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Two ways of particle exposure for ecotoxicological testing with *Gammarus roeseli* as representative river-dwelling organism

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Preface

In this thesis, some of the challenges in aquatic plastic particle toxicity research are addressed and solutions are presented. In the Introduction (Chapter 3), the reader is introduced to the background of ecotoxicological research and the need of covering all trophic levels and compartments in this practice. Then, the why and how of plastic particle toxicity research including the challenges of plastic particle handling during the processes is addressed. These explanations lead to aims and objectives of this thesis presented in in chapter 4. In Chapter 5, the general study design and the methods to reach the objectives are explained.

Chapter 6 to 8 are the core of this thesis and present the studies to reach the objectives. First, a standardized bioassay for the river-dwelling shredder *Gammarus roeseli* is developed to fill a gap in the usually examined sea- and lake-dwelling organisms and address the importance of rivers regarding the transport and sink function for plastic particles. In the same step (Chapter 6), a dietary exposure tool for particles is examined to overcome the challenge of suspending the plastic particles in water and the resulting unpredictable distribution and exposure.

Both evaluated applications are then used to examine some scientific issues for plastic particles. The impact of three different particle types, natural particle, biodegradable particle, and non-biodegradable particle is examined in two concentrations and four sizes from 30 nm to 1000 nm on *G. roeseli* via dietary exposure in chapter 7. After the examination of those essential information for basic risk assessment, the experiment in chapter 8 is used to extend this knowledge with an examination of the assumed vector function of plastic particles for chemicals in the environment by using the common exposure via the water phase.

At last, chapter 9 discusses the developed procedures and investigated impacts of plastic particles regarding risk assessment. It highlights the need of adjustments for particle exposure studies as well as the importance of considering the objectives to choose the right exposure scenario for particles.

This thesis presents solutions to overcome the challenges in plastic particle risk assessment and can be used to examine the effects of particles on a wide range of shredders and grazers by simultaneously standardizing these investigations.

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List of Abbreviations

ANOVA ..... *Analysis of Variance*
DDT ..... *dichlorodiphenyltrichloroethane*
dw_F ..... :
dw_I ..... *Initial dry weight*
ECHA ..... *Europäische Chemikalienagentur/ European Chemicals Agency*
MWL ..... *Mean weight loss*
OECD ..... *Organisation for Economic Co-operation and Development*
PA ..... *Polyamide*
PCB ..... *Polychlorinated biphenyl*
PE ..... *Polyethylene*
PET ..... *Polyethylene terephthalate*
PLA ..... *Polylactide*
PMMA ..... *Polymethylmethacrylate*
POP ..... *Persistent Organic Pollutant*
PP ..... *Polypropylene*
PS ..... *Polystyrene*
PVC ..... *Polyvinylchlorid*
REACH .. *regulation for the Registration, Evaluation, Authorisation and Restriction of Chemicals*
tdw ..... *tab dry weight*
WFD/WRRL ..... *water framework directive/Wasserrahmenrichtlinie*
ww_I ..... *Initial wet weight*

1. Summary

Plastics are a pollution of great social and political concern which is thought to rise in the future because of the plastics persistence. Therefore, not only the impact of huge macroplastic like nets and bottles came into focus of ecotoxicological risk assessment but also the emergence, occurrence and effects of micro and nanoplastic are examined. Although the sampling and detection methods still struggle with the assessment of the occurrence of plastic particles in the environment, it is evident that plastic particles are ubiquitous. As the aquatic environment is the major sink for the plastic particles, ecotoxicological research is mostly conducted for aquatic species. Most of the studies investigated the impact to marine organisms followed by freshwater organisms, often from standing waters like lakes. Fortunately, the examination of river-dwelling organisms is rising since few years.

The manifold factors regarding the plastic particle research, like plastic type depending properties, make risk assessment for plastic particles difficult in various ways. This is made even more complicated as the common toxicity tests (e.g., OECD-Guidelines) are based on soluble substances and still need adjustments for particles.

This thesis presents in chapter 6 a standardized easy-to-conduct bioassay for the river-dwelling *Gammarus roeseli*, which is often examined and sensitive to many pollutants but is lacking of a standardized bioassay. To overcome this lack, the applicability of a more standardized food source instead of conditioned leaves is investigated for laboratory treatments and adjusted to the nutritional needs of *G. roeseli*. In addition, a bioassay is developed on the basis of previous guideline-preparing studies for *Gammarus* spp. After this, the dietary exposure of plastic particles with the new food source as alternative to the unpredictable water exposure of the particles is evaluated and discussed in detail. The dietary exposure is a great alternative to the aqueous exposure because the particle uptake can be controlled and a more realistic dose-response-analysis can be applied.

The developed bioassay and the deep evaluation of dietary particle exposure enables the comprehensive examination of particle toxicity with three different factors to *G. roeseli* in chapter 7. In contrast to this examination, chapter 8 provides the investigation of the common exposure via water phase in combination with a chemical. In both chapters, the possible impact of plastic nano and microparticles to

the population of *G. roeseli* is discussed regarding, size, type, concentration, and vector function. As no effect by plain particles over the course of three weeks and no vector function for the lipophilic chemical was determined, no plain plastic particles induced impact was assumed for the *Gammarus* populations in the environment.

In the general discussion in chapter 9, the future role of the developed bioassay including the advantages of the new standardized food source and the resulting possibility of dietary particle exposure is discussed. Likewise, the absent effect of the plastic micro and nanoparticles in combination with the new dietary particle exposure and the developed bioassay as contribution to the risk assessment is addressed. As studies with no-effect observations are still rare, this is a good addition for risk assessment. The findings correspond to the risk assessments with today's plastic concentration in the environment stating that plastic particles are not hazardous to the environment nowadays. The calculated, reliable particle exposure concentration which is often not provided when common exposure via water phase is used is another great addition for good risk assessment. For particle toxicity examination, it will be helpful when the dietary exposure is used to examine the effects which enables a great dose-response-analysis. Nevertheless, the aquatic exposure is likewise helpful to address some scientific questions like vector function of the particles. Both exposure scenarios should be carefully chosen with regard to the scientific question that will be investigated.

This thesis highlights the need of and provides a solution for a standardized laboratory test for particles by offering an improvement with dietary exposure and simultaneously adding a standardized bioassay with a river-dwelling organism. Through the application of both developments in two toxicity tests, the risk assessment gains comprehensible and reproducible outcomes for plastic toxicity.

2. Zusammenfassung

Die großflächige Umweltverschmutzung durch Plastik ist seit Jahren von großem öffentlichem und politischem Interesse. Auf Grund des langen Verbleibs des Plastiks in der Umwelt und der langsamen Degradation zu Mikro- und Nanopartikeln wird angenommen, dass der Anteil der Plastikverschmutzung in Zukunft noch anwachsen wird. Neben den inzwischen deutlichen negativen Auswirkungen von Makroplastik rücken auch das Vorkommen in der Umwelt und die Effekte auf die Organismen von Mikro- und Nanoplastik in den Fokus der Wissenschaft. Denn obwohl die heutigen Nachweismethoden noch verfeinert werden, steht fest, dass Plastikpartikel inzwischen überall in der Umwelt zu finden sind. Die toxikologischen Untersuchungen zu Plastikpartikeln sind allerdings erschwert, unter anderem durch die vielen unterschiedlichen Eigenschaften der verschiedenen Plastiktypen. Die üblichen aquatischen Untersuchungen, die meistens auf den OECD-Guidelines für Chemikalien basieren, müssen in der Herangehensweise angepasst werden, da sich Partikel im Wasser anders verteilen als lösliche Chemikalien.

Die vorliegende Arbeit stellt in Kapitel 6 einen standardisierten und einfach durchführbaren Bioassay für den in Flüssen lebenden *Gammarus roeseli* vor. Im gleichen Zuge wird eine Alternative zur üblichen Exposition im Wasser präsentiert. Die Art *G. roeseli* wurde bereits oft in toxikologischen Studien untersucht und reagiert sensitiv auf verschiedene Chemikalien, allerdings fehlt ein allgemein angewendeter standardisierter Bioassay. Dieser Flussbewohner ist eine gute Ergänzung zu den häufig untersuchten marine und stehende Gewässer bewohnenden Tieren und Organismen. Durch die Optimierung eines Futterpellets für die Gammariden können so die sonst verwendeten konditionierten Blätter durch ein sehr viel einheitlicheres Futter ersetzt werden. Zusammen mit einem Bioassay, der unter Zuhilfenahme verschiedener vorhergehender Studien zum Thema Laborversuche mit *Gammarus* spp. erstellt wurde, entsteht eine einfache, standardisierte Versuchsdurchführung. Nach der Entwicklung dieses Bioassays wird das Futterpellet als mögliche Matrix für eine Exposition der Partikel über das Futter untersucht und diskutiert. Dies stellt eine gute Alternative zur Exposition über das Wasser dar, da die Partikel sich im Pellet nicht unkontrolliert verteilen, sondern gezielt dosiert verabreicht werden können.

Der entwickelte Bioassay für Gammariden und die im Detail überprüfte Exposition

über das Futter ermöglichen so eine umfangreiche Untersuchung der Toxizität von Plastikpartikeln gegenüber *G. roeseli* in Kapitel 7. In diesem Kapitel wird durch das Einbetten der Partikel eine gezielte Exposition erreicht und dadurch die Faktoren Partikeltyp, -größe und -konzentration untersucht. Erweiternd dazu wird in Kapitel 8 die Vektorfunktion der Plastikpartikel mit einer Chemikalie untersucht. Dafür werden die Partikel statt der Fixierung in der Nahrung wieder zusammen mit der Chemikalie im Wasser suspendiert. In beiden Kapiteln werden die möglichen Auswirkungen der Faktoren auf die Population von *G. roeseli* diskutiert. Beide Untersuchungen konnten keine Effekte durch Plastikpartikel innerhalb von drei Wochen nachweisen, auch eine mögliche Vektorfunktion für die lipophile Chemikalie blieb aus. Daher konnte geschlossen werden, dass in umweltrelevanten Konzentrationen die untersuchten Plastikpartikel keine Auswirkungen auf die *Gammarus*-Population in der Umwelt haben.

Im Anschluss wird in der generellen Diskussion in Kapitel 9 die mögliche Verwendung des Bioassays inklusive der Vorteile durch das optimierte Futterpellet und die daraus resultierende alternative Exposition über die Nahrung in der weiteren Forschung mit *Gammarus* spp. diskutiert. Ebenfalls wird das Ausbleiben der Effekte durch Plastikpartikel in den mit der Exposition über die Nahrung angewendeten Bioassays in Hinblick auf die Risikoanalysen betrachtet. Solche Studien ohne negative Effekte werden immer noch selten publiziert und sind daher ein wichtiger Beitrag zu einer umfassenden Risikoanalyse. Die Ergebnisse unterstützen die Erkenntnisse aus jüngsten Risikoanalysen zur heutigen Umweltkonzentration von Plastikpartikeln, die besagen, dass die Menge an Plastikpartikeln heutzutage nicht gefährlich für die Umwelt und deren Bewohner sind.

Auch die kalkulierbare Partikelexposition bringt eine Verbesserung für die Experimente für die nötige Risikoanalyse. Allerdings zeigt die vorliegende Arbeit auch, dass die Fragestellung der Untersuchung unbedingt berücksichtigt werden muss. Um die Toxizität der reinen Partikel zu untersuchen, eignet sich die Exposition über die Nahrung besser, da eine Dosis-Wirkungs-Analyse mit der gezielten Exposition zuverlässiger erstellt werden kann. Um zum Beispiel die Vektorfunktion zu untersuchen, ist eine Exposition über Wasser allerdings vorzuziehen, da sich das Einbetten der Partikel auf die realistische Analyse der Partikel-Chemikalie-

Organismus-Interaktion auswirken würde.

Die vorliegende Arbeit zeigt auf, dass eine neue Herangehensweise an die Untersuchung mit Partikeln nötig ist und liefert gleichzeitig auch eine Lösung dieses Problems in Form eines im Detail überprüften Expositionsszenarios. Gleichzeitig wird die allgemeine Risikoanalyse um einen Bioassay mit einem Organismus erweitert, der im Gegensatz zu den üblicherweise untersuchten Organismen im Fluss lebt. Durch die Anwendung beider Entwicklungen - Bioassay und alternatives Expositionsscenario für Partikel - konnten in zwei Toxizitätsversuchen nachvollziehbare und reproduzierbare Beobachtungen der Risikoanalyse für Plastikpartikel hinzugefügt werden.

3. General introduction

3.1. The rising of the attention for chemical toxicity in the environment

Since the first humans used rudimentary tools, humankind was changing and influencing the environment to its needs. Some changes are still clearly visible in the environment: forests were cleared, rivers were straightened and chemicals like herbicides or insecticides were applied for specific benefits without consideration of the adverse consequences to the environment. The first remarkable change in thinking was in the 1960, when the heavy use of dichlorodiphenyltrichloroethane (DDT) led to the near extinction of the bald eagles (Fent 2013), of which the effects to the whole ecosystem were made available to the public by the popular roman *Silent spring* from the biologist Rachel Carson (1962).

The following environmental catastrophes made by human like the nuclear disaster of Chernobyl in 1986 (Smith and Beresford 2005) or the oil spill from the sinking of the Exxon Valdez in 1989 (Peterson et al. 2003) increased the attention to the anthropogenic influence and consequences to the environment. As a result, the research field of ecotoxicology has been developed to examine the adverse effects of the Anthropocene, focusing on the effects of chemicals and other contaminants applied to the environment by humans (Fent 2013).

3.1.1. Development of regulatory policies

Two noteworthy strategic developments to remediate and reduce anthropogenic impact are the Water Framework Directive (WFD/WRRL) from 2000 for the improvement of European river quality and the European REACH regulation for the Registration, Evaluation, Authorization and Restriction of Chemicals. The aim of REACH is to prevent environmental catastrophes, like with DDT in 1962, with the specification that possible effects of each produced chemical must be examined by the producer before usage. The application of the chemicals is regulated by the results of the investigations (ECHA 2007). It was clear, that the ecosystem services of each aquatic compartment are linked to the well-being of the ecosystem and therefore, must be maintained by the restriction of pollution (Grizzetti et al. 2019).

For the examination of contaminants and their effects to the environment including terrestrial, marine and limnic compartments and the organisms living there, the

Organization for Economic Co-operation and Development (OECD) developed and evaluated many standard test guidelines for comparable and reproducible results. Out of the collection, three standard test procedures for aquatic exposure distilled as most applied and best comparable within laboratories. The usually investigated organisms are *Daphnia magna* (OECD-Guideline 2004), *Danio rerio* (OECD-Guideline 2013), and *Lemna* sp. (OECD-Guideline 2006), as representatives for different trophic levels. The three organisms revealed to be sensitive to a wide range of chemicals and the test procedures are easy to reproduce. Nevertheless, the three organisms are only common in freshwater lakes or waters with low flow rate.

3.1.2. The need of representative organisms in rivers

Crux of the matter is the prediction of hazard by extrapolation from the three often examined organisms as habitat-sharing organisms. Although each of them represents a different trophic level, other compartments than the standing freshwaters are underrepresented. It is commonly known that the characteristics of rivers and lakes vary in chemical and physical properties, surrounding area as well as their inhabitants (Canning and Death 2019; Drakou et al. 2008). Rivers are more regularly used by humans for shipping and trading, flow through urban areas and are the receiving waters for waste water treatment plants or other industries (Grizzetti et al. 2019). Therefore, the investigation specifically of river-dwelling organisms should likewise be considered when anthropogenic impact assessment is intended.

The amphipod *Gammarus* spp. fits many of the requirements for a representative river-dwelling organism. The genus *Gammarus* includes 204 freshwater species distributed to Europe, China and North America (Väinölä et al. 2008). Besides their high abundance and widespread distribution, they are keystone species in the food web (Gerhardt et al. 2011). Further, G*ammarus* spp. were often examined regarding the variation in natural behavior and development impacted by abiotic factors (Gergs et al. 2014; Glazier 2000; Plaistow et al. 2003). They have also been shown to be sensitive to many contaminants (Besse et al. 2013; Brock and van Wijngaarden 2012). Finally, some studies attempted to develop standard toxicity testing protocols with gammarids (Kunz et al. 2010; Maltby et al. 2002) and an official standard protocol exists (US EPA 2016). A detailed explanation of *Gammarus* spp. life cycle and function

in the environment and in ecotoxicological tests is given in chapter 3.3 "*Gammarus* spp. – a representative species for ecotoxicological research".

3.2. When (plastic) particles were detected in the environment

Beside the development of a sophisticated testing regulation for chemicals and the resulted emergence of standard testing protocols (OECD, ECHA, REACH) in the laboratory, methods were also established to measure the concentration of these chemicals in the environment. The wide range of monitoring projects, evolving identification and quantification methods led more and more to a decrease of the detection limits for concentration and size classes (Reichel et al. 2020; Schwaferts et al. 2021). With the improvement of detection methodologies in micro and recently nanoscale, a shift of interest from only chemicals to the effects of small particulates occurred. Especially plastic particles as particulates with an anthropogenic origin and their environmental concentration came into focus as humans are responsible for any adverse effect in the environment originating from plastic particles.

The plastic particles were classified by size, whereby the size ranges differed sometimes. In this thesis, the classification for particles size classes was in accordance with the classification from the project “plastics in the environment” (see Bertling et al. (2022)). Plastic microparticles are those in size from 1 - 1000 µm and nanoparticles are less than 1 µm.

3.2.1. Detection of micro and nanoplastic

Plastic microparticles in the environment were first reported by Carpenter and Smith (1972) and were since then mainly studied in marine ecosystems (Akdogan and Guven 2019). In the recent years, the idea rose that rivers are the main pathway for plastic particles to enter the sea and therefore an increasing amount of studies also dealt with the occurrence of plastic microparticles in rivers and lakes (Bellasi et al. 2020). It is predicted that the environmental concentration of plastic particles will increase due to high production load and the persistence of the various compounds (Enders et al. 2015; Koelmans et al. 2016). For such microparticles, the methods for detection in environmental samples are not readily established (Anger et al. 2018) and are often discussed regarding reliability (Hidalgo-Ruz et al. 2012; Löder and Gerdts 2015; Song et al. 2015). The same holds true for nanoparticles, where methods for detection and identification are still at the development stage (Lehner et al. 2019; Nguyen et al. 2019; Schwaferts et al. 2020). In the current state, it was not possible to find studies

trying to give first measurements of plastic nanoparticle occurrence in the environment (Tamayo-Belda et al. 2022).

As a consequence, another approach was to demonstrate the formation of nanoparticles by weathering in laboratory experiments. It describes the degradation of bigger particles to smaller ones by biotic and abiotic processes, such as mechanistic grinding or photodegradation (Mattsson et al. 2015). It was demonstrated in laboratory but environmental relevant conditions, that PET, PLA and PS are downsized to micro- and nanoparticles by photodegradation and the inevitable conclusion was that the number of particles increase exponentially proportional to the decreasing size (Annenkov et al. 2021; Gigault et al. 2016; Lambert and Wagner 2016). Modelling studies came to the same conclusion that the smallest plastic particles must occur more frequent in the environment than plastic macro and microparticles (Eriksen et al. 2014).

3.2.2. How many plastic particles are in the environment?

Both, micro and nanoparticles, can enter the environment as primary particles. For example, as supplement in products over the waste water treatment plants, accidentally released from the factory or degrade to secondary particles by weathering processes over time (da Costa et al. 2016; Duis and Coors 2016). Therefore, the aquatic environmental concentration was rising from around 3,500 plastic microparticles (290 grams) per square kilometer sea surface in 1972 (Carpenter and Smith) up to a hundred thousand per square kilometer in many marine environmental samples (Bakir et al. 2020). A modelling estimation assumes a minimum amount of 5.25 trillion particles (268,940 tons) floating on the marine sea surface (Eriksen et al. 2014), whereas the suspended particles under the surface are not considered. In freshwater compartments, a high amount of microparticles was also reported in the Lake Garda with 2.5×10^{10} particles per square meter or detected in Rhine and Elbe River in average between $1 - 9 \times 10^{5}$ particles per square kilometer (Bellasi et al. 2020; Triebskorn et al. 2019). At last, the amount of plastic debris from the macro to the nanoscale will increase further when high production load and incorrect plastic handling are maintained (Eriksen et al. 2014).

The increasing concentration and decreasing size of plastic particles in the environment give rise to the questions whether and when plastic particles are toxic to

the organisms in the contaminated compartments. In the last years, many researchers tried to answer these questions and a short impression of their findings is given in chapter 3.4 “State of the ecotoxicological research”.

The investigations are made difficult by the manifold factors that are influencing the outcomes of the toxicity studies, which leads to contradictory or incomprehensible results and risk assessments. Additionally, the properties of plastics are completely different to the chemicals the standardized OECD-guidelines are adjusted for. This is a challenge many researchers are struggling with and nowadays it is evident, that the “old” guidelines cannot be applied in particle toxicity research. In chapter 3.5 “Current difficulties in ecotoxicological research” the challenges and recent solution strategies are presented.

3.3. *Gammarus* spp. – a representative species for ecotoxicological research

The typical standard organisms in aquatic ecotoxicology are the fish *Danio rerio*, the crustacean *Daphnia magna* and the alga *Lemna* sp., all three live in lakes. There is a clear lack in standard organisms for marine compartments and rivers. The in this thesis examined and here presented crustacean *Gammarus* spp. is nowadays an often-considered alternative standard organism in ecotoxicological tests for the field of river-dwelling organisms (Kunz et al. 2010; McCahon and Pascoe 1988b), although there is still no established and often-used standard testing guideline.

First, the morphology and life cycle of the organisms as well as possibilities to manipulate this cycle are described in this chapter to illustrate the benefits for laboratory use of *Gammarus* spp. Second, a short overview about the functional role in the environment and sensitivity of this species is given to show the relevance of *Gammarus* spp. for ecotoxicological research. Third, the current investigations and approaches for ecotoxicological testing with *Gammarus* spp. are shortly outlined.

3.3.1. Morphology and life cycle of *Gammarus* spp.

The genus of *Gammarus* relates to the order of Amphipoda and includes over 6,000 species all over the world (Eggers and Martens 2001). They can be found in terrestrial, marine and limnic environments, especially in freshwater habitats like rivers and lakes (Eggers and Martens 2001). The family *Gammaridae* includes 304 freshwater species, while the genus *Gammarus* includes 204 freshwater species distributed to Europe, China and North America (Väinölä et al. 2008).

The body of the gammarids is divided into cephalon, thorax and abdomen with overlapping segments of their carapax (Figure 3.1). The rounded to reniform eyes, two pairs of antennae and the mandibles are located at the cephalon (Karaman and Pinkster 1977b). The mouthparts can vary according to the specific diet of the species (Haley 1997). Behind the head capsule begins the thorax with two pereiopods per segment followed by the abdomen with two pleiopods at each of the first three segments, uropods and the telson (Karaman and Pinkster 1977a; Karaman and Pinkster 1977b).

Figure 3.1: Picture of a *Gammarus roeseli* (by S. Beggel).

Gammarids sexual dimorphism is observable in many aspects. Females of the same age like the males are for example smaller in size and have shorter antennae. Male gammarids often have bigger gnathopods because they use them to clench to the back of the female during mating (Karaman and Pinkster 1977b). The most reliable method to determine sex is to search for the genital papillae between the 5th pereiopod as described in chapter 5.2.4 or the brood plate located at the basis of the coxal plates (Constable 2014). On the one hand, sex determination is necessary for species identification as the females often do not have the distinguishing morphological characteristics mentioned in identification guides (Constable 2014). On the other hand, there is evidence that the sex impacts the sensitivity to stressors (Sornom et al. 2010).

Another sensitivity influencing factor is the life stage (McCahon and Pascoe 1988b; Pöckl 1992). Life stage can be determined by the body length and the number of antennal segments, which are also closely correlated with the culture temperature (McCahon and Pascoe 1988b). For example, in 13 °C cold tap water cultured *Gammarus pulex* juveniles of 1 day have a body length of 2.04 ± 0.09 mm and 5.00 ± 0.00 antennal segments, while 110 days old are 8.59 ± 1.27 mm in size and have

19.37 ± 2.13 antennal segments. A simulation study revealed temperature dependent life history, where *G. fossarum* and *G. roeseli* live around 500 days at 11.5 °C and longer with decreasing temperature (Pöckl et al. 2003).

For female gammarids, the molt cycle and the embryo development cycle are closely linked. The molt cycle stages are postmolt, intermolt, premolt and ecdysis. The postmolt stage is right after molting, where the cuticle is flexible and pale. Over intermolt and premolt, the new skeleton is built up and consolidates until the cycle ends again with the ecdysis stage, where the gammarid molts and the cycle restarts (Geffard et al. 2010; Hyne 2011). During these cycles, the energy reserves containing glycogen, lipids and proteins vary depending on molt stage for females (Charron et al. 2014) and also the different stages can have an impact to the females sensitivity to pollutants (McCahon and Pascoe 1988a).

Sexual maturity is reached around 130 days for *G. pulex* at 13 °C under steady conditions in the laboratory (McCahon and Pascoe 1988b), but took 550 days for *G. fossarum* and *G. roeseli* in a 5.6 °C cold river and less than 150 days in a river with 10.9 °C in mean (Pöckl et al. 2003). Egg survival in the laboratory is 40 - 60 % and fecundity is also dependent from the culture temperature and species (Pöckl 1993). One female of *G. fossarum* produces theoretically in maximum 194 eggs in her lifetime, while one female *G. roeseli* can produce 333 eggs (Pöckl 1993).

When a female is in the maturation stage, a male catches her at the back with his gnathopods and starts guarding her until the female molts and external fertilization occurs (Hartnoll and Smith 1978; Sutcliffe 1992). This stage is called precopula. Although this behavior of the male is cost effective, it seems necessary because the moment when a female is ready for fertilization is very short (Plaistow et al. 2003). The fertilized eggs of *G. pulex* develop within 22 to 26 days at 15 °C under the brood plates between the pereiopods until the embryo hatches (Sutcliffe 1992).

Depending on the light:dark cycle or rather season, the females and males of *Gammarus* enter the diapause, where no mating occurs and no eggs are produced. This diapause can be manipulated in the laboratory. Reproduction is continuous during long daylength (18:6 h light:dark), while diapause starts when short daylength is given (8:16 light:dark) (Sutcliffe 1992).

The well-known development of the gammarids from embryos to adult organisms with

the possibility to manipulate the life cycle and fecundity makes them great culturable organism for laboratory studies.

3.3.2. Function of *Gammarus* spp.

Regarding the function in the environment, *Gammarus* spp. were classified as detritus feeder (MacNeil et al. 1997). Gammarids belong to the functional feeding group of so called “shredder”, referring to their feeding on detritus and vegetable food like leaves, making it more available for other invertebrates and bacteria (MacNeil et al. 1997). It has been shown that gammarids mainly get their needed nutrients from so named “conditioned leaves”. Those Leaves, overgrown and partly decomposed by biofilms, especially fungi, are preferred and easier digested (Bärlocher and Kendrick 1975; MacNeil et al. 1997). Nevertheless, gammarids are also known to be predators of various macroinvertebrates, e.g. *mayfly nymph* (Kelly et al. 2002; MacNeil et al. 1997). In laboratories as well as in the field, cannibalism of juveniles by adults or freshly molted gammarids by bigger ones has been observed (Bartonitz et al. 2020; MacNeil et al. 1997). Consequently, gammarids should be rather seen as “opportunistic generalists” than as specialized herbivorous detritus feeder, having a lasting effect on all parts of the food web in their habitats (MacNeil et al. 1997).

Next to the importance of *Gammarus* spp. as shredder and predator in the food web, many organisms are predators of the small crustaceans. Freshwater communities with gammarids are often dominated by them due to high biomass and abundance, which makes them a rich resource as prey for fish or birds as well as some invertebrates (MacNeil et al. 1999). For example, various species of *Gammarus* are prey for salmonids and trout, sometimes depending on the season when the hunting grounds change or the abundance of other invertebrates is low. Gammarids were also found in the digestive tract of sea- and shorebirds like dugs, gulls and dipper (MacNeil et al. 1999).

As a result, the decline of a *Gammarus*-population in their habitats will not only affect decomposition rates but also the abundance of prey and predator populations. Thus, the position and function in the food web as well as their opportunism makes them a

keystone organism in river compartments and therefore a great representative species for ecotoxicological research.

3.3.3. Toxicological research with *Gammarus* spp.

Overall, the relative high reproduction rate, the well-known life cycle, the ability of cultivation in the laboratory and the unselective feeding behavior makes gammarids a useful candidate as standard organism for ecotoxicological assessments regarding rivers. Their life cycle can easily be manipulated and the life stages are distinguishable, which allows therefore a detailed examination of the impact of various pollutants on different sensitivity stages in laboratory tests. In addition, the functional role of *Gammarus* spp. in their habitats is of great importance, wherefore the impact on these organisms is a great starting point for risk assessments of aquatic pollutions. In the following, a short overview of the efforts to develop standard toxicity tests and some recent studies with gammarids is given to show the current state of the work in the laboratory.

One of the first studies dealing with the establishment of toxicity tests with *Gammarus* spp. was made by McCahon and Pascoe (1988a). They described the culture of *Gammarus pulex* in aquaria with dechlorinated tap water and steady conditions of 13 °C, 239 µs/cm, pH 7.7, 115 mg/L hardness and a steady flow rate where 99 % water was replaced within 4 h. The dark:light cycle was 12:12 h. The culture was started with 100 precopula pairs and 100 pregnant females placed in a breading container with pore diameter of 0.5 mm. By removing the breading container each day and transferring it to a new aquarium, a selection of gammarids in the same age is possible. Under these culture conditions, 70 % of the juvenile gammarids survived and reached sexual maturity after 130 days. This is a benefit for toxicity tests because the life stages and therefore sensitivity stages are easy to collect. Nevertheless, most laboratories do not have the ability and space to culture gammarids in various aquaria and conduct toxicity tests at the same time.

The US EPA (2016) provides an official guideline for *Gammarus* spp. to test chemicals in acute experiments with a minimum duration of 96 h. The guideline recommends juveniles of same size and age, preferably newly-released and taken from a laboratory culture. Acclimatization to the test conditions should be done gradually over 48 h to

the test medium and at last 7 days for the acclimatization to the test temperature that should preferably be constant at 18 ± 1 °C. Feeding is not planned in this approach during the short test duration, but during acclimatization aged leaves or pray can be offered as food. Light:dark cycle can be between 12:12 h or 16:8 h. One test vessel includes ten gammarids and two replicates per treatment including control and solvent control. The guideline considers each test procedure: static, static-renewal and flow-through techniques without directly specifying the volume or type of used medium, excluding dechlorinated tap water. The guideline works with recommendations enabling a wide range of test procedures, which may lower the comparability.

Another experimental approach came up with a circulatory system for long-term toxicity tests in 2016 (Feiner et al.). It provides a steady water flow through in a 14 cm wide and 5 cm high vessel with manual water exchange and steady conditions at 10 ± 1 °C and 16:8 h light:dark cycle. Organisms of *Gammarus roeseli* were caught one week before the experiment and only exposed to the natural water from the specific sampling site during the procedure. Conditioned black alder leaves served as food for 15 organisms per test vessel. This setup provides low mortality (< 20 %) within 21 days and revealed comparable and reproducible results in a copper toxicity test.

The next point for laboratory toxicity testing was to find the best feeding conditions for gammarids. Since they were classified as shredders (MacNeil et al. 1999), the most often offered food is dried leaves (Agatz and Brown 2014; Blarer and Burkhardt-Holm 2016; Blockwell et al. 1998; McCahon and Pascoe 1988a). Also, some researchers tried to conduct bioassays with gammarids feeding on prey (Kelly et al. 2002). The options for dried leaves are manifold ranging from e.g., beech leaves (Dietrich et al. 2010) over alder leaves (Agatz and Brown 2014; Besse et al. 2013; Coulaud et al. 2011) to maple leaves (Gust 2006) collected in the field. All leaves have in common that they should be conditioned before the test, meaning slightly pre-decomposed by fungi or bacteria, reached by manifold procedures to make the leaves more palatable for the gammarids (Agatz and Brown 2014; Bärlocher and Kendrick 1975). However, a standardized food source is still under development and urgently needed for better comparison and reproducibility of toxicity testing procedures with *Gammarus* spp.

