# Effects of microplastics on freshwater organisms: A laboratory approach

Dissertation
der Mathematisch-Naturwissenschaftlichen Fakultät
der Eberhard Karls Universität Tübingen
zur Erlangung des Grades eines
Doktors der Naturwissenschaften
(Dr. rer. nat.)

vorgelegt von
Diplom-Biologin Saskia Rehse
aus Biberach an der Riß

Tübingen

2019

Gedruckt mit Genehmigung der Mathematisch-Naturwissenschaftlichen Fakultät der Eberhard Karls Universität Tübingen.

| | |
|---|---|
| Tag der mündlichen Qualifikation: | 23. Juli 2019 |
| Dekan: | Prof. Dr. Wolfgang Rosenstiel |
| 1. Berichterstatter: | Jun. Prof. Dr. Christiane Zarfl |
| 2. Berichterstatter: | Prof. Dr. Werner Kloas |

# Summary

The ubiquitous pollution of freshwater systems with particularly small plastics, i.e. microplastics (< 5 mm Moore 2008), became increasingly evident within the last years (e.g. Eriksen et al. 2013, Faure et al. 2015, Free et al. 2014). Microplastics have been shown to be able to adversely affect aquatic organisms by themselves (physical effects, e.g. Wright et al. 2013b as overview) but are also discussed to act as vector for chemical pollutants, which are associated with microplastics such as additives or contaminants sorbed to the microplastic material (chemical effects, e.g. Teuten et al. 2009). The potential risk that microplastics may pose to freshwater organisms is still difficult to estimate due to uncertainties of environmental exposure and because the majority of studies in the past focused on marine organisms. The aim of this thesis is to systematically analyse how microplastics potentially adversely affect freshwater organisms by themselves and in combination with chemical pollutants. The presented results were obtained from an experimental laboratory approach, which was based on established ecotoxicological methods. Juvenile water fleas (*Daphnia magna*), as representative for limnic zooplankton, and tadpoles of the African clawed frog (*Xenopus laevis*), as representative for amphibians, were first exposed to pristine microplastics alone, before combining them with selected chemical pollutants in the next step. High concentrations of both, microplastics and chemical pollutants, were used for exposures in order to get a better mechanistic understanding of potential adverse impacts and to identify critical concentrations of microplastics for freshwater organisms.

Physical effects of the mere microplastic material itself were induced by the smallest analysed microplastic type in *D. magna*. Exposure of water fleas to a high concentration range (25-400 mg $L^{-1}$) of polyethylene (PE) particles with 1-4 µm in diameter (1 µm PE particles) with a regular round shape for 72 and 96 hours and rapid ingestion led to immobilisation. Immobilisation rates increased with dose and time resulting in an $EC_{50}$ of 57.43 mg $L^{-1}$ after 96 hours (95% confidence intervals in mg $L^{-1}$, lower: 32.76, upper: 100.69). In contrast, PE particles with 90-106 µm in diameter (100 µm PE) in the same concentration range as 1 µm PE did not induce adverse impacts in daphnids after exposure for up to 96 hours. Exposure to polyamide (PA) particles with 15-20 µm mean diameter and an irregular fragmented shape for 48 hours did not induce immobilisation in daphnids at high concentrations (25-250 mg $L^{-1}$). In tadpoles, a relatively low and high concentration (1 and 100 mg $L^{-1}$) of the same type of PA particles did neither adversely affect general development nor elevate stress hormone levels significantly after one and three weeks of exposure.

In daphnids, the presence of PA particles (200 mg $L^{-1}$) reduced immobilisation induced by a range of concentrations of bisphenol A (BPA, 2.5-40 mg $L^{-1}$). Sorption of BPA to PA particles, prior to exposure until sorption equilibrium was reached, led to a decrease of aqueous BPA. During exposure, microplastics loaded with BPA were ingested by

daphnids but microplastics did not contribute substantially to the overall uptake of BPA. The removal of BPA from the most bioavailable fraction, i.e. water, was most crucial for immobilisation rates. In tadpoles, estrogenic effects of the endocrine disruptor ethinylestradiol (EE2) on sexual development and on mRNA expression of different biomarkers from the brain, gonad and liver were not statistically significant altered by the presence of two concentrations of PA particles (1 and 100 mg $L^{-1}$) after three weeks of exposure. A trend for elevated mRNA expression of vitellogenin in the presence of PA particles, however, indicated potential enhanced exposure to EE2, especially in males and for the high concentration of microplastics (100 mg $L^{-1}$). The trend for elevated vitellogenin mRNA levels point towards a potential vector effect of PA particles i.e. facilitated uptake of EE2 from loaded PA particles. This potential vector effect could be attributed to the high sensitivity of tadpoles to detect also small differences of exposure to estrogenic substances. Physiological conditions within the tadpoles such as longer gut residence times of ingested particles and different gut regimes compared to e.g. daphnids have to be considered as well.

This thesis provides evidence that small-sized microplastics in the range of a few micrometres at high concentrations in the unit range of mg $L^{-1}$ are most likely to pose a risk for freshwater organisms, in this case freshwater zooplankton. Removal of a chemical contaminant from the aqueous phase can lead to reduced effect rates of a chemical contaminant in organisms in an equilibrated system when water is the most important uptake pathway, as shown in daphnids. The vector effect of microplastics may most likely play a role for contaminants which affect organisms at very low concentrations such as endocrine disruptors, as indicated by a trend for enhanced exposure to EE2 in the presence of microplastics in tadpoles. Both, physical and chemical effects of microplastics in this study were observed only at high concentrations which are presumably above present environmental concentrations. Breakdown of bigger plastic items and ongoing emission of microplastics, though, can be expected to increase the abundance of microplastics in rivers and lakes. The results presented in this study can be compared to recent and future environmental exposure scenarios and can help to estimate the effects of microplastics on freshwater organisms.

# Zusammenfassung

Die allgegenwärtige Verschmutzung von Binnengewässern mit besonders kleinen Plastikteilchen, Mikroplastik (< 5 mm, Moore 2008), wurde in den vergangenen Jahren immer deutlicher (z.B. Eriksen et al. 2013, Faure et al. 2015, Free et al. 2014). Es wurde gezeigt, dass Mikroplastik dazu in der Lage ist, adverse Effekte in aquatischen Organismen auszulösen (physikalische Effekte, z.B. Wright et al. 2013b als Übersicht). Außerdem wird die Rolle von Mikroplastik als Vektor für chemische Schadstoffe, wie zum Beispiel Additive und Schadstoffe die an Mikroplastik binden, diskutiert (chemische Effekte, z.B. Teuten et al. 2009). Das potentielle Risiko, das Mikroplastik für Süßwasserorgansimen darstellt, ist allerdings immer noch schwer abzuschätzen, da Unsicherheiten bezüglich der Umweltexposition bestehen und die meisten Studien in der Vergangenheit marine Organismen im Fokus hatten. Ziel dieser Dissertation ist es, systematisch zu analysieren, inwiefern Mikroplastik an sich und in Kombination mit chemischen Schadstoffen Süßwasserorganismen beeinflusst. Die dargestellten Ergebnisse wurden experimentell im Labor basierend auf etablierten ökotoxikologischen Methoden gewonnen. Jungtiere des Großen Wasserflohs (*Daphnia magna*) als Vertreter für limnisches Zooplankton und Kaulquappen des Afrikanischen Krallenfroschs (*Xenopus laevis*) als Vertreter für Amphibien wurden zuerst mit reinem Mikroplastik und dann mit Mikroplastik in Kombination mit ausgewählten chemischen Schadstoffen exponiert. Für die Exposition wurden jeweils hohe Mikroplastik- und Schadstoffkonzentrationen gewählt, um ein besseres mechanistisches Verständnis der potentiellen adversen Effekte von Mikroplastik zu gewinnen und um für Süßwasserorganismen kritische Mikroplastikkonzentrationen zu ermitteln.

Physikalische Effekte des reinen Mikroplastikmaterials an sich wurden durch den kleinsten verwendeten Mikroplastiktyp bei *D. magna* induziert. Die Exposition der Wasserflöhe mit hohen Konzentrationen (25-400 mg $L^{-1}$) kugelförmiger Polyethylen (PE)-Partikel mit einem Durchmesser von 1-4 µm (1 µm PE-Partikel) für 72 und 96 Stunden führte zur schnellen Aufnahme der Partikel in den Verdauungstrakt und zur Immobilisierung der Tiere. Die Immobilisierungsrate stieg mit der Dosis und der Expositionsdauer und resultierte in einem $EC_{50}$ von 57.43 mg $L^{-1}$ nach 96 Stunden (95 % Konfidenzintervalle in mg $L^{-1}$, unteres: 32.76, oberes: 100.69). PE-Partikel mit einem Durchmesser von 90-106 µm (100 µm PE) im gleichen Konzentrationsbereich wie 1 µm PE-Partikel induzierten nach bis zu 96 Stunden dagegen keine adversen Effekte in *D. magna*. Die Exposition der Wasserflöhe mit hohen Konzentrationen (25-250 mg $L^{-1}$) unregelmäßig geformter Polyamid (PA)-Partikel mit durchschnittlich 15-20 µm im Durchmesser für bis zu 48 Stunden induzierte ebenfalls keine adversen Effekte. Die Exposition der Kaulquappen mit einer eher niedrigen und einer hohen Konzentration (1 und 100 mg $L^{-1}$) derselben Sorte an PA-Partikeln für eine und drei Wochen beeinflusste weder die allgemeine Entwicklung der Tiere noch die Plasmaspiegel der Stresshormone.

Immobilisierungsraten, welche von unterschiedlichen Bisphenol A-Konzentrationen (BPA, 2.5-40 mg $L^{-1}$) in Wasserflöhen induziert wurden, waren in Kombination mit einer hohen Konzentration von PA-Partikeln (200 mg $L^{-1}$) reduziert. Die Sorption von BPA an PA-Partikel bis zum Erreichen des Sorptionsgleichgewichts vor Beginn der Exposition führte zu einer Reduzierung von in Wasser gelöstem BPA. Mit BPA beladene PA-Partikel wurden während der Exposition zwar von den Wasserflöhen aufgenommen, die Aufnahme von BPA über die PA-Partikel war dabei aber nicht maßgeblich. Die Reduzierung von BPA in der bioverfügbarsten Fraktion, nämlich Wasser, war entscheidend für die Immobilisierungsraten. Östrogene Effekte des endokrinen Disruptors Ethinylestradiol (EE2) auf die Geschlechtsentwicklung und die mRNA-Expression verschiedener Biomarker aus den Gehirnen, Gonaden und Lebern der Kaulquappen waren nach einer dreiwöchigen Exposition mit zwei Konzentrationen an PA-Partikeln (1 und 100 mg $L^{-1}$) nicht in statistisch signifikantem Maß messbar. Ein Trend für erhöhte mRNA-Expression des sensitivsten Biomarkers Vitellogenin deutet jedoch auf eine erhöhte Exposition mit EE2 hin, wenn die Kaulquappen mit EE2 in Kombination mit PA-Partikeln exponiert wurden. Dieser Trend war vor allem für Männchen und bei einer hohen Konzentration an PA-Partikeln (100 mg $L^{-1}$) zu beobachten und deutet auf einen potentiellen Vektoreffekt, also auf die erleichterte Aufnahme von EE2 durch beladene PA-Partikel, hin. Die hohe Sensibilität der Kaulquappen selbst auf kleinste Unterschiedene in der Exposition zu östrogenartig wirkenden Substanzen anzusprechen, könnte für diesen potentiellen Vektoreffekt eine Rolle spielen. Ebenfalls zu berücksichtigen sind physiologische Charakteristika der Kaulquappen mit längerer Verweildauer aufgenommener Partikel im Darm und unterschiedlichen Darmregimes im Vergleich zu – beispielsweise – Wasserflöhen.

Die Ergebnisse dieser Dissertation zeigen auf, dass kleine Mikroplastikteilchen im Bereich von wenigen µm bei hohen Konzentrationen im Bereich von mehreren mg $L^{-1}$ am ehesten ein Risiko für Süßwasserorganismen, hier Zooplankton, darstellen. Wie bei Wasserflöhen gezeigt, kann die Reduktion der Konzentration eines chemischen Schadstoffs im Wasser bei einem System im Sorptionsgleichgewicht und mit Wasser als wichtigsten Aufnahmepfad zu reduzierten Effektraten führen. Der Trend für erhöhte Exposition der Kaulquappen mit EE2 durch Mikroplastik deutet darauf hin, dass der Vektoreffekt von Mikroplastik voraussichtlich eher eine Rolle für Schadstoffe spielt, die Organismen bereits bei sehr niedrigen Konzentrationen beeinträchtigen, zum Beispiel endokrine Disruptoren. In der vorliegenden Studie wurden sowohl physikalische als auch chemische Effekte von Mikroplastik ausschließlich bei sehr hohen Mikroplastikkonzentrationen beobachtet, welche vermutlich über momentanen Konzentrationen in Binnengewässern liegen. Es kann allerdings erwartet werden, dass die Fragmentierung größerer Kunststoffteile und die fortwährende Emission von Mikroplastik zu einem Anstieg von Mikroplastikkonzentrationen in Flüssen und Seen führen. Die Erkenntnisse der vorliegenden Dissertation können mit derzeitigen und zukünftigen Expositionsszenarios

in der Umwelt verglichen werden und können so dazu beitragen, die Effekte von Mikroplastik auf Süßwasserorganismen besser abzuschätzen.

# Declaration of contributions to joint work

Parts of this thesis (Chapter 2, Chapter 3 and Chapter 4) are based on joint work with Christiane Zarfl, Werner Kloas, Andrea Ziková, Wibke Kleiner, Nadine Poßnien, Antje Tillack, Angela Krüger and Claudia Theel. Details on the contributions of each author can be found at the beginning of the respective chapters. The majority of all listed steps was performed by Saskia Rehse.

# Acknowledgements

I would like to thank the German Federal Environmental Foundation (DBU) for giving me a personal scholarship (20013/283) including funding as well as participation in the scholarship program. Special thanks for the additional support within the "Stipendienschwerpunkt Integrierte Chemikalienbewertung" including funding for the International Conference on Chemistry and the Environment (ICCE) in Leipzig in 2015. I very much enjoyed being part of the DBU community. The meetings always encouraged me to carry on and to set my research into a broader perspective.

I also thank the German Academic Exchange Service (DAAD) for funding me for two international conferences, the Annual meeting of the Society of Environmental Toxicology and Chemistry (SETAC Europe) in Barcelona in 2015 and the ICCE in Oslo in 2017.

I would like to thank my supervisors, Christiane Zarfl and Werner Kloas, for supervising my thesis and for giving me the opportunity to conduct my research at the Leibniz-Institute of Freshwater Ecology and Inland Fisheries (IGB) with the support from the Center for Applied Geoscience (ZAG, University of Tübingen). I appreciate their ongoing support, enthusiasm, encouragement and all fruitful discussions, especially on how to tackle this newly emerging topic with all the challenges which came along. Special thanks to Christiane Zarfl for always being open to discuss the approaches and results of this thesis from a systemic point of view and for introducing me to new perspectives, which complemented my understanding of the topic. Special thanks to Werner Kloas for always providing me with everything I needed to conduct my research at IGB and for sharing his ecotoxicology know-how with me, including his broad hands-on support during experimental procedures for the *Xenopus* project.

I appreciate the support of many people during experimental and laboratory work. Thank you to Alexander Wacker from the University of Potsdam who provided daphnids for the establishments of a stock and to the group of Justyna Wolinska at IGB, especially Ursula Neven, Johanna Griebel and Manja Saebelfeld for helping setting up the *Daphnia* stock. I thank Thomas Meinelt for providing space for the *Daphnia* cultures and experimental setups as well as Sascha Behrens and Wibke Kleiner for their ongoing general support and for looking after my *Daphnia* culture from time to time. A special thanks to Andrea Ziková, Wibke Kleiner, Antje Tillack and Nadine Poßnien for their extensive work for the *Xenopus* project, which would not have been possible without them. I would also like to thank Claudia Lorenz for introducing me into cutting tissue sections, Angela Krüger and Claudia Theel for their help with analytical measurements as well as Sara Kleindienst and Claudia Tominski from the Geomicrobiology group of Andreas Kappler at ZAG for taking pictures of microplastics.

I thank Kirsten Pohlmann for checking with me the statistics for my second paper and Julia Kleinteich from ZAG for her suggestions on how to improve the second mansucript.

It is my great pleasure to thank all my colleagues and friends who accompanied and supported me during this special time, including the people from the Ecophysiology and Aquaculture department at IGB, the Environmental Systems Analysis group at ZAG and especially Oleksandra Shumilova and Maja Grubisic, who also gave me helpful feedback for the introduction of this thesis, as well as Barbara Behounek, Romain Clément, Therese Kettner, Abel de Souza Machado, my office mate Gregor Kalinkat, Laura Tydecks, Andrea Ziková, Fengzhi He, Clara Romero and Thomas Fuß.

Last, I would like to thank my family as well as Florian and his family for their never-ending support.

# Table of contents

# List of figures



**Appendices**

# List of tables



**Appendices**

# 1 General introduction

Plastic pollution is one of the most perceived environmental issues in the 21st century. The presence of plastics in all environmental compartments has been shown by various scientific studies but its full extent is still under investigation (e.g. Eriksen et al. 2014, Faure et al. 2015, Jambeck et al. 2015). It is also not fully clarified yet how plastic pollution influences the environment, from a broad perspective of ecosystems to organism-based levels. The effects of other environmental issues, such as climate change, chemical pollution and land-use change, are not fully clarified yet as well, but overall there is no doubt, that anthropogenic actions have lasting impacts on the environment (Meybeck 2004, Sala 2000, Steffen et al. 2011).

What is perceived as natural has been already altered by human civilisations in most areas in the world, e.g. by land-use and deforestation (Turner et al. 1995). These often obvious changes are accompanied by changes which are sometimes more difficult to perceive, e.g. environmental pollution (Singer 1970). Anthropogenic impacts on the environment are diverse and so can be the consequences from its interventions. The impacts of humanity on the environment are so severe, that a new geological era, the Anthropocene (Crutzen 2002), was proposed to follow the Holocene. The presence of plastics is discussed as one indicator for the Anthropocene, because of its longevity and ubiquitous presence in various compartments such as terrestrial, marine and freshwater sediments, even in remote regions (e.g. Barnes et al. 2009, Zalasiewicz et al. 2016). During the era of the Holocene, humanity developed under relatively stable conditions (Petit et al. 1999). Over thousands of years, various biotic and abiotic interactions have led to the development of a complex system with interdependencies and interrelationships, the "web of life" (Capra 1997). Humanity developed within this diverse environment as a result of evolution as driving force of the biosphere and it needs natural resources and relatively stable conditions to survive and thrive. The concept of "planetary boundaries" was proposed to define factors that are crucial for sustaining the Earth system and thresholds for each of them (Rockström et al. 2009a, Rockström et al. 2009b). Exceeding these thresholds is hypothesized to weaken fundamental environmental systems, resulting in instability of the relatively narrow range of favourable conditions established in the Holocene. This concept received a lot of attention not only in the scientific community, but also in the public, while being critically discussed at the same time, e.g. the quantifiability of the proposed factors (Montoya et al. 2018). Pollution, e.g. with endocrine disruptors and plastics, was included as one factor in the first published proposal of the concept but could not be quantified and was included in the factor "novel entities" in a later version (Steffen et al. 2015). This factor includes entirely new substances and altered forms of substances or life forms with the potential to influence geophysical and biological processes. Persistence and widespread distribution of novel entities are considered to be crucial for potential effects on the Earth system and both apply to microplastics as pollutants. The

inclusion of plastics in the concepts of both, the Anthropocene and Planetary boundaries illustrates the need to get a more detailed understanding about plastic pollution in the environment in order to identify potential effects on ecosystems down to organisms.

## 1.1 Concerns about freshwater ecosystems

Freshwater is one of the most important resources for life, which is why concerns such as water scarcity and safety of drinking water are highly relevant for the well-being of humans. Apart from these direct dependencies, humans also rely on the functioning of ecosystems e.g. for fundamental ecosystem services such as the production of food (Daily 1997). In freshwater systems, pollution was identified as crucial stressor and is assumed to be one reason for the more rapid worldwide decline of freshwater biodiversity compared to terrestrial systems (e.g. Dudgeon et al. 2006, Sala 2000). Amphibians, which spent at least a part of their lifetime in freshwater during early development, are threatened worldwide, but not all processes behind their rapid decline are identified yet (Stuart et al. 2004). The presence of hormonal active synthetic compounds, i.e. endocrine disruptors, is assumed to be one reason for decreasing amphibian populations (Carey and Bryant 1995). The presence of synthetic particulate matter, such as nanoparticles and plastics in freshwaters, along with pollution with chemicals, has been increasingly recognized as potential new threat to freshwater organisms within the last years (Eerkes-Medrano et al. 2015, Howard 2010).

## 1.2 Ambivalence of plastic material

### 1.2.1 Plastic as versatile material

The discovery and further development of polymer material in the beginning of the early 20$^{th}$ century represents a significant step for industrial and technological progress leading to numerous societal benefits e.g. in the health sector (Andrady and Neal 2009). Natural-occurring polymers such as cellulose or silk have been used long before their molecular structures have been identified. The German chemist Herrmann Staudinger was one of the first scientists to elucidate chemical characteristics of polymers. He postulated that polymers are long chains of molecules (i.e. macromolecules, Staudinger 1920) and won the Nobel prize in chemistry for his accomplishments in polymer science in 1953. His macromolecular hypothesis was confirmed some years later leading to the discovery of polymers such as nylon, known as polyamide (PA, Sperling 2005). Spinned PA was one of the first produced synthetic fibres. The repeating units of PA are linked with amide bonds as in the natural-occurring polymer silk. The group of polyethylene (PE) is an example for the high diversity of polymers. PE was found to have a simple structure with the alkane ethylene as monomer, several thousands of carbon atoms without side chains and strong covalent bond connections. Its characteristics are highly variable, e.g.

crystallinity, density and molecular weight, and result in different properties for its diverse applications (Andrady 2017).

Based on their molecular structure, synthetic polymers can be classified into thermoplastics, elastomers and thermosets (Young and Lovell 2011). Thermoplastics (e.g. PE) have a linear or branched molecular structure and turn liquid when treated with heat, which is why they can be easily moulded. Elastomers are rubbery and can be stretched due to their crosslinked molecular structure (e.g. polyisobutylene, PIB). Thermosets have a highly crosslinked structure leading to high rigidity of the material (e.g. polyurethane, PUR). The term "plastic" originally represents only one subgroup of synthetic polymers by chemical definition, i.e. thermoplastics, but is broadly used to refer to synthetic polymers in general. Thus, plastic is used as the latter in this thesis.

A total of 335 million tonnes of plastics have been produced in 2016 with an increasing future trend (Plastics Europe 2017). By far, most of the produced plastics which are processed by plastic converters are used for packaging (about 40% in mass), followed by building and construction (about 20%) as well as automotive industry (about 10%). The group of PE is amongst the most demanded polymer types (around 14.5 million tonnes in 2016). Followed by PE, the demands for polypropylene (PP, about 9.5 million tonnes) and poly vinyl chloride (PVC, around 5 million tonnes per) are the highest compared to other plastic types in Europe in 2016. PE is used e.g. for packaging of food and cosmetics, production of pipes, toys and reusable bags. Some applications for PP are packaging, automotive parts, microwave-safe containers and bank notes. PVC is applied for the production of floor and wall coverings, inflatable pools, window frames and many others. Other important resin types for plastic converters in Europe are PU, PA, polyethylene terephthalates (PET), polystyrenes (PS) and polycarbonates (PC). The possibility to specifically design polymers depending on their application makes them so versatile and the preferred choice of material for many products including technological and medicinal items. Their numerous applications and ubiquitous presence in everyday-life, however, make their release into the environment more likely.

### 1.2.2 Plastic as environmental pollutant

Pollution of the environment with plastics increased since the start of mass production of plastics in the 1950's and has been described with main focus on the oceans within the last decades, e.g. at shores and beaches worldwide (Barnes et al. 2009, Browne et al. 2011, Carpenter et al. 1972). High abundance of floating plastics in ocean gyres was first quantified by Moore et al. (2001) for the North Pacific Central Gyre with a mean mass of 5114 g $km^{-2}$ and a mean abundance of 334,271 plastic pieces per $km^2$. Other ocean gyres have been identified as hotspots for marine plastic pollution to date (e.g. Law et al. 2010). Furthermore, numerous studies have been published within the last years illustrating the widespread pollution of the ocean with plastic debris (e.g. Auta et al. 2017 as overview). The growing number of reports about plastic pollution corresponds to the increasing

amount of plastics which have been produced and also released into the environment (Geyer et al. 2017). Based on estimations on a global scale, a total of 7800 Mt of plastics have been produced until 2015, more than half of which within the last thirteen years. If the trend of increasing use of plastics continues, more and more plastics will be produced in the future. A total of 6300 Mt of plastic waste was ever generated out of which 60% ended up in landfills or were released into the environment. One of the biggest causes of the rapid generation of plastic waste is the short lifetime of many plastic items of about one year or even single use of disposable items like packaging. A small minority of only 9% of the total mass of plastic waste entered the recycling cycle. Efforts to recycle plastics are noteworthy since the 1980's, but downgrading of the quality of recycled plastics, so-called "downcycling", makes recycling still challenging on a broader scale (McDonough and Braungart 2010). Recycling is discussed only as part-time solution to the generation of plastic waste, because it only delays final disposal and does not fully avoid plastic waste in the future (Geyer et al. 2016). Plastic production broadly relies on limited fossil resources which is often overlooked by customers. Being relatively cheap to produce and used for various items with a relatively short lifetime, polymer material is often not perceived as resource itself which can hamper recycling efforts. Finally, the longevity of plastics is one of the main reasons for the widespread presence of plastics in the environment in addition to the continuous generation of new plastic waste and its release into the environmental. Plastics are designed to be durable which makes them relatively persistent also as pollutants.

## 1.3 Microplastics as newly emerging pollutants

### 1.3.1 Definitions of microplastics

First attention for pollution with small plastics in particular, besides plastic pollution in general, was reported from Carpenter and Smith (1972) at the Sargasso Sea in the North Atlantic Ocean. The rising awareness of the presence of a broad scale of sizes of plastic waste led to first definitions of size classes, i.e. macro- (> 20 mm in diameter), meso- (2-20 mm) and microplastics (< 2 mm, Ryan et al. 2009). The term "microplastics" is used for a high variety of different materials with various characteristics. From the term itself, both the small size and the plastic material can be derived. The size spectrum of microplastics is very broad with a factor of $10^3$-$10^4$ between the upper and the lower limit. The upper size limit of microplastics varies depending on the definition with 1 mm (Browne et al. 2008), 2 mm (Ryan et al. 2009) or 5 mm (Arthur et al. 2009, Moore 2008). More recently, the GESAMP (2016) decided to adopt 5 mm as commonly defined upper limit. Thus, 5 mm is considered as upper size limit in this thesis as well. The lower size limit goes down to the micrometre scale with a threshold of 1 µm defined in an earlier report of the GESAMP (2015) or 0.1 µm according to other sources (e.g. Duis and Coors 2016). The lower size limit is not consistently defined also because the size spectrum of

nanoplastics has not been clearly classified yet. Either 1 µm or 0.1 µm have been suggested as upper size limits for nanoplastics (e.g. Gigault et al. 2018, Koelmans et al. 2015). Both, the upper and the lower size limits of microplastics relate to at least one dimension, e.g. a fibre as thin as 10 µm in diameter but with 10 cm length also falls into the category of microplastics. Besides a large size range, the variety of polymer types is as diverse as their intended applications, which again illustrates the diversity of the group of microplastic material.

### 1.3.2 Sources of microplastics

Different sources for microplastics in the environment have been identified. Release of plastics, especially small pellets in the range of a few mm, from a plastic production site was reported in the river Danube (Lechner and Ramler 2015). Pellets are used as raw material for further processing such as forming of plastic products. They are relatively easy to perceive with the naked eye and were found at shores and beaches worldwide, as well as in rivers and lakes (e.g. Carpenter et al. 1972, Mato et al. 2001, Moore et al. 2011, Zbyszewski and Corcoran 2011). Plastic items which are produced in the size spectrum of microplastics such as these pellets or polymer beads are called primary microplastics. They often consist of polystyrene (PS) or PE (Cole et al. 2011). Other primary microplastics are produced e.g. for industrial air blasting (Gregory 1996), as abrasives in cosmetics (Zitko and Hanlon 1991) or as plastic resin powders (Mato et al. 2001). In some areas, such as the state of California, plastic beads have been banned after the rising awareness of microplastic pollution and related concerns in the public. Some international companies like L'Oreal decided to proactively waive the use of polymer beads or powders in their wash-off products and use natural equivalents instead to meet customer concerns. In contrast to primary microplastics, secondary microplastics form as the result of fragmentation of bigger plastic items that are released into the environment (Cole et al. 2011). Plastics that have already been in the environment for a long time can become brittle e.g. due to exposure to UV radiation (Ter Halle et al. 2016). Floating microplastics are in particular prone to UV radiation. This aging of plastic material can weaken its stability, making it easier for mechanical forces such as waves to break it down into smaller pieces (Corcoran et al. 2009). Fragments of polymer material with a glassy, solid structure can be expected to be sharp-edged, while other polymer materials like foams such as expanded polystyrene disintegrate into relatively smooth-edged particles. Sources for disintegrating plastic items are numerous, ranging from manufactural to societal release, e.g. plastic waste from landfills (Horton et al. 2017). Some types of microplastics are directly emitted into freshwaters, e.g. fibres from synthetic fabrics that are released with every washing process, or microplastics from cosmetics. These can end up in relatively high quantities in wastewater. A relatively high proportion of these microplastics is removed by already existing cleaning processes in wastewater treatment plants (e.g. around 98%, Murphy et al. 2016). In spite of high removal rates, the large volumes of processed wastewater still result in significant amounts of microplastics

in final effluents which are discharged into freshwaters (Browne et al. 2011, Carr et al. 2016). Finally, sewage sludge contains microplastics retained during sewage treatment. Deposition and application of sewage sludge as agricultural fertilizer is still applied in some countries and leads to microplastic contamination of soils (Browne et al. 2011, de Souza Machado et al. 2018). Via erosion and runoff, contaminated soils can act as terrestrial source for microplastics for freshwaters.

### 1.3.3 Characteristics of microplastics and their fate in the environment

The characteristics of plastic materials do not only determine their intended applications but also influence the behaviour of microplastics in the environment. Physical properties of the polymer material such as density, for example, are crucial for the buoyancy of the material in water. Density can range between 0.8 and 1.4 kg $L^{-1}$ for consumer products. Polymer material with a density lower than water (1 kg $L^{-1}$ for freshwater) has a positive buoyancy and tends to accumulate on the water surface (Ryan et al. 2009), while polymer material with a higher density than water tends to sink and is more likely to accumulate in sediments (Wright et al. 2013b). In addition to physical properties, plastics also have variable chemical characteristics. For manufacturing of polymers different chemicals are used as starting material for polymerisation (e.g. bisphenol A) or as additives for adjusting properties of the polymer depending on its intended application (e.g. colourants, plasticizers, UV stabilizers). These chemicals can desorb from the polymer matrix and leach into the environment, e.g. during aging. At the same time, some polymer types tend to sorb chemicals, especially hydrophobic organic chemicals, from their environment. Microplastics are particularly prone to both, leaching and sorption, due to their high surface mass ratios.

The characteristics and behaviour of microplastics can also be influenced by environmental factors. The longer the microplastics such as fragments are exposed to e.g. mechanical forces, the more they can round and lose their edgy shape or break down into even smaller pieces. Thus, microplastics initially categorized as primary microplastics can break down and become secondary microplastics. Weathering of microplastics can not only lead to fragmentation, but also to a change of colour. Polymer types such as PVC tend to become yellowish because of oxidation during aging (Andrady 2017). Biotic factors can also influence the behaviour of microplastics. Colonization of microplastics by biofilms, for example, can increase the overall weight of the particles. Hence, initially floating microplastics may sink and accumulate either in the water column or the sediment (Zettler et al. 2013). *Vice versa*, remobilisation of settled microplastics in sediments can occur due to turbulences or currents (Lattin et al. 2004).

### 1.3.4 Abundance of microplastics in freshwater systems

Similar to marine microplastic debris (e.g. Browne et al., 2011), population density and land use correlate with high abundance of microplastics in freshwaters (Eerkes-Medrano et al. 2015, Eriksen et al. 2013, Faure et al. 2015, Yonkos et al. 2014) but also in remote regions with the lack of proper wastewater treatment (Free et al. 2014). Irregular shaped plastic fragments and very small microplastics of only a few micrometres seem to make a big proportion of the overall amount of microplastics in surface waters and beach sediments (Faure et al. 2015, Free et al. 2014, Imhof et al. 2016). Some polymer types are more abundant than others in the environment. In beach sediments of Lake Garda in Italy, PE and PA were found to be the most abundant polymer types for microplastics between 1 and 500 µm, while bigger sized microplastics (between 500 µm and 5 mm) consisted mainly of PS. In surface waters of Swiss rivers and lakes about 60% of microplastics consisted of PE.

Quantifying microplastics is still challenging, leading to an incomplete picture of microplastic pollution in freshwaters. By now, mainly surface waters, beach and shoreline sediments have been analysed, while other compartments, e.g. the free water zone, are understudied. Sampling and detection techniques applied in previous studies automatically exclude some microplastic types or identify natural materials as microplastics (Hidalgo-Ruz et al. 2012, Song et al. 2015). Nets with mesh sizes around 300 µm are widely used to collect microplastics from surface waters and in principle exclude smaller microplastics. This is even more relevant when considering that these small microplastics are expected to be most abundant in freshwaters (Imhof et al. 2016). Further improved sampling methods and techniques for analysis of microplastics are currently developed and discussed in the scientific community (Dümichen et al. 2017, e.g. Imhof et al. 2012, Löder and Gerdts 2015, Song et al. 2015). Advanced detection and identification techniques, e.g. FTIR and Raman spectroscopy, are still expensive and time-consuming and are discussed to not be reliable for e.g. natural fibres (Comnea-Stancu et al. 2017). Finally, individual studies are difficult to compare because of differing units. Results are reported as numerical or mass concentrations per area or volume and cannot always be interconverted due to different sampling techniques. Both, mass and numerical concentrations of microplastics seem to be in a similar range in rivers and lakes. Mass concentrations are rarely reported and are expressed in different units with $10^{-3}$ – $10^{-1}$ mg $m^{-3}$ for rivers and $10^{-3}$ – $10^{-1}$ mg $m^{-2}$ in lakes (Faure et al. 2015, Yonkos et al. 2014). Highest pollution with microplastics was reported from Asian rivers with up to $10^{3}$ mg $m^{-3}$ in Yangtze river (Lebreton et al. 2017, Zhao et al. 2014). Reported numerical concentrations of microplastics in rivers and lakes both go up to $10^{1}$ pieces $m^{-3}$ for surface water (Faure et al. 2015, McCormick et al. 2016, Yonkos et al. 2014). Microplastic loads in beach sediments are in the range of $10^{2}$ pieces per $kg^{-1}$ for rivers and lakes (Fischer et al. 2016, Vaughan et al. 2017). River sediments from the Rhein and Main were shown to include high abundance of microplastics mass fractions of up to $10^{3}$ pieces $kg^{-1}$ (Klein et al. 2015).

Highly polluted marine sediments e.g. from the Venetian lagoon, were reported to include similar high masses of microplastics (Vianello et al. 2013). In rivers, concentrations and composition of the microplastic material can be influenced by hydrological characteristics, i.e. with increasing variety of polymer types after confluences and higher concentrations downstream driven by transport from tributaries to mainstreams (Klein et al. 2015). In addition to experimental studies, model-based studies can help get a better understanding of environmental abundance and distribution of microplastics by including mechanistic processes e.g. about the fate of microplastics in the environment. In a model-based study the size of microplastics was shown to be crucial for aggregation with other material and retention of microplastics in river sediments (Besseling et al. 2017a). According to the calculations, microplastics in the size range of 1-50 µm are likely to be transported from rivers to oceans, while bigger or smaller microplastics are likely to be retained in rivers. In lakes, wind and surface circulation are discussed as important factors for microplastic distribution (Fischer et al. 2016, Free et al. 2014, Imhof et al. 2013).

## 1.4 Microplastics as potential threat for organisms

Adverse impacts of plastics were first studied for bigger sized plastics such as mesoplastics. Sea birds were one of the first organisms shown to be affected by plastic pollution (e.g. Ryan 1987). In birds with a high loading of plastics in their intestines uptake of food can be impeded by blockage of the intestines by plastic items (Parslow and Jefferies 1972, Ryan 1989). Organisms in the ocean were reported to also get disentangled in larger plastics, e.g. in abandoned fishing gears or nets, leading to injuries or even death (Laist 1987, 1997). These effects which are based on the plastic material itself and on mechanical forces are categorized as physical effects (Wright et al. 2013b, Zarfl et al. 2011). Sea birds which ingested high amounts of plastics were found to have elevated tissue concentrations of chemical pollutants such as polychlorinated biphenyls (PCBs, Ryan et al. 1988). This led to the conclusion that plastics may act as carrier for chemicals to aquatic organisms. Effects by chemicals associated to the plastic material, either chemicals from the surrounding accumulating in the microplastic material or additives being part of the material itself, are categorized as chemical effects (Yamashita et al. 2011, Zarfl et al. 2011). Based on the observations on harmful effects of larger plastic material on organisms, the question arose whether these effects can also be induced by microplastic material. Being a subcategory of plastic in general, microplastics share characteristics with larger plastics, e.g. specific characteristics of the polymer material. However, the small size of microplastics can be expected to influence their physical and chemical behaviour as shortly mentioned before. Small particle size, for example, may alter chemical characteristics of microplastics due to their larger surface-volume ratio leading to enhanced leaching of additives and sorption of chemicals to microplastic particles (Teuten et al. 2007).

Ultimately, the potential risk of microplastics for aquatic organisms in the environment highly depends on the exposure scenario. First, the organisms need to be in contact with microplastics. Organisms which collect food from the water surface (e.g. fish) can be expected to be prone to floating microplastics, while benthic organisms (e.g. macroinvertebrates) are mostly exposed to microplastics accumulating in sediments. Microplastics which are distributed in the water column are available for pelagic organisms (e.g. fish), especially for filtering organisms such as zooplankton. The abundance of microplastics including numeric and mass concentrations are crucial for their risk assessment as well. The more microplastics are present in the environment, the higher the potential risk for organisms to be influenced by their presence. Ecotoxicological approaches are based on this assumption of dose-dependency; however, this principle has been challenged in some studies (e.g. de Souza Machado et al. 2017). Ecotoxicological methods aim to determine potential adverse impacts of mainly chemical pollutants on different organisms which are representatives of important functional groups in ecosystems. In these studies, accurately defined endpoints, which are characteristic for the tested organisms and known to impair them, are qualified and quantified. Acute effects impair individuals that come in contact with the substance within a relatively short time and often with severe consequences, e.g. death (e.g. Jorgensen 2010). Chronic effects are not as immediate but manifest in a later life-stage or even in upcoming generations. Model organisms with well-known characteristics such as physiology or life-history traits are often used in ecotoxicological studies. Controlled exposure of model organisms to pollutants facilitates the analysis and interpretation of obtained empirical data and the comparison of the results between different studies. Experiments are often performed in a laboratory environment under well-defined and stable conditions to be able to adjust or alter the exposure scenario independently from environmental factors. Apart from studying effects of chemical pollutants on organisms, systematic ecotoxicological analysis in the laboratory can be a valuable approach to study effects of microplastics in order to develop a first baseline on the potential impacts of microplastic material itself. So far, potential effects of microplastics have been analysed in laboratory studies on representatives of some key taxa such as zooplankton and fish (e.g. Desforges et al. 2015, Lu et al. 2016).

### 1.4.1 Uptake of microplastics

Physical effects of microplastics via entanglement and damage of external structures, which are similar to physical impacts of bigger-sized plastics, were observed for microplastic fibres in daphnids (Ziajahromi et al. 2017). Concerns about the potential threats of microplastics, however, mostly focus on the uptake of microplastics from the surrounding, e.g. via ingestion or gill breathing, as a precondition of possible adverse impacts. Early reports from the marine and freshwater environment revealed that organisms such as fur seals (*Arctocephalus* spp.) and wild gudgeons (*Gobio gobio*) ingest microplastics (Eriksson and Burton 2003, Sanchez et al. 2014). Different feeding

experiments under laboratory conditions showed uptake of microplastics by invertebrates including mussels, worms and zooplankton (e.g. Browne et al. 2008, Lee et al. 2013) as well as vertebrates such as fish (e.g. Mazurais et al. 2015, Rochman et al. 2013). Thus, both benthic and pelagic organisms were shown to be able to ingest microplastics under laboratory conditions. Trophic transfer of microplastics via the food chain was hypothesized because of the presence of microplastics in faeces of animals of a higher trophic level, i.e. fur seals (Eriksson and Burton 2003). The transfer of microplastics via the diet was later observed in laboratory studies, e.g. for crabs feeding on mussels (Farrell and Nelson 2013) and fish feeding on *Artemia* nauplii (Batel et al. 2016), both of which were pre-contaminated with microplastics they previously ingested. In fish, ingested microplastics were observed to accumulate in the gut (Lu et al. 2016) and also retained in the mucus of intestines (Batel et al. 2016). At the same time, fish exposed to microplastics were shown to be able to egest microplastics relatively fast after ingestion (Batel et al. 2016, Jovanović et al. 2018).

### 1.4.2 Physical effects of microplastics

Different effects of microplastic exposure in general and ingestion in particular have been reported in the literature for invertebrates and vertebrates. In lugworms, the presence of microplastics led to reduced food uptake followed by weight loss (Besseling et al. 2013). However, microplastics did not accumulate within the organisms leading to the conclusion that microplastics did not obstruct the digestive tract. The authors suggested that lugworms needed to process a higher amount of sediment to get the same amount of food in sediments with microplastics, compared to clean sediments. The additional volume of microplastics diluted the food in the sediments leading to an overall lower food uptake. In marine copepods, microplastics were shown to be egested as well, but some microplastics were retained in intestines for up to seven days after exposure (Cole et al. 2013). Ingestion rates of copepods decreased in the presence of microplastics, which was hypothesized to be based on a change in the feeding strategy to avoid ingestion of microplastics (Cole et al. 2015). More drastic effects, i.e. reduced survival and fecundity and even negative impacts on following generations, were induced after microplastic ingestion in copepods in another study (Lee et al. 2013). In amphipods, higher mortality after the exposure and ingestion of microplastics was accompanied by chronic effects, i.e. hampered growth and reproduction (Au et al. 2015). In microalgae, growth was decreased after exposure to microplastics as well (Sjollema et al. 2016). Stress, indicated by glycogen depletion and oxidative stress, as well as inflammation processes in the liver were induced after microplastic uptake in different fish species (Lu et al. 2016, Rochman et al. 2013). Not only uptake into the digestive tract but also contact of microplastics with gills can induce adverse effects in gill-breathing organisms. Histopathological changes in gills of catfish were observed after exposure to microplastics, although no microplastics were detected in gill tissue (Karami et al. 2016). In intestines of fish, histopathological damage was indicated by cracked villi and split enterocytes as well as thickened intestinal

epithelium after microplastic exposure (Lei et al. 2018, Romano et al. 2018). Translocation of microplastics in the range of a few micrometres from the intestines to other parts of the organism was hypothesized as potential additional risk of microplastics and first shown for marine mussels with translocation into haemolymph (Browne et al. 2008) and later for zebra fish with translocation into liver tissue (Lu et al. 2016). Overall, smaller microplastics (e.g. in the lower µm-range) were found to induce more drastic effects, than larger microplastics (e.g. in the mm-range).

### 1.4.3 Chemical effects of microplastics

Ingestion is not only hypothesized to enhance physical effects of microplastic particles, but also chemical effects by associated pollutants that could leach from the polymer material itself or which are sorbed to it (e.g. Teuten et al. 2007, Teuten et al. 2009). Dietary exposure in laboratory setups to microplastics loaded with organic pollutants confirmed that microplastics can act as carrier for chemical pollutants (Browne et al. 2013, Chua et al. 2014, Rochman et al. 2013). Fish (Japanese medaka), for example, were fed with PE microplastics loaded with organic chemical pollutants including polycyclic aromatic hydrocarbons (PAHs), poly-brominated diphenyls (PBDEs) and PCBs that are also present in the environment. The chemical pollutants were found to accumulate in fish after ingestion of loaded microplastics. In some studies, plastics have been considered as a substantial source for especially hydrophobic organic compounds to organisms because of their high affinity to sorb to polymer material (Browne et al. 2013, Teuten et al. 2007). The crucial question is, however, if microplastics substantially facilitate the transfer of organic contaminants in environmental scenarios. Internal factors within organisms, such as temperature and pH, especially in the intestinal system, were proposed to enhance desorption of organic pollutants from ingested microplastics (Bakir et al. 2014). However, the role of ingested microplastics as source for organic pollutants was calculated to be negligible when internal factors in the gut were considered in addition to environmental relevant concentrations of the chemical pollutants (Bakir et al. 2016). Other model-based studies also hypothesized that plastics constitute no crucial source of organic pollutants compared to other uptake pathways (Gouin et al. 2011, Koelmans et al. 2014). Re-evaluation of available empirical data with new calculations in another modelling study provided a more holistic and consistent framework (Koelmans et al. 2016). The authors concluded that natural carriers for organic pollutants, e.g. prey and water, play a much bigger role for bioavailability of organic pollutants than microplastics for most environmental scenarios in the ocean. In some scenarios, microplastics were hypothesized to even reduce effects of chemical pollutants, namely when sorption to microplastics with a relatively low loading of organic pollutants i.e. lower than expected from the equilibrium partition coefficient or pristine microplastics, leads to reduced concentrations in organisms. According to calculations, most microplastics in the marine environment can be expected to be in an equilibrium state. This aspect, however, was rarely considered in studies investigating the potential vector effect of microplastics,

especially for freshwater. This is why there was a call for empirical data to tests this hypothesis, i.e. that microplastics are negligible uptake pathway for chemical pollutants if other uptake pathways are considered, especially in an equilibrated system.

## 1.5 Objectives and structure of this thesis

The objective of this thesis is to assess how microplastics affect freshwater organisms. More specifically, this thesis provides new insights about potential adverse effects of the microplastic material itself and the influence of microplastics on the effect patterns of chemical pollutants. It aims to fill the following knowledge gaps for freshwater organisms: (i) whether the microplastic material itself induces adverse physical impacts in a dose-dependent manner, (ii) whether microplastics can reduce the effects of chemical pollutants, (iii) to provide first empirical data on potential physical and chemical effects of microplastics on amphibians, including their potential role as vector for chemical pollutants, i.e. an endocrine disruptor.

A laboratory approach was chosen to investigate these key questions systematically under controlled and stable conditions. Effects of microplastics alone and in combination with chemical pollutants were analysed using established ecotoxicological methods. Two established freshwater model species, i.e. *Daphnia magna* as representative for limnic zooplankton and *Xenopus laevis* as representative for amphibians, were confronted with microplastics and chemical pollutants in a series of experiments. Limnic zooplankton was chosen as model functional group because of its crucial role in lake ecosystems, e.g. in the limnic food web where zooplankton consumes primary producers and represents an important prey for organisms on a higher trophic level itself (e.g. for fish). As previous studies mainly focused on marine species this thesis aims to assess whether the presence of microplastics adversely affects zooplankton as important functional group also in freshwaters. There is a paucity of studies which examined if microplastics can adversely affect amphibians, which is why there is a need to analyse this important group especially in the light of worldwide declining amphibian populations. *X. laevis* is an established model for endocrine disruption in vertebrates. These reasons make *X. laevis* a highly suitable candidate to study potential effects of microplastics on freshwater vertebrates in this thesis in order to set a first basis for further investigations.

