umweltfaktoren und deren Wechselwirkungen Rückschlüsse über den tatsächlichen Einfluss

der Arzneimittel auf das aquatische Ökosystem zu ziehen. So konnten auch in unserer großräumig angelegten Freilandstudie (Kapitel 5) nur Vermutungen über die Auswirkungen von eingeleiteten Schadstoffen auf die Gemeinschaft benthischer Makroinvertebraten angestellt werden.
Dies könnte sich jedoch zukünftig durch den Einsatz von Mesokosmenstudien ändern (Richards et al. 2004). Dabei wird das gesamte Ökosystem einschließlich der darin vorkommenden Interaktionen betrachtet, zusätzlich können aber bestimmte Faktoren gezielt manipuliert werden. Somit könnten die durch die Arzneimittel hervorgerufene Effekte auf sämtlichen trophischen Ebenen detektiert werden und somit die Konsequenzen für das Ökosystem verdeutlicht werden.

**Reduktion des Arzneistoffeintrags**

Wasser ist das höchste Gut der Menschheit und muss in seiner Reinheit bewahrt werden. Daher muss der Eintrag von Schadstoffen so gering wie möglich gehalten werden. Da heutzutage der Einsatz von Arzneistoffen in unserer Gesellschaft jedoch nicht mehr wegzudenken ist, scheint nur eine Verbesserung der Eliminierungseffizienz von Kläranlagen die Belastung der aquatischen Ökosysteme zu vermindern. Dies könnte durch Techniken wie der Aktivkohlefiltration und der Ozonierung oder durch den Einsatz von Membranverfahren erreicht werden (Heberer 2002, Reungoat et al. 2009). Da die Umrüstung konventioneller Kläranlagen mit extremen Kosten verbunden ist, könnten zunächst extrem belastete Abwässer aus Kliniken und Pflegeheimen diesen neuen Techniken unterzogen werden. Nach und nach sollten auch die Kläranlagen größerer Städte nachgerüstet werden. Zugleich gilt es aber, an die Vernunft der Bevölkerung bezüglich der fachgerechten Entsorgung von Altmedikamenten zu appellieren und deren Bewusstsein für den Erhalt der Natur zu schärfen.

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# LEBENSLAUF

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## Ausbildung

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| seit 01/2007 | Promotion an der Ludwig-Maximilians-Universität (LMU) München „Einfluss von Arzneistoffen auf aquatische Invertebraten“ |
| 03/2006 | Abschluss als Diplom-Biologin |
| 06/2005 - 03/2006 | Diplomarbeit an der LMU München „Einfluss verschiedener Bewirtschaftungsoptionen auf die aquatische Biozönose in Abwasserklärteichen des Ismaninger Teichgebiets“ |
| 10/2000 - 03/2006 | Studium der Biologie an der LMU München<br>Hauptfach: Ökologie<br>Nebenfächer: Mikrobiologie, Neurobiologie, Wildbiologie |
| 09/1991 - 06/2000 | Gymnasium Starnberg |

## Praktika

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| 07/2004 | Institut für Wildtierkunde und Ökologie der Veterinärmedizinischen Universität Wien, Graubünden, Schweiz: Ökophysiologie von Alpenmurmeltieren |
| 06/2004 | Landesbund für Vogelschutz, Starnberg: Beringungsaktion von Flussseeschwalben |
| 03/2004 - 05/2004 | Aquatic Research Group of the Department of Zoology, University of Otago, Dunedin, New Zealand: Labortätigkeiten, Freilandarbeiten |
| 07/2003 - 08/2003 | Landesbund für Vogelschutz, Garmisch-Partenkirchen: Kartierungs- und Beobachtungsprojekte, Umweltbildung, Öffentlichkeitsarbeit |

## Berufserfahrung

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| seit 01/2010 | Wissenschaftliche Mitarbeiterin in der Arbeitsgruppe „Evolutionsökologie“ an der LMU München |

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| 09/2009 | Freiberufliche Tätigkeit im Büro für Naturschutz, Gewässer- und Fischereifragen, Pähl: Freilandarbeiten |
| 12/2001 - 12/2006 | Wissenschaftliche Hilfskraft am Botanischen Institut und Ökologischen Institut der LMU München: sterile Arbeitstechniken mit Zellkulturen, Aufbereitung und Auswertung von Invertebraten- und Algenproben, Freilandarbeiten |
| 05/2000 - 12/2005 | Max-Planck-Institut für Ornithologie, Seewiesen: Jungvogelaufzucht, Versuchsvorbereitungen und -durchführungen (Vogelzug) |

**Präsentationen**

| | |
|---|---|
| 10/2009 | EES Conference, München: „Detrimental effects of a pharmaceutical mixture on *Daphnia magna*“ (Vortrag) |
| 10/2009 | Jahrestagung der Deutschen Gesellschaft für Limnologie, Oldenburg: „Auswirkungen von Arzneistoffen in umweltrelevanten Konzentrationen auf life-history Parameter von *Daphnia magna*: ein Multigenerationsexperiment“ (Vortrag) |
| 04/2009 | Interact PhD Symposium, München: „Chronic effects of a pharmaceutical mixture on *Daphnia magna*“ (Poster) |
| 12/2008 | Doktorandenforum der Studienstiftung des deutschen Volkes, Köln: „Einfluss von Arzneistoffen auf aquatische Invertebraten“ (Vortrag) |
| 09/2008 | Jahrestagung der Deutschen Gesellschaft für Limnologie, Konstanz: „Auswirkungen von Arzneistoffgemischen auf life-history Parameter von *Daphnia magna* nach chronischer Exposition: Multigenerations- und Lebenszyklusexperimente“ (Vortrag) |
| 09/2006 | Jahrestagung der Deutschen Gesellschaft für Limnologie, Dresden: „Einfluss verschiedener Bewirtschaftungsformen auf die aquatische Biozönose von Abwasserklärteichen und ihre Nutzung durch Wasservögel“ (Vortrag) |