In sum, there exist some approaches for assays with gammarids. An *in situ* feeding

assay for gammarids has been presented for the evaluation of water quality in the field (Coulaud et al. 2011; Gerhardt 2011; Maltby et al. 2002). Likewise, an *in situ* biomonitoring for chemicals and metals in caged gammarids (Besse et al. 2013). At last, Kunz et al. (2010) or Gerhardt (2011) provided a good overview and start for standardized bioassays with *Gammarus* spp., but, yet it is more than one decade ago, standardization is still in progress and widely accepted guidelines are missing.
Nevertheless, many studies have been conducted with gammarids demonstrating their sensitivity to a high range of pollutants (Beketov and Liess 2008; Blarer and Burkhardt-Holm 2016; Blockwell et al. 1998) and abiotic factors (Pöckl 1992; Sornom et al. 2010; Sroda and Cossu-Leguille 2011). Also, gammarids often proved to be more sensitive than other organisms or even similar to the often-investigated *Daphnia magna*, which is greatly shown by species sensitivity distribution analyses (Liu et al. 2015; Park et al. 2018).

In this chapter, six worthy factors for the applicability of *Gammarus* spp. in ecotoxicological investigations have been described: (I) The life cycle is well-known and (II) can be manipulated easily to the laboratory needs. (III) The various species of *Gammarus* spp. are found in many different compartments and in high abundance, which (IV) makes them keystone species in the food web. (V) They proved to be sensitive to a wide range of pollutants and changes in the environment. At last, (VI) there exist some basic guidelines for ecotoxicological testing. Overall, *Gammarus* spp. are promising organisms for ecotoxicological testing regarding the field of stream invertebrates.

3.4. State of the ecotoxicological research of plastic micro and nanoparticles

The global occurrence of plastic micro and nanoparticles in the environment and the prediction of increasing plastic concentration in the future led to the question whether and how the plastic particles affect the environment. The first studies dealing with the impact of plastic to organisms in the marine environment occurred in 2003 and investigations about the effect on organisms in the freshwater environments 11 years later (Akdogan and Guven 2019). First, the ingestion of plastic from macro (> 5 mm) to microscale by various marine and limnic organisms has been reported (Cole et al. 2013; Cole and Galloway 2015; Ryan 1987; van Cauwenberghe et al. 2015). Many of them refer to the accidently uptake of plastic particles, mistaken as food or strangulation for example with meshes. However, the exposure of aquatic organisms to plastic was proven so far and therefore the possible consequences came into focus. Organisms can be affected by plastics due to three mechanisms (Bouwmeester et al. 2015; Shen et al. 2019; Triebskorn et al. 2019): by plastic as a particle, by leaching additives from the plastic particle or by plastic particles as carrier for ad/absorbed environmental pollutants (Figure 3.2). The toxicity of the plastic particles itself can be induced by manifold physical properties, including shape, size, surface area, crystallinity and others (Lambert et al. 2017). Whereas the additives are generally metals (Pb, Al, Mn, etc.) or organic compounds (Bisphenol A, benzothiazole, etc.) with the primary function to give plastics the desired properties. Leaching of those additives into organisms or to the surrounding medium occurs over time and is facilitated by degradation processes (Gunaalan et al. 2020). The function of plastic particles as vectors, carrier or more likely as passive sampler for dissolved chemicals depends on the properties of the particle, which can facilitate or impede bioaccumulation of the chemicals in the organisms (Besseling et al. 2019).

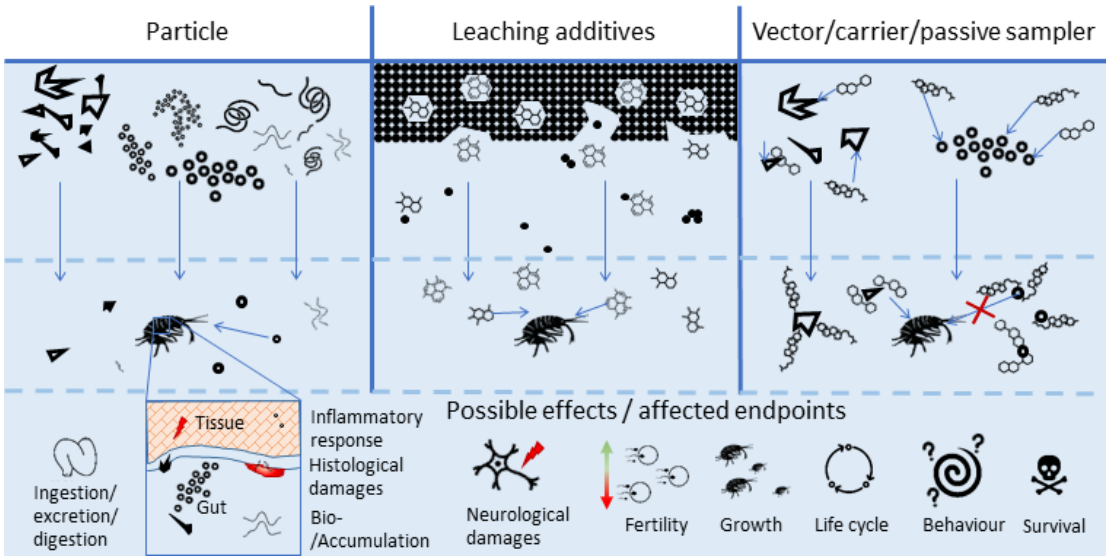

Figure 3.2: Exemplary abstract of the three possible effect mechanisms of plastic particles in the aquatic environment. The possibly measured effects can be induced by the plastic particle, the leached additives or by the carrier function.

In the following, a short overview of detected plastic particle effects to aquatic invertebrates is given, sorted by the three possible mechanisms. The most often examined plastics were polystyrene (PS), polyethylene (PE), polyvinylchloride (PVC), polypropylene (PP), polyethylene terephthalate (PET) and polyamide (PA) (de Ruijter et al. 2020).

Effects of plain particles

PE microparticles were found after 3 h accumulated in the lysosomal system and gills of the mussel *Mytilus edulis* and caused after 6 h histological damages as well as inflammatory responses (von Moos et al. 2012b). After 52 days, attachment strength and hemolymph proteome of *M. edulis* were affected from PE microparticles, too (Green et al. 2019). PS nanoparticles with sulfate groups on the surface caused lowered filter activity and increased pseudofeaces production of *M. edulis* within 8 h (Wegner et al. 2012). On the other hand, when exposed for 14 days to PS microspheres in concentrations higher than environmental relevant concentrations, no adverse effects on the energy content were detected (van Cauwenberghe et al. 2015). Also, no change in feeding or growth of the pacific oyster *Crassostrea gigas* was observed within 8 days when exposed to PS nano and microspheres (Cole and Galloway 2015). The reproduction cycle and feeding was negatively impacted by PS microspheres after two months (Sussarellu et al. 2016).

The exposure of *Dreissena polymorpha* over 6 days to PS microbeads revealed no

oxidative stress or genetic damage, only the activity of catalase and glutathione peroxidase was modulated and the abundance of dopamine was increased (Magni et al. 2018).

After one month, the lugworm *Arenicola marina* did not ate less on sediment spiked with microparticles of polylactide (PLA), PE and PVC and no wet weight change was measurable. High doses of PVC resulted in a higher oxygen consumption (Green et al. 2016). Beside this, the energy content was not affected after 14 days exposure to PS microspheres (van Cauwenberghe et al. 2015).

The mudsnail *Potamopyrgus antipodarum* was also not influenced by microparticles of any of five plastic types (PA, PET, polycarbonate, PS, PVC) concerning morphological changes or juvenile development (Imhof and Laforsch 2016).

PS nanobeads functionalized with carboxylic groups were found to induce malformations in *Daphnia magna* and reduce the number and body size of neonates within 21 days (Besseling et al. 2014). No immobility was induced within two days from polymethylmethacrylate (PMMA) nanoparticles (Booth et al. 2016). Another study reported a range of adverse effects to *D. magna* induced by fragmented PE microparticles in contrast to PE microbeads and kaolin particles (Ogonowski et al. 2016).

The exposure of *Gammarus fossarum* to PA fibers and microbeads resulted only for fibers in lowered assimilation efficiency within 28 days, but for both shapes no change in feeding rates or wet weight occurred (Blarer and Burkhardt-Holm 2016). *G. fossarum* were also exposed to polyhydroxybutyrate and PMMA for 28 days. The plastic particle exposed organisms lost weight during the experiment compared to those exposed to silica particles (Straub et al. 2017). One study was examining the impact of PS microparticles on *Gammarus pulex*, *Hyallella Azteca* and *Asellus aquaticus* within 28 days and only for *G. pulex* growth was affected (Redondo-Hasselerharm et al. 2018b). In contrast, PET did not induce any effects on *G. pulex* regarding survival, development, metabolism and feeding activity after 48 days (Weber et al. 2018).

Effects of leached additives

The additive diisononylphthalate leached from the flexible PVC during a 31-day experiment, increased the body length and reduced the number of offspring of *D. magna*, while the plain PVC did not induce any effects (Schrank et al. 2019). The

same negative impact by PVC exposure on *D. magna* was observed for reproductive output, reproduction cycle and body length after 21 days. In contrast to PVC, the toxicity of the plastic types polyurethan and the biodegradable PLA can be assigned to the particle and not to the additives (Zimmermann et al. 2020). The study from Redondo-Hasselerharm et al. (2018a) revealed that the zinc in car tires is just in low concentrations bioavailable and does not induce effects to four macroinvertebrates during 28 days without forced leaching. The review by Gunaalan et al. (2020) summarizes various investigations exploring the effects of leachates from plastics to marine and freshwater organisms covering all trophic levels. It elucidated the high impact of the leachates on organisms mainly regarding the reproduction cycle, e.g. by reducing algal growth or delaying embryo development.

Effects in combination with ad/absorbed chemicals

The property of plastic particles as carrier for chemicals was investigated in seawater and by the simulation of gut conditions regarding pH and temperature. This modelling experiment revealed enhanced desorption rates under gut conditions compared to seawater for four persistent organic pollutants (POPs) from PVC and PE microparticles (Bakir et al. 2014a), suggesting a vector function of the two plastic types. Nevertheless, the comparison of the bioavailability of polychlorinated biphenyls (PCBs) sorbed to PP particles or coal, biochar and wood particles led to the conclusion that the proportion of plastic particles as carrier in the environment is negligible compared to the sorption and vector function of natural particles (Beckingham and Ghosh 2017). Further, the early assumption that particle size or hydrophobicity are decisive for the carrier function and plastic particles are predestined as carrier for hydrophobic chemicals could not completely be supported as the interaction is much more complex (Marchant et al. 2022).

Nevertheless, the simultaneous exposure of the bivalve *Corbicula fluminea* to the antimicrobial florfenicol and microparticles (only named polymer by the manufacturer, precise plastic type unknown) for 96 h led to higher toxicity regarding feeding, neurotoxicity and oxidative stress compared to the separate exposure to both (Guilhermino et al. 2018).

In contrast, the exposure of *Daphnia magna* neonates to PA microparticles in combination with the common additive bisphenol A resulted in lower immobilization

rates compared to the immobilization rates induced by the chemical alone (Rehse et al. 2018).

This overview highlights the variations in the impact of plastic particles to aquatic organisms regarding each of the three mechanisms. Depending on the properties of the investigated particle, organism and exposure scenario (Besseling et al. 2019) and even more factors, the outcome of a study and interpretations are likewise manifold (Kögel et al. 2020; Paul et al. 2020). The discussion whether plastic particles are hazardous remains therefore controversial and it seems that the answer is as multifaceted as plastics are.

3.5. Current difficulties in ecotoxicological research regarding plastics

Nowadays, many challenges of toxicity research with plastic particles are well-documented. The various applied strategies to deal with these problems and the subsequent decreased comparability in the outcomes make the risk assessment difficult. In this chapter, some of the most basic problems are described and current applied or discussed solutions are presented.

3.5.1. Particle origin and preparation

The most often addressed problem in aquatic plastic particle toxicity research is the variation in particle preparation (Eitzen et al. 2019; Hartmann et al. 2015; Heinrich et al. 2020). This includes the purchase from producers (e.g. Micromod, BS-Partikel, Molecular Probes, Cospheric, Polyscience) as primary spherical particles suspended in water with or without surfactants or other solvents (Bhattacharya et al. 2010; Gorokhova et al. 2018; Jeong et al. 2016; Ogonowski et al. 2018b; Redondo-Hasselerharm et al. 2018b). The generation of often fragmented particles in the laboratory by milling (Eitzen et al. 2019; Straub et al. 2017; Weber et al. 2018), sonication (von der Esch et al. 2020) or other processes (Booth et al. 2016; Redondo-Hasselerharm et al. 2018a; Yardy and Callaghan 2020) is also possible. While the plastic particles of the manufacturer are usually homogeneous in size and spherical in shape, the self-produced plastic particles have a greater and variable size distribution and various fragmental shapes (Eitzen et al. 2019; von der Esch et al. 2020). Latter rises the environmental relevance of the tests as the plastic particles in the environment likewise occur in every size and shape but lowers the comparability and reproducibility (Heinrich et al. 2020).

Both procedures are additionally impaired by the individual strategies to suspend the plastic particles. Depending on the strategy, particle properties, distribution in the aqueous media used in the experiments and maybe even the effects can change (Hartmann et al. 2015; Heinrich et al. 2020). The oxidation of plastic particles was tested as an alternative for surfactants, and indeed, the procedure lowered aggregation and improved suspension of the plastic particles but simultaneously induced leaching of additives or chemical formations (Eitzen et al. 2019). On the other hand, a study with manufactured particles stabilized in solution with sodium azide revealed that some endpoints were just affected by the surfactant and not by the

dialyzed nanoparticles (Pikuda et al. 2019). Therefore, the individual pre-treatment or rather preparation of plastic particles for experiments makes comparisons between the outcomes of the studies impossible due to changes in the particle properties, distribution and subsequently effects (Heinrich et al. 2020). There is still need for standardization although experiments with low doses of ethanol as surfactant for plastic particles were recently carefully recommended (Hartmann et al. 2015; Heinrich et al. 2020).

Another point against pre-treatment by abiotic processes like oxidation or mechanical abrasion is that they can likewise significantly change the chemical and physical properties of the plastic particles (Lambert et al. 2017; Zhang et al. 2021). This mechanism is named aging and occurs also in the environment by natural degradation processes (Zhang et al. 2021). The aging can lead to a change in the toxicity mechanism and impact of the particles (Vroom et al. 2017) and must be seen as a further aspect in particle toxicity (Heinrich et al. 2020; Jahnke et al. 2017). Therefore, these processes should be avoided when the effect of a primary plastic particle effect is under question but may be recommended when the effect of secondary plastic particles is examined (Heinrich et al. 2020). In conclusion, the preparation procedure including the production and suspension of plastic particles is still very variable and impeded by many factors that need to be considered even before the experiment can be started (Hartmann et al. 2015; Heinrich et al. 2020; Lambert et al. 2017).

3.5.2. Exposure concentration

Once the preparation procedure was chosen and the particles were suspended in the exposure medium, the actual exposure of the organisms is uncertain (Heinrich et al. 2020). The overall approach for particle exposure often relies on the established OECD-Guidelines for testing chemicals without consideration that plastic particles are not soluble like chemicals. As a result, the plastic particle concentrations are given as external concentrations, for example particles/L (Besseling et al. 2014; Blarer and Burkhardt-Holm 2016; Green et al. 2019), as this is the established information to be given for toxicity tests. Instead, it can be assumed that the particles do not distribute homogeneously in the aquatic medium like the soluble substances. Depending on their properties, particles rather sink to the ground, swim on the surface, attach to the test vessel and agglomerate, which can lower or increase the effective exposure

concentration and dose (Eitzen et al. 2019; Heinrich et al. 2020). Therefore, the established indication of nominal test concentration is inappropriate and does not reflect the concentration the organisms are exposed to. The inner and therefore effective particle exposure concentration occurs to be much lower and variable for each tested organism.

3.5.3. The natural particles

Further, early studies neglect the comparison of natural and anthropogenic particles which leads to the question whether the observed effects are really based on the plastic in the particle or just particle driven. Some particle effects may be standard stress for many organisms and thus, some reported adverse effects may be negligible as the population is used to deal with it. Organisms can be adapted to the exposition with the ubiquitous particles like bivalves (Lummer et al. 2016) or even depend on them as habitat like the lugworm (Zebe and Schiedek 1996). Only in recent times, since 2016, researchers request a simultaneous exposure and comparison of plastic particles with a standard reference particle, for example silica. Despite this request, just a few studies can be found considering this issue in their experiments but with an increasing course (Ogonowski et al. 2018b; Scherer et al. 2020; Schür et al. 2020; Straub et al. 2017).

In conclusion, the approaches of plastic particle testing need to be updated regarding the assumptions of preparation, handling, exposure scenario and even positive or negative controls. These assumptions are most often based on the established guidelines for soluble chemicals although many aspects influencing the particle toxicity research are now well-known and just have to be considered. For a much more detailed overview of the essential aspects in particle toxicity research, the review of Heinrich et al. (2020) is recommended.

4. Aims and objectives

The research of plastic particle toxicity in the aquatic environment is challenging and often set back due to inappropriate or missing information or assessments leading to controversial outcomes. Therefore, this study is built up on two aims. The first aim is to revise and adjust the approaches for aquatic investigations of plastic particle toxicity where necessary. The second aim is to synthesize the measured effects of two different exposure scenarios for a risk assessment of plastic particles with *Gammarus roeseli*.

To reach these aims, an alternative to the inaccurate aqueous particle exposure is developed by embedding the particles in a food matrix for dietary exposure (Figure 4.1). This food matrix is adjusted in the same process to the nutritional needs of the chosen freshwater organism *G. roeseli*. As a result, a bioassay is established with the exposure scenario via the food which bypass many of the challenges in this research field (Chapter 6).

With this improved exposure scenario, the effects on the shredder *G. roeseli* by dietary exposure to plain non-aged spherical plastic particles is measured. The observations and measured effects are likewise collected for the evaluations of an appropriate exposure scenario as well as for the risk assessment of plastic particles in the aquatic environment (Chapter 7).

To give a contrast in the exposure scenarios and to investigate the vector/carrier function of plastic particles, a bioassay with the simultaneous aqueous exposure of *G. roeseli* to plain mechanically aged plastic particles and a hydrophobic chemical is also conducted. The observations and measured effects are likewise collected for the evaluation of an appropriate exposure scenario as well as for the risk assessment of plastic particles in the aquatic environment (Chapter 8).

Overall, this thesis addresses following questions:

I. Is *Gammarus* spp. suitable to be a representative river-dwelling organism for standard toxicity tests in the laboratory?
    a. Is it usable for acute toxicity tests as well as for long-term experiments?
    b. Is it sensitive and are the measurements of the endpoints reproducible?

II. Is the oral exposure of plastic particles a possible alternative to the aqueous exposure?
    a. Which difficulties can be managed with this tool?
III. Do plastic micro and nanoparticles affect *Gammarus roeseli*?
    a. Is there a difference between natural and plastic particles regarding effects to *G. roeseli*?
    b. Do the plastic particles work as carrier or as passive sampler for solved chemicals?

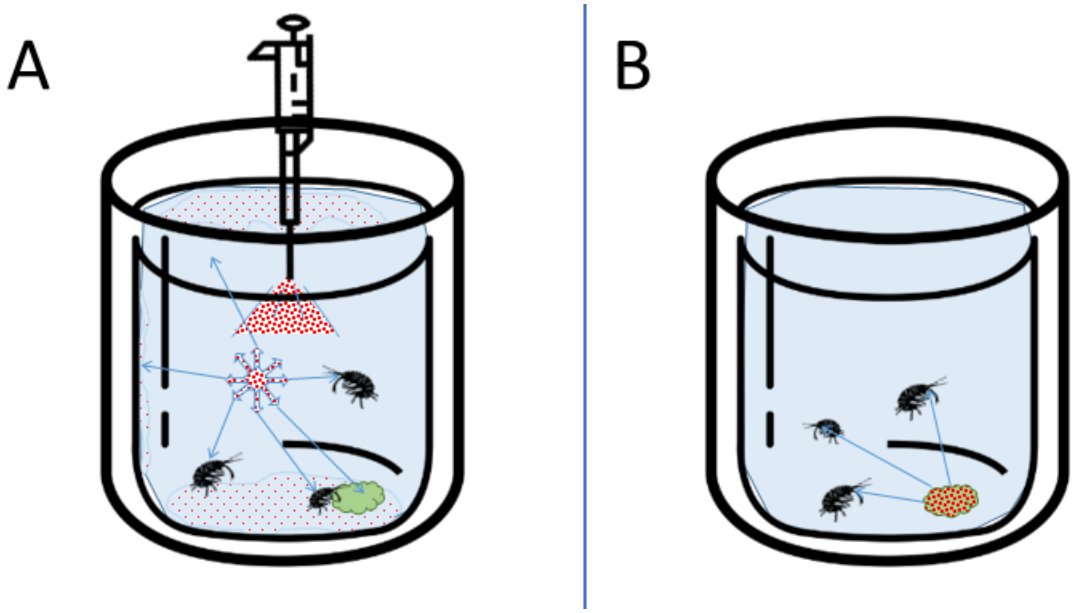

Figure 4.1: Conceptual framework for outlining the assumed differences between the particulate exposure via (A) water phase or (B) a dietary tool. When the exposure scenario is planned via water phase, the particles distribution is quite unpredictable as it depends on too many factors. Particles (red) can (blue arrows) settle down, aggregate, float on the surface, stick to the glass of the test vessel or to the organism instead of being ingested. The idea of the alternative dietary exposure was to stabilize the particles homogeneously distributed in a food matrix to ensure particle exposure by feeding the organisms.

5. General materials and methods

5.1. General study design

In order to answer the questions of this thesis, a straight process in the laboratory with various materials and specific methods is applied. Each step of the process is shown in Figure 5.1 and is conducted in the laboratory. First, the handling of *Gammarus roeseli* during the experiments as well as the measurement of the various endpoints are established to reach reproducibility of the procedures and the applicability of *G. roeseli* as standard organism regarding rivers or lotic compartments. This includes the collection of the gammarids, optimal feeding, set up for swimming behavior and the methods for size and sex determination as well as the establishment and evaluation of energy reserves measurement. Second, the method for particle exposure is investigated. Two particle exposures are examined; the exposure via water phase and the exposure via food. Both exposure pathways have advantages over another and this thesis aims to consider both for risk assessment.

Third and last step is to combine the processes and methods established before and investigate the effects of the plastic particles of polystyrene, polylactide and the natural particles (sediment or silica) in different sizes and concentrations on *G. roeseli*. Also, the vector function of polyamide for phenathrene as an example for a hydrophobic plastic and a hydrophobic chemical is investigated by examining modulated effects on *G. roeseli*. The so reached information is then used to answer the questions of this thesis.

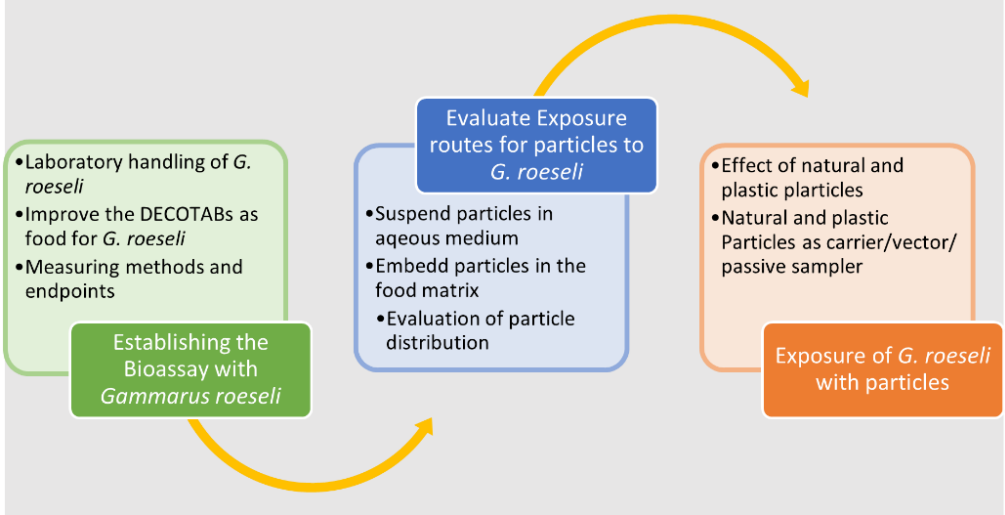

Figure 5.1: General overview of this thesis process to examine the exposure and toxicity of plastic micro and nanoparticles to the newly upcoming standard organism for ecotoxicological assessments *Gammarus*.

5.2. The experimental design

5.2.1. Food preparation

The decomposition and consumption tablets (DECOTABs) were developed by Kampfraath et al. (2012) to have a standardized and modifiable food source for decomposition monitoring in the field or for laboratory experiments. They originally consist of water, agar and cellulose and can be poured into any shape during production. The developer recommends to replace a variable amount of the cellulose with a supplement to adjust the DECOTABs to the specific experiment.

The process to find an optimal food supplement for gammarids is described in chapter 6, which resulted in the replacement of 70 % cellulose weight with phyll (Tetra GmbH, Figure 5.2). The production process of this formulation is described below. Phyll is a dried mixture of various algae and crustaceans with standardized nutrients, which are mainly 46 % crude protein and 9 % crude oil. The mixture also contains vitamin A and D3 as well as manganese, zinc, and iron.

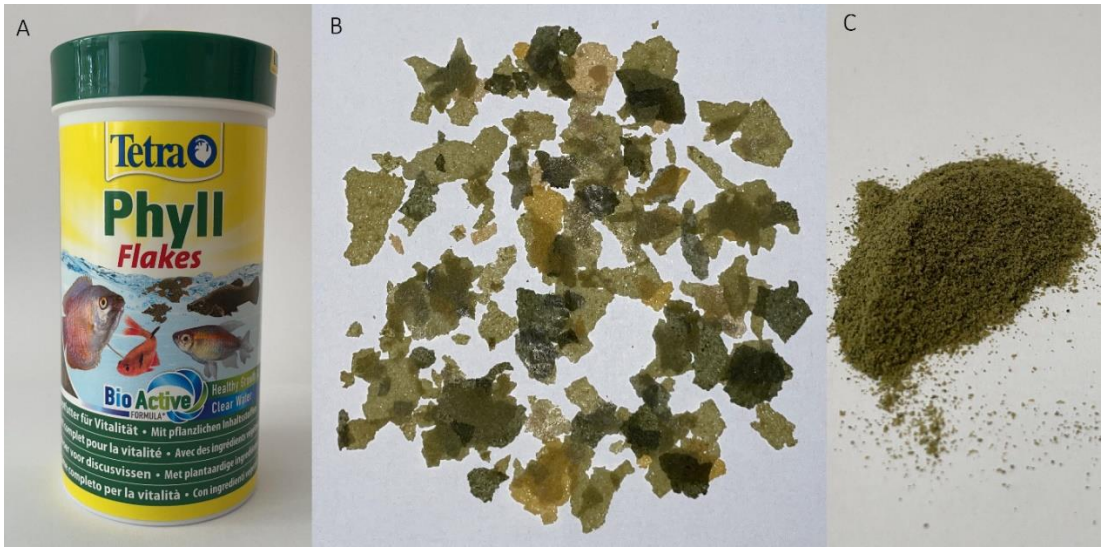

Figure 5.2: (A + B) Phyll Flakes from Tetra GmbH grounded with a mortar to (C) fine powder. The phyll flakes are a standardized source made of algae and crustaceans for fish feeding. Pictures taken by D. Gonzalez.

Next to the purpose as food source for the gammarids in the experiment, the modified DECOTABs, further named phyll-tabs, can be used to measure the decomposition performance or feeding rate of the organisms (Kampfraath et al. 2012). The feeding rate is an indicator for the function of *Gammarus* as shredder, predator and detritus feeder in their habitat. When the function of the gammarids in their food web is

disturbed, it can additionally have an indirect negative impact on all other organisms in the habitat.

The standard production procedure of the final phyll-tabs is like follows: The purchased phyll flakes are grounded to powder and weighed to 4.2 g. The weight of cellulose is 1.8 g and agar is 2 g. 100 mL water is prepared in a 250 mL beaker with an agitator. In a first step, the agar is added to the water and both are heated in a microwave at 360 W (Panasonic) for around one to two minutes. The heating process is instantly stopped when the mixture starts to foam. The beaker is then placed on a magnetic plate and the mixture is stirred for two minutes with the agitator at 700 rpm. After 2 minutes, the phyll powder and cellulose are carefully added and the rotational speed of the agitator is increased to the maximum (> 1100 rpm). After another 2 min, the viscous mixture is poured into a stainless steel form with 152 cylindrical molds of 1 cm diameter and 0.5 cm height. The supernatant is wiped off with a plate and the form is placed in a refrigerator at 6 °C for at least 5 min. When the mixture cooled down, the gummy phyll-tabs are removed from the mold with a glass stick and placed on the round site in a petri dish. At last, the tabs are dried for one to three days at 45 °C. After drying, the tabs can be stored in the refrigerator at 6 °C or instantly used in the experiments.

For the application in the experiments, stored phyll-tabs need to be dried again for at least one day in a drying cabinet at 45 °C until no weight change can be measured. After this, the tabs are weighed on a fine scale (Satorius) to the nearest 0.01 mg for initial dry weight and separately placed into 50 mL deionized water for 1 day to make them easier palatable. When the test starts, the single tabs are allocated to a specific beaker according to the treatment in order to be able to connect the initial dry weight with the final dry weight of each phyll-tab. After several days in the experiment, but at last after one week, the phyll-tabs can be removed and dried again for one to three days at 45 °C in the drying cabinet. After weighing for the final dry weight, the weight loss per day and gammarid can be calculated with respect to the natural weight loss as described in chapters 6 to 8.

The calculation method varies in the experiments in chapter 6, 7 and 8. In chapter 8, the initial dry weight is calculated from the mean dry weight of 150 phyll-tabs from a

preceding weighing experiment. As this method includes a small measurement inaccuracy for each tab, the individual initial phyll-tab dry weight is used in the experiments in chapter 6 and 7.

5.2.2. Bioassay

The gammarids for the studies of this thesis are collected in the River Moosach near the Aquatic Systems Biology Unit in Freising. Collection occurred either with nets or traps. The traps are filled with plants of the habitat for more attractive hiding possibilities and trout food as bait. The caught gammarids are carefully washed off the net or plants with river water into buckets and instantly transported to the laboratory.

At the laboratory, the gammarids are size selected by passage of a cascade of two sieves. One sieve has 3 mm and one has 1.5 mm pores, which leads to three size classes. Juvenile gammarids smaller than around 9 mm are able to pass both sieves and old gammarids greater than approximately 10.5 mm can not pass the upper sieve with 3 mm pores. The middle size class with young adult gammarids from about 9 to 10.5 mm size is chosen for the studies and is therefore reviewed and classified into the wanted *Gammarus roeseli* and the other gammarids. The other gammarids not corresponding to the middle size class or the species *G. roeseli* are released back to the River Moosach.

Acclimatization is conducted for one week in a climate chamber with steady conditions of 13 ± 0.5 °C and a 16:8 h light:dark cycle. In the first experiments, the individuals of *G. roeseli* were not fed and slowly acclimatized in two steps of three days to artificial water (ISO 6341 2012) but later experiments were conducted with tap water and the acclimatization was instantly started with the new medium to avoid stress during water exchange and transfer to new aquaria. In addition, the gammarids are fed ad libitum with phyll-tabs. Independent of the used water, it is aerated and cooled in the climate chamber conditions for at last one day before the gammarids were transferred into the aquaria. In the first experiments, the aquaria were filled with around 6 L of the water. Later, plants and glass stones for hiding were added to increase the well-being of the gammarids.

The bioassay is conducted with 5 individuals per 1 L beaker in the first experiments (chapter 8), but after a small experiment revealed greater food competition for five

individuals feeding on the phyll-tabs, the number was reduced to three gammarids per beaker (chapter 6 and 7). Each beaker contains three glass stones for hiding to reduce the stress and cannibalism as well as one phyll-tab for feeding.

The number of replicates per treatment was five to seven. In addition to the exposure treatments, a control and a solvent control (ethanol) each are added to the experiments. In the first experiments, beakers were aerated constantly, but after a small experiment without steady aeration showed just minor changes in the periodically measured parameters oxygen, pH, conductivity and temperature, aeration was omitted to avoid disturbance of particle distribution and organisms. For longer experiments, water and food are completely exchanged weekly by transferring the gammarids into new prepared beakers with the same conditions. The beakers are randomly placed in the climate chamber to avoid position dependent responses (e.g., by nearer and therefore possibly colder position of the beaker at the ventilator).