Two different microplastic types in different concentrations were used for exposure of *D. magna* and *X. laevis*. These comprised different specific characteristics (i.e. size, polymer material, shape), which addressed the high diversity of microplastics detected in freshwater environments. Moreover, they represent the most abundant polymer types and size classes for microplastics. The microplastic material chosen for exposure is in the lower size-range of microplastics, i.e. micrometre-sized. The microplastic concentrations used for exposure of the animals were relatively high in favour of high sensitivity of potential effects, both, physical and chemical. High concentrations and the use of small

microplastics also reflect the trend for expected increasing concentrations of small microplastics in the future. The exposure of *X. laevis* and *D. magna* to the same type of microplastic in one case allowed comparing the responses to microplastics on an organism-level.

Chapter two aims to fill in the research gap described in (i), namely to examine whether the mere microplastic material itself induces adverse impacts in limnic zooplankton and whether these effects are dose-dependent. In this study, *D. magna* was exposed to PE beads of two sizes (1 and 100 µm in diameter) in a series of concentrations (12.5-400 mg $L^{-1}$) in order to examine acute effects resulting from short-term exposure to microplastics and their potential dose-dependency. Uniformly shaped PE beads were presented to new-born daphnids for up to 96 hours following an established ecotoxicological method aligned to OECD standards. This short-term exposure aimed to qualify and quantify acute effects with immobilisation as criterion for an adverse impact. Using a broad set of microplastic concentrations allowed for calculating an effective concentration for pristine microplastics without any additives for the first time. This threshold concentration can be used for risk-assessment of microplastics in the environment in the future, when more representative data on exact concentrations of microplastics in freshwater systems are available.

Chapter three describes the second study on *D. magna*, which aimed to investigate physical effects of another type of microplastics (i.e. PA particles) to address the first knowledge gap (i) and if the presence of microplastics modulates acute effects of a chemical pollutant, i.e. bisphenol A (BPA), as described in the second knowledge gap (ii). Neonates of *D. magna* were exposed to a range of PA particle concentrations (25-250 mg $L^{-1}$), to a broad range of BPA concentrations (5-15 mg $L^{-1}$) and the same range of BPA concentrations in combination with one concentration of PA particles (200 mg $L^{-1}$). The specific effects of BPA, namely the dose-dependency of immobilisation of daphnids, could be compared between the treatments with and without microplastics to give a clear indication for a potential influence of the PA fragments on the effects of BPA. The interpretation of immobilisation data was facilitated by the additional calculation of the mass distribution of BPA in the test system. Sorption equilibrium and different uptake pathways for chemical pollutants, which were rarely considered in earlier empirical studies, were included in this approach. Freshwater systems are sinks for BPA and its effects on daphnids are well described, which makes it a suitable candidate for this approach. The same OECD aligned approach as in chapter two was used in this chapter.

In chapter four the findings of an empirical study, which aims at assessing physical and chemical effects of microplastics on amphibians are described. This chapter addresses all previously identified research gaps (i, ii, iii). Tadpoles of *X. laevis* were exposed to a low (1 mg $L^{-1}$) and a high (100 mg $L^{-1}$) concentration of PA fragments for a short- (seven days) and long-time (21 days) in order to assess short-term and long-term exposure effects. Tadpoles were exposed to microplastics alone and to microplastics in combination with

the chemical pollutant 17-beta-ethinylestradiol (EE2, at $10^{-8}$ M) during a sensitive life-stage of early development. EE2 was selected as model chemical pollutant because of its high relevance as endocrine disruptor for amphibians in the environment. The rate of effects induced by given concentrations for EE2 is known and thus the effect of microplastics on these specific effects could be assessed. The exposure with only microplastics allowed for analysing if physical effects can be induced by the microplastic material itself and if these effects are dose-dependent for amphibians (i, iii). The general development and stress hormone levels of tadpoles were determined in order to quantify potential adverse impacts of the microplastic material itself. Sexual development and the mRNA expression of a number of biomarkers were assessed to quantify potential influences of the microplastic material on specific estrogenic effects of EE2 (ii, iii).

In chapter five, the key findings that were presented in the previous chapters are discussed in an overall context and set into a broader perspective. The contribution of the presented results to risk assessment of microplastics is outlined in this section of the thesis together with the knowledge gaps which need to be addressed in further studies for the risk assessment of microplastics.

# 2 Effects of pristine microplastics on limnic zooplankton

*This chapter is based on joint work with:*

*Christiane Zarfl, Center for Applied Geosciences, Eberhard Karls Universität Tübingen*

*Werner Kloas, Leibniz-Institute of Freshwater Ecology and Inland Fisheries; Department of Endocrinology, Institute of Biology, Humboldt-Universität Berlin*

*This joint work yielded the following publication:*

*This article was published in Chemosphere 153; Rehse S, Kloas W, Zarfl C; Short-term exposure with high concentrations of pristine microplastic particles leads to immobilisation of Daphnia magna; 91–99; Copyright Elsevier (2016)*

*Individual contributions:*

***Saskia Rehse**, Christiane Zarfl and Werner Kloas designed the study; **Saskia Rehse** collected and analysed the data; **Saskia Rehse** wrote the manuscript with the support of Christiane Zarfl and Werner Kloas.*

## 2.1 Introduction

The European Environment Agency (EEA) identified "Increasing Environmental Pollution" as one of the eleven global megatrends on European resource systems. Among our ecosystems, freshwaters are facing a five times greater decline of biodiversity than terrestrial ecosystems (e.g. Ricciardi and Rasmussen 1999). Main stressors are not only habitat degradation and overexploitation, but also water pollution (e.g. Dudgeon et al. 2006, Sala 2000). A better understanding of these stressors and their interactions is crucial to develop effective management strategies in a system-oriented approach to save freshwater as an important resource for humans and functioning of ecosystems. Therefore, past and ongoing research addresses the distribution, accumulation and effects of chemicals and their degradation products (e.g. El-Shahawi et al. 2010, Staples et al. 1998) .

Recent studies underline water pollution by synthetic particulate materials, which often cannot be identified by standard monitoring and risk assessment procedures (Eerkes-Medrano et al. 2015, Howard 2010). This includes nanomaterials like titanium dioxide ($TiO_2$) but also microplastics, synthetic polymer particles smaller than 1 mm (Browne et al. 2008) or 5 mm depending on the definition (Moore 2008). These microplastic particles can be of different size, polymer type and shape (beads, fragments, fibres) and can be found both, in marine (Barnes et al. 2009, Browne et al. 2011, Carpenter and Smith 1972) and freshwater systems (Eriksen et al. 2013, Moore et al. 2005). The shape of microplastics in the environment is often linked to the emission source. Small polymer beads, especially polyethylene (PE) and polystyrene (PS), are produced in the µm-range (primary microplastics, Cole et al. 2011) as contents in personal care and cosmetic products (Zitko and Hanlon 1991), as plastic resin powders (Mato et al. 2001) or pellets for industrial air blasting (Gregory 1996). Together with fibres washed out from synthetic fabrics they are directly emitted into freshwaters via wastewater treatment plants (Browne et al. 2011) . Mechanical abrasion or UV-radiation of bigger synthetic polymer products that have been emitted into the environment also lead to smaller plastic fragments (secondary microplastics, Cole et al. 2011). In addition, plastic production sites located close to river systems may accidently release pristine plastic material into freshwaters (Lechner and Ramler 2015, Mato et al. 2001). Since plastic products are produced to be long-lasting it is not surprising that plastic debris including microplastics can be transported by air and water and are thus detected in the environment ubiquitously, even in remote regions (do Sul et al. 2009, Free et al. 2014). So far, research has mainly focussed on microplastics in marine systems where it was detected on the ocean surface (Colton et al. 1974, Eriksen et al. 2014), in estuaries (Sadri and Thompson 2014), along shorelines (Browne et al. 2010), and even in deep-sea sediments (van Cauwenberghe et al. 2013, Woodall et al. 2014).

Only recently, microplastics have also been reported in lakes and rivers in Europe (Faure et al. 2015, Imhof et al. 2013, Klein et al. 2015, Lechner et al. 2014), North America (Castañeda et al. 2014, Eriksen et al. 2013, Zbyszewski and Corcoran 2011, Zbyszewski et

al. 2014), South America (Rech et al. 2015), Africa (Biginagwa et al. 2016) and Asia (Free et al. 2014, Zhang et al. 2015). The observed abundances can reach up to several hundreds of thousands of microplastic items per $km^2$ in the surface water in size classes < 5 mm. High abundance of microplastics in river sediments was shown in two German rivers (Klein et al. 2015). In this study, mass fractions of microplastics with up to 1 g $kg^{-1}$ were similar to sediments from marine systems e.g. from the Venetian lagoon (Vianello et al. 2013). It was shown that, similar to marine microplastic debris (e.g. Browne et al. 2011), high abundance of microplastics in freshwater can be observed in correlation with population density and land use, e.g. industrial areas (Eerkes-Medrano et al. 2015, Eriksen et al. 2013, Yonkos et al. 2014).

In addition, the fate of microplastics within the environment and especially in the freshwater environment is hardly understood. Depending on environmental conditions, they may be prone to degradation (e.g. by UV light, Andrady 2011) or, depending on their density, to sedimentation (Cole et al. 2011). The densities of plastics used for consumer products range from 0.85 to more than 1.4 kg $L^{-1}$. Having lower densities than water, most polymer types like polypropylene (PP) and polyethylene (PE) thus tend to float on the water surface (Ryan et al. 2009), while plastic types having higher densities than water, like polystyrene (PS) or polyethylene terephthalate (PET), might end up in benthic environments (Wright et al. 2013b). Biofilms that colonise microplastics may also increase particle density and thus induce sedimentation of the particles (Zettler et al. 2013). Turbulence could finally remobilize deposited microplastics (Lattin et al. 2004).

Impacts of microplastics have mainly been studied in marine organisms, both in laboratory as well as in field experiments. Ingestion of microplastics from the marine environment has been observed for animals of different trophic levels like zooplankton (Desforges et al. 2015), fish (Lusher et al. 2013), corals (Hall et al. 2015), fur seals (Eriksson and Burton 2003) or whales (Besseling et al. 2015, Lusher et al. 2015). Presence of microplastics in organisms on a higher food chain level like in fur seals indicates microplastic uptake also through trophic transfer in the environment (Eriksson and Burton 2003). Studies under laboratory conditions confirmed ingestion of microplastics by organisms from different marine habitats, i.e. benthic organisms like mussels (Browne et al. 2008, Farrell and Nelson 2013) and amphipods (Wright et al. 2013a), for pelagic species like zooplankton (Lee et al. 2013, Setälä et al. 2014) and fish (Mazurais et al. 2015) as well as for an isopod species representing sub- and eulitoral isopods (Hämer et al. 2014). Trophic transfer of microplastics under laboratory conditions was shown from mussels to crabs (Farrell and Nelson, 2013) as well as from zooplankton to mysid shrimps (Setälä et al. 2014).

Ingestion of microplastics by freshwater organisms has only recently been studied for invertebrates and fish. Freshwater fish from French streams (Sanchez et al. 2014), the African Great Lakes (Biginagwa et al. 2016) and the Brazos River Basin (Peters and Bratton 2016) were shown to accumulate microplastics in their intestines. In laboratory

experiments between 32% and 100% of the studied invertebrates ingested microplastics (Imhof et al. 2013). Japanese medaka, which are known to live in freshwater as well as in brackish water, were also shown to ingest PE fragments under laboratory conditions (Rochman et al. 2013). Uptake of PE particles via the food web was observed for another model organism for freshwater fish (*Danio rerio*) by uptake of *Artemia* nauplii which already ingested the particles before (Batel et al. 2016).

If the presence of microplastics in general and the ingestion in particular pose a direct risk to aquatic organisms in the environment is still under discussion. However, initial laboratory studies on marine organisms show physical effects like increased immune response, reduced food consumption, reduced fecundity, negative impacts on next generations and depletion of energy reserves of the respective organisms after ingestion of microplastics (Cole et al. 2013, Cole et al. 2015, Lee et al. 2013, von Moos et al. 2012, Wright et al. 2013a). First implications for freshwater species indicate that medaka show signs of stress if fed with pristine microplastics (Rochman et al. 2013).

An additional impact is hypothesized to occur via the vector function of microplastics for chemical substances (Teuten et al. 2009). Plastics strongly accumulate organic pollutants from the environment and sorption of organic contaminants may be higher than to natural sediments (Teuten et al. 2007). Additionally, the desorption of organic contaminants from synthetic polymer material is slower than for natural particles, which may lead to long term storage of the contaminants in the plastic material. Long-term storage in the polymer matrix may decrease concentrations of pollutants in the water column in the presence of microplastics. On the other hand, if organisms take up polluted microplastics, conditions within the organism e.g. in intestines, can be different to environmental conditions (e.g. increased temperature, lower pH) and can result in an increased release of pollutants directly within the organs (Bakir et al. 2014). In addition, many plastic products already contain additives like colorants, plasticizers or UV-stabilizers that desorb from the plastic matrix (Fries et al. 2013, Lithner et al. 2011). Accumulation of microplastics along the food chain may thus implicate an additional pollutant pressure by the simultaneous uptake of sorbed (organic) compounds. There are indications from experiments with lugworms (Besseling et al. 2013, Browne et al. 2013), amphipods (Chua et al. 2014) and fish (Khan et al. 2015, Rochman et al. 2013, Rochman et al. 2014) that microplastics can facilitate the transport of pollutants to organisms both, in marine and freshwater environments. However, at the moment it is still not clear if the exposure of pollutants carried by microplastics is a relevant pathway to organisms in the environment where pollutants are already present. Moreover, it is not clarified in detail to which extent and at which concentrations effects on freshwater organisms can also be induced by the microplastic material itself (physical effects). Before addressing questions about chemical effects by sorbed pollutants, we need to identify systematically which characteristics of the material itself are critical for organisms.

In order to provide a better understanding of the impacts of microplastics on freshwater organisms we show results of a systematic effect study aligned to OECD standards on *Daphnia mag*na, a well-known model species representative for limnic zooplankton, which represents one of the lower levels of the food chain. The aim of this study was to investigate and analyse physical effects (i.e. excluding chemical effects) of two different size classes of pristine microplastic particles covering a broad concentration range each. We analysed (1) if small microplastics (1 µm) are ingested by limnic zooplankton, and (2) if acute effects (i.e. immobilisation) can be observed on zooplankton when exposed to microplastics as well as if effects differ with size of the particles (1 µm and 100 µm). Since the knowledge on concentrations of microplastics in freshwaters, especially for particles smaller than 300 µm, is still very limited this approach follows the precautionary principle and aims at identifying critical concentrations first before evaluating the risk at environmental concentrations in retrospect. This is beneficial especially for small microplastics such as 1 µm particles, which are very difficult to analyse qualitatively and quantitatively with currently available methods. This study thus provides a basis for future research on microplastics impacts in limnic ecosystems, especially in combination with food chain accumulation.

## 2.2 Methods

We conducted short-term exposure experiments with *D. magna*. By exposing the animals to pristine microplastic material produced with defined properties for laboratory applications we excluded chemical effects e.g. induced by additives. To avoid contamination of the microplastic material by plastics from laboratory items, we used glass material for handling of particles, culturing of daphnids and for exposure experiments. All material was covered to reduce airborne contamination.

### 2.2.1 Characteristics and behaviour of particles in aqueous solution

Before exposing the daphnids, we first analysed the characteristics of both dry powders and their behaviour in water. To confirm the size and the shape of the particles we examined both particle types with a stereomicroscope and photographed them (Zoom Stereo Microscope System SZH with ColorView III, Olympus). We also weighed 2 mg of both particle sizes separately into glass vials, added 20 ml of *Daphnia* culture medium (ADaM medium; Klüttgen et al. 1994), vigorously shook the vials with a vortex mixer, treated the mixtures in an ultrasonic bath for 15 min and shook them again. The components of the ADaM medium are listed in Appendix Table 1. Shaking was performed to facilitate continuous particle distribution in the water column and according to mixing events in the environment. Ultrasonic treatment minimized aggregation of the particles. We also observed the behaviour of particle mixtures during exposure experiments i.e. turbidity of tested mixtures.

### 2.2.2 Exposure of *D. magna*

The testing procedure was based on the OECD guideline *Daphnia* sp. Acute Immobilisation Test (OECD guideline 202, Organization for Economic Co-operation and Development 2004). This guideline aims at identifying critical acute effect and threshold concentrations of chemicals towards daphnids. According to the guideline, the fraction of immobilised organisms is the criterion for acute adverse effects. Like suggested in literature for testing of particulate material (e.g. Baumann et al. 2014), exposure length was prolonged from 48 to 96 hours in all treatments. Pre-tests confirmed that neonates from our culture younger than 24 hours survive for 96 hours in the absence of food without showing immobilisation or abnormal behaviour like being trapped at the water surface.

In order to identify in which concentration range observable effects of microplastics can be induced and whether size of the particles is a determining factor, we tested both particle size classes (1 µm and 100 µm) on *D. magna* with six concentrations each (12.5 mg $L^{-1}$, 25 mg $L^{-1}$, 50 mg $L^{-1}$, 100 mg $L^{-1}$, 200 mg $L^{-1}$, 400 mg $L^{-1}$; for number of particles $L^{-1}$ at each concentration see Table 1). Pre-tests showed immobilisation of daphnids at concentrations between 25 and 200 mg $L^{-1}$. To cover an even broader range we therefore chose concentrations between 12.5 and 400 mg $L^{-1}$ in a geometrical series. Although these concentrations are very high, there are first implications that concentrations of microplastics in sediment core water might be as high as 162 mg $L^{-1}$ (Besseling et al. 2014).

Exposure experiments were conducted with a clone of *D. magna* (originally isolated from Großer Binnensee, Lampert 1991). Neonates came from a healthy stock without signs of stress cultured under the same conditions as used in the tests (dark cycle of 16:8 h, temperature 20-22 °C). The animals were raised in ADaM medium, a well-defined medium with good reproduction output (Klüttgen et al. 1994). Semi-sterile cultured algae of *Scenedesmus obliquus* (SAG Göttingen) were fed to the stocks *ad libitum* every second day. A concentrated algae suspension was prepared by centrifugation of harvested algae followed by resuspension in *Daphnia* culture medium. According to the guideline, the animals were not fed during exposure with microplastics.

Following the test design from the guideline, a total of 20 neonates younger than 24 hours (second or third brood) were exposed for up to 96 hours to each tested concentration and the control. For testing, we used 50 ml glass beakers with 40 ml test solution (*Daphnia* culture medium and microplastics) with five animals per beaker leading to four replica for each tested concentration and the control (n=4). Since stable suspensions of particles in water could not be established without adding additional substances, we directly weighed the needed amount of particles for each test concentration (0.5, 1, 2, 4, 8, 16 mg), added 10 ml *Daphnia* culture medium, vigorously shook the mixtures followed by treatment in an ultrasonic bath for 15 min and shook again. All mixtures were prepared directly before the start of the experiments and filled into test beakers. Preparation vessels

were subsequently rinsed with *Daphnia* culture medium three times to ensure complete decanting of microplastic mixtures. After transferring five neonates to each test beaker by releasing them below the water surface, the medium was carefully topped to 40 ml each. According to the guideline, test beakers were not aerated during exposure to avoid disturbance. Beakers were loosely covered with glass petri dishes to reduce evaporation and airborne contamination. Each test vessel was checked for immobilised individuals and abnormal behaviour or appearance (e.g. being trapped at the water surface, malformation) after 24, 48, 72 and 96 hours. Animals were considered as immobilised if they were not able to swim within 15 seconds after gentle agitation of the test beakers (see OECD 202). The test is valid if the immobilisation of daphnids in the control is not exceeding 10%. Presence of ingested particles was assessed qualitatively also after 24, 48, 72 and 96 hours by observing the animals in the test beakers under a microscope. In order to avoid external disturbance of the experiments, beakers were moved carefully and animals were not removed during this procedure. After 96 hours animals from each treatment were removed in order to take photos under the microscope (Zoom Stereo Microscope System SZH with ColorView III, Olympus).

*Table 1* *Mass and numerical concentrations of PE particles*
Number of 1 µm (1-4 µm in diameter) and 100 µm (90-106 µm in diameter) PE particles in each tested mass concentration during exposure of *D. magna*.

| Concentration (mg $L^{-1}$) | 1 µm particles (Number $L^{-1}$) | 100 µm particles (Number $L^{-1}$) |
|---|---|---|
| 12.5 | $3.9x10^{8}$ - $2.5x10^{10}$ | $2.1x10^{4}$ - $3.4x10^{4}$ |
| 25 | $7.8x10^{8}$ - $5.0x10^{10}$ | $4.3x10^{4}$ - $6.8x10^{4}$ |
| 50 | $1.6x10^{9}$ - $1.0x10^{11}$ | $8.5x10^{4}$ - $1.3x10^{5}$ |
| 100 | $3.1x10^{9}$ - $2.0x10^{11}$ | $1.7x10^{5}$ - $2.7x10^{5}$ |
| 200 | $6.2x10^{9}$ - $4.0x10^{11}$ | $3.4x10^{5}$ - $5.4x10^{5}$ |
| 400 | $1.2x10^{10}$ - $8.0x10^{11}$ | $6.8x10^{5}$ - $1.1x10^{6}$ |

### 2.2.3 Calculation of $EC_{50}$ and statistics

$EC_{50}$ including 95% confidence intervals after 96 hours were calculated with the EPA trimmed Spearman-Karber (TSK) program (Version 1.5). Immobilisation rates after 24, 48 and 72 hours were too low to calculate the corresponding $EC_{50}$. In order to analyse if the exposure with 1 µm particles was related to observed immobilisation rates, we calculated odds ratios and their associated confidence intervals with Excel 2010. We compared each concentration with its associated control and each concentration individually between different exposure durations (24, 48, 72 and 96 hours).

## 2.3 Results

### 2.3.1 Behaviour of particles in aqueous solution

Microscopic examination of both particle sizes (Appendix Figure 1 and Appendix Figure 2) confirmed their relatively uniform spherical shape and size spectrum (1-4 µm for 1 µm particles, 90-106 µm for 100 µm particles). Observations on the behaviour of the PE particles in aqueous solutions showed that both dry powders, but especially 1 µm particles, tend to attach to surfaces. Due to the density of the investigated plastic (0.96 g $cm^{-3}$) particles of both size classes floated close to the water surface after having added the *Daphnia* culture medium to the weighed particles. After shaking and ultrasonic treatment, size determined the particle behaviour in the aqueous solution. Particles of 1 µm remained in the water column but also floated at the water surface and attached to the inner surface of the glass beakers at the interface between glass and water surface. This was still observed also after stirring the mixture intensively for up to 24 hours with a stir bar. Ultrasonic treatment minimized formation of aggregates, but could not fully prevent it. Particles of 100 µm were floating back to the water surface immediately after mixing. The 100 µm particles aggregated less than the 1 µm particles and formed a thin and relatively closed layer at the water surface in the centre of the glass vials.

During exposure experiments, 1 µm particles in the mixtures were also not equally distributed in the water column, meaning that some particles were floating on the water surface of the test beakers. Visible turbidity of the mixtures increased along the nominal test concentration gradient (12.5-400 mg $L^{-1}$). By the end of the test (96 hours) the increase of turbidity along the concentration gradient was less pronounced than at the beginning of the test, in particular for 400 mg $L^{-1}$ mixtures which looked less turbid than 200 mg $L^{-1}$ mixtures.

### 2.3.2 Exposure of *D. magna*

#### 2.3.2.1 Exposure with 1 µm PE particles

Guts of the animals in the control series looked transparent greenish. Ingested particles, in contrast, were identified by their whitish appearance under the microscope. After 24 hours of exposure to *D. magna*, 1 µm particles were present in the guts of the animals for every tested particle concentration (12.5-400 mg $L^{-1}$). This was also observed after 48, 72 and 96 hours. Closer examination after 96 hours illustrated that exposed daphnids from all tested concentrations had whitish transparent or bright whitish intestines. As shown in Figure 1, the particle structure in the intestines of some individuals looked a bit lumpy, but whitish colour of the intestines confirmed high uptake of 1 µm particles in all concentrations. Possible differences in the exact amounts of ingested particles at different concentrations or exposure times could not be quantified by visual examination under

the microscope. During the exposure experiments we also observed that particles could be egested again together with faeces. Adherence to external structures of the daphnids like the carapax or appendages were not observed at any concentration.


*Figure 1* *Appearance of daphnids at the end of exposure*
Individuals of *D. magna* after 96 hours in the control (A) and exposed to 400 mg $L^{-1}$ of 1 µm PE particles (B).

Within the first 48 hours, no immobilisation exceeding 10% in all treatments (1 µm particles and control) was observed (Figure 2), which was considered a non-significant effect according to OECD guideline 202. After 72 hours of exposure, a maximum mean of 35% of exposed daphnids were immobile (for 200 mg $L^{-1}$, Figure 2). Means showed a slight increase in immobilisation following the concentration gradient except for 400 mg $L^{-1}$. Immobilisation increased until the end of the complete test series (96 hours) to 25% for the lowest concentration (12.5 mg $L^{-1}$) and 75% for 200 mg $L^{-1}$. For both timelines, 72 and 96 hours, means of immobilisation at concentrations of 400 mg $L^{-1}$ were smaller than for all lower concentrations after 72 hours and equal to immobilisation for 25 mg $L^{-1}$ after 96 hours.

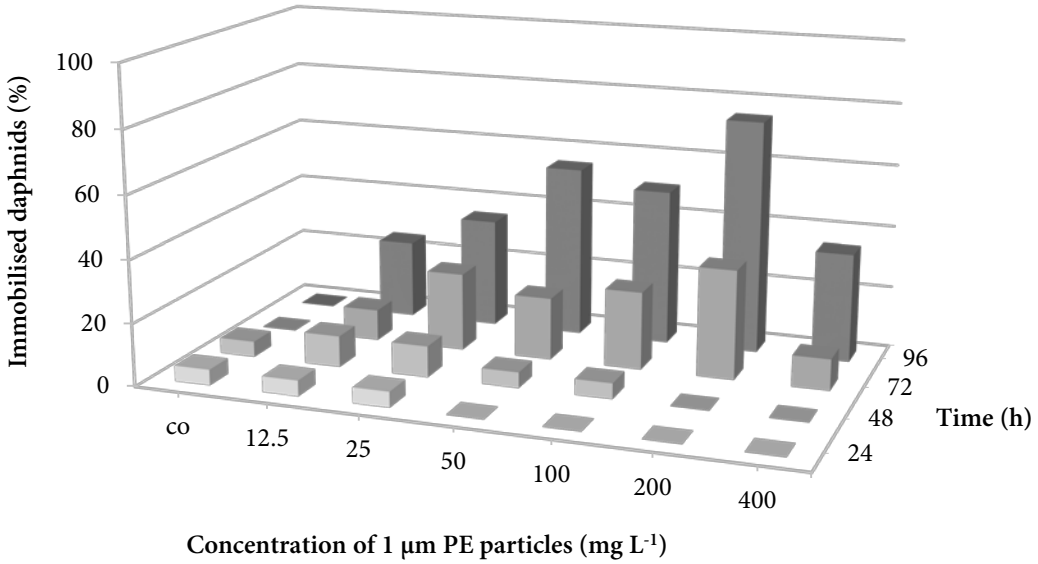



*Figure 2* *Immobilisation of daphnids in treatments with 1 µm PE particles*
Percentage of immobilised individuals of *D. magna* as means (n=4) after 24, 48, 72 and 96 hours exposed to different concentrations of 1 µm PE particles between 12.5-400 mg $L^{-1}$ and in the control (co).

Excluding the results for 400 mg $L^{-1}$ because of the higher dissipation rate, $EC_{50}$ after 96 hours was 57.43 mg $L^{-1}$ (95 CI, lower: 32.76, upper: 100.69). $EC_{50}$ values for 24, 48 and 72 hours could not be calculated due to low immobilisation rates which indicate no observable effects within this timeframe. Pairwise comparison (odds ratios) of immobilisation at different concentrations with the corresponding control showed significant differences at 200 mg $L^{-1}$ after 72 hours (Table 2). After 96 hours, all concentrations between 25 and 400 mg $L^{-1}$ differed significantly from the control. Odds ratios of each concentration compared individually with itself after 24, 48, 72 and 96 hours showed significant effects between 24 and 96 hours for all concentrations but the lowest with 12.5 mg $L^{-1}$ (Table 2). Most significant differences were found for 200 mg $L^{-1}$ when comparing immobilisation at different exposure times.

*Table 2* *Statistical significances for immobilisation in 1 µm PE treatments*
Percentages of means of immobilised daphnids exposed in each treatment (co: control) with standard error (SE, n=4). Unequal letters indicate significant differences calculated by odds ratios with normal letters for dose response (columns individually at one time) and capital letters for time dependency (rows individually for one treatment).

| | **Means of immobilised daphnids (%) ± SE** | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| **Conc. (mg $L^{-1}$)** | **24 h** | | **48 h** | | **72 h** | | **96 h** | |
| co | 5 ± 1 | a *X* | 5 ± 1 | b *X* | 0 | c *X* | 0 | e *X* |
| 12.5 | 5 ± 1 | a *B* | 10 ± 2 | b *B* | 10 ± 2 | cd *B* | 25 ± 1.91 | ef *B* |
| 25 | 5 ± 1 | a *C* | 10 ± 2 | b *CD* | 25 ± 1 | cd *CD* | 35 ± 1 | f *D* |
| 50 | 0 | a *E* | 10 ± 1 | b *E* | 20 ± 1.63 | cd *E* | 55 ± 1 | fg *F* |
| 100 | 0 | a *G* | 5 ± 1 | b *G* | 25 ± 2.52 | cd *GH* | 50 ± 2.58 | fg *H* |
| 200 | 0 | a *I* | 0 | b *I* | 35 ± 3 | d *J* | 75 ± 1 | g *K* |
| 400 | 0 | a *L* | 0 | b *L* | 10 ± 1.15 | cd *LM* | 35 ± 1.91 | fg *M* |

#### 2.3.2.2 Exposure with 100 µm PE particles

In contrast to experiments with 1 µm particles, 100 µm particles exposed to daphnids did not cause any immobilisation that exceeded 10% at any time or concentration. Focussing on the organism behaviour, we could observe that some individuals swam into the particle layer at the surface from time to time. The particles that were afterwards attached to the carapax came off again after some swimming movements.

## 2.4 Discussion

### 2.4.1 Characterisation of particles

With a density of 0.96 g $cm^{-3}$ the PE particles used in our experiments belong to the group of high density polyethylene (HDPE) which is known to be very stable, tensile as well as corrosion and abrasion resistant. Its numerous applications (e.g. grocery sacks, pipes) make PE in general one of the most demanded polymers in Europe (Plastics Europe

2015), which is also reflected by its high abundance in the environment (Browne et al. 2011). Similar to the particles used in our experiments, PE particles of around 100 µm in diameter or smaller are known to be used in cosmetic products (e.g. Fendall and Sewell 2009).

Due to our results on short-term fate of pristine PE particles, not only density of the plastic material but also particle size determined the behaviour of the particles in aqueous solution. In contrast to the 100 µm particles, which formed a thin layer on the water surface after shaking and ultrasonic treatment, 1 µm particles remained in the water column but also tended to form more aggregates. These results imply that even particles of the same polymer but of different sizes are heterogeneously distributed in the water column after mixing events in the environment.

### 2.4.2 Exposure of *D. magna*

Different behaviour of 1 and 100 µm particles in the aqueous phase led to different exposure scenarios for the test organisms because of their presence either in the water column (1 µm) or at the water surface (100 µm). With increasing concentrations an increasing amount of 1 µm particles was distributed in the water column, for 100 µm particles the diameter of the thin particle layer at the surface increased. While daphnids in experiments with 1 µm particles were constantly exposed to the microplastics in the water column, daphnids in experiments with 100 µm particles just came into direct contact with the particles by swimming to the water surface. Therefore, by using 100 µm particles, not only the potential impacts of particles in the water column (1 µm particles), but also of microplastics floating at the water surface was included in our study.

#### 2.4.2.1 Ingestion of 1 µm PE particles

Similar to other studies on microplastics (e.g. Imhof et al. 2013, Rosenkranz et al. 2009) our results demonstrate that *D. magna* is also able to ingest 1 µm PE particles. We conclude that the presence of the 1 µm particles in the water column made them available for a filtering organism like *D. magna*. Since food algae ranging from 0.7 µm (Gophen and Geller 1984) to 70 µm (Burns 1968) in size can be ingested by *D. magna*, the size of the 1 µm microplastic particles was no hindering factor for ingestion. Although photographs of daphnids indicate no distinct differences in particle loads of the intestines between concentrations and exposure times, it cannot be fully excluded if this was due to the two dimensional view of the intestines, which is very limited for quantitative analysis of particle load. Egestion which we observed for 1 µm particles, was also described after microplastic ingestion by marine zooplankton like copepods (Cole et al. 2013, Setälä et al. 2014).

#### 2.4.2.2 Dose and time effects for 1 µm PE particles

In agreement with a study on nanoparticle toxicity (Dabrunz et al. 2011), immobilisation exceeding 10% was not observed within the recommended exposure time of 48 hours (OECD guideline 202), but after 72 and 96 hours. The pronounced increase in toxicity if exposure time is prolonged to 96 hours is similar to another study (Baumann et al. 2014) and underlines the need for adaptations and different testing procedures on impacts of particles in general and microplastics in particular.

Since daphnids in the controls did not show immobilisation after 72 and 96 hours, immobilisation of daphnids exposed to 1 µm PE particles cannot be exclusively explained by a lack of food during experiments. It is possible, though, that daphnids in the experiments are more vulnerable to other stressors like microplastics if food is absent for a longer time (here 72 and 96 hours). It is known from daphnids that they can regulate their filtering activity depending on the abundance of food (McMahon and Rigler 1965). High abundance or in general presence of food can increase filtering activity, causing also higher ingestion rates of pollutants. Since food was not supplied in any of the treatments in our study, ingestion rates cannot have been altered by different amounts of food. Only if daphnids identified the 1 µm particles as potential food, higher ingestion rates could be expected for higher concentrations. This could also explain higher immobilisation rates with increasing concentrations. Further studies are needed to analyse in more detail how filtering activity, amount of ingested particles, egestion of particles and overall particle load in the intestines are linked to negative impacts of microplastic particles.

An increased number of immobilised test individuals when exposed to higher particle concentrations (after 72 and 96 hours) indicates that the dose of the tested particles does influence the extent of adverse impacts. This was not the case for 400 mg $L^{-1}$ and can be related to a decrease of equally distributed particles in the water column, as the suspensions in the test beakers with 400 mg $L^{-1}$ looked less turbid than the suspensions in lower concentrations. Dissipation of particles from the test medium is also described for nanoparticles with faster dissipation for higher concentrations (Dabrunz et al. 2011) and discussed to result from the tendency of the particles to aggregate and to attach e.g. to the inner glass surface of the test vessels. From a biological perspective, high concentrations of particles could potentially block the filtering apparatus of the daphnids which would lead to a decreased ingestion of the particles. This might also be a reason for lower immobilisation of daphnids exposed to 400 mg $L^{-1}$ and could be analysed more specifically in further studies.

To the best of our knowledge, this is the first study providing $EC_{50}$ of PE particles (here after 96 hours) for limnic zooplankton when chemical effects are excluded. In addition to dose-dependency especially after 96 hours, time dependency of the observed impacts for concentrations between 25 and 200 mg $L^{-1}$ was even more pronounced with continuously increasing immobilisation rates and significant differences. Altogether, an $EC_{50}$ of 57.43

mg $L^{-1}$ after 96 hours indicates that very high concentrations and a relatively long exposure time are needed to cause negative acute effects in daphnids. In order to estimate the resulting risk on limnic zooplankton by physical effects of microplastics in the environment, we first need to gain more knowledge about the plastic particle distribution and abundance in freshwaters. Although first estimations indicate concentrations of microplastics as high as 162 mg $L^{-1}$ in sediment pore water, future studies need to show if we can expect actual environmental concentrations for microplastics in the size range of about 1 µm equal to the tested concentrations in our study.

#### 2.4.2.3 Mechanisms causing adverse effects of 1 µm PE particles

Although leachates from HDPE are ranked as moderate toxic in general (Lithner et al. 2011), leachates of HDPE were found to cause acute toxic effects on *D. magna* (Lithner et al. 2012). However, as we used particles without any additives (manufacturer information) and did not apply any surfactants for the preparation of the test suspensions, we exclude chemical effects "carried" by the microplastic particles.

Since we did not observe adhesion of 1 µm particles to appendages like antennae or the carapax, we also exclude inhibition of movements by adhered particles, which was the reason for negative impacts both on marine copepods exposed to PS beads (Cole et al. 2013) and *D. magna* exposed to $TiO_2$ nanoparticles (Dabrunz et al. 2011). Although particles could be egested again, we conclude that physical effects by ingestion cause higher immobilisation rates when *D. magna* is exposed to small microplastic particles like 1 µm PE. However, further studies are needed to unravel mechanisms causing immobilisation induced by 1 µm PE particles in detail. Mechanical forces are already known to induce inflammatory processes in blue mussels (*Mytilus edulis*) after ingestion and translocation of > 0-80 µm HDPE particles to the digestive gland and the lysosomal system (von Moos et al. 2012). This indicates that microplastic particles have the potential to enter tissues and cells and to induce immune responses. In another study, translocation of PS-particles from intestines to the haemolymph cavity of blue mussels showed the potential of microplastic particles to be transported to other organs via the circulatory system (Browne et al. 2008). In the common goby (*Pomatoschistus microps*) red coloured PE particles in the range of 1-5 µm were shown to inhibit acetylcholinesterase activity for 22% in average after 96 hours of exposure (Oliveira et al. 2013). In a study with *D. magna* there was evidence, that both 20 nm and 1 µm carboxylated PS particles are not only accumulated in the intestines but also cross gut epithelial layers and can be incorporated in storage lipid droplets already after short term exposure (Rosenkranz et al. 2009). As these droplets are important for daphnids in the environment in times of starvation, particles are likely to be mobilised again from lipid droplets (Goulden and Hornig 1980).

#### 2.4.2.4 Impact of 100 µm PE particles

During exposure with 100 µm particles, individuals of *D. magna* were observed to swim into the particle layer, which was increasing in diameter with increasing concentrations

of particles. Although some particles attached to the carapax afterwards, which impeded swimming for some of the individuals, the particles detached again after some swimming movements of the daphnids. In contrast to surface coating by PS beads (Cole et al. 2013) or nanoparticles (Dabrunz et al. 2011), 100 µm PE particles did not attach permanently to the carapax of *D. magna*. Further studies need to analyse if also smaller *Daphnia* species, which are in general more easily trapped by the surface tension, can detach again from the water surface in the presence of floating microplastic particles. In the environment, daphnids move down- and upward in the water column depending on e.g. light and temperature (Gerritsen 1982). However, because of a very limited water depth under laboratory conditions in general, it is not clear to what extent floating particles potentially affect daphnids in the environment.

## 2.5 Conclusions

The results of our study on impacts of pristine microplastic particles on daphnids show that (1) 1 µm PE particles can be ingested by limnic zooplankton and (2) that the ingestion of 1 µm particles results in immobilisation of daphnids at high concentrations. Characteristics of microplastics (i.e. density and size) influence the fate of the particles in the water column, which leads to different exposure scenarios for freshwater organisms. When microplastic particles are distributed in the water column (1 µm particles) and can be ingested, both dose- and time-dependent effects can be observed. Since we can exclude chemical effects by additives and pollutants attached to the microplastic particles and adherence of particles to outer structures of the daphnids, immobilisation can be related to physical effects by ingestion. Floating particles, which cannot be ingested (100 µm particles) due to their size and availability to the organisms, do not cause any adverse effects. Although 100 µm floating particles can attach to the carapax of *D. magna*, they can detach again and do not result in immobilisation. In view of further analysis of the potential effects of microplastics on limnic organisms, our results underline the need of information on particle characteristics including their behaviour in water as well as the necessity of adapted testing procedures, e.g. prolongation of the test duration. We show that physical effects by pristine microplastics should be taken into account in future studies which also aim at distinguishing between the effect of the microplastic particles and the chemical effects by the vector function of microplastics for pollutants. As concentrations used in our study are very high, the risk of pristine PE particles seems to be relatively low for daphnids in the environment. However, further analysis including e.g. different plastic types, sizes and chronic exposure with lower microplastics concentrations are needed to understand potential adverse effects of microplastics in more detail. Also more complex exposure scenarios, including e.g. chemical burden, availability of food and a comparison to effects of natural particulate material, need to be taken into account to be able to estimate the environmental risk of microplastics. Since these processes are very complex in the environment, experimental approaches in the

laboratory are needed to isolate processes and to unravel systematically which parameters define the potential of microplastics to induce negative effects on (limnic) organisms.

# 3 Altered effects of a chemical pollutant by microplastics on limnic zooplankton

*This chapter is based on joint work with:*

*Christiane Zarfl, Center for Applied Geosciences, Eberhard Karls Universität Tübingen*
*Werner Kloas, Leibniz-Institute of Freshwater Ecology and Inland Fisheries; Department of Endocrinology, Institute of Biology, Humboldt-Universität Berlin*
*Angela Krüger, Leibniz-Institute of Freshwater Ecology and Inland Fisheries*
*Claudia Theel, Leibniz-Institute of Freshwater Ecology and Inland Fisheries*

*This joint work yielded the following publication:*

*Rehse S, Kloas W, Zarfl C (2018) Microplastics reduce short-term effects of environmental contaminants. Part I: Effects of bisphenol A on freshwater zooplankton are lower in presence of polyamide particles. Int J Environ Res Public Health 15(2): 280.*

*Individual contributions:*

***Saskia Rehse**, Christiane Zarfl and Werner Kloas designed the study; **Saskia Rehse** collected the data, analytical measurements were supported by Angela Krüger and Claudia Theel; **Saskia Rehse** and Christiane Zarfl analysed the data, Christiane Zarfl contributed to the calculation of the mass distribution of bisphenol A; **Saskia Rehse** wrote the manuscript with the support of Christiane Zarfl and Werner Kloas.*

## 3.1 Introduction

The presence of different stressors, like pollutants and their interactions, leads to complex scenarios in the environment. Freshwater systems are not only polluted by chemical substances, but also by microplastics (plastic particles < 5 mm, Moore 2008). Microplastics have been considered as potentially harmful to freshwater organisms (e.g. Eerkes-Medrano et al. 2015 for an overview). In particular, concurrent effects of microplastics and other pollutants are challenging to evaluate, because of diverse causalities under environmental conditions (Koelmans et al. 2016). Therefore, it is important to systematically identify not only effects of microplastic material itself, but also interactions of microplastics with other pollutants, and their potential combined effects on freshwater organisms.

In the past, rivers have been considered as a source for marine litter, including plastics (Williams and Simmons 1997). More recent studies confirmed that rivers and lakes are ubiquitously polluted with microplastics (Faure et al. 2015, Klein et al. 2015, Moore et al. 2011, Zbyszewski and Corcoran 2011). Besides the high variety of characteristics of microplastics, some sizes, shapes, and polymer types are reported to be more abundant in freshwater. Irregular shaped plastic fragments and very small microplastics, in the range of only a few to some hundreds of micrometres, make up a big proportion of the observed overall amount of microplastics in surface waters and beach sediments (Faure et al. 2015, Free et al. 2014, Imhof et al. 2016). Polyamide (PA) and polyethylene (PE) are among the most abundant polymer types found in environmental samples. Due to limits in sampling and evaluation techniques, especially data on concentrations of microplastics in the range of a few micrometres, and microplastics in the free water zone are still scarce, and sometimes not directly comparable, because of different reference units. Results are often either given as number of microplastic particles or mass of microplastics per area (e.g., $m^2$) or per volume (e.g., $m^3$). Monitoring so far has shown that mass concentrations of microplastics in surface waters range from $10^{-3}$ to $10^{-1}$ mg $m^{-3}$ for rivers, and from $10^{-3}$ to $10^{-1}$ mg $m^{-2}$ for lakes (Faure et al. 2015, Yonkos et al. 2014). Particle numbers of microplastics in both, rivers and lakes, go up to 10 particles $m^{-3}$ in samples from surface water (Faure et al. 2015, McCormick et al. 2016, Yonkos et al. 2014) Asian rivers seem to be the most polluted, with up to 1000 microplastic particles $m^{-3}$ and 1000 mg $m^{-3}$ in Yangtze river (Lebreton et al. 2017, Zhao et al. 2014).

Until now, the majority of studies about the potential harm of organisms by microplastics focused on marine species (e.g. Wright et al. 2013b for an overview). First results on freshwater organisms showed that microplastics may harm these in a similar way like they do with marine organisms, especially species of similar functional groups or with comparable food acquisition strategies, e.g., filtering organisms (Eerkes-Medrano et al. 2015, Ziajahromi et al. 2017). By filtering surrounding water, organisms like zooplankton or mussels are prone to water contaminants, in particular. Daphnids play an important role in lake ecosystems at the base of the food web as effective consumers of algae and

bacteria, and are an important prey for, e.g., fish larvae. Similarly to bigger sized plastics, raw microplastic material can have negative impacts on freshwater organisms by itself (physical effects), especially after ingestion (Au et al. 2015, Jemec et al. 2016, Rehse et al. 2016, Sjollema et al. 2016). However, only high concentrations of some microplastic types induced negative effects in laboratory experiments. Effects range from acute effects, like increased mortality in amphipods and daphnids, and immobilisation in daphnids, to chronic effects like decreased growth in algae and lower reproduction rates in amphipods. If small enough, microplastics can translocate within the body and enter tissues, as shown for 1 µm polystyrene (PS) particles in oil storage droplets of daphnids (Rosenkranz et al. 2009). Being some orders of magnitude smaller than microplastics, nanoplastics were shown to have a bigger negative impact on aquatic organisms than microplastics (Ma et al. 2016).

For the risk assessment of microplastics, not only physical effects but also interactions with organic pollutants need to be considered (chemical effects). Hydrophobic organic pollutants (HOC) are of special concern for freshwater ecosystems (Malaj et al. 2014). Being lipophilic, they tend to sorb to natural organic material like sediments, and to bioaccumulate in aquatic organisms. Some organic pollutants are associated with the production of plastics. Various chemicals are used as starting material for polymerisation (e.g., bisphenol A, BPA), or as additives for adjusting properties of the polymer depending on its intended application (e.g., colourants, plasticizers, UV stabilizers). Similar to natural organic material, microplastics tend to sorb organic pollutants from the water column, but also leach chemicals used for manufacturing (Teuten et al. 2009). This is why microplastics are considered as vectors for pollutants to aquatic organisms in general, especially if microplastics are ingested (Bakir et al. 2014). Due to the high affinity of HOC to plastics, microplastics are considered as vectors for HOC in particular (Browne et al. 2013, Teuten et al. 2007). BPA is used for the production of polymer types like polycarbonates and epoxy resins, and was shown to sorb into microplastics in freshwater with mean concentrations of 16.6 ng $g^{-1}$ (Faure et al. 2015). It is hormonally active in freshwater vertebrates, with disruption of larval development and the thyroid system in amphibians (Heimeier and Shi 2010, Levy et al. 2004). This is why BPA is classified as an endocrine disruptor. In freshwater zooplankton, BPA induces moderate acute toxicity which is indicated by an $EC_{50}$ after 48 hours of 10 mg $L^{-1}$ (Chen et al. 2002). BPA has been shown to leach from products used in households, including products associated with food consumption, and is considered as a potential direct threat to humans (Brotons et al. 1995).