**Veröffentlichungen**

**Dietrich S.**, Dammel S., Ploessl F., Bracher F., Laforsch C. (2010): Effects of a pharmaceutical mixture at environmentally relevant concentrations on *Gammarus fossarum*. Marine and Freshwater Research 61: 196–203.

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**Lehrtätigkeit an der Universität**

| | |
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| Praktikum | „Basics in aquatic ecology“ (Lehrauftrag) |
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| Praktikum | „Grundlagen der Limnologie“ (Lehrauftrag) |
| Praktikum | „Methoden der Fließgewässerökologie“ |
| Praktikum | „Bachelorkurs Ökologie I“ |
| Praktikum | „Bachelorkurs Ökologie II“ |
| Praktikum | „Basiskurs Ökologie und Evolutionsbiologie“ |
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**Auszeichnungen**

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| 06/2000 | Carl Friedrich von Martius Umweltpreis für Facharbeit im Leistungskurs Biologie „Beobachtungen zum Brutverhalten solitär lebender Bienen und Wespen an Nisthilfen“ |

**Stipendien**

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| 02/2007 - 12/2009 | Promotionsstipendium der Studienstiftung des deutschen Volkes |
| 08/2005 | Oskar-Karl-Forster Bücherstipendium der LMU München |
| 09/2000 - 07/2006 | Online-Stipendium e-fellows.net |

**Kenntnisse, Fähigkeiten, Interessen**

| | |
|---|---|
| Sprachen | Deutsch (Muttersprache), Englisch (sehr gut), Französisch (gut), Latein (Latinum) |
| EDV | Sehr gute Kenntnisse von Microsoft Office<br>Gute Kenntnisse von Statistiksoftware (z.B. SPSS, Past, Primer), Adobe Photoshop |
| Fischerei | Fischereischein, Bedienungsschein für Elektrofischfanggeräte |
| Interessen | Berg- und Ausdauersport, Violine (Symphonieorchester) |

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# BEITRAG DER AUTOREN AN DEN ARTIKELN

Alle Paper und Manuskripte basieren vorwiegend auf meiner eigenen Arbeit, allerdings wurde ich dabei von einigen Coautoren unterstützt:

Artikel 1:
Wilfried Gabriel berechnete die Populationswachstumsrate und war am Verfassen des Manuskripts beteiligt. Florian Plössl und Franz Bracher stellten die Arzneimittel zur Verfügung und führten Vorversuche zum Abbauverhalten der verwendeten Stoffe durch. Christian Laforsch unterstützte mich bei der Konzeption des Versuches und bei der Interpretation der Ergebnisse. Zudem war er am Verfassen des Manuskripts beteiligt.

Artikel 2:
Florian Plössl und Franz Bracher waren für die Durchführung der Arzneistoffanalysen zuständig und wirkten am Verfassen des Material und Methodenteils „Chemical analysis" mit. Christian Laforsch war an der Konzeption des Versuches, bei der Interpretation der Ergebnisse sowie am Verfassen des Manuskripts beteiligt.

Artikel 3:
Shana Dammel war im Rahmen ihrer Diplomarbeit an der Durchführung der Experimente beteiligt und wirkte bei der Datenaufnahme mit. Florian Plössl und Franz Bracher stellten die Arzneimittel zur Verfügung und testeten das Abbauverhalten der verwendeten Stoffe in Vorversuchen. Zudem halfen sie beim Verfassen des Diskussionsabschnittes „Chemical compounds". Christian Laforsch war an der an der Konzeption des Versuches, bei der Interpretation der Ergebnisse sowie am Verfassen des Manuskripts beteiligt.

Artikel 4:
Anne-Kathrin Bothe war im Rahmen ihrer Diplomarbeit an den Freilandarbeiten beteiligt und wirkte bei der Auswertung der Experimente (Auszählen der Invertebratenproben) mit. Volker Witte half bei den statistischen Analysen und der Interpretation der Ergebnisse. Michael Effenberger unterstützte mich bei der Versuchskonzeption sowie bei der Durchführung im Freiland. Florian Plössl führte die begleitende chemische Analytik durch. Christian Laforsch wirkte bei der Interpretation der Ergebnisse sowie beim Verfassen des Manuskriptes mit.

## EHRENWÖRTLICHE VERSICHERUNG UND ERKLÄRUNG

Diese Promotion wurde im Sinne des § 12 der Promotionsordnung von PD Dr. Christian Laforsch betreut.

Hiermit erkläre ich, dass die vorliegende Dissertation von mir selbständig und ohne unerlaubte Hilfe angefertigt wurde. Zudem wurden keine anderen als die angegebenen Quellen verwendet.

Ich versichere, dass die Dissertation keiner anderen Prüfungskommission vorgelegt wurde und ich mich nicht anderweitig einer Doktorprüfung ohne Erfolg unterzogen habe.

Maising, 20. Mai 2010

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Sabine Bernhard