5.2.3. Swimming behavior determination

The ability to swim is essential for gammarids to search for food or hiding places against predators, avoid unpleasant pollutions and for reproduction. Therefore, the determination of their swimming behavior and velocity after exposure to a pollution is one way to examine survival probability and healthiness of the organism and population. This is much more sensitive than the determination of mortality as a slow or immobile gammarid is an earlier indication for negative impact of pollutions.

For the determination of the velocity of the gammarids before, during and after the exposure, the program Ethovision XT, versions 9 or 11, from Noldus is used. The gammarids are tracked at test start to have a standard velocity marking the point, when the gammarids are in best state. It is also possible to determine velocity every time during the experiment, but the experiment should end with the last determination for the comparison of best state and last state after exposure.

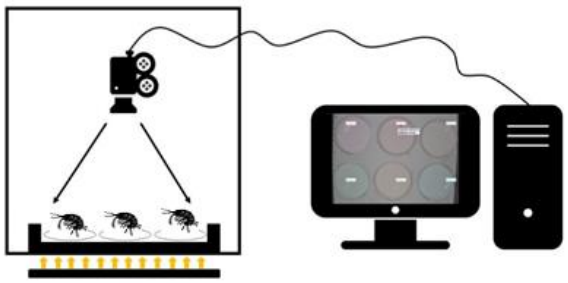

Figure 5.3: Schematic illustration for the setup to determine swimming behavior (by G. Thomas). The gammarids are individually placed in 5.5 cm petri dishes on a light board with 10 mL of their exposure medium under a camera. The camera films 6 organisms in their specific area for 10 minutes with 25 frames per second and the program Ethovision XT (Version 9 or 11) tracks their movement simultaneously. The results are given in moved distance (cm) and velocity (cm/s).

The setup for the tracking is schematically illustrated in Figure 5.3. Petri dishes with 5.5 cm in diameter are defined as arenas and filled with 10 mL of the artificial water used in the beaker where the gammarid is from. Afterwards, each single organism is caught carefully with a long spoon and allocated to the prepared arena according to beaker and treatment.

The arenas are placed on light boards (M.Way, China) 30 cm under a camera. The setup with light board, arena and camera is permanently placed in a completely closable dark box to avoid light movement interruptions which could disturb the precise tracking. This dark box is attached on a table in the climate chamber where the

whole experiment is conducted to provide the same temperature conditions (Figure 5.4).

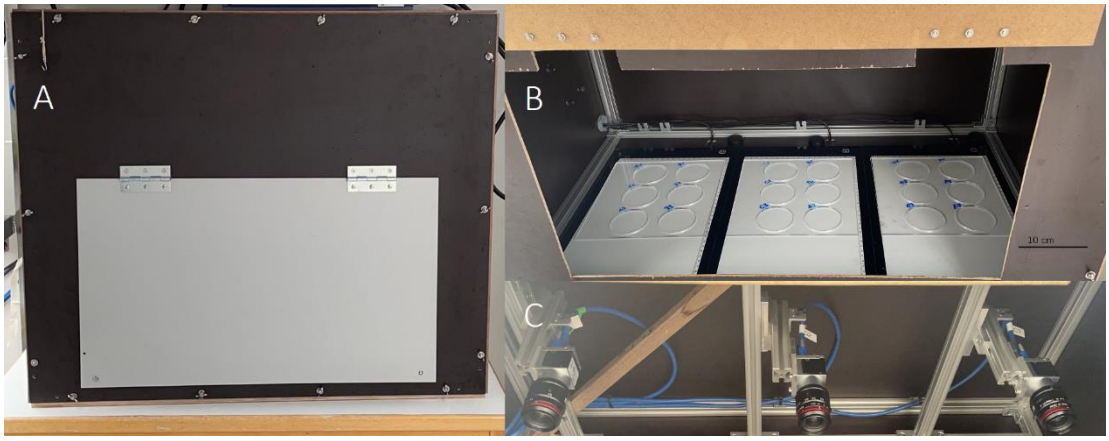

Figure 5.4: Setup for swimming determination. (A) The box is placed in the climate chamber where the experiments are conducted to provide same temperature conditions. The grey lid can be opened for test preparation and closed during measurement. The petri dishes with the gammarids are placed on the predefined areas on the light boards (B) und the camera (C). Pictures are taken by D. Gonzalez.

The camera films the gammarids on the enlightened background for 10 minutes with 25 frames per second (Figure 5.5). The video is constantly sent to the computer. The program Ethovision XT 9 (later 11, Noldus) can simultaneously track the moved distance for each gammarid by recording the coordinates and calculating it with a predefined scale to cm. The results are given as distance moved in cm and velocity in cm/s for each gammarid.

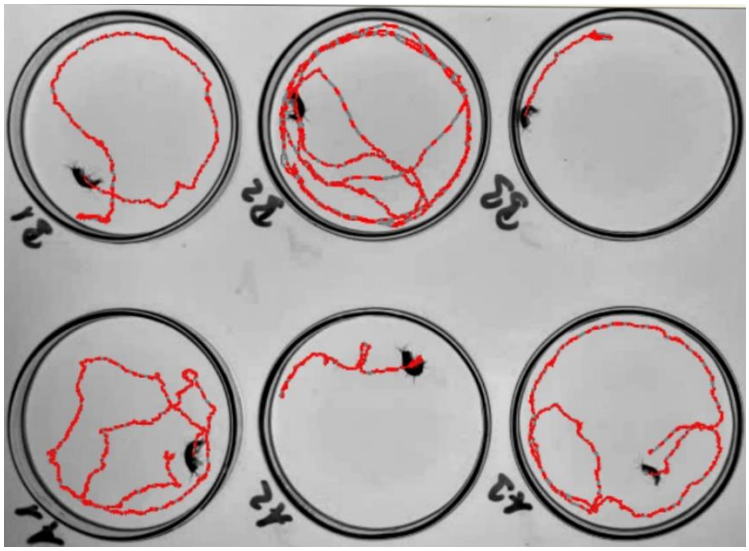

Figure 5.5: An exemplary picture of a video filmed by a camera placed 30 cm above the arenas. One petri dish is one arena. The dishes are placed on a light board to give a high contrast to the gammarids for precise tracking. One gammarid is placed in one arena and tracked for velocity measurement (red and grey dots). The grey dots mark extrapolations of the gammarids movements, the red dots mark the successful tracking points.

5.2.4. Sex determination on *Gammarus* spp.

Sex for gammarids can easily be determined at the end of the experiment. It can be worthy to examine whether the sex of the gammarids in the test vessel has any effect on the observed endpoints or how the ratio of female and male gammarids was.

Therefore, the remaining living gammarids are anaesthetized in carbon dioxide. The immobile gammarids are then turned upon their back and the pleopods are turned to the telson. Male gammarids have two papillae at the basal end of the 5th pereiopod (Figure 5.6). Female gammarids can be determined by the brood plates at the basal end of the first three pereiopods.

It is recommendable to determine sex for recently died gammarids as soon as possible. The degradation of the gammarids body occurs very fast even when stored at -20 °C and subsequently, sex determination becomes more difficult.

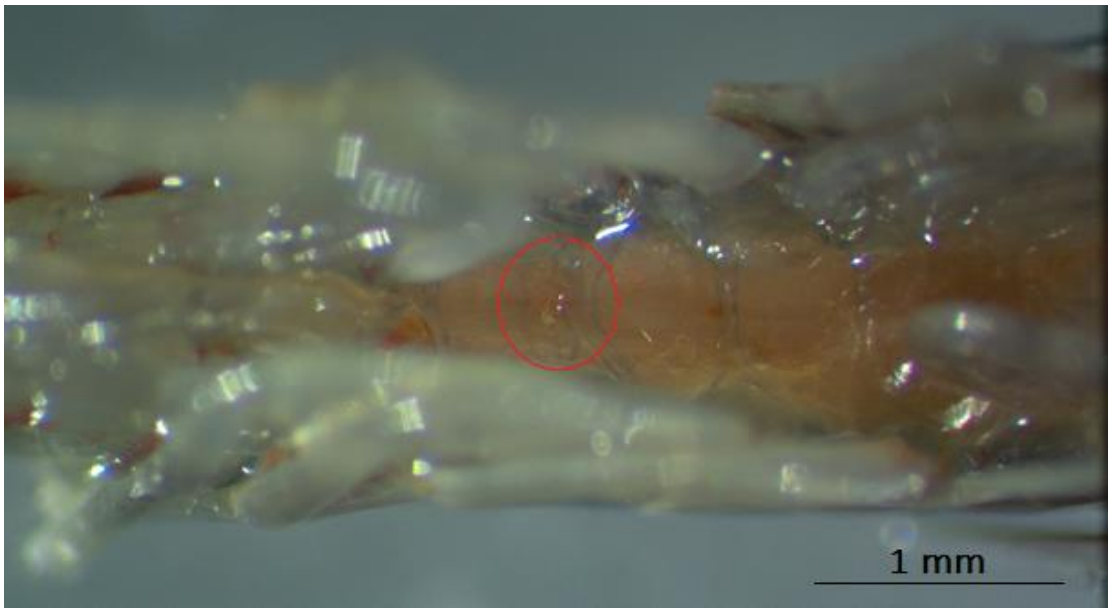

Figure 5.6: Penes papillae (red circle) of male *Gammarus roeseli* at the dorsal end of the 5th pereiopod. The gammarid was anaesthetized in carbon dioxide and turned on his back. The pleopods were turned to the telson to uncover the papillae on the last segment before the pleopods.

5.2.5. Size measurement on *Gammarus* spp.

Size measurement is useful for a vague age determination or as a benchmark like dry weight for comparisons of the endpoints like energy reserves. It further correlates with biomass and can be used like dry weight for dose-response analyses. Therefore, each gammarid is measured in length for potential relations or ratios. A picture of each gammarid is taken with a camera installed on a stereomicroscope with which the measurement can be conducted time-independent and without movement of the gammarid.

Size measurement is based on the instructions of Burgherr and Meyer (1997). There are two options for size determination. First option, the head capsule is measured from the basal end of the antennae to the dorsal end of the head segment as shown in Figure 5.7. The second and applied option because of the best correlation between size and weight was body size. This is measured from the basal end of the antennae along the segments down to the telson. The gut is used for orientation as it lies close to the segments mid (Figure 5.7). This procedure allows an approximate accurate body size measurement independent of the body curvature.

Figure 5.7: Example for the measurement of head capsule and body length (red line). Both measurements start at the basal end of the antennae. The head capsule is then measured to the dorsal end of the head segment, while the body length is measured over the segments along the gut near to the mid of each segment to the telson.

5.2.6. Gut preparation from *Gammarus* spp.

In chapter 7, the gut of the gammarids is carefully dissected from the gammarids before transfer to the freezer, and measurement of energy reserves for subsequent microbiome analyses is not included in this thesis. Nevertheless, to show that there is no or minimal loss of body remains for energy reserves, the dissection method is shortly described.

The gammarids are anesthetized in carbonized water before dissection and placed on a petri dish. To dissect the gut, the gammarid is carefully fixated with a tweezer at one of the last segments by sliding with one spike under the segment and holding it with slight pressure. The next segment is grabbed with another tweezer and carefully torn from the front part of the gammarid. The gut slides thereby with the last segments out of the body without destroying anything of the front part (Figure 5.8). Sometimes, the glands are dissected with the gut and can be easily removed from the gut and added to the sample for energy reserves.

Figure 5.8: Dissected gut (mid) with telson (left) and front part (right) of the gammarid.

5.2.7. Energy reserves measurement

Energy reserves including lipids, protein, glycogen, and glucose are already established molecular endpoints in bioassays. It is assumed, that species build up or use their energy reserves during life cycle and under stress conditions. This molecular endpoint is an earlier indicator of stress than mortality or changes in behavior. Therefore, the measurement of energy reserves is a helpful and comprehensible step for toxicological assessment. Especially when no mortality is expected like in the toxicity assessment for plastic particles. Therefore, the assay is integrated in the experiments of chapter 6 and 7.

An assay for the energy reserves of lipid, glucose, and glycogen in mosquitos was established by van Handel (1985a, b) and Charron et al. (2015) modified the assay for gammarids. The modified method has been adapted and examined for variability induced by changes during test conduction. This includes the stability of the reagents and the usability of different wavelengths or dilution of the suspension (see Appendix 13.1). van Handel (1985 a, 1985b) recommends diluting solutions or measuring them with a different wavelength when solutions are too highly concentrated for reliable measurement.

The assays for lipid and glucose/glycogen are extended in chapter 7 with the measurement of proteins according to Bradford (1976) and Walker (1996).

The energy reserves measurement is conducted at the end of the experiment. The gammarids are taken from the beaker and anesthetized with carbon water. Before the gammarids are stored separately in 1.5 mL tubes with conical end at -20 °C, pictures can be taken for size measurement (chapter 5.2.5). For each assay, a maximum of 20 Tubes, corresponding to 20 gammarids, are taken from the freezer and dried for one day at 45 °C in a drying cabinet. This is necessary to receive the dry weight of each gammarid, which is more precise than the wet weight as this parameter is falsified by probably remaining water. The dry weight of each gammarid is determined on a small alu piece with a fine scale to the nearest 0.01 ± 0.02 mg (Satorius).

Afterward, the 20 gammarids are grind with a mortar shaped like the conical end of the tubes and suspended in 900 µL methanol (95 % v/v). In chapter 6 the mortar was used by hand while in chapter 7 the same mortar was fixed in a Dremel which

improved the grinding by a faster rotation speed resulting in finer powder. To receive ideally fine powder, the gammarids body is first grinded dry and, in the assay in chapter 7, in a second step with 200 µL methanol. This suspension is then filled up to 900 µL. Aliquots for lipid, protein, and glucose/glycogen measurement are taken from this stock suspension and transferred into new 1.5 mL tubes for lipid and glucose/glycogen and into glass tubes for protein measurement. In chapter 6, only lipid and glucose/glycogen assays were conducted. The measurement for protein was newly added in chapter 7.

In chapter 6, between 200 and 400 µL were taken for the lipid aliquot and between 400 and 600 µL were taken for the glucose/glycogen aliquot depending on the gammarid size to avoid measurements above the range of the calibration curve. In chapter 7, the aliquot proportions were fixed to 300 µL each for lipid and glucose/glycogen assay and 50 µL twice for protein assay.

The further process of the assays is precisely conducted as in chapter 6 and 7 described and illustrated in the flowchart, excluding the protein assay (appendix 13.2).

Glucose/glycogen measurement

Sodium sulfate (2 %, 200 µL) is added to the glucose/glycogen aliquot (300 - 600 µL) to precipitate the glycogen and mixed on a vortexer. Afterwards, the suspension is cooled for 20 min at 4 °C and centrifuged for 4 min at 11 000 *g* (Centrifuge 5430R; Eppendorf). The precipitated glycogen gathers in the pellet while the supernatant contains the glucose. The supernatant is carefully and completely transferred into a new 1.5 mL tube. The remaining glycogen pellet is resuspended with distilled water according to the amount of aliquot. E.g., when 400 µL was taken as glucose/glycogen aliquot, the pellet was diluted in 400 µL distilled water.

The same volume is separately transferred from each suspension to glass test tubes. After the addition of 5 mL anthrone-reagent (yellow color), the suspensions are heated at 95 °C for 17 min. A color change from yellow to green allows a photometric measurement. Photometric measurement was performed using the photometer UVIKON 930 at 625 nm against the reagent as blank. The amount of absorption shows the amount of glucose and glycogen on the basis of a calibration curve. Glucose and glycogen are calculated in micrograms per milligram dry weight and the provided

energy is converted with 17,500 mJ/mg glucose and glycogen according to de Coen and Janssen (1997).

Lipid measurement

Chloroform is added to the lipid aliquot in a 2:1 ratio to extract the lipid, and mixed with a vortexer. The suspension is then cooled for 20 min at 4 °C and 600 µL (chapter 6) or 400 µl twice for duplicates (chapter 7) are transferred to glass test tubes. A water bath at 95 °C is used to heat the suspension for 5 min to evaporate the solvent. The glass tubes are shortly removed from the water bath to add 200 µL sulfuric acid (95 % v/v), and then the suspension is heated for 10 min again. After cooling the samples in an ice bath, 5 mL (chapter 6) or 2.5 mL (chapter 7) vanillin-phosphoric acid as a reagent is added. A color change from colorless to pink was achieved. Photometric measurement was performed using an UVIKON 930 photometer at 525 nm against the reagent as blank. The amount of absorption shows the amount of lipids on the basis of a calibration curve. Lipids are calculated in micrograms per milligram dry weight of the gammarid. The provided energy reserves are calculated according to de Coen and Janssen (1997) by converting the energy per milligram with 39,500 mJ/mg lipids.

Protein measurement

The protein aliquot is diluted with 50 µL distilled water in each glass test tube. As blank, 50 µL of distilled water and 50 µL of the used solvent methanol were pipetted into an extra glass test tube. Finally, 1 mL the Bradford reagent is added to each glass test tube. A color change from brown to blue allows the photometric measurement after 2 min at 595 nm against the blank. The proteins are calculated per mg dry weight of the gammarid using a calibration curve.

5.3. Evaluation of exposure routes for particles

Two possible exposure routes are accounted for in this thesis; the common and more environmentally relevant exposure via the water phase and the reliable and quantifiable exposure via dietary feeding. For both exposure pathways the application methods were tested and, in case the of dietary exposure, evaluated in detail.

5.3.1. Examination of suspension preparation for (plastic) particles

An overview of ecotoxicological studies working with plastic particle suspensions revealed many different methods of suspension preparation (see chapter 3.5). To find an appropriate method for plastic particle powder suspension, a preceding test with various approaches was conducted. Therefore, red polystyrene microparticles with sizes from 10 - 200 µm were used as they were easy to see in the artificial water used in bioassays. All approaches were conducted in 250 mL beakers filled with 200 mL artificial water and in duplicates. This experiment was not published but is worth mentioning for this thesis. The implementation and outcomes are described in chapter 13.1.2.

The examination revealed that suspension of the particles should be done methanol or ethanol. After evaluating the resulting particle concentration in the suspension, the according amount was pipetted directly into the artificial water or the liquid tab matrix during production.

5.3.2. Embedding particles into the food matrix

The phyll-tabs are also intended to be used as a tool for dietary exposure of particles in the experiments. To reach this, particles are suspended in methanol. The suspension is constantly shaken on a vortexer and pipetted into the mixture two minutes after cellulose and phyll are added. After short but intensive stirring, the mixture is poured into the molds and handled as described in chapter 5.2.1. This method is evaluated in chapter 6 and applicated in chapter 7.

Evaluation of particle distribution within the food matrix

Homogeneous particle distribution in the food source as a dietary exposure tool is checked with fluorescent polystyrene micro and nanoparticles (chapter 6). The fluorescence allows to a certain extent the detection and identification of those particles in a mixture with the ingested food or other natural non-fluorescent particles.

The relation of particles per mg dry weight in the food and the mass eaten by the organism gives a more relevant exposure concentration than the concentration in the test vessel (see chapter 6). A homogeneous distribution of the particles in the mixture is a prerequisite for this relation.

Dried phyll-tabs with embedded fluorescent particles are sliced with a cryostat microtome HM 505 E (microm, Figure 5.9). The tabs are fixated on a basis by embedding it completely in Tissue-Tek® O.C.T.™ Compound (scienservices.de) in the working station of the microtome at -18 to -22 °C. The basis with the sample is then installed on the fixation spot and the position and angle of the sample must be modulated to fit the position of the knife. By turning the crank outside the microtome the sample can be sliced in the preferred thickness. Thickness can be changed with the section thickness control. Various slices of thickness between 1 - 80 µm were taken until sections of 50 µm were chosen for further analysis with the fluorescence microscope.

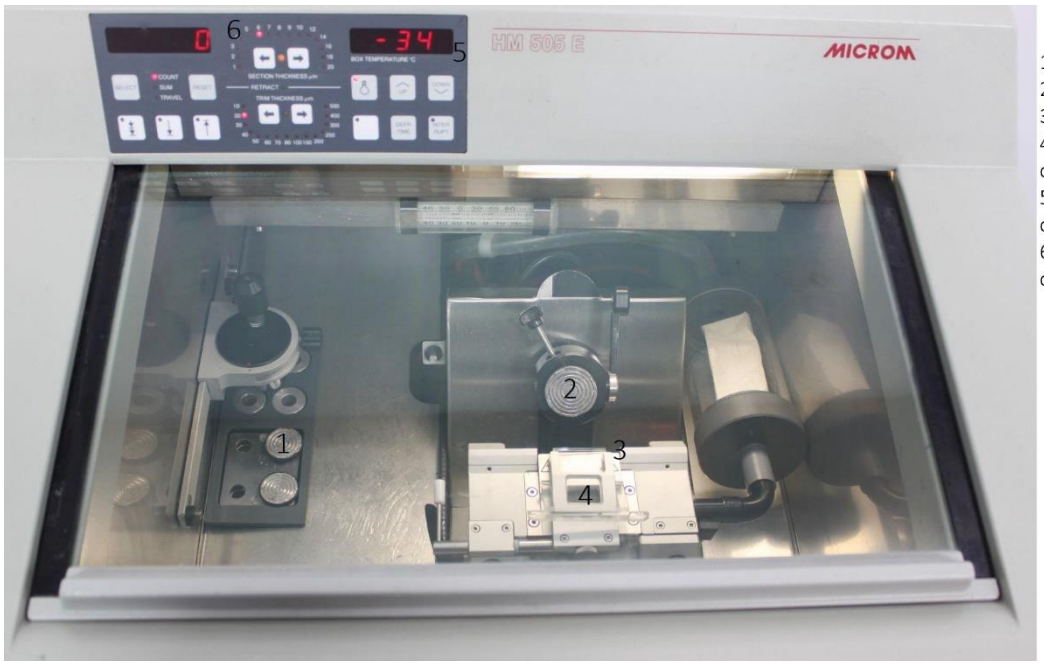

Figure 5.9: Working station of the cryostat microtome HM 505 E (Microm). The basis (1) with the sample is fixated in the fixation spot (2) and adjusted to the position of the knife (3). The sample slices are collectable at the tray (4) with microscope slices. During the procedure it is possible to control temperature (5) and slice thickness (6).

The chosen sections of 50 µm from the dried tabs are mounted on the microscope slices and stitched with the fluorescent microscope. This method results in a complete picture of the individual slices with the fluorescent particles made visual (chapter 6).

5.4. Statistical analysis

The analysis of the data is conducted with R (latest version 4.1.0) and RSstudio (RStudio 2015), and with some exceptions with Jamovi (latest version 1.6.23, Jamovi 2019) which is also based on R. All endpoint data are tested for normal distribution with the Shaprio-Wilk-test followed by the Levene's test in chapter 8 or the robust Filgner-Kileen-test in chapter 6 and 7 for homogeneity in variance. Due to the high amount of non-normal distributions in all endpoint data, the Kruskal-Wallis-test is mainly chosen for group comparisons, otherwise, Analysis of Variance (ANOVA) is used. Statistical significance is seen as given at a significance level of $\alpha = 0.05$ for all tests. When significant differences are found, the groups are analyzed with the pairwise Wilcoxon-test with Benjamini-Hochberg-correction (Benjamini et al. 1998) for independent or dependent samples as a post hoc test.

Dose-response models for mortality, feeding rate, and velocity are calculated in R and Rstudio with the log-logistic regression and the best model is chosen based on the Likelihood-ratio-test and the Akaike-information-criterion.

Further, particle distribution analysis is conducted in PAST (latest version 4.01, Hammer et al. 2001) with point pattern analysis with nearest neighbor classification and wrap-around edge correction. Correlations are conducted with R and Rstudio giving the correlation coefficient *r*. $r = 0$ implies no correlation, whereas $r = 1$ relates to a strong positive and $r = -1$ to a strong negative correlation.

6. Moving Toward Standardized Toxicity Testing Procedures with Particulates by Dietary Exposure of Gammarids

A similar version of this chapter has also been published in the Journal "*Environmental Toxicology and Chemistry*" as Götz A, Geist J, Imhof HK, Beggel S (2021) Moving Toward Standardized Toxicity Testing Procedures with Particulates by Dietary Exposure of Gammarids. *Environmental Toxicology and Chemistry* 40(5):1463-1476. doi: 10.1002/etc.4990

Abstract

Ecotoxicological effect assessment of particulate materials and sparingly soluble substances is an emerging field. Current standard toxicity tests of aquatic organisms are based on soluble substances which are added to the aqueous phase. Although soluble substances distribute homogeneously, particles can form aggregates, resulting in inhomogeneous distribution and unpredictable exposure. Therefore, test scenarios need to be adapted to overcome these uncertainties. We present a dietary particle exposure tool for the toxicity testing of sparingly soluble substances or particles in combination with a standardizable food source for gammarids based on decomposition and consumption tablets (DECOTABs). Four food supplements in the DECOTAB formulation were compared to test their influence on the energy reserves of gammarids. Although feeding rate was constant for most supplements, mortality and energy reserves revealed clear differences. Tabs supplemented with algae-based phyll or animal protein-based trout food best met all of the requirements. Fluorescent plastic microparticles (10 - 65 µm) were homogenously distributed and stable in the DECOTABs. Constant feeding was observed, and the number of ingested microparticles by *Gammarus roeseli* was quantified in relation to the consumed food. The developed method provides a realistic and methodologically reliable uptake from the oral pathway and allows the quantification of inner exposition via feeding rate, providing a promising tool for standardized dietary exposure scenarios with particles.

Keywords: Ingestion; Particle; Toxicity testing; Oral pathway; Feeding rate; Dietary exposure

6.1. INTRODUCTION

In the context of environmental risk assessment, it is necessary to conduct standardized ecotoxicological bioassays that can be linked to realistic exposure scenarios (Connon et al. 2012). Such investigations are well established for many organisms from aquatic environments and are available as Organization for Economic Co-operation and Development guidelines, mainly for cladocera, algae, and fish. However, the present guidelines target the exposure with soluble substances, limiting their applicability for toxicity testing of particles or sparingly soluble substances. In contrast to soluble substances, particles or sparingly soluble substances have other chemical properties, resulting in entirely different distribution in the test medium and therefore affecting uptake routes (Rufli et al. 1998; Hartmann et al. 2015; European Centre for Ecotoxicology and Toxicology of Chemicals 2018; Eitzen et al. 2019). In addition, reliable methods for prediction of resulting particle distributions and exposure are lacking (Rist and Hartmann 2018; Eitzen et al. 2019). This lowers the general comparability of the aquatic ecotoxicological studies on particle effects (Hartmann et al. 2015) and stresses the need for ecotoxicological bioassays that account for particle-specific properties. With respect to sparingly soluble substances, it is more likely that exposure via the food is the main uptake route compared to the surrounding water column (Cole et al. 2011; Bundschuh et al. 2019; Toussaint et al. 2019), which is thereby often neglected.

Therefore, a testing procedure which allows a standardized and systematic testing of particulates or chemicals via the oral pathway is needed (Bundschuh et al. 2019). Such a dietary exposure system would need to fulfill specific prerequisites to ensure a standardized and quantifiable uptake of the substances during the feeding process. This includes comparability in production, homogeneity of the substrate, stability, and minimal weight variation during the test. Further prerequisites are a homogenous distribution of the sparingly soluble substances in the food without aggregate formation, adjustable dosimetry, and minimal leaching of embedded sparingly soluble substances. Also, constant feeding by the test organisms is necessary to allow quantification of embedded and ingested particulates.

Decomposition and consumption tablets (DECOTABs; Kampfraath et al. 2012) can meet these prerequisites and have been successfully used as a food source in aquatic

toxicity tests (Straub et al. 2017; Raths et al. 2020). It was recommended by the authors, and applied by several studies, that DECOTABs can be amended by adding food supplements for specific applications or loading them with chemicals during production (Kampfraath et al. 2012). Some exposure studies have already used loaded tabs (Zhai et al. 2018) or similar matrices (Imhof et al. 2013; Hämer et al. 2014; Imhof and Laforsch 2016; Fenoy et al. 2020; Yardy and Callaghan 2020). Therefore, DECOTABs are a promising tool for a standardized toxicity testing procedure with organisms from different functional feeding groups such as shredders and grazers.

Decomposition and consumption tablets already meet some of the stated prerequisites such as minimal weight variation and quantifiable uptake, which helps to ensure a high comparability between different experimental runs (Kampfraath et al. 2012). Nevertheless, a deeper examination concerning the needs of the targeted organism regarding nutritional value and survival by adding food supplements is still required. Subsequent upcoming prerequisites are stability of the supplemented DECOTABs, the distribution of the particles in the DECOTABs, and the leaching of particles to the surrounding medium.

In the present study, we focus on a river organism because many sensitive organisms of lotic environments are underrepresented in established guidelines (Feiner et al. 2016). Suitable organisms for ecotoxicity testing of river ecosystems are *Gammarus* spp., which are already often examined and known to be sensitive to many pollutants (Gerhardt et al. 2011; Brock and van Wijngaarden 2012). The characteristics of *Gammarus* spp., like a wide trophic repertoire, foraging plasticity, and migration ability, make this keystone species in the food web a representative benthic river organism (Gerhardt et al. 2011; Boeker and Geist 2015). In addition, a basic guideline for ecological testing with gammarids is provided by the US Environmental Protection Agency (2016).

With the objective of moving toward a standardized protocol for toxicity testing with gammarids, we first evaluated the suitability of DECOTAB formulations with different food supplements as a food source for *Gammarus roeseli*. This was tested based on food source properties, gammarid response in feeding behavior, and energy content. In a second step, to adapt the protocol for testing with particulates, we evaluated the applicability of the test system as a dietary exposure tool of particles or, with regard

to aggregates and homogeneous distribution, sparingly soluble substances by testing the practicability of loading the adjusted food source with microparticles and the constant and quantifiable supply of particles over the oral exposure pathway.

6.2. MATERIAL AND METHODS

6.2.1. Evaluation of DECOTABs with food supplements

To examine a nutritionally valuable and standardizable food source for toxicity testing with gammarids, *G. roeseli* were fed over the course of 21 d with different formulations based on the DECOTAB as originally described by Kampfraath et al. (2012). This formulation, filled with cellulose and 4 DECOTAB formulations modified with specific food supplements, was tested for variability in weight, based on size and initial dry weight, and stability over time. Stability in water was measured during a watering experiment for 3 and 4 d. Next to mortality and the feeding behavior of the gammarids, their energy reserves were monitored by the measurement of lipids, glycogen, and glucose.

The DECOTABs

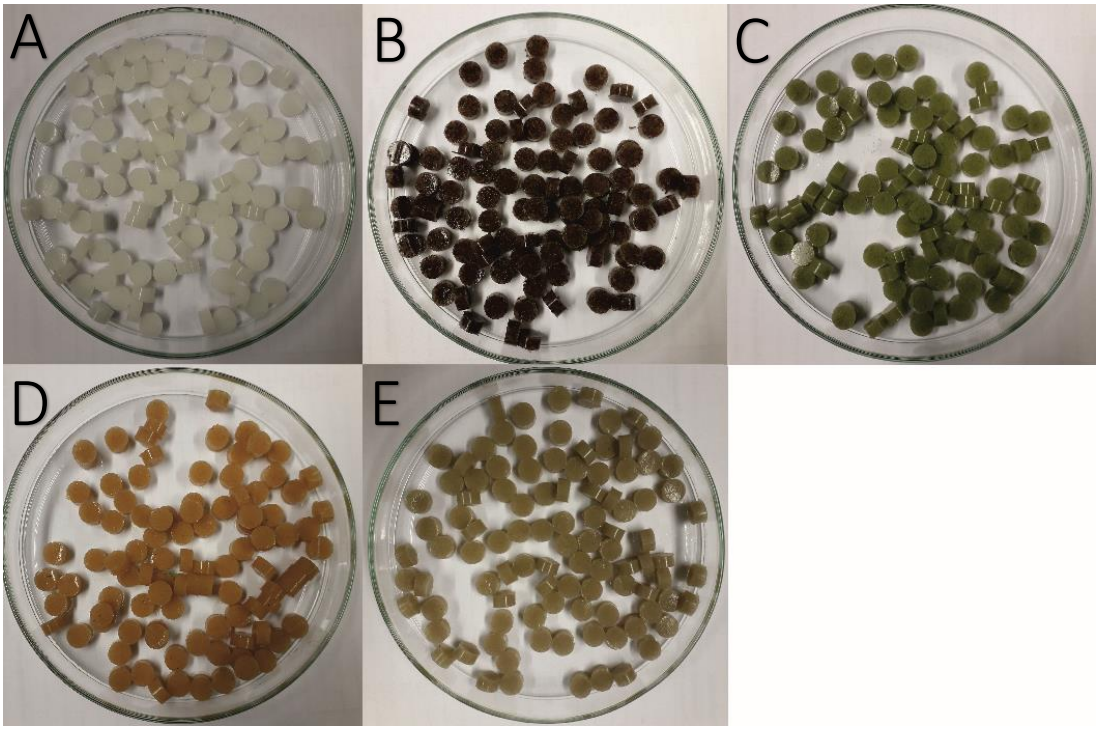

Figure 6.1: Prepared decomposition and consumption tablets before first drying. (A) Cellulose-tabs, (B) beech-tabs, (C) phyll-tabs, (D) gammarus-tabs, and (E) trout food-tabs.