That microplastics can act as vector for organic pollutants and modulate effects of pollutants was demonstrated in laboratory feeding experiments with fish (Karami et al. 2016, Rochman et al. 2013, Wardrop et al. 2016). Trophic transfer of microplastics and sorbed pollutants was shown in zebrafish (Batel et al. 2016). While this identifies microplastics as a potential source for pollutants in general, the relative importance of

microplastics as vector is not clarified. After exposure of bivalves with environmentally relevant concentrations of microplastics spiked with polychlorinated biphenyls (PCBs), no PCBs could be detected in the bivalves, and in fish which were feeding on the bivalves (Rochman et al. 2017). Model-based studies indicate that the vector function of microplastics is negligible in the environment compared to other uptake pathways and that sorption equilibrium can be assumed for most marine microplastics on relevant timescales (Bakir et al. 2016, Besseling et al. 2017b, Koelmans et al. 2016). This is also supported by a modelling study showing that leaching of additives from microplastics is not a relevant exposure pathway for lugworms (Koelmans et al. 2014). Validation by empirical data is needed to raise credibility for this evidence. On a microorganism level, Kleinteich et al. (2018) show that the effect of polycyclic aromatic hydrocarbons on bacterial community composition was reduced in the presence of microplastics. Recent studies with marine organisms indicate no or only low impact of microplastics as carrier for pollutants (Beckingham and Ghosh 2017, Besseling et al. 2017b, Devriese et al. 2017, Paul-Pont et al. 2016). Due to similar modes of action, this highlights the need for studies with experimental evidence in freshwater organisms.

The underlying hypothesis of this study is that microplastic particles do not increase, but rather reduce the effect of a pollutant that is already available in the aqueous phase. Assuming a system in equilibrium, sorption of the contaminant to microplastics leads to removal from the aqueous, i.e., bioavailable, fraction. To test these hypotheses freshwater zooplankton (*Daphnia magna*) was exposed to BPA and PA particles as model compounds in equilibrium batch systems. Immobilisation was analysed as criterion for negative effects for (1) PA particles alone, (2) BPA alone, and (3) BPA in presence of PA particles. Results of this approach aim to entangle the discussion on the potential vector effect of microplastics for environmental pollutants in freshwater systems.

## 3.2 Methods

### 3.2.1 Microplastic material and chemicals

Polyamide particles (PA particles) were purchased as powder from Goodfellow (Nylon 6, AM306010; Goodfellow GmbH, Bad Nauheim, Germany). The particles had an irregular shape, a mean diameter of 15–20 µm (min. 5 µm, max. 50 µm) and a polymer material density of 1.13 g $cm^{-3}$. Bisphenol A (BPA, ≥ 99%, CAS number 80-05-7) was purchased from Sigma-Aldrich (Sigma-Aldrich Chemie GmbH, Munich, Germany). Stock solutions of BPA with 40 mg $L^{-1}$ were prepared with freshly prepared artificial *Daphnia* culture medium (ADaM medium, Klüttgen et al. 1994), and stored for a maximum of two days at 7 °C. Glass material was used for, e.g., preparation of test solutions whenever possible.

### 3.2.2 Study design

To test our hypotheses, the experimental setup for the exposure of daphnids needed to meet several predefined conditions. For analysing the potential vector effect of microplastics, the sorption of a quantifiable proportion of BPA to the PA particles, and the uptake of the particles by the daphnids, in general, needed to be assured. Sorption and desorption processes of BPA on PA particles needed to be in an equilibrium state. Finally, the particles themselves should cause neither chemical (e.g., by leaching additives) nor physical acute effects on daphnids, that could confound effects of the pollutant itself.

#### 3.2.2.1 Sorption characteristics and BPA content of PA particles

To analyse sorption characteristics of BPA to PA particles and determine when sorption equilibrium is reached, a batch experiment was performed with one concentration of BPA (10 mg $L^{-1}$) and PA particles (microplastics, MP; 200 mg $L^{-1}$). Mixtures contained BPA together with PA particles (BPA + MP), and were compared to mixtures with only BPA as control (BPA alone). A batch with only PA particles (MP alone) was analysed in addition, to make sure that no BPA was leaching out of the PA particles themselves. Bisphenol A (BPA) solutions with an initial concentration of 10 mg $L^{-1}$ were prepared by dilution of the stock solution with AdaM medium. For treatments containing PA particles (BPA + MP, MP alone), 50 mg of PA particles were weighed into 20 ml glass flasks, rinsed three times with either BPA solution (BPA + MP) or ADaM medium (MP alone), and mixed thoroughly. The mixtures were decanted to 500 ml glass bottles, which were then filled up to a total volume of 250 ml with BPA solution (BPA + MP, BPA alone) or ADaM medium (MP alone), and shaken at 200 rpm. All treatments were run in duplicates. The concentration of BPA dissolved in water was measured regularly for up to 72 hours (after 0, 6, 24, 48, 72 hours) after removing the PA particles with a syringe filter (Whatman Spartan HPLC cert. syringe filter, 13 mm diameter, 0.45 µm pore size) via high performance liquid chromatography (HPLC) with fluorescence detection (Dionex Ultimate 3000 HPLC with Nova-Pak C18 column). The filtrate was diluted to reach a nominal concentration of 2 mg $L^{-1}$ and filled in 1.5 ml glass vials, which were then placed into the autosampler of the HPLC. Samples were measured within 24 hours. Results were multiplied by the corresponding dilution factor. HPLC measurement accuracy of 0.1 mg $L^{-1}$ led to increasing inaccuracies of the measured values with increasing concentrations of BPA, due to dilution. Concentrations of BPA in water were continuously lower with PA than without (Appendix Table 2). BPA concentrations in the solution with BPA alone ranged from 9.5–10.0 mg $L^{-1}$. For BPA in combination with microplastics, the concentration of BPA decreased with reaching equilibrium, after 48 hours at 7.5 mg $L^{-1}$. In batches with microplastics alone, no BPA was detected above the detection limit of 0.1 mg $L^{-1}$. Sorption of BPA to glass surfaces and degradation of BPA were assumed to be negligible, because of good recovery rates in batches with only BPA.

Assuming equilibrium after 48 hours, the partition coefficient, defined as (Schwarzenbach et al. 2016)

$$K_{pa,w} = \frac{c_{pa}}{c_w}$$

was calculated with $c_{pa}$ as equilibrium concentration of BPA adsorbed to PA particles (in mg kg$^{-1}$), and $c_w$ as equilibrium concentration of BPA in water measured by HPLC (7.5 mg L$^{-1}$, Appendix Table 2). $c_{pa}$ was calculated from the BPA mass balance as follows:

$$c_{pa} = \frac{m_{total} - c_w * V_w}{M_{pa}}$$

with $m_{total}$ as total mass (in mg) of BPA in the system, $V_w$ as volume of water in the beakers (in L), and $M_{pa}$ as mass of PA particles (in kg) added to the beakers.

#### 3.2.2.2 Pre-exposure with single substances

A clone of *D. magna* (originally isolated from Großer Binnensee, Lampert 1991) from a healthy laboratory stock was cultured according to Rehse et al. (2016). Potential uptake and effects of pristine PA particles alone were studied by exposing daphnids not older than 24 hours (neonates) to a broad range of concentrations of PA particles (25–250 mg L$^{-1}$; n=3 per concentration with five daphnids each). Glass beakers (total volume of 50 ml) were filled with 40 ml ADaM medium spiked with either PA particles or BPA followed by careful transfer of five new born daphnids not older than 24 hours per beaker. Beakers were covered with glass petri dishes and daphnids exposed at culturing conditions (temperature 20 ± 2 °C, 16:8 light–dark period) for up to 48 hours. Immobilisation was the criterion for acute negative effects. The particles were dispersed in the water column at the beginning of the exposure, but settled at the bottom of the test beakers shortly after. Ingestion of particles not only from the water column, but also from settled material, was observed within the first 24 hours (Horton et al. 1979, Lampert 1987). Ingestion of particulate matter as potential food for daphnids is size-dependent, with an optimum range between 0.7–70 µm in diameters (Burns 1968, Gophen and Geller 1984). Daphnids are unselective filter feeders, so ingestion of PA particles with a size range between 5–50 µm in diameter could be expected. No daphnids were immobilised after 24 or 48 h of exposure to PA particles at any concentration (25–250 mg L$^{-1}$). Therefore, both physical and chemical effects i.e., by leaching additives, can be excluded for an exposure time of up to 48 hours, which was later also applied for exposure experiments with mixtures of PA particles and BPA. If the exposure time was prolonged to 96 hours, only in the treatment with the highest concentration of PA particles more than 10% of the daphnids were immobilised after 96 hours (around 30%).

For finding the range of BPA concentrations relevant for acute toxicity, daphnids were also exposed to a broad concentration range of BPA alone (2.5–40 mg L$^{-1}$). The concentration at which 50% of the daphnids were immobilised (effective concentration, $EC_{50}$) was

calculated as benchmark. If the $EC_{50}$ value is lower for one treatment, daphnids are assumed to be more sensitive towards the tested pollutant. The $EC_{50}$ of 7.6 mg $L^{-1}$ after 48 hours is similar to others reported in the literature (Brennan et al. 2006), suggesting similar or slightly higher sensitivity of our daphnid stock.

### 3.2.3 Exposure experiments with mixtures of BPA and PA particles

To analyse how the presence of PA particles modulates the effects of BPA, daphnids were exposed to treatments with five different initial nominal concentrations of BPA (5, 7.5, 10, 12.5, and 15 mg $L^{-1}$). Each concentration of BPA was tested alone (BPA alone) and with PA particles (BPA + MP) with a constant concentration of PA particles (200 mg $L^{-1}$), leading to five pairs of treatment combinations.

#### 3.2.3.1 Procedure for exposure of *D. magna*

Test solutions were prepared as described for the pre-experiment on sorption characteristics of PA particles. One control treatment contained ADaM medium only. All test solutions were shaken in glass bottles as batches for 48 hours prior to exposure experiments, to ensure sorption equilibrium. BPA concentrations in the water of batches were checked after 0, 24, and 48 hours of shaking via HPLC, to validate sorption equilibrium. Each test beaker was then filled with 40 ml of test solution. A total of 25 neonates in groups of five animals were exposed to each treatment (n=5 per treatment) following the *Daphnia* sp. Acute Immobilisation Test, for full assessment of acute toxicity (OECD guideline 202, Organization for Economic Co-operation and Development 2004). According to the guideline, immobilisation after 24 and 48 hours was the criterion for negative effects. If daphnids were not able to swim within 15 s after gentle agitation of the test vessel, individuals were considered to be immobile. Daphnids were not fed during exposure. Temperature, pH, and oxygen were measured in an extra beaker without daphnids, with one beaker for each treatment and processed the same way. Measurements were all in the same range after 24 and 48 hours of exposure (22.6 °C, pH 7.5, 8.6 mg $O_2$ $L^{-1}$).

#### 3.2.3.2 Concentrations of aqueous BPA and $EC_{50}$ values

Concentrations of BPA dissolved in water were measured via HPLC in the test beakers at the beginning (0 hours) and at the end of exposure (48 hours). The mass balance of BPA was used based on physiochemical characteristics to analyse the distribution of BPA within the different compartments (water, PA particles, organisms) and determine the theoretical BPA concentration in water $c_w$ in both experimental setups, i.e., for BPA alone:

$$c_w = \frac{m_{total}}{V_w + BCF * M_{org}}$$

and for BPA in combination with microplastic particles:

$$c_w = \frac{m_{total}}{V_w + BCF * M_{org} + K_{pa,w} * M_{pa}}$$

with $m_{total}$ as total mass (in mg) of BPA in the system, $V_w$ as volume of water in the beakers (in L), the bioconcentration factor (BCF), i.e. the partition coefficient between the organic phase (organisms) and water (in L $kg^{-1}$), $K_{pa,w}$ as partition coefficient between PA particles and water (in L $kg^{-1}$), and $M_{pa}$ as mass of PA particles (in kg). BCF was calculated according to Veith et al. (1980), leading to 225.95 L $kg^{-1}$. The partition coefficient of $K_{pa,w} = 1666\ L\ kg^{-1}$ was calculated from pre-experiments on sorption characteristics.

Measured concentrations of BPA in water in the test beakers before exposure were compared to calculated concentrations. $EC_{50}$ values were calculated with immobilisation rates and measured BPA concentrations after 48 hours of exposure for BPA alone and BPA in combination with microplastics.

### 3.2.4 Statistics

Treatments with BPA alone and BPA in combination with microplastics were tested for significant differences with two-sided Fisher's exact test with the software GraphPad Prism (version 4.03) in pairwise comparison for nominal BPA concentrations. The EPA trimmed Spearman–Karber (TSK) program (version 1.5; U.S. Environmental Protection Agency, Washington, DC, USA) was used for calculating $EC_{50}$ values, including 95% upper (UC) and lower (LC) confidence intervals.

## 3.3 Results

### 3.3.1 Impacts of PA particles during co-exposure with BPA

We observed that the intestines of the daphnids appeared to be whitish after 24 hours already in all treatments with BPA combined with microplastics, indicating ingestion of particles. Being equally distributed within the water column at the beginning of exposure, the particles sank to the bottom of the test beakers, forming a thin layer within the first 24 hours. Immobilisation rates of daphnids after 24 and 48 hours increased, following the gradient of the nominal BPA concentration (Figure 3). Immobilisation after 48 hours of exposure increased in comparison to 24 hours. Treatments with BPA in combination with microplastics always caused reduced immobilisation compared to BPA alone. After 24 and 48 hours, some treatments differed significantly when directly comparing treatments with the same nominal BPA concentration in the presence (BPA + MP) and absence of particles (BPA alone).

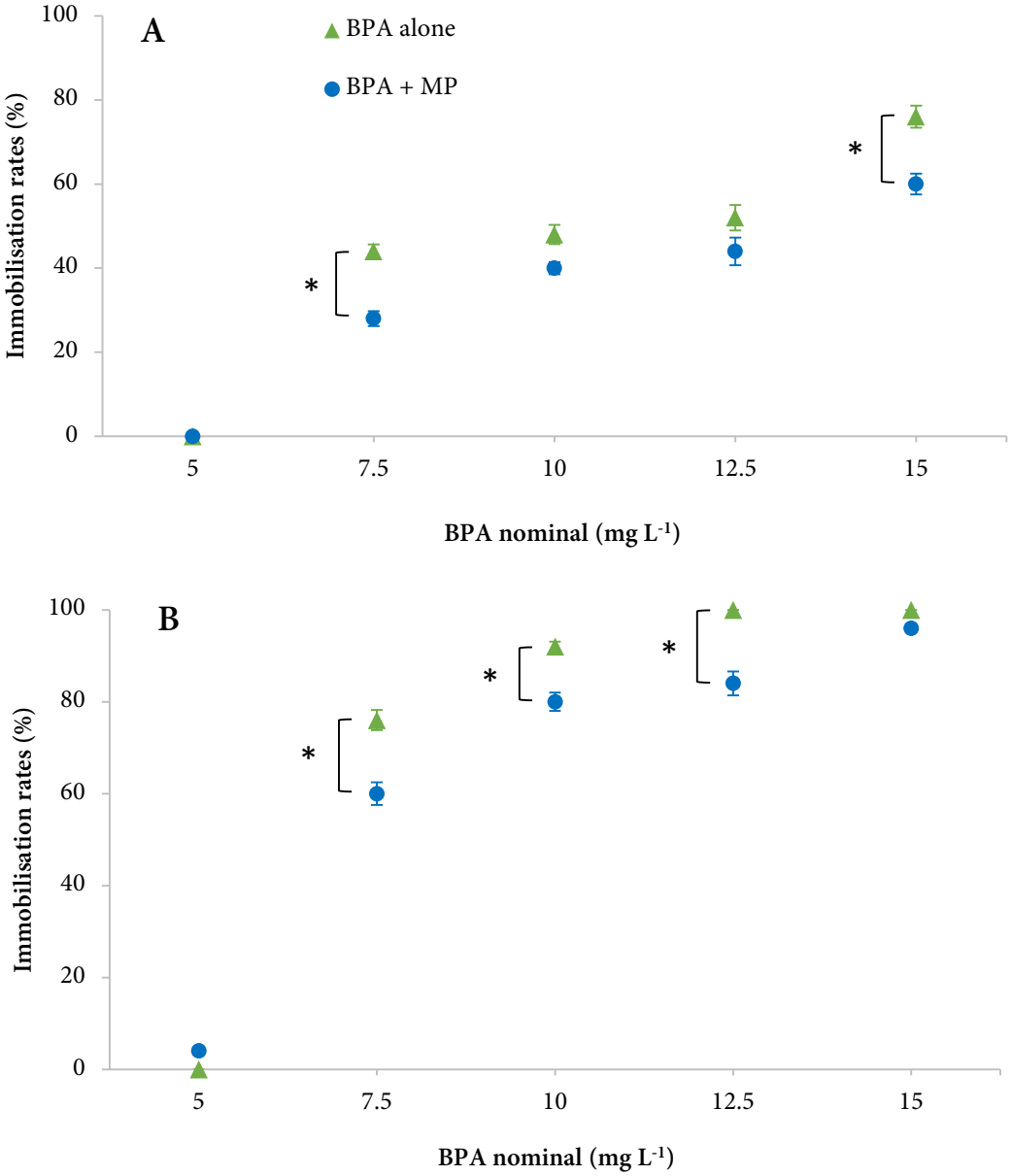



*Figure 3* *Immobilisation for BPA alone and in combination with PA particles*
Immobilisation of daphnids after (A) 24 hours and (B) 48 hours of exposure with bisphenol A (BPA) and microplastics (MP), in different treatments with increasing nominal BPA concentrations for BPA alone and BPA in combination with microplastics (BPA + MP; 5–15 mg $L^{-1}$), and one concentration of microplastics for BPA + MP (200 mg $L^{-1}$; mean ± SE, n=5). Brackets marked with asterisks indicate significant differences (Fisher's exact test, $p < 0.05$) between treatments with BPA alone and BPA + MP.

### 3.3.2 Concentrations of aqueous BPA

The concentrations of BPA in water, measured by HPLC, were lower for BPA in combination with microplastics, compared to BPA alone, during exposure of daphnids (Appendix Table 3). Concentrations in water were stable until the end of exposure after 48 hours, with only a small decrease of BPA, which could be due to degradation of only a small proportion of BPA in the presence of daphnids.

Calculated BPA concentrations in water ($c_{w,calculated}$) are close to measured values ($c_{w,measured}$), confirming accuracy of the measurements and that sorption equilibrium was reached (Figure 4).

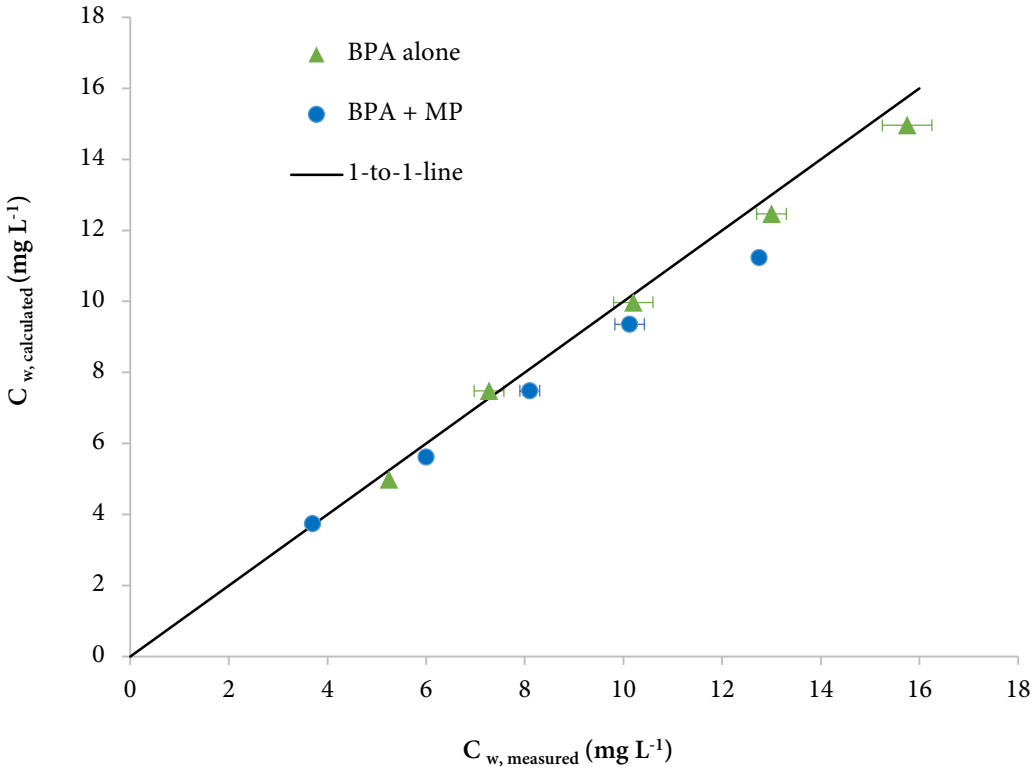



*Figure 4* *Comparison of measured and calculated aqueous BPA*
Relationship between measured (means ± standard deviations, n=5) and calculated concentrations of bisphenol A (BPA) in water for treatments with BPA alone and BPA combined with microplastics (MP) (treatment BPA + MP).

According to the calculations, sorption of BPA to microplastics led to a reduction of nearly 25% of BPA in water, in BPA combined with microplastic treatments, compared to BPA alone (Table 3). Taking 0.28% of BPA in daphnids from BPA alone as reference, daphnids in BPA combined with microplastics hold 25% less.

*Table 3* *Calculated distribution of BPA in the system*
Mass distribution of bisphenol A (BPA) for all relevant compartments in the test system calculated for BPA alone and BPA in combination with microplastics (MP) (treatment BPA + MP).

| | Mass Distribution of BPA (%) | |
|---|---|---|
| **Compartment** | **BPA alone** | **BPA + MP** |
| water | 99.72 | 74.85 |
| organisms | 0.28 | 0.21 |
| PA-particles | – | 24.94 |

### 3.3.3 $EC_{50}$ values for measured and calculated aqueous BPA

$EC_{50}$ based on measured BPA concentrations in water is lower for BPA in combination with microplastics (BPA + MP; 5.54, LC: 4.98, UC: 6.15 in mg $L^{-1}$) than for BPA alone (6.4, LC: 5.94, UC: 6.87 in mg $L^{-1}$; Figure 5). Overlap of confidence intervals indicates no significant differences between the $EC_{50}$ values.

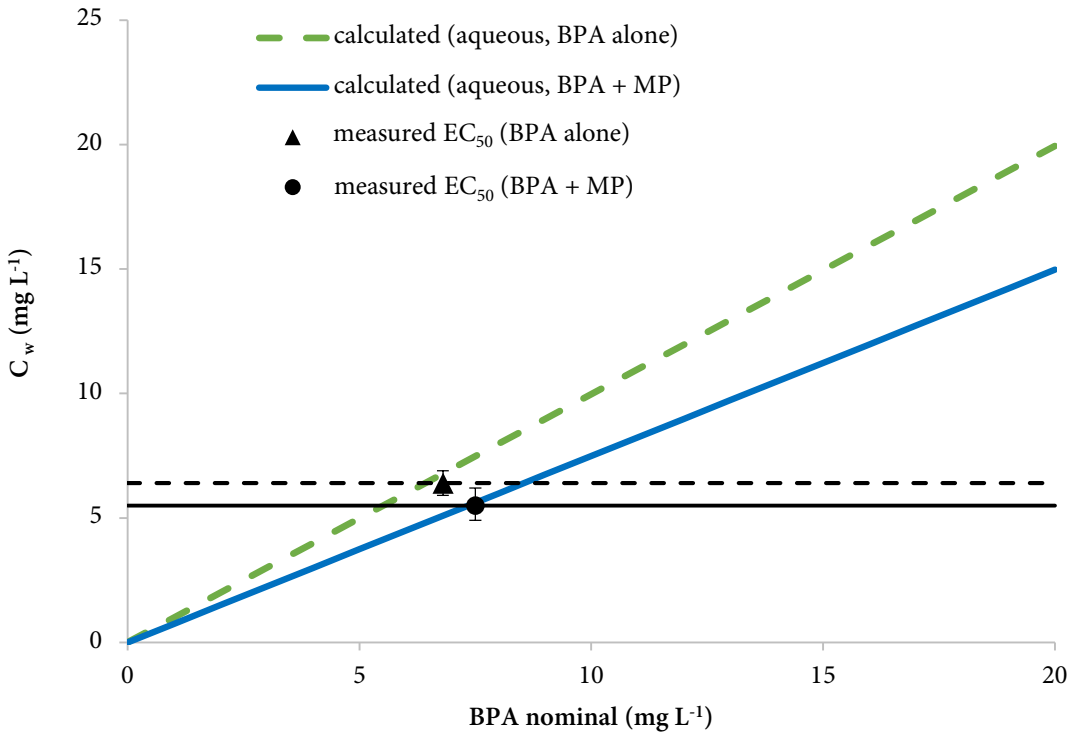



*Figure 5* *Comparison of $EC_{50}$ values for measured and calculated aqueous BPA*
Calculated concentrations of bisphenol A (BPA) in water for BPA alone and BPA in combination with microplastics (MP) (treatment BPA + MP) represented as coloured lines, together with $EC_{50}$ values based on measured concentrations of BPA in water and corresponding $EC_{50}$ lines.

## 3.4 Discussion

In contrast to studies that assign microplastics an important role as pollutant vector, this experimental study found no evidence for this. Having the same total mass of BPA in the system, even fewer daphnids were immobilised in the presence of PA particles than without. This was despite that clear evidence could be provided for sorption of BPA to the particles and ingestion of the PA particles by the daphnids.

### 3.4.1 Study design with predefined conditions

Recently, there was a call for more complex experimental setups with scenarios likely encountered in the environment for the risk assessment of microplastics (Besseling et al. 2017b, Koelmans et al. 2016). Including only selected predefined parameters under well controlled laboratory conditions in the setup of this study provided the opportunity to focus on a more mechanistic understanding of single relevant aspects. In many previous studies, the potential vector effect of microplastics was analysed by measuring uptake rates of the pollutants assessed by tissue concentrations as proxy (Khan et al. 2015, Ma et al. 2016). Other studies analysed pollutant effects, e.g., biomarker activity or histopathological changes (Paul-Pont et al. 2016, Rochman et al. 2014). This study focused on analysing how the acute effects of a pollutant are modified by microplastics, rather than measuring uptake rates and tissue concentrations of the pollutant within the daphnids. Experimental results on immobilisation in daphnids and analytical measurements of BPA in water were complemented by calculations of the mass distribution of BPA in the test system.

Sorption behaviour of pollutants to microplastics is crucial for their potential vector effect. Batch experiments showed that PA particles are an intermediate strong sorbent for BPA with fast sorption equilibration. Shaking the mixtures for 48 hours prior to testing assured that sorption processes of BPA to PA particles were in an equilibrium state. In most previous studies, microplastics loaded with organic pollutants were directly fed to the test animals without reaching sorption equilibrium in the test system before exposure (Devriese et al. 2017, Rochman et al. 2013). Even if microplastic pollutant mixtures have been pre-equilibrated prior to exposure to ensure sorption of the pollutant to microplastics in some studies, dilution of the mixtures led to a non-equilibrium state at the beginning of exposure (Khan et al. 2015, Rochman et al. 2013, Wardrop et al. 2016). While this non-equilibrium state is also a relevant environmental scenario with contaminated microplastics emitted into the aqueous system, e.g., via point sources, sorption equilibrium for microplastics and pollutants can be expected for microplastics being in the environment for a longer time (Koelmans et al. 2016), and allows estimation of the contaminant distribution within the experimental setup without additional kinetic studies. In marine systems, the majority of microplastics are expected to be in the environment for 2–4 years at, or close to sorption equilibrium. Rivers were shown to act

as an emission compartment of microplastics ending up in the oceans (Lebreton et al. 2017). Being close to emission sources (e.g. wastewater treatment plants), microplastics in freshwater systems might be more abundant than in the oceans. Shorter residence times of microplastics in freshwater systems could lead to a smaller proportion of microplastics at, or close to sorption equilibrium. Time to reach equilibrium depends on properties of the microplastic material, the pollutant, and characteristics of the water (Koelmans et al. 2016). Sorption capacity is influenced by the properties of the microplastic material itself (e.g. size, polymer type, shape) and of the pollutants (e.g., physicochemical characteristics, hydrophobicity, Teuten et al. 2009). Fast sorption is expected for pollutants like hydrophobic organic pollutants (HOC) and for small microplastics (Endo et al. 2013, Koelmans et al. 2016). Sorption equilibrium within 48 hours for BPA to PA particles is relatively fast. The equilibrium partition coefficient indicates similar sorption characteristics, like sorption of phenanthrene to polyvinylchloride in seawater with equilibrium sorption within 24 hours (Teuten et al. 2007). Log $K_{PA,w}$ of 3.22 corresponds to a log $K_{ow}$ of BPA of 3.4 measured in an earlier study (Staples et al. 1998), indicating that hydrophilicity of BPA is a good estimate for sorption capacity of BPA to PA particles. The partition coefficient ($10^3$ L $kg^{-1}$) is within the range of HOC sorption to microplastics in seawater ($10^2$–$10^7$ L $kg^{-1}$) as indicated by Lee et al. (2014). Competitive sorption by other pollutants and leaching of additives influence sorption behaviour as well, but were not analysed in this study (Bakir et al. 2012).

### 3.4.2 Exposure experiments with mixtures of BPA and PA particles

Besides sorption behaviour, also organism dependent factors need to be considered for the potential vector effect of microplastics, i.e., uptake of microplastics, as well as conditions and processes within the organism. Two possible uptake pathways for BPA were included in the experiments: direct uptake by BPA dissolved in water, and vector-based uptake by ingestion of PA particles loaded with BPA. Microplastics tested in most studies were the only uptake pathway for the pollutants (Rochman et al. 2013). Other media, e.g., water, prey and detritus, which were shown to also hold a fraction of the pollutants, have not been included (Koelmans et al. 2016). Since daphnids are organisms living in the water column, an important uptake pathway of nutrients, but also pollutants, is water. This is why water was selected as an additional uptake pathway for BPA in the simplified exposure scenario of this study. Non-suspended microplastics (e.g., aggregated at the water surface or settled) were discussed to reduce interactions of test organisms, leading to reduced effects of microplastic associated pollutants (Khan et al. 2015). In this study, grazing by daphnids on settled PA particles from the bottom of the test beakers was observed. We consider high ingestion rates of microplastics by the daphnids, because intestines were observed to be filled up with PA particles within the first 24 hours until the end of the test. Grazing with high uptake rates ensured availability of PA particles, which could then potentially act as vector for BPA. Daphnids were also able to egest PA

particles. Quantification of exact ingestion and egestion rates was beyond the focus of this study, but would allow getting a deeper understanding of the processes within the daphnids. The scope of furthermore refined studies could be to analyse, e.g., if the time span of the microplastic particles within the daphnids is related to sorption and desorption kinetics.

Immobilisation of daphnids was analysed as an experimental endpoint to directly determine the influence of microplastics on pollutant toxicity. The pairwise comparison of immobilisation rates with the same nominal concentration of BPA directly compares scenarios with the same overall mass of BPA without taking the distribution of BPA into account. The increase of immobilisation rates in a dose-dependent manner for nominal concentrations follows the known acute toxicity pattern for BPA. The same dose-dependent pattern for BPA, in combination with microplastics but with overall lower immobilisation rates, shows that the presence of PA particles reduced immobilisation in daphnids.

Water seems to be the most bioavailable fraction, as hypothesized. Analytical measurements showed that decreased immobilisation for BPA in combination with microplastics was associated with lower BPA concentrations in water compared to BPA alone. Sorption of BPA to PA particles led to lower actual concentrations of BPA in water, already during preparation of test solutions. Sorption of PCB to microplastics (PE, 10–180 µm) with dilution of PCB in water was shown to compensate possible vector effects, contributing to bioaccumulation of PCB in lugworms (Besseling et al. 2017b). That sorption of phenanthrene to microplastics (unplasticized polyvinyl chloride, 200–250 µm) can lead to reduced effect rates of the compound was shown by biomarker activity in zebra fish larvae (Sleight et al. 2017). Vector-based uptake by ingestion was excluded, because larvae did not develop mouthparts yet. Larvae interacted with microplastics only by dermal contact. Even though PA particles loaded with a fraction of the pollutant were ingested by the daphnids, this vector-based uptake of the pollutant does not seem to compensate reduced uptake from water. The overall effect of the pollutant seems to depend mainly on the amount of dissolved pollutant in water, if sorption equilibrium is assumed.

While comparing the same nominal concentrations of BPA clearly showed a reduction of immobilisation in the presence of PA particles, it cannot be excluded that a fraction of BPA causing immobilisation was associated to PA particles. How much the single fractions (water, microplastics) contributed to the overall effect of BPA can be addressed by comparing $EC_{50}$ values. $EC_{50}$ values were calculated with immobilisation rates and actual concentrations of BPA measured by HPLC in water. If only BPA dissolved in water is determining the rate of immobilisation, $EC_{50}$ values of BPA alone, and BPA in combination with microplastics, can be expected to be in the same range. A lower $EC_{50}$ for BPA in combination with microplastics would indicate higher sensitivity of daphnids to BPA if PA particles are present. Although the $EC_{50}$ for BPA in combination with

microplastics is lower than for BPA alone, the overlapping confidence intervals indicate no significant difference. Thus, we consider the contribution of PA particles as a source for BPA to the overall immobilisation rate to be negligibly small.

Tissue concentrations of pollutants were measured in most studies to analyse the potential vector effect of microplastics. While we did not measure internal conditions, including tissue concentrations, calculations based on physiochemistry give an indication for the overall uptake of BPA. Calculations on the mass distribution of BPA indicate that not only less BPA is partitioning into water, but also, less BPA is distributed into the daphnids. Lower calculated body burden with BPA corresponds to observed lower immobilisation rates. In adult zebra fish tissue, concentrations of silver (Ag) were reduced, if Ag could sorb to microplastics during 96 hours incubation prior to exposure (Khan et al. 2015). In another study, whole body concentrations of phenanthrene in daphnids were not different between treatments with microplastics or phenanthrene alone (Ma et al. 2016). Only nanoplastics enhanced phenanthrene uptake in this study, which stresses the bigger vector potential of plastics below micro scale.

Different factors have been discussed to influence desorption of pollutants within organisms between ingestion and egestion of microplastics, i.e., pre-exposure with pollutants, biological conditions, and processes. The concentration gradient in this study was not influenced by pre-experimental BPA burden in daphnids, because the daphnids have not been exposed to BPA before exposure. When organisms have already accumulated pollutants in their body, remobilization of the pollutant from microplastics can be expected to be lower because of smaller concentration gradients. Ingestion of relatively clean microplastics is discussed to reduce pollutant burden in organisms, if partition coefficients are higher for the plastic material (Koelmans et al. 2013). Three week exposure of lobsters with microplastics loaded with PCBs and incorporated in food had no effect on PCB concentrations in tail tissue (Devriese et al. 2017). The lobsters had been pre-exposed to PCBs prior to experiments in the environment, which resulted in a smaller concentration gradient compared to clean organisms. After a depuration phase of one week with ingestion of clean microplastics, PCB concentrations were the same, indicating no cleaning effect of microplastics.

Based on experimental evidence, physiological conditions in the gut, like gut surfactants, pH, and temperature, were discussed to enhance remobilization of absorbed pollutants on microplastics (Bakir et al. 2014). Faster desorption rates were found only for warm-blooded organisms. Model-based studies on marine organisms hypothesize that desorption of organic pollutants from microplastics is negligible, even if physiological factors are included (Bakir et al. 2016). Interactions between organismal tissue and ingested microplastics loaded with pollutants also depend on gut passage time. Higher remobilization rates of pollutants from microplastics can be expected for longer gut passage times. Gut passage time in daphnids for food particles is relatively short, with egestion within minutes (Murtaugh 1985). Thus, remobilization from loaded

microplastics which pass through the digestive system might be limited. Smaller microplastics which are able to pass tissue or even cell barriers, might be of more importance for acting as vectors, while bigger microplastics can be egested more easily (Ma et al. 2016). In addition to microplastics in the intestinal tract of daphnids, translocation of PA particles within the body, like observed for 1 µm microplastics (Rosenkranz et al. 2009), cannot be excluded.

Besides tissue concentration, also the location of a pollutant within the body and depuration was shown to be influenced by microplastics. A bigger proportion of Ag was found to be located in the intestines in the presence of microplastics in zebra fish, although overall Ag concentration was lower compared to exposure without microplastics (Khan et al. 2015). Gut content was not separated from organismal tissue for analysis. This is why the higher proportion could be due to microplastics still carrying Ag, rather than higher concentrations of Ag in organismal tissue.

Similar to the results of this study, the role of microplastics as vector seemed small as soon as other uptake pathways than microplastics were included in recent studies. In a sediment-living marine worm, PCB uptake from microplastics was lower than from sediment (Beckingham and Ghosh 2017). Gut solubilisation potential was relatively low compared to natural material, i.e., wood and biochar, indicating the limited role of microplastics in pollutant transfer. In marine mussels, a mixture of fluoranthene (Flu), microplastics (PS, mix of 2 and 6 µm), and food algae did not change the concentration of Flu in digestive glands after seven days, compared to Flu and algae without microplastics (Paul-Pont et al. 2016). By incubating Flu with microplastics and food algae prior to exposure, different uptake pathways were included in this study (water, microplastics, algae). A fraction of Flu which was held by algae was transferred to microplastics during incubation, due to the higher partition coefficient. During a seven day period without any exposure, depuration was lower if mussels had been receiving mixtures, including microplastics, beforehand. Negative effects on detoxification and impairment of the filter activity were discussed as reasons. Also, remaining microplastics loaded with Flu could not be excluded. While concentrations of a pollutant within the organism indicate uptake associated to microplastics, it is necessary to also analyse specific effects of the pollutant on the organism. Even if pollutants desorb from microplastics, a negative effect only manifests if the pollutant reaches the target tissue. Tissue concentrations of a pollutant, especially the whole body burden, do not necessarily reflect the extent of a net pollutant effect. Studies including effects of pollutants, e.g., toxicity, can help to identify the actual influence of microplastics on organisms. Interestingly, toxic effects of Flu on mussels were enhanced for treatments including microplastics, although concentrations of Flu in tissue were not different (Paul-Pont et al. 2016). More histopathological damage and higher activity of antioxidant markers were found.

Besides studies showing low evidence for microplastics as carriers for pollutants, there are also reports about enhanced pollutant body burden and negative effects after ingestion of loaded microplastic (Karami et al. 2016, Rochman et al. 2013, Wardrop et al. 2016). Different conclusions have been made about the role of microplastics as vector, because of different outcomes of experimental studies. Experimental approaches in these studies differed a lot, but re-evaluation including equilibrium sorption showed that most studies indicate no, or only low relevance for microplastics as pollutant vector (Koelmans et al. 2016). In a recent study, model and experimental approaches were combined to analyse the vector effect of microplastics (PE) for PCB on marine lugworms (Besseling et al. 2017b). Uptake fluxes from all exposure pathways were quantified to comply with environmental relevant exposure conditions. Experimental and model approaches both go along with the general results of our study, that the role of microplastics as vector for organic pollutants is small.

## 3.5 Conclusions

The exposure scenario in this study addressed selected requirements for an environmentally relevant exposure, i.e., sorption equilibrium and water, as an additional uptake pathway in addition to microplastics. All BPA concentrations used in this experiment greatly exceed concentrations of BPA detected in rivers and lakes, e.g., with a maximum of 16 ng $L^{-1}$ in WWTP effluent (Kuch and Ballschmiter 2001). The concentration of the PA particles is also above expected values in freshwater environments (Faure et al. 2015, Imhof et al. 2016). Nevertheless, high concentrations of BPA and PA particles, exposure of clean daphnids, and high uptake rates of PA particles, created a scenario in favour of high sensitivity to detect the potential vector effect of PA particles in general. Supporting model-based studies, a vector effect as shown in other experimental studies only plays a minor role when experiments are carried out under more environmentally relevant conditions, and under the assumption of sorption equilibrium (e.g. Koelmans et al. 2016). These findings help to systematically identify how freshwater organisms are harmed by pollution of chemicals and microplastics, and support a more mechanism-based risk assessment. Further experimental studies could analyse, e.g., how higher sorption capacity of microplastics and other additional uptake pathways might influence a potential vector effect of microplastics, and if other organisms respond in a similar way. Natural particulate matter seems to make a big proportion of particles in aquatic systems. Thus, analysing their potential vector function in comparison to microplastics could help to set the role of microplastics as pollutant vectors into perspective.

# 4 Effects of microplastics and an endocrine disruptor on amphibians

*This chapter is based on joint work with:*

*Christiane Zarfl, Center for Applied Geosciences, Eberhard Karls Universität Tübingen*
*Werner Kloas, Leibniz-Institute of Freshwater Ecology and Inland Fisheries; Department of Endocrinology, Institute of Biology, Humboldt-Universität Berlin*
*Andrea Ziková, Leibniz-Institute of Freshwater Ecology and Inland Fisheries*
*Wibke Kleiner, Leibniz-Institute of Freshwater Ecology and Inland Fisheries*
*Nadine Poßnien, Leibniz-Institute of Freshwater Ecology and Inland Fisheries*
*Antje Tillack, Leibniz-Institute of Freshwater Ecology and Inland Fisheries*
*Angela Krüger, Leibniz-Institute of Freshwater Ecology and Inland Fisheries*
*Claudia Theel, Leibniz-Institute of Freshwater Ecology and Inland Fisheries*

*The content of this chapter was neither published nor submitted as a manuscript yet.*

*Individual contributions:*

***Saskia Rehse**, Christiane Zarfl and Werner Kloas designed the study with the support of Andrea Ziková and Wibke Kleiner; **Saskia Rehse**, Werner Kloas, Andrea Ziková, Wibke Kleiner and Nadine Poßnien performed experiments and sampling; **Saskia Rehse** and Wibke Kleiner performed laboratory analysis with the support of Nadine Poßnien; **Saskia Rehse**, Antje Tillack und Nadine Poßnien produced histological sections; Angela Krüger and Claudia Theel performed analytical measurements; **Saskia Rehse** analysed the data; **Saskia Rehse** wrote the text and discussed its content with Christiane Zarfl and Werner Kloas.*

## 4.1 Introduction

Aquatic and terrestrial ecosystems are ubiquitously polluted with plastics in general and microplastics (< 5 mm, Moore 2008) in particular (e.g. Auta et al. 2017, Horton et al. 2017 as an overview). Improved methodological approaches in the last years led to a more detailed picture of microplastic pollution, although exact quantities are still challenging to analyse in environmental matrices, especially for small microplastics in the range of only a few micrometres and below (Imhof et al. 2016, Vandermeersch et al. 2015). Rivers were identified as one important emission source of plastic waste into the ocean with 1.15-2.41 million tonnes per year on a global scale (Lebreton et al. 2017). This highlights the need to get a better understanding of microplastic pollution and its effects on wildlife in freshwater systems besides marine systems.

Concerns about potential effects of microplastics in the environment have been addressed in numerous studies with focus on mainly marine but also freshwater species (e.g. Anbumani and Kakkar 2018). Studies on the effects of microplastics on terrestrial species are still scarce (de Souza Machado et al. 2018, e.g. Huerta Lwanga et al. 2016). Some organisms spend only parts of their lifetime in water or move between terrestrial and aquatic systems. Amphibians spend the time of their early development obligatory in water, before some of them extend their mobility towards terrestrial areas. During their early development, amphibians are particularly sensitive to stressors like chemical pollutants (e.g. Kloas et al. 2009). Exposure to chemical pollutants such as endocrine disruptors during this critical time was shown to harm amphibians in the environment (e.g. Hayes et al. 2002). Endocrine disruptors are hormonal active substances, which can be of natural and anthropogenic origin (e.g. Kloas 2002). Humic substances, for instance, are released from plant material by degradation and have been shown to act estrogenic in frogs (Lutz et al. 2005a). Pharmaceuticals with endocrine effects are released via wastewater effluents and can alter physiological processes within aquatic organisms already at very low concentrations (e.g. Jones et al. 2001). Some chemicals associated to the production of plastics, e.g. additives, can be expected to have hormonal effects in wildlife as well (Teuten et al. 2009).

In frogs, endocrine disruptors with estrogenic effects lead to feminisation with genotypic males having phenotypic female sex characteristics, i.e. characteristics of ovaries or mixed gonads with sections characteristic for ovaries and testes, also at environmental relevant concentrations (Bögi et al. 2002, Hu et al. 2008, Pettersson and Berg 2007). These mixed or sex reversed ovaries were shown to be persistent in adults resulting in altered sex ratios in populations (Pettersson et al. 2006). The oral contraceptive 17-beta-ethinylestradiol (EE2) can be detected in surface waters, especially close to wastewater discharge sites and acts estrogenic (e.g. Belfroid et al. 1999, Ternes et al. 1999). Amphibians share similar hormonal systems with other vertebrates including humans (Kloas et al. 2009). The natural estrogen 17-beta-estradiol (E2) is the same in all vertebrates, which is the reason why results of (anti)estrogenic effects of endocrine disrupting substances can be

transferred from lower to higher vertebrates and *vice versa*. The African clawed frog (*Xenopus laevis*), an anuran species, is an established model organism for endocrine disruption in vertebrates including reproduction *in vitro* and *in vivo* (Bögi et al. 2002, Kloas et al. 1999, Kloas 2002, Lutz et al. 2005b). High plasticity during early development, when metamorphosis and sexual differentiation take place, characterises *X. laevis* as sensitive model organism (Kloas et al. 2009). The gonads of *X. laevis* are bipotential. Their differentiation into ovaries or testes is regulated by genotypic sex determination and the action of endogenous steroids. The hypothalamic-pituitary-gonadal (HPG) axis is the endocrine system in anurans responsible for sexual differentiation in larvae and reproduction maintenance in adults (Kloas and Lutz 2010). The HPG axis can be regulated physiologically by steroid hormones, which influence the secretion of gonadotropins via feedback mechanisms. This is why pollutants mimicking steroid hormones, i.e. endocrine disruptors, have the potential to alter processes on the HPG axis. Endocrine disruptors are assumed to contribute to the worldwide decline of amphibian populations (Carey and Bryant 1995). Some underlying processes for this decline, such as loss and overutilization of habitats and pollutants, have been identified, but the processes being responsible for nearly 50% of all declining amphibian species are not clear yet (Stuart et al. 2004). It needs to be clarified, if microplastics contribute as additional stressor to the rapidly declining populations in the environment.

In principle, microplastics can have adverse effects by the pristine microplastic material itself (physical effects) and by chemicals associated to microplastics (chemical effects). Chemical effects can either be based on chemicals leaching from the microplastic material, i.e. additives, or on chemicals in their surroundings interacting with microplastics, i.e. sorption and desorption of these chemicals (Teuten et al. 2009). Organisms which misinterpret microplastics as food ingest microplastics (e.g. fish, Lusher et al. 2013), others take up microplastics from the water column unspecifically (e.g. daphnids, Rehse et al. 2016). Filter feeders like tadpoles are prone to microplastics in the water column likewise. The uptake of microplastic particles in the range of a few micrometres (1 and 10 µm) was observed in *X. laevis* tadpoles within one hour (Hu et al. 2016). The particles were present in gills and intestines of the tadpoles and were egested again. Potential adverse effects of the microplastic material were not analysed in this study. In another study, body growth and swimming activity of *X. laevis* larvae in an early life stage were not affected by the exposure to microplastics in an laboratory setup in petri dishes without feeding (de Felice et al. 2018). Uptake of pristine microplastic material at high concentrations has been shown to induce stress and cause inflammation in aquatic vertebrates like fish (Lu et al. 2016, Rochman et al. 2013). In principle, microplastics can act as vector for sorbed chemicals to organisms, especially if they are ingested (e.g. Rochman et al. 2013). However, as soon as additional uptake pathways for the chemical pollutants are included, microplastics seem to play only a minor role for pollutant uptake compared to e.g. water (e.g. Koelmans et al. 2016, Rehse et al. 2018). An influence of microplastics on the effects of chemical pollutants can be expected most likely within

environmental scenarios with high concentrations of microplastics and chemicals. Effects of microplastics described in the literature are heterogeneous not only for physical, but also for chemical ones, such as an influence of microplastics on pollutants. Microplastics were shown to enhance effects of chemical pollutants, however, also decreased or unchanged responses to chemical pollutants in the presence of microplastics were described (e.g. Karami et al. 2016, Oliveira et al. 2013). Microplastics seem to have a selective influence on the toxicity of chemical pollutants. The mixture of phenanthrene and microplastics only affected some biomarkers in catfish in the study by Karami et al. (2016). Even within one experiment, some endpoints can show increased biomarker responses, while others indicate decreased effects, when chemical pollutants are presented together with microplastics compared to chemical pollutants alone (Oliveira et al. 2013). Mortality induced by pyrene was shown to be delayed for several hours if presented together with PE particles. At the same time, the mixture of pyrene and microplastics decreased $NADP^+$-dependent isocitrate dehydrogenase activity, which is likely to have adverse impacts on energy production.