Formulation and preparation. The DECOTABs (Kampfraath et al. 2012) were produced with 80 mL distilled water and 1.6 g agar (Sigma-Aldrich). This mixture was heated for approximately 1.5 min until it foamed and homogenized with an agitator. Then 4.8 g cellulose (Sigma-Aldrich) was added and homogenized for 1 min. The mixture was poured into 50 cylindrical molds of 1 cm diameter and 0.5 cm height (custom-built

stainless steel device; Chair of Process Systems Engineering, Technical University of Munich). The supernatant was scraped using an even scraper. After 15 min in the refrigerator at 6 °C, the DECOTABs were removed from the molds and dried in a drying cabinet (U 40; Memmert) at 45 °C for 24 h. After this, the DECOTABs were placed into a desiccator for 30 min, and initial dry weight (dw_I) was measured with a Sartorius R200D Analytical Balance (Sartorius; 0.01 ± 0.02 mg). Before the transfer to the experiment, the DECOTABs were prewetted in the test medium for 48 h in the climate chamber at 13 ± 0.5 °C.

For the modified DECOTABs, the same procedure was used, but 70 % of the cellulose weight was replaced with the particular food supplement. This results in 1.44 g cellulose and 3.36 g of the food supplement added to 80 mL distilled water. The following 4 DECOTAB formulations with specific food supplements were tested in addition to the cellulose-tab, solely containing cellulose and agar: phyll-tabs (ground flakes of the algae-based fish food Phyll; Tetra), gammarus-tabs (powdered dried gammarids; Dehner), trout food-tabs (ground pellets of the commercial trout food Advance, 0.2 - 0.3 mm; Alltech Coppens), and beech-tabs (dried and powdered beech leaves; Figure 6.1).

Variability (size, volume, dry weight)

To evaluate the effect of drying and prewetting on the DECOTABs and to evaluate dry weight stability, 150 cellulose-tabs were weighed at different steps of handling using a fine scale (Sartorius; 0.01 ± 0.02 mg). Initial wet weight (ww_I) of freshly prepared but not dried DECOTABs was measured for three production iterations (n = 50) to evaluate wet weight variation. In addition, the dw_I and the final dry weight (dw_F) after 48 h prewetting were measured to examine the dry weight variation within and between the dry weight states. Further, the wet weight after prewetting of dried DECOTABs for 24 and 48 h was measured to verify whether their ability to absorb the water again.

Stability in water

Weight loss of the different DECOTAB formulations in water without further impact was monitored to evaluate the stability during bioassays. Therefore, 14 DECOTABs of each food supplement were weighed to examine dw_I, transferred into tap water, and prewetted for 2 days in the climate chamber (13 ± 0.5 °C) prior to the experiment. To

mimic the food replacement cycle of the bioassay with gammarids, the experimental procedure was as follows. After prewetting, 7 DECOTABs of each food supplement were placed into glass beakers (1 L) filled with 500 mL tap water, 3 glass stones for hiding, but no gammarids and kept in a climate chamber with a 16:8 h light:dark cycle and 13 ± 0.5 °C. After 4 days, the DECOTABs were replaced for another 3 days by the remaining 7 DECOTABs. After the experiment, the DECOTABs were dried again for 24 h, placed into a desiccator for 30 min, and weighed with a Sartorius R200D Analytical Balance (0.01 ± 0.02 mg) to measure the dw_F. The difference between dw_I and dw_F as a percentage per day gives the stability for each DECOTAB formulation and allows comparisons between the treatments. In addition, the dry weight difference in milligrams per day was used as an adjustment factor for the feeding rate of the gammarids.

The bioassay

Gammarus roeseli were caught from the River Moosach at the Aquatic Systems Biology Unit in Freising, Germany, with plant-filled traps and trout food as bait in November 2019. Subsequently, gammarids were size-selected (9.9 ± 1.5 mm, n = 534) by sieve passage (Beggel et al. 2016), and their body size was determined as described below (see Gammarid length and dry weight).

To assess their natural energy reserve state, 100 *G. roeseli* were directly transferred to liquid nitrogen and stored at -20 °C until further analysis.

For acclimatization to the test conditions, 500 gammarids were evenly allocated to the 5 treatments and transferred to aerated 5 L glass beakers with 5 L of tap water and glass stones. Acclimatization occurred in a climate chamber for 1 week with a 16:8 h light:dark cycle and 13 ± 0.5 °C. The 100 gammarids per treatment were fed ad libitum with 10 DECOTABs of the formulation corresponding to the treatment. For determination of energy reserves after the acclimatization period (acclimatization state), 20 randomly chosen gammarids from each treatment were transferred to liquid nitrogen and stored at -20 °C until further analysis.

After the acclimatization period, three randomly chosen gammarids were placed into each of the 21 glass beakers (1 L) per treatment filled with 500 mL tap water, 3 glass stones for hiding, and one dried and preweighed DECOTAB. The experiment took place under the same conditions as the acclimatization period, except aeration. Instead,

water change was conducted once per week and oxygen content, conductivity, temperature, and pH were monitored. All parameters were constant throughout the experiment, and oxygen concentration was always >7.8 mg/L. Gammarids were fed with one DECOTAB of the corresponding formulation per experimental beaker in an alternating exchange interval of 4 and 3 days. Because the remains of approximately 67 ± 7 % of the DECOTABs were found in the glasses after 3 or 4 days, ad libitum feeding can be anticipated. Mortality was controlled daily, and dead gammarids were removed from the beaker and stored at -20 °C until further examination.

After 1, 2, and 3 weeks, 7 replicates were removed from the experiment; and the corresponding individuals were transferred to liquid nitrogen and stored at -20 °C until measurement of the energy reserves. In addition, 42 gammarids were starved for 16 days, then transferred to liquid nitrogen, and stored at -20 °C until further examination of the energy reserves from starvation state.

Feeding rate

To determine the feeding rate, DECOTABs were dried for 24 h to measure dw_I, then they were watered again for 48 h prior to the transfer to the experimental beakers. The DECOTABs were replaced by new ones in an alternating cycle of 4 and 3 days. The used DECOTABs were again dried for 24 h to determine dw_F. The feeding rate (FR) per day and gammarid (gd) was calculated with dw_I and dw_F of the specific DECOTAB, adjusted by the number of feeding days and the mean milligrams of weight loss per day (MWL) calculated from the stability measurement:

$$FR\left[\frac{mg}{gd \times day}\right] = \frac{\frac{dw_I\,[mg] - dw_F\,[mg]}{\text{feeding days}} - MWL\left[\frac{mg}{day}\right]}{n\ (\text{living gammarids})}$$

Gammarid length and dry weight

Prior to further processing of the gammarids, samples were defrosted, and pictures were taken with a stereomicroscope (M3Z; Wild Heerbrugg; equipped with an SC180 camera; Olympus). The size of the gammarids was determined by measuring the length from the base of the first antenna to the end of the uropod by a polyline along the gut using an image analysis system (CellSens Entry, Ver 1.18; Olympus) according to Burgherr and Meyer (1997). After the measurements, gammarids were dried at

45 °C for 24 h, placed into a desiccator for 30 min, and weighed with a Sartorius R200D Analytical Balance (0.01 ± 0.02 mg) to examine gammarid dry weight.

Energy reserve determination

The energy storage assay followed the original protocol by van Handel (1985) and the modifications by Charron et al. (2014), with some further adjustments. The assay for lipids and the carbohydrates glycogen and glucose is described in brief; a schematic view is available in the Supplemental Data (appendix, 13.2). Dried gammarids were frozen with liquid nitrogen in a 1.5 mL centrifuge tube and ground to a fine powder with a stainless-steel pistil. Powdered gammarids were suspended in 900 µL methanol. Aliquots were transferred into new 1.5 mL tubes for the lipid assay and the glucose/glycogen assay. Energy reserves were calculated according to de Coen and Janssen (1997) using the energy per milligram of storage substance with 39,500 mJ/mg lipids and 17,500 mJ/mg glucose or glycogen.

Lipid assay. Chloroform was added to the lipid aliquot (2:1 ratio) to extract the lipids from the powder. After mixing thoroughly, the suspension was cooled for 20 min at 4 °C, and 600 µL were transferred to glass test tubes. The suspension was heated for 5 min at 95 °C until the solvent evaporated. After adding 200 µL sulfuric acid (95 % v/v), the suspension was heated again at 95 °C for 10 min and cooled in an ice bath. By adding 5 mL vanillin-phosphoric acid a color change from colorless to pink was achieved. Photometric measurement was performed using a UVIKON 930 photometer at 525 nm against the reagent as blank. The amount of absorption shows the amount of lipids on the basis of a calibration curve. Lipids were calculated in micrograms per milligram dry weight.

Glucose and glycogen assay. Sodium sulfate (2%, 200 µL) was added to the glucose/glycogen aliquot to precipitate the glycogen. After mixing thoroughly, the suspension was cooled for 20 min at 4 °C and centrifuged for 4 min at 11 000 *g* (Centrifuge 5430R; Eppendorf). The supernatant with the glucose was transferred into a new 1.5 mL tube. The remaining glycogen pellet was resuspended with 400 µL distilled water. Then, 400 µL from each suspension were separately transferred to glass test tubes. After the addition of 5 mL anthron reagent, the suspensions were heated at 95 °C for 17 min. A color change from yellow to green allowed a photometric

measurement. Photometric measurement was performed using a UVIKON 930 at 625 nm against the reagent as blank. The amount of absorption shows the amount of glucose and glycogen on the basis of a calibration curve. Glycogen and glucose were calculated in micrograms per milligram dry weight.

6.2.2. DECOTABs as oral exposure tool for particles

Decomposition and consumption tablets were examined as a dietary exposure tool for particle testing. Therefore, a suspension of red fluorescent polystyrene microparticles (10 - 65 µm) was prepared and added to the DECOTABs during preparation. These particles were tracked in sections of the DECOTABs by fluorescence microscopy, and the concentration per milligram and volume was calculated. Afterward, gammarids were exposed to blue fluorescent microparticle-filled phyll-tabs, and the inner particle concentration was monitored by gut dissection and subsequent fluorescence microscopy. Spherical fluorescent polystyrene nanoparticles of 1000 nm were likewise embedded in phyll-tabs and imaged to get first impressions of nanoparticle distribution.

Particle distribution in the DECOTABs

To receive red fluorescent polystyrene microplastic particles with a fraction of 10 - 65 µm, 200 mg of fluorescent microparticles generated by centrifugal milling (0 - 125 µm, Ultra Centrifugal Mill Type ZM 200; Retsch) were suspended in 54 mL ethanol (96 %, undenatured) and centrifuged according to Correia and Loeschner (2018) for 44 s at 8.5 *g* to separate particles at 65 µm. The supernatant was transferred into new centrifuge tubes and centrifuged again for 1 min at 95 *g* (Labofuge 400; Heraeus Instruments). Supernatant with the 10 - 65 µm particles was removed by pipetting, and the pellet was resuspended in 8 mL ethanol to a concentration of approximately 31 particles/µL. The size distribution (Appendix, Figure 13.11) was verified using a Mastersizer S longbed (Malvern).

Stock suspension (3200, 1600, or 160 µL) was added during the DECOTAB production after the homogenization of the formulation for 1 min (for detailed production, see above, *The DECOTABs*). The suspension was again homogenized for approximately 30 s and poured into the molds. Further DECOTAB handling was as described.

One to 3 horizontal sections with 50 µm thickness from 2 dried DECOTABs for the

lowest and middle and 3 from the highest concentration were generated with a microtome cryostat HM 505 E (Microm) and from one DECOTAB per concentration in the vertical direction. Sections were mounted on microscopic slides with the use of VECTASHIELD Antifade Mounting Medium (Vector Labs) and imaged under a Leica DMi8 with a CoolLED Pe4000 light source (Leica Microsystems) with green fluorescent protein excitation for green phyll-tab fluorescence and rhodamine (Rhod) excitation for red particle fluorescence. Images were exported, and extended depth-offield was calculated from the approximately 20 Z-planes to project all particles into one single layer using FiJi (Schindelin et al. 2012) and the method of Forster et al. (2004). Fluorescence channels were merged and stitched using the method of Preibisch et al. (2009). The number of fluorescent microplastic particles was counted to particles per cubic millimetre and particles per milligram dry weight, and their distribution within the sections was analyzed.

For an exemplary examination of the distribution of polystyrene beads in the DECOTABs, 250 µL of a 5 wt % polystyrol 1000 nm (nominal mean, mean diameter 1294 nm) fluorescent beads solution (BS-Partikel) was added during phyll-tab production. One horizontal section with resulting 10 µm thickness was cut with the microtome (adjusted to 2 µm slice thickness) and imaged with the Leica Thunder imaging system (DM6B-Z microscope, DFC9000GT camera, and LAS X software, Ver 3.0.2.7506) with large-volume computational clearing.

Particle leaching from the DECOTABs

Six particle-loaded phyll-tabs with polystyrene microparticles with a fraction of 10 - 65 µm, were dried, transferred into tap water, and prewetted for 2 d in the climate chamber (13 ± 0.5 °C) prior to the experiment. After prewetting, the loaded phyll-tabs were placed into glass beakers (1 L) filled with 500 mL tap water, 3 glass stones for hiding, but no gammarids and kept in a climate chamber with a 16:8 h light:dark cycle and 13 ± 0.5 °C. After 4 days, the DECOTABs were removed and the water was filtered through a 0.8 µm filter (47 Ø; Merck Millipore). Beakers and filter stations were flushed twice with distilled water to remove particles attached to the glass. The whole surface of each filter was examined with a fluorescence microscope (Laborlux S; Leitz; equipped with a DP74 camera with CellSens Standard, Ver 1.18) to count the leached particles.

Uptake of the particles via DECOTABs

Phyll-tabs were loaded with blue fluorescent polystyrene microparticles of 10 to 60 µm with 1 % particle weight of tab weight, resulting in approximately 40 000 particles per tab. Estimation is based on particle concentration and the amount of stock suspension used. Loaded phyll-tabs were used in an exposure experiment with *G. roeseli* to characterize the particle uptake and compare it with the feeding rate. Therefore, gammarids were exposed to 1) particle-free phyll-tabs as a negative control, and 2) particle-loaded phyll-tabs as a positive control. This experiment was conducted under the same conditions as described in above (see *The Bioassay*), only the starvation during the acclimatization was conducted as recommended by the US Environmental Protection Agency (2016).

After 1 week of acclimatization, 3 randomly chosen individuals were placed in each beaker with 500 mL tap water and 3 glass stones for hiding. Dried and preweighed DECOTABs were changed every day to measure the feeding precisely. Also, gammarids were transferred to new beakers in daily rhythm to minimize the uptake of leached particles from the surrounding medium. Every day for 2 weeks, 5 replicates were taken from the negative and the positive controls, and the gammarids were anesthetized with carbon dioxide and fixed in 7.5 % formaldehyde with 7.5 g/100 mL glucose. Used particle-loaded phyll-tabs were dried and weighed again. Feeding rate was calculated as described above (see *The Bioassay*) and compared between the negative and positive controls. Guts were removed from fixed gammarids, mounted on microscopic slides with VECTASHIELD Antifade Mounting Medium, and imaged with a stereomicroscope (M3Z; Wild Heerbrugg; equipped with an SC180 camera; Olympus) to measure the gut length with CellSens Entry (Ver 1.18). The ingested particles were counted under fluorescence excitation at 340 to 380 nm (Laborlux S; Leitz; equipped with a DP74 camera with CellSens Standard, Ver 1.18). Further, feeding rate and particles per centimeter of gut were set in relation to validate whether the inner particle concentration can be calculated by the measurement of the mean mass eaten alone.

6.2.3. Statistical analysis

Statistical analyses were conducted with Rstudio (Rstudio 2015). Normal distribution was tested with the Shapiro-Wilk test, followed by the robust Fligner test for homogeneity of variance. Gammarid dry weight per millimeter body length was normally distributed and homogeneous in variance; therefore, further analysis was conducted with analysis of variance. All other endpoints including feeding, lipids, glucose content, glycogen content, and energy reserves as well as ingested particles were tested with the Kruskal-Wallis test, followed by a post hoc pairwise Wilcoxon test with the Benjamini-Hochberg correction (Benjamini et al. 1998). Differences in risk for mortality were tested by survival analysis with the Kaplan-Meier model and log-rank test using Jamovi (Jamovi Project 2019) based on the R language. Particle distribution in the DECOTABs was examined based on the XY coordinates with PAST, Ver 4.01 (Hammer et al. 2001) and point pattern analysis with nearest neighbor classification and wrap-around edge correction. Clustered points give ratio $R < 1$, Poisson patterns give R of approximately 1, whereas overdispersed points give $R > 1$. Correlation of particles per centimeter of gut and feeding rate was conducted with Rstudio with the Kendall method. A correlation coefficient of $r = 0$ implies no correlation, whereas $r = 1$ or -1 shows a strong positive or negative correlation, respectively. The p values for summarized comparisons are given as a minimum p-value for non-significant comparisons and a maximum p value for significant comparisons.

6.3. RESULTS

6.3.1. Evaluation of DECOTABs with food supplements

The DECOTABs

Variability (size, volume, weight)

The size of the freshly prepared DECOTABs was 10 mm in diameter and 5 mm in height before drying. When dried, they shrank to 4.0 to 4.5 mm in diameter and 2.0 to 2.5 mm in height but nearly retained their shape. The production iteration of cellulose-tabs revealed variable ww_I (8.5 % relative standard deviation) within the iterations ($p = 0.004$), but the dw_I remained stable ($p = 1.000$) and was on average 10.5 ± 0.5 % of the ww_I.

A comparison of the dry weight of the cellulose-tabs with the other formulations revealed that the cellulose-tabs varied more in dry weight than the modified tabs ($p < 0.001$; Table 6.1). Trout food-tabs were lightest, and variation of the dry weight was lowest. The dry weight of phyll-, gammarus-, and beech-tabs was nearly the same as for the cellulose-tabs ($p = 0.061$); but the dry weight variation of phyll-tabs was 50 % smaller than the variation of the cellulose-tabs. Beech- and gammarus-tabs had medium dry weights and variations 75 % lower than the cellulose-tabs.

If watered again, the DECOTABs readsorbed water and reached a maximum wet weight of 60 % compared to the ww_I independent of the watering duration ($p = 0.461$). When dried a second time after watering, the dw_F of the DECOTABs was still 10.1 % of the ww_I and thus, the dry weight before and after watering was the same ($p = 0.747$).

Table 6.1: Initial dry weight variability and stability of DECOTABs with different food supplement.

DECOTAB	initial dry Weight [mg]	variation coefficient	weight loss [%/day]	variation coefficient
original	43.2 ± 12.2	0.28	2.0 ± 2.2	1.12
beech leaves	37.2 ± 2.2	0.06	2.9 ± 1.1	0.38
phyll	40.1 ± 4.9	0.12	6.4 ± 2.2	0.35
gammarus	35.1 ± 2.0	0.06	8.1 ± 1.8	0.22
trout food	33.9 ± 0.7	0.02	6.3 ± 1.2	0.18

Stability in water

All DECOTABs were relatively stable in water, with a daily weight loss between 2.0 ± 2.2 and 8.1 ± 1.8 % (Table 6.1). Cellulose- and beech-tabs revealed the lowest weight loss per day ($p < 0.001$). The least stable DECOTABs were those with gammarids as a supplement ($p = 0.003$). Phyll- and trout food-tabs lost weight to the same extent of approximately 6.3 % ($p = 0.324$) and are thus in the middle between the other DECOTABs. Remarkably, the trout food-tabs had the lowest variation in weight loss per day.

The Bioassay

Mortality

Feeding the gammarids with cellulose-tabs resulted in the lowest risk for mortality over the course of 3 weeks, with 11.5 % after 21 days (Figure 6.2). The risk for mortality was also very low for gammarids fed with phyll-tabs (23.3 %, $p = 0.061$). Feeding gammarids with trout food-tabs resulted in nearly the same low risk for mortality as those fed with phyll-tabs (26.4 %, $p = 0.684$). Although the survival for gammarids fed with beech-tabs was similar to phyll- and trout food-tab-fed gammarids in the first 2 weeks, the risk for mortality increased to 61.6 % after the experimental duration of 21 days. Feeding gammarids with gammarus-tabs resulted in high mortality of approximately 40 % already within the first 2 weeks. At the end of the experiment a similar high risk for mortality compared with beech-tab-fed gammarids of 53.4 % was seen ($p = 0.334$).

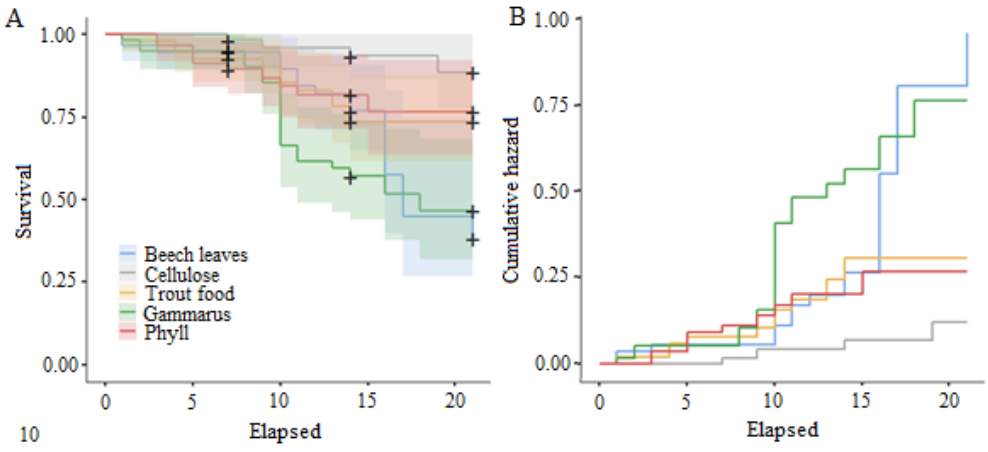

Figure 6.2: (A) Kaplan-Meier Survival curve and (B) cumulative hazard function for gammarids fed with the specific DECOTAB formulation over the course of 21 days. Survival analysis was conducted with the R based statistic software Jamovi v. 1.19.0.

Feeding rate

All gammarids accepted the offered DECOTAB formulations as a food source, though no tab was fully consumed after feeding periods of 3 or 4 days. Constant feeding rates were observed in the 3 treatments with beech-tabs ($p = 1.000$, 0.31 ± 0.29 mg/d), trout food-tabs ($p = 0.140$, 0.34 ± 0.41 mg/d), and phyll-tabs ($p = 0.059$, 0.40 ± 0.49 mg/d). This resulted in an equal amount of food eaten after 21 d ($p = 0.410$) of 12.60 ± 5.59 mg in sum for trout food-tab-fed, 12.60 ± 2.32 mg in sum for phyll-tab-fed, and 10.00 ± 4.43 mg in sum for beech-tab-fed gammarids.

Although, the highest DECOTAB mass was consumed from the gammarus-tabs with 27.10 ± 11.10 mg ($p < 0.001$), the feeding rate was very variable and decreased or increased over time ($p < 0.001$), with minimum feeding of 0.352 ± 0.240 mg/d in week 1 to a maximum feeding of 1.99 ± 1.11 mg/d in week 2 (Figure 6.3). Likewise, the feeding rate on the cellulose-tabs increased from the beginning (0.44 ± 0.29 mg/d) until the end (2.39 ± 0.69 mg/d, $p < 0.001$) of the experiment. After 21 days, gammarids were in sum consuming the second highest amount of the cellulose-tabs ($p = 0.052$, 25.30 ± 7.66 mg).

Additional weight loss occurred for the cellulose-tabs because they lose material if touched under water, for example, by the gammarids while swimming or feeding. Also, gammarids were observed pulling bigger pieces of the grounded beech leaves out of the beech-tabs but not ingesting them afterward. This weight loss was not calculable and is still included in the results; thus, it rather mirrors a shredding rate than a consumption rate.

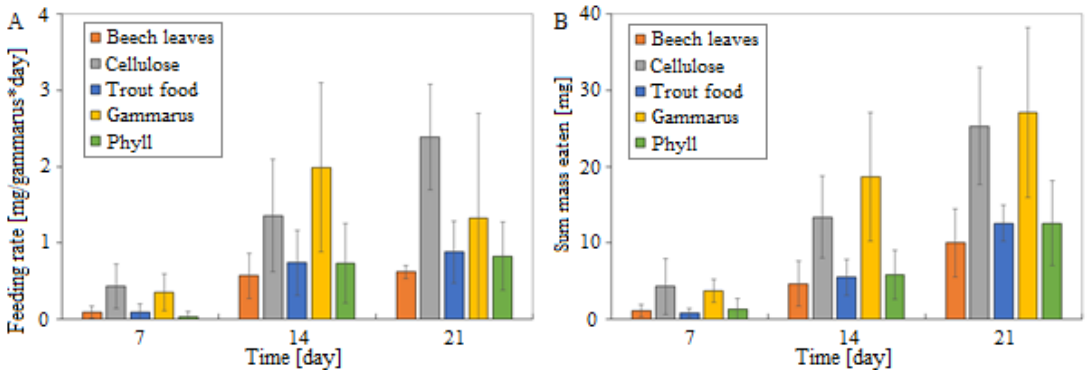

Figure 6.3: (A) Feeding rate per day and gammarid on the DECOTABs, separated by the different food supplements and (B) added up for each day to sum mass eaten over time. Error bars symbolize standard deviation.

Gammarid dry weight

The dry weight per millimeter of gammarid length decreased for the beech-tab-fed gammarids within 21 days ($p = 0.045$) and resulted in the lowest dry weight per millimeter at the end of the experiment (Table 1). Reduction in dry weight per millimeter over time was also observed for cellulose-tab-fed gammarids ($p = 0.044$), but at the end of the experiment they still had the same dry weight as the other treatments. The gammarids fed with phyll- ($p = 0.865$), gammarus- ($p = 0.577$), or trout food-tabs ($p = 0.664$) did not change in milligrams of dry weight per millimeter within the 3 weeks (Table 6.2) and had comparable dry weight per millimeter at the end of the experiment ($p = 0.135$). Overall, the dry weight per millimeter of all 5 treatments was between the dry weight per millimeter of the natural and the starved states ($p = 0.126$). Only the natural-state gammarids had a higher weight than the starved ones ($p < 0.001$).

Energy reserve determination

Energy reserves. The energy reserves per milligram of dry weight were the same for all acclimatization treatments and the natural state gammarids ($p = 0.130$). The starved gammarids had approximately 35 % less energy reserves than the natural-state gammarids and the gammarids acclimatized with beech-, trout food-, phyll-, or cellulose-tabs ($p = 0.007$). In contrast, those fed with gammarids had with 3260 ± 1173 mJ/mg dry weight the same energy reserves as both states ($p = 0.108$). Beside the differences in the natural and starvation states, the energy reserves between the treatments were the same for the acclimatization state ($p = 0.193$; Appendix, Figure 13.12) at approximately 3500 ± 235 mJ/mg dry weight.

Comparing the treatments after 3 weeks, only the phyll- and the trout food-tab-fed gammarids still had 30 % more energy reserves per milligram of dry weight than the starved gammarids ($p = 0.035$). The treatments with gammarus- and cellulose-tabs led to energy reserves in the gammarids between the natural ($p = 0.560$) and starvation ($p = 0.067$) states. It has to be noted that beech-tab-fed gammarids lost 40 % of their energy reserves compared to the acclimatized gammarids ($p = 0.002$) and had the lowest energy reserves out of the 5 treatments (Table 6.2). This results in lower energy reserves than the natural state ($p = 0.031$) and is comparable to starved organisms ($p = 0.551$).

Glucose. All treatments, including the natural-state gammarids, had from 90 to 320 % more glucose per milligram of dry weight than the starved ones ($p = 0.001$; Table 1). The gammarids acclimatized with cellulose-tabs or those with beech leaves, phyll, or trout food had at least 50 % more glucose than those in the natural state ($p = 0.011$); but those acclimatized on gammarus-tabs had the same glucose content as those in the natural state ($p = 0.806$). The gammarids acclimatized on the cellulose-tabs had the most glucose per milligram of dry weight, approximately 40 % more compared to the other acclimatization treatments ($p = 0.065$), except for the gammarids acclimatized with beech-tabs ($p = 0.501$), which had only slightly lower glucose content. Gammarus-tab-acclimatized gammarids had 16.0 ± 10.7 µg glucose/mg dry weight and therefore the lowest glucose content (Table 6.2).

After 3 weeks, glucose content in gammarids was stable for 3 out of 4 treatments ($p = 0.113$). The gammarids fed with trout food- or gammarus-tabs had 80 % more glucose per milligram of dry weight than those in the natural state ($p = 0.028$) and 235 % more than those in the starvation state ($p < 0.001$). In addition, phyll-tab-fed gammarids had 21.0 ± 10.6 µg glucose/mg dry weight and, thus, 180 % more glucose than those in the starvation state ($p < 0.001$) and the same as those in the natural state ($p = 0.087$). Overall, gammarids from all 3 treatments had similarly high glucose content ($p = 0.463$).

The glucose content of the beech-tab-fed gammarids was reduced by up to 80 % ($p = 0.002$) and, thus, was the same as those in the starvation ($p = 0.616$) and lower than those in the natural state ($p = 0.033$). Also, gammarids fed with cellulose-tabs contained after 3 weeks 40 % less glucose than after acclimatization ($p = 0.018$) and consequently the same amount as those in the natural state ($p = 0.125$) yet still more glucose than the starved gammarids ($p < 0.001$). Overall, beech-tab-fed gammarids contained 6.4 ± 3.0 µg glucose/mg dry weight and had the lowest glucose content compared to the other treatments at the end of the experiment ($p = 0.007$).

Glycogen. In sum, natural-state and starved gammarids had the same glycogen content ($p = 0.969$) of approximately 9.2 ± 5.4 µg glycogen/mg dry weight, and 4 out of the 5 acclimatization treatments had more than twice the amount of glycogen than both states ($p < 0.001$). The glycogen content (13.5 ± 7.2 µg/mg dry weight) of gammarus-tab-fed gammarids was lowest but still the same as for those in the natural

state ($p = 0.066$). Glycogen per milligram of dry weight was highest for gammarids acclimatized with cellulose-tabs ($p < 0.001$).

After 3 weeks, the cellulose-fed gammarids contained 30.6 ± 12.7 µg glycogen/mg dry weight and had still the highest glycogen content compared to the other treatments ($p = 0.013$), including the natural and starvation states ($p = 0.003$). As with the glucose content, the gammarids fed with trout food-, gammarus-, or phyll-tabs had all approximately 84 ± 14 % more glycogen than those in the natural and starvation states ($p = 0.003$). In contrast, the beech-tab-fed gammarids were not different in their glycogen content from natural state and starved gammarids ($p = 0.221$), having the lowest glycogen content compared to the other treatments (Table 6.2). Overall, glycogen content was stable in all 5 treatments over the course of 3 weeks ($p = 0.074$).

Lipids. Gammarids from the natural state and acclimatization fed with trout food- or phyll-tabs had approximately 30 % more lipids per milligram of dry weight than those from the starvation state ($p = 0.042$; Table 6.2). In contrast, the lipid content of gammarids acclimatized with cellulose-, gammarus-, or beech-tabs was the same as for those in the starvation state ($p = 0.080$). Beside these differences, the 5 treatments were mainly similar ($p = 0.061$), except trout food-tab- and cellulose-tab-fed gammarids ($p = 0.024$). This is because trout food-tab-fed gammarids contained the most lipids (81.7 ± 29.9 µg/mg dry wt), and those fed with cellulose-tabs contained the lowest amount (56.7 ± 14.7 µg/mg dry wt). After 3 weeks, the lipid content of the gammarids was stable within the 4 treatments ($p = 0.487$). Only the beech-tab-fed gammarids lost 30 % lipids per milligram of dry weight over the course of 3 weeks ($p = 0.026$). The lipid content of gammarids fed with beech- or cellulose-tabs was approximately 51.2 ± 11.4 µg/mg dry weight, which was the lowest and similar to the starvation state ($p = 0.444$). The gammarus-, trout food-, or phyll-tab-fed gammarids had same amount of lipids per milligram of dry weight as those in the natural state, but only trout food-tab-fed gammarids had a higher lipid content than those in the starvation state ($p = 0.024$; Table 6.2). Nevertheless, the gammarids of these 3 treatments had together the highest amount of lipids per milligram of dry weight ($p = 0.217$) of approximately 66 ± 17.1 to 76 ± 17 µg lipids/mg dry weight.