The aim of this study was to get a better mechanistic understanding of potential effects of microplastics on amphibians. Both, effects of the mere microplastic material itself and the influence of microplastic on the effects of an environmental pollutant, i.e. endocrine disruptor, were studied in tadpoles of *X. laevis*. The exposure scenario was designed in order to analyse in principle, whether microplastics can harm amphibians in their early development. High concentrations of both, microplastics and the chemical pollutant, were chosen to achieve basic knowledge about potential effects, rather than representing a scenario likely encountered in the environment. The systematically laboratory approach was based on established criteria to identify adverse effects including general and more specific endpoints. The general development, stress hormones and histological analysis of the heads including gills and intestines of tadpoles were chosen as endpoints for physical effects of the microplastic material itself. Sexual development and the mRNA expression of biomarkers in the brain, liver and gonads were analysed in order to identify an influence of microplastics on specific effects of an endocrine disruptor. Polyamide (PA) particles in the µm-range and in the shape of fragments and EE2 were chosen as model compounds because of their environmental relevance. Irregular shaped microplastics, i.e. fragments, and small microplastics in the µm-range were found to be one of the most abundant microplastic types in the freshwater systems (Faure et al. 2015, Free et al. 2014, Imhof et al. 2016). PA is one of the most detected polymer types in the environment, e.g. in beach sediments of lakes. Tadpoles were exposed to a low and a high concentration of PA particles alone and in combination with one concentration of EE2 (more details are given in the method section). This is the first study about physical and chemical effects of microplastics on amphibians from an early larval stage until completion of sexual differentiation, but some general hypothesis can be proposed. First, if PA particles induce adverse impacts on tadpoles, the high concentration of PA particles is expected to cause more pronounced effects, i.e. altered general development, stress

hormone levels and histological changes, than the low concentration. Second, specific effects of EE2, i.e. sexual development and biomarker expression, are expected to be modified rather by the high concentration of PA particles, than the low concentration. Modulation of the effects of EE2 can most likely be expected for the most sensitive biomarkers, i.e. sexual differentiation and expression of vitellogenin mRNA. The results of this study serve as first basis to identify potential impacts of microplastics on amphibians and help to estimate the potential risk microplastics may pose to amphibians in the environment. Results on effects of the microplastic material itself, which have been addressed only rarely in amphibians in earlier studies (de Felice et al. 2018), were obtained after short- and long-term exposure. In the light of declining amphibian populations in the environment it is crucial to not only analyse the effects of new emerging contaminants such as microplastics themselves, but also to obtain an understanding of mixed effects of these contaminants with already known stressors such as endocrine disruptors. To the best of our knowledge this study addressed for the first time how effects of a chemical pollutant, i.e. endocrine disruptor, can be modulated by microplastics in amphibians. Finally, the obtained results are also relevant in a broader context, i.e. for vertebrates in general, due of the high similarity of their endocrine systems.

## 4.2 Methods

### 4.2.1 Animals

The animals were derived from a healthy laboratory stock of the Leibniz-Institute of Freshwater Ecology and Inland Fisheries (IGB), Berlin, Germany. Human chorionic gonadotropin (Sigma, Deisenhofen, Germany) was injected to adult frogs to induce spawning (Kloas et al. 1999). Larvae were grown in aerated tanks with deionized water containing 0.25 g $L^{-1}$ commercial sea salt ("Tropic Marin Meersalz", Tagis, Dreieich, Germany) as holding water with a photoperiod of 12 hours light and 12 hours dark. As soon as the larvae reached the free-swimming stage they were fed *ad libitum* three times a day with commercial food (Sera Micron, Heinsberg, Germany).

### 4.2.2 Model microplastic material and endocrine disruptor

PA powder was chosen as model microplastic material and purchased from Goodfellow (Goodfellow GmbH, Bad Nauheim, Germany). The particles had a mean diameter of 15-20 µm with an overall range of a minimum of five to a maximum of 50 µm and an irregular shape. The density of the PA material is 1.13 g $cm^{-3}$ (Nylon 6, AM306010). EE2 (≥ 98%, CAS number 57-63-6) was purchased from Sigma Aldrich (Sigma-Aldrich Chemie GmbH, Munich).

### 4.2.3 Experimental setup

Individuals of *X. laevis* were exposed semi-static to PA particles (microplastics, MP) and EE2 for seven days (short-term) and 21 days (long-term). A total of 420 tadpoles were separated and randomly distributed into 14 aquaria (10 L total water volume) with 30 animals per aquarium seven days prior to exposure to acclimatize to experimental conditions. The aquaria were aerated with a constant airflow through glass pipettes under the same conditions as described before (holding water, photoperiod). Water temperature (22 ± 0.5 °C) was regulated by setting the aquaria up in channels filled with water heated with a heating rod. Exposure experiments started as soon as the tadpoles reached developmental stage 51 according to Nieuwkoop and Faber (1958), at which the hind limbs of the tadpoles have a regular conical shape with some melanophores.

Tadpoles were exposed to seven different treatments with two aquaria per treatment (n=30 per aquarium, n=60 per treatment). Microplastic specific effects were studied by exposing the tadpoles to two different concentrations of PA particles alone (1 mg $L^{-1}$ and 100 mg $L^{-1}$) and in combination with one concentration of EE2 ($10^{-8}$ M ≙ 2.96 µg $L^{-1}$). PA particles were presented alone to analyse the effects of the microplastic material itself, while the combination of the PA particles was presented to analyse the influence of microplastics on the specific effects of EE2. The seven treatments consisted of (1) the low concentration of PA particles (MP low) and (2) the high concentration of PA particles (MP high), (3) one concentration of EE2 ($10^{-8}$ M) alone as pollutant control (EE2), (4) EE2 combined with the low concentration of PA particles (MP low, EE2) and (5) EE2 combined with the high concentration of PA particles (MP high, EE2), (6) PA particles loaded with EE2 prior to exposure in an EE2 solution with the same concentration of EE2 as for EE2 alone (MP loaded) and (7) a solvent control treatment (control). All procedures were reviewed and approved by the German State Office of Health and Social Affairs (LaGeSo, Berlin Germany; Reg 0013/016).

### 4.2.4 Preparation of treatment specific conditions

PA particles were weighed into glass flasks (10 mg for MP low and 1 g for MP high) and mixed with an equivalent of the holding water from the associated aquaria that were intended for the treatments with microplastics (MP low; MP high; MP low, EE2; MP high, EE2), thoroughly mixed, sonicated for one minute and added to the aquaria. Flasks were rinsed three times with holding water to assure full transfer of particles. A stock solution with EE2 (296 mg $L^{-1}$) was prepared with dimethyl sulfoxide (DMSO) to ensure that the stock solution remained stable. 100 µl of the stock solution were pipetted into the aquaria for treatments that included EE2 (EE2; MP low, EE2; MP high, EE2). For treatments that included both, EE2 and PA particles (MP low, EE2; MP high, EE2), EE2 was added after adding the PA particles. For the treatment with pre-loaded PA particles (MP loaded), 10 mg of PA particles were added to glass bottles (0.5 L total volume) together with 200 ml of holding water and 2 µl of EE2 stock solution resulting in the same initial aqueous

concentration of EE2 during pre-loading of particles ($10^{-8}$ M or 2.96 µg $L^{-1}$) as directly applied for the other treatments including EE2 (EE2; MP low, EE2; MP high, EE2). After 24 hours of shaking at 250 rpm, mixtures stood still for two hours to allow the particles to settle down at the bottom of the bottles. Subsequently, the EE2 solution was decanted carefully to an overall volume of 10 ml remaining solution including the loaded PA particles. The decanted part was fully transferred to the aquaria and rinsed three times with holding water to assure full transfer. The control treatment contained only holding water and 100 µl DMSO, which resulted in the corresponding concentration of DMSO in treatments with EE2 (10 µl $L^{-1}$).

Aeration of aquaria led to a fast equal distribution of PA particles, EE2 and the solvent in the water column. Three times a week the water was changed entirely in all aquaria, which were then cleaned carefully and refilled according to the different treatments. Animals were fed three times a day *ad libitum* with Sera Micron. After seven days of exposure, ten out of thirty individuals were separated from each aquarium (n=10 per aquarium, n=20 per treatment). Twenty tadpoles remained for long-term exposure per aquarium (n=20 per aquarium, n=40 per treatment) for sampling after 21 days.

### 4.2.5 Water parameters

The temperature, pH and oxygen content in each aquarium were measured before every water change with a probe (HQ40D Digital Multimeter Kit, Hach Lange GmbH, Düsseldorf, Germany). Nitrate, nitrite and ammonium contents in each aquarium were checked twice, in the first (day 4) and the third week of exposure (day 18) with LCK Cuvette Tests (Hach Lange GmbH, Düsseldorf, Germany).

### 4.2.6 Measured EE2 concentrations

Concentrations of EE2 in water were checked exemplarily twice during exposure, in the first (day 4) and in the third week of exposure (day 18). Water was sampled from all treatments including EE2 (EE2; MP low, EE2; MP high, EE2; MP loaded) directly (0 hour), 3 hours, 6 hours and 18 hours after changing the water. 500 ml of water per aquarium were filled into glass beakers. During a resting phase of one hour, the PA particles settled at the bottom of the beakers. About 400 ml of the water were carefully decanted into glass bottles (2 L total volume) without the PA particles and stored at -20 °C until further processing. The water was filtered, before EE2 was extracted from 350 ml of water by solid-phase extraction (Thermo Scientific Dionex AutoTrace 280, Thermo Fisher Scientific, Berlin, Germany) equipped with silica-based cartridges (Dionex SolEx 0.5 g C18 silica-based SPE cartridges, Thermo Fisher Scientific, Berlin, Germany). EE2 concentrations were measured with a Q-TOF LC/MS system (Agilent 6550 I funnel Q-TOF LC/MS ESI negative, Agilent, Waldbronn, Germany) equipped with a Zorbax

Eclipse separation column (Zorbax Eclipse XDB-C18 3.0x100mmx 1.8 micron, Agilent, Waldbronn, Germany).

### 4.2.7 Partition coefficient and mass balance of EE2

The partition coefficient for sorption of EE2 to PA particles was calculated based on results of a study on the removal of EE2 from water by sorption to different polyamides including polyamide 6 (PA6) particles in a similar size range (20 µm in diameter, Han et al. 2012).

Assuming equilibrium, the partition coefficient defined as (Schwarzenbach et al. 2016):

$$K_{pa,w} = \frac{c_{pa}}{c_w}$$

was calculated with values from Han et al. (2012) including $c_{pa}$ as equilibrium concentration of EE2 sorbed to PA particles (in mg $kg^{-1}$), and $c_w$ as equilibrium concentration of EE2 in water after 24 hours measured by Han et al. (230 µg $L^{-1}$). $c_{pa}$ was calculated from the EE2 mass balance as follows:

$$c_{pa} = \frac{m_{total} - c_w * V_w}{M_{pa}}$$

with $m_{total}$ as total mass (in mg) of EE2 in the system, $V_w$ as volume of water (in L), and $M_{pa}$ as mass of PA6 particles (in kg).

The partition coefficient based on Han et al. (2012) was used for the following calculations. Apart from the partition coefficient, all other values were taken from the present study. The concentration of EE2 in water after 24 hours in the batches in the present study for the treatment with loaded PA particles (PA loaded) prior to exposure of tadpoles was calculated with

$$c_w = \frac{m_{total}/V_w}{1 + K_{pa,w} * M_{pa}/V_w}$$

The mass of EE2 sorbed to PA particles (10 mg) in each batch was calculated with

$$m_{pa} = m_{total} - m_w$$

The theoretical concentrations of EE2 in water for all batches with EE2 (EE2; MP low, EE2; MP high, EE2), except for the loaded PA particles (MP loaded) at day seven and 18 of exposure were calculated based on the mass balance of EE2. For EE2 alone the concentration in water was calculated as follows

$$c_w = \frac{m_{total}}{V_w + BCF * M_{org}}$$

and for the treatments with the low and the high concentration of PA particles (PA low, EE2; PA high, EE2)

$$c_w = \frac{m_{total}}{V_w + BCF * M_{org} + K_{pa,w} * M_{pa}}$$

with $m_{total}$ as total mass (in mg) of EE2 in the system, $V_w$ as volume of water in the aquaria (in L), the bioconcentration factor (BCF), i.e. the partition coefficient between the organic phase (tadpoles) and water (in L $kg^{-1}$), $K_{pa,w}$ as partition coefficient between PA particles and water (in L $kg^{-1}$) as calculated above (3043 L $kg^{-1}$), and $M_{pa}$ as mass of PA particles (in kg; 1 mg $L^{-1}$ for PA low and 100 mg $L^{-1}$ for PA high). The BCF of EE2 for tadpoles was calculated according to Veith et al. (1980) based on $K_{o,w}$ (4677; Yamamoto and Liljestrand 2004) leading to 362.4 L $kg^{-1}$. $M_{org}$ as mass of the tadpoles measured during sampling after seven and 21 days (means per treatment).

### 4.2.8 Survival and general fitness

In general, all tadpoles were visually checked daily for dead animals or any abnormal behaviour, e.g. swimming movements and food consumption. Handling during water changes allowed checking all single individuals in particular.

### 4.2.9 Sampling and analysed endpoints

Various general and specific endpoints were analysed in order to identify effects of microplastics alone and their influence on the effects of EE2 on tadpoles of *X. laevis*. The general development of all individuals was assessed by analysing growth indicated by morphological parameters and developmental stage (Nieuwkoop and Faber 1958) after seven and 21 days. Histological sections of the head of two individuals per aquarium (n=4 per treatment) including the intestines after seven days and of four individuals (n=8 per treatment) after 21 days including only the intestines were prepared. This method aimed at analysing if particles might induce morphological changes and damages in the tissues and organs that were in direct contact with PA particles (e.g. gills and the intestinal epithelium). The amount of the stress hormones corticosterone and aldosterone was assessed for four individuals per aquarium (n=8 per treatment) after seven and 21 days according to Ziková et al. (2013). EE2 specific endpoints after 21 days were included to analyse how the presence of the PA particles influenced specific effects of EE2 as endocrine disruptor. Sexual differentiation was assessed by gross-morphology and histology for all individuals. Genotypic sex of all individuals was determined by DNA analysis of tail tissues. Gene expression of hypophyseal gonadotropins, i.e. luteinizing hormone (LHbeta, LH), follicle stimulating hormone (FSHbeta, FSH), aromatase (Aro), gonadal enzymes for steroidogenesis, i.e. steroidogenic acute regulatory protein (StAR), cytochrome P450 (P450scc, P450), steroid 5-alpha reductase type 1 (Srd5a1; S1), steroid

5-alpha reductase type 2 (Srd5a2; S2) and hepatic vitellogenin (Vit) expression was analysed in eight individuals per aquarium (n=16 per treatment).

### 4.2.10 General development

During sampling after seven and 21 days all individuals were weighed. Total body length, snout-vent length after 21 days and in addition hind limb length after seven days were measured under a binocular. Nieuwkoop and Faber (1958) defined 66 clearly differentiated developmental stages (NF) for *X. laevis* beginning with the fertilized egg until complete metamorphosis (NF 66). The scheme allows quantifying the general development. Sexual differentiation of gonads can be assessed from NF 56 on, when testes and ovaries can be already morphologically distinguished. The developmental stage of each individual after seven and 21 days was assessed according to schemes of Nieuwkoop and Faber.

### 4.2.11 Histology of heads and guts

Whole heads including the gills together with the guts of tadpoles after seven days and only guts of tadpoles after 21 days were put in formalin (Carl Roth GmbH + Co. KG, Karlsruhe, Germany) for fixation of the tissues immediately after removal from the tadpoles and stored until further processing. The samples were dehydrated with an automatic tissue processor (Shandon ExcelsiorTM ES, Thermo Fisher Scientific, Berlin, Germany) by rinsing the samples first in a graded series of ethanol (ethanol > 99.8%, denatured, Carl Roth GmbH + Co. KG, Karlsruhe, Germany) once each with 75%, 90%, twice with 95% and twice with 99% ethanol at room temperature for 60 minutes each. Samples were embedded in Technovit 7100 according to the manufacturer's instructions (Kulzer GmbH, Wehrheim, Germany). Technovit 7100 is a resin based on 2-hydroxyethyl methacrylate. It was used, because its solid, hard structure was expected to retain microplastic particles, which could potentially be present in the samples, better than paraffin during slicing. All reagents and material used for Technovit embedding are listed in Appendix Table 4.

In the first step, the samples were pre-infiltrated in a mixture of liquid Technovit 7100 with 96% absolute ethanol (1:1; Carl Roth GmbH + Co. KG, Karlsruhe, Germany) for two hours. For infiltration, 1 g of hardener I was mixed with 100 ml base liquid for ten minutes. All samples rested in the mixture overnight to allow infiltration into the tissues. After infiltration, heads and guts were placed separately in the moulds of the histoforms. For polymerisation, 1 ml hardener II was mixed with 15 ml of the base liquid and, depending on the size of the sample, 1-3 ml of the mixture were added to each histoform mould. Polymerisation with hardening of the substance took place within minutes (maximum of ten minutes). Histoblocs were attached to each specimen with Technovit 3040 in the last step. For this, Technovit 3040 powder was mixed quickly with the base

liquid (2:1) with a glass rod. After placing a histobloc on each mould, the mix was carefully poured at the back of each histobloc. The mixture hardened within ten minutes and histoblocs could carefully be removed from the moulds together with the specimens embedded in the polymer. Heads and guts were sliced in 5 µm thin sections with a Supercut 2065 microtome (Superfrost, Carl Roth GmbH + Co. KG, Karlsruhe, Germany) and placed on glass slides. The sections were stained with pre-mixtures of blueing acid ethanol containing hydrochloric acid (1% HCl, Carl Roth GmbH + Co. KG, Karlsruhe, Germany) and ethanol (70%, Carl Roth GmbH + Co. KG, Karlsruhe, Germany) as well as with hematoxylin (hematoxylin solution according to Harris, ready to use; Carl Roth GmbH + Co. KG, Karlsruhe, Germany) and eosin (concentrate for microscopy, Merck, Darmstadt, Germany; all pre-mixtures listed in Appendix Table 5). In addition, ethanol (Ethanol > 99.8%, denatured, Carl Roth GmbH + Co. KG, Karlsruhe, Germany) and xylol (98%, for histology, Carl Roth GmbH + Co. KG, Karlsruhe, Germany) were used. All single steps for staining are listed in Table 4. Sections were examined under an Axiovert 200 microscope (Zeiss, Berlin, Germany) equipped with a Show View II digital camera (Olympus, Hamburg, Germany).

*Table 4* *Staining procedure of head and gut sections*
Single steps applied for head and gut sections with all reagents and incubation times.

| **Reagent** | **Incubation time** |
|---|---|
| hematoxylin | 25 min |
| running tap water | 5 min |
| blueing acid ethanol | dipping 3 times |
| distilled water | dipping 5 times |
| 0.1% eosin solution | 15 min |
| 96% EtOH | dipping 5 times |
| 100% EtOH | dipping 5 times |
| xylol | dipping 2 times |

### 4.2.12 Stress hormone levels

Corticosteroids influence osmotic and metabolic changes and the action of thyroid hormones during larval development of amphibians (Hanke and Leist 1971, Kaltenbach 1958). Aldosterone and corticosterone can be used as an indicator for stress in amphibians and were already measured with the same method, i.e. ELISA kits, in another study in whole tissues of tadpoles (Ziková et al. 2013). Stress hormones were determined with enzyme immunoassays for the quantitative determination of corticosterone and aldosterone (Corticosterone ELISA kit and Aldosterone ELISA kit, IBL International GmbH, Hamburg, Germany), which is based on competitive binding on solid phase enzyme-linked immunosorbent assays (ELISA). For the extraction of the steroids whole

animals were homogenized in glass vials with 1 ml distilled water. Six ml of diethyl ether were added to each vial, which was shaken for one hour on a horizontal shaker. The vials rested in a deep freezer at -80 °C for 30 minutes to separate the water phase from the organic phase holding the steroids. In the next step, the liquid organic phase was carefully decanted into new glass vials, while the frozen water phase rested at the bottom of the vials. The extraction procedure was repeated two more times with the water phase. The vials with the extracts were placed on a heating panel with 30 °C to evaporate the organic solvent. The vials were stored at -20 °C until analysis of the steroids. Steroids were resolved in 1 ml ethanol (5%) and processed according to the manufacturer's instructions of the immunoassay kits (all reagents in Appendix Table 6 and Appendix Table 7). All steps were done at room temperature.

For corticosterone, 20 µl of each standard, control and samples were pipetted into the microtiter wells from the kit. 200 µl enzyme conjugate were added to each well, the plates shaken for ten seconds and incubated for one hour. The content of the wells was quickly shaken out and wells rinsed three times with 400 µl wash solution. 100 µl substrate solution were added to each well, which were then incubated for 15 minutes. The enzymatic reaction was stopped by adding 50 µl stop solution to the wells. The optical density (OD) at 450 +/- 10 nm was measured with a microplate reader (infinite® M200, Tecan, Crailsheim, Germany) within ten minutes. For aldosterone, 50 µl of each standard, control and sample were added into the wells of the microtiter plate. 150 µl of enzyme conjugate were added to each well, which were subsequently mixed for ten seconds and incubated for 60 minutes. After shaking out the content of the wells, wells were washed five times with 400 µl wash buffer. 200 µl of TMB substrate solution were pipetted to each well followed by 30 minutes incubation. The reaction was stopped by adding 100 µl TMB stop solution to each well and OD measured as before at 450 nm within ten minutes.

### 4.2.13 Sexual development

#### 4.2.13.1 Gross-morphology and histology of gonads

The gonad-kidney complex of each animal after long-term exposure (21 days) was examined gross-morphologically during preparation of the organs. Gonad-kidney complexes of all animals (with only half of the complex for animals of which biomarkers were analysed) were fixed in Bouin's fluid (Carl Roth GmbH + Co. KG, Karlsruhe, Germany), dehydrated, embedded in paraffin, sliced in thin sections and stained to evaluate the morphological sex of the tadpoles. Dehydration was done with an automatic tissue processor (Shandon ExcelsiorTM ES, Thermo Fisher Scientific, Berlin, Germany) by rinsing the samples first in a graded series of ethanol (Ethanol > 99.8%, denatured, Carl Roth GmbH + Co. KG, Karlsruhe, Germany), once with 75% and 90% Ethanol each, twice with 95% and 99% Ethanol each at room temperature for 60 minutes each. In the second step, samples were rinsed three times with xylene (98%, for histology; Carl Roth GmbH + Co. KG, Karlsruhe, Germany) at room temperature for 60 minutes each. In the last step,

the samples were treated with paraffin (Paraplast, Carl Roth GmbH + Co. KG, Karlsruhe, Germany) at 60 °C for 80 minutes. Finally, each gonad-kidney complex was placed in embedding moulds with melted paraffin and cooled down to solidify the paraffin. The gonads were sliced in 5 µm thin sections with a Supercut 2065 microtome (Superfrost, Carl Roth GmbH + Co. KG, Karlsruhe, Germany) and placed on glass slides. The slides were incubated at 60 °C overnight to remove paraffin. Hematoxylin (hematoxylin solution according to Harris, Carl Roth GmbH + Co. KG, Karlsruhe, Germany) and eosin (concentrate for microscopy; Merck, Darmstadt, Germany; mixed as listed in Appendix Table 5 for heads and guts) were used for staining. Single steps of staining are listed in Table 5. Sections were examined under an Axiovert 200 microscope (Zeiss, Berlin, Germany) equipped with a Show View II digital camera (Olympus, Hamburg, Germany).

*Table 5* *Staining procedure of gonad-kidney sections*
Single steps applied for staining of gonad-kidney complex sections with all reagents and incubation times.

| **Reagent** | **Incubation time** |
|---|---|
| xylene (twice) | 5 min |
| 100% EtOH | 2 min |
| 96% EtOH | 2 min |
| 70% EtOH | 2 min |
| 40% EtOH | 2 min |
| distilled water | 2 min |
| hematoxylin | 2:30 min |
| running tap water | 10 min |
| eosin (0.1%) | 2:30 min |
| distilled water | short rinse |
| 80% EtOH | short rinse |
| 100% EtOH | 0:30 min |
| 100% EtOH | 2 min |
| xylene (twice) | 5 min |

#### 4.2.13.2 Genotypic sex determination

In *X. laevis* heterogametic sex chromosones (ZW for females, ZZ for males) determine the sex (Chang and Witschi 1956). DM-W is a gene associated with the sex chromosone W and crucial for the development of the ovaries (Yoshimoto et al. 2008, Yoshimoto et al. 2010). The gene DMRT1 (doublesex and mab-3 related transcription factor 1) is associated to the Z chromosone and crucial for the development of the testicles in males. DM-W and DMRT1 are exclusively expressed during sexual differentiation. According to the presence of sex chromosones, DM-W is only expressed in females, while DMRT1

is expressed in both sexes. DM-W and DMRT1 are transcription factors with nearly identical DNA binding domains. This is why both can bind to the same DNA sequence. In contrast to DMRT1, DM-W lacks a transactivation domain. If DM-W binds to the DNA, the transcription is not activated. Binding of DM-W blocks the binding of DMRT1 to the same DNA sequence, which means that DM-W hinders the transcription of genes essential for testicle development. To sum up, the suppression of the transcription of male sex specific genes leads to the development of ovaries. To analyse the genetic sex of tadpoles, DMRT1 and DM-W were analysed as in Tamschick et al. (2016). First, DNA from the tail tip was extracted. Sex specific DNA fragments in the samples were then amplified in a polymerase chain reaction (PCR), separated via gel electrophoresis and evaluated according to their fluorescence pattern.

#### 4.2.13.3 Extraction of DNA from tail tissue

The peqGOLD Tissue DNA Mini Kit (PEQLAB Biotechnologie GmbH, Erlangen, Germany; all components listed in Appendix Table 8) was used for the DNA extraction from the tail tissue. This method combines the selective and reversible binding properties of a silica-based matrix with the micro centrifugation technique. All reagents used are from the kit and all steps were done at room temperature according to the manufacturer's instructions. In a first step, DNA was homogenized and lysed. Columns were loaded with the lysate where DNA bound to the silica membrane. In two washing steps, proteins and other contaminations were removed. Finally, the DNA was eluted in PCR water and stored until further use.

Lysis of DNA was done by mixing 400 µl DNA lysis buffer T, 20 µl proteinase K and 15 µl RNase A (20 mg $ml^{-1}$) and vortexing for 10 sec. The samples were incubated at 50 °C for three hours with continuously shaking on a thermo shaker and centrifuged for five minutes at 15.000 rpm at room temperature to pellet unsolvable debris. The supernatant was carefully transferred into a 1.5 ml vial and mixed with 200 µl DNA binding buffer and 400 µl DNA lysis buffer T. The PerfectBind DNA columns were loaded with the mixture including precipitates with 2 ml collection tube attached to the column and centrifuged at 10.000 rpm for two minutes. The column discharge was discarded. Columns were washed by pipetting 650 µl of the DNA wash buffer (buffer concentrate plus 1.5 volume absolute ethanol) followed by centrifugation of the columns at 10.000 x g for 1 minute. Again, the discharge was discarded and washing repeated once again. After attaching a 2 ml collection tube, the columns were centrifuged at 12.00 rpm for four minutes to dry. 100 µl PCR water were pipetted on each column, columns incubated for three minutes and centrifuged at 10.00 rpm for one minute to elute the extracted DNA. This step was repeated once.

#### 4.2.13.4 PCR of sex specific DNA sequences

To identify sex specific DNA fragments (DMRT1 and DM-W) the extracted DNA was diluted to 10 ng $µl^{-1}$. 2 µl diluted DNA of each sample was mixed with 22.4 µl of the

reaction mixture shown in Appendix Table 9 and 0.2 µl of each primer (25 µM stock; Table 9). PCR was performed with specific primers for DMRT1 and DM-W (Table 6) taken from Tamschick et al. (2016) and under the conditions shown in Table 7.

*Table 6* *Primers for sexing*
Primer sequences for sex specific DNA fragments used for PCR including forward and reverse sequences, NCBI accession numbers and product sizes.

| Gene | forward (F) and reverse (R) primer (5'→3') | NCBI accession | Product size (bp) |
|---|---|---|---|
| DM-W | F: CCACACCCAGCTCATGTAAAG | AB365520 | 260 |
| | R: GGGCAGAGTCACATATACTG | | |
| DMRT1 | F: AACAGGAGCCCAATTCTGAG | AB259777 | 206 |
| | R: AACTGCTTGACCTCTAATGC | | |

*Table 7* *PCR program for sexing*
PCR conditions for amplification of sex specific DNA fragments including cycle number, temperature and time for each step.

| Cycles | Temperature (°C) | Time | Process |
|---|---|---|---|
| 1 | 95 | 4 min | initial denaturation, polymerase activation |
| 40 | 95 | 1 min | incubation, |
| 40 | 60 | 1 min | annealing of sex specific primers |
| 40 | 72 | 1 min | elongation |
| 1 | 72 | 10 min | incubation |

#### 4.2.13.5 Gel electrophoresis of PCR products

The PCR products were separated subsequently by gel electrophoresis in an agarose gel including ethidium bromide. The agarose gel was prepared with 1.35 g agarose (Agarose GTQ for DNA/RNA electrophoresis, Roth, Karlsruhe, Germany) in 80 1*TAE buffer heated up for three minutes at 600 W in a microwave (Appendix Table 10; all reagents from Carl Roth GmbH + Co. KG, Karlsruhe, Germany). Subsequently the gel was chilled for ten minutes down to 50 °C before adding 9 µl ethidium bromide. The gel solidified in a special mould (Agagel, Biometra, Göttingen, Germany), which was then placed in an electrophorese chamber (Biometra, Göttingen, Germany).

3 µl amplified DNA of each sample were pipetted together with 1 µl bromophenol blue into gel pockets. A standard with a specific molecular weight (DNA standard, 100-1000 bp length, Sigma, Deisenhofen, Germany) was analysed in parallel to simplify the identification of the size of the PCR products. Separation of the products occurred at 70 mV during 45 minutes. The evaluation of the gels was done with the gel documentation system Gel Doc XR (Bio-Rad, Munich, Germany). In principle, fluorescence of ethidium

bromide bound between base pairs makes the DNA fragments visible. Female tadpoles show two bands for both genes at 206 pb for DMRT1 and 260 bp for DM-W, while male tadpoles only show a band at 206 bp for DMRT1.

### 4.2.14 Genexpression of biomarkers

The HPG axis is organized as a cascade of different mechanisms including different organs for synthesis and as target for related products like hormones (Kloas and Lutz 2010, schematic of the HPG axis included in this publication). The central nervous system (CNS), especially the hypothalamus and the pituitary, the gonads and the liver are organs related to the HPG axis (Kloas and Lutz 2010, Norris and Carr 2013). Endogenous and exogenous stimuli trigger specialized cells in the hypothalamus, which orchestrates the synthesis and secretion of gonadotropin releasing hormones (GnRHs, Zohar et al. 2010). Excreted GnRHs from the hypothalamus are transported via portal veins to the pituitary and stimulate the synthesis and release of gonadotropins, i.e. luteinizing hormone (LH) and follicle stimulating hormone (FSH), in the pituitary. LH and FSH are released into the blood circulation and, in turn, stimulate the release of gonadal sex steroids, i.e. androgens and estrogens. The step-wise biosynthesis of sex steroids is done with the help of specialized enzymes, which can be found mainly in the gonads, but also in the CNS. Cholesterol is the starting molecule for all steroids (Urbatzka et al. 2007a). The steroidogenic acute regulatory protein (StAR) and the side chain cleavage enzyme (P450) are important factors for steroidogenesis. In the first step, StAR mediates the transport of cholesterol from the outer into the inner mitochondrial membrane. P450, which is located at the inner mitochondrial membrane, converts cholesterol into pregnenolone subsequently (Arukwe 2008). Testosterone (T) is synthesized from progesterone in the next step and can be either processed into 17 beta-estradiol (E2) by the enzyme aromatase (Aro, Urbatzka et al. 2007a) or into dihydrotestosterone (DHT) by the enzyme steroid-5-alpha-reductase (srd5a1 and srd5a2). In *X. laevis*, E2 is the most potent estrogen and DHT the most potent androgen (Bögi et al. 2002, Kloas 2002). DHT and E2 influence the differentiation of the gonads into testes or ovaries, i.e. sexual differentiation, as well as spermatogenesis and oogenesis, i.e. maintenance of sexual functions. DHT and E2 have opposite effects, while DHT acts masculinizing, E2 acts feminizing. However, DHT and E2 can be found in both sexes. Aromatase activity, which is crucial for the synthesis of E2, is much lower in testes than in ovaries. In females, high activity of aromatase in the ovaries leads to about 20-fold higher E2 levels, compared to males. Relatively high DHT levels are present in females as well, but finally the ratio of DHT and E2 is crucial for their specific effects on sexual differentiation. For the transport of sex steroids to target organs, i.e. gonads, liver and the CNS, via the blood, sex steroids bind to specific sex steroid-binding proteins. When located in the target tissue, androgens and estrogens bind to cytosolic receptors, which mediate their biological actions. After binding of the sex steroids to cytosolic receptors, the sex steroid-receptor-complex translocates into the mitochondrial nucleus, where it binds to specific gene regions and initiates sex steroid-dependent gene

expression. Finally, synthesized androgens and estrogens cause a negative feedback for the production of the gonadotropins LH and FSH, which leads to a downregulation of the step-wise cascade of steroidogenesis (Urbatzka et al. 2006). The liver is one of the most important target organs for E2, besides the gonads and the CNS (Nimrod and Benson 1996). In egg-laying vertebrates, the synthesis of vitellogenin in the liver is induced by E2 (Perlman et al. 1984). Vitellogenin, the yolk promoting protein, is an established biomarker for exposure with estrogens (Kloas et al. 1999, Palmer and Palmer 1995). Sex steroids also reach peripheral target tissues via blood circulation and are crucial for important physiological functions, e.g. functions of the immune system (Grossman 1985). The activity of steroidogenic enzymes can provide information about the effects of exogenous substances, i.e. endocrine disruptors, on reproductive biology including sexual differentiation. The mRNA expression of the steroidogenic enzymes StAR, P450, Aro, srd5a1 and srd5a2 can act as biomarkers for endocrine disruption of reproductive biology in vertebrates. Gene expression of biomarkers analysed by real-time PCR is a highly sensitive method to detect endocrine disruption such as estrogenic activity of EE2 and other pollutants (Kloas et al. 1999). Information about expressed target genes, especially semi-quantitative results, is a powerful tool to monitor response of organisms already on a molecular level, even though mRNA expression does not necessarily reflect the responses on the protein level.

For analysis of mRNA expression, as measure for transcription of the genes, eight randomized chosen animals were sacrificed with Tricaine methanesulfonate (MS-222). A part of the brain containing the pituitary and diencephalon, the gonad-kidney complex and the liver were dissected, immediately frozen in liquid nitrogen and stored at -80 °C until further processing. Gene expression via mRNA of the luteinizing hormone (LHbeta) and the follicle stimulating hormone (FSHbeta) from the pituitary and cytochrome P450 (P450scc), aromatase (Aro), steroidogenic acute regulatory protein (StAR), steroid 5-alpha reductase type 1 (Srd5a1; S1) and the steroid 5-alpha reductase type 2 (Srd5a2; S2) from the gonad-kidney complex and vitellogenin from the liver were analysed to characterize sexual differentiation and specific effects of EE2. In order to determine mRNA expression, mRNA needed to be first reversely transcribed into robust complementary DNA (cDNA). The reverse transcriptase (RT), a RNA dependent DNA polymerase, acted as catalyst for reverse transcription. The cDNA synthesis occurred only based on RNA by using a poly-dT-primer in a reverse transcription polymerase chain reaction (RT-PCR). After transcription of mRNA in cDNA, the cDNA was amplified using real-time PCR. In addition to producing millions of copies of a sequence as in PCRs in general, real-time PCRs also quantify the amount of DNA copies, which can be used to predict the activity of the target gene.

#### 4.2.14.1 Extraction of RNA from pituitaries, gonads and livers

Total mRNA from the brains including the pituitary was extracted with the RNeasy-Plus-Micro-Kit (Qiagen, Hilden, Germany; all components are listed in Appendix Table 11),

which includes the removal of DNA, according to the protocol of the manufacturer. To sum up, the tissue was lysed and homogenized in a buffer, which inactivated RNases. The lysate was added to a spin column (gDNA Eliminator spin column) in which DNA was removed. In the next step, ethanol was added to enhance binding of RNA before adding the lysate to another spin column (RNeasy MinElute spin column). Next, the RNA was washed with washing buffer and ethanol and eluted in RNase-free water.

In detail, the tissue was lysed and homogenized by adding 350 µl RLT buffer followed by two times homogenisation in a Tissue Lyser (Qiagen, Retsch, Hilden, Germany) at 18 rpm for two minutes. The samples were centrifuged for three minutes at 12.000 rpm at room temperature (Biofuge Fresco, Heraeus, Hanau, Germany). The supernatant was added to a gDNA eliminator column, which was centrifuged at 10.000 rpm for 15 seconds. 350 µl 70% ethanol were mixed with the supernatant in a new vial and 700 µl of the suspension added into an RNeasy MiniElute column, which ass centrifuged for 15 seconds at 10.000 rpm. The column was washed with wash buffer in three steps, twice with RW1 buffer (first with 700 µl, second with 500 µl) and once with 500 µl RPE buffer, each with centrifuging the column at 10.000 rpm for 15 seconds after adding the buffer. In a final washing step, the columns were washed with 500 µl 80% ethanol and centrifuged at 10.000 rpm for two minutes. After discarding the eluate, the columns were centrifuged with open caps for five minutes at 12.000 rpm to remove all ethanol. RNA was eluted in 14 µl Rnase free water with incubation for one minute, followed by centrifugation for one minute at 12.000 rpm. The eluate was added to the column again, followed by one minute incubation and centrifugation for one minute at 12.000 rpm.

Total RNA from gonads was extracted using the ReliaPrep™ RNA Tissue Miniprep System (Promega, Mannheim, Germany; all components are listed in Appendix Table 12). The extraction procedure was performed according to the manufacturer's instructions and includes DNase treatment to reduce DNA contamination. In principle, tissue was homogenized in lysis buffer and RNA precipitated by adding isopropanol. Specific washing buffers (RNA Wash Solution) were used to wash RNA retained at the silica membrane of spin column. DNase digestion was included in the stepwise protocol. RNA was re-dissolved in RNase-free water. Single steps of the extraction are described in the next paragraph.

In detail, samples were homogenized in a Tissue Lyser (Qiagen Retsch, Hilden, Germany) after adding 250 µl LBA buffer mixed with 1-Thioglycerol. The homogenates were cleared by centrifugation for three minutes at 14.000 x g and transferred to clean vials. 85 µl isopropanol were added and samples vortexed for five seconds. Each lysate was transferred to a mini column in a collection tube and centrifuged at 12.000-14.000 x g for 30 seconds. The discharge in the collection tube was discarded. DNase I treatment was prepared by gently mixing 24 µl yellow core buffer, 3 µl $MnCl_2$ and 3 µl DNase I per sample. 30 µl of this mix was applied to every mini column and incubated for 15 minutes at 20-25 °C. 200 µl column wash solution mixed with ethanol were added in the next step,

followed by centrifugation at 12.000-14.000 x g for 15 seconds. Columns were washed twice with RNA wash solution, first with 500 µl followed by centrifugation at 12.000-14.000 x g for 30 seconds, second with 300 µl and centrifugation at full speed for two minutes. Subsequently the mini columns were placed into an elution tube and 15 µl nuclease free water added to each column. Finally, the columns were centrifuged at 12.000-14.000 x g for one minute to release the RNA extracts into the elution tubes.

Total RNA from the livers was extracted with TRIzol (Thermo Fisher Scientific, Berlin, Germany) according to the manufacturer's instructions. In principle, this procedure included the following steps. The samples were homogenized in TRIzol. By adding chloroform to the aqueous phase containing the RNA, the RNA was separated from the organic phase containing DNA and proteins. The RNA was precipitated in the aqueous phase with isopropanol and washed before being re-suspended in RNase-free water. Details are given in the next paragraph.

First, the livers were homogenized in 700 µl TRIzol with a TissueLyser for 1:30 minutes at 18 rpm (Qiagen Retsch, Hilden, Germany) and centrifuged at 12.000 g for ten minutes at 4 °C. 650 µl of the supernatant were mixed with 150 µl of fresh TRIzol in a new vial. 160 µl chloroform (Roth, Karlsruhe, Germany) were added and the vials vortexed for 15 seconds followed by ten minutes incubation at room temperature and centrifugation at 12.000 g for 15 minutes at 4 °C. During this step, the aqueous phase containing the RNA was separated from the organic phase containing DNA and proteins. 300 µl of the aqueous phase with the RNA were mixed with 300 µl isopropanol (Roth, Karlsruhe, Germany) to precipitate the RNA in the aqueous phase. The samples were incubated first for ten minutes at room temperature, second for one hour at -20 °C and third for ten minutes at room temperature. The incubated samples were centrifuged at 12.000 g for twelve minutes at 4 °C, the supernatant discarded, the pellet with the RNA washed by adding 300 µl ice cold 70% ethanol and centrifuging at 12.00 g for six minutes at 4 °C. Again, the supernatant was discarded, while the RNA in the pellet was dried in a vacuum centrifuge for three minutes and finally re-suspended in 40 µl RNAse free water (Thermo Fisher Scientific, Berlin, Germany). Possible contamination with genomic DNA was removed from RNA samples from livers with DNase I (Amplification Grade, Invitrogen, Thermo Fisher Scientific, Berlin, Germany; reagent listed in Appendix Table 13) at room temperature. A working solution with 1 µl reaction buffer and 1 µl DNase was added to 1 µg total RNA in a 10 µl reaction. DNase I was immediately inactivated by adding 1 µl EDTA solution to the mixture, followed by incubation at 65 °C for ten minutes.

RNA extracts from the liver were diluted to 125 ng $\mu l^{-1}$, to 4 ng $\mu l^{-1}$ from the brain and to 0.125 ng $\mu l^{-1}$ from gonads with RNase-free water and stored at -80°C until further processing.

#### 4.2.14.2 RNA concentrations and RIN value

RNA concentrations were determined in replicates by measuring UV absorbance at 230 nm, 260 nm and 280 nm using a Nano-Drop ND-100 spectrophotometer (Thermo Fisher Scientific, Berlin, Germany). The RNA integrity number (RIN) was assessed for a part of the samples with the Bioanalyzer 2100 (Agilent, Waldbronn, Germany) according to the manufacturer's protocol to determine RNA quality. The RIN value is calculated by the Bioanalyzer based on the proportion of ribosomal 18S and 28S rRNA. RIN values between five and eight are considered as good, values higher than eight as high quality. RIN values of all samples from all sampled tissues indicate good to high quality of RNA (7.6-9.1 for RNA from brain, 8.5-9.7 for RNA from liver, 8.3-9.7 for RNA from gonads).

#### 4.2.14.3 cDNA synthesis by reverse transcription

Preparation of samples and cDNA synthesis was done on ice. cDNA was synthesized by reverse transcription with a thermal cycler (Biometra, Göttingen, Germany) with different protocols for samples from pituitaries, gonads and livers. Reagents used for the protocols are summed up in Appendix Table 14. After cDNA synthesis, all samples were stored at -20 °C until further processing. In addition to samples from the tadpole experiments, control RT-PCR samples without reverse transcriptase were included in the cDNA synthesis.

Two pre-mixtures were prepared for cDNA synthesis of pituitary RNA from tadpoles (Appendix Table 15). In the first step, 1.5 µl of Premix I were added to 12.5 µl RNA from the brain (4 ng $\mu l^{-1}$). Mixtures were incubated in the thermocycler for five minutes at 65 °C for primer annealing and subsequently cooled down slowly at room temperature for ten minutes. In the second step, 6 µl of Premix II were added and incubated for 60 minutes at 42 °C in the thermocycler. To inactivate the transcriptase (RT) the samples were heated up for 15 minutes at 70 °C.

9 µL RNA solution from gonads (0.125 ng $\mu l^{-1}$) were incubated with 1 µL oligo(dT) primer (1:10) for two minutes at 65°C and samples cooled down on ice. In the next step, 4.5 µL PCR water, 2 µL MMLV- reaction buffer, 2 µl DTT, 1 µL dNTPs and 0.5 µL MMLV - RT were added for cDNA synthesis. The mixture was incubated for 60 minutes at 37 °C, before being heated up for five minutes to 85 °C to end the reaction.

For primer annealing 8 µL RNA solution from the livers (0.125 ng $\mu l^{-1}$) were incubated with 1 µL PCR water and 1 µL oligo(dT)primer at 65°C for 2 minutes and samples chilled on ice afterwards. In a second reaction, cDNA synthesis was performed by adding a reaction mix with 4.5 µl RNase/DNase free water, 2 µL MMLV- reaction buffer, 2 µL DTT, 1 µL dNTPs and 1 µL MMLV- RT. The resulting mixture was incubated at 37°C for 60 min, before the reaction was terminated by heating for 5 minutes to 85°C and immediately cooled down to 10°C.

#### 4.2.14.4 Real-time polymerase chain reaction for biomarkers

The cDNA sequences specific for the analysed biomarker genes were amplified with real-time polymerase chain reaction (PCR). The PCR conditions, e.g. the temperature for annealing of gene specific primers, were optimized for all PCRs in different pre-experiments to maximize cDNA yields. The efficiency of all used primers was analysed with a dilution series of the cDNA with six different dilution steps. Efficiency was between 98-113% for all primers. All preparation steps were performed on ice. Amplification was done in a Mx3005P qPCR cycler (Stratagene, Agilent, Waldbronn, Germany). All biomarker specific primers used for PCR were taken from previous publications (Kloas et al. 1999, Urbatzka et al. 2009) and are listed in Table 8. All reagents, pre-mixtures and the thermal programs for PCRs of pituitary, gonad and liver samples are listed in Appendix Table 16 to Appendix Table 19 as well as Table 9. cDNA from the different organs was diluted in different proportions with RNase-free water (1:5 for pituitaries and gonads, 1:15 for livers). 2 µl of each diluted cDNA sample were pipetted into 96 well plates (Agilent), before adding 18 µl of PCR premix according to the specific gene. All samples were amplified by PCR in duplicates. For the control samples, which were included earlier in cDNA synthesis without adding reverse transcriptase, no amplification should take place later during real-time PCR. Samples in which reverse transcriptase was replaced by PCR water were analysed in addition to confirm the absence of genomic contamination. Specificity of target gene cDNA amplification was checked by adding controls, in which the cDNA was replaced with PCR water. A calibrator sample containing pooled cDNA was run in triplicate and PCR efficiency of the target and the normalizing gene assay corrected to determine relative quantities of target transcripts. The efficiency for the target gene assays ranged between 86% and 100%. The expression of the housekeeping gene elongation factor 1a (EF-1a) was analysed in parallel as reference to normalize target gene expression according to Pfaffl (2001).