Table 6.2: Mean and standard deviation of the dry weight ratio and the energy storage substances including energy reserves for gammarids in natural (-7 days), starvation (16 days), and acclimatization state (0 days), and after 3 weeks (21 days)[a].

time (days)	treatment	dry weight ratio (mg/mm)	glucose (µg/mg dw)	glycogen (µg/mg dw)	lipid (µg/mg dw)	energy reserves (mJ/mg dw)
0	natural state	0.50 ± 0.12	13.90 ± 8.18	9.32 ± 6.52	76.10 ± 32.00	3395 ± 1293
	starvation state	**0.38 ± 0.12**	**7.46 ± 3.97**	9.10 ± 4.28	**59.20 ± 21.50**	**2628 ± 853**
	cellulose	0.47 ± 0.09	***31.50 ± 14.30***	***36.30 ± 15.50***	**56.70 ± 14.70**	*3428 ± 872*
	beech leaves	0.45 ± 0.10	***27.40 ± 16.50***	***21.00 ± 11.90***	68.80 ± 17.80	*3558 ± 863*
	phyll	0.44 ± 0.08	***21.00 ± 10.60***	***18.20 ± 7.33***	*74.40 ± 25.00*	*3625 ± 1097*
	gammarus	0.50 ± 0.13	*16.00 ± 10.70*	*13.50 ± 7.16*	69.50 ± 26.20	3260 ± 1173
	trout food	0.46 ± 0.10	***22.20 ± 10.30***	***18.20 ± 7.75***	*81.70 ± 29.90*	*3935 ± 1234*
21	cellulose	0.41 ± 0.09	*19.00 ± 10.60*	***30.60 ± 12.70***	**54.00 ± 13.40**	3000 ± 575
	beech leaves	0.36 ± 0.09	**6.37 ± 2.97**	11.90 ± 5.03	**48.40 ± 9.44**	**2232 ± 412**
	phyll	0.45 ± 0.11	*19.50 ± 10.90*	***15.90 ± 6.98***	66.00 ± 17.10	*3225 ± 777*
	gammarus	0.49 ± 0.11	***28.40 ± 20.00***	***18.60 ± 8.69***	72.30 ± 28.70	3679 ± 1368
	trout food	0.42 ± 0.13	***22.40 ± 11.60***	***16.30 ± 6.42***	*76.40 ± 17.00*	*3695 ± 836*

[a]**Bold** values indicate significant difference from natural state, *italic* values indicate significant difference from starvation state, underlined values indicate difference from acclimatization state

6.3.2. DECOTABs as oral exposure tool for particles

Phyll-tabs were loaded with red fluorescent polystyrene microparticles of a size range from 10 to 65 µm. Randomly selected DECOTABs were sliced into 50 µm layers to evaluate the particle distribution and concentration. Further, adult *G. roeseli* were fed with the particle-loaded DECOTABs, and the feeding rate as well as the particles per centimeter of gut were measured to examine the usability of the DECOTABs as a dietary exposure tool.

Particle distribution in the DECOTABs

Analysis of the particle distribution in the particle-loaded DECOTABs revealed a random distribution or overdispersion. In all vertical slices (n = 1 - 3 per concentration), the particles were found distributed in a random pattern over the entire height of the DECOTAB ($p = 0.129$) for all tested particle concentrations (Figure 6.4; Appendix, Figure 13.13). Overdispersion occurred in only one (1600 µL) out of the 7 sections ($p = 0.017$, $R = 1.123$).

In 50 % of the horizontal sections at each concentration (n = 1 - 3 per concentration), particle distribution was random ($p = 0.062$; Appendix, Figure 13.13) and 42 % revealed overdispersion ($p = 0.004–0.042$). Only one horizontal section out of 14 contained clustered but not aggregated particles ($p < 0.001$, $R = 0.827$).

Because the particles were randomly distributed or overdispersed, it was possible to calculate the number of particles per DECOTAB dry weight out of the number of particles per slice of 50 µm thickness. The addition of 3200 µL of the particle stock suspension to the DECOTAB formulation resulted in a final particle concentration of 456 ± 124 particles/mm³ and 381 ± 103 particles/mg dry weight of the DECOTAB. Preparation with 1600 µL reduces the particle concentration to 243 ± 48 particles/mm³ and 203 ± 40 particles/mg dry weight, which is a reduction of 53 % compared to the highest concentration and corresponds to the added amount of particles. Also, the addition of 160 µL (5 % of the highest amount added) resulted in 17 ± 8 particles/mm³ and 14 ± 6 particles/mg dry weight, which is approximately 3.6 % of the highest concentration.

The section from the tab loaded with fluorescent 1000-nm beads revealed also a homogeneous particle distribution, which has to be verified with further statistics. At first sight, neither agglomerations nor clustered particles were observed (Figure 6.5).

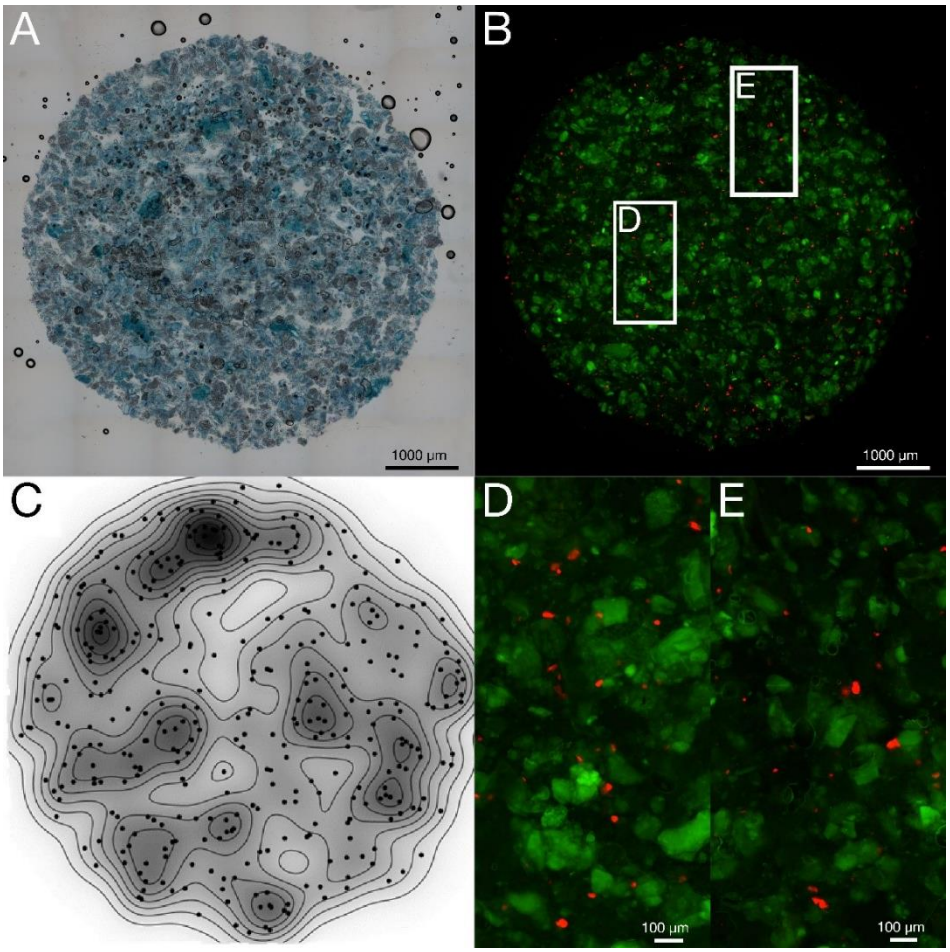

Figure 6.4: Particle distribution in one horizontal section of 50 µm thickness from a phyll-tab filled with 3200 µL of the prepared 10 - 65 µm microplastic suspension. This section contained 376 particles. (A) Brightfield image of the phyll-tab with red fluorescent polystyrene particles. (B) Fluorescence microscopic image of phyll-tab with red fluorescent particles with extended depth-of-field calculation of 15 Z-sections. (C) Nearest neighbors method plot. Dots mark particle position, and gray scaling shows particle density. (D, E) Magnification of (B).

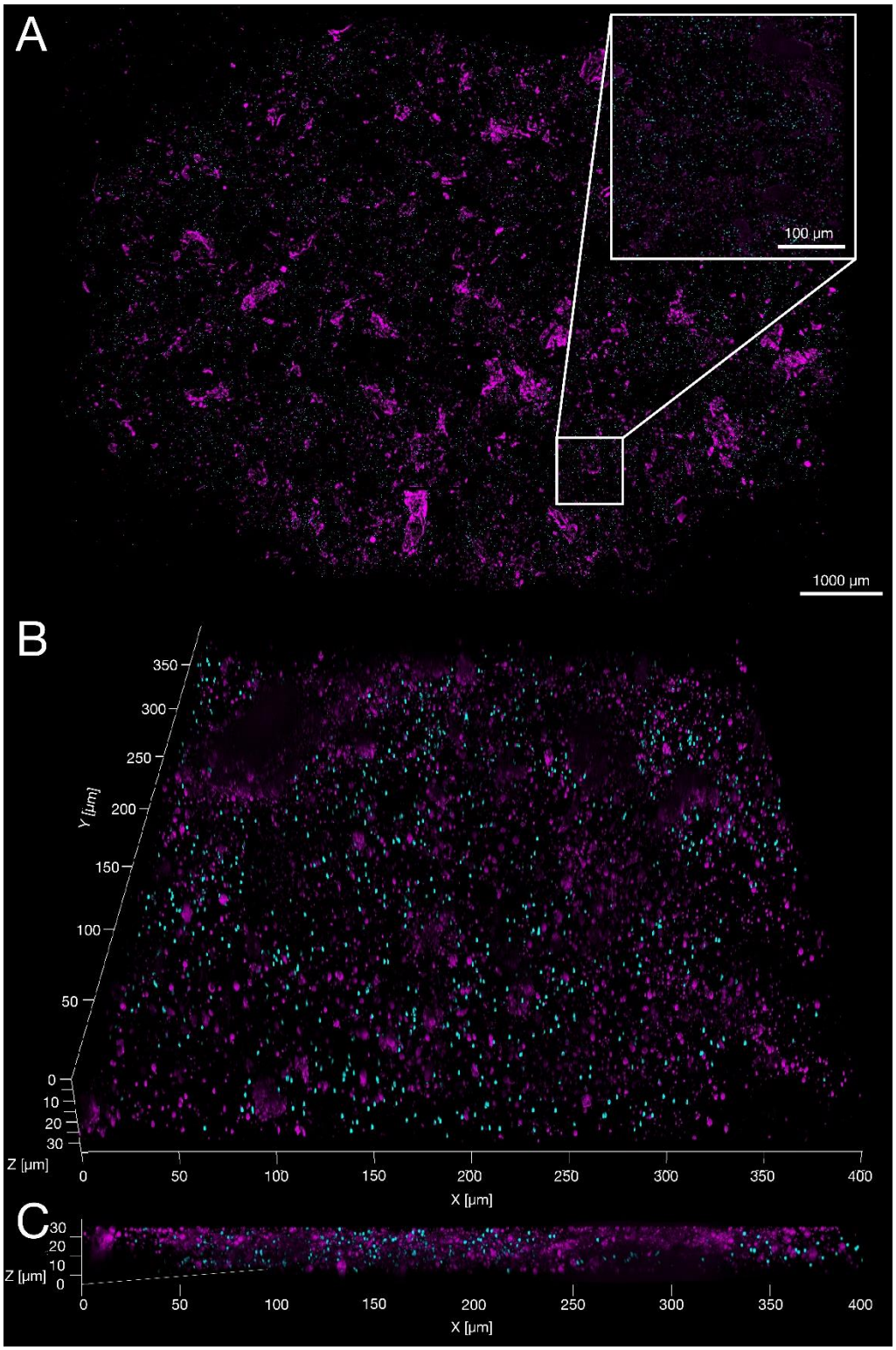

Figure 6.5: Exemplary examination of one horizontal slide (10 µm) of a decomposition and consumption tablet loaded with 1000-nm beads. Fluorescence images were taken under a Leica Thunder imaging system (DM6B-Z microscope, DFC9000GT camera, and LAS X software, Ver 3.0.2.7506) with large-volume computational clearing. Cyan fluorescent protein (CFP) excitation was used for the blue fluorescent particles (cyan) and Texas red (TxRed) excitation for the underlying red fluorescence of the phyll-tab (magenta). (A) Overview image with maximum projection imaged with a ×20 HC PLAPO CS2 20×/0.75 IMM UV objective. Cutout represents a magnified extract with 2 × 2 tiles imaged with a ×63 HC PL FLUOTOAR 63×/1.10 IMM and 81 Z-planes. (B, C) Volume visualization (3D) of a small 2 × 2 area of the slide imaged with a ×63 HC PL FLUOTOAR 63×/1.10 IMM and 81 Z-planes.

Particle leaching from the DECOTABs

No leached particles were detected after the prewetted DECOTABs were left for 4 days in water.

Uptake of the particles via the DECOTABs

The feeding rate of gammarids exposed with microparticle-loaded phyll-tabs was like the feeding rate of gammarids fed with particle-free tabs ($p = 0.192$). Further, the feeding rate in both treatments did not change over time ($p = 0.127$ for control, $p = 0.057$ for particle treatment), except for the first day when feeding rate was higher ($p = 0.035$; Figure 6.6A). As expected, no particles were detected in the gammarids fed with particle-free tabs (negative control, $p = 1.000$), whereas a significant amount of particles (70 ± 35 particles/cm gut) was found in the guts of the gammarids fed with particle-loaded tabs ($p = 0.042$; Figure 6.6B) within 1 day. The number of particles per centimeter of gut was stable over time ($p = 0.189$) and proportional to the feeding rate ($p < 0.001$, $r = 0.33$).

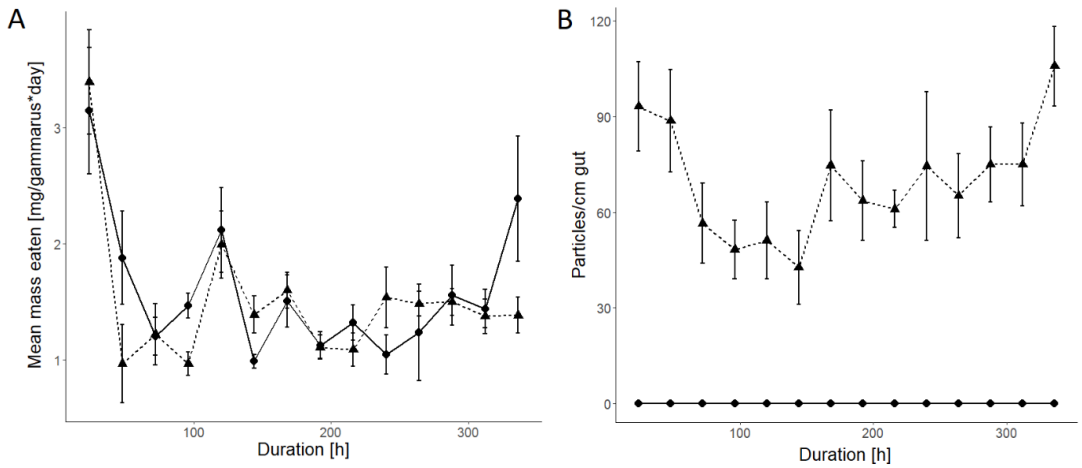

Figure 6.6: (A) Feeding rate of the provided decomposition and consumption tablets by one gammarid per day and (B) mean particle count in gammarid gut over the course of 2 weeks. Feeding rates for the first 3 days were calculated with a batch-specific tab standard weight (initial dry weight = 33.4 ± 0.2 mg, n = 330).

6.4. DISCUSSION

6.4.1. Evaluation of DECOTABs with food supplements

We were able to identify 2 food supplements that allow an increase of the nutritional value of the cellulose-tabs originally published as DECOTABs by Kampfraath et al. (2012) to facilitate their use as suitable food in ecotoxicological short- and long-term bioassays. Best-suited supplements for *G. roeseli* were the algae-based phyll and the animal protein-based aquaculture trout food. This was demonstrated by dietary properties and behavioral responses including mortality and constant feeding of gammarids as well as the characteristics of the supplemented DECOTABs themselves. Important prerequisites of using the tabs in standardized dietary exposure experiments such as stability, low dry weight variation, the homogenous distribution of embedded particles, and the possibility to calculate the feeding rate with high accuracy make them a suitable tool for controlled ecotoxicological experiments.

The main need for the supplementation of the cellulose-tabs was to increase the nutritional value and adapt it to the needs of shredding organisms like gammarids to minimize a loss in fitness and mortality. The nutritional value was determined by the change of the organismal wide level of lipids, glucose, and glycogen. While glucose and lipids are primarily metabolized by gammarids during starvation for 14 days, glycogen seems to be utilized only when glucose is nearly depleted (Semsar-Kazerouni et al. 2020). The cellulose-tabs were not able to meet the nutritional demand because gammarids lost weight per millimeter when feeding on them for 3 weeks and had to use their glucose reserves, although they were fed ad libitum. The same was true for the gammarids fed with tabs supplemented with beech leaves, although conditioned leaf discs are an established standard food in *Gammarus* spp. assays (Blockwell et al. 1998; Gergs and Rothhaupt 2008; Blarer and Burkhardt-Holm 2016). This observed reduction of the glucose level was similar to reduction after a starvation period of 16 days in our experiments and was likewise observed by Charron et al. (2014) after a starvation period of 23 days.

Phyll-, gammarus-, or trout food-tabs were chosen as alternative food supplements because they are based on algae or animal proteins. Although gammarids fed nearly twice as much on the gammarus- than on the trout food- or phyll-tabs, they did not build up more energy reserves. This indicates that gammarids as a food supplement

provides a lower food quality compared to the algae-based phyll or animal protein-based trout food, which might be compensated by higher feeding rates (Bärlocher and Kendrick 1975; Gergs and Rothhaupt 2008; Agatz et al. 2014). Next to the lower food quality, a fast increasing risk for mortality up to > 50 % within the 21 days when fed with gammarus-tabs highly exceeded the acceptable range.

In contrast to the cellulose-tabs or the supplementation with beech leaves or ground and dried gammarids, trout food or phyll proved suitable for long-term feeding of *G. roeseli*. After feeding on DECOTABs with both supplements, the gammarids were able to build up energy reserves higher than starved gammarids, and the feeding rate was constant over 21 days. Another remarkable fact is the slowly increasing and low risk for mortality up to a maximum of 25 % after 3 weeks, which is particularly crucial in establishing valid control groups in any ecotoxicological testing.

The addition of food supplements can lead to a varying weight loss when exposed to water or a variation in dry weight after production, which interferes with the aim of a high standardization. Moreover, stability in dry weight of the food tabs during exposure is essential for the application of embedded substances and the determination of feeding rates. Although beech- and cellulose-tabs were the most stable ones compared to the other formulations when submerged into water, both revealed an incalculable weight loss when gammarids were feeding on them. The dried cellulose-tabs were very brittle and had a tendency to lose material solely through touching by the gammarids. The beech-tabs still contained larger leaf parts, which were entirely removed from the tab by the gammarids but not ingested. In contrast, the tabs supplemented with phyll, gammarids, or trout food revealed a consistent dry weight after production; and no additional weight loss was observed during feeding experiments with organisms. This would make phyll, trout food, and gammarids suitable as food supplements with an acceptable material loss in the aquatic environment of only 6 to 8 % if only the stability of the DECOTABs is considered as an important prerequisite. In contrast, beech and cellulose were inappropriate in this respect.

An additional positive aspect of trout food or phyll is the commercial availability of these supplements. On the one hand, this ensures a constant quality and subsequently reproducible feeding rates. On the other hand, it greatly increases comparability

between different exposures and laboratories. The latter is a strong advancement in contrast to the common use of conditioned leaf discs as a food source. Herein, a strong variation in food quality can occur because conditioning is typically performed by using stream water (Blarer and Burkhardt-Holm 2016), organically enriched dechlorinated water (Blockwell et al. 1998), or bacterial cultures (Agatz and Brown 2014). Because of the high nutritional value gammarids can gain from the tabs with phyll or trout food as a supplement, no pre-treatment of the DECOTABs is necessary, making their use easier and more convenient. Overall, supplementation of the DECOTABs with phyll or trout food promises the best conditions for standardized toxicity testing.

6.4.2. DECOTABs as oral exposure tool for particles

During aqueous exposure, every particle or other sparingly soluble substance distributes differently (Cole et al. 2011; Oliveira and Almeida 2019), leading to fundamental problems in toxicity testing of an inhomogeneous distribution in the medium and the wrong assumption of equilibrium between the medium and the organism (European Centre for Ecotoxicology and Toxicology of Chemicals 2018; Eitzen et al. 2019). Actually, the uptake of a particle in aqueous exposure-oriented experiments is mainly random (Cole et al. 2011; Bartonitz et al. 2020) and therefore not calculable. This is further complicated by the often incomplete or unfeasible tracking of the particles in the exposure system (Tiede et al. 2009; Correia and Loeschner 2018; Triebskorn et al. 2019). We wanted to explore an alternative way to aqueous exposure focusing on the oral exposure of organisms with their food, which bypasses these toxicity testing problems targeting particles or sparingly soluble substances. Moreover, oral exposure is a more realistic pathway for the uptake of such substances because they tend to adsorb to surrounding material (Werner et al. 2002; Bundschuh et al. 2019). Different approaches of particle exposure via a food matrix can be found, for example, for the mudsnail *Potamopyrgus antipodarum* (Imhof 2013; Imhof and Laforsch 2016), the amphipod *Gammarus pulex* (Imhof 2013), and the isopod *Idotea emarginata* (Hämer et al. 2014), however without deeper evaluation of the properties of the matrix and their respective influences on the uptake of the tested substances. Therefore, we demonstrated the fulfilled prerequisites for such an oral exposure tool regarding homogenous distribution of particles as well as their permanent fixation in the matrix. Also, the necessary predictable and constant particle

exposure based on the feeding rate was verified.

We were able to prove the applicability of loaded tabs as a dietary exposure tool by adding fluorescent polystyrene microparticles with a size of 10 to 65 µm. Examination of tab slices revealed homogenous distribution of the microparticles without aggregation in phyll-tabs. The same seems true for 1000 nm PS beads, as shown by exemplary visualization in the present study. Because of the standardized production procedure and formulation of the DECOTABs, it was possible to ensure a comparable and quantifiable exposure of *G. roeseli* with particles based on the feeding rate calculation and the particle concentration in the DECOTAB. Particle preparation before addition to the tabs is a remaining challenge that still should be harmonized and standardized (Hartmann et al. 2015; Eitzen et al. 2019), but no complex particle resuspension seems necessary for particles like those used in the present study where an oral exposure tool is used.

The exposure of *G. roeseli* with microparticle-loaded DECOTABs led to a constant inner particle concentration resulting from a steady and fast ingestion as well as from a continuous egestion of the particles with the food. Complete egestion of particles with a size of 10 to 65 µm from the gut was observed, corresponding with the limited transfer of this size of particles into cells (Triebskorn et al. 2019). For the particles in the nanometer size class, such a transfer and resulting accumulation are more likely and need to be examined. Finally, we were able to verify that the dietary exposure as performed by Zhai et al. (2018) and Yardy and Callaghan (2020) solves the often-claimed inhomogeneous distribution and unpredictable exposure of the particles in aquatic toxicity tests.

Further, we were able to link the feeding on the microparticle-loaded tab with the amount of particles in the gut of the gammarids, enabling a more realistic dose-response assessment. The inner particle concentration can be determined without a direct examination of the gut content, which can only be quantified at the end of the experiment (e.g., Straub et al. 2017). Instead, the inner particle concentration, which is more relevant for the observed effects than the outer particle concentration (European Centre for Ecotoxicology and Toxicology of Chemicals 2018), becomes continuously calculable via the feeding rate. This gains an adjustable dosimetry for each experiment, which is of great advantage not only for a standardized particle

testing via the oral pathway but also for a more realistic dose-effect assessment for particulate substances.

Nevertheless, leaching of particulates or other embedded chemicals from the DECOTAB into the surrounding medium should be considered because it can affect the exposure pathway. The DECOTABs suggested in the present study did not leach any particles when watered for several days, whereby it must be recognized that gammarids are shredding their food during feeding and, thus, DECOTAB pieces with particles can spread in the test vessel. However, in our experiments, uptake via this route was negligible. Concerning the exposure with soluble substance via DECOTABs or if particles with adsorbed pollutants are embedded, a validation of potentially leached substance via chemical analysis of the medium is highly recommended because the partitioning between food source, medium, and organism might vary (Moermond et al. 2013; US Environmental Protection Agency 2016).

Another essential prerequisite for oral application is that the embedded substance does not influence the uptake by the organism. This could be proven as the embedded microparticles did not change the feeding of the gammarids on the particle-loaded phyll-tabs compared to particle-free ones. We observed a higher feeding rate on day 1, which can be interpreted as a response to starvation during the acclimatization period. This consequently leads to the conclusion that the acclimatization and the test periods should be conducted with a constant ad libitum feeding. Despite the compensatory feeding after starvation, the feeding rate normalized within 1 day and remained constant with and without microparticles during the entire test period.

6.4.3. Recommendations for future studies using DECOTABs as an advanced food source and/or oral exposure tool

With regard to the applicability in further studies, we want to give some recommendations concerning test duration, feeding time, and tab handling as well as the possibility of using the tabs for other organisms.

The test duration is mainly dependent on the feeding behavior of the test organism. The organisms should feed at least several days on the tabs to allow a reliable detection of tab dry weight changes. For one day exposures, the weighing error and weight loss are sometimes higher than the feeding of the gammarids, which may lead to undetectable effects on the feeding behavior (Bartonitz et al. 2020). Further, if the

transfer of particles is intended, a certain amount of time is necessary to allow subsequent exposure of the particles. In addition, experiments with particulates often require a longer duration because no acute toxicity has been shown so far (Triebskorn et al. 2019).

The maximum feeding time on one DECOTAB can be extended. In our experiment, the DECOTABs were stable for over 4 days. However, to enable ad libitum feeding, the DECOTAB should be replaced on a regular basis depending on their dry weight before they are eaten completely. We replaced them after 3 or 4 days, which corresponds to a weight loss of 11.5 ± 3.6 mg with 3 untreated gammarids (∼30 % of our phyll-tab dry weight). Considering the overall duration of the experiment, no constraints exist if the DECOTABs are replaced regularly considering the above-mentioned minimum and maximum exposure times of a single DECOTAB. Most important for measuring the feeding rate is the use of the individual tab weight before and after the experiment because this increases the sensitivity of the endpoint instead of using an average standard weight. Calculation of feeding rate should include the mean daily weight loss of the tabs and the initial and final dry weight for every single tab. We also recommend the examination of a suitable food supplement for the investigated organism including stability of the resulting tab.

Although not specifically tested in the present study, it is likely that supplemented DECOTABs can also be used to feed organisms other than *G. roeseli* (Kampfraath et al. 2012). Decomposition and consumption tablets with and without supplements were already successfully used to feed other amphipods, like *Gammarus fossarum* (Straub et al. 2017) or in experiments with *Hyalella azteca* (Raths et al. 2020) and microbes (Hunting et al. 2017); but standardization of production and homogeneous distribution of the particles and tabs as a sufficient nutrient supply were not extensively tested. Also, mixtures containing similar ingredients and supplements were successfully used to provide food for the caddisfly *Allogamus mortoni* (Fenoy et al. 2020). Consequently, we assume the suitability of the DECOTABs supplemented with either phyll or trout for gammarids or even another species-dependent supplement during short- and long-term exposures for multiple functional feeding groups, particularly for shredder and grazer organisms in aquatic and terrestrial environments.

6.5. CONCLUSION

In the present study we describe a step toward a more standardized operational framework for toxicity testing procedures with particulate substances during short- and long-term bioassays with aquatic organisms. Focusing on the oral exposure pathway, we were able to provide an alternative to inhomogeneous aqueous particle exposure, which is difficult to standardize because of inhomogeneous distribution and unpredictable uptake. Moreover, a more realistic dose-response assessment of dietary particle exposures over the course of the entire experiment is feasible by combining the advantages of the homogenous distribution of particles and sparingly soluble substances in a stable food matrix with the possibility of a reliable feeding rate determination and consequently controllable exposure.

Supplementation of DECOTABs to meet the requirements of a test organism is already common practice. However, the deeper evaluation of the nutritional value in the present study provides one of the most standardized and verified food sources which is adapted to the specific needs of gammarids. It allows short- and long-term bioassays while maintaining a healthy state of the test organisms. Regular application of such supplemented DECOTABs in ecotoxicity testing procedures will improve the determination of feeding rates as a sensitive endpoint because of the high stability and low dry weight variation of the tabs. It also will increase comparability between studies by providing a constant health status and lowering possible stress of the gammarids, resulting in more realistic endpoint measurement and effect assessment. By varying the supplementation, tabs have a great potential of being used in toxicity tests with several organisms from different functional feeding groups.

Future research should focus on establishing clear relationships between the bioavailable fraction of particles or sparingly soluble substances in aquatic environments and observed effects. Studies concerning the partitioning of particles and other sparingly soluble substances to digestible sources are rare as well as particle concentration in the organism and subsequently real particle exposure for each functional feeding group. We further emphasize the mandatory comparison between anthropogenic plastic particles and natural particles, to avoid the confusion of mechanical and chemical effects. In addition, the uptake pathways (oral, dermal, pulmonal) of particles and sparingly soluble substances clearly must be considered in

ecotoxicological research because exposure from the water column is negligible, and guidelines must be updated accordingly (Bundschuh et al. 2019; Oliveira and Almeida 2019). Our approach provides a template for further studies in this direction.

7. Dietary exposure to four sizes of spherical polystyrene, polylactide and silica nanoparticles does not affect mortality, behavior, feeding and energy assimilation of *Gammarus roeseli*

A similar version of this chapter has also been published in the Journal "*Ecotoxicology and Environmental Safety*" as Götz A, Beggel S, Geist J (2022) Dietary exposure to four sizes of spherical polystyrene, polylactide and silica nanoparticles does not affect mortality, behaviour, feeding and energy assimilation of *Gammarus roeseli*. *Ecotoxicology and Environmental Safety* 238:113581. doi: 10.1016/j.ecoenv.2022.113581

Abstract

The abundance and persistence of plastic nanoparticles in aquatic habitats are considered a threat to marine and freshwater biota. However, the risk assessment of plastic particles is complicated due to various factors that need to be considered, including composition, size and environmental abundance. This study investigated the behavioral response of a key river species, *Gammarus roeseli*, to dietary exposure of plain biodegradable and non-biodegradable plastic as well as to natural small micro- and nanoparticles. Mortality, feeding, swimming velocity and energy assimilation endpoints were examined by considering four particle sizes ranging from 30 to 1000 nm in two concentrations. Contrary to our expectations, neither decreasing size nor increasing abundance of each tested particle impacted any of the examined endpoints. Likewise, dietary exposition with biodegradable plain polylactide did not induce other or stronger effects than non-biodegradable plain polystyrene or natural silica micro- and nanoparticles, as all three particle types did not lead to adverse effects on *G. roeseli.* These findings also suggest that the functional role of *Gammarus roeseli* as a shredder is not impaired due to particle occurrence within the exposure range of this study.