*Table 8* *Primers for all analysed biomarkers*
Primer sequences of the elongation factor 1- α and all analysed biomarkers including forward and reverse sequences, NCBI accession numbers and product sizes.

| Gene | Forward (F) and reverse (R) primer (5'→3') | NCBI accession | Product size (bp) |
|---|---|---|---|
| Elongation factor 1- α chain (EF-1α) | F: ACCGCACAGGTTATCATC<br>R: CAACAATGGCAGCATCTC | M25504 | 286 |
| Aromatase (Aro) | F:CGGTTCCATATCGTTACTTCC<br>R:GCATCTTCCTCTCAATGTCTG | AB031278 | 140 |
| P450 side chain cleavage enzyme (P450) | F: CAGTGTTGGCCAGGATTTTGT<br>R: GCGGAAGAGCTCATTGGTCAG | XM_002934562 | 97 |
| steroid 5-α reductase, type 1 (Srd5a1, S1) | F: CTGAACCTCTTGGCTATG<br>R: GATGCCTAACTCGGATTG | NM_001098696 | 201 |
| steroid 5-a reductase, type 2 (Srd5a2; S2) | F: CTTATCCTGCTGCTTATG<br>R: AGTCCTTTGGAAATAGTG | NM_001017113 | 203 |
| steroidogenic acute regulatory protein (StAR) | F: AACCCAAATGTCAAGGAAGTCAAG<br>R: ACAAAATCCCGGGCCCCTACAATA | AF220437 | 113 |
| Follicle stimulating hormone (FSH) | F: TGCTCGTTCTGTGTTGGAAGATG<br>R: CCTGTTTGATGAGTGGATGCTTTG | AB175888 | 171 |
| Luteinizing hormone beta (LH) | F: CACTGACGCTTCTGGGGTTCTAC<br>R: GATTGGGCAGTCGTCTTTCTCT | AF360397 | 101 |
| Vitellogenin (Vit) | F: CGGCTATATCAAACTTTTTGGC<br>R: GTTTTCTTGTTGAAATGGAGGC | Y00354 | |

*Table 9* *PCR program for gonad, pituitary and liver samples*
Conditions for amplification of cDNA from gonad, pituitary and liver samples in real-time PCR including cycle number, temperature and time for each step.

| Cycles | Temperature (°C) | Time (gonad, pituitary) | Time (liver) | Process |
|---|---|---|---|---|
| 1 | 95 | 7.40 min | 3 min | initial denaturation, polymerase activation |
| 40 | 95 | 17 sec | 10 sec | denaturation cDNA strands |
| 40 | 62 | 25 sec | 25 sec | primer annealing |
| 40 | 72 | 25 sec | 25 sec | elongation |

### 4.2.15 Statistics

All data were checked for normal distribution by Shapiro-Wilk test, except for developmental stages according to the nature of these data. Depending on the output, ANOVAs or Kruskal-Wallis tests with corresponding post-hoc tests were run with each aquarium as single unit to pre-check, if aquaria of one treatment (two aquaria per treatment) differed significantly from each other. None of the tests revealed significant differences between the two aquaria of one treatment, which is why further analysis was done with each treatment as one unit. Treatment specific differences within one sampling were checked by ANOVAs or Kruskal-Wallis test with corresponding post-hoc tests. Morphological data and stress hormone levels were analysed for all animals per treatment, mRNA gene expression of biomarkers was analysed for male and females separately.

## 4.3 Results

### 4.3.1 Water parameters

Temperature, pH and dissolved oxygen content were stable throughout the exposure within a narrow range with means and standard deviations of 21.7 ± 0.3 °C, pH of 6.9 ± 0.2 and 6.4 ± 0.9 mg of dissolved oxygen. Total nitrogen of nitrate, nitrite and ammonium are expressed as $NO_3$-N, $NO_2$-N and $NH_4$-N respectively (Table 10). $NO_3$-N, $NO_2$-N and $NH_4$-N levels were all higher in the last week of exposure, compared to the first week. No-observed-adverse-effect levels (NOAEC) of nitrate- and ammonium-nitrogen for *X. laevis* after chronic exposure were shown to be in the range of $10^2$ mg $L^{-1}$ (Schuytema and Nebeker 1999), which is why nitrate- and ammonium-nitrogen measured in the present study can be considered to be harmless for the tadpoles. Median lethal concentrations ($LC_{50}$) of nitrite for larvae of five amphibian species were shown to range between 0.6 and 1.8 mg $L^{-1}$ $NO_2$-N, which is above measured values in the present study (Marco et al. 1999). The good general performance and fitness of the tadpoles supports the assumption that no of the measured nitrogen compounds influenced the tadpoles adversely.

*Table 10 Nitrate, nitrite and ammonium contents in holding water*
Nitrate-nitrogen ($NO_3$-N), nitrite-nitrogen ($NO_2$-N) and ammonium-nitrogen ($NH_4$-N) content during exposure of tadpoles after four and 18 days as means and standard deviations (SD) in mg $L^{-1}$.

| | $NO_3$-N (mg $L^{-1}$) | | $NO_2$-N (mg $L^{-1}$) | | $NH_4$-N (mg $L^{-1}$) | |
|---|---|---|---|---|---|---|
| **Exposure time** | **Mean** | **SD** | **Mean** | **SD** | **Mean** | **SD** |
| day 4 | 0.18 | 0.02 | 0.02 | 0.00 | 0.98 | 0.06 |
| day 18 | 0.30 | 0.08 | 0.05 | 0.01 | 1.53 | 0.51 |

### 4.3.2 Measured EE2 concentrations

Analytical measurements showed exemplarily, that EE2 concentrations in water in the first week of exposure (4 days) were similar and stable (between 0 and 18 hours) for the treatment with EE2 alone and in combination with the low concentration of PA particles (EE2; MP low, EE2; Figure 6). In contrast, the concentration of EE2 in the treatment with EE2 combined with the high concentration of PA particles (MP high, EE2) decreased about 40% after 18 hours compared to 0 hour. Only trace amounts of EE2 were detected in the water from the treatment with loaded PA particles (MP loaded), both in the first and third week of exposure (after 18 days). In the third week of exposure EE2 concentrations in all other treatments declined more quickly and already between the first three hours until 18 hours after water change. EE2 combined with the high PA particle concentration led to a lower concentration from the beginning on (0 hours).

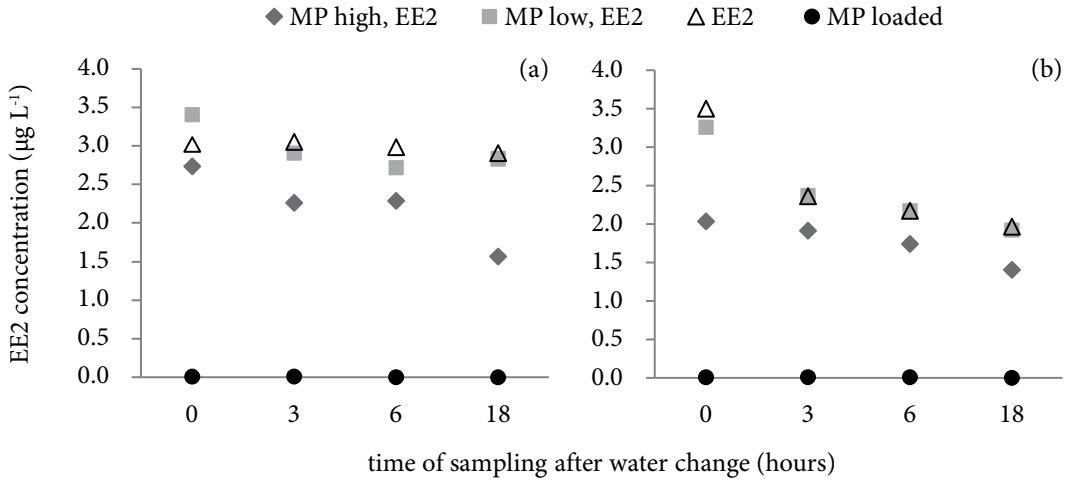



*Figure 6* *Measured aqueous EE2*
Measured concentrations of EE2 in water in the (a) first and (b) third week of exposure after 0, 3, 6 and 18 hours respectively for the treatment with EE2 in combination with the high concentration of PA particles (MP high, EE2), EE2 in combination with the low concentration of PA particles (MP low, EE2), only EE2 (EE2) and PA particles loaded with EE2 (MP loaded).

### 4.3.3 Partition coefficient and mass distribution of EE2

The calculated partition coefficient for sorption of EE2 to PA particles as the basis for all further calculations was 3043 L $kg^{-1}$. For the treatment with the pre-loaded PA particles (MP loaded) the mass of EE2 sorbed to the PA particles (10 mg per aquarium) after incubation in $10^{-8}$ M EE2 solution for 24 hours was 0.0782 µg (corresponding to 7.82 mg EE2 $kg^{-1}$ PA particles). Due to the 10 ml EE2 solution added to the aquaria in addition to the loaded PA particles, a total of 0.1039 µg of EE2 were added to 10 L water in each of the aquaria belonging to the treatment with loaded PA particles (for PA loaded). This results in a nominal concentration of 0.010 µg $L^{-1}$ for the treatment with loaded PA particles, if all EE2 would be solved in the water compared to a nominal EE2 concentration of 2.96 µg $L^{-1}$ in all other treatments including EE2 (EE2; MP low, EE2; MP

high, EE2). The nominal concentration of EE2 or the total mass of EE2 in the system for the treatment with loaded particles (MP loaded) is lowered by a factor of approximately 300 compared to all other treatments including EE2 (EE2; MP low, EE2; MP high, EE2).

The calculated concentrations of EE2 relate to the concentrations after seven and 21 days, because the mass of the tadpoles after seven and 21 days were used for the calculations (Table 11). EE2 concentrations in water within seven and 21 days of exposure are very similar for the treatment with only EE2 (EE2) and combined with the low concentration of PA particles (MP low, EE2). In the treatment with EE2 combined with the high concentration of PA particles (MP high, EE2), about 17% less EE2 is solved in water, compared to EE2 alone (EE2) or in combination with the low concentration of PA particles (MP low, EE2) after seven days. Only trace amounts of EE2 in water were calculated for the treatment with loaded PA particles (MP loaded), due to the lower total mass of EE2 added, both after seven and 21 days. After 21 days, EE2 concentrations in all treatments except for the loaded particles are around 1 µg $L^{-1}$. Calculated concentrations of EE2 in water are similar but overall lower than measured concentrations (first week of exposure: factor 1.5 for EE2 and MP low, EE2; second week of exposure: factor 2 for EE2 and factor 1.5 for MP low, EE2).

*Table 11* *Calculated aqueous EE2*
Calculated concentrations of EE2 after seven and 21 days of exposure for the treatments with EE2 alone (EE2), the low concentration of PA particles with EE2 (MP low, EE2), the high concentration of PA particles with EE2 (MP high, EE2) and PA particles loaded with EE2 (MP loaded).

| | **$c_w$ calculated (µg $L^{-1}$)** | |
|---|---|---|
| **Treatment** | **7 days** | **21 days** |
| EE2 | 1.788 | 1.042 |
| MP low, EE2 | 1.785 | 1.041 |
| MP high, EE2 | 1.510 | 0.941 |
| MP loaded | 0.006 | 0.004 |

### 4.3.4 Survival and general fitness

In total nine out of 420 individuals died within the three weeks of exposure, which represents a very low mortality rate. Four individuals died in the treatment with loaded PA particles (MP loaded), two individuals in the treatment with only EE2 (EE2) and in the treatment with EE2 in combination with the high concentration of PA particles (MP high, EE2), respectively, and one individual in the treatment with only the high concentration of PA particles (MP high). No abnormal behaviour of the tadpoles was observed in any treatment for the whole time of exposure.

## 4.3.5 General development

### 4.3.5.1 Morphological parameters

All morphological parameters of tadpoles measured after seven days (body weight, total length, snout-vent length and the hind limb length) did not differ between the treatments (Table 12). Overall means for all treatments were 0.603 +/- 0.153 g body weight, 53.0 +/- 4.7 mm total length, 20.3 +/- mm snout-vent length and 3.4 +/- 0.6 limb length.

*Table 12 Morphological parameters of tadpoles after seven days*
Body weight (g), total length, snout-vent length and limb length (all in mm) in tadpoles after seven days exposure in different treatments with two concentrations of microplastics and one concentration of EE2 alone (MP low; MP high; EE2) and in combination with each other (MP low, EE2; MP high, EE2; MP loaded) and one solvent control (control) as means +/- standard deviations (SD).

| | Body weight (g) | | Total length (mm) | | Snout-vent length (mm) | | Limb length (mm) | |
|---|---|---|---|---|---|---|---|---|
| **Treatment** | **Mean** | **SD** | **Mean** | **SD** | **Mean** | **SD** | **Mean** | **SD** |
| Control | 0.610 | 0.186 | 53.1 | 5.3 | 20.4 | 2.2 | 3.6 | 0.7 |
| MP low | 0.669 | 0.153 | 55.4 | 4.8 | 21.0 | 1.9 | 3.6 | 0.6 |
| MP high | 0.668 | 0.170 | 53.7 | 5.1 | 20.9 | 1.7 | 3.6 | 0.6 |
| EE2 | 0.563 | 0.140 | 52.0 | 4.2 | 20.0 | 1.7 | 3.4 | 0.5 |
| MP low, EE2 | 0.574 | 0.094 | 52.5 | 3.8 | 20.1 | 1.4 | 3.2 | 0.4 |
| MP high, EE2 | 0.566 | 0.116 | 51.9 | 4.2 | 19.8 | 1.6 | 3.2 | 0.4 |
| MP loaded | 0.575 | 0.142 | 52.6 | 4.8 | 19.7 | 1.7 | 3.4 | 0.7 |

After 21 days, body weight and total length were slightly higher for treatments including EE2 (EE2; MP low, EE2; MP high, EE2; MP loaded; Table 13), but not significantly different from all other treatments and the control. Only snout-vent length of two treatments including EE2 were significantly increased compared to the control (EE2; MP high, EE2; Figure 7). At the same time, the snout-vent length for the high concentration of PA particles combined with EE2 (MP high, EE2) was significantly lower than the high concentration of particles alone (MP high). Means of all treatments were 1.694 +/- 0.480 g body weight, 68.7 +/- 13.3 mm total length and 24.0 +/- 1.8 mm snout-vent length.

*Table 13 Morphological parameters of tadpoles after 21 days*

Body weight (g), total length, snout-vent length and limb length (all in mm) in tadpoles after 21 days exposure in different treatments with two concentrations of microplastics and one concentration of EE2 alone (MP low; MP high; EE2) and in combination with each other (MP low, EE2; MP high, EE2; MP loaded) and one solvent control (control) as means +/- standard deviations (SD).

| | Body weight (g) | | Total length (mm) | | Snout-vent length (mm) | |
|---|---|---|---|---|---|---|
| **Treatment** | **Mean** | **SD** | **Mean** | **SD** | **Mean** | **SD** |
| Control | 1.556 | 0.556 | 64.4 | 15.6 | 22.9 | 2.0 |
| MP low | 1.577 | 0.576 | 63.2 | 16.8 | 23.0 | 2.3 |
| MP high | 1.490 | 0.516 | 63.1 | 18.4 | 22.9 | 2.1 |
| EE2 | 1.771 | 0.354 | 74.1 | 4.4 | 24.3 | 1.3 |
| MP low, EE2 | 1.844 | 0.307 | 73.8 | 4.2 | 24.2 | 1.3 |
| MP high, EE2 | 1.805 | 0.334 | 73.6 | 5.8 | 24.4 | 1.3 |
| MP loaded | 1.825 | 0.535 | 68.4 | 12.9 | 24.0 | 1.8 |

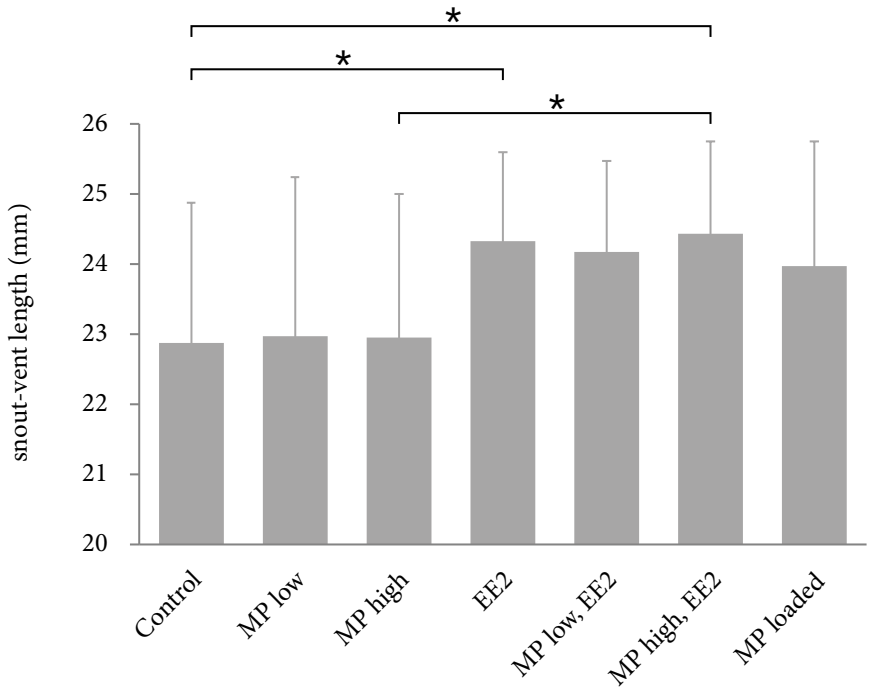



*Figure 7 Snout-vent lengths after 21 days*

Snout-vent lengths of tadpoles after 21 days of exposure in different treatments with two concentrations of microplastics and one concentration of EE2 alone (MP low; MP high; EE2) and in combination with each other (MP low, EE2; MP high, EE2; MP loaded) and one solvent control (control) as means +/- standard deviations.

#### 4.3.5.2 Developmental stages

After seven days of exposure, the developmental stages of tadpoles in all treatments were in the same narrow range between 53 and 56 (Figure 8). After 21 days, tadpoles in all treatments were in a broad range of developmental stages between 57 and 65. Treatments including EE2 (EE2; MP low, EE2; MP high, EE2; MP loaded) did only reach stage 63 and 64 with on overall lower trend except for the treatment with microplastics loaded with EE2 (MP loaded).

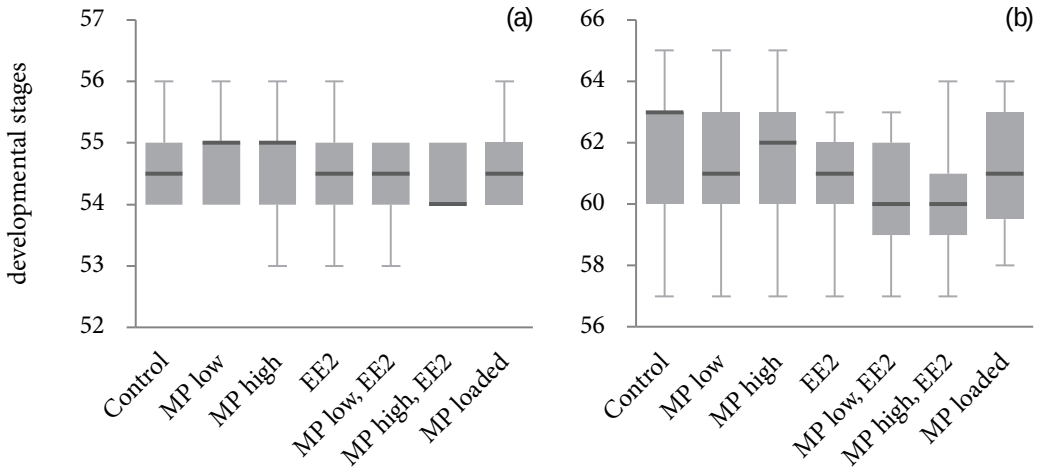



*Figure 8 Developmental stages after seven and 21 days*
Developmental stages of tadpoles after (a) seven days and (b) 21 days exposure in different treatments with two concentrations of microplastics and one concentration of EE2 alone (MP low; MP high; EE2) and in combination with each other (MP low, EE2; MP high, EE2; MP loaded) and one solvent control (control) as means +/- standard deviations as boxplots with medians (black line), 25% and 75% percentiles and the minimum and maximum values.

### 4.3.6 Histology of heads and guts

During dissection of organs after seven and 21 days intestines of tadpoles were noticed to have whitish lumps within intestinal convolutions in treatments including microplastics. Neither morphological abnormalities in the tissues nor microplastic particles could be identified in gills after seven days exposure and intestines after seven and 21 days exposure in the histological sections.

### 4.3.7 Stress hormone levels

Neither aldosterone nor corticosterone levels differed statistically significantly between the treatments in males and females after seven and 21 days (Table 14). While overall aldosterone levels were in a similar range after seven and 21 days (means for all treatments of 0.29 +/- 0.19 ng $g^{-1}$ body weight after seven days and 0.18 +/- 0.13 ng $g^{-1}$ body weight after 21 days), corticosterone levels were up to about four times elevated after seven days compared to 21 days (means for all treatments of 23.51 +/- 7.16 ng $g^{-1}$ body weight after

seven days and 6.90 +/- 3.03 ng $g^{-1}$ body weight after 21 days). Corticosterone levels after 21 days were slightly elevated for treatments with microplastics, but not statistically significant.

*Table 14 Stress hormone levels after seven and 21 days*
Aldosterone and corticosterone plasma levels (in ng $g^{-1}$ body weight) after seven and 21 days of exposure with two concentrations of microplastics and one concentration of EE2 alone (MP low; MP high; EE2) and in combination with each other (MP low, EE2; MP high, EE2; MP loaded) and one solvent control (control) as means +/- standard deviations (SD).

| | Aldosterone | | | | Corticosterone | | | |
|---|---|---|---|---|---|---|---|---|
| | 7 days | | 21 days | | 7 days | | 21 days | |
| **Treatment** | **Mean** | **SD** | **Mean** | **SD** | **Mean** | **SD** | **Mean** | **SD** |
| Control | 0.29 | 0.25 | 0.15 | 0.12 | 22.96 | 10.00 | 4.71 | 2.41 |
| MP low | 0.26 | 0.17 | 0.21 | 0.20 | 23.50 | 5.81 | 6.44 | 2.15 |
| MP high | 0.26 | 0.19 | 0.19 | 0.12 | 21.80 | 8.32 | 7.84 | 2.79 |
| EE2 | 0.26 | 0.08 | 0.15 | 0.07 | 20.64 | 8.49 | 5.97 | 3.37 |
| MP low, EE2 | 0.37 | 0.09 | 0.22 | 0.18 | 23.96 | 5.05 | 8.83 | 4.01 |
| MP high, EE2 | 0.34 | 0.35 | 0.21 | 0.11 | 25.99 | 7.46 | 8.15 | 2.97 |
| MP loaded | 0.22 | 0.11 | 0.14 | 0.04 | 25.74 | 4.57 | 6.35 | 1.90 |

### 4.3.8 Sexual development

In the control treatment slightly more animals were identified as females than as males (62.5% in females; Figure 9). All individuals identified as mixed or shift sex by histological analysis were genotypic males except for one individual in the treatment with EE2 alone (EE2) with one genotypic female having mixed sex gonads. Individuals with morphological abnormalities of gonads indicating mixed sex were exclusively found in the treatments with only EE2 and the low concentration of microplastics in combination with EE2 (EE2; MP low, EE2). Most males with a sex shift were found for the treatments with only EE2 and EE2 in combination with both concentrations of microplastics each, between 40% and nearly 60% in proportion to the overall number of males per treatment (47.1% for EE2; 40.0% for MP low, EE2; 58.8% for MP high, EE2). Only one individual for the treatments with microplastics loaded with EE2 (MP loaded) was identified as sex shift.

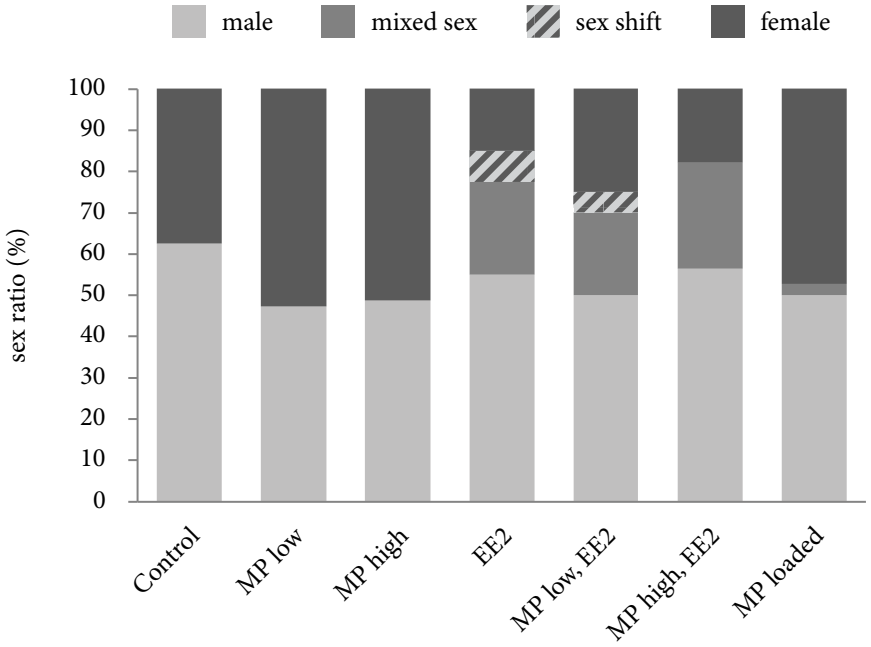



*Figure 9* *Sex ratios after 21 days*
Sex ratios (%) in all treatments after 21 days of exposure with two concentrations of microplastics and one concentration of EE2 alone (MP low; MP high; EE2) and in combination with each other (MP low, EE2; MP high, EE2; MP loaded) and one solvent control (control).

### 4.3.9 Gene expression of biomarkers

Biomarker mRNA expression was evaluated separately for males and females to include sex specific effects and only for individuals matching genotypic and histomorphological sex. Individuals that were identified as mixed sex or with reversed sex were excluded from further comparisons of treatment specific effects in biomarker expression. Gene expression of mixed and reverse sex individuals were all in the same range as clearly identified males and females, except for one genotypic female with mixed gonads for vitellogenin. More details are given in the paragraph about vitellogenin.

#### 4.3.9.1 mRNA gene expression in pituitaries

In males, LH mRNA levels were increased in treatments including EE2 (EE2; MP low, EE2; MP high, EE2) except for microplastics loaded with EE2 (MP loaded; Figure 10). Statistical significant differences compared to the control were found for EE2 alone and the low concentration of microplastics combined with EE2 (EE2; MP low, EE2). The mean LH mRNA expression in females was slightly elevated for the high concentration of MP combined with EE2 (MP high, EE2) compared to the other treatments, but also showed the highest standard deviation indicating high variability between individuals in the treatments. FSH mRNA levels in both sexes did not differ significantly between any treatment. The control treatment had highest mean levels of FSH mRNA but also high individual variability.

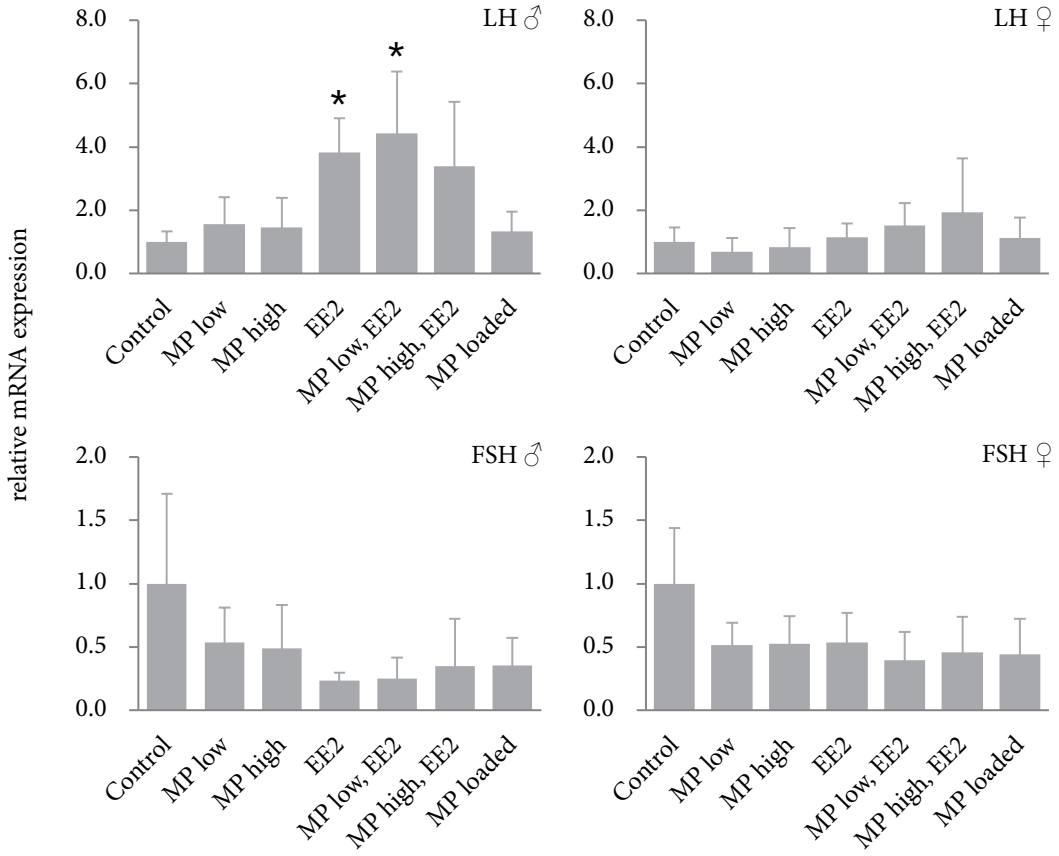



*Figure 10 mRNA expression of LH and FSH after 21 days*

LH and FSH mRNA expression in male and female tadpoles after 21 days of exposure with two concentrations of microplastics and one concentration of EE2 alone (MP low; MP high; EE2) and in combination with each other (MP low, EE2; MP high, EE2; MP loaded) and one solvent control (control) relative to the elongation factor (EF) and the control treatment as means +/- standard deviations, significant differences ($p < 0.05$) compared to the control are marked by asterisks.

#### 4.3.9.2 mRNA gene expression in gonads

StAR mRNA expression in both sexes was in a uniform range between treatments (Table 15). In males, levels of P450 mRNA were in a similar range for all treatments, while P450 mRNA levels in females were significantly lower for the treatment with EE2 alone (EE2) compared to the control (Figure 11 and Appendix Table 20). mRNA expression of aromatase was highly variable among individuals in all treatments indicated by high standard deviations, especially in males (Table 15). Aromatase mRNA levels in males were about 3-8 times elevated in the treatment with only EE2 and both concentration of MP combined with EE2 (EE2; MP low, EE2; MP high, EE2), but not significantly different among treatments. mRNA expression of S1 was in a uniform range for all treatments (Table 15). Influence of microplastics alone on biomarker activity was measured exclusively for S2 (Figure 12 and Appendix Table 20). More specifically, in males, both microplastics concentrations alone (MP low; MP high) induced significant higher mRNA levels than in the control. In females, S2 mRNA levels of microplastics alone were also

elevated, but the difference was less pronounced than in males and not statistical significant to the control.

*Table 15 mRNA expression of StAR, aromatase and S1 after 21 days*
StAR, aromatase and S1 mRNA expression in male and female tadpoles after 21 days of exposure with two concentrations of microplastics and one concentration of EE2 alone (MP low; MP high; EE2) and in combination with each other (MP low, EE2; MP high, EE2; MP loaded) and one solvent control (control) relative to the elongation factor (EF) and the control treatment as means +/- standard deviations (SD).

| | | Males | | Females | |
|---|---|---|---|---|---|
| **Biomarker** | **Treatment** | **Mean** | **SD** | **Mean** | **SD** |
| StAR | Control | 1.00 | 0.37 | 1.00 | 0.46 |
| | MP low | 1.16 | 0.40 | 0.66 | 0.18 |
| | MP high | 0.92 | 0.25 | 0.87 | 0.45 |
| | EE2 | 1.54 | 0.14 | 0.80 | 0.33 |
| | MP low, EE2 | 0.87 | 0.12 | 0.72 | 0.20 |
| | MP high, EE2 | 0.80 | 0.21 | 0.66 | 0.23 |
| | MP loaded | 1.20 | 0.41 | 0.95 | 0.30 |
| Aromatase | Control | 1.00 | 0.79 | 1.00 | 0.61 |
| | MP low | 1.11 | 0.86 | 0.70 | 0.35 |
| | MP high | 0.95 | 0.47 | 0.92 | 0.45 |
| | EE2 | 3.38 | 3.32 | 0.86 | 0.39 |
| | MP low, EE2 | 8.34 | 5.19 | 0.98 | 0.36 |
| | MP high, EE2 | 3.76 | 3.78 | 0.70 | 0.46 |
| | MP loaded | 0.80 | 0.38 | 1.20 | 0.52 |
| S1 | Control | 1.00 | 0.46 | 1.00 | 0.31 |
| | MP low | 1.42 | 0.46 | 0.96 | 0.43 |
| | MP high | 1.14 | 0.30 | 0.82 | 0.37 |
| | EE2 | 1.20 | 0.16 | 0.61 | 0.16 |
| | MP low, EE2 | 1.23 | 0.54 | 0.76 | 0.14 |
| | MP high, EE2 | 1.07 | 0.18 | 1.05 | 0.34 |
| | MP loaded | 1.14 | 0.40 | 0.87 | 0.24 |

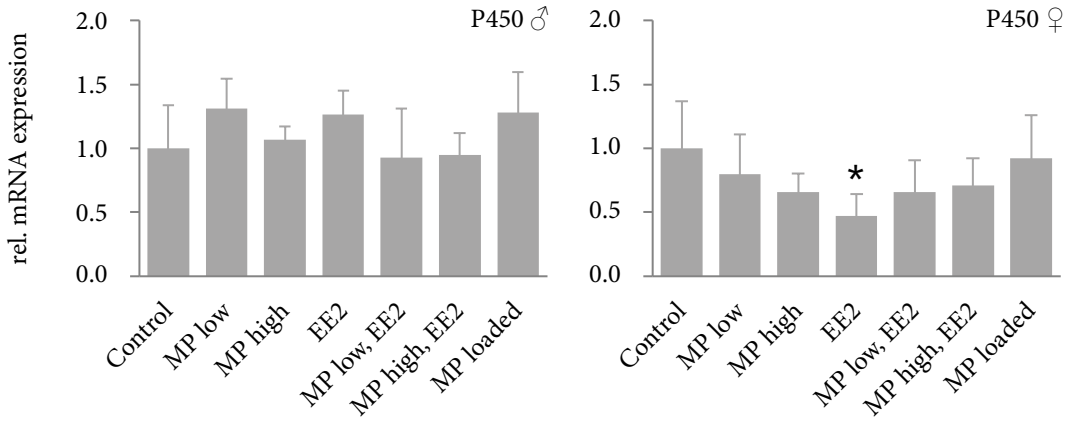



*Figure 11 mRNA expression of P450 after 21 days*

P450 mRNA expression in male and female tadpoles after 21 days of exposure with two concentrations of microplastics and one concentration of EE2 alone (MP low; MP high; EE2) and in combination with each other (MP low, EE2; MP high, EE2; MP loaded) and one solvent control (control) relative to the elongation factor (EF) and the control treatment as means +/- standard deviations, significant differences ($p<0.05$) compared to the control are marked by asterisks.

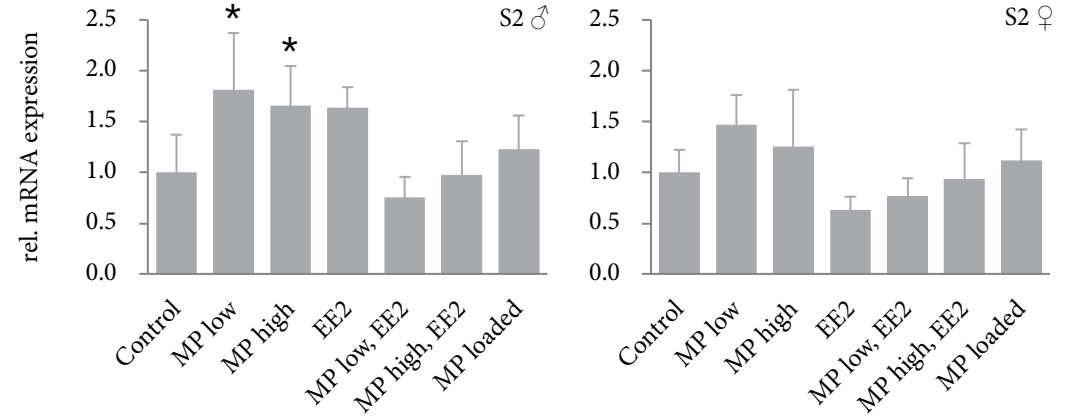



*Figure 12 mRNA expression of S2 after 21 days*

S2 mRNA expression in male and female tadpoles after 21 days of exposure with two concentrations of microplastics and one concentration of EE2 alone (MP low; MP high; EE2) and in combination with each other (MP low, EE2; MP high, EE2; MP loaded) and one solvent control (control) relative to the elongation factor (EF) and the control treatment as means +/- standard deviations, significant differences ($p<0.05$) compared to the control are marked by asterisks.

#### 4.3.9.3 mRNA gene expression in livers

In general, vitellogenin mRNA expression compared to the control groups was higher in males than females for all treatments (Figure 13 and Appendix Table 21). Vitellogenin mRNA levels were elevated exclusively for treatments including EE2 (EE2; MP low, EE2; MP high, EE2) compared to all other treatments, except for microplastics loaded with EE2 (MP loaded) in both sexes. Again, high individual variabilities within the treatments groups were indicated by high standard deviations. In males, the combination of

microplastics and EE2 (MP low, EE2; MP high, EE2) induced higher mRNA levels of vitellogenin than EE2 alone (EE2), i.e. about ten times more for the low (MP low, EE2) and about sixteen times more for the high microplastic concentration (MP high, EE2), though these differences were not significantly different due to high standard deviations. In females, mRNA levels were more similar for EE2 alone (EE2) and in combination with microplastics (MP low, EE2; MP high, EE2) and all were significantly different from the control. Both concentrations of microplastic in combination with EE2 (MP low, EE2; MP high, EE2) differed significantly compared to both concentrations of microplastics alone (MP low; MP high) and to microplastics loaded with EE2 (MP loaded).

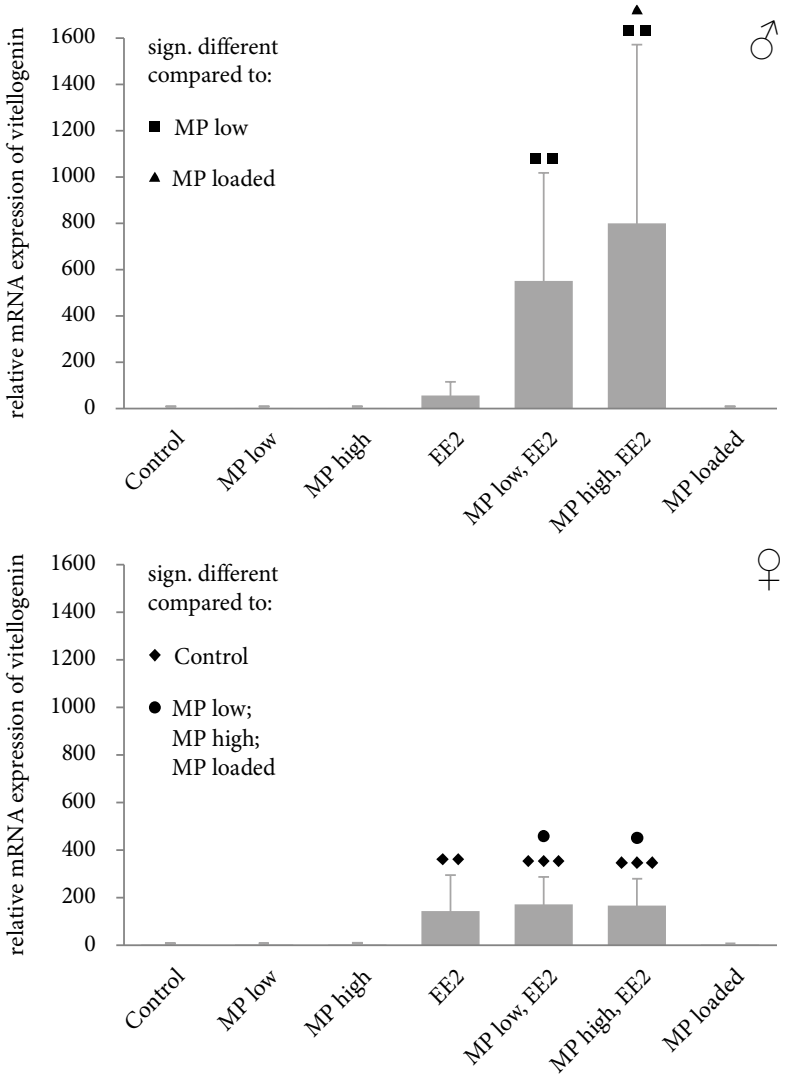



*Figure 13 mRNA expression of vitellogenin after 21 days*
Vitellogenin mRNA expression in male and female tadpoles after 21 days of exposure with two concentrations of microplastics and one concentration of EE2 alone (MP low; MP high; EE2) and in combination with each other (MP low, EE2; MP high, EE2; MP loaded) and one solvent control (control) relative to the elongation factor (EF) and the control treatment as means +/- standard deviations, significant differences ($p<0.05$) compared to the control are marked according to the legends within the diagrams.

In one individual from treatment EE2, which was excluded from treatments specific comparisons because it was identified as genotypic female with a mixed sex gonad, mRNA expression of vitellogenin was elevated compared to the overall mean of both males and females in this treatment (1362.7 relative mRNA expression).

## 4.4 Discussion

The results of this study show that the mere microplastic material itself (PA particles) did not have adverse impacts on tadpoles, both, after short- and long-term exposure. Neither the general development nor the stress hormone levels were significantly influenced if PA particles were presented to the tadpoles alone. However, microplastics with and without EE2 increased the stress hormone corticosterone moderately but due to high standard deviations, no significance could be determined. Exposure to PA particles caused no morphological abnormalities in intestines and gills. The presence of microplastics did not alter specific effects of EE2 significantly, i.e. sex ratios and biomarker mRNA expression. Elevated mRNA levels of vitellogenin levels in male tadpoles nevertheless indicated higher exposure to EE2 in combination with the PA particles.

### 4.4.1 Exposure scenario

The laboratory approach of this study was chosen to get a better mechanistic understanding of how microplastics potentially affect amphibians during larval development. The conditions represent an extreme exposure scenario, which aimed at identifying processes rather than reflecting a realistic environmental scenario. Both, the two microplastic concentrations and the EE2 concentration used for the different treatments exceed concentrations which can be expected in the environment. Mass concentrations of microplastics in surface waters with differing units, i.e. mass per cubic metre in rivers and mass per square metre in lakes, were reported between $10^{-3}$ to $10^{-1}$ mg $m^{-3}$ for rivers and $10^{-3}$ to $10^{-1}$ mg $m^{-2}$ for lakes (Faure et al. 2015, Yonkos et al. 2014). Concentrations of EE2 in surface waters were detected at concentrations in the range of several ng $L^{-1}$, e.g. up to 42 ng $L^{-1}$ in wastewater effluents (Belfroid et al. 1999, Ternes et al. 1999). The exposure scenario in the present study can be most likely compared to an environmental scenario with high concentrations of both, endocrine disruptors and microplastics, e.g. at discharge sites for wastewater effluents.

The distribution of PA particles within the water column due to aeration of the aquaria ensured that the particles were available for direct contact with the tadpoles. While EE2 was first only dissolved in water at the beginning after renewal of exposure solutions (for EE2; MP low, EE2; MP high EE2), it can be expected to distribute between the different compartments of the test system such as food particles, PA particles, if present, as well as tadpoles, until reaching sorption equilibrium. In the treatment with pre-loaded PA particles (MP loaded), a smaller overall mass of EE2 was introduced in the aquaria

compared to the other treatments with EE2 (EE2; MP low, EE2; MP high, EE2) leading to lower exposure of tadpoles with EE2. Regarding sorption processes, EE2 pre-sorbed to PA particles can be expected to desorb from the particles towards sorption equilibrium (for MP loaded). In the first week of exposure, lower measured EE2 levels in the treatment including the high concentration of microplastics (MP high, EE2) compared to the other treatments with EE2 (MP low, EE2; MP loaded; EE2) indicate fast sorption of EE2 to PA particles. Fast sorption is in accordance with the study of Han et al. (2012), which was used as basis for calculating the sorption capacity of PA particles ($K_{pa,w}$). That this difference seems to be less pronounced in the third week of exposure could be due to the higher body mass of tadpoles with about three times more mean mass per individual than in the first week. The higher body mass of tadpoles in the third week of exposure leads to a higher proportion of the overall mass of EE2 being associated with the tadpoles if compared to the first week. At the same time, EE2 can be expected to be more diluted in the individuals. The increase of the overall body mass of the tadpoles leads to both, a dilution of EE2 in water and the tadpoles. The proportion of EE2 in the tadpoles, in addition, can be expected to be heterogeneously distributed depending on physiochemical characteristics such as lipid content of tissues and biological processes in target organs.

A relatively high sorption capacity of the PA material was indicated by $K_{pa,w}$ (3043 L $kg^{-1}$). The calculated BCF (362.4 L $kg^{-1}$), which was based on $K_{o,w}$, is comparable to the BCF reported from Lai et al. (2002) of fish (332 L $kg^{-1}$). Calculated concentrations of EE2 in water were in a similar range as measured values, but most of them were lower than the measured concentrations. Only the aqueous EE2 concentration in the treatment with EE2 in combination with the high concentration of PA particles (MP high, EE2) was the same for calculated and measured values in the first week of exposure. Different processes could be the reason for higher measured than calculated EE2 values in the other treatments (EE2; MP low, EE2). First, methodological uncertainties in laboratory measurements, e.g. during extraction of EE2, could lead to either over- or underestimation of EE2 levels. Second, calculations are based on estimations, which could lead to e.g. overestimation of sorption of EE2 to PA particles (high $K_{pa,w}$ value) and the bioconcentration factor (BCF). Third, all calculations are based on the assumption of sorption equilibrium in the test system, however, different mechanisms like sedimentation of PA particles after some hours and metabolisation of EE2 by the tadpoles may interfere with a stable state of sorption equilibrium. Fourth, sorption of EE2 to food particles and glass surfaces as well as metabolisation of EE2 by the tadpoles was not addressed for the calculations. However, these mechanisms would lead to lower measured EE2 levels compared to calculated EE2 levels, which is the opposite to measured concentrations being higher. Last and presumably most important, calculated values relate to EE2 concentrations at day seven and 21, because measured masses of tadpoles during sampling were used for the calculations, while measured EE2 concentrations relate to day four and 18, when water was sampled for analytical analysis. At the time of measuring EE2 concentrations at days

four and 18, the mass of the tadpoles can be expected to be lower than at days seven and 21. In principle, this leads to an underestimation of calculated EE2 concentrations in water. The difference between the masses of the tadpoles is not quantifiable, but may be one of the main reasons for higher measured EE2 concentrations in water. That the calculated and measured EE2 levels after four and seven days are in the same range could be based on the fact, that within the first week of exposure, the mass of the PA particles was most crucial for the mass distribution of EE2 in the system. This is why inaccuracies in the mass of tadpoles are presumably not as pivotal, as in the third week of exposure leading to similar calculated and measured values.

### 4.4.2 Uptake pathways for PA particles and EE2

Quantification of uptake of EE2 and PA particles was beyond the focus of this study, but some observations during exposure and dissection of tadpoles for PA particles implied some general information. In principle, EE2 can enter the body by uptake from dissolved EE2 in water and from EE2 sorbed to either PA particles or food particles. Dissolved EE2 can be internalized via different pathways, i.e. through the skin, by gills during filtering water for oxygen uptake and by ingestion of particles during food acquisition. PA particles in the range of 20-50 µm are not expected to enter the body through the skin, but by filtering of water and ingestion. Hence, the gills and the intestines are the main potential targets for effects of the PA particles, both, physically and chemically. However, also attached particles on the skin could potentially interact with the tadpoles, i.e. chemically. The transfer of persistent organic pollutants via attached microplastics at epithelia was shown in zebrafish embryos (Batel et al. 2018).