Keywords: Dietary exposure; Nanoplastic; Biodegradable plastics; Effect assessment; Nanoparticle

7.1. INTRODUCTION

Plastic particles in the environment are considered an emerging threat due to their various chemical compositions, increased use, and inappropriate disposal (Lambert et al., 2014; Shen et al., 2019). Particles can have a three-fold effect on organism survival: the direct effect of the plastic particle, the leaching of additives from the plastic particle, and the potential vector effect for ab- /adsorbed chemicals and pathogens (Shen et al., 2019; Triebskorn et al., 2019). Regarding the first aspect, particle size and abundance are two key factors that govern uptake, exposure and potential ecotoxicological effects of plastic particles in the environment (Kögel et al., 2020; Zimmermann et al., 2020). There is evidence that particle sizes can range from macro- to nanoscale due to degradation processes (Lambert and Wagner, 2016). With further ongoing degradation of plastic in the environment, the abundance of very small particles in the micro- and nanoscale is expected to increase in the future (Kögel et al., 2020; Lambert and Wagner, 2016). Plastic nanoparticles are believed to be more hazardous than macro- and microparticles. Especially, the increasing abundance of plastic nanoparticles has become the focus of research. Finer particles can be ingested by smaller and thus more diverse groups of organisms (Besseling et al., 2019; Kögel et al., 2020; Shen et al., 2019), in which a greater diversity of exposure pathways such as pulmonary inhalation and dermal uptake can occur (Kögel et al., 2020; Stapleton, 2019). Once in the organism, it is assumed that nanoparticles < 1 µm can be transferred into gut cells and induce cytotoxicity (Firdessa et al., 2014; Pitt et al., 2018; Shen et al., 2019; Triebskorn et al., 2019). Likewise, when transferred into cells, particle excretion is delayed for an indefinite period. The higher possibility of ingestion and intracellular uptake contribute to the greater potential of nanoparticles to accumulate in the food web (Pitt et al., 2018).

To prevent overestimation or misinterpretation of plastic particle toxicity, their comparison with natural particles of the same size and shape is necessary (Götz et al., 2021; Ogonowski et al., 2018a). Natural particles are typically present in the environment at much higher concentrations (Triebskorn et al., 2019) and are thus adaptable by most organisms. Some species even depend on certain concentrations to provide required levels of turbidity for feeding and predator avoidance (Evan Ward and Shumway, 2004; Lummer et al., 2016; Hasenbein et al., 2013). Direct comparisons

between exposure effects of plastic nanoparticles with naturally occurring particles can distinguish effects of the material from those that are simply a result of physical (e.g. shape-related) properties.

Further, most studies have focused on high particle concentrations viable for hazard assessment, but not environmentally relevant for risk assessment. The observed effects appeared at the cellular and organism levels. For example, reduced survival rate and locomotion behavior were observed, and gene expression and cell-stress-response were upregulated (Prüst et al., 2020). Observed effects also include reduced cell viability in algae, and impacts on the physiology of three aquatic invertebrates (González-Pleiter et al., 2019; Hazeem et al., 2020). Zimmermann et al. (2020) investigated the toxicity of three plastic particle types, including the biodegradable polylactide, differentiating the toxicity of plastic with additives, leached additives and plain particles. They found that plastic toxicity is not always chemically driven, because they observed that the plain polylactide particle - but not the leached chemicals - reduced survival in *D. magna*.

In addition, studies reporting no effects of plastic micro- and nanoparticles are rare, possibly reflecting a rejection of publishing such results (De Sá et al., 2018). The effect assessment of plastic particles is also impeded by the number and complexity of factors that are compared (Kögel et al., 2020; Paul et al., 2020). This makes the observed effects inconsistent, leading to a controversy about the hazard potential and real ecotoxicological risk of plastic particles. The classification is even less strict regarding plastic nanoparticles because the database is poorly filled (Paul et al., 2020).

This study's primary objective was to assess the ecotoxicological effects of micro- and nanoparticles on a key species of stream ecosystems, *Gammarus roeseli*. In this context, four hypotheses were examined regarding size-, concentration- and composition-related plastic particle toxicity. In addition, general natural- and not plastic-specific particle toxicity was determined. In particular, the following four hypotheses were tested: (I) smaller nanoparticles < 100 nm cause more harm than bigger ones due to cytotoxicity and enhanced accumulation; (II) higher concentrations of micro- and nanoparticles lead to earlier and stronger toxicity responses in aquatic organisms like *Gammarus roeseli*; (III) biodegradable plastics induce other toxicity than non-biodegradable plastics; and, (IV) plastics are more toxic than natural particles of

the same size, shape and concentration.

Therefore, we investigated the dietary exposure of particles for two weeks to the shredder *Gammarus roeseli*. It is a key species in riverine systems (Boeker and Geist, 2015) and has been recently recognized as a standard invertebrate species for freshwater river-dwelling organisms for laboratory risk assessment (Kunz et al., 2010) and stressor assessment in the wild occurring in high numbers in streams of the study region (Pander et al., 2022).

7.2. MATERIAL AND METHODS

The four hypotheses were examined by considering lethal and sublethal endpoints based on a comparison of two plastic types and one natural particle of the sizes 1000, 500, 100 and 30 nm, at two concentrations. The examined particle types were (1) plain polystyrene as a non-biodegradable and one of the most investigated plastic particle types for easier comparisons with other studies; (2) plain polylactide because of its biodegradable properties and supposed different toxicity mechanism than polystyrene and as a rare examined plastic particle type; (3) silica as a natural reference particle to exclude false-positive general particle effect observations.

7.2.1. Organism and acclimatization

Gammarus roeseli were sampled in April 2021 using a dip net in the River Moosach near the Aquatic Systems Biology Unit of TUM in Freising (Germany) and transported in river water to the laboratory. Afterwards, they were size-selected by sieve passage with a diameter of 1.5 and 3 mm. A homogenous size class (8.3 ± 1.8 mm, based on a subsample of n = 40) was chosen for the experiment according to Beggel et al. (2016). After size selection, gammarids were acclimatized for one week in an aquarium filled with aerated artificial water (EN ISO, 1998) and glass stones to the steady conditions of the climate chamber with 13 °C ± 0.5 °C and a 16:8 h light:dark cycle. The water parameters were measured weekly. The artificial water was prepared a day before use and aerated for one day in the climate chamber. Parameters before use were 9.8 ± 0.1 mg O2/L, 13.5 ◦C ± 0.3 °C, pH = 7.9 ± 0.1 and 669 ± 5 µS/cm. Phyll-tabs (Götz et al., 2021) and Phyll-flakes (Tetra GmbH, Germany) were fed ad libitum during acclimatization. Based on pre-experiments which revealed no sex-related differences, animals were then randomly distributed into the test units.

7.2.2. Particle concentration

Three spherical particle types in four sizes were tested in this experiment: plain polystyrene (PS, 1000/500/100/30 nm), plain polylactide (PLA, 2000/500 nm) and plain silica (Silica, 1000/500/100/30 nm). All particles were provided in suspension in water without further detergents of 50, 25 or 10 mg/mL by the company micromod (Germany, Rostock), except for the 30 nm PS particles which were provided in suspension by BS Partikel (Germany, Mainz). According to the manufacturer's

protocol, plain silica particles have a hydrophilic surface with terminal Si-OH-groups without additional functional groups. The plain PS particles have no additional surface coating or functional groups, but can have rare negative loaded sulphate groups on the surface. In the absence of reliable environmental nanoparticle concentrations, the concentration used was based on Triebskorn et al. (2019) to match reported concentrations of 100–900 plastic microparticles/L in the River Elbe of which 1.8 % were PS, mostly (90 %) with a mean diameter of 20 µm. The mean environmental concentration of 10 PS microparticles/L with 20 µm size and an approximate spherical shape was assumed to mirror a realistic concentration.

Therefore, ten 20 µm PS-particles/L were set as environmental relevant concentration. This corresponds in theory to a weight of 4.31 ng based on the density 1.03×10^{-9} mg/µm^3 of PS specified by micromod. Subsequently, the same mass was used for the environmentally relevant concentration for each particle size. This decision was based on the simplified assumption that the microparticles degrade to nanoparticles without mass loss. Thus, two concentrations were chosen: one environmentally relevant concentration (ERC) of 4.31 ng PS (low concentration = L) and one concentration of 431 ng, 100-fold higher than the environmentally relevant concentration (high concentration = H). The particles were homogeneously embedded in the food matrix for dietary exposure, as described by Götz et al. (2021). The tabs consist of agar, cellulose and Phyll (Tetra GmbH) and can be customized with supplements of interest. This method provides a stable distribution of the particle during the experiment without leaching and the possibility to connect the mass of consumed food to the amount of ingested particles.

G. roeseli were exposed to 4.31 ng/mg tab dry weight (tdw) of each size of the PS nanoparticles. The theoretically equivalent particle concentrations are listed in Table 7.1. The environmental concentration of silica as natural reference particles is supposed to be much higher than that of PS, while PLA is assumed to occur at substantially lower concentrations. To use comparable concentrations, the ERC for these two particle types was based on the particle concentrations of PS and their specific density provided by micromod (Table 7.1). To ensure similar particle concentration, 4.19 ng PLA/mg tdw and 8.38 ng silica/mg tdw were used for exposure. Subsequently, the 100-fold higher concentration was 431 ng PS/mg tdw, 419 ng

PLA/mg tdw and 838 ng silica/mg tdw. The particle suspensions were pipetted into the DECOTAB mixture during production (Götz et al., 2021) or, for the ERC, previously diluted in distilled water.

Table 7.1: Density of the three particle types and the theoretical particle number of the used particle weights provided by the manufacturer. Particles were embedded in phyll-tabs, and concentrations are given in ng particle per mg dry weight of the tab.

Density of [mg/µm³]	Polystyrene	1.03×10^{-9}
	Silica	2.00×10^{-9}
	Polylactide	1.00×10^{-9}
Environmental relevant Concentration (ERC, L) [ng/mg tdw]	Polystyrene	4.31
	Silica	8.38
	Polylactide	4.19
100-fold concentration (H) [ng/mg tdw]	Polystyrene	431
	Silica	838
	Polylactide	419
Size [nm]	Volume/Particle [µm³]	Particle number in ERC
20,000	4188.8	10
2,000	4.188	10,000
1,000	0.5236	80,000
500	0.0654	640,000
100	0.00052	80,000,000
30	0.000014	2,962,000,000

7.2.3. Bioassay

To test the four hypotheses, the experiment comprised a total of 22 treatments (Table 7.2). To minimize stress originating from predation or competition for food, the setup consisted of smaller numbers of three acclimatized gammarids per beaker, but a higher number of six replicates per treatment including the control, according to Götz et al. (2021). Three glass stones were placed into each beaker as a refuge for the gammarids, to prevent cannibalism and to reduce stress. The 1-L beakers were filled with 500-mL artificial water and placed randomly into the climate chamber. Eighteen gammarids were allocated to starvation treatment for test duration and separated into a 250-mL beaker with 150 mL artificial water and two glass stones to avoid cannibalism. These gammarids were not fed and remained unexposed to particles. Exposure time was two weeks. Water was completely changed once a week. After the organisms were allocated to a beaker, exposure was initiated by adding the particle-loaded phyll-tabs according to the respective treatments. The tabs were exchanged

weekly. For the measurement of the status of the acclimatized gammarids right before the start of the experiment, 40 specimens from the gammarids, pre-acclimatized for one week, were instantly prepared for energy assimilation measurement and stored at -20 °C until analysis.

7.2.4. Measurements

Mortality

Mortality was monitored daily, and dead gammarids were removed from the test. Gammarids were counted as dead when no movement of antennae or pleopoda was observed.

Feeding

To determine the mass of food consumed, the loaded phyll-tabs were dried for three days at 45 °C in a drying cabinet (Memmert GmbH), and initial dry weight (dw_I) was determined with a fine scale (Sartorius, 0.01 ± 0.02 mg). Afterwards, tabs were pre-wetted in distilled water for a day for easier feeding and transferred into the allocated treatment. After one week of exposure, the tabs were removed, exchanged with new tabs (pre-weighed and pre-wetted) and dried for two days at 45 °C in the drying cabinet. The final dry weight (dw_F) was also determined. Simultaneously, tabs of each treatment were handled the same but without gammarids in the beaker to determine weight loss during the week independent of gammarids feeding on the tabs (dw_L). The mass eaten per week of all living gammarids in the beaker was calculated by subtracting the percentual proportion of the calculated dw_L from the dw_I and then subtracting the dw_F. Subsequently, the mass eaten per gammarid and day was calculated by dividing the mass eaten per week by the sum of the number of feeding gammarids on each day.

Table 7.2: Summary of the 22 treatments with shortcuts and combinations. The first indicator stands for the treatment, C = Control, H = Hunger/Starvation, Silica = silica, PS = polystyrene, PLA = polylactide. The second indicator stands for the particle size in nm. The last indicator gives the concentration. "L" in the shortcut or "Low" is the environmentally relevant concentration of 4.31 ng/L PS, 4.19 ng/L PLA and 8.38 ng/L silica, and "H" in the shortcut or "high" is the 100-fold higher concentration.

Treatment	Particle type	Particle size [nm]	Conc.
C.0.0 (Control)	None	-	-
H.0.0 (Starvation)	None	-	-
Silica.1000.H	Silica	1000	High
Silica.1000.L	Silica	1000	Low
Silica.500.H	Silica	500	High
Silica.500.L	Silica	500	Low
Silica.100.H	Silica	100	High
Silica.100.L	Silica	100	Low
Silica.30.H	Silica	30	High
Silica.30.L	Silica	30	Low
PS.1000.H	Ploystyrene	1000	High
PS.1000.L	Ploystyrene	1000	Low
PS.500.H	Ploystyrene	500	High
PS.500.L	Ploystyrene	500	Low
PS.100.H	Ploystyrene	100	High
PS.100.L	Ploystyrene	100	Low
PS.30.H	Ploystyrene	30	High
PS.30.L	Ploystyrene	30	Low
PLA.2000.H	Polylactide	2000	High
PLA.2000.L	Polylactide	2000	Low
PLA.500.H	Polylactide	500	High
PLA.500.L	Polylactide	500	Low

Swimming behavior

The swimming behavior of the individual gammarids was monitored at the beginning of the experiment and subsequently every seventh day (Bartonitz et al., 2020). First, individual gammarids were carefully removed with a spoon from the beaker and transferred into a Petri dish with a diameter of 5.5 cm filled with 10 mL of artificial water. The dishes were placed on light boards and at a 30 cm distance under a camera.

The gammarids were tracked with Ethovision XT 11 (Noldus, Netherlands) for 10 min and with a sample rate of 25 frames per second. Thus, for each gammarid, the velocity in cm/s was measured and summarized in an Excel sheet. Afterwards, gammarids were transferred using a spoon into a new beaker according to the treatment.

Dry weight determination

The dry weight of the gammarid tissue (except head and gut) was measured before the energy assimilation determination to allow connection with the other endpoints. Tissues were dried for one day in a drying cabinet (U 40, Memmert, Germany) at 45 °C and weighed after being cooled down for 30 min in a desiccator.

Energy assimilation

The determinations of lipid, glucose and glycogen were based on the assays from Charron et al. (2014) with some modifications. The determination of protein content was based on the Bradford assay (Bradford, 1976) as described by Walker (2002). The dried gammarid tissue (without head and gut) were homogenized in a 1.5 mL tube with a mortar fixated in a Dremel (Micromod 50/e, Proxxon). Then, 200 µL methanol was added, and the remains were homogenized the second time. Another 700 µL methanol was rinsed over the mortar into the tube to wash any sample residues. After homogenization using a vortexer, the sample was divided into three aliquots: 300 µL was transferred into a 1.5 mL tube for lipid measurement, 300 µL was transferred into the second 1.5 mL tube for glucose and glycogen measurement. Next, 2 × 50 µL was transferred to two glass test tubes for protein measurement.

Lipid and glucose/glycogen measurement assays were further conducted as described in Götz et al. (2021) with some adjustments. The lipid aliquot was divided into two samples of 400 µL after cooling for 20 min for measurements in duplicate. The used amount of reagents in each assay was 2.5 mL instead of 5 mL. For protein measurement, 50 µL distilled water was added to 50 µL of the sample in each glass test tube. Also, 50 µL of distilled water and 50 µL of methanol were pipetted into an extra glass test tube as blank. Finally, 1 mL Bradford reagent was added to each glass test tube. After 2 min, the color change from brown to blue allows the photometric measurement of the absorption at 595 nm against the blank. The amount of proteins was calculated using a calibration curve.

7.2.5. Statistical analysis

R (v.4.1.0), Rstudio (RStudio, 2015) and Jamovi (The jamovi project, 2021) were used for statistical analyses. Each treatment per measurement was tested for normal distribution with the Shapiro–Wilk test and, due to some treatments without normal distribution and the small sample number, tested with the robust Fligner–Kileen test for homogeneity in variance. Based on the results, The Friedman test for repeated measurements and, in case of significance, the Durbin–Conover post hoc test was used to test for changes within each treatment over time. To test for differences between treatments, the parameter-free Kruskal–Wallis test was conducted. When differences were observed ($p < 0.05$), the Wilcoxon for pairwise comparisons for unpaired measurements with Bonferroni–Holm continuity correction was used as a post hoc test to find the specific treatments, which were different from each other.

7.3. RESULTS

Mortality

There was a significant increase in mortality for each single treatment over time ($p < 0.001$). Despite this result, there was no difference between the treatments after one week ($p = 0.921$) and two weeks ($p = 0.781$). Furthermore, 61.1 % ± 32.8 % of the gammarids in control and 44.4 % ± 50.2 % of the starved gammarids died within the two weeks. In the particle-exposure treatments, mortality was between 39.9 % ± 25.1 % (Silica.30.L) and 83.3% ± 27.9% (PS.1000.L) after two weeks. The other treatments also varied within this range.

Feeding

Overall, the gammarids in each treatment constantly fed during the two weeks (within treatment comparisons between week one and two, every $p > 0.05$) and significantly more than the starved gammarids ($p = 0.031$). In the first week, gammarids fed with Phyll-tabs loaded with a high concentration of 500 nm silica particles slightly consumed less than those from the treatments with PLA.2000.H, PS.100.H, PS.500.H, Silica.100.H and Silica.1000.H ($p = 0.048$). There were no further differences in the amount of mass eaten by the treatments in the first and second week, including the control. Each control gammarid fed with unloaded Phyll-tabs consumed on average 184.6 ± 68.7 µg/d*gammarid in the first and 237.6 ± 78.6 µg/d*gammarid in the second week. In the particle treatments, mean mass consumption ranged from 141.5 ± 39.3 (Silica.500.H) to 335.2 ± 140.9 (PS.100.L) µg/d*gammarid in the first week. There was more variation in feeding within the treatments in the second week including some outliers (Figure 7.1); the minimum consumed food was 231.8 ± 85.1 µg/d*gammarid (PS.1000.H) and the maximum was 1164.1 ± 1638.7 µg/d*gammarid (PS.500.H). A table with the mean mass eaten and the corresponding ingested amount of particles per gammarid and day for each treatment is provided in the supplementary information (Appendix, Table 13.1). The calculation of the amount of ingested particles was based on the findings of Götz et al. (2021), where the uptake of the particles embedded into the food has been systematically examined. In this context, it is known that leaching of particles from the tab can be neglected. As an example for the calculation of the ingested amount of particles: In

the treatment PLA.2000.H, the consumption per gammarid in one of the beakers was in theory 291.3 µg/d of the Phyll-tab in the first week. The tab was loaded with 419 ng PLA-particles/mg, which leads to a calculated mass of 122.1 ng PLA-particles ingested per day. A gammarid from the treatment PLA.2000.L with nearly the same amount of food consumed (297.48 µg/d*gammarid) ingested around 1.3 ng PLA-particles per day. Overall, the gammarids treated with the low dose ingested particles in the one-digit nanogram level, while those fed with the high dose ingested around 100-fold higher amounts of particles in the lower three-digit nanogram range.

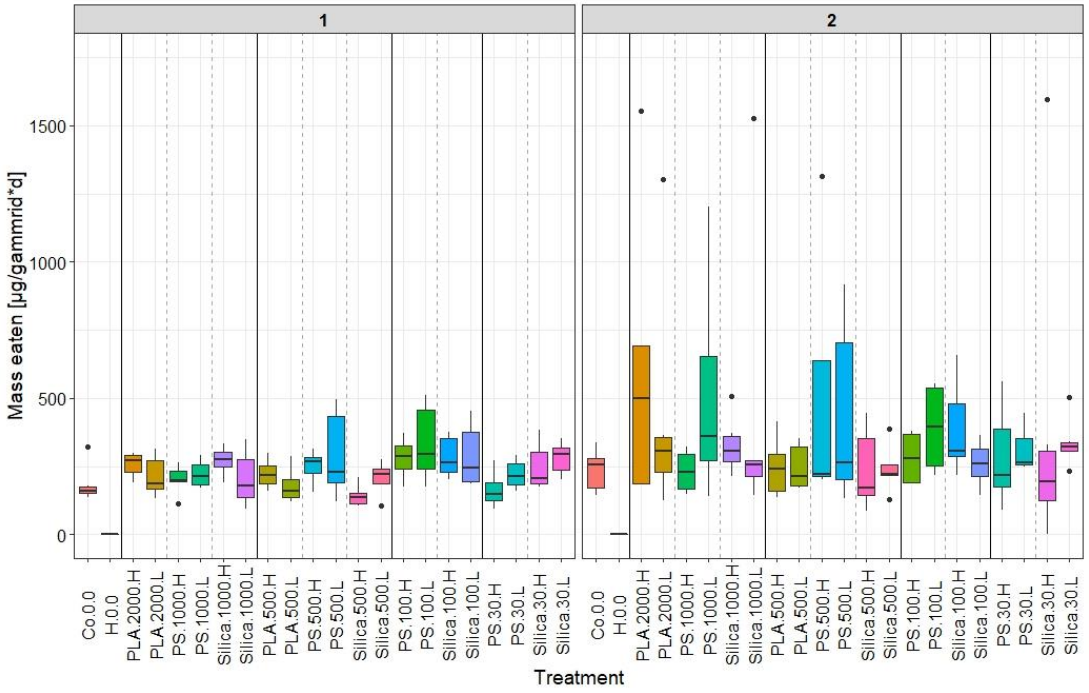

Figure 7.1: Mass eaten per day and gammarid in week one (1) and two (2). Gammarids were exposed to four sizes (30, 100, 500 and 1000 nm) and two concentrations, High = H, Low = L, of spherical micro- and nanoparticles of plain polystyrene, polylactide and silica. The number of measured gammarid samples per treatment ranged from n = 3 (PS.1000.L) to n = 11 (Silica.30.L).

Swimming behavior

None of the gammarids in any treatment swam significantly different to those in other treatments at test start (p = 0.339), after one week (p = 0.267) or after two weeks (p = 0.071), including the comparison of the treatments to the control. Comparing the velocity within each treatment from start to end of the test, the starved gammarids swam slower after two weeks (p = 0.008), while the velocity of the gammarids from the control did not change (p = 0.368). The gammarids from the particle treatments did not change their swimming speed over the two weeks of exposure, except the gammarids treated with PLA.500.L (p = 0.014) and Silica.1000.L (p = 0.007). In both

treatments, the gammarids swam significantly faster after two weeks. Control gammarids swam between 0.71 ± 0.32 and 0.43 ± 0.38 cm/s. The other treatments varied in the same range from minimum (0.50 ± 0.24 cm/s; H.0.0) to maximum (0.82 ± 0.77 cm/s; PLA.500.L) at test start. After two weeks, the range of the velocity of the gammarids in the treatments varied from 0.24 ± 0.14 cm/s (H.0.0) to 0.91 ± 0.30 cm/s (Silica.1000. L).

Dry weight determination

Particle exposure did not influence dry weight of the dissected gammarids ($p = 0.852$, $n = 3$ (PS.1000. L) to $n = 11$ (Silica.30.L) and $n = 39$ for acclimatization). The dried body remains mean weight was 2.30 ± 0.39 mg. Gammarids from the Silica.1000.H treatment weighed the most with 2.95 ± 0.93 mg, and gammarids from the Silica.500.H treatment weighed only 1.54 ± 1.19 mg.

Energy assimilation

The four storage substances, protein, lipid, glucose and glycogen, were measured in the body of the gammarids without head and gut. None of the four substances changed due to the particle treatments ($p = 0.130$ - 1.000) after the two weeks exposure and, hence, were the same as in gammarids from the control, starvation and acclimatization (Figure 7.2).

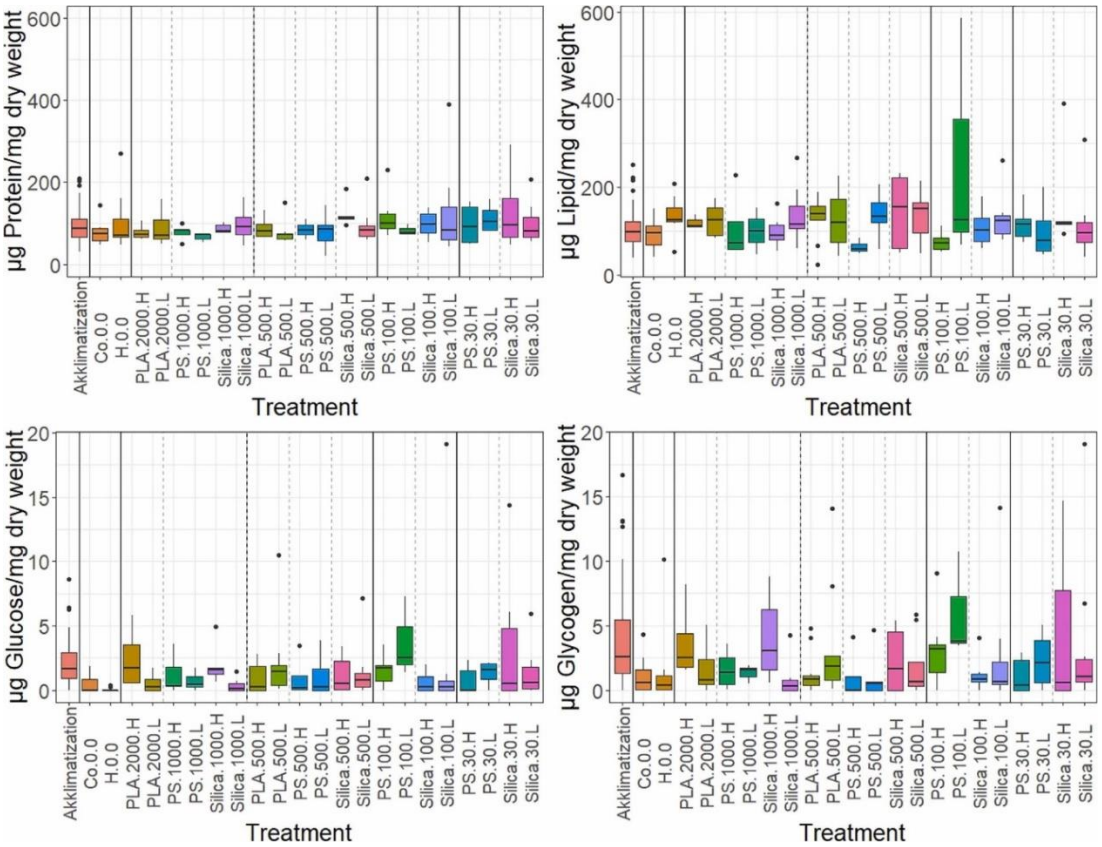

Figure 7.2: The amount of protein, lipid, glucose and glycogen in µg per mg dry weight of the gammarid bodies exposed to different sizes and concentrations of three micro- and nanoparticles after acclimatization and two weeks. The particle types were spherical polystyrene, polylactide and silica in 30, 100, 500 and 1000 nm. The number of measured gammarid samples per treatment ranged from n = 3 (PS.1000.L) to n = 11 (Silica.30.L) and 39 for acclimatization.

7.4. DISCUSSION

To our knowledge, this is the first study which examines nanoparticle effects on *Gammarus roeseli* based on comparing dietary exposures to mineral particles, non-biodegradable and biodegradable plastic particles. Surprisingly, exposure to small nanoparticles of 30 nm did not result in increased signs of toxicity on the organism level compared with larger particles up to 1000 nm. In contrast to our hypotheses, none of the four tested particle sizes induced any adverse effect in the examined endpoints independent of whether the environmentally relevant ng concentration or the 100-fold higher concentration was used. Our further hypotheses were likewise not confirmed. Primarily, there was no observable difference in the reaction of *G. roeseli* to the plastic and the natural particles. Also, the biodegradable PLA did not induce higher or other toxicity responses in the investigated endpoints than the non-biodegradable PS. The dietary exposure did not result in alterations in survival, feeding, swimming behavior or energy assimilation of *G. roeseli* within the experimental duration of two weeks. Therefore, no concentration, particle type or size-dependent effects were evident. This suggests that *G. roeseli* will not be negatively affected in their performance and behavior if it ingests micro- and nanoscale particles in the environment. Thus, even if the nanoparticles translocate into tissues (Firdessa et al., 2014; Triebskorn et al., 2019), there is no effect showing up to the organism level.

The gammarids fed with Phyll-tabs without particles consumed on average half as much food as observed by Götz et al. (2021). However, the consumption is still in the acceptable range, like for the other to micro- and nanoparticles exposed gammarids. This suggests that the functional role of *Gammarus* as a shredder is not impaired due to particle occurrence. For the interpretation of the energy reserves, it should be noted that the lack of significance of the differences between treatments might be due to the relatively small sample size of some treatments. Nevertheless, the amount of measured lipids in the dissected gammarids from each treatment is similar to the amount of lipids of the natural state and Phyll-tab-fed gammarids of another study (Götz et al., 2021). Only glucose and glycogen were consistently less stored in the present gammarids independent of the treatment, including acclimatization, than in previously tested gammarids (Götz et al., 2021). The small amount of glucose stored

in the gammarids is the same or lesser as in gammarids starved for 16 (Götz et al., 2021) and 23 d (Charron et al., 2015). This discrepancy is likely due to an additional environmental stressor affecting the gammarids´ survival. Mortality induced by the setup can be excluded as the controls in preceding experiments of gammarids from the same population in well-fed states resulted in low mortality (Bartonitz et al., 2020; Götz et al., 2021). In contrast, it indicates a preceding malnutrition of the gammarids of our study (Charron et al., 2015; Götz et al., 2021), which seems to continue over the experiment as the time of two weeks may not be enough to fill up the reserves. The gammarids were caught in April in contrast to those from Götz et al. (2021), which were caught in November. In this spring, the habitat contained remarkably less vegetation and detritus, which most likely led to the malnutrition of the gammarids. This additional stressor due to preceding starvation affects the survival, but not the behavior relating to the investigated endpoints. In contrast, the gammarids consumed the offered tabs and subsequently ingested the micro- and nanoparticles as intended. Therefore, particle uptake und subsequent exposition of the gammarids can be considered as given since particle uptake via the DECOTAB was confirmed in a previous study by Götz et al. (2021). Additionally, organisms treated with more than one stressor normally react more sensitively than they would with a particular one (Marcogliese and Pietrock, 2011; Vellinger et al., 2012). Consequently, the pre-starved population of the caught and tested gammarids would likely not additionally be affected regarding swimming, feeding and energy assimilation by the occurrence of those particles as tested here. In addition, multiple stressors affecting gammarids in the wild can also result in sub-optimal conditions and high mortalities in natural exposures (Pander et al., 2022). From an ecotoxicological point of view, comparing treatments of defined stressor exposure in non-optimal states can exacerbate the effect strength. The absence of such effects in our study may be indicative that no larger effects would occur under optimal conditions.

Generally, the risk of negative environmental consequences caused by nanoparticles is rising since the degradation and disintegration of plastic debris will most likely increase the abundance of smaller-sized particle fractions. This probability for increased risk is based on the assumption that nanoscale particles can pass epithelial barriers and translocate into tissues, which induces cytotoxicity (Shen et al., 2019).

Hence, we wanted to evaluate whether environmentally relevant concentrations of nanoparticles induce toxicity affecting the population of *G. roeseli* correlating with the decreasing size from 1000 to 30 nm. Due to the dietary exposure, the uptake and subsequent exposure of the particles can be predicted when feeding occurred (Götz et al., 2021). However, the ingested nanoparticles were not documented by detection methods in our study. The lack of effects does not correspond with those from other studies, as 70 nm PS nanoparticles induced mortality and delayed development for *D. pulex* (Liu et al., 2019), and 20 - 500 nm PS nanoparticles reduced cell viability, chlorophyll-*a* concentration and increased levels of reactive oxygen species (ROS) in *Chlorella vulgaris* (Hazeem et al., 2020). Nevertheless, most studies greatly exceed the environmental concentrations we examined in our study in the nanogram range and used milligrams of the particles. Our observations suggest that PS and PLA nanoparticles do not impact the individuals' behavior and, very likely, the performance of a population of *G. roeseli* in the mass concentrations that are currently assumable for PS nanoparticles in the environment. These observations do not correspond with other studies, where biodegradable plastic particles were found to be more toxic than non-biodegradable plastic or mineral particles. For example, secondary nanoparticles (75–200 nm) of the biodegradable plastic polyhydroxybutyrate (PHB) reduced cellular growth and affected physiological parameters of *Anabaena* sp. and *Chlamydomonas reinhardtii* within three days of exposure and *Daphnia magna* after two days (González-Pleiter et al., 2019). Furthermore, fragmented PLA microparticles (< 59 µm) at a high mg concentration reduced survival and reproduction of *Daphnia magna*, being 35-times more toxic than the natural kaolin microparticles, which also reduced the reproduction rate but only in a much higher concentration (Zimmermann et al., 2020). The EC50 was calculated as 23.6 mg PLA/L, and the smallest investigated concentration of PLA microparticles in their study did not reduce reproduction. The latter finding corresponds with ours, as the examined concentrations in the ng range did not affect the investigated endpoints.

Still, the missing significance in the effects may also be due to a variability in the response originating from the undifferentiated sexes of the gammarids, which is known to be a possible factor (Sornom et al., 2010; Charron et al., 2014). Nevertheless, the conclusion seems likely that the environmentally relevant nanoparticle mass

concentrations in the lower nanogram range calculated from the microparticle mass concentrations from Triebskorn et al. (2019) used in our study will not impact *Gammarus roeseli* when exposed to them in their habitat.

Finally, we assessed whether the plastic particle toxicity is similar to the toxicity of naturally occurring particles. The direct comparison showed that neither plastic particles nor mineral particles impacted the examined organisms. Although this could be interpreted as evidence that the in other studies interpreted plastic particle toxicity could likewise be a general particle toxicity, this assumption needs to be verified with effect-inducing concentrations. Nevertheless, most of those studies did not compare their observed responses with responses to natural nanoparticles of the same size and shape (González-Pleiter et al., 2019; Hazeem et al., 2020; Liu et al., 2019). Therefore, not a specific plastic nanoparticle effect but rather a general nanoparticle effect was proven. Studies investigating plastic particle effects compared with natural particles are occasional but important (Triebskorn et al., 2019). The study of Zimmermann et al. (2020) is the first but rare evidence that the toxicity of biodegradable plastic particles is different to natural particles.

Regarding the assessed endpoints, most of the studies examine mechanistic and metabolic relevant endpoints. The focus of our study was on ecologically relevant endpoints for risk assessment reflecting the performance of particle-exposed organisms in the environment. The future, integration of additional physiological endpoints and microindicators reflecting metabolic pathways and gene or protein expression patterns with the same setup may make a useful addition and contribute to a more mechanistic understanding of the underlying factors. In the context of the reviewed literature, our study is a rare example that micro- and nanoparticles of polystyrene and biodegradable polylactide do not induce adverse effects on aquatic keystone organisms. With this study we provide another basic dataset for further plastic nanoparticle risk assessment by examining a realistic ERC.

8. Modulation of PAH toxicity on the freshwater organism *G. roeseli* by microparticles

A similar version of this chapter has also been published in the Journal "*Environmental pollution*" as Bartonitz A, Anyanwu IN, Geist J, Imhof HK, Reichel J, Graßmann J, Drewes JE, Beggel S (2020) Modulation of PAH toxicity on the freshwater organism *G. roeseli* by microparticles. *Environmental Pollution* 260:113999. doi: 10.1016/j.envpol.2020.113999

Abstract

Polycyclic aromatic hydrocarbons are widespread and environmentally persistent chemicals that readily bind to particles in air, soil and sediment. Plastic particles, which are also a ubiquitous global contamination problem, may thus modulate their environmental fate and ecotoxicity. First, the acute aqueous toxicity of phenanthrene in adult *Gammarus roeseli* was determined with a LC_{50} of 471 µg/L after 24 h and 441 µg/L after 48 h. Second, considering lethal and sublethal endpoints, effects of phenanthrene concentration on *G. roeseli* were assessed in relation to the presence of anthropogenic and natural particles. The exposure of gammarids in presence of either particle type with phenanthrene resulted after 24 and 48 h in reduced effect size. Particle exposure alone did not result in any effects. The observed reduction of phenanthrene toxicity by polyamide contradicts the discussion of microplastics acting as a vector or synergistically. Especially, no difference in modulation by plastic particles and naturally occurring sediment particles was measured. These findings can most likely be explained by the similar adsorption of phenanthrene to both particle types resulting in reduced bioavailability.

Keywords: Phenanthrene; Gammarus; Toxicology; Microplastic; Bioavailability

8.1. INTRODUCTION

Polycyclic aromatic hydrocarbons (PAH) are widespread and environmentally persistent chemicals of concern. Their sources and distribution are well understood and documented (Abdel-Shafy and Mansour, 2016; Baek et al., 1991; Lima et al., 2005; Rabodonirina et al., 2015). Small quantities of the PAHs can be of biological origin, but most enter the environment from anthropogenic sources (Menzie et al., 1992). The highest quantity of PAHs is emitted into the atmospheric environment, where further distribution to soil and water occurs. Because of their lipophilic characteristics, PAHs readily bind to particles in air, soils and sediments, and tend to be rarely found dissolved in water (Abdel-Shafy and Mansour, 2016; Baek et al., 1991; Srogi, 2007).

PAHs are known to cause potential adverse health effects for humans and animals (Abdel-Shafy and Mansour, 2016) and a wide range of toxicity-tests for various PAHs have been conducted (Barata Martí et al., 2005; Besse et al., 2013; McConkey et al., 1997). With respect to the lipophilic characteristics and known distribution of PAHs (Srogi, 2007), many of these studies focused on PAHs adsorbed to natural particles like sediment (Landrum et al., 1994; Lankadurai et al., 2011; Lotufo and Fleeger, 1997; Verrhiest et al., 2001). Thus, the effects of PAHs sorbed to sediment particles are well-known for many species. Due to the increasing occurrence in the environment, research has recently focused on the effects of chemicals associated with anthropogenic plastic particles (Kleinteich et al., 2018; Karami et al., 2016; Browne et al., 2013).

Likewise to PAHs, plastic particles are ubiquitous in lake and stream ecosystems worldwide (Rezania et al., 2018; Sharma and Chatterjee, 2017; Strungaru et al., 2019). As reviewed by Dris et al., (2018), a median of 0.0026 particles/L surface water with a size of >50 µm was found in 63 rivers. In beach sediments, even 333 particles/kg dry weight in a size range between 0.5 and 5 mm were detectable. Water samples from anthropogenically influenced sites proved to be more burdened with microplastic in concentrations around 2.5 and 2.9 particles/L in China and 7.9 ± 7.3 microparticles/L in Germany compared to natural sites (Dris et al., 2018; Schmidt et al., 2018).

PAHs adsorb to and desorb from plastic (Alimi et al., 2017; Bakir et al., 2014a; Browne et al., 2013; Rios et al., 2010). Comparing the chemical properties, sorption capacity of microplastic seems to be slightly higher than for sediment, while adsorption kinetics

of PAHs on microplastic or sediment can vary depending on many properties such as the chemicals hydrophobicity or sediment organic carbon content. Like for sediment, partitioning coefficient of the specific chemicals is one of the main drivers for sorption on microplastic and vice versa. However, the release of the chemicals from plastic particles seems to be faster (Wang and Wang, 2018). As a result, a discussion about possible synergistic effects of PAH and plastic particles evolved, specifically concerning the role of plastic particles to act as a vector (Alimi et al., 2017). Nowadays, nearly 80 % of examined PE-microparticles from the north pacific central gyre were associated with PAHs (Rios et al., 2010). In samples from the Bejing river in the Feilaixia reservoir, all the microplastic particles found were associated with PAHs ranging from 282.4 ng/g for PP and 364.2 ng/g for PE (Tan et al., 2019). As many organisms ingest plastic particles (Cole et al., 2013; Cole and Galloway, 2015; Foley et al., 2018; van Cauwenberghe et al., 2015), a high potential for adverse effects exists evoked by transported chemicals due to the large surface of microparticles (Bakir et al., 2014a; Batel et al., 2016; Browne et al., 2013). However, modelling studies assume a negligible impact from plastic particles as vector, because of their insignificant small amount in the environment (Bakir et al., 2016; Koelmans et al., 2013). Yet, a tipping point may be reached at which the role of plastic in sorption, cleaning and transport of chemicals can become important (Koelmans et al., 2016) depending on the plastics' characteristics (Lee et al., 2018; Sun et al., 2019; Wang and Wang, 2018).

Studies simultaneous investigating the chemicals toxicity in the presence of anthropogenic particles, like microplastic, and natural particles are rare (Alimi et al., 2017). The studies investigating the effect of chemical-pollution-combined particles mainly addressed one aspect, e.g. toxicity of one chemical-associated-particle (sediment: Landrum et al., 1994; Lotufo and Fleeger, 1997, microplastic: Browne et al., 2013; Kleinteich et al., 2018). The comparison of the PAH-toxicity when dissolved or sorbed to different particle types remains largely unattended (Alimi et al., 2017). Due to the sorption characteristics of microplastic (described by Wang and Wang, 2018) and the previous studies with contaminant-associated particles, the combined effect of PAH and microplastic particles in the same compartment has to be tested and compared with natural microparticles. Therefore, the main goal of the present study was to compare the modulation of sublethal PAH-effects on gammarids by the

presence of anthropogenic and natural microparticles.

A preceding acute toxicity test for exposure of phenanthrene through water was performed to determine the LC_{50} via this exposure route and to determine the concentration range for sublethal toxicity measurements. On the assumption that sublethal endpoints are more sensitive and an earlier response than lethality (Maltby et al., 2002), *G. roeseli* were exposed to a range of sublethal phenanthrene concentrations with and without combination of polyamide (PA) or sediment microparticles (SP). These tests were intended to show the change in effect strength of phenanthrene to the gammarids by the presence of natural and anthropogenic microparticles. The measured endpoints were mortality, swimming behavior and feeding.

8.2. MATERIAL AND METHODS

Sampling and acclimatization of test organisms

Because of its environmental relevance, *G. roeseli* was chosen as test species. They have a widespread distribution, form a majority of biomass in stream ecosystems and play an important role in the food web (Gerhardt et al., 2011; Kelly et al., 2002). Furthermore, a high sensitivity towards a wide range of pollutants has been reported (Brock and van Wijngaarden, 2012; Hunting et al., 2016; Zhai et al., 2018). *G. roeseli* were collected from the river Moosach in Freising, Germany (48°23'38.8''N 11°43'26.1''E) between May and June 2018 and size selected (9.6 ± 1 mm, n = 50) by sieve passage.

Acclimatization occurred in a climate chamber with constant temperature of 13 ± 0.5 °C and a 16:8 h light:dark cycle. First, individuals were kept in aerated aquaria filled with a mix of 50 % bank filtrate from the river Moosach and 50 % artificial water (ISO 6341 2012) for at least three days. During this part of the acclimatization period, gammarids were fed ad libitum with modified DECOTABs (Kampfraath et al., 2012). 10 % of cellulose was replaced with ground Tetraphyll (Tetra GmbH, Germany), which was added as a supplementary food source (DECOTAB size 1 cm Ø, 0.5 cm high, dry weight 30.8 ± 3.0 mg (n = 150)). The subsequent acclimatization phase in 100 % artificial water (ISO 6341, 2012) started three days before the test. Gammarids were not fed during this step (US EPA, 2016). Mortality during acclimatization did not exceed 5 %.

Test substances

Phenanthrene was chosen as a standard model PAH. It is one of the smallest PAHs with three benzene rings and a known low toxicity to humans compared to other PAHs (GESTIS Substance Database, 2019; Samanta et al., 2002). Due to its well-described chemical and toxicological characteristics (Verbruggen and van Herwijnen, 2012) it is often used as standard PAH (Barata Martí et al., 2005; Verrhiest et al., 2001; Zhang et al., 1997). Water solubility is 1.15 mg/L at 25 °C, so stock solution was made with methanol (99.8%). The Log KOW is 4.52 (safety data sheet, IFA substance data base) and KOC is around 2.97 x 10^4 mL/g (Zhang et al., 2009). Phenanthrene for toxicity testing (Phe, CAS: 85-01-8) and phenanthrene-d10 (CAS: 1517-22-2) for chemical analysis was purchased from Sigma-Aldrich (Germany), and stored at 8 °C. Methanol

for chemical analysis of phenanthrene concentration in the artificial medium was purchased from VWR chemicals (Germany).

Polyamide (PA), which has a high market share (Scheibitz and Spies, 2016), was used as an exemplary anthropogenic particle, while sediment particles from the river Moosach were used as natural reference particles. Granulates of PA were purchased from Lanxess (Cologne, Germany) as "Durethan C38F" (ISO 16396-PA 6/66, E, S14-020). The microparticles were generated by centrifugal milling the PA granulates and sieving (Ultra Centrifugal Mill Type ZM 200, Retsch, Germany). Particles were 40 - 63 µm in size, had a fragmental shape and were stored at room temperature (Figure 8.1B).

With respect to Hartmann et al. (2015), stock solutions were prepared in 10 mL methanol, resulting in a concentration of 2.5 mg/mL for each stock solution. PA is stable in 100 % methanol according to Bürkle GmbH (2011).

Sediment microparticles (SP) were obtained from wet-sieved and dried fine sediment samples from the river Moosach in Freising, Germany. Particles were 45 - 63 µm in size, had a fragmental shape and were stored at room temperature (Figure 8.1 A). A gravimetric measurement with Element Analyser EA 4000 (Analytik Jena) revealed total organic carbon of 1.64 % (balancing method), limit of determination was 0.1 %.

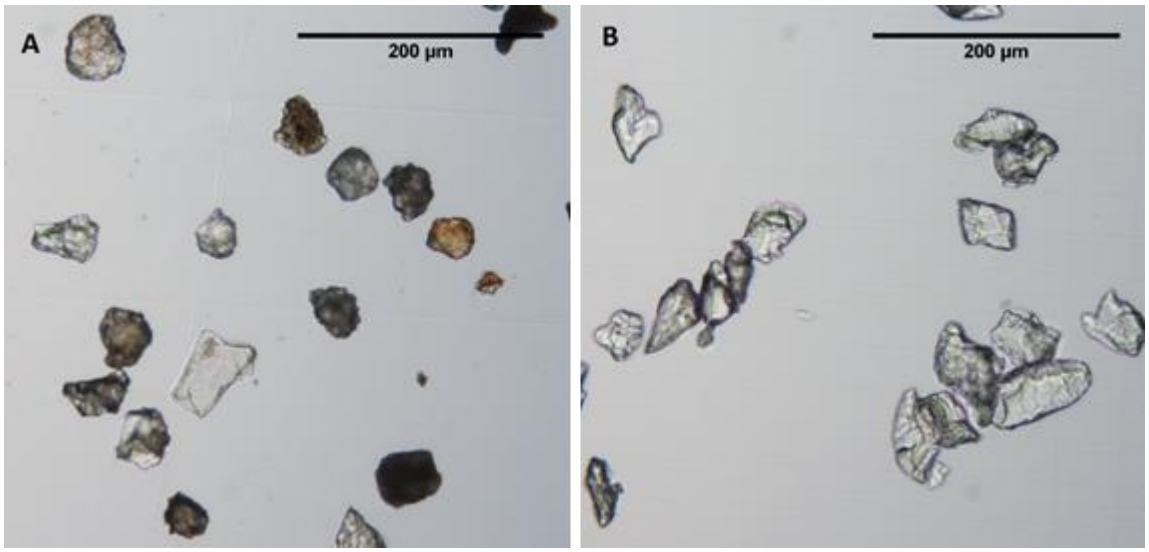

Figure 8.1: Microscopic images of the microparticles used during the exposure experiments with a size of 40 - 63 µm and a fragmental shape. (A) Sediment microparticles representing natural particles, (B) Microparticles of polyamide (PA) representing anthropogenic particles.

8.2.1. Chemical analysis

Instrumentation. Stir bars (Gerstel Twister®) coated with polydimethylsiloxane (PDMS, film thickness 0.5 mm, length 10 mm) were purchased from Gerstel GmbH & Co. KG (Germany). For incubation, 20 mL Headspace vials (Agilent, USA) with Sil/PTFE coated septa (Thermo Fisher, USA) were used. The Twisters were stirred on a multiple position magnetic stirrer (15 positions) from Thermo Fisher (USA). The thermal extraction/desorption-gas chromatography/mass spectrometry (TD-GC/MS) analysis was performed using a Gerstel ThermalDesorptionUnit (TDU) 2 equipped with a Gerstel Multi-PurposeSampler (MPS) robotic^{pro}, a Cooled Injections System (CIS 4) with C506 and an Agilent 7890B gas chromatograph equipped with an DB-5MS (Agilent) column combined with an Agilent 5977B MSD mass spectrometer.

Sample preparation and incubation. Standard solutions of phenanthrene and phenanthrene-d10 were dissolved in methanol. To obtain a calibration curve, the standard solutions were diluted in artificial water (ISO 6341 2012), adding 0.01 mg/L phenanthrene-d10 as an internal standard to a final volume of 10 mL. Samples containing sediment and microplastic particles were centrifuged before analysis. Water samples and 0.01 mg/L phenanthrene-d10 were diluted to a final volume of 10 mL in 20 mL headspace vials together with one stir bar and stirred at 24 °C, 1000 rpm for 60 min. The stir bar was removed, washed with LC/MS water, and dried with a lint-free tissue. Subsequently the stir bar was transferred into the thermodesorption tube. The limit of detection (LOD) for phenanthrene was 2.5 x 10^{-3} µg/L.

Chemical analysis. The concentration of phenanthrene was determined with stir bar sorptive extraction via TED-GC/MS. All measurements were conducted in triplicate. The TDU method was adopted from Ochiai et al. (2005). The TDU was set at an initial temperature of 20 °C (delay time 0.30 min; initial time 1 min) and a following ramp at 50 °C/min to an end temperature of 280 °C, hold time 1 min. The desorbed compounds were cryo-focused at -100 °C. Transfer from the stir bar to the cold trap was done in split-less mode. The CIS was programmed from −100 °C to 280 °C (held for 5 min) at 10 °C/s. Injection was done in a 1:100 split mode and the GC oven temperature gradient was as follows: 70 °C (2 min)/25 °C/min → 150 °C/3 °C/min →

200 °C/8 °C/min → 300 °C. Helium was used as carrier gas. The mass spectrometer was operated in the full scan mode (m/z range 40 - 550) with electron impact ionization (70V). Calibration concentrations were 50 µg/L to 0.05 µg/L.

Table 8.1: Nominal and measured concentration of phenanthrene only and with microparticles after 0 and 48 h. * Label for samples not centrifuged, the other samples were centrifuged to remove microparticles. LOD for Phe was 2.5 x 10^{-3} µg/L.

Treatment	Nominal concentration [µg/L]	Measured concentration 0 h [µg/L] ± SD	Measured concentration 48 h
Control	< LOD	< LOD	< LOD
Solvent control	< LOD	< LOD	< LOD
Sediment control	< LOD	< LOD	< LOD
Plastic control	< LOD	< LOD	< LOD
Phe A	50	92.1 ± 0.1 *	19.9 ±1.4 *
Phe B	75	78.9 ± 8.1 *	16.3 ± 0.5 *
Phe C	100	115.3 ± 2.2 *	21.1 ± 0.3 *
Phe D	250	329.3 ± 22.1 *	52.8 ± 10.6 *
Phe E	500	601.3 ± 42.8 *	141.8 ± 6.5 *
Plastic + Phe A	50	0.3 ± 0.4	0.0 ± 0.0
Plastic + Phe B	75	7.9 ± 5.9	2.2 ± 0.0
Plastic + Phe C	100	25.1 ± 3.3	4.6 ± 0.2
Plastic + Phe D	250	131.1 ± 3.4	28.3 ± 2.4
Plastic + Phe E	500	209.4 ± 5.3	34.1 ± 1
Sediment + Phe A	50	10.8 ± 0.3	0.9 ± 0.1
Sediment + Phe B	75	33.0 ± 0.6	1.5 ± 0.1
Sediment + Phe C	100	24.5 ± 0.6	4.3 ± 0.6
Sediment + Phe D	250	138.7 ± 3.4	27.0 ± 0.6
Sediment + Phe E	500	325.2 ± 10.1	24.4 ± 0.2

8.2.2. Bioassay

Toxicity determination was conducted in a two-step approach. As the main goal was to determine microparticle-induced changes in sublethal effects, an acute toxicity assay for phenanthrene was performed first to determine lethal thresholds for adult *G. roeseli*. Then, the main toxicity assay with sublethal concentrations was performed, based on the results from acute toxicity assay. Sublethal toxicity of Phe alone was compared with presence of polyamide (PA) or sediment microparticles (SP) and microparticles alone as described below.

The validity criteria and test conditions of all bioassays followed the Gammarid Amphipod Acute Toxicity Test (US EPA, 2016) with minor modifications in feeding protocol and test medium. Assays were conducted in 1 L appropriate aerated artificial water (ISO 6341, 2012) in glass beakers under same conditions as acclimatization. Each beaker contained three glass stones as hiding places to reduce stress for the gammarids.

While gently mixing the stock suspension/solution to ensure a homogenous dispersion, Phe, PA and SP concentrations were pipetted directly into the medium. Following, five individuals were transferred into each prepared beaker and fed ad libitum with one modified DECOTAB.

The parameters of the static assays were measured at the end of each treatment after 24 and 48 h with portable pH meter MultiLine® Multi 3630 IDS SET G (WTW, Germany). The exposure concentration was intentionally not maintained stable during the exposure period to show the reduction due to the adsorption behavior of the chemical, which is also a more environmentally realistic scenario.

Acute toxicity

Acute toxicity of phenanthrene was determined with a control, solvent control (methanol, 1 mL/L) and five concentrations of phenanthrene (nominal concentrations: 5, 50, 500, 1,000, 1500 µg/L, n = 3). Mortality was recorded after 24, 48, 72 and 90 h in the corresponding three replicates, which were then removed from the test. Therefore, the other treatments were not disturbed or stressed. Individual gammarids were classified as dead when they were not moving (including movement of legs), unbent or/and body color changed from white/grey to orange.

Sublethal toxicity

In order to determine the modulation of the sublethal phenanthrene toxicity in presence or absence of different microparticles, *G. roeseli* were exposed to eight sublethal exposure concentrations of phenanthrene in three exposure scenarios over 24 and 48 h: (1) phenanthrene alone (Phe), (2) phenanthrene and PA microparticles (Phe + PA), and (3) phenanthrene and natural microparticles (Phe + SP) in the water column. Nominal concentrations from 50 to 500 µg/L were chosen for the sublethal toxicity experiment in order to cover a range with expected sublethal effect concentrations. The nominal phenanthrene concentrations were: 50, 75, 100, 250, 315, 375, 435, and 500 µg/L. In consequence of the chemical analysis (8.2.2), the nominal concentrations were replaced by measured concentrations (Table 8.1) throughout the analyses and illustrations.

Both, the sediment and PA microparticles were added at a concentration of 500 µg/L. Each treatment was replicated five times. Four treatments were added to serve as controls: medium only (control), medium with solvent methanol (solvent control) and two microparticle controls with SP and PA, respectively (SP control, PA control). Experimental setup was as described in chapter 8.2.1.

After 24 and 48 h exposure, three endpoints were assessed including mortality, feeding rate and swimming behavior. The feeding rate, swimming behavior and chemical analysis (see 8.2.1) were determined for five test concentrations (50, 75, 100, 250 and 500 µg/L). Pooled samples for chemical analysis were taken after 0 and 48 h and stored at -20 °C until analysis.

Feeding rate

For feeding rate determination, DECOTAB leftovers were removed with a spoon at the end of the experiment (24 and 48 h) and transferred to small pre-dried aluminum dishes. The leftovers were dried for one day in a drying cabinet at 45 °C and then weighed with a Sartorius R200D Analytical Balance (Sartorius GmbH, Germany, 0.01 ± 0.02 mg). For the standard DECOTAB-weight, 150 freshly made DECOTABs were also dried for one day and weighed (30.8 ± 3.0 mg). To calculate feeding rate, leftover dry weight was subtracted from the standard DECOTAB dry weight. Estimated loss of weight was divided by the amount of living gammarids at the end of the exposure and calculated per day.

$$\text{Feeding rate} = \frac{\text{DECOTAB standard dry weight [g] - DECOTAB leftover dry weight [g]}}{\text{living gammarids x test duration [d]}}$$

Swimming behavior

A camera-based automated tracking system with the software Ethovision XT 9 (Noldus, Germany) was used for the measurement of velocity. Recording time was 10 min with a sample rate of 25 frames per second. The measurement setup was made of three cameras positioned 30 cm above 15 arenas. The five organisms of each beaker were transferred to five glass petri dishes (Ø 5.5 cm) filled with 10 mL of treatment water and placed under the camera in programmed arena position to ascertain individual swimming behavior. To avoid interfering influences of moving light or shadows a cardboard box was pulled over and a lightboard (M.Way, China) was placed under the setup. A ruler of 10 cm served as scale calibration standard in the videos. After automated tracking of *G. roeseli*, videos were manually checked for false detections and corrected if reasonable.

8.2.3. Statistical analysis

Statistical analyses were conducted with RStudio (RStudio, 2015). Data were tested for normality using the Shapiro-Wilk test and homogeneity of variance with the Levene's Test. Due to low replicate number, non-normality and heterogeneity of variance, mortality was tested with exact Fisher-test (EF) for significance of deviation between the treatments to estimate whether the observed mortality is independent of the treatment and/or the microparticles. When EF showed a significant p-value (<0.05) pairwise Wilcoxon test with Benjamini-Hochberg correction (pW-BH, Benjamini et al., 1998) was used as post hoc test to identify those treatments significantly different to the control. After this, log-logistic regression (LL.2.5, LL.3, LL.4 and LL.5) was conducted to identify the best model fi t. The models were compared with Likelihood Ratio-Test (LRT) and Akaike-Information-criterion (AIC). For swimming behavior, influence of beaker was tested first with ANOVA. For this, the model ‘y = $ß_{00}$ + $ß_1$ x treatment x beaker´ was chosen because of assumed interaction of beaker and phenanthrene concentration. Since the model resulted in normal distribution and homogeneity of variance, ANOVA could be used. ANOVA showed just in one case out of five significant influence from beaker on the effect, which lead to the further exclusion of beaker effects. Due to resulting non-normality and heterogeneity,

Kruskal-Wallis-test (KW) was conducted to test differences between the three exposure scenarios, followed by pairwise Wilcoxon test (pW). One-way ANOVA by Welch (AW) was used to test significant difference between the treatments. As post-hoc test pW-BH was used. Furthermore, linear regression was used to fit a model between concentration and velocity. Feeding rate was analyzed with KW to test for differences between the three exposure scenarios. Additionally, AW was used to test for significant differences in the exposure scenarios, followed by the post-hoc test pW-BH. Furthermore, linear regression was used to fit a model between concentration and feeding rate. Data from GC/MS were acquired with MassHunter Workstation Software (Ver.B.08.000 from Agilent Technologies). Ion chromatograms were extracted for m/z 178.2 for phenanthrene and 188.2 for phenanthrene-d10. Integration was conducted automatically, and software supported. Data analysis was conducted with Microsoft Excel 2016.

8.3. RESULTS

Water chemistry

Physicochemical parameters in medium measured at start and termination of the exposure period were within the acceptable range for the test organism for all experiments and treatments (US EPA, 2016). The measured mean values (± standard deviation) were: Temperature 12.7 ± 0.6 °C; oxygen 101% ± 1.5 %; pH 7.6 ± 0.2, and conductivity 677 ± 13 µS/cm.

8.3.1. Chemical analysis

The chemical analysis of the water samples from the sublethal toxicity assay indicated a discrepancy between predicted nominal and measured concentrations at the beginning of the experiment, with around 20 % more phenanthrene in the treatment with Phe (Table 8.1). Phenanthrene concentration in all control treatments was below limit of detection (2.5×10^{-3} µg/L).

Exposure scenarios of Phe with microparticles showed a reduction of phenanthrene in the water column by 60 % in the highest concentrations and 99 % in the lowest concentrations compared to the Phe in samples from the start of the test. Compared to exposure scenario with Phe, concentration was constantly (0 and 48 h) more than 50 % lower (mean 24 ± 18 %) with microparticles in the water column. Phenanthrene concentration reduction was similar in plastic and sediment particle treatments.

After 48 h, the measured concentration of phenanthrene revealed a decrease down to around 20 % ± 3 % of the measured initial concentrations in the Phe exposures and 17 % ± 10 % (Phe + PA) and 11 % ± 7 % (Phe + SP) of initial concentrations with microparticles.

8.3.2. Bioassay

Acute toxicity

The preceding test for acute toxicity was based on nominal test concentrations and revealed an increasing mortality with increasing phenanthrene concentration at all four timepoints (24, 48, 72 and 90 h). Phenanthrene revealed very similar effect responses, with low mortality at 5 (<10 %) and 50 µg/L (7 % and 13 %), followed by a strong increase to more than 60 % up to nearly 100 % in the concentrations 500, 1000 and 1500 µg/L. LC_{50} was reached at a nominal phenanthrene concentration of

471.9 µg/L for 24 h, 441.1 µg/L for 48 h, 125.9 µg/L for 72 h and 83.6 µg/L for 90 h (Figure 8.2 and Table 13.2).

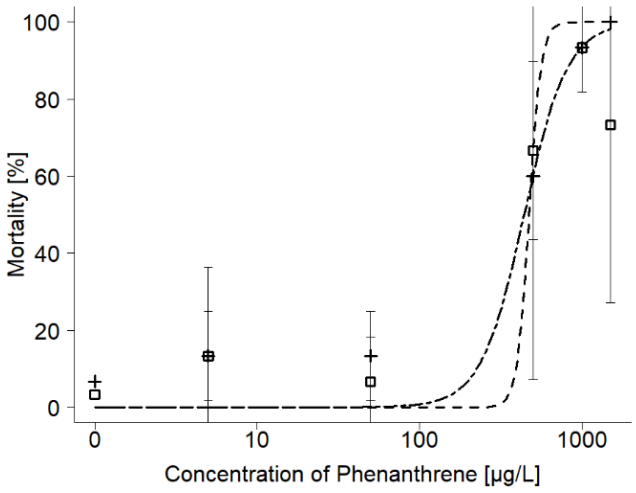

Figure 8.2: Acute mortality of *G. roeseli* after 24 h (square) and 48 h (plus) exposure to phenanthrene in artificial water with concentrations of 5 to 1500 µg/L. Data are shown in means of replicates with standard deviation (error bars). Dose-response-fits (24 h dashed and 48 h twodash) for the exposure scenarios. AIC for 24 h regression was 193.6 with 2 degrees of freedom and for 48 h 191.1 with 3 degrees of freedom). Mortality for 72 and 90 h has been excluded to focus on 24 and 48 h because of the subsequent tests.

Sublethal toxicity

Based on the LC_{50} of the preceding acute toxicity test, nominal concentrations with maximum 500 µg/L were chosen for the subsequent sublethal toxicity assay. Whole analysis was conducted with the measured concentrations of Phe after 0 h (Table 8.1).

Mortality

Phe exposure to expected sublethal concentrations resulted in a low mortality of *G. roeseli* not exceeding 20 %, except for a phenanthrene concentration of 601 m g/L after 48 h (84 %, pW, $p = 0.01$).

Exposure scenarios with microparticles alone did not result in any significant differences in mortality of *G. roeseli* (pW, $p > 0.7$) irrespective of microparticle species (PA or SP) and exposure time (24 h and 48 h, Figure 8.3).

The mortality of *G. roeseli* exposed to Phe with additional microparticles (Phe + PA and Phe + SP) did not significantly differ neither to the Phe exposure nor among each other (EF, $p > 0.2$). The combined exposure resulted in lowered mortalities <20 % after 24 h (EF, $p > 0.1$). Only after 48 h at the highest concentration a significantly increased mortality occurred (Phe + SP, 36 %, pW, $p < 0.05$). Though, models show clearly lower courses in mortality with microparticles added, especially for 48 h.