Tadpoles have high filtering and ingestion rates, so PA particles can be expected to have been internalized fast. On the other hand, also egestion rates are high. PA particles seemed to be included in faeces indicated by its partly whitish appearance. Sinking faeces may have enhanced sedimentation of PA particles in addition to sedimentation by aggregation with e.g. food particles or by the particles themselves. Besides egestion of PA particles, PA particles observed in intestinal convolutions during sampling and three days after the last water change show that either PA particles remained in the intestines from uptake of particles before sedimentation or that tadpoles still ingested remaining or remobilized particles from the water column. The absence of PA particles in histological sections of gills and intestines could be due to the preparation of the tissues including several washing steps with inorganic (e.g. ethanol) and organic solvents (e.g. xylol). Staining of the samples was done with focus on the tissues, while PA particles may need special staining to be visible in the sections under the microscope. Fluorescently labelled microplastics used in other studies (e.g. Batel et al. 2016) would have facilitated localisation in tissues but were avoided in this study because of potential chemical effects of the fluorescent dye. Relatively fast uptake and egestion of microplastics was reported in a study with *X. tropicalis* tadpoles at stage 45 (Hu et al. 2016). The tadpoles had been exposed to high

concentrations (0.1-$10^5$ particles $ml^{-1}$) of polystyrene particles (1 and 10 µm in diameter). Within one hour of exposure, particles were found in both, gills and intestines. After six hours, particles were observed in faeces as well. Transfer of the tadpoles into clean water after exposure for 48 hours led to a fast significant decrease of particles only when food was present. Feeding of tadpoles seems to facilitate egestion of microplastics, which can be expected in the present study as well. Fast egestion of a high quantity of ingested microplastics is also known from laboratory studies with fish (e.g. Batel et al. 2016, Jovanović et al. 2018). However, a small fraction of ingested microplastics were found to get trapped in livers of fish indicating that microplastics can potentially translocate within the body (Jovanović et al. 2018, Lu et al. 2016) .

### 4.4.3 Effects of PA particles themselves

The results of the present study do not indicate adverse impacts of the PA particles themselves on general development, as neither the low nor the high concentrations of PA particles influenced growth and developmental stages significantly. In contrast, ingestion of microplastics reduced feeding activity followed by weight loss and energy depletion in lugworms (Besseling et al. 2013, Wright et al. 2013a). Overall, microplastics seem to mostly pass intestinal tracts in fish and can be egested again (e.g. Batel et al. 2016), as assumed for the present study as well. In spite of high ingestion of PA particles, gills and intestines of tadpoles did not show signs of obvious physical damage in histological sections. However, micro-injuries or inflammational processes cannot be excluded. Adverse impacts like inflammation processes and physical damage in organs being in direct contact with microplastics were observed in earlier studies (e.g. Karami et al. 2016, Rochman et al. 2013, von Moos et al. 2012). In mussels, inflammation parameters were increased after ingestion of high amounts of microplastics. Inflammation processes were discussed as result of mechanical abrasion, especially of sharp-edged or irregular shaped plastic fragments which were also used in the present study. In catfish, histopathological changes in gills were observed as a result of exposure to microplastics (PE, 0.3-138 µm in diameter), although no microplastics could be detected in gill tissue. No PAHs, PCBs, heavy metals and phthalates could be detected in the virgin microplastics, which is why physical effects rather than chemical effects were suggested as reason for the changes in the gills. Reduced acetylcholinesterase (AChE) activity was found in goby fish as a result of microplastic exposure (PE, 1-5 µm in diameter) with potential adverse impacts on neurofunctions (Oliveira et al. 2013). Accumulation of lipids and inflammation in livers of zebra fish indicated adverse impacts of microplastics themselves in another study with PS particles (5 and 20 µm, Lu et al. 2016). In a study by Lu et al. (2016) microplastics accumulated in the gills, liver and the gut. After microplastic exposure for 14 days with indirect uptake of microplastics from *Artemia* nauplii containing microplastics in another study, a small fraction of microplastics were found to be retained within the mucus in intestines, but did not induce physical damage in zebra fish (Batel et al. 2016). Histopathological damages in intestines of zebra fish, i.e. cracked villi and split

enterocytes, were induced by different polymer types including PVC, PA, PE and PP with about 70 µm in diameter after 10 days (Lei et al. 2018). After short-term exposure (96 hours) of fish with PVC fragments (0.1-1000 µm) no severe damage in intestines like lesions were observed, but the epithelium was thickened (Romano et al. 2018). Presumably as result of the thickened epithelium, protease activity was found to be elevated as well.

The absence of significant differences also for corticosteroid levels between all treatments including the control after seven and 21 days indicate, that PA particles did not induce severe stress in tadpoles neither alone nor in combination with EE2. However, due to elevated mean values of corticosteroids microplastic exposure seemed to cause some slight or moderate stress, which is not statistically significant though. The levels of corticosterone and aldosterone were in similar ranges as in other studies (Kloas et al. 1997, Ziková et al. 2013). That corticosterone levels were higher for all treatments including the control after seven days than after 21 days is in accordance to the previous studies and does not represent a specific effect. In a study with African catfish, lower cholesterol plasma levels were discussed to be a result of increased corticosteroidogenesis in response to the exposure with virgin microplastics (Karami et al. 2016) Interestingly, this effect was only observed for a relatively low microplastic concentration (50 µg $L^{-1}$), but not for a higher concentration (500 µg $L^{-1}$). Upregulation of corticosteroidogenesis could not be explicitly confirmed in the present study, because slightly elevated levels of corticosterone after 21 days of exposure in treatments with microplastics were not statistically significant. In fish, stress was indicated by glycogen depletion in the liver after microplastic exposure (e.g. Rochman et al. 2013). Oxidative stress in fish was found in another study after exposure with PS particles (5 and 20 µm, Lu et al. 2016).

Besides the shape, the size of microplastics seems to be crucial for their potential effects. That even smaller plastics in the nano-scale (nanoplastics, uncoated polystyrene, 50 nm in diameter) have potential teratogenic effects in amphibians during very early development was shown in embryos of *X. laevis* (Tussellino et al. 2015). After microinjection or contact exposure, embryos showed e.g. malformations and slower growth and modified gene expression of different biomarkers. Mortality was not affected by nanoplastic exposure. The particles were able to enter cells which was indicated by their presence in gut cells, cytoplasm and the nucleus. Most importantly, only relatively high concentrations between $10^{-3}$ to $10^{1}$ mg $L^{-1}$ were able to induce adverse direct effects in most studies. The concentrations used in the present study are in the upper range and above with 1 and 100 mg $L^{-1}$ used for the experiments, which leads to a higher potential to detect adverse impacts of the particles on the tadpoles. The lack of clear evidence that microplastics have severe physical effects is in accordance with other studies, which studied the effects of pristine microplastics on different endpoints such as growth and biomarker gene expression in fish (e.g. Jovanović et al. 2018, Karami et al. 2017). First results on potential effects of pristine microplastics at relatively high concentrations ($10^{-}$

$^{1}$-$10^{2}$ mg $L^{-1}$) on *X. laevis* larvae during early-life stages did not indicate severe impacts on growth and swimming activity as well (de Felice et al. 2018).

### 4.4.4 Influence of PA particles on specific effects of EE2

Sexual development and biomarker activities were analysed as endpoints in order to clarify if PA particles have the potential to influence specific effects of EE2 as endocrine disruptor. The treatment with only EE2 served as baseline for the specific effects, the treatments with only PA particles (MP low; MP high) allowed to exclude effects of the microplastic material itself on EE2 specific endpoints. Results on general development are shortly discussed as background for more specific effects of EE2.

#### 4.4.4.1 General development

Effects of EE2 alone on general development were only observed for snout-vent lengths and development stages after 21 days. Slightly retarded development as result of exposure to estrogens such as EE2 and E2 indicated by lower developmental stages is known from other studies (Lutz et al. 2008, Tompsett et al. 2012), while slightly increased growth has not been reported explicitly in other studies and seems to be rather spontaneous. Snout-vent lengths in the treatment with only the high concentration of PA particles (MP high) were significantly lower than in the combination with EE2 (MP high, EE2) indicating a developmental effect of EE2 in this treatment towards higher snout-vent lengths rather than an effect of the PA particles themselves.

#### 4.4.4.2 Sexual development

The presence of PA particles in addition to EE2 did not change estrogenic effects of EE2 indicated by similar sex ratios and numbers of sex shifted individuals. An influence of PA particles on effects of EE2 can most likely be interpreted for the treatment with the high concentration of PA particles in addition to EE2 (MP high, EE2), because of the total absence of mixed sex individuals.

The ratio of genotypic females to genotypic males was in a similar range for all treatments and the control according to the randomized distribution of tadpoles at the beginning of the exposure. That in the control slightly more females were found compared to all other treatments seems to be a randomized effect due to the relatively low number of individuals (n=60) per treatment. EE2 exposure shifted the sex ratios towards phenotypic females, which could be expected because of its estrogenic effects. In tadpoles of *X. laevis*, sex is pre-determined by the genotype, but the presence of estrogens can result in the development of ovaries or mixed stages with characteristics of both, testes and ovaries in genotypic males (Kloas et al. 1999, Kloas 2002, Pettersson et al. 2006, Wolf et al. 2010). The complete sex shift of one genotypic female to a morphological male in the treatment with only EE2 is regarded as rare spontaneous shift. That only one individual was identified as sex reversed in the treatment with pre-sorbed PA particles (MP loaded) can

be explained by the lower overall mass of EE2 compared to the other treatments with EE2 (EE2; MP low, EE2; MP high, EE2). PA particles alone did not influence sexual development, i.e. did not change sex ratios in any treatment with only microplastics (MP low; MP high), thus microplastics themselves did not act as endocrine disruptor concerning sexual differentiation.

#### 4.4.4.3 Biomarker activities

Expression of the selected biomarkers in the brain, gonad and liver was highly variable within the treatments. High standard deviations were the consequence from high individual variability in all treatments including the control. The relatively broad range of developmental stages (NF 57-65) in the present study may partly explain the high individual variability of mRNA expression in general. Tadpoles of different developmental stages (NF 58 compared to NF 66) were shown to have altered sensitivity to hormonal active substances in some cases (Urbatzka et al. 2014).

##### 4.4.4.3.1 mRNA expression in pituitaries

In females, both, LH and FSH levels were in a similar range without significant patterns for all treatments and the control. Only in males, significant differences for LH mRNA expression were found, while FSH mRNA levels were not significantly different. FSH in the present study displayed a reversed pattern to LH like a negative feedback to EE2 but the difference was not statistically significant. Elevated levels of LH mRNA in males for two of the treatments with EE2 (EE2; MP low, EE2) indicate stimulated secretion of LH from the pituitary. In contrast, LH mRNA expression in adult male frogs in a similar study was found to be decreased after four weeks of exposure with EE2 at the same concentration as in the present study ($10^{-8}$ M) and was interpreted as a negative feedback mechanism (Urbatzka et al. 2006). This is why elevated levels of LH in the present study were not expected. Induction of LH, as in the present study, was reported as a response to the exposure with finasteride (FIN), an inhibitor of 5α-reductase enzyme activity (Urbatzka et al. 2009). Higher LH levels in males were interpreted to stimulate steroidogenesis in the gonads. Exposure to flutamide (FLU) significantly increased LH mRNA levels as well, if compared to the control treatment in another study (Urbatzka et al. 2014). In the study by Urbatzka et al. (2006) adult frogs were exposed to EE2, while in the present study juvenile frogs with developmental stages between 57 and 65 were exposed. Hence, the question is, if the maturity of the gonads might be a reason for different response patterns of LH levels to EE2 exposure of the present study and the study by Urbatzka et al. (2006). The maturity of the gonads was also found to be crucial for the response of gonadotropins to steroids in fish (Sohn et al. 2001). In general, basal levels of LH in male *X. laevis* during larval development are highest at NF 56 during pro-metamorphosis and decrease until completion of metamorphosis at NF 66 (Urbatzka et al. 2010). The mRNA expression levels of the LH and FSH from the pituitary as well as StAR and P450 from the gonad-kidney complex were analysed after exposure to a set of

different hormonal active substances for tadpoles at NF 58 and NF 66 to compare response patterns between different developmental stages (Urbatzka et al. 2014). Interestingly, response patterns of LH and FSH in males were not distinctively different between the two stages. FLU, for example, was elevated both at NF 58 and 66 compared to the control. Exposure to E2 did not change mRNA expression of LH and FSH statistically significant, which shows that exposure to an estrogen, does not necessarily lead to a reduction of LH mRNA expression in males. LH mRNA was even slightly increased in males at NF 58 in response to E2 exposure, which shows a similar trend as in the present study. In contrast to males, response patterns of females to the different hormones were slightly different between NF 58 and 66, i.e. more pronounced at NF 66 in most cases. For example, E2 exposure induced lower expression of LH and FSH in females only at NF 66 and not in NF 58. *Vice versa*, steroidogenic genes (StAR, P450) in males responded more pronounced to the different exposures at NF 58 compared to NF 66 in male tadpoles, indicating a higher sensitivity of tadpoles during pro-metamorphosis. In summary, response patterns in males seem to be stage-dependent rather for steroidogenic enzymes than gonadotropins. However, it cannot be fully excluded that the maturity of the gonads did play a role for different response patterns of LH in the present study. In conclusion, an expected negative feedback of EE2 on gonadotropins could not be confirmed, which might be due to a relatively low sensitivity of tadpoles to react to sexual steroids, although further studies are needed to fully clarify this.

#### 4.4.4.3.2 mRNA expression in gonads

Results of the analysis of the steroidogenic genes StAR, P450, Aro, S1 and S2 in the gonads show only minor effects of EE2 and the PA particles alone and in combination. An increase of LH mRNA expression in male tadpoles in response to exposure with FIN was accompanied with upregulation of P450 and StAR in another study (Urbatzka et al. 2009). This pattern can be explained by the stepwise organisation of steroidogeneses as described earlier, however, it cannot be confirmed in the present study for biomarkers in the gonads, because neither P450 nor StAR mRNA were upregulated. Downregulation of P450 mRNA in response to EE2 alone in females is rather small with less than 50%, but may be interpreted as feedback mechanism to the estrogenic effects of EE2. Elevated levels of S2 in males for the treatments with only PA particles indicate a weak effect of the particles themselves. Expression of S2 has been suggested as regulatory feedback after the exposure with FIN and its inhibiting effect of 5α-reductase enzyme activity (Urbatzka et al. 2009). Higher S2 mRNA levels in response to exposure with only the PA particles may point to chemical effects of the particles, although the PA particle material is expected to be inert according to the manufacturer's information. However, S2 mRNA levels are elevated less than factor two in the present study, which can be assumed to be rather small for biomarker expression. Differences for S2 are assumed to be most likely random rather than an effect of the PA particles themselves, but chemical effects of the particles cannot be fully excluded. Altered biomarker expression after exposure to only microplastics (PE, 0.3-138 µm in diameter) at a concentration (0.5 mg $L^{-1}$) in a similar range as the low

concentration of microplastics used in the present study (1 mg $L^{-1}$), was reported from a study on catfish (Karami et al. 2016). A biomarker for the regulation of stress (11 β-hydroxysteroiddehydrogenasetype 2, Alderman and Vijayan 2012) was upregulated, while a biomarker which is crucial for the serotonin system (tryptophan hydroxylase 2, Walther and Bader 2003) was downregulated. Leaching of several main groups of chemicals from the microplastic material could be excluded in this study. The authors suggested physical effects or the release of monomers from the PE particles as potential reason for altered gene expression.

#### 4.4.4.3.3 mRNA expression livers

Induction of vitellogenin mRNA by EE2 with higher sensitivity of males compared to females is in accordance with a study by Urbatzka et al. (2007b). These sex specific differences were discussed as a result of males not being able to deposit vitellogenin in the testes, like females do in their ovaries. Vitellogenin induction in response to estrogens, which was found to be accompanied with a decrease of testosterone in fish (Folmar et al. 1996), is suggested to be based on negative-feedback mechanisms on the HPG axis (Urbatzka et al. 2007b).

The liver, which is crucial for detoxification, was shown to be amongst the most vulnerable organs for physical and chemical effects of microplastics (e.g. Lu et al. 2016, Rainieri et al. 2018, Rochman et al. 2013). Exposure to microplastics alone was shown to result in oxidative stress, glycogen depletion and inflammational processes in livers of fish. In zebrafish, gene expression of different biomarkers from the liver were most affected, if a mixture of chemical pollutants, including e.g. PCBs, were presented in combination with microplastics (LDPE, 120-250 µm; Rainieri et al. 2018). White formations, which point towards histopathological changes, were exclusively found in livers of fish exposed to the mixture of chemical pollutants and microplastics. Means of vitellogenin mRNA levels in males show a trend for higher induction in the range of two orders of magnitude, when EE2 is presented to the tadpoles in combination with the PA particles compared to EE2 alone (EE2), despite the differences were not statistically significant. The high individual variability within tadpoles and relatively low samples size may lead to an underestimation of the differences. The trend for elevated mRNA expression of vitellogenin mRNA in males points towards higher exposure of the tadpoles to EE2 leading to an enhanced effect of EE2 if microplastics are present. According to former studies, different mechanisms within the tadpoles could be the reason for higher exposure to EE2 in the presence of microplastics. Sorption of EE2 to PA particles followed by uptake of the loaded particles could lead to an additional uptake pathway for EE2 resulting in altered bioavailability of EE2 in tadpoles. That microplastics can act as vectors for endocrine disruptors and other chemical pollutants in vertebrates in general was shown in studies with e.g. fish (e.g. Rochman et al. 2013). However, as soon as different uptake pathways such as water were considered in experimental and model based studies, microplastics seem to only contribute a small proportion to the overall uptake of chemical

pollutants (e.g. Besseling et al. 2017a, Koelmans et al. 2016). Uptake of chemical pollutants sorbed to microplastics was discussed to be enhanced by internal factors within the organism, e.g. temperature and pH in the intestines, especially in vertebrates like fish (Bakir et al. 2014, Teuten et al. 2009). The results of a more recent study indicate, that the role of these internal factors can be considered as rather small (Bakir et al. 2016). However, other factors such as gut content and pre-exposure to the chemical pollutant are vital to estimate the role of microplastics as uptake pathway for chemical pollutants as well (Mohamed Nor and Koelmans 2019). Gut regimes in tadpoles are presumably similar as in fish. Uptake rates of EE2 and tissue concentrations were beyond the focus of this study, but a contribution of the PA particles for the overall uptake of EE2 needs to be considered. As already stated, released chemicals such as additives from the PA particle material itself cannot be fully excluded. In principle, released additives with estrogenic effects could also lead to higher induction of mRNA expression of vitellogenin as well.

That the differences for the mixtures of EE2 with PA particles (MP low, EE2; MP high, EE2) were not pronounced enough to be statistically significant could be based on the exposure scenario in the third week. In the first week of exposure, the high concentration of PA particles (MP high, EE2) resulted in different distribution of EE2 in the system compared to the other treatments with EE2 (EE2; MP low, EE2; MP loaded) due to sorption of EE2 to the PA particles, as indicated by analytical measurements and calculations. At the time of sampling livers for analysing mRNA expression of vitellogenin after 21 days, the mass of the tadpoles was most crucial for the mass distribution of EE2 in the system. The potential role of the PA particles to act as vectors for EE2 at this time was lower compared to the beginning of exposure, e.g. in the first week. This may partly explain, why differences for induction of vitellogenin mRNA were not statistically significant. In studies showing upregulation of vitellogenin mRNA by estrogenic compounds, adult frogs were exposed (Urbatzka et al. 2007b). The tadpoles in the present study did not fully complete metamorphosis as indicated by developmental stages. Thus, response to EE2 exposure in general and to the mixtures including PA particles in particular may have been less pronounced compared to adults. Physical effects of the microplastics themselves were also discussed to enhance effects of biomarkers besides chemical effects (Karami et al. 2016). Facilitated uptake of phenanthrene by mechanical abrasions in tissues was suggested as reason for changed biomarker responses in catfish, although no microplastics could be detected in histological sections. In the present study, histological sections of gills and intestines did not show any signs of obvious histopathological changes. Nevertheless, micro lesions or inflammation processes induced by the PA particles, also in the liver, cannot be fully excluded. However, an influence of physical damage by the PA particles on the uptake of EE2 is rather unlikely, because the high lipophilicity of EE2 results in fast and efficient uptake via membranes. Overall, further studies are needed to clarify the potential of microplastics to enhance effects of chemical pollutant in general and endocrine disruptors in particular in amphibians. This is vital, especially because enhanced exposure to EE2 in the presence of

microplastics does not follow assumptions on the mass distribution of EE2 in the system in an equilibrium state.

## 4.5 Conclusions

This is the first study on potential physical and chemical effects of microplastics on amphibians during their development until sexual differentiation. A broad set of indicators was analysed in order to identify adverse effects of microplastics themselves and their influence of effects of a chemical pollutant, i.e. the endocrine disruptor EE2. The results of this study indicate that PA particles alone do not induce drastic adverse impacts on tadpoles, although PA particles were ingested. Neither the low nor the high concentration of PA particles caused higher mortality, inhibited development indicated by growth and developmental stage or statistical significant higher levels of stress hormones. Physical effects of microplastic fragments did not crucially impair the tadpoles. The first hypothesis, that microplastics at high concentrations are more likely to induce adverse effects is not confirmed, as no statistically significant effects were observed at all. The influence of microplastics on the toxicity pattern of the endocrine disruptor EE2 was limited. However, a trend for upregulation of vitellogenin mRNA expression in males indicated higher exposure to EE2. The upregulation was more pronounced for the higher concentration of PA particles together with EE2, which follows the second hypothesis, that higher concentrations of microplastics have a higher potential to influence specific effects of EE2. Vitellogenin is the most sensitive biomarker for disruption of sexual development including sexual differentiation in amphibians besides histomorphology of the gonads (Kloas et al. 2009). This is why a response of vitellogenin, even though not statistically significant different in the present study, needs to be considered as potential marker for enhanced endocrine effects, i.e. estrogenic effects, of pollutants like EE2 by microplastics. The results of this study are a first basis for further studies which aim at identifying the potential harm of microplastics for amphibians. Further studies with bigger sample sizes are needed to clarify, if microplastics can alter effects of chemical pollutants which can disrupt organisms already at small concentrations such as endocrine disruptors.

# 5 General discussion

The rising recognition of plastics as environmental pollutants within the last years led to concerns about potential impacts of plastic material on wildlife. Numerous studies within the last two decades pointed at the ubiquitous presence of particularly small plastics, i.e. microplastics (< 5 mm, Moore 2008), which have been considered only rarely in earlier studies (Barnes et al. 2009, Eriksen et al. 2013, Free et al. 2014). Concerns about pollution by microplastics are mainly based on potential risks for organisms being in direct contact with microplastic material with consequences such as physical blockage of intestines and trophic transfer within the food chain (e.g. Wright et al. 2013b as overview). The actual potential risk that microplastics may pose to organisms in the environment, however, is still challenging to estimate. One reason is the high diversity of microplastic types including a broad range of sizes and polymer material, but also scarcity of data about environmental exposure. Both, environmental concentrations of microplastics and their potential to cause adverse effects are not fully clarified yet, especially in freshwater systems. Scientific studies pointed out that microplastics can in principle induce adverse effects in organisms by themselves (e.g. Cole et al. 2015, Lee et al. 2013). Chemical effects of microplastics, which are based on plastic associated chemicals such as additives and especially hydrophobic organic compounds which sorb to the microplastic material, have been discussed extensively especially within the last years (Koelmans et al. 2016, e.g. Teuten et al. 2007). Most studies in the past focused on marine organisms because plastics were first identified as environmental issue in the oceans. Only within the last years, freshwater systems are studied to a greater extent. This thesis sheds light on how microplastics potentially affect freshwater organisms in response to the scarcity of scientific studies. A mechanism-based laboratory approach with microplastic types being relevant for environmental scenarios was chosen to address these knowledge gaps. More specifically, new insights on the effects of the microplastic material itself and the influence of microplastics on the effects of chemical pollutants are provided for a representative functional group of lake ecosystems, i.e. limnic zooplankton, and a representative of a specifically threatened group of vertebrates which spend the time of early development obligatory in freshwater, i.e. amphibians.

## 5.1 Overall discussion of key findings

The results presented in this thesis show that microplastics can induce adverse effects in freshwater zooplankton by themselves depending on the microplastic type. An influence of microplastics on the effects of chemical pollutants, i.e. the vector function of microplastics for chemical pollutants, seems to be limited as soon as other uptake pathways of the chemical pollutant are included in addition to microplastics both, in freshwater zooplankton and in amphibians.

### 5.1.1 Effects of the microplastic material itself

One of the research questions which were aimed to be filled in this thesis, whether the mere microplastic material itself can induce adverse physical impacts in a dose-dependent manner, can be answered for freshwater zooplankton represented by the model species *Daphnia magna* and amphibians represented by the model species *Xenopus laevis*. The results of this thesis show that the microplastic material itself is able to cause acute adverse effects in limnic zooplankton in principle. Physical effects were induced at high concentrations in the range of 25-400 mg $L^{-1}$ by one type of microplastics, i.e. translucent PE particles with 1-4 µm in diameter (referred to as 1 µm PE particles) in the shape of beads, in *D. magna* (Chapter 2). Fast ingestion and egestion of the 1 µm PE particles was observed within the first 24 hours and throughout exposure. Immobilisation, as criterion for acute adverse effects, was increasing with time and dose and was above the immobilisation rate in the control treatment after 72 and 96 hours. The effective concentration after 96 hours at which 50% of the daphnids were immobile ($EC_{50}$) was 57.43 mg $L^{-1}$ (95% confidence interval, lower: 32.76, upper: 100.69) or in the range of $10^8$-$10^9$ pieces of PE particles $L^{-1}$. Bigger sized PE particles with 90-106 µm in diameter (referred to as 100 µm PE particles) which could not be ingested by daphnids did not induce immobilisation at any presented concentration. Interestingly, PA particles with 15-20 µm mean diameter (total range of 5-50 µm, referred to as PA particles) at a high concentration (200 mg $L^{-1}$; Chapter 3) did not induce immobilisation in daphnids, although PA particles were also quickly ingested as observed for the 1 µm PE particles. Tadpoles of *X. laevis* were also observed to ingest PA particles indicated by white lumps in their intestines (Chapter 4), but neither short- nor long-term exposure (seven and 21 days) to a low and high concentration of PA particles (1 and 100 mg $L^{-1}$) induced statistically significant adverse impacts. Only elevated levels of the stress hormone corticosterone after 21 days of exposure to treatments with microplastic indicated a trend for adverse impacts in tadpoles. The general development of the tadpoles was not influenced by the presence of microplastics and no histopathological abnormalities in gills and intestines were observed. Overall, PA particles did not induce statistically significant adverse effects neither in daphnids (Chapter 3) nor in tadpoles (Chapter 4).

In some of the most recent studies other microplastic types were also shown to only cause minor adverse effects in both, daphnids and tadpoles. Morphological parameters such as body length and life history traits such as reproductive output were not or only little affected by the exposure to a mixture of different microplastic types with a similar size (40 µm mean diameter) as PA particles in the present study (Imhof et al. 2017). In another study, the presence of food seemed to be more crucial for mortality and reproduction output of *D. magna* rather than the presence of microplastics (Aljaibachi and Callaghan 2018). In tadpoles at an earlier developmental stage (NF 36-NF 46) than in the present study (NF 51-NF 65) neither body growth nor swimming activity were influenced by

exposure to a similar concentration of microplastics in the range of $10^{-1}$-$10^{2}$ mg $L^{-1}$ (PS, 3 µm, de Felice et al. 2018).

The results presented in Chapter 2 provide evidence that high concentrations are needed to cause direct adverse acute impacts based on physical effects in daphnids. This is in accordance with other recent studies on freshwater organisms (e.g. Au et al. 2015, Ogonowski et al. 2016, Sjollema et al. 2016, Ziajahromi et al. 2017). At the time of publication of the present results (Rehse et al. 2016), the $EC_{50}$ provided for 1 µm PE particles was the first reported effective concentration for the effects of the microplastic material itself without any additives which could potentially cause additional chemical effects in freshwater organisms. In a study by Au et al. (2015) an effective lethal concentration (LC) of microplastic particles for limnic amphipods was reported to be in the range of $10^{7}$ particles $L^{-1}$ after 10 days exposure. However, the microplastic type used in this study contained fluorescent colour, which was avoided in the present study to exclude potential chemical effects of the colour itself. In the meantime, the results of some studies on adverse effects of microplastics themselves on water fleas also showed dose dependent patterns (e.g. Frydkjær et al. 2017, Ogonowski et al. 2016, Ziajahromi et al. 2017), while the effect rates in other studies (e.g. Jemec et al. 2016) did not clearly indicate dose-dependency. Jemec et al. (2016) assumed that sedimentation of microplastics differed between exposure concentrations leading to unequal exposure conditions and that this may be the reason for the lack of clearly dose-dependent effect patterns in *D. magna*. Exposure for 48 hours of another water flea species, i.e. *Ceriodaphnia dubia*, with a similar microplastic type (white PE beads with 1-4 µm in diameter) as in the present study with a concentration range of $10^{-1}$-$10^{2}$ mg $L^{-1}$ , in contrast, led to clearly dose-dependent impacts on survival (Ziajahromi et al. 2017). The calculated lethal concentration at which 50% of the daphnids did not survive ($LC_{50}$) after exposure for 48 hours was 2.2 mg $L^{-1}$ ($10^{4}$ microplastic beads $L^{-1}$), which is in a similar range of concentration (mg $L^{-1}$) as in the present study after 96 hours. That the $LC_{50}$ for *C. dubia* after 48 hours is one order of magnitude below the $EC_{50}$ calculated in the present study for *D. magna* and that a clear dose-dependent toxicity pattern was already induced after 48 hours may be based e.g. on different sensibilities of the daphnid species or on properties of the microplastic material. More specifically, the PE beads used in the study of Ziajahromi et al. (2017) were white coloured, while the PE beads in the present study did not contain any colourants. According to the manufacturer's information (Cospheric, California, USA), titanium dioxide ($TiO_2$) is used as colourant for white PE beads used in the study by Ziajahromi et al. (2017). $TiO_2$ nanoparticles are known to impair daphnids (e.g. Lovern and Klaper 2006), which is why leaching of $TiO_2$ could potentially adversely affect daphnids in addition to the mere physical effects of the PE beads. Exposure of *D. magna* to PE fragments in the size range of 10-75 µm led to an $EC_{50}$ after 48 hours of 65 mg $L^{-1}$ (Frydkjær et al. 2017) which is not only in the same order of magnitude and unit range but very similar to the $EC_{50}$ after 96 hours in the present study. Interestingly, regular shaped white coloured PE beads (10-106 µm in diameter) induced immobilisation only

at much higher concentrations, which is indicated by an $EC_{50}$ of 5 g $L^{-1}$. This lower potential of bigger sized microplastics up to a size range of $10^2$ µm is in accordance to the results of the present study.

The results of this thesis show that the potential of microplastics to cause physical effects in freshwater organisms depends on the microplastic type as one type of PE particles (1 µm) caused clearly dose-dependent adverse effects in daphnids, while two other types of microplastics, i.e. PE particles (100 µm) and PA particles (15-20 µm), caused either no or only minor effects in daphnids and tadpoles. Different mechanisms can be considered to influence the varying potentials of the microplastic types to adversely affect the exposed organisms. First, different exposure scenarios which are based on different behaviour of the microplastic types due to their characteristics have to be considered. Second, specific characteristics of the microplastic types could explain why adverse impacts are induced only by some microplastic types after direct contact, e.g. ingestion. Hereafter, the roles of exposure scenarios and specific characteristics of microplastics are discussed.

Direct exposure, i.e. direct contact, was ensured for all microplastic types used in this thesis, although the microplastic types behaved differently in water resulting in different distribution of microplastics in the test vessels. Initially, the buoyancy of the microplastic particles followed expected buoyancy according to the density of the polymer material. PE particles with a lower density than water (Chapter 2) initially floated on the water surface after being freshly added to the water. PA particles with a higher density than water (Chapter 3 and 4), in contrast, sank to the bottom, especially if no water movements due to aeration were present. The size of microplastic particles influenced the floating or sinking behaviour of the microplastic particles after mixing as well, which was most obvious for the PE particles. Smaller PE particles (1 µm) remained for a certain time in the water column, while bigger PE particles (100 µm) directly floated back to the surface of the test vessels after mixing. The question is, whether different exposure scenarios altered e.g. ingestion rates of daphnids. Quantification of ingestion rates was beyond the focus of this study but it can be assumed that ingestion rates were relatively high for all microplastic types, except for the 100 µm PE beads, which could not be ingested by *D. magna* at all because of their relatively big size. Intestines of daphnids seemed to be entirely filled with microplastics, both, during exposure to 1 µm PE particles as well as PA particles. Thus, different behaviour of microplastic types is not assumed to vary the ingestion of microplastics to a great extent in this study. Overall, the potential to cause adverse effects for one type of microplastics (PE, 1 µm) and no significant effects of another type (PA, 15-20 µm) is not expected to only be based on different exposure scenarios leading to highly variable ingestion.

The potential risk of microplastics to induce adverse impacts after direct contact was shown to depend on the shape of the microplastic type in other studies. Microplastics with an irregular shape and sharp edges such as fragments and microplastic fibres were shown to have bigger negative impacts on organisms than relatively smooth rounded

microplastics such as beads (e.g. Au et al. 2015, Frydkjær et al. 2017, Ogonowski et al. 2016, Ziajahromi et al. 2017). Prolonged gut-residence time of fibres compared to microplastic particles was discussed to cause higher mortality induced by fibres (Au et al. 2015). Lower egestion rates of fragments compared to regular shaped beads shown for *D. magna* were discussed to explain higher adverse effect rates of fragments (Frydkjær et al. 2017). In the present study, however, PA particles with a fragmented shape and a similar size spectrum as analysed by Frydkjær et al. (2017) did not induce significant adverse impacts, while uniformly shaped PE beads with 1 µm in diameter did.

Ultimately, differences in the potential of the microplastic types to induce adverse impacts by themselves seem to be most likely based on their different sizes. First of all, the size of microplastics needs to be considered relative to the size of the organism in order to identify potential risks posed by microplastic material (Windsor et al. 2019). If microplastics are considerably larger than the organism, external interactions such as entanglement may play a role, while relatively small microplastics which can be ingested by the organism may result in blockage of intestines. In the present study, ingestion was observed for 1 µm PE particles as well as for PA particles, while no adverse impacts on the outer structure of the daphnids resulting e.g. in disentanglement were observed. Small microplastics in the range of a few micrometres and even smaller plastics in the nanometre size-range were shown to induce higher adverse effect rates than bigger sized plastics in other recent studies (e.g. Rist et al. 2017). This is in accordance with the present study with dose-dependent adverse effects only for the smallest microplastic type. The higher potential of smaller microplastics to induce adverse effects in organisms is discussed to be based on their ability to be transferred into other tissues. PS particles with 1 µm in diameter were shown to translocate into lipid droplets after ingestion by daphnids (Rosenkranz et al. 2009). It was not addressed how 1 µm PE particles in the present study translocated within the daphnids but that could be an explanation for immobilisation of daphnids after ingestion. The bigger size of PA particles used for exposure of daphnids (Chapter 3) and tadpoles (Chapter 4) seems to be crucial for the lack of significant adverse impacts of the particles themselves even after ingestion and at very high concentrations up to hundreds of mg $L^{-1}$.

### 5.1.2 Modulation of the effects of chemical pollutants by microplastics

A reduction of the adverse effects of the chemical pollutant bisphenol A (BPA) by the presence of microplastics, i.e. PA particles, was shown in the freshwater zooplankton species *D. magna* (Chapter 3), which answers one of the main research questions of this thesis, i.e. whether the presence of microplastics can reduce the effects of chemical pollutants. Immobilisation rates, which were analysed as criterion for adverse effects of BPA in *D. magna*, were lower for all treatments in the presence of PA particles compared to the exposure with BPA alone with statistically significant differences both, after 24 and 48 hours. Reduced effects were accompanied by a reduction of the concentration of BPA

in water, due to the sorption of BPA to the PA particles. Ingestion of PA particles by the daphnids made sure that PA particles could act as uptake pathway for attached BPA in principle. PA particles contributed a relatively high mass to the overall mass in the system during exposure. The body mass of the daphnids themselves was much smaller compared to the mass of the PA particles. Thus, the potential of the PA particles to act as uptake pathway for BPA was relatively high given the high loading of the PA particles with BPA in sorption equilibrium. Calculations on the mass distribution of BPA in the test vessels showed that a smaller fraction of the overall mass of BPA was hold by the daphnids if PA particles were present compared to BPA alone which is in accordance with lower effect rates induced by BPA in the presence of the PA particles. A comparison of $EC_{50}$ values between BPA alone and BPA combined with PA particles, which were calculated based on the actual measured concentrations in water, addressed if the loaded PA particles contributed to the overall effect of BPA in daphnids. According to this comparison, PA particles did slightly contribute to the overall immobilisation of BPA indicated by the lower $EC_{50}$ for BPA in the presence of PA particles compared to BPA alone. The lack of statistical significance, though, showed that the contribution of PA particles as source for BPA is negligibly small. Based on general assumptions of a system in sorption equilibrium, continuous sorption processes of the chemical contaminant between all compartments of the system are balanced leading to a specific fraction of the chemical pollutant being associated to each compartment including the organisms. Within the balanced processes of partitioning microplastics may contribute a fraction of the chemical pollutant to the organism, thus, act as one uptake pathway. The crucial question is, however, if this contribution is higher than expected from balanced processes of partitioning. For daphnids, water seems to be the most bioavailable fraction which follows the hypotheses proposed especially by earlier model-based studies, namely that other uptake pathways of chemical contaminants are more important than uptake via microplastics (e.g. Koelmans et al. 2016). The relative contribution of microplastics to total exposure of daphnids is rather small. The removal of aqueous BPA by sorption of BPA to pristine PA particles is more essential than uptake of sorbed BPA from PA particle if sorption equilibrium is assumed. Microplastics are no substantial uptake pathway for BPA in daphnids in the present study.

Experimental evidence for the limited role of microplastics as uptake pathway for chemical pollutants has been given also by other recent laboratory studies (e.g. Beckingham and Ghosh 2017, Besseling et al. 2017b, Frydkjær et al. 2017, Kleinteich et al. 2018). Reduced uptake of polychlorinated biphenyls (PCB) via microplastics (polypropylene spheres, 35 µm) compared to the uptake via natural sediment was shown for a sediment-living limnic worm (Beckingham and Ghosh 2017). Uptake of PCBs in a marine lugworm by microplastics (PE spheres, 10-180 µm) was small when all environmental relevant exposure pathways were included in both, model and experimental approaches (Besseling et al. 2017b). Reduced effects of chemical pollutants in the presence of microplastics were also shown on a different organism level by

Kleinteich et al. (2018). Bacterial community composition was less affected by polycyclic aromatic hydrocarbons in the presence of microplastics. Exposure of daphnids with a mixture of microplastics (PE fragments, 10-75 µm) with a chemical pollutant (phenanthrene) in an experimental setup which is similar to the setup in this thesis (Chapter 3) showed that toxicity of phenanthrene was not significantly altered by the presence of microplastics (Frydkjær et al. 2017).

The results presented in chapter four show how microplastics influence the effects of a chemical pollutant, i.e. an endocrine disruptor, in an amphibian species which fills two of the knowledge gaps identified at the beginning of this thesis (ii, iii, Chapter 1). Namely, whether microplastics can reduce effects of chemical pollutants (ii) in an amphibian species (iii). More specifically, the results show that sorption of the endocrine disruptor to the microplastic material does not reduce the effects of the endocrine disruptor 17-beta-ethinylestradiol (EE2), which is in contrast to the reduction of effect rates of BPA in daphnids. Specific effects of EE2 on tadpoles of *X. laevis* were not statistically significant influenced by the presence of microplastics, i.e. PA particles. Sexual differentiation and mRNA expression of most biomarkers were not altered by a relatively low and high concentration of PA particles combined with EE2, but a trend for elevated mRNA expression of a biomarker in the liver point towards an enhanced effect of EE2 if microplastics are present. Upregulation of the most sensitive biomarker, i.e. vitellogenin mRNA expression in the liver, with a factor of $10^2$-$10^3$ after three weeks of exposure indicated higher exposure of the tadpoles to EE2 in the presence of both concentrations of PA particles. The trend was more pronounced for the higher concentration of PA particles and male tadpoles, which are known to be most sensitive for estrogenic effects of endocrine disruptors such as EE2 (Urbatzka et al. 2007b). The increase of vitellogenin mRNA expression was not statistically significant due to high individual variability. Nevertheless, upregulation of mRNA calls for further investigations in order to clarify the potential of microplastics to enhance effects of chemicals, especially endocrine disruptors, in amphibians. Interestingly, the potential of the PA particles to act as uptake pathway for EE2, i.e. the loading of the PA particles with EE2, can be expected to be rather low based on assumptions about the mass distribution of EE2 in the equilibrated system. The presence of PA particles reduced the concentration of EE2 measured in water, especially for the high concentration of PA particles and in the first week of exposure. Thus, a fraction of EE2 can be considered to be sorbed to PA particles in treatments including PA particles. PA particles were observed to be ingested by the tadpoles, which in principle made them a potential uptake pathway for sorbed EE2. The concentration differences of EE2 in water in the third week of exposure, though, were not as pronounced between the treatments as in the first week. Thus, at the time of the analysis of mRNA expression including vitellogenin in the third week, a relatively small fraction of the overall mass of EE2 can be considered to be sorbed to the PA particles. The relatively high mass of the tadpoles in the third week of exposure shifted the distribution of EE2 towards the tadpoles. Given the relatively low potential of PA particles to act as uptake pathway from

the perspective of mass distribution of EE2 in the system, the trend for enhanced estrogenic effects of EE2 in tadpoles indicated by elevated mRNA expression of vitellogenin seems to be even more noteworthy. Hormonal active substances such as EE2 have very specific modes of action and can have significant effects on organisms also at very low concentrations, especially when exposed for a long-term as in this study.

To the best of my knowledge this is the first reported indication in amphibians for a potential enhanced effect of a chemical pollutant in general and an endocrine disruptor in particular by the presence of microplastics. Experimental evidence for a significant increase of the effects of organic pollutants by the presence of microplastics was given in some of the most recent publications with dietary exposure of zebra fish. Histopathological changes, i.e. white formations, were most prominent in livers of fish feeding on a diet with microplastics (rounded PE fragments, 125-250 µm) pre-sorbed with a mixture of persistent organic compounds including e.g. PCB compared to food with persistent organic compounds alone (Rainieri et al. 2018). Gene expression of biomarkers in the liver indicating metabolism of exogenous compounds, detoxification and a response to oxidative stress was also most pronounced for the diet with pre-sorbed organic compounds. Similar results were obtained in a study on the dietary exposure of zebra fish with nanoplastics (PS, 50 nm) pre-sorbed with BPA (Chen et al. 2017). Both uptake and neurotoxic effects of BPA were increased in the presence of nanoplastics. The authors concluded that BPA associated to nanoplastics, i.e. the carrier function of nanoplastics, could be the reason for this. Dietary exposure with presumably fast ingestion of contaminated food and the lack of additional uptake pathways, however, represents another exposure scenario than in the present study with different uptake pathways of EE2 for tadpoles. The key question, whether microplastics contribute substantially to the overall exposure of organisms via the vector function, remains to be answered in studies with only dietary exposure.

The vector or carrier function of microplastics for chemical pollutants in general and hydrophobic organic contaminants in particular has been extensively discussed within the last years (e.g. Bakir et al. 2016, Diepens and Koelmans 2018, Koelmans et al. 2016, Teuten et al. 2007, Teuten et al. 2009). The question has been raised, if microplastics facilitate the transfer of hydrophobic organic pollutants which are associated to the plastic material either by earlier sorption from the surrounding or by incorporation within the polymer material itself during manufacturing, e.g. additives. Pristine microplastic materials used in this thesis can be expected to not contain additives which could leach out of the polymer material according to the information of the two manufacturers. The focus of this part of the thesis was to analyse the modulation of effects of chemical pollutants which sorbed to microplastics from the surrounding. The vector effect of microplastics goes beyond their mere potential to act as uptake pathway or carrier for transport of chemicals. Only if the transfer of chemical pollutants is facilitated, i.e. enhanced, by microplastics in an exposure scenario with different uptake pathways,

which are assumed for most environmental scenarios, microplastics act as substantial source (Koelmans et al. 2016). In daphnids, PA particles were no substantial uptake pathway for BPA as immobilisation rates followed expected exposure based on estimations of the mass distribution of BPA in the system. For the vector effect, the overall mass of the chemical being associated with the organism needs to exceed the mass which can be expected to be associated with the organism according to the mass distribution in the equilibrated system. The concept of the vector effect herein exceeds the concept of plastic materials used for passive dosing. Passive dosing can be used to ensure stable aqueous concentrations of hydrophobic organic compounds during exposure of organisms (e.g. Mayer et al. 1999). Plastic materials used for passive dosing act as source for organic compounds, but release of the compounds from the plastic material is solely following concentration gradients. Microplastics do not only need to act as transport medium for chemical contaminants but also need to increase the mass which is transferred to the organism compared to partitioning in order to act as substantial source. Such microplastic-facilitated transfer is mainly associated with microplastics with high concentrations of chemical pollutants which exceed concentrations at equilibrium (Koelmans et al. 2016). These non-equilibrium states are most relevant for freshly introduced, already loaded microplastics and microplastics carrying contaminants which only slowly desorb from the polymer material, e.g. additives which are incorporated in the polymer material. Ultimately, the vector function of microplastics after ingestion only plays a role for aquatic organisms if chemical pollutants also desorb from the microplastic material within the organisms. Physiological conditions in the gut, e.g. pH, temperature and gut passage time, were discussed to enhance remobilisation of organic chemicals associated to microplastics (e.g. Bakir et al. 2014, Teuten et al. 2009). In a follow-up study by Bakir et al. (2016) uptake of organic pollutants from microplastics was calculated to be negligible compared to other uptake pathways, also if gut surfactants, pH and temperature were implemented in a model. One of the most recent studies shows that remobilisation of organic compounds from microplastics after ingestion depends on concentration gradients between ingested microplastics and gut content (Mohamed Nor and Koelmans 2019). Hence, clean organisms, which have not been exposed to the organic compound before as in the present study, are more prone to remobilisation of chemicals from microplastics. Finally, an increase of effect rates of the organic chemicals within the organisms only comes into place, when the chemicals are also bioavailable. After potential uptake from microplastics the chemicals need to reach target tissues in order to be able to detect an influence of microplastics on effect rates of organic pollutants.

To sum up, in contrast to reduced effects of BPA in daphnids, vitellogenin mRNA as indicator for estrogenic effects of EE2 tended to be elevated in the presence of PA particles in tadpoles in spite of the rather low potential of the PA particles to act as uptake pathway according to their relatively low loading with EE2. The relatively high mass of the tadpoles in the third week of exposure was a decisive factor for the distribution of EE2 in the system. This is why removal of EE2 from the water by sorption to PA particles was not as

crucial as removal of aqueous BPA by sorption to PA particles during exposure of daphnids. Estimations about EE2 exposure of tadpoles, which are based on equilibrium partitioning, match with statistical significant results of all endpoints. PA particles played a minor role for the distribution of EE2 in the system, which is in accordance with similar effect rates of EE2 in the absence and presence of PA particles. The trend for elevated vitellogenin mRNA levels, however, goes beyond expected exposure, which points towards a potential vector effect of PA particles in tadpoles. The potential vector function of PA particles may be attributed to the different, more complex physiology of the tadpoles with presumably longer gut-residence times and a different gut regime compared to e.g. daphnids. Most importantly, highly specific modes of actions of estrogens such as EE2 resulting in high sensitivity to also detect small differences in EE2 exposure could be an explanation. The high sensitivity of tadpoles to detect small differences of exposure to endocrine disruptors makes them a helpful model organism to study the potential vector effect of microplastics in vertebrates in further studies.

## 5.2 Implications for risk assessment of microplastics

### 5.2.1 Methods for assessment of effects of microplastics

Methods on how to identify adverse effects microplastics and how to estimate their potential risk for wildlife are being discussed within the scientific community more frequently within the last years (e.g. Karami 2017, Koelmans et al. 2017, Lenz et al. 2016, Syberg et al. 2015). There is a call for analysing potential adverse impacts of microplastics with exposure scenarios likely encountered in the environment. This includes exposure to actual concentrations of microplastics in environmental compartments, i.e. relatively low concentrations, but also exposure to more complex scenarios, e.g. by including food and other stressors. In the present study, however, a simplified laboratory exposure was chosen in order to systematically identify potential impacts of microplastics as baseline for further studies. Established ecotoxicological methods were applied to gain new insights on potential adverse impacts of microplastics on freshwater organisms. Both, effects of the microplastic material itself and the modulation of effect patterns of chemical pollutants by microplastics, i.e. the vector effect, have been addressed.

The results of this thesis illustrate that standardized ecotoxicological approaches can be useful for the risk assessment of the microplastic material itself if exposure scenarios are carefully considered. Exposure scenarios include the behaviour of the microplastic particles and their resulting availability for aquatic organisms. Ecotoxicological approaches have been developed specifically to assess adverse effects of chemical substances. The particulate nature of microplastics leads to their breakdown into smaller pieces in time instead of being dissolved in water as chemicals. Solubility of chemical pollutants, however, is required as criterion for exposure of aquatic organisms to

chemicals according to established ecotoxicological methods (e.g. OECD guideline 202 as applied in Chapter 2 and 3). If the chemical pollutant is entirely dissolved in water, it can be assured that it is fully available for aquatic organisms. This principle can be adapted to particulate material by making sure that the organisms come in contact with the microplastic material during exposure, which is why the behaviour of the particles as part of the exposure scenario is crucial to be considered. Exposure of the organisms to a range of high microplastic concentrations allowed calculating an $EC_{50}$ for 1 µm PE particles, which can be useful for further studies and for risk assessment of microplastics. Koelmans et al. (2017) proposed a systematic framework for risk assessment of plastics in the environment which comprises assessment of threshold concentrations, as provided for one microplastic type in the present thesis, as one fundamental aspect. Both, the use of high concentrations of microplastics and the use of a wide range of microplastic concentrations, are needed to obtain such threshold concentrations.

Established ecotoxicological approaches can also be applied to analyse if adverse effects of chemical pollutants on aquatic organisms are influenced by the presence of microplastics, i.e. due to sorption processes leading to microplastics as potential vector for chemicals. In fact, the specific design of these approaches to quantify adverse effects of chemical pollutants makes them suitable to detect the modulation of effect rates by the presence of microplastics. The relative importance of microplastics for pollutant uptake can be determined by including different uptake pathways of the chemical pollutant, i.e. water as in the present study. If, different from the present study for PA particles used for co-exposure with chemical pollutants, the microplastic material itself does already induce adverse impacts on the organisms this needs to be considered in addition. Model-based approaches can help to complement experimental results by getting a better understanding of sorption processes. If sorption equilibrium can be assumed during exposure, the contaminant distribution in the experimental setup can be estimated without the need for additional kinetic studies. The mass distribution of the chemical pollutant in the system can give an indication for the mass of the chemical pollutant in the different compartments, e.g. organisms, water and microplastics. Analytical measurements of the concentration of the chemical pollutant in water, which are often required within the standard protocols of ecotoxicological approaches, can be compared to the calculated concentration in water to verify pre-assumptions about sorption equilibrium.

Based on the approach and results of the present study the following recommendations can be proposed for further studies which aim at analysing adverse effects of the microplastic material itself and its influence on the effects of chemical pollutants based on ecotoxicological methods. First, it is crucial to describe the exposure scenario including the behaviour of the microplastic particles in water during exposure in addition to e.g. water parameters, which are already required in standardized methods. Second, the exposure scenarios need to be carefully considered for the interpretation of adverse

impacts. More specifically, the distribution of microplastics within the test system and changes of behaviour need to be taken into account. Third, the established ecotoxicological approaches might need to be adapted as illustrated by physical effects of PE particles in chapter two only after a prolonged exposure time of 72 and 96 hours. Prolongation of exposure has been already proposed for e.g. nanomaterials (Baumann et al. 2014). Fourth, the calculation of mass distribution of the chemical pollutant in the test system is a helpful tool for studying the modulation of effects of chemical pollutants by microplastics. Calculations can complement analytical measurements, especially when these are only done for the concentration of the chemical pollutant in water.

### 5.2.2 Contribution to risk assessment of microplastics

The results of this thesis contribute to the basic understanding of potential adverse impacts of microplastics on freshwater organisms and hence contribute to the further understanding of the actual risks microplastics may pose to freshwater organisms in the environment. The presence and absence of adverse effects in the investigated model organisms, *D. magna* and *X. laevis*, illustrate that effects of pristine microplastics themselves on organism level can neither completely be excluded nor in general expected. The potential of microplastics to cause adverse effects in juvenile daphnids and amphibians was shown to depend on the microplastic type. The size seems to be one of the most crucial characteristics as only the smallest microplastic type (PE 1 µm) induced significant adverse effects in daphnids (immobilisation). The dose-dependency of effect rates in daphnids for this microplastic type shows that microplastic material itself can induce adverse impacts in a similar manner as chemical pollutants.

The diversity of microplastic types makes it challenging to estimate an overall risk for microplastics in general. The concentration range needed to induce adverse impacts in the present study illustrates that only very high concentrations in the range of 10-100 mg $L^{-1}$ are able to induce immobilisation. An actual risk of microplastics themselves for organisms in the environment, however, can only be expected if environmental concentrations are in this high concentration range. Until now, concentrations up to $10^{-3}$ – $10^{-1}$ mg $m^{-3}$ (= $10^{-6}$ – $10^{-4}$ mg $L^{-1}$) of microplastics in the size range of 300 µm to 5 mm for rivers and $10^{-3}$ – $10^{-1}$ mg $m^{-2}$ in lakes have been reported from freshwater systems (Faure et al. 2015, Yonkos et al. 2014). The actual risk that pristine microplastic material itself may pose to limnic organisms by physical effects seems to be relatively low because concentrations in freshwater systems are some orders of magnitude lower than in the present study. The $EC_{50}$ for 1 µm PE particles can be compared to environmental concentrations now and in the future, when more reliable data about small microplastics in general and in different compartments such as the water column is available.

One of the key questions is, if physical effects of microplastics differ from effects of natural occurring particulate matter which can be expected to be abundant in freshwater environments, e.g. suspended solids. Adverse effects of natural particles in aquatic

organisms, e.g. reduced survival and blockage of guts (e.g. Bilotta and Brazier 2008as overview), seem to be similar to effects of microplastics (Scherer et al. 2018). In *Daphnia pulex*, suspended silt and clay was observed to reduce not only feeding activity but also population growth (McCabe and O'Brien 1983). The concentration range of natural suspended solids ($10^2$-$10^3$ mg $L^{-1}$), which was observed to induce adverse impacts in different aquatic taxa, is in a similar range as microplastic concentrations used in the present thesis (Hogg and Norris 1991). Concentrations of suspended solids can go up to this concentration range e.g. after storm events. The high abundancy of suspended solids sets the potential risk which may be posed by presumably lower concentrations of microplastics into perspective.

The modulation of effect rates of chemical pollutants due to sorption to the microplastic material seems to be rather limited if sorption equilibrium can be assumed. Water seems to be the most crucial uptake pathway of chemical pollutants as predicted by calculations based on physiochemical characteristics in both organisms, *D. magna* and *X. laevis*. A reduction of effect rates due to a reduction of the concentration of the chemical pollutant in water as shown in daphnids was not observed for effect rates in tadpoles. In contrast, exposure to the endocrine disruptor EE2 tended to be elevated by the presence of microplastics indicated by the most sensitive endpoint which points towards potential facilitated, i.e. vector-based, uptake of EE2 by microplastics. The vector effect of microplastics was discussed to be most relevant for non-equilibrium states with high loadings of microplastics with chemicals, such as additives leaching from the microplastic material itself (e.g. Teuten et al. 2009). In a model-based study, ingestion of plastics by lugworms and fish led to the uptake of additives, i.e. BPA and nonylphenol, via leaching from plastic material in general (Koelmans et al. 2014). The authors argue, however, that this plastic-associated transfer is negligible because exposure via other uptake pathways in the environment can be expected to be higher according to environmental concentrations. In addition, low diffusivities of additives such as BPA and NP have been discussed to limit exposure of organisms because of rather low leaching rates (e.g. Berens 1997, Koelmans et al. 2014). Apart from this, facilitated desorption needs to be considered e.g. for aged plastic material, specific gut regimes and organisms with long gut retention times (e.g. Bakir et al. 2014, Koelmans et al. 2013, Koelmans et al. 2014, Teuten et al. 2009). It needs to be clarified, if higher exposure of amphibians to endocrine disruptors such as EE2 by the vector function of microplastics is substantial by using a higher sample size which was limited in the present thesis. Physiological characteristics such as gut regimes and gut retention times in tadpoles could be considered in future studies.

## 5.3 Future perspectives

The potential of particularly small microplastics to induce physical effects showed in this thesis is of special relevance because small microplastics in the range of only a few µm were shown to be most abundant in freshwater systems (Faure et al. 2015, Free et al. 2014,

Imhof et al. 2016). Fragmentation of plastics which were already induced in the environment can be expected to lead to increasing concentrations of microplastics and a higher proportion of small microplastics even if new release of plastics in general and microplastics in particular will be reduced or prevented in the future. Further fragmentation of microplastics to nanoplastics, which were not included in the impact analysis of plastics in this study, will be the next step. Actual concentrations of especially small microplastics in the environment including rivers and lakes are still hard to estimate, however, they are ultimately needed to assess the actual risk microplastics may pose to freshwater organisms. Robust data about environmental concentrations in freshwaters is needed to compare with threshold concentrations for adverse impacts of microplastics as provided in this thesis ($EC_{50}$). Thus, it is one of the most crucial further research goals to get a more detailed understanding of environmental concentrations of microplastics and their size distributions in environmental compartments in general and in the water column for freshwater systems in particular, especially for microplastics in the µm size-range. Both abundance and distribution of microplastics need to be clarified with the help of experimental but also model based approaches.

Future projects also need to clarify which other microplastic types are more likely to induce adverse impacts in organisms than others. A systematically approach with a high variety of microplastic types is needed to address this knowledge gap. Furthermore, more experimental data is needed to analyse the sensitivity of other freshwater organisms which have mostly been neglected in earlier studies, e.g. amphibians. It is also important to consider that the risk which is potentially posed to organisms by the microplastic material depends on the relative size of the microplastic particles compared to the organism (Windsor et al. 2019). The size is determining e.g. if microplastics can be ingested by the exposed organism. In this thesis, this is illustrated by 100 µm PE particles which were simply too big to be ingested by daphnids because of morphological constraints. Other aquatic organisms such as fish on the other hand, would be able to ingest microplastics of this size range and may show adverse impacts after ingestion. This is why one microplastic type may cause adverse impacts in one organism but not in another. One of the most challenging goals for further studies will be to address the complexity of environmental exposure scenarios including e.g. exposure to several stressors such as a variety of chemical pollutants at the same time. Finally, potential impacts of microplastics on populations and ecosystems need to be addressed and clarified besides impacts on organism-level, which were in the focus of most studies in the past.

The results of chapters three and four provide basic experimental evidence for the limited role of microplastics for the uptake of chemical pollutants relative to other uptake pathways if sorption equilibrium is assumed. Further studies could include chemical pollutants with higher sorption capacities and compare the potential vector effect of microplastics to natural particulate matter to take the results of this study into perspective. Furthermore, it needs to be addressed how the effects of chemical pollutants which affect

organisms already at very low concentrations, e.g. endocrine disruptors, can be altered by the presence of microplastics. Experimental evidence is also needed in addition to model-based evidence (Koelmans et al. 2014) to clarify the potential vector effect of microplastic material with compounds that are already incorporated into the plastic matrix during manufacturing, i.e. additives, especially in regard of leaching kinetics that might determine transport distances and uptake risks. Last, the mechanisms which caused immobilisation in daphnids after exposure to 1 µm PE microplastics remain to be determined. Further studies could focus on potential inflammatory responses as indicated by earlier studies (e.g. von Moos et al. 2012) and translocation of microplastics into other tissues as shown for 1 µm PS particles in daphnids (Rosenkranz et al. 2009).

Finally, the question can be raised which consequences need to be drawn from the scientific evidence about the potential risk of microplastics given in this thesis, i.e. that microplastics only have a limited potential to adversely impair freshwater organisms both physically and chemically if relatively low environmental concentrations are considered. The ubiquitous pollution of freshwater systems with plastics in general and microplastics in particular and the persistence of already released plastic material are pivotal criteria for political and societal decisions on how to deal with plastic pollution in the future. Microplastics themselves are just a snapshot in time, because some of them origin from the defragmentation of bigger-sized plastics and all of them can be expected to disintegrate into even smaller-sized plastics such as nanoplastics. Both, plastics above the size range of microplastics and nanoplastics were already shown to impair organisms (e.g. Besseling et al. 2014, Derraik 2002). The rising awareness of plastic pollution in the environment led to different initiatives to deal with this recent environmental issue. Some initiatives aim at reducing the use of plastic products and release of plastics into the environment, e.g. wave of disposable one-use plastic bags or replacement of plastic particles in cosmetics, others aim at directly reducing the amount of plastics which have already been released into the environment, e.g. collection of plastics at beaches. The present momentum for action against plastic pollution can be used in order to apply the pre-cautionary principle regarding uncertainties of potential impacts of microplastics and to address adverse impacts of plastics in general which are already known.

## 5.4 Final conclusions

This thesis provides evidence that microplastics have only limited potential to adversely impair freshwater organisms both physically and chemically, especially when relatively low environmental concentrations of microplastics are considered. The obtained results contribute to the basic understanding on how high concentrations of microplastics can potentially affect freshwater organisms. This approach follows the pre-cautionary principle by first identifying potential adverse effects and critical microplastic concentrations before evaluating the risk at environmental concentrations in retrospect. This systematic approach is beneficial in the light of uncertainties of environmental

concentrations of microplastics in the lower size range and rising microplastic concentrations in the future due to new release and fragmentation of plastics. The knowledge gaps identified at the beginning of this thesis, i.e. (i) whether the microplastic material itself induces adverse physical impacts in a dose-dependent manner, (ii) whether microplastics can reduce the effects of chemical pollutants, (iii) to provide first empirical data on potential physical and chemical effects of microplastics on amphibians, were filled. Microplastics can adversely affect limnic zooplankton and those effects are dose-dependent at high microplastic concentrations in the range of 10-100 mg $L^{-1}$ and depending on the microplastic type, while other microplastic types do not adversely affect limnic zooplankton and amphibians (i and iii). The $EC_{50}$ provided for immobilisation of daphnids for 1 µm PE particles (57.43 mg $L^{-1}$, lower 95% CI: 32.76, upper 95% CI: 100.69) after 96 hours exposure is one of the first reported threshold concentrations for freshwater organisms and will help to estimate the potential risks of microplastics in the future. Effect rates of organic chemical pollutants in limnic zooplankton and amphibians were shown to not be significantly enhanced in the presence of microplastics in a sorption equilibrium state; on the contrary, immobilisation was reduced in daphnids in the presence of microplastics (ii and iii). A trend for elevated stress response after exposure to only microplastics and elevated mRNA expression of the most sensitive biomarker after exposure to microplastics in combination to an endocrine disruptor, however, point at potential impacts of microplastics on amphibians, which need to be clarified in future studies (iii). These new insights help to disentangle recent questions about this highly perceived environmental issue and act as basis for subsequent studies. The complexity of environmental factors influencing the exposure scenarios as well as the diversity of microplastic material call for more interdisciplinary approaches in order to identify how organisms are potentially threatened by microplastics now and in the future.

# References


Alderman SL, Vijayan MM (2012) 11β-Hydroxysteroid dehydrogenase type 2 in zebrafish brain: A functional role in hypothalamus-pituitary-interrenal axis regulation. J Endocrinol 215(3): 393–402

Aljaibachi R, Callaghan A (2018) Impact of polystyrene microplastics on *Daphnia magna* mortality and reproduction in relation to food availability. PeerJ 6: e4601

Anbumani S, Kakkar P (2018) Ecotoxicological effects of microplastics on biota: A review. Environ Sci Pollut Res Int 25(15): 14373–14396

Andrady AL, Neal MA (2009) Applications and societal benefits of plastics. Philos Trans R Soc Lond , B, Biol Sci 364(1526): 1977–1984

Andrady AL (2011) Microplastics in the marine environment. Mar Pollut Bull 62(8): 1596–1605

Andrady AL (2017) The plastic in microplastics: A review. Mar Pollut Bull 119(1): 12–22

Arthur C, Baker J, Bamford H (eds) (2009) Proceedings of the international research workshop on the occurrence, effects, and fate of microplastic marine debris, September 9-11, 2008. NOAA Technical Memorandum NOS-OR&R-30

Arukwe A (2008) Steroidogenic acute regulatory (StAR) protein and cholesterol side-chain cleavage (P450scc)-regulated steroidogenesis as an organ-specific molecular and cellular target for endocrine disrupting chemicals in fish. Cell Biol Toxicol 24(6): 527–540

Au SY, Bruce TF, Bridges WC, Klaine SJ (2015) Responses of *Hyalella azteca* to acute and chronic microplastic exposures. Environ Toxicol Chem 34(11): 2564–2572

Auta HS, Emenike CU, Fauziah SH (2017) Distribution and importance of microplastics in the marine environment: A review of the sources, fate, effects, and potential solutions. Environ Int 102: 165–176

Bakir A, Rowland SJ, Thompson RC (2012) Competitive sorption of persistent organic pollutants onto microplastics in the marine environment. Mar Pollut Bull 64(12): 2782–2789

Bakir A, Rowland SJ, Thompson RC (2014) Enhanced desorption of persistent organic pollutants from microplastics under simulated physiological conditions. Environ Pollut 185: 16–23

Bakir A, O'Connor IA, Rowland SJ, Hendriks AJ, Thompson RC (2016) Relative importance of microplastics as a pathway for the transfer of hydrophobic organic chemicals to marine life. Environ Pollut 219: 56–65

Barnes DKA, Galgani F, Thompson RC, Barlaz M (2009) Accumulation and fragmentation of plastic debris in global environments. Philos Trans R Soc Lond , B, Biol Sci 364(1526): 1985–1998

Batel A, Linti F, Scherer M, Erdinger L, Braunbeck T (2016) Transfer of benzoapyrene from microplastics to *Artemia* nauplii and further to zebrafish via a trophic food web experiment: CYP1A induction and visual tracking of persistent organic pollutants. Environ Toxicol Chem 35(7): 1656–1666

Batel A, Borchert F, Reinwald H, Erdinger L, Braunbeck T (2018) Microplastic accumulation patterns and transfer of benzoapyrene to adult zebrafish (*Danio rerio*) gills and zebrafish embryos. Environ Pollut 235: 918–930

Baumann J, Sakka Y, Bertrand C, Köser J, Filser J (2014) Adaptation of the *Daphnia sp.* acute toxicity test: Miniaturization and prolongation for the testing of nanomaterials. Environ Sci Pollut Res Int 21(3): 2201–2213

Beckingham B, Ghosh U (2017) Differential bioavailability of polychlorinated biphenyls associated with environmental particles: Microplastic in comparison to wood, coal and biochar. Environ Pollut 220: 150–158

Belfroid AC, van der Horst A, Vethaak AD, Schäfer AJ, Rijs GBJ, Wegener J, Cofino WP (1999) Analysis and occurrence of estrogenic hormones and their glucuronides in surface water and waste water in The Netherlands. Sci Total Environ 225(1-2): 101–108

Berens AR (1997) Predicting the migration of endocrine disrupters from rigid plastics. Polym Eng Sci 37(2): 391–395

Besseling E, Wegner A, Foekema EM, van den Heuvel-Greve MJ, Koelmans AA (2013) Effects of microplastic on fitness and PCB bioaccumulation by the lugworm *Arenicola marina* (L.). Environ Sci Technol 47(1): 593–600

Besseling E, Wang B, Lürling M, Koelmans AA (2014) Nanoplastic affects growth of *S. obliquus* and reproduction of *D. magna*. Environ Sci Technol 48(20): 12336–12343

Besseling E, Foekema EM, van Franeker JA, Leopold MF, Kühn S, Bravo Rebolledo EL, Heße E, Mielke L, IJzer J, Kamminga P, Koelmans AA (2015) Microplastic in a macro filter feeder: Humpback whale *Megaptera novaeangliae*. Mar Pollut Bull 95(1): 248–252

Besseling E, Quik JTK, Sun M, Koelmans AA (2017a) Fate of nano- and microplastic in freshwater systems: A modeling study. Environ Pollut 220(Pt A): 540–548

Besseling E, Foekema EM, van den Heuvel-Greve MJ, Koelmans AA (2017b) The effect of microplastic on the uptake of chemicals by the lugworm *Arenicola marina* (L.) under environmentally relevant exposure conditions. Environ Sci Technol 51(15): 8795–8804

Biginagwa FJ, Mayoma BS, Shashoua Y, Syberg K, Khan FR (2016) First evidence of microplastics in the African Great Lakes: Recovery from Lake Victoria Nile perch and Nile tilapia. J Great Lakes Res 42(1): 146–149

Bilotta GS, Brazier RE (2008) Understanding the influence of suspended solids on water quality and aquatic biota. Water Res 42(12): 2849–2861

Bögi C, Levy G, Lutz I, Kloas W (2002) Functional genomics and sexual differentiation in amphibians. Comp Biochem Physiol B 133(4): 559–570

Brennan SJ, Brougham CA, Roche JJ, Fogarty AM (2006) Multi-generational effects of four selected environmental oestrogens on *Daphnia magna*. Chemosphere 64(1): 49–55

Brotons JA, Olea-Serrano MF, Villalobos M, Pedraza V, Olea N (1995) Xenoestrogens released from lacquer coatings in food cans. Environ Health Perspect 103(6): 608–612

Browne MA, Dissanayake A, Galloway TS, Lowe DM, Thompson RC (2008) Ingested microscopic plastic translocates to the circulatory system of the mussel, *Mytilus edulis* (L.). Environ Sci Technol 42(13): 5026–5031

Browne MA, Galloway TS, Thompson RC (2010) Spatial patterns of plastic debris along estuarine shorelines. Environ Sci Technol 44(9): 3404–3409

Browne MA, Crump P, Niven SJ, Teuten E, Tonkin A, Galloway T, Thompson R (2011) Accumulation of microplastic on shorelines woldwide: Sources and sinks. Environ Sci Technol 45(21): 9175–9179

Browne MA, Niven SJ, Galloway TS, Rowland SJ, Thompson RC (2013) Microplastic moves pollutants and additives to worms, reducing functions linked to health and biodiversity. Curr Biol 23(23): 2388–2392

Burns CW (1968) The relationship between body size of filter-feeding Cladocera and the maximum size of particle ingested. Limnol Oceanogr 13(4): 675–678

Capra F (1997) The web of life: A new scientific understanding of living systems. Anchor, New York

Carey C, Bryant CJ (1995) Possible interrelations among environmental toxicants, amphibian development, and decline of amphibian populations. Environ Health Perspect 103: 13

Carpenter EJ, Smith KL (1972) Plastics on the Sargasso Sea surface. Science 175(4027): 1240–1241

Carpenter EJ, Anderson SJ, Harvey GR, Miklas HP, Peck BB (1972) Polystyrene spherules in coastal waters. Science 178(4062): 749–750

Carr SA, Liu J, Tesoro AG (2016) Transport and fate of microplastic particles in wastewater treatment plants. Water Res 91: 174–182

Castañeda RA, Avlijas S, Simard MA, Ricciardi A (2014) Microplastic pollution in St. Lawrence River sediments. Can J Fish Aquat Sci 71(12): 1767–1771

Chang CY, Witschi E (1956) Genic control and hormonal reversal of sex differentiation in Xenopus. Proc Soc Exp Biol Med 93(1): 140–144

Chen M-Y, Ike M, Fujita M (2002) Acute toxicity, mutagenicity, and estrogenicity of bisphenol-A and other bisphenols. Environ Toxicol 17(1): 80–86

Chen Q, Yin D, Jia Y, Schiwy S, Legradi J, Yang S, Hollert H (2017) Enhanced uptake of BPA in the presence of nanoplastics can lead to neurotoxic effects in adult zebrafish. Sci Total Environ 609: 1312–1321

Chua EM, Shimeta J, Nugegoda D, Morrison PD, Clarke BO (2014) Assimilation of polybrominated diphenyl ethers from microplastics by the marine amphipod, *Allorchestes compressa*. Environ Sci Technol 48(14): 8127–8134

Cole M, Lindeque P, Halsband C, Galloway TS (2011) Microplastics as contaminants in the marine environment: A review. Mar Pollut Bull 62(12): 2588–2597

Cole M, Lindeque P, Fileman E, Halsband C, Goodhead R, Moger J, Galloway TS (2013) Microplastic ingestion by zooplankton. Environ Sci Technol 47(12): 6646–6655

Cole M, Lindeque P, Fileman E, Halsband C, Galloway TS (2015) The impact of polystyrene microplastics on feeding, function and fecundity in the marine copepod *Calanus helgolandicus*. Environ Sci Technol 49(2): 1130–1137

Colton JB, Knapp FD, Burns BR (1974) Plastic particles in surface waters of the Northwestern Atlantic. Science 185(4150): 491–497

Comnea-Stancu IR, Wieland K, Ramer G, Schwaighofer A, Lendl B (2017) On the identification of rayon/viscose as a major fraction of microplastics in the marine environment: Discrimination between natural and manmade cellulosic fibers using fourier transform infrared spectroscopy. Appl Spectrosc 71(5): 939–950

Corcoran PL, Biesinger MC, Grifi M (2009) Plastics and beaches: A degrading relationship. Mar Pollut Bull 58(1): 80–84

Crutzen PJ (2002) Geology of mankind. Nature(415): 23

Dabrunz A, Duester L, Prasse C, Seitz F, Rosenfeldt R, Schilde C, Schaumann GE, Schulz R (2011) Biological surface coating and molting inhibition as mechanisms of TiO2 nanoparticle toxicity in *Daphnia magna*. PLoS One 6(5): e20112

Daily GC (1997) Nature's services. Societal dependence on natural ecosystems, vol 1. Island Press, Washington

de Felice B, Bacchetta R, Santo N, Tremolada P, Parolini M (2018) Polystyrene microplastics did not affect body growth and swimming activity in *Xenopus laevis* tadpoles. Environ Sci Pollut Res Int 25(34): 34644–34651

de Souza Machado AA, Zarfl C, Rehse S, Kloas W (2017) Low-dose effects: Nonmonotonic responses for the toxicity of a *Bacillus thuringiensis* biocide to *Daphnia magna*. Environ Sci Technol 51(3): 1679–1686

de Souza Machado AA, Zarfl C, Kloas W, Hempel S, Rillig MC (2018) Microplastics as an emerging threat to terrestrial ecosystems. Glob Chang Biol 24(4): 1405–1416

Derraik JGB (2002) The pollution of the marine environment by plastic debris: A review. Mar Pollut Bull 44(9): 842–852

Desforges J-PW, Galbraith M, Ross PS (2015) Ingestion of microplastics by zooplankton in the Northeast Pacific Ocean. Arch Environ Contam Toxicol 69(3): 320–330

Devriese LI, Witte B de, Vethaak AD, Hostens K, Leslie HA (2017) Bioaccumulation of PCBs from microplastics in Norway lobster (*Nephrops norvegicus*): An experimental study. Chemosphere 186: 10–16

Diepens NJ, Koelmans AA (2018) Accumulation of plastic debris and associated contaminants in aquatic food webs. Environ Sci Technol 52(15): 8510–8520

do Sul JAI, Spengler A, Costa MF (2009) Here, there and everywhere. Small plastic fragments and pellets on beaches of Fernando de Noronha (Equatorial Western Atlantic). Mar Pollut Bull 58(8): 1236–1238

Dudgeon D, Arthington AH, Gessner MO, Kawabata Z-I, Knowler DJ, Lévêque C, Naiman RJ, Prieur-Richard A-H, Soto D, Stiassny MLJ, Sullivan CA (2006) Freshwater biodiversity: Importance, threats, status and conservation challenges. Biol Rev Camb Philos Soc 81(2): 163–182

Duis K, Coors A (2016) Microplastics in the aquatic and terrestrial environment: Sources (with a specific focus on personal care products), fate and effects. Environ Sci Eur 28(1): 2

Dümichen E, Eisentraut P, Bannick CG, Barthel A-K, Senz R, Braun U (2017) Fast identification of microplastics in complex environmental samples by a thermal degradation method. Chemosphere 174: 572–584

Eerkes-Medrano D, Thompson RC, Aldridge DC (2015) Microplastics in freshwater systems: A review of the emerging threats, identification of knowledge gaps and prioritisation of research needs. Water Res 75: 63–82

El-Shahawi MS, Hamza A, Bashammakh AS, Al-Saggaf WT (2010) An overview on the accumulation, distribution, transformations, toxicity and analytical methods for the monitoring of persistent organic pollutants. Talanta 80(5): 1587–1597

Endo S, Yuyama M, Takada H (2013) Desorption kinetics of hydrophobic organic contaminants from marine plastic pellets. Mar Pollut Bull 74(1): 125–131

Eriksen M, Mason S, Wilson S, Box C, Zellers A, Edwards W, Farley H, Amato S (2013) Microplastic pollution in the surface waters of the Laurentian Great Lakes. Mar Pollut Bull 77(1-2): 177–182

Eriksen M, Lebreton LCM, Carson HS, Thiel M, Moore CJ, Borerro JC, Galgani F, Ryan PG, Reisser J (2014) Plastic pollution in the World's oceans: More than 5 trillion plastic pieces weighing over 250,000 tons afloat at sea. PLoS One 9(12): e111913

Eriksson C, Burton H (2003) Origins and biological accumulation of small plastic particles in fur deals from Macquarie Island. Ambio 32(6): 380–384

Farrell P, Nelson K (2013) Trophic level transfer of microplastic: *Mytilus edulis* (L.) to *Carcinus maenas* (L.). Environ Pollut 177: 1–3

Faure F, Demars C, Wieser O, Kunz M, Alencastro LF de (2015) Plastic pollution in Swiss surface waters: Nature and concentrations, interaction with pollutants. Environ Chem 12(5): 582

Fendall LS, Sewell MA (2009) Contributing to marine pollution by washing your face: Microplastics in facial cleansers. Mar Pollut Bull 58(8): 1225–1228

Fischer EK, Paglialonga L, Czech E, Tamminga M (2016) Microplastic pollution in lakes and lake shoreline sediments - A case study on Lake Bolsena and Lake Chiusi (central Italy). Environ Pollut 213: 648–657

Folmar LC, Denslow ND, Rao V, Chow M, Crain DA, Enblom J, Marcino J, Guillette LJ (1996) Vitellogenin induction and reduced serum testosterone concentrations in feral male carp (*Cyprinus carpio*) captured near a major metropolitan sewage treatment plant. Environ Health Perspect 104(10): 1096–1101

Free CM, Jensen OP, Mason SA, Eriksen M, Williamson NJ, Boldgiv B (2014) High-levels of microplastic pollution in a large, remote, mountain lake. Mar Pollut Bull 85(1): 156–163

Fries E, Dekiff JH, Willmeyer J, Nuelle M-T, Ebert M, Remy D (2013) Identification of polymer types and additives in marine microplastic particles using pyrolysis-GC/MS and scanning electron microscopy. Environ Sci Process Impacts 15(10): 1949–1956

Frydkjær CK, Iversen N, Roslev P (2017) Ingestion and egestion of microplastics by the cladoceran *Daphnia magna*: Effects of regular and irregular shaped plastic and sorbed phenanthrene. Bull Environ Contam Toxicol 99(6): 655–661

Gerritsen J (1982) Behavioral response of *Daphnia* to rate of temperature change: Possible enhancement of vertical migration. Limnol Oceanogr 27(2): 254–261

GESAMP (2015) Sources, fate and effects of microplastics in the marine environment: A global assessment. Rep. Stud. GESAMP

GESAMP (2016) Sources, fate and effects of microplastics in the marine environment: Part two of a global assessment. Rep. Stud. GESAMP

Geyer R, Kuczenski B, Zink T, Henderson A (2016) Common Misconceptions about recycling. J Ind Ecol 20(5): 1010–1017

Geyer R, Jambeck JR, Law KL (2017) Production, use, and fate of all plastics ever made. Sci Adv 3(7): e1700782

Gigault J, Halle AT, Baudrimont M, Pascal P-Y, Gauffre F, Phi T-L, El Hadri H, Grassl B, Reynaud S (2018) Current opinion: What is a nanoplastic? Environ Pollut 235: 1030–1034

Gophen M, Geller W (1984) Filter mesh size and food particle uptake by *Daphnia*. Oecologia 64(3): 408–412

Gouin T, Roche N, Lohmann R, Hodges G (2011) A thermodynamic approach for assessing the environmental exposure of chemicals absorbed to microplastic. Environ Sci Technol 45(4): 1466–1472

Goulden CE, Hornig LL (1980) Population oscillations and energy reserves in planktonic cladocera and their consequences to competition. Proc Natl Acad Sci U S A 77(3): 1716–1720

Gregory MR (1996) Plastic 'scrubbers' in hand cleansers: A further (and minor) source for marine pollution identified. Mar Pollut Bull 32(12): 867–871

Grossman C (1985) Interactions between the gonadal steroids and the immune system. Science 227(4684): 257–261

Hall NM, Berry KLE, Rintoul L, Hoogenboom MO (2015) Microplastic ingestion by scleractinian corals. Mar Biol 162(3): 725–732

Hämer J, Gutow L, Köhler A, Saborowski R (2014) Fate of microplastics in the marine isopod *Idotea emarginata*. Environ Sci Technol 48(22): 13451–13458

Han J, Qiu W, Meng S, Gao W (2012) Removal of ethinylestradiol (EE2) from water via adsorption on aliphatic polyamides. Water Res 46(17): 5715–5724

Hanke W, Leist KH (1971) The effect of ACTH and corticosteroids on carbohydrate metabolism during the metamorphosis of *Xenopus laevis*. Gen Comp Endocrinol 16(1): 137–148

Hayes T, Haston K, Tsui M, Hoang A, Haeffele C, Vonk A (2002) Atrazine-induced hermaphroditism at 0.1 ppb in American leopard frogs (*Rana pipiens*). Laboratory and field evidence. Environ Health Perspect 111(4): 568–575

Heimeier RA, Shi Y-B (2010) Amphibian metamorphosis as a model for studying endocrine disruption on vertebrate development: Effect of bisphenol A on thyroid hormone action. Gen Comp Endocrinol 168(2): 181–189

Hidalgo-Ruz V, Gutow L, Thompson RC, Thiel M (2012) Microplastics in the marine environment: A review of the methods used for identification and quantification. Environ Sci Technol 46(6): 3060–3075

Hogg ID, Norris RH (1991) Effects of runoff from land clearing and urban development on the distribution and abundance of macroinvertebrates in pool areas of a river. Mar Freshwater Res 42(5): 507

Horton AA, Walton A, Spurgeon DJ, Lahive E, Svendsen C (2017) Microplastics in freshwater and terrestrial environments: Evaluating the current understanding to identify the knowledge gaps and future research priorities. Sci Total Environ 586: 127–141

Horton PA, Rowan M, Webster KE, Peters RH (1979) Browsing and grazing by cladoceran filter feeders. Can J Zool 57(1): 206–212

Howard AG (2010) On the challenge of quantifying man-made nanoparticles in the aquatic environment. J Environ Monit 12(1): 135–142

Huerta Lwanga E, Gertsen H, Gooren H, Peters P, Salánki T, van der Ploeg M, Besseling E, Koelmans AA, Geissen V (2016) Microplastics in the Terrestrial Ecosystem: Implications for *Lumbricus terrestris* (Oligochaeta, Lumbricidae). Environ Sci Technol 50(5): 2685–2691

Hu F, Smith EE, Carr JA (2008) Effects of larval exposure to estradiol on spermatogenesis and in vitro gonadal steroid secretion in African clawed frogs, *Xenopus laevis*. Gen Comp Endocrinol 155(1): 190–200

Hu L, Su L, Xue Y, Mu J, Zhu J, Xu J, Shi H (2016) Uptake, accumulation and elimination of polystyrene microspheres in tadpoles of *Xenopus tropicalis*. Chemosphere 164: 611–617

Imhof HK, Schmid J, Niessner R, Ivleva NP, Laforsch C (2012) A novel, highly efficient method for the separation and quantification of plastic particles in sediments of aquatic environments. Limnol Oceanogr: Methods 10(7): 524–537

Imhof HK, Ivleva NP, Schmid J, Niessner R, Laforsch C (2013) Contamination of beach sediments of a subalpine lake with microplastic particles. Curr Biol 23(19): R867-R868

Imhof HK, Laforsch C, Wiesheu AC, Schmid J, Anger PM, Niessner R, Ivleva NP (2016) Pigments and plastic in limnetic ecosystems: A qualitative and quantitative study on microparticles of different size classes. Water Res 98: 64–74

Imhof HK, Rusek J, Thiel M, Wolinska J, Laforsch C (2017) Do microplastic particles affect *Daphnia magna* at the morphological, life history and molecular level? PLoS One 12(11): e0187590

Jambeck JR, Geyer R, Wilcox C, Siegler TR, Perryman M, Andrady A, Narayan R, Law KL (2015) Marine pollution. Plastic waste inputs from land into the ocean. Science 347(6223): 768–771

Jemec A, Horvat P, Kunej U, Bele M, Kržan A (2016) Uptake and effects of microplastic textile fibers on freshwater crustacean *Daphnia magna*. Environ Pollut 219: 201–209

Jones OA, Voulvoulis N, Lester JN (2001) Human pharmaceuticals in the aquatic environment a review. Environ Technol 22(12): 1383–1394

Jorgensen E (2010) Ecotoxicology. Academic Press, New York

Jovanović B, Gökdağ K, Güven O, Emre Y, Whitley EM, Kideys AE (2018) Virgin microplastics are not causing imminent harm to fish after dietary exposure. Mar Pollut Bull 130: 123–131

Kaltenbach JC (1958) Direct steroid enhancement of induced metamorphosis in peripheral tissues. Anat Rec 3(131): 569–570

Karami A, Romano N, Galloway T, Hamzah H (2016) Virgin microplastics cause toxicity and modulate the impacts of phenanthrene on biomarker responses in African catfish (*Clarias gariepinus*). Environ Res 151: 58–70

Karami A, Groman DB, Wilson SP, Ismail P, Neela VK (2017) Biomarker responses in zebrafish (*Danio rerio*) larvae exposed to pristine low-density polyethylene fragments. Environ Pollut 223: 466–475

Karami A (2017) Gaps in aquatic toxicological studies of microplastics. Chemosphere 184: 841–848

Khan FR, Syberg K, Shashoua Y, Bury NR (2015) Influence of polyethylene microplastic beads on the uptake and localization of silver in zebrafish (*Danio rerio*). Environ Pollut 206: 73–79

Klein S, Worch E, Knepper TP (2015) Occurrence and spatial distribution of microplastics in river shore sediments of the Rhine-Main area in Germany. Environ Sci Technol 49(10): 6070–6076

Kleinteich J, Seidensticker S, Marggrander N, Zarfl C (2018) Microplastics reduce short-term effects of environmental contaminants. Part II: Polyethylene particles decrease the effect of polycyclic aromatic hydrocarbons on microorganisms. Int J Environ Res Public Health 15(2): 287

Kloas W, Reinecke M, Hanke W (1997) Stage-dependent changes in adrenal steroids and catecholamines during development in *Xenopus laevis*. Gen Comp Endocrinol 108(3): 416–426

Kloas W, Lutz I, Einspanier R (1999) Amphibians as a model to study endocrine disruptors: II. Estrogenic activity of environmental chemicals in vitro and in vivo. Sci Total Environ 225(1-2): 59–68

Kloas W (2002) Amphibians as a model for the study of endocrine disruptors. Int Rev Cytol 216: 1–57

Kloas W, Urbatzka R, Opitz R, Würtz S, Behrends T, Hermelink B, Hofmann F, Jagnytsch O, Kroupova H, Lorenz C, Neumann N, Pietsch C, Trubiroha A, van Ballegooy C, Wiedemann C, Lutz I (2009) Endocrine disruption in aquatic vertebrates. Ann N Y Acad Sci 1163: 187–200

Kloas W, Lutz I (2010) Hypothalamic pituitary gonadal axis amphibians. In: Eldridge JC, Stevens JT (eds) Endocrine Toxicology, 3rd ed. CRC press, Boca Raton, pp 352–364

Klüttgen B, Dülmer U, Engels M, Ratte HT (1994) ADaM, an artificial freshwater for the culture of zooplankton. Water Res 28(3): 743–746

Koelmans AA, Besseling E, Wegner A, Foekema EM (2013) Plastic as a carrier of POPs to aquatic organisms: a model analysis. Environ Sci Technol 47(14): 7812–7820

Koelmans AA, Besseling E, Foekema EM (2014) Leaching of plastic additives to marine organisms. Environ Pollut 187: 49–54

Koelmans AA, Besseling E, Shim WJ (2015) Nanoplastics in the aquatic environment. Critical review. In: Bergmann M, Gutow L, Klages M (eds) Marine anthropogenic litter. Springer, Berlin, pp 325–340

Koelmans AA, Bakir A, Burton GA, Janssen CR (2016) Microplastic as a vector for chemicals in the aquatic environment: Critical review and model-supported reinterpretation of empirical studies. Environ Sci Technol 50(7): 3315–3326

Koelmans AA, Besseling E, Foekema E, Kooi M, Mintenig S, Ossendorp BC, Redondo-Hasselerharm PE, Verschoor A, van Wezel AP, Scheffer M (2017) Risks of Plastic Debris: Unravelling Fact, Opinion, Perception, and Belief. Environ Sci Technol 51(20): 11513–11519

Kuch HM, Ballschmiter K (2001) Determination of endocrine-disrupting phenolic compounds and estrogens in surface and drinking water by HRGC–(NCI)–MS in the picogram per liter range. Environ Sci Technol 35(15): 3201–3206

Lai KM, Scrimshaw MD, Lester JN (2002) Prediction of the bioaccumulation factors and body burden of natural and synthetic estrogens in aquatic organisms in the river systems. Sci Total Environ 289(1-3): 159–168

Laist DW (1987) Overview of the biological effects of lost and discarded plastic debris in the marine environment. Mar Pollut Bull 18(6): 319–326

Laist DW (1997) Impacts of marine debris: Entanglement of marine life in marine debris including a comprehensive list of species with entanglement and ingestion records. In: Coe JM, Rogers DB (eds) Marine debris. Sources, Impacts, and Solutions. Springer, New York, pp 99–139

Lampert W (1987) Feeding and nutrition in *Daphnia*. Mem Ist Ital Idrobiol 45: 143

Lampert W (1991) The dynamics of *Daphnia magna* in a shallow lake. Verh - Int Ver Theor Angew Limnol 24(2): 795–798

Lattin GL, Moore CJ, Zellers AF, Moore SL, Weisberg SB (2004) A comparison of neustonic plastic and zooplankton at different depths near the southern California shore. Mar Pollut Bull 49(4): 291–294

Law KL, Morét-Ferguson S, Maximenko NA, Proskurowski G, Peacock EE, Hafner J, Reddy CM (2010) Plastic accumulation in the North Atlantic subtropical gyre. Science 329(5996): 1185–1188

Lebreton LCM, van der Zwet J, Damsteeg J-W, Slat B, Andrady A, Reisser J (2017) River plastic emissions to the world's oceans. Nat Commun 8: 15611

Lechner A, Keckeis H, Lumesberger-Loisl F, Zens B, Krusch R, Tritthart M, Glas M, Schludermann E (2014) The Danube so colourful: A potpourri of plastic litter outnumbers fish larvae in Europe's second largest river. Environ Pollut 188: 177–181

Lechner A, Ramler D (2015) The discharge of certain amounts of industrial microplastic from a production plant into the River Danube is permitted by the Austrian legislation. Environ Pollut 200: 159–160

Lee H, Shim WJ, Kwon J-H (2014) Sorption capacity of plastic debris for hydrophobic organic chemicals. Sci Total Environ 470-471: 1545–1552

Lee K-W, Shim WJ, Kwon OY, Kang J-H (2013) Size-dependent effects of micro polystyrene particles in the marine copepod *Tigriopus japonicus*. Environ Sci Technol 47(19): 11278–11283

Lei L, Wu S, Lu S, Liu M, Song Y, Fu Z, Shi H, Raley-Susman KM, He D (2018) Microplastic particles cause intestinal damage and other adverse effects in zebrafish *Danio rerio* and nematode *Caenorhabditis elegans*. Sci Total Environ 619-620: 1–8

Lenz R, Enders K, Nielsen TG (2016) Microplastic exposure studies should be environmentally realistic. Proc Natl Acad Sci U S A 113(29): E4121-2

Levy G, Lutz I, Krüger A, Kloas W (2004) Bisphenol A induces feminization in *Xenopus laevis* tadpoles. Environ Res 94(1): 102–111

Lithner D, Larsson A, Dave G (2011) Environmental and health hazard ranking and assessment of plastic polymers based on chemical composition. Sci Total Environ 409(18): 3309–3324

Lithner D, Nordensvan I, Dave G (2012) Comparative acute toxicity of leachates from plastic products made of polypropylene, polyethylene, PVC, acrylonitrile-butadiene-styrene, and epoxy to *Daphnia magna*. Environ Sci Pollut Res Int 19(5): 1763–1772

Löder MGJ, Gerdts G (2015) Methodology used for the detection and identification of microplastics - A critical appraisal. In: Bergmann M, Gutow L, Klages M (eds) Marine anthropogenic litter. Springer, Berlin, pp 201–227

Lovern SB, Klaper R (2006) *Daphnia magna* mortality when exposed to titanium dioxide and fullerene (C60) nanoparticles. Environ Toxicol Chem 25(4): 1132–1137

Lusher AL, McHugh M, Thompson RC (2013) Occurrence of microplastics in the gastrointestinal tract of pelagic and demersal fish from the English Channel. Mar Pollut Bull 67(1-2): 94–99

Lusher AL, Hernandez-Milian G, O'Brien J, Berrow S, O'Connor I, Officer R (2015) Microplastic and macroplastic ingestion by a deep diving, oceanic cetacean: The True's beaked whale *Mesoplodon mirus*. Environ Pollut 199: 185–191

Lutz I, Jie Z, Opitz R, Kloas W, Ying X, Menzel R, Steinberg CEW (2005a) Environmental signals: Synthetic humic substances act as xeno-estrogen and affect the thyroid system of *Xenopus laevis*. Chemosphere 61(8): 1183–1188

Lutz I, Blödt S, Kloas W (2005b) Regulation of estrogen receptors in primary cultured hepatocytes of the amphibian *Xenopus laevis* as estrogenic biomarker and its application in environmental monitoring. Comp Biochem Physiol C 141(4): 384–392

Lutz I, Kloas W, Springer TA, Holden LR, Wolf JC, Krueger HO, Hosmer AJ (2008) Development, standardization and refinement of procedures for evaluating effects of endocrine active compounds on development and sexual differentiation of *Xenopus laevis*. Anal Bioanal Chem 390(8): 2031–2048

Lu Y, Zhang Y, Deng Y, Jiang W, Zhao Y, Geng J, Ding L, Ren H (2016) Uptake and accumulation of polystyrene microplastics in zebrafish (*Danio rerio*) and toxic effects in liver. Environ Sci Technol 50(7): 4054–4060

Malaj E, Ohe PC von der, Grote M, Kühne R, Mondy CP, Usseglio-Polatera P, Brack W, Schäfer RB (2014) Organic chemicals jeopardize the health of freshwater ecosystems on the continental scale. Proc Natl Acad Sci U S A 111(26): 9549–9554

Marco A, Quilchano C, Blaustein AR (1999) Sensitivity to nitrate and nitrite in pond-breeding amphibians from the Pacific Northwest, USA. Environ Toxicol Chem 18(12): 2836–2839

Mato Y, Isobe T, Takada H, Kanehiro H, Ohtake C, Kaminuma T (2001) Plastic resin pellets as a transport medium for toxic chemicals in the marine environment. Environ Sci Technol 35(2): 318–324

Ma Y, Huang A, Cao S, Sun F, Wang L, Guo H, Ji R (2016) Effects of nanoplastics and microplastics on toxicity, bioaccumulation, and environmental fate of phenanthrene in fresh water. Environ Pollut 219: 166–173

Mayer P, Wernsing J, Tolls J, Maagd PG-J de, Sijm DTHM (1999) Establishing and controlling dissolved concentrations of hydrophobic organics by partitioning from a solid phase. Environ Sci Technol 33(13): 2284–2290

Mazurais D, Ernande B, Quazuguel P, Severe A, Huelvan C, Madec L, Mouchel O, Soudant P, Robbens J, Huvet A, Zambonino-Infante J (2015) Evaluation of the impact of polyethylene microbeads ingestion in European sea bass (*Dicentrarchus labrax*) larvae. Mar Environ Res 112: 78–85

McCabe G d., O'Brien WJ (1983) The effects of suspended silt on feeding and reproduction of *Daphnia pulex*. Am Midl Nat 110(2): 324–337

McCormick AR, Hoellein TJ, London MG, Hittie J, Scott JW, Kelly JJ (2016) Microplastic in surface waters of urban rivers: Concentration, sources, and associated bacterial assemblages. Ecosphere 7(11): e01556

McDonough W, Braungart M (2010) Cradle to cradle: Remaking the way we make things. North point press, New York

McMahon JW, Rigler FH (1965) Feeding rate of *Daphnia magna* Straus in different foods labeled with radioactive phosphoros. Limnol Oceanogr 10(1): 105–113

Meybeck M (2004) The global change of continental aquatic systems: dominant impacts of human activities. Water Sci Technol 49(7): 73–83

Mohamed Nor NH, Koelmans AA (2019) Transfer of PCBs from microplastics under simulated gut fluid conditions is biphasic and reversible. Environ Sci Technol 53(4): 1874–1883

Montoya JM, Donohue I, Pimm SL (2018) Why a planetary boundary, if it is not planetary, and the boundary is undefined? A reply to Rockström et al. Trends Ecol Evol 33(4): 234

Moore CJ, Moore SL, Leecaster MK, Weisberg SB (2001) A comparison of plastic and plankton in the North Pacific Central Gyre. Mar Pollut Bull 42(12): 1297–1300

Moore CJ, Lattin GL, Zellers AF (2005) A brief analysis of organic pollutants sorbed to pre and post-production plastic particles from the Los Angeles and San Gabriel river Watersheds. Proceedings of the Plastic Debris Rivers to Sea Conference, Algalita Marine Research Foundation, Long Beach, CA

Moore CJ (2008) Synthetic polymers in the marine environment: A rapidly increasing, long-term threat. Environ Res 108(2): 131–139

Moore CJ, Lattin GL, Zellers AF (2011) Quantity and type of plastic debris flowing from two urban rivers to coastal waters and beaches of Southern California. J Integr Coast Zone Manag 11(1): 65–73

Murphy F, Ewins C, Carbonnier F, Quinn B (2016) Wastewater treatment works (WwTW) as a source of microplastics in the aquatic environment. Environ Sci Technol 50(11): 5800–5808

Murtaugh PA (1985) The influence of food concentration and feeding rate on the gut residence time of *Daphnia*. J Plankton Res 7(3): 415–420

Nieuwkoop PD, Faber J (1958) Normal table of *Xenopus Laevis* (Daudin). A systematical and chronological survey of the development from the fertilized egg till the end of metamorphosis. Q Rev Biol 33(1): 85

Nimrod AC, Benson WH (1996) Environmental estrogenic effects of alkylphenol ethoxylates. Crit Rev Toxicol 26(3): 335–364

Norris D, Carr JA (2013) Vertebrate Endocrinology, 5th Edition. Academic Press, New York

Ogonowski M, Schür C, Jarsén Å, Gorokhova E (2016) The effects of natural and anthropogenic microparticles on individual fitness in *Daphnia magna*. PLoS One 11(5): e0155063

Oliveira M, Ribeiro A, Hylland K, Guilhermino L (2013) Single and combined effects of microplastics and pyrene on juveniles (0+ group) of the common goby *Pomatoschistus microps* (Teleostei, Gobiidae). Ecol Indic 34: 641–647

Organization for Economic Co-operation and Development (2004) OECD guidelines for the testing of chemicals, section 2, test no. 202: *Daphnia* sp. acute immobilisation test

Palmer BD, Palmer SK (1995) Vitellogenin induction by xenobiotic estrogens in the red-eared turtle and African clawed frog. Environ Health Perspect 103: 19–25

Parslow JLF, Jefferies DJ (1972) Elastic thread pollution of puffins. Mar Pollut Bull 3(3): 43–45

Paul-Pont I, Lacroix C, González Fernández C, Hégaret H, Lambert C, Le Goïc N, Frère L, Cassone A-L, Sussarellu R, Fabioux C, Guyomarch J, Albentosa M, Huvet A, Soudant P (2016) Exposure of marine mussels *Mytilus* spp. to polystyrene microplastics: Toxicity and influence on fluoranthene bioaccumulation. Environ Pollut 216: 724–737

Perlman AJ, Wolffe AP, Champion J, Tata JR (1984) Regulation by estrogen receptor of vitellogenin gene transcription in *Xenopus* hepatocyte cultures. Mol Cell Endocrinol 38(2-3): 151–161

Peters CA, Bratton SP (2016) Urbanization is a major influence on microplastic ingestion by sunfish in the Brazos River Basin, Central Texas, USA. Environ Pollut 210: 380–387

Petit JR, Jouzel J, Raynaud D, Barkov NI, Barnola J-M, Basile I, Bender M, Chappellaz J, Davis M, Delaygue G, Delmotte M, Kotlyakov VM, Legrand M, Lipenkov VY, Lorius C, PÉpin L, Ritz C, Saltzman E, Stievenard M (1999) Climate and atmospheric history of the past 420,000 years from the Vostok ice core, Antarctica. Nature 399: 429

Pettersson I, Arukwe A, Lundstedt-Enkel K, Mortensen AS, Berg C (2006) Persistent sex-reversal and oviducal agenesis in adult *Xenopus* (Silurana) tropicalis frogs following larval exposure to the environmental pollutant ethynylestradiol. Aquat Toxicol 79(4): 356–365

Pettersson I, Berg C (2007) Environmentally relevant concentrations of ethynylestradiol cause female-biased sex ratios in *Xenopus tropicalis* and *Rana temporaria*. Environ Toxicol Chem 26(5): 1005–1009

Pfaffl MW (2001) A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res 29(9): e45

Plastics Europe (2015) Plastics - the facts 2014/2015: An analysis of European plastics production, demand and waste: 34 pp

Plastics Europe (2017) Plastics - the facts 2017: An analysis of European plastics

Rainieri S, Conlledo N, Larsen BK, Granby K, Barranco A (2018) Combined effects of microplastics and chemical contaminants on the organ toxicity of zebrafish (*Danio rerio*). Environ Res 162: 135–143

Rech S, Macaya-Caquilpán V, Pantoja JF, Rivadeneira MM, Campodónico CK, Thiel M (2015) Sampling of riverine litter with citizen scientists - Findings and recommendations. Environ Monit Assess 187(6): 335

Rehse S, Kloas W, Zarfl C (2016) Short-term exposure with high concentrations of pristine microplastic particles leads to immobilisation of *Daphnia magna*. Chemosphere 153: 91–99

Rehse S, Kloas W, Zarfl C (2018) Microplastics reduce short-term effects of environmental contaminants. Part I: Effects of bisphenol A on freshwater zooplankton are lower in presence of polyamide particles. Int J Environ Res Public Health 15(2): 280

Ricciardi A, Rasmussen JB (1999) Extinction rates of North American freshwater fauna. Conserv Biol 13(5): 1220–1222

Rist S, Baun A, Hartmann NB (2017) Ingestion of micro- and nanoplastics in *Daphnia magna* - Quantification of body burdens and assessment of feeding rates and reproduction. Environ Pollut 228: 398–407

Rochman CM, Hoh E, Kurobe T, Teh SJ (2013) Ingested plastic transfers hazardous chemicals to fish and induces hepatic stress. Sci Rep 3: 3263

Rochman CM, Kurobe T, Flores I, Teh SJ (2014) Early warning signs of endocrine disruption in adult fish from the ingestion of polyethylene with and without sorbed chemical pollutants from the marine environment. Sci Total Environ 493: 656–661

Rochman CM, Parnis JM, Browne MA, Serrato S, Reiner EJ, Robson M, Young T, Diamond ML, Teh SJ (2017) Direct and indirect effects of different types of microplastics on freshwater prey (*Corbicula fluminea*) and their predator (*Acipenser transmontanus*). PLoS One 12(11): e0187664

Rockström J, Steffen W, Noone K, Persson Å, Chapin III FS, Lambin EF, Lenton TM, Scheffer M, Folke C, Schellnhuber HJ, Nykvist B, Wit CA de, Hughes T, van der Leeuw S, Rodhe H, Sörlin S, Snyder PK, Costanza R, Svedin U, Falkenmark M, Karlberg L, Corell RW, Fabry VJ, Hansen J, Walker B, Liverman D, Richardson K, Crutzen P, Foley JA (2009a) A safe operating space for humanity. Nature 461: 472

Rockström J, Steffen WL, Noone K, Persson Å, Chapin III FS, Lambin E, Lenton TM, Scheffer M, Folke C, Schellnhuber HJ (2009b) Planetary boundaries: Exploring the safe operating space for humanity. Ecol Soc 14(2): 32

Romano N, Ashikin M, Teh JC, Syukri F, Karami A (2018) Effects of pristine polyvinyl chloride fragments on whole body histology and protease activity in silver barb *Barbodes gonionotus* fry. Environ Pollut 237: 1106–1111

Rosenkranz P, Chaudhry Q, Stone V, Fernandes TF (2009) A comparison of nanoparticle and fine particle uptake by *Daphnia magna*. Environ Toxicol Chem 28(10): 2142–2149

Ryan PG (1987) The incidence and characteristics of plastic particles ingested by seabirds. Mar Environ Res 23(3): 175–206

Ryan PG, Connell AD, Gardner BD (1988) Plastic ingestion and PCBs in seabirds: Is there a relationship? Mar Pollut Bull 19(4): 174–176

Ryan PG (1989) The effects of ingested plastic and other marine debris on seabirds. In: Shomura RS, Godfrey ML (eds) Proceedings of the Second International Conference on Marine Debris, 2-7 April 1989, Honolulu, Hawaii, pp 623–634

Ryan PG, Moore CJ, van Franeker JA, Moloney CL (2009) Monitoring the abundance of plastic debris in the marine environment. Philos Trans R Soc Lond , B, Biol Sci 364(1526): 1999–2012

Sadri SS, Thompson RC (2014) On the quantity and composition of floating plastic debris entering and leaving the Tamar Estuary, Southwest England. Mar Pollut Bull 81(1): 55–60

Sala OE (2000) Global biodiversity scenarios for the year 2100. Science 287(5459): 1770–1774

Sanchez W, Bender C, Porcher J-M (2014) Wild gudgeons (*Gobio gobio*) from French rivers are contaminated by microplastics: Preliminary study and first evidence. Environ Res 128: 98–100

Scherer C, Weber A, Lambert S, Wagner M (2018) Interactions of microplastics with freshwater biota. In: Wagner M, Lambert S (eds) Freshwater Microplastics. Springer International Publishing, Cham, pp 153–180

Schuytema GS, Nebeker AV (1999) Comparative toxicity of ammonium and nitrate compounds to pacific treefrog and African clawed frog tadpoles. Environ Toxicol Chem 18(10): 2251–2257

Schwarzenbach RP, Gschwend PM, Imboden DM (2016) Environmental organic chemistry, 3rd edition. John Wiley & Sons, Hoboken

Setälä O, Fleming-Lehtinen V, Lehtiniemi M (2014) Ingestion and transfer of microplastics in the planktonic food web. Environ Pollut 185: 77–83

Singer SF (1970) Global effects of environmental pollution. Eos Trans AGU 51(5): 476–478

Sjollema SB, Redondo-Hasselerharm P, Leslie HA, Kraak MHS, Vethaak AD (2016) Do plastic particles affect microalgal photosynthesis and growth? Aquat Toxicol 170: 259–261

Sleight VA, Bakir A, Thompson RC, Henry TB (2017) Assessment of microplastic-sorbed contaminant bioavailability through analysis of biomarker gene expression in larval zebrafish. Mar Pollut Bull 116(1-2): 291–297

Sohn YC, Kobayashi M, Aida K (2001) Regulation of gonadotropin β subunit gene expression by testosterone and gonadotropin-releasing hormones in the goldfish, *Carassius auratus*. Comp Biochem Physiol B 129(2-3): 419–426

Song YK, Hong SH, Jang M, Han GM, Rani M, Lee J, Shim WJ (2015) A comparison of microscopic and spectroscopic identification methods for analysis of microplastics in environmental samples. Mar Pollut Bull 93(1-2): 202–209

Sperling LH (2005) Introduction to physical polymer science. John Wiley & Sons, Hoboken

Staples CA, Dome PB, Klecka GM, Oblock ST, Harris LR (1998) A review of the environmental fate, effects, and exposures of bisphenol A. Chemosphere 36(10): 2149–2173

Staudinger H (1920) Über Polymerisation. Ber Dtsch Chem Ges 53(6): 1073–1085

Steffen W, Persson Å, Deutsch L, Zalasiewicz J, Williams M, Richardson K, Crumley C, Crutzen P, Folke C, Gordon L (2011) The Anthropocene: From global change to planetary stewardship. Ambio 40(7): 739

Steffen W, Richardson K, Rockström J, Cornell SE, Fetzer I, Bennett EM, Biggs R, Carpenter SR, Vries W de, Wit CA de, Folke C, Gerten D, Heinke J, Mace GM, Persson LM, Ramanathan V, Reyers B, Sörlin S (2015) Planetary boundaries: Guiding human development on a changing planet. Science 347(6223): 1259855

Stuart SN, Chanson JS, Cox NA, Young BE, Rodrigues ASL, Fischman DL, Waller RW (2004) Status and trends of amphibian declines and extinctions worldwide. Science 306(5702): 1783–1786

Syberg K, Khan FR, Selck H, Palmqvist A, Banta GT, Daley J, Sano L, Duhaime MB (2015) Microplastics: Addressing ecological risk through lessons learned. Environ Toxicol Chem 34(5): 945–953

Tamschick S, Rozenblut-Kościsty B, Ogielska M, Lehmann A, Lymberakis P, Hoffmann F, Lutz I, Kloas W, Stöck M (2016) Sex reversal assessments reveal different vulnerability to endocrine disruption between deeply diverged anuran lineages. Sci Rep 6: 23825

Ter Halle A, Ladirat L, Gendre X, Goudouneche D, Pusineri C, Routaboul C, Tenailleau C, Duployer B, Perez E (2016) Understanding the fragmentation pattern of marine plastic debris. Environ Sci Technol 50(11): 5668–5675

Ternes TA, Stumpf M, Mueller J, Haberer K, Wilken R-D, Servos M (1999) Behavior and occurrence of estrogens in municipal sewage treatment plants - I. Investigations in Germany, Canada and Brazil. Sci Total Environ 225(1-2): 81–90

Teuten EL, Rowland SJ, Galloway TS, Thompson RC (2007) Potential for plastics to transport hydrophobic contaminants. Environ Sci Technol 41(22): 7759–7764

Teuten EL, Saquing JM, Knappe DRU, Barlaz MA, Jonsson S, Björn A, Rowland SJ, Thompson RC, Galloway TS, Yamashita R, Ochi D, Watanuki Y, Moore C, Viet PH, Tana TS, Prudente M, Boonyatumanond R, Zakaria MP, Akkhavong K, Ogata Y, Hirai H, Iwasa S, Mizukawa K, Hagino Y, Imamura A, Saha M, Takada H (2009) Transport and release of chemicals from plastics to the environment and to wildlife. Philos Trans R Soc Lond , B, Biol Sci 364(1526): 2027–2045

Tompsett AR, Wiseman S, Higley E, Pryce S, Chang H, Giesy JP, Hecker M (2012) Effects of 17α-ethynylestradiol on sexual differentiation and development of the African clawed frog (*Xenopus laevis*). Comp Biochem Physiol C 156(3-4): 202–210

Turner B, Skole DL, Sanderson S, Fischer G, Fresco L, Leemans R (1995) Land-use and land-cover change

Tussellino M, Ronca R, Formiggini F, Marco ND, Fusco S, Netti PA, Carotenuto R (2015) Polystyrene nanoparticles affect *Xenopus laevis* development. J Nanopart Res 17: 70

Urbatzka R, Lutz I, Opitz R, Kloas W (2006) Luteinizing hormone, follicle stimulating hormone, and gonadotropin releasing hormone mRNA expression of *Xenopus laevis* in response to endocrine disrupting compounds affecting reproductive biology. Gen Comp Endocrinol 146(2): 119–125

Urbatzka R, Lutz I, Kloas W (2007a) Aromatase, steroid-5-alpha-reductase type 1 and type 2 mRNA expression in gonads and in brain of *Xenopus laevis* during ontogeny. Gen Comp Endocrinol 153(1-3): 280–288

Urbatzka R, Bottero S, Mandich A, Lutz I, Kloas W (2007b) Endocrine disrupters with (anti)estrogenic and (anti)androgenic modes of action affecting reproductive biology of *Xenopus laevis*: I. Effects on sex steroid levels and biomarker expression. Comp Biochem Physiol C 144(4): 310–318

Urbatzka R, Watermann B, Lutz I, Kloas W (2009) Exposure of *Xenopus laevis* tadpoles to finasteride, an inhibitor of 5-alpha reductase activity, impairs spermatogenesis and alters hypophyseal feedback mechanisms. J Mol Endocrinol 43(5): 209–219

Urbatzka R, Lorenz C, Lutz I, Kloas W (2010) Expression profiles of LHbeta, FSHbeta and their gonadal receptor mRNAs during sexual differentiation of *Xenopus laevis* tadpoles. Gen Comp Endocrinol 168(2): 239–244

Urbatzka R, Lorenz C, Wiedemann C, Lutz I, Kloas W (2014) Steroid exposure during larval development of *Xenopus laevis* affects mRNA expression of the reproductive pituitary-gonadal axis in a sex- and stage-dependent manner. Comp Biochem Physiol C 160: 1–8

van Cauwenberghe L, Vanreusel A, Mees J, Janssen CR (2013) Microplastic pollution in deep-sea sediments. Environ Pollut 182: 495–499

Vandermeersch G, van Cauwenberghe L, Janssen CR, Marques A, Granby K, Fait G, Kotterman MJJ, Diogène J, Bekaert K, Robbens J, Devriese L (2015) A critical view on microplastic quantification in aquatic organisms. Environ Res 143(Pt B): 46–55

Vaughan R, Turner SD, Rose NL (2017) Microplastics in the sediments of a UK urban lake. Environ Pollut 229: 10–18

Veith GD, Macek KJ, Petrocelli SR, Carroll J (1980) An evaluation of using partition coefficients and water solubility to estimate bioconcentration factors for organic chemicals in fish. In: Eaton JG, Parrish PR, Hendricks AC (eds) Aquatic Toxicology. ASTM International, West Conshohocken, p 116-116-14

Vianello A, Boldrin A, Guerriero P, Moschino V, Rella R, Sturaro A, Da Ros L (2013) Microplastic particles in sediments of Lagoon of Venice, Italy. First observations on occurrence, spatial patterns and identification. Estuarine, Coastal Shelf Sci 130: 54–61

von Moos N, Burkhardt-Holm P, Köhler A (2012) Uptake and effects of microplastics on cells and tissue of the blue mussel *Mytilus edulis* L. after an experimental exposure. Environ Sci Technol 46(20): 11327–11335

Walther DJ, Bader M (2003) A unique central tryptophan hydroxylase isoform. Biochem Pharmacol 66(9): 1673–1680

Wardrop P, Shimeta J, Nugegoda D, Morrison PD, Miranda A, Tang M, Clarke BO (2016) Chemical pollutants sorbed to igested microbeads from personal care products accumulate in fish. Environ Sci Technol 50(7): 4037–4044

Williams AT, Simmons SL (1997) Estuarine litter at the river/beach interface in the Bristol Channel, United Kingdom. J Coastal Res 13(4): 1159–1165

Windsor FM, Durance I, Horton AA, Thompson RC, Tyler CR, Ormerod SJ (2019) A catchment-scale perspective of plastic pollution. Glob Chang Biol 25: 1207–1221

Wolf JC, Lutz I, Kloas W, Springer TA, Holden LR, Krueger HO, Hosmer AJ (2010) Effects of 17 beta-estradiol exposure on *Xenopus laevis* gonadal histopathology. Environ Toxicol Chem 29(5): 1091–1105

Woodall LC, Sanchez-Vidal A, Canals M, Paterson GLJ, Coppock R, Sleight V, Calafat A, Rogers AD, Narayanaswamy BE, Thompson RC (2014) The deep sea is a major sink for microplastic debris. R Soc Open Sci 1(4): 140317

Wright SL, Rowe D, Thompson RC, Galloway TS (2013a) Microplastic ingestion decreases energy reserves in marine worms. Curr Biol 23(23): R1031-R1033

Wright SL, Thompson RC, Galloway TS (2013b) The physical impacts of microplastics on marine organisms: A review. Environ Pollut 178: 483–492

Yamamoto H, Liljestrand HM (2004) Partitioning of selected estrogenic compounds between synthetic membrane vesicles and water: Effects of lipid components. Environ Sci Technol 38(4): 1139–1147

Yamashita R, Takada H, Fukuwaka M-a, Watanuki Y (2011) Physical and chemical effects of ingested plastic debris on short-tailed shearwaters, *Puffinus tenuirostris*, in the North Pacific Ocean. Mar Pollut Bull 62(12): 2845–2849

Yonkos LT, Friedel EA, Perez-Reyes AC, Ghosal S, Arthur CD (2014) Microplastics in four estuarine rivers in the Chesapeake Bay, U.S.A. Environ Sci Technol 48(24): 14195–14202

Yoshimoto S, Okada E, Umemoto H, Tamura K, Uno Y, Nishida-Umehara C, Matsuda Y, Takamatsu N, Shiba T, Ito M (2008) A W-linked DM-domain gene, DM-W, participates in primary ovary development in *Xenopus laevis*. Proc Natl Acad Sci U S A 105(7): 2469–2474

Yoshimoto S, Ikeda N, Izutsu Y, Shiba T, Takamatsu N, Ito M (2010) Opposite roles of DMRT1 and its W-linked paralogue, DM-W, in sexual dimorphism of *Xenopus laevis*: implications of a ZZ/ZW-type sex-determining system. Development 137(15): 2519–2526

Young RJ, Lovell PA (2011) Introduction to polymers. CRC press, Boca Raton

Zalasiewicz J, Waters CN, Ivar do Sul JA, Corcoran PL, Barnosky A d., Cearreta A, Edgeworth M, Gałuszka A, Jeandel C, Leinfelder R, McNeill JR, Steffen W, Summerhayes C, Wagreich M, Williams M, Wolfe AP, Yonan Y (2016) The geological cycle of plastics and their use as a stratigraphic indicator of the Anthropocene. Anthropocene 13: 4–17

Zarfl C, Fleet D, Fries E, Galgani F, Gerdts G, Hanke G, Matthies M (2011) Microplastics in oceans. Mar Pollut Bull 62(8): 1589–1591

Zbyszewski M, Corcoran PL (2011) Distribution and degradation of fresh water plastic particles along the beaches of Lake Huron, Canada. Water Air Soil Pollut 220(1-4): 365–372

Zbyszewski M, Corcoran PL, Hockin A (2014) Comparison of the distribution and degradation of plastic debris along shorelines of the Great Lakes, North America. J Great Lakes Res 40(2): 288–299

Zettler ER, Mincer TJ, Amaral-Zettler LA (2013) Life in the "plastisphere": Microbial communities on plastic marine debris. Environ Sci Technol 47(13): 7137–7146

Zhang K, Gong W, Lv J, Xiong X, Wu C (2015) Accumulation of floating microplastics behind the Three Gorges Dam. Environ Pollut 204: 117–123

Zhao S, Zhu L, Wang T, Li D (2014) Suspended microplastics in the surface water of the Yangtze Estuary System, China: First observations on occurrence, distribution. Mar Pollut Bull 86(1-2): 562–568

Ziajahromi S, Kumar A, Neale PA, Leusch FDL (2017) Impact of microplastic beads and fibers on waterflea (*Ceriodaphnia dubia*) survival, growth, and reproduction: Implications of single and mixture exposures. Environ Sci Technol 51(22): 13397–13406

Ziková A, Lorenz C, Lutz I, Pflugmacher S, Kloas W (2013) Physiological responses of *Xenopus laevis* tadpoles exposed to cyanobacterial biomass containing microcystin-LR. Aquat Toxicol 128-129: 25–33

Zitko V, Hanlon M (1991) Another source of pollution by plastics: Skin cleaners with plastic scrubbers. Mar Pollut Bull 22(1): 41–42

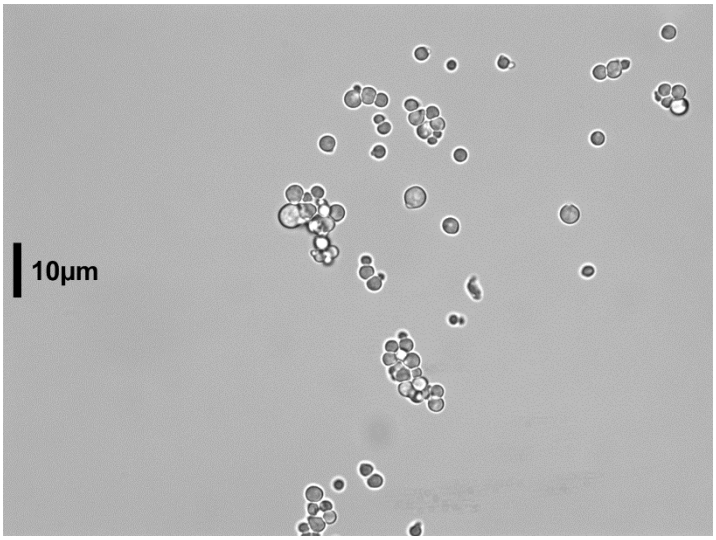
Zohar Y, Muñoz-Cueto JA, Elizur A, Kah O (2010) Neuroendocrinology of reproduction in teleost fish. Gen Comp Endocrinol 165(3): 438–455

# Appendices

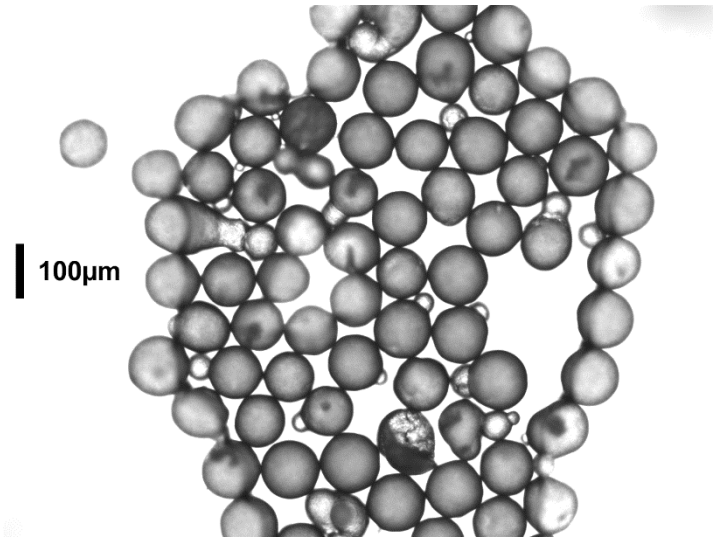
## A.1 Appendix for Chapter 2

*Appendix Table 1* *Reagents for the Aachener Daphnien Medium*
List of ingredients for the Aachener Daphnien Medium (AdaM) after Klüttgen et al. (1994) used for preparation of 1 µm and 100 µm mixtures and as *Daphnia* culture medium; * Wimex hw Marinemix professinal, Wiegandt GmbH, Krefeld, Germany.

| **Reagent** | **Amount** |
|---|---|
| Synthetic sea salt* | 0.333 g $L^{-1}$ |
| $CaCl_2$-solution, 0.8 mol $L^{-1}$ ($CaCl_2$ . $2H_20$ ) | 2.3 ml $L^{-1}$ |
| $NaHCO_3$-solution, 0.3 mol $L^{-1}$ ($NaHCO_3$) | 2.2 ml $L^{-1}$ |
| $SeO_2$-solution, 0.013 mol $L^{-1}$ ($SeO_2$) | 0.1 ml $L^{-1}$ |




*Appendix Figure 1* *Image of 1 µm PE particles*
Stereomicroscopic image of 1 µm PE particles confirming the spherical shape and the size of the particles (between 1-4 µm in diameter).




*Appendix Figure 2* *Image of 100 µm PE particles*
Stereomicroscopic image of 100 µm PE particles confirming the spherical shape and the size of the particles (between 90-106 µm in diameter).

# A.2 Appendix for Chapter 3

*Appendix Table 2* *Sorption characteristics of BPA to PA particles*

Measured concentrations (mg $L^{-1}$) of bisphenol A (BPA) in water via HPLC after 0, 6, 24, 48 and 72 hours of shaking with and without polyamide particles (microplastics, MP) as means and standard deviations (SD, n=2).

| | **Measured BPA Concentration (mg $L^{-1}$ ± SD)** | |
|---|---|---|
| **Time (hours)** | **BPA** | **BPA + MP** |
| 0 | 9.5 ± 0.0 | 9.0 ± 0.5 |
| 6 | 9.5 ± 0.0 | 8.0 ± 0.5 |
| 24 | 10.0 ± 0.0 | 8.0 ± 0.5 |
| 48 | 10.0 ± 0.0 | 7.5 ± 0.0 |
| 72 | 9.5 ± 0.0 | 7.5 ± 0.0 |

*Appendix Table 3* *Measured aqueous BPA in test vessels*

Measured bisphenol A (BPA) concentrations (mg $L^{-1}$) in water in test vessels at the beginning and the end of exposure experiments as means ± standard deviation (SD, n=5).

| | **Measured BPA Concentration (mg $L^{-1}$ ± SD)** | | | |
|---|---|---|---|---|
| **BPA Nominal (mg $L^{-1}$)** | **BPA alone** | | **BPA + MP** | |
| | **0 h** | **48 h** | **0 h** | **48 h** |
| 5 | 5.3 ± 0.0 | 4.8 ± 0.1 | 3.7 ± 0.1 | 3.4 ± 0.1 |
| 7.5 | 7.3 ± 0.3 | 6.8 ± 0.2 | 6.0 ± 0.0 | 5.6 ± 0.0 |
| 10 | 10.2 ± 0.4 | 9.9 ± 0.2 | 8.1 ± 0.2 | 7.5 ± 0.3 |
| 12.5 | 13.0 ± 0.3 | 13.0 ± 0.3 | 10.1 ± 0.3 | - |
| 15 | 15.8 ± 0.5 | 15.8 ± 0.0 | 12.8 ± 0.0 | 12.0 ± 0.0 |

## A.3 Appendix for Chapter 4

*Appendix Table 4* *Components for Technovit embedding*
Reagents and material used for embedding of heads and guts of tadpoles in Technovit 7100 (Kulzer GmbH, Wehrheim, Germany).

| Reagents and material | Description |
|---|---|
| Technovit 7100 | base liquid, 500 ml |
| hardener I | powder, 5 x 1 g |
| hardener II | liquid, 40 ml |
| Technovit 3040 yellow | 100 g powder, 80 ml liquid |
| histoform | block with moulds for embedding of samples |
| histoblocs | plastic blocs to attach to embedded samples |

*Appendix Table 5* *Reagents for staining of head and gut sections*
Pre-mixtures which were prepared for staining of head and gut sections of tadpoles.

| Mixed reagent | Description |
|---|---|
| blueing acid | 1 ml 1% HCl mixed with 99 ml 70% ethanol |
| 0.1% eosin solution | 1 g eosin dissolved in 10 ml distilled water, diluted 1:100 with distilled water |

*Appendix Table 6* *Components for analysis of corticosterone*

Reagents and materials which were included in the corticosterone ELISA kit (IBL International GmbH, Hamburg, Germany).

| **Reagent** | **Description** |
|---|---|
| microtiter wells | 12 x 8 (break apart) strips, 96 wells,<br>wells coated with an anti-corticosterone antibody (polyclonal) |
| standard (0-6) | 7 vials with 1 ml,<br>concentrations: 0, 5, 15, 30, 60, 120, 240 nmol $L^{-1}$,<br>conversion: 1 nmol $l^{-1}$=34.646 ng $dl^{-1}$== 0.34646 ng $ml^{-1}$ |
| control low & high | 2 vials with 1 ml |
| enzyme conjugate | 250x concentrate, 1 vial with 150 µl,<br>corticosterone conjugated to horseradish peroxidase,<br>diluted 1:250 with conjugate diluent |
| conjugate diluent | 1 vial with 25 ml |
| substrate solution | 1 vial with 25 ml,<br>tetramethylbenzidine (TMB) |
| stop solution | 1 vial with 14 ml,<br>contains 0.5M $H_2SO_4$ |
| wash solution | 1 vial with 30 ml (40x concentrated),<br>30 ml diluted with 1170 ml deionized water |

*Appendix Table 7* *Components for analysis of aldosterone*

Reagents and materials which were included in the aldosterone ELISA kit (IBL International GmbH, Hamburg, Germany).

| **Reagent** | **Description** |
|---|---|
| microtiter wells | 12 x 8 (break apart) strips, 96 wells,<br>coated with antibodies against aldosterone (polyclonal) |
| standard A-F | 0; 20; 80; 200; 500; 1000 pg $ml^{-1}$,<br>each dissolved with 1 ml distilled water,<br>conversion: 1 pg $ml^{-1}$ corresponds to 2.77 pmol $l^{-1}$,<br>contains aldosterone, in protein-containing buffer,<br>non-mercury preservatives |
| positive control | lyophilized,<br>contains: aldosterone in protein-containing buffer,<br>non-mercury preservatives |
| negative control | lyophilized,<br>contains: aldosterone in protein-containing buffer,<br>non-mercury preservatives |
| enzyme conjugate | contains: aldosterone-HRP conjugate,<br>in protein-containing buffer,<br>non mercury preservatives |
| TMB substrate solution | 1 vial with 25 mL<br>contains: Tetramethylbenzidine (TMB) |
| TMB Stop Solution | 0.5 M $H_2SO_4$ |
| wash Buffer | concentrate (40x),<br>dilution 1:40 with distilled water |

*Appendix Table 8* *Components for DNA extraction from tails*
Materials in the peqGOLD Tissue DNA Mini Kit (PEQLAB Biotechnologie GmbH, Erlangen, Germany) for DNA extraction from tail tissue.

| **Component** |
|---|
| PerfectBind DNA Columns |
| 2 ml Collection Tubes |
| DNA Lysis Buffer T |
| DNA Binding Buffer |
| DNA Wash Buffer |
| Elution Buffer (10 mM Tris-HCl, pH 9.0) |
| Proteinase K |
| RNase A (20 mg $ml^{-1}$) |
| 10 mM TE Buffer |

*Appendix Table 9* *PCR mixture for sexing*
Description and amounts of reagents used for a mixture for the PCR of gene specific DNA fragments.

| **Reagent** | **Description** | **Supplier** | **Amount** |
|---|---|---|---|
| PCR water | RNase/DNase free water | ThermoFisher | 19.25 |
| 10XTaq reaction buffer | includes 1,5mM $MgCl_2$ | Qiagen | 2.5 |
| dNTP- Mix | stock 10 mM per dNTP | Biometra | 0.25 |
| Taq | Taq-polymerase 5 U $\mu l^{-1}$ | Qiagen | 0.2 |

*Appendix Table 10* *Mixtures for gel electrophoresis for sexing*
Description and amounts of reagents used for the mixtures for gel electrophoresis of sex specific DNA fragments.

| **Reagent** | **Description and amount** |
|---|---|
| TAE buffer | Tris-Acetate-EDTA-buffer 50 fold with<br>242 g Tris HCl,<br>37.2 g $Na_2EDTA$,<br>57.1 ml glacial acetic acid in 1 l aqua bidest., pH 7.5<br>and diluted again to 1 fold |
| bromophenol blue solution | 600 µl RNase/DNase free water<br>600 µl glycerine<br>6 µl bromophenol blue |

*Appendix Table 11* *Components for mRNA extraction from pituitaries*
Reagents and material included in the RNeasy-Plus-Micro-Kit (Qiagen, Hilden, Germany) which was used for extraction of mRNA from pituitaries.

| **Component** | **Description** |
|---|---|
| gDNA Eliminator Spin Columns | remove DNA |
| RNeasy Mini Spin Columns | purify total RNA |
| Collection Tubes | collect discharge from columns |
| RNase-free water | water without RNase |
| RLT buffer | lysis buffer |
| RW1 buffer | wash buffer |
| RPE buffer | wash buffer |

*Appendix Table 12* *Components for mRNA extraction from gonads*
Reagents and material included in the ReliaPrep™ RNA Tissue Miniprep System (Promega, Mannheim, Germany) which was used for extraction of mRNA from gonads.

| **Component** | **Description** |
|---|---|
| ReliaPrep™ Mini columns | purify total RNA |
| Collection Tubes | collect discharge from columns |
| Elution Tubes | collect extracted RNA |
| LBA Buffer | LBA, used for homogenisation |
| Column Wash Solution | CWE, washing component |
| DNase I | lyophilized, remove DNA |
| $MnCl_2$ | 0.09M, component for DNA removal |
| Yellow Core Buffer | buffer for DNA removal |
| RNA Wash Solution | RWA |
| 1-Thioglycerol | added to the LBA buffer |
| Nuclease-Free Water | used to elute extracted RNA |
| RNA Dilution Buffer | RDB |

*Appendix Table 13* *Components for DNA removal in liver samples*
Reagents and material included in the DNase I, Amplification Grade kit (Invitrogen, Thermo Fisher Scientific, Berlin, Germany) which was used to remove possible DNA contamination from the mRNA samples from the livers.

| **Reagent** | **Amount (µl)** |
|---|---|
| DNase I | Amplification Grade, 100 U |
| DNase I Reaction Buffer | 10x, 1000 µl |
| ethylenediaminetetra-acetate (EDTA) | 25 mM, pH 8.0, 200 µl |

*Appendix Table 14* *Reagents for cDNA synthesis*

Descriptions of reagents used for cDNA synthesis in pituitary, gonad and liver RNA from tadpoles.

| Reagent | Description | Supplier |
|---|---|---|
| PCR water | RNase/DNase free water | Thermo Fisher Scientific, Berlin, Germany |
| oligo-dT-primer | 2.5 µM; sequence: CCTGAATTCTAGAGCTCA(T)17 | Biometra, Göttingen, Germany |
| poly-dT-Primer | 1:5 diluted; sequence: 5´CCTgAATTCTAgAgCTCA(T)17-3´ | Biometra, Göttingen, Germany |
| dNTPs | 10 mM each dNTP | Qiagene, Hilden, Germany |
| MMLV (Kit) | MMLV High Performance reverse transcriptase (RT), Reaction buffer (10 fold concentrate), Dithiothreitol (DTT; 100 mM) | Biozym, Hessisch Oldendorf, Germany |
| Affinity-Script-RT (Kit) | Affinity Script Multiple Temperature Reverse Transcriptase (RT), Affinity-Script reaction buffer (10 fold), Dithiothreitol (DTT; 100 mM) | Stratagene (Agilent), Waldbronn, Germany |

*Appendix Table 15* *Pre-mixtures for cDNA synthesis of pituitaries*

Amounts of each component used to prepare pre-mixtures for cDNA analysis of pituitaries in tadpoles.

| Reagent | Description | Supplier |
|---|---|---|
| Pre-mixture I | 1,5 µl poly-dT-Primer 1:5 dissolved with PCR water, 5´CCTgAATTCTAgAgCTCA(T)17-3´ | Biometra, Göttingen, Germany |
| Pre-mixture II | 1 µl Affinity Script Multiple Temperature Reverse Transcriptase | Stratagene (Agilent), Waldbronn, Germany |
| | 2 µl 10* Affinity Script RT buffer, | " |
| | 2µl DTT 100mM, | " |
| | 1 µl dNTP-solution, 10mM per dNTP, | Biometra, Göttingen, Germany |

*Appendix Table 16* *Reagents for real-time PCR of all samples*
Descriptions of all components used for real-time PCR of pituitary, gonad and liver samples.

| Reagent | Description | Supplier |
|---|---|---|
| PCR water | RNase/DNase free water | Thermo Fisher Scientific, Berlin, Germany |
| oligo-dT-primer | 2.5 µM; sequence: CCTGAATTCTAGAGCTCA(T)17 | Biometra, Göttingen, Germany |
| poly-dT-Primer | 1:5 diluted; sequence: 5´CCTgAATTCTAgAgCTCA(T)17-3´ | " |
| dNTPs | 10 mM each dNTP | Biometra, Göttingen, Göttingen |
| MMLV (Kit) | MMLV High Performance reverse transcriptase (RT);<br>Reaction buffer (10 fold concentrate);<br>Dithiothreitol (DTT; 100 mM) | Biometra, Göttingen, Germany |
| Affinity-Script-RT (Kit) | Affinity Script Multiple Temperature Reverse Transcriptase (RT);<br>Affinity-Script reaction buffer (10 fold concentrate);<br>Dithiothreitol (DTT; 100 mM) | Stratagene (Agilent), Waldbronn, Germany |
| Phire Hot Start II (Kit) | Phire Hot Start II DNA Polymerase;<br>Phire Reaction Buffer;<br>$MgCl_2$ 50mM | Biozym, Hessisch Oldendorf, Germany |
| Biozym qPCR Mix Separate ROX | qPCR 2xMix | " |
| SYBR Green DNA I dye | 1:200 diluted | Invitrogen (Thermo Fisher Scientific), Berlin, Germany |
| Platinum Taq polymerase | 5 U $\mu l^{-1}$ | " |

*Appendix Table 17* *Pre-mixtures for real-time PCR of pituitary samples*
Reagents and corresponding amounts for the pre-mixtures used for real-time PCR of pituitary samples for mRNA expression of EF, FSH and LH.

| Reagent | Amount (µl) for EF/FSH | Amount (µl) for LH |
|---|---|---|
| PCR water | 13.19 | 13.19 |
| 10x buffer for Taq DNA-Polymerase | 4.00 | 4.00 |
| dNTP-mixture | 0.20 | 0.20 |
| Sybr – 200 | 0.11 | 0.11 |
| forward primer EF/FSH (25-50 µM) | 0.15 | - |
| reverse primer EF/FSH (25-50 µM) | 0.15 | - |
| forward primer LH (25-50 µM) | - | 0.15 |
| reverse primer LH (25-50 µM) | - | 0.15 |
| Phire Taq Polymerase | 0.20 | 0.20 |
| $MgCl_2$ | - | 0.40 |

*Appendix Table 18* *Pre-mixture for real-time PCR of gonad samples*
Reagents and corresponding amounts for the pre-mixture used for real-time PCR of gonad samples for mRNA expression of aromatase, StAR, P450, S1 and S2.

| Reagent | Amount (µl) |
|---|---|
| PCR water | 14.00 |
| reaction buffer (10x) | 2.00 |
| $MgCl_2$ (50 mM) | 1 .00 |
| dNTPs (10 mM each) | 0.17 |
| SYBR Green DNA I dye (1:200) | 0.11 |
| forward primer (25-50 µM) | 0.20 |
| reverse primer (25-50 µM) | 0.20 |
| Platinum® Taq polymerase (5 U $\mu L^{-1}$) | 0.20 |

*Appendix Table 19* *Pre-mixture for real-time PCR of liver samples*
Reagents and corresponding amounts for the pre-mixture used for real-time PCR of samples for mRNA expression of vitellogenin.

| Reagent | Amount (µl) |
|---|---|
| PCR water | 7.70 |
| qPCR-Mix | 10.00 |
| Sybr – 200 | 0.11 |
| forward primer (25-50 µM) | 0.15 |
| reverse primer (25-50 µM) | 0.15 |

*Appendix Table 20* *mRNA expression of P450 and Aro after 21 days*

P450 and S2 mRNA expression in male and female tadpoles after 21 days of exposure with two concentrations of microplastics and one concentration of EE2 alone (MP low; MP high; EE2) and in combination with each other (MP low, EE2; MP high, EE2; MP loaded) and one solvent control (control) relative to the elongation factor (EF) and the control treatment as means +/- standard deviations (SD).

| | | Males | | Females | |
|---|---|---|---|---|---|
| **Biomarker** | **Treatment** | **Mean** | **SD** | **Mean** | **SD** |
| P450 | Control | 1.00 | 0.34 | 1.00 | 0.37 |
| | MP low | 1.31 | 0.23 | 0.80 | 0.31 |
| | MP high | 1.07 | 0.10 | 0.66 | 0.14 |
| | EE2 | 1.27 | 0.19 | 0.47 | 0.17 |
| | MP low, EE2 | 0.93 | 0.39 | 0.66 | 0.25 |
| | MP high, EE2 | 0.95 | 0.17 | 0.71 | 0.21 |
| | MP loaded | 1.28 | 0.32 | 0.92 | 0.34 |
| S2 | Control | 1.00 | 0.37 | 1.00 | 0.22 |
| | MP low | 1.81 | 0.56 | 1.47 | 0.29 |
| | MP high | 1.66 | 0.39 | 1.26 | 0.56 |
| | EE2 | 1.63 | 0.21 | 0.63 | 0.13 |
| | MP low, EE2 | 0.76 | 0.20 | 0.77 | 0.18 |
| | MP high, EE2 | 0.98 | 0.33 | 0.94 | 0.35 |
| | MP loaded | 1.23 | 0.33 | 1.12 | 0.31 |

*Appendix Table 21* *mRNA expression of vitellogenin after 21 days*

Vitellogenin mRNA expression in male and female tadpoles after 21 days of exposure with two concentrations of microplastics and one concentration of EE2 alone (MP low; MP high; EE2) and in combination with each other (MP low, EE2; MP high, EE2; MP loaded) and one solvent control (control) relative to the elongation factor (EF) and the control treatment as means +/- standard deviations (SD).

| | Vitellogenin | | | |
|---|---|---|---|---|
| | Males | | Females | |
| **Treatment** | **Mean** | **SD** | **Mean** | **SD** |
| Control | 1.00 | 0.34 | 1.00 | 0.58 |
| MP low | 0.90 | 0.36 | 1.13 | 0.68 |
| MP high | 1.50 | 1.34 | 1.43 | 0.75 |
| EE2 | 54.92 | 61.20 | 144.76 | 149.67 |
| MP low, EE2 | 549.52 | 468.55 | 171.79 | 115.53 |
| MP high, EE2 | 800.24 | 772.77 | 166.45 | 113.53 |
| MP loaded | 1.19 | 0.59 | 1.25 | 0.33 |

# Statement of academic integrity

I hereby declare that I alone wrote the doctoral work submitted here under the title "Effects of microplastics on freshwater organisms: A laboratory approach", that I only used the sources and materials cited in the work, and that all citations, whether word for word or paraphrased are given as such. I declare that I adhered to the guidelines set forth by the University of Tübingen to guarantee proper academic scholarship (Senate Resolution 25.05.2000). I declare that these statements are true and that I am concealing nothing. I understand that any false statements can be punished with a jail term of up to three years or a financial penalty.

Berlin, 2nd April 2019

Saskia Rehse