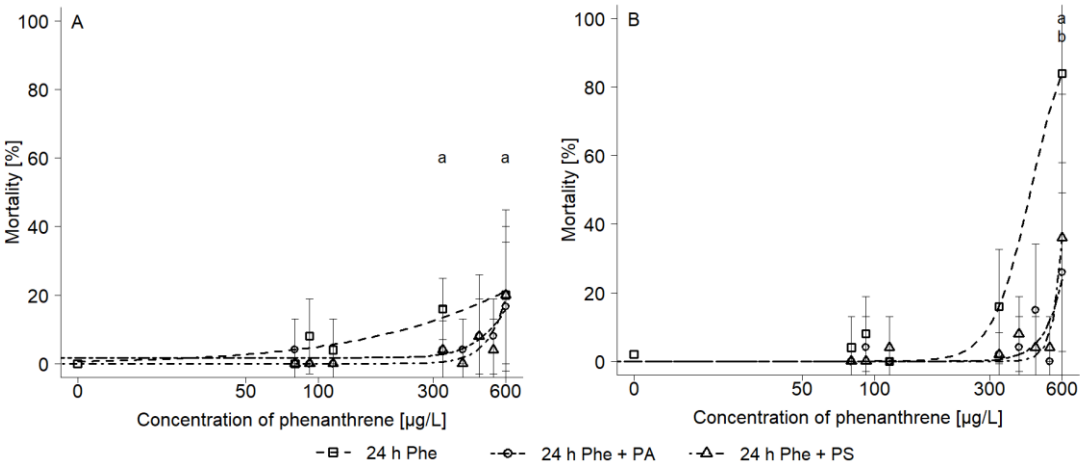

Figure 8.3: Mean mortality of *G. roeseli* after 24 (A) and 48 h (B) exposure to phenanthrene alone (Phe) or in combination with PA or SP of 40 - 63 µm. Mortality was counted for dead (including eaten) gammarids and calculated per beaker (n = 5). Concentration range of phenanthrene (measured concentration 80 - 600 µg/L) was pre-selected from the acute toxicity test for sublethal effect concentrations. Dose-response-curves for exposure scenarios are shown by lines. Labels a (Phe), b (Phe + PA) and c (Phe + SP) mark significant differences to solvent control. Statistical information for regression is given in the appendix (Table 13.3). Error bars represent standard deviation.

Feeding rate

Feeding rate was independent from microparticle exposure scenario, i.e. neither Phe nor the combination with SP or PA after 24 and 48 h influenced the gammarids' feeding (KW, χ^2 = 0.72 for 24 h/0.68 for 48 h, df = 2, $p > 0.5$). Control feeding rates varied from 0.88 to 1.40 mg/gammarid*day within 24 h and 0.65 - 1.15 mg/gammarid x day within 48 h. Also, feeding rates within the treatments varied randomly (Figure 8.4). In Phe exposures, there was no change in feeding rate with increasing phenanthrene concentration compared to the controls (AW, F(6,11.12) 24 h, F(6,12.13) 48 h, $p > 0.5$).

Variation of feeding rates occurred only for Gammarids exposed to Phe + SP after 24 h (AW, F(7, 13.15), $p < 0.001$) at concentrations 80, 92 with higher feeding rate and 329 µg/L with lower feeding rate (pW, $p < 0.05$) and after 48 h at the highest concentration resulting in lower feeding rate (pW, $p = 0.28$).

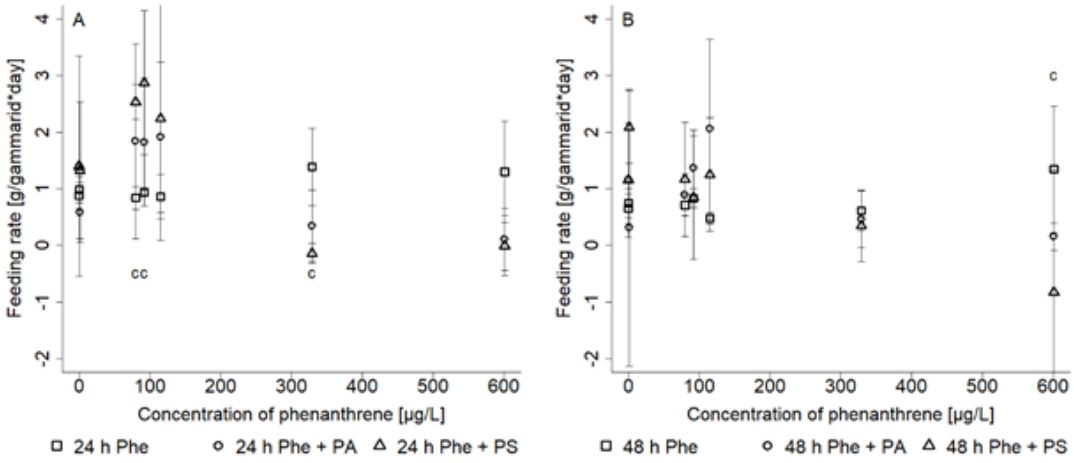

Figure 8.4: Mean feeding rates of *G. roeseli* after 24 (A) and 48 h (B) exposure to phenanthrene alone and in combination with PA or SP. DECOTAB final weight was measured and feeding rate was calculated for alive gammarids at the end of the test per beaker. Error bars represent standard deviation. Labels a (Phe), b (Phe + PA) and c (Phe + SP) mark significant differences to solvent control.

Swimming behavior

Swimming behavior did not change after exposure to microparticles compared to controls and was on average 1.5 ± 0.7 cm/s and 1.8 ± 0.7 cm/s after 24 and 48 h (pW, $p > 0.5$ (PA) and $p > 0.4$ (SP)). Effect of phenanthrene on velocity after 24 and 48 h was significantly different in the presence of microparticles (KW, χ^2 = 59.8 (24 h)/35.0 (48 h), df = 2, $p < 0.001$). Swimming velocity during exposure with Phe was lower than in exposures with Phe and microparticles (pW, $p < 0.001$) after 24 and 48 h. Phe with microparticle exposures (Phe + PA and Phe + SP) resulted in similar decreasing velocity (pW, p = 0.77 for 24 h, 0.93 for 48 h).

Swimming velocity of *G. roeseli* decreased significantly with increasing phenanthrene concentration for the Phe exposure (Fig. 2, AW, F(6, 40.7/16.1), $p < 0.001$). At lowest concentration (80 µg/L), Phe resulted in decreased velocity of 0.3 cm/s slower than the control after 24 h (pW, p = 0.018) and 0.6 cm/s slower after 48 h (pW, $p < 0.001$). Velocity reduction coincided with increasing concentration. At highest phenanthrene concentration, gammarids swam with a speed of 0.27 (24 h) and 0.35 cm/s (48 h), which equals a reduction by 0.84 and 1.16 cm/s from control to 601 µg/L. The same negative trend occurred in exposures with Phe + PA (AW, F(7, 40.7/33.0), $p < 0.001$) and Phe + SP (AW, F(7, 78.3/14.3), $p < 0.001$). Although velocity was reduced even at lowest concentrations for both exposure scenarios and timepoints, significant difference was detected from 115 m g/L for Phe + PA (pW, $p < 0.01$ for 24 and 48 h) and from 92 µg/L for Phe + SP (pW, $p < 0.01$ for 24 and 48 h). Gammarids exposed for

24 h to phenanthrene and microparticle swam 0.21 (Phe + PA) and 0.17 (Phe + SP) cm/s at 601 µg/L, which is slightly slower than in the Phe exposure, while exposure over 48 h resulted in a comparable or faster speed in highest concentration (0.33 (PA) and 0.48 (SP)). Velocity reduction followed a linear relationship (Figure 8.5).

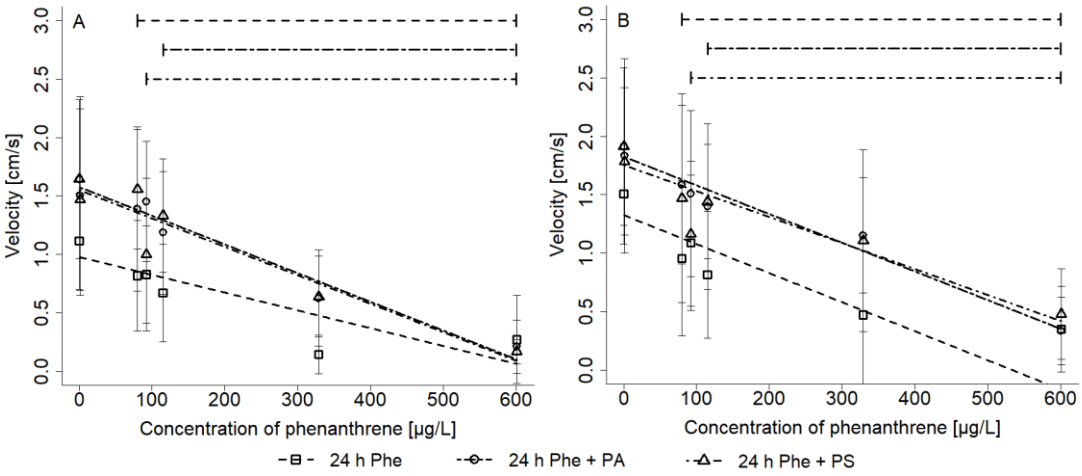

Figure 8.5: Mean Velocity of *G. roeseli* after 24 (A) and 48 h (B) exposure to Phe and in combination with PA or SP. Error bars represent standard deviation. Linear regression for exposure scenarios are shown by lines. Horizontal Lines for Phe (dashed), Phe + PA (twodash) and Phe + SP (dotdash) mark significant differences to control. Velocity of individual *G. roeseli* was measured with Ethovision XT 9. Dead gammarids were excluded. Statistical information for regression is given in the appendix (Table 13.4).

8.4. DISCUSSION

Against the common hypothesis, the measured influence of microparticles on the ecotoxicity of phenanthrene was reducing, ruling out their role as vectors. Furthermore, the potential of plastic particles acting as a more effective sorbent and carrier than natural microparticles has been relativized for polyamide and phenanthrene, because they did not modulate effects different to naturally occurring inorganic sediment particles based on effects on gammarids. Instead, the presence of microparticles revealed a reduction of mortality as well as changes in sublethal endpoints such as swimming behavior. This impact can be explained by adsorption of phenanthrene to the microparticles, resulting in a reduced bioavailability of phenanthrene.

Acute toxicity determination revealed delayed rapid mortality due the baseline toxicity of the hydrophobic chemical (Mayer and Reichenberg, 2006). Phe exposure resulted in mortality of adult *G. roeseli* following a sigmoidal dose-response curve over the course for 24, 48, 72 and 90 h. Acute toxicity thresholds (LC_{50}) of phenanthrene were 471.9 µg/L after 24 h and 441.1 µg/L after 48 h and further decreased over time. Thereby*, G. roeseli* was more sensitive to phenanthrene compared to other crustaceans like *Daphnia magna* (24 h LC_{50}: 678.4 µg/L - 853.7 µg/L (Verrhiest et al., 2001)) or copepods (*Oithona davisae*, 24 h LC_{50} at 440.2 µg/L - 604.2 µg/L (Barata Martí et al., 2005)). The sudden and strong increase in mortality after a steady low mortality can be explained by a critical concentration of the hydrophobic chemical that is reached in the lipid membranes (Mayer and Reichenberg, 2006; Mayer and Holmstrup, 2008). Phenanthrene might be tolerated by adult *G. roeseli* until a certain threshold is exceeded.

Additionally, phenanthrene is hydrophobic with a log K_{OW} of 4.16 - 4.67 and most likely adsorbed to surrounding compartments such as food, particles or the organism (Teuten et al., 2007). As a consequence, there was after some time less phenanthrene available to the test organisms than at the beginning of the experiment, but at higher concentrations effective doses of phenanthrene remained. Also, Lee et al. (2014) concluded a reduction of dissolved chemicals concentration and bioavailability due to sorption kinetics for hydrophobic chemicals and microplastics, but with the condition of a sufficient microplastic concentration. According to Bakir et al. (2014a) the plastic

particle - water equilibrium of phenanthrene and polyethylene or polyvinylchloride can be reached within 24 h at a temperature of 18 °C. Therefore, this sorption is likely to happen in a relevant timescale. This is further supported by the measured phenanthrene concentrations in the sublethal toxicity experiment which were reduced remarkably after 48 h. It is thus likely that organisms not affected in the first hours of the exposure might not suffer from the bound phenanthrene in low concentrations but from remaining phenanthrene in higher concentrations.

The measurement of sublethal endpoints such as swimming behavior during the exposure of *G. roeseli* with sublethal phenanthrene concentrations proved to be highly sensitive, even at the lowest concentrations. The most important observation was the reducing toxic effect of both used microparticle types to phenanthrene, which follows a strong interaction of the contaminant with the present natural and anthropogenic microparticles. This effect is most likely due to a reduced bioavailability of adsorbed phenanthrene. Concerning mortality and swimming behavior, another observation was that the degree of reduction was independent of added particle type. As supported by chemical analysis of the artificial water with and without particles, these outcomes can be explained by a fast and, most important, similar adsorption to both particle types.

Also, with regard to the sublethal toxicity assay and as initially intended, mortality was very low in all exposure scenarios with phenanthrene (including cannibalism). Due to the low mortality, no change in mortality due to microparticles was observed. This is comparable to results of exposure with phenanthrene in combination with sediment on *Diporeia* spp., in which a low mortality around 12 ± 3 % at a phenanthrene concentration of 110.5 µg/g sediment was found (Landrum et al., 1994). Moreover, when exposed to sediment-associated phenanthrene (max. conc. 333 mg/kg) for ten days (Gust, 2006), *Hyalella azteca* also showed low mortality (5 ± 8 %). Therefore, focus in this assay was on the sublethal endpoints swimming behavior and feeding. The responses by sublethal endpoints are more sensitive compared to mortality and made an earlier detection of adverse effects possible.

The exposure to polyamide and sediment particles in the used particle concentration without phenanthrene did not induce any adverse responses on the tested endpoints, as expected. Both, polyamide and sediment particles are very likely non-toxic, but it is

possible that the potential toxicity of the plastic particles is not severe enough to be detected using these endpoints on the organism level. The molecular level could be a more sensitive level than organism level (Blarer and Burkhardt-Holm, 2016; Karami et al., 2016). Plastic particle toxicity has been reported in other species exposed to a comparable size-class and concentration as used in this study. Karami et al. (2016) for instance reported damage on inner organs in *Clarias gariepinus* after exposure to < 60 µm sized low-density polyethylene particles in concentrations of 50 and 500 µg/L. Also, Blarer and Burkhardt-Holm (2016) did not observe any impact on feeding rate and wet weight change, but in assimilation efficiency for *Gammarus fossarum* after application of polyamide fibers (2680 fibers/beaker) and polystyrene beads (12,500 beads/mL). This is in line with other studies not finding effects of plastic microparticles even at very high concentrations and relevant particle sizes according to endpoints from molecular to organism level (reviewed by Burns and Boxall, 2018). Consequently, it can be anticipated that there was no measurable effect from 40 - 63 µm sized polyamide microparticles as from sediments for *G. roeseli* at the tested concentration.

Effect size of phenanthrene is lowered in the presence of particles due to adsorption to the particles. In this context, a strong influence of microparticles on phenanthrene-exposed gammarids' swimming velocity has been shown for 24 and 48 h. When exposed to phenanthrene and microparticles, gammarids swam in mean 0.5 cm/s faster than when exposed to Phe. Apart from this, feeding was not influenced by microparticles. Reduction of swimming velocity does not correspond with results reported by Ma et al. (2016) with *Daphnia magna* tested for 10 days with 0.05 - 1.2 mg/L phenanthrene. They found no impact at a concentration of 500 mg particles/L (10 µm) on the phenanthrene-induced effect. Though, phenanthrene-loaded low-density polyethylene particles < 60 µm resulted in higher molecular stress response than only phenanthrene exposure on catfish (Karami et al., 2016). The highly sensitive changes on the molecular level can thereby be seen as early warning signals, but cannot be interpreted regarding their adverse effects without a linkage to phenotypic consequences (Beggel et al., 2011; Beggel et al., 2012). Hence, rather than an undetected counteracting positive effect induced by the particles, because none was found in the particle control, this result indicates a decreasing bioavailability of

phenanthrene due to adsorption to the microparticles. Consequentially, as the chemical potential of phenanthrene is decreased when adsorbed to microparticles, the measured lowered effect is most likely caused by the remaining dissolved chemical or from the chemical in the food (Koelmans et al., 2016; Kwon et al., 2017).

Several studies dealt with the distribution of PAHs and found concentrations ranging from mg adsorbed to sediment and µg to ng dissolved in water (Kafilzadeh, 2015; Luo et al., 2008; Rabodonirina et al., 2015). Thus, the tendency of PAHs to adsorb to plastic has already been proven (Alimi et al., 2017; Bakir et al., 2014a; Wang and Wang, 2018) and sorption equilibrium of phenanthrene to polyethylene was within 24 h (Bakir et al., 2014a). This is also confirmed by the analytically determined concentrations of the phenanthrene test solutions in this study. The artificial medium clearly exhibited a fast decline of phenanthrene concentration within 48 h to 20 % of initial concentration with phenanthrene only and a mere fraction with microparticles rapidly after test start. More studies with varying particle species and sizes need to be conducted to verify if the particle type is an important factor for sorption and resulting effect modulation. Nevertheless, as stated by Bakir et al. (2014a) and shown by Rochman et al. (2017) or Lee et al. (2014), it depends on both polymer and pollutant properties. Therefore, the chemical potential and toxic effect of a contaminant is modulated by the presence of microparticles in the aquatic environment and this possibly depends on the respective particles surface quality, internal diffusion and chemical properties. An open question is whether a toxic phenanthrene concentration is also reached in the organism if the substance is transported by ingested phenanthrene-loaded particles to the organism. The study by Bakir et al. (2016) indicates to some degree that the ingestion of loaded plastic is a negligible additional pathway compared to chemical uptake from the surrounding compartment or food. Ingestion and egestion of particles via food in µm size by *G. roeseli* is very fast and nearly in equilibrium with 6 and 8 ± 5 particles per minute, while uptake from surrounding medium was lower (unpublished data). Tracking of fluorescent particles showed egestion rates within 24 h comparable to Straub et al. (2017). Whilst sorption equilibrium of phenanthrene to polyvinyl chloride and polyethylene is reached within 24 h, desorption is slower with 5 days to reach equilibrium in seawater again (Bakir et al., 2014b). Although desorption of phenanthrene is faster under simulated gut conditions (Bakir et al., 2014a), it is

likely that egestion of phenanthrene-associated particles is too fast for phenanthrene to desorb in an appreciable amount or even reach equilibrium to cause significant effects (Mohamed Nor and Koelmans, 2019). Even the assumed faster desorption from microplastic (Wang and Wang, 2018) does not intervene. Additionally, uptake of particles via the water is very low compared to ingestion via food, which lowers the possibility of phenanthrene uptake with particles again. Even if the particles are ingested, the release of the hydrophobic contaminant is very unlikely, intensified by the plastics characteristics like size and intraparticle diffusion (Seidensticker et al., 2019). Due to minimal ingestion and slow desorption kinetics, it can be concluded that suspended particles are negligible carriers for sorbed chemicals compared to the surrounding compartment.

The used amount of solved phenanthrene and suspended polyamide particles in medium was higher and that of sediment particles lower than the known concentrations in freshwater. Consequently, especially environmental concentrations of phenanthrene, around 3 - 45 ng/L (Kafilzadeh, 2015; Luo et al., 2008), are most likely not leading to acute mortality or change in behavior of *Gammarus* spp. Partly, particle concentrations could represent a scenario where sediment and sand become a rare source and plastic particle concentration in environment rises as discussed by Koelmans et al. (2013) or Enders et al. (2015).

In view of environmental relevance, this study focused more on effect and modulation detection than environmental concentrations. Due to the aim of the study to see modulated effects, the higher concentrations were needed. Thus, with respect to their environmental occurrence, it seems that the impact of microplastic compared to natural microparticles is approximately equivalent. In cases like the observed, where the chemical adsorbs to microplastic and sediments to a similar extend, the higher bioavailability due to higher abundance of sediments makes sediment much more hazardous. There is an uneven higher probability for sediment to be ingested and release the chemical than for microplastic. Further, the adsorption and desorption of chemicals highly depends on the environmental parameters like abundance of organic matter (Seidensticker at al. 2017), pH or salinity (Wang et al., 2018). Also, after ingestion, the potential of the plastic particle to act as a vector and release the chemical highly depends on particle and chemical characteristics (Seidensticker et al.,

2019). This indicates possible different behavior of the chemical in the environment than in static laboratory experiments and it has to be mentioned that the effects and modulation by microparticles could differ with the varying combinations. However, many recent model-based studies evaluated that the role of microplastic as a vector for anthropogenic pollutants not added to plastics is a common misconception (Mohamed Nor and Koelmans 2019; Koelmans et al., 2019). Nevertheless, it is necessary to examine a broad empirical area for chemical and (plastic) particle toxicity, as it is rising in the last years, to generate a stronger basis for risk assessment and regulation.

9. General discussion

This thesis focused on the evaluation and establishment of a long-term bioassay for freshwater organisms linked with the investigation of two different particle exposure scenarios regarding exposure pathways and induced effects on the organism level.

In this context, it first highlighted the need for guidelines for more diverse species and habitats for risk assessments by simultaneously providing a comprehensive evaluation of a standardized bioassay with *Gammarus* spp., especially for the river-dwelling *Gammarus roeseli,* in chapter 6. The practicability was shown in laboratory experiments conducted in chapter 7 and 8. As a result, the on the basis of preceding studies developed bioassay can now be conducted in a test battery to extend the risk assessment analyses with every possible impact on *Gammarus* spp. For a detailed discussion see chapter 9.1.

Secondly, the performance of the standard test guidelines in aquatic risk assessment was examined regarding particle exposure. Hence, the for aquatic experiments most conventional exposure via the water phase in chapter 8 provides other insights than the less applied alternative dietary exposure of particles in chapter 7. Both are effective approaches to examine the particles regarding dose-response-analysis like in chapter 7 or the vector function in chapter 8. The investigations would be challenging when the respective other scenario might be chosen. For risk assessment, booth scenarios are important. This topic is discussed in chapter 9.2.

Third, the plastic particle toxicity to *G. roeseli* was examined. The possible effects induced by plastic particles resulting from size, concentration, shape and type were examined. As well as the functions as vector/carrier, for chemicals was investigated and refuted at least for polyamide and PAHs. The investigated plastic and natural nanoparticles (chapter 7), equally to the used fragmented plastic and natural microparticles (chapter 8), did not induce any changes in the examined behavior endpoints. This topic will be discussed in chapter 9.3.

9.1. Establishment and evaluation of a bioassay for *Gammarus* spp.

The examination of adverse effects induced by human products has become obligate since REACH. To fulfill the requirements of REACH, the OECD-guidelines (OECD) are commonly used for the experiments. These guidelines focus on a few freshwater species including lake-dwelling fish, daphnia, and algae, despite some others like the lugworm for example. Although the trophic levels are well covered, the compartments, marine, lotic and freshwater, are lacking regarding river-dwelling organisms like *Gammarus* spp. Species of lotic compartments should be considered as well as marine and freshwater species and *Gammarus* spp. seems to fill this gap perfectly (Kunz et al. 2010). With respect to that, this thesis offers an advanced draft for standardized toxicity testing with *Gammarus roeseli*, expandable for each *Gammarus* species and other organisms.

The properties of the species *Gammarus* are well documented and therefore a great basic knowledge for bioassay conduction is provided (Geffard et al. 2010; Haley 1997; Hartnoll and Smith 1978). Many aspects of ecotoxicity testing were already tested regarding possible endpoints for gammarids and toxicity of chemicals to gammarids (Kunz et al. 2010). This, and some pioneering tries from e.g., McCahon and Pascoe (1988b), Kunz et al. (2010), Gerhardt (2011) and Feiner et al. (2016), made it possible to start with a predefined laboratory bioassay supported by the validity criteria by US EPA (2016).

Some fixed prerequisites were adopted from those preliminary studies and were applied in all tests conducted in chapter 6, 7 and 8: (1) acclimatization to the test conditions for one week. (2) The conditions of 16:8 h light:dark cycle and water temperature. (3) The exposition of a few individuals together in one test vessel as they are social organisms and need others around them (Gerhardt 2011; US EPA 2016). (4) Aeration of the medium before adding it to the test vessel (US EPA 2016).

Other conditions were added: (1) the modified food matrix by (Kampfraath et al. 2012) instead of conditioned leaves (Gerhardt 2011; US EPA 2016) to provide a standardized food source without conditioning. (2) Glass stones for hiding to reduce stress and cannibalism. (3) The sensitivity to food competition has been noticed (Chapter 8) and was reduced by adding only three organisms per beaker instead of five (Chapter 6 and 7). (4) Feeding during acclimatization became necessary (Chapter 6, 7 and 8) as a diet

(US EPA 2016) falsifies the feeding at the start of the test (Chapter 6).

Some conditions were not implemented: (1) a circulatory system (Feiner et al. 2016) was not used in the bioassay to simplify the already complex exposition with the plastic micro and nanoparticles via the water phase (e.g. Chapter 8). (2) Instead of steady aeration (Gerhardt 2011), weekly measurement of oxygen content and water exchange was conducted to reduce stress for the gammarids and interference with the particle as the distribution may be influenced by the bubbles (Chapter 6, 7 and 8). (3) Stream water (Gerhardt 2011) was not applied although many tests were successful with the water the organisms originally were caught as there can be unpredicted pollutions or fluctuations in its composition, artificial water increases reproducibility (chapter 7 and 8).

All the adaptations or modifications built a great and easy-to-apply procedure for ecotoxicological tests with chemicals. The in chapter 6 developed and evaluated bioassay with *G. roeseli* is applicable for the common chemical toxicity testing (chapter 8) as well as the newly aimed particle toxicity testing (chapter 7 and 8). Most important is the standardized dietary exposure tool modified to the needs of the gammarids in chapter 6. The phyll-tab does not only allow a standardized and effective diet (chapter 8) but also is a great tool to calculable expose the shredder to the embedded particles (chapter 7). By applying this bioassay with the used or other endpoints, it is now possible to examine a great variety of particle impacts on *G. roeseli* as a river-dwelling organism.

9.1.1. Possible extensions

The developed bioassay is easy to conduct with low effort. The establishment in a new laboratory does not require much time, as most or all components are usually available in the laboratories. The effects seen from the endpoints can be significant and the organism is sensitive. The testing of either chemicals or particles is feasible. Over all, there is no further update necessary, but possible. These extensions should not be missed and can be added to the bioassay with a little more effort.

One interesting point for the extension of the bioassay is the culture of *Gammarus* spp. in the laboratory. If possible, it seems to be a desirable procedure as it allows access to the most sensitive life stage, juveniles (Gerhardt 2011; McCahon and Pascoe

1988a). It is challenging to sort juveniles caught in the environment to the correct life stage as they are very small. Therefore, juveniles hatched in culture, can be sorted directly to the right stage by transferring them from the culture aquarium to a new one each day (Gerhardt 2011; McCahon and Pascoe 1988b). Furthermore, culturing gammarids by considering the appropriate light:dark cycle enables the control of the maturity status of the females (Sutcliffe 1992) and brings them to maturity twice a year or holds them in the diapause, for example. The sensitivity of females is presumable higher right after fertilization as the development of eggs is very cost-effective. If possible, this should be considered in further experiments and adjusted in light:dark cycle (McCahon and Pascoe 1988b).

Another aspect is the testing procedure with non-static flow-through set ups as developed by Feiner et al. (2016). The in this thesis developed bioassay for *Gammarus* spp. is meant to add a river-dwelling organism to the standard toxicity testing as rivers are different in many properties to seas and lakes (Canning and Death 2019; Drakou et al. 2008). Therefore, it might be recommendable to fit this procedure to the habitat-specific conditions including, and maybe most importantly, the flowing compartment.

As Kunz et al. (2010) recommended, the in this thesis established bioassays with gammarids combine visual, non-invasive endpoints like swimming and feeding with the sophisticated endpoint of energy reserve measurement. It is even possible to extend the examination with more non-invasive endpoints like species interaction or ventilatory activity with some more helping hands. Kunz et al. (2010) summarize many possible, already investigated endpoints for tests with gammarids ranging from feeding to invasive mode-of-action examinations and biomarkers. It has to be noted, that the addition of further invasive endpoints next to the in this thesis investigated energy reserves seems to require the addition of many more organisms to separate them into groups for the measurements. The energy reserve measurement completely consumes the body sample and it seems not simple to take more samples from it. Nevertheless, the head and gut were dissected in chapter 7 before the energy reserves measurement and the energy reserves did not remarkably differ from the measurement with head and gut in chapter 6. The gut was dissected for gut microbiome analysis and the head was dissected for molecular biomarker analysis. To date, the analyses were not performed and published yet. Thus, the presented

bioassay shows a possible way to add more invasive molecular endpoints to the procedure but this needs to be further evaluated. Nevertheless, the here developed bioassay provides great reproducible and sensitive endpoints and does not necessarily need to be adapted regarding these possible extensions.

9.2. Risk assessment of particles related to exposure scenarios

The difference in the investigation of insoluble or sparingly soluble substances like particles compared to soluble substances is a challenge in the aquatic risk assessment (Rufli et al. 1998). Despite the nearly uncontrollable dispersion in aqueous media, the uptake of and subsequent exposure with particles to an organism is quite different from soluble substances. Therefore, the need to adapt the common testing procedures became obvious (de Ruijter et al. 2020). The dietary exposure (Chapter 7) is a great alternative when the effect assessment targets the dose-response-analysis. While the classical exposure via the water phase like in chapter 8 represents the more common pathway of plastic to enter the water and can be used for environmental-impact-analysis when the quality criteria for particle testing are fulfilled (de Ruijter et al. 2020). Both scenarios have advantages over each other and it is useful to carefully choose the scenario based on the scientific issue.

Organisms are most likely exposed to ingested particles and may not even notice most of the particles in the water passing them. Even ingestion is very variable per measuring interval, and depends as much on the investigated organism (Cole and Galloway 2015). Still, reviews find that mostly the nominal particle concentration in the aqueous media is given in the descriptions and exposure assessment is neglected (de Ruijter et al. 2020). The aqueous particle exposure scenario like in chapter 8 leads to an unknown and very variable particle exposure concentration during the experiment. In this scenario, particle exposure remains unknown unless particle concentration in the test vessel is determined considering all partitioning and distribution possibilities or organisms are removed from the experiment to measure ingested particles. The latter requires either (I) high amounts of organisms when they are constantly removed for dissection during the experiment or (II) only reflects one moment of exposure when organisms are only dissected at the end of the experiment. Both detection methods are complicated compared to the dietary particle exposure in chapter 7. Due to the fixation of the particles in a food matrix, dissection of the organisms is not necessary as the number of ingested particles is related to the amount of food eaten (chapter 6). The feeding rate can be measured at any time of the experiment and set in relation to the ingested particles, which is a great advantage. Therefore, dietary exposure is advisable for dose-response-analyses as the dose the

organisms are exposed to can be calculated even during the experiment without removing organisms from the test. The used tabs are a matrix that can serve as food for many organisms (Kampfraath et al. 2012), is easy to produce and modified with supplements to the nutritional needs (Kampfraath et al. 2012, chapter 6), and makes feeding rates comparable between tests with the same organisms (chapter 6 and 7). Depending on the frequency of feeding rate determination, a dose can be given for example each week and plotted with the at the same time observed responses (chapter 7). The exposure can be nearly constantly monitored as required for good test quality (de Ruijter et al. 2020). The dietary particle exposure scenario might be a more satisfactory solution than the exposure via water phase, especially for two scientific issues: At which concentration in the organism are adverse effects caused by particles (?) like in chapter 7., or does bioaccumulation occur (?) like investigated for *Hyalella azteca* by Kuehr et al. (2021).

This advantage of dietary particle exposure over exposure via the water phase results from the stable and homogenous distribution of the particles in the food matrix (chapter 6). Which is for the named scientific issues an advantage is a disadvantage for other questions regarding particle toxicity. The fixation does not allow to examine any related possible impact due to particle distribution in the aqueous compartment in the environment. With dietary exposure, the feeding organisms are inevitably exposed to the particles regardless of whether they would be exposed to in the environment. Hence, the question of whether an organism is exposed or not can only be answered with a field experiment or a great conducted laboratory experiment with a huge effort to meet the prerequisites (de Ruijter et al. 2020) and represent best the environmental conditions. Another example of necessary aqueous exposure is given in chapter 8, where the function of plastic and natural particles as vector or passive sampler was investigated. The fixation of particles and chemicals in the food matrix would have been counterproductive for this examination.

Thus, the scientific issues that can be answered with the aqueous exposure and not with a dietary exposure are for example: Are the aquatic organisms exposed to particles in the water and how is particle fate during exposure? (Booth et al. 2016; von Moos et al. 2012a). Or does the presence of particles in the water modulate the toxicity of soluble substances (?) (vector/carrier/passive sampler function, chapter 8).

This thesis exemplarily presents two effective approaches to examine particle influence on aquatic organisms when the aimed question is carefully considered. For risk assessment, booth scenarios are important. For the assessment of the impact of particles alone, it must be considered whether the organisms are exposed to the particles in the water and if so, the effect according to the number of particles the organisms are likely to be exposed to in the environment can be examined. In chapter 6 and 7, tests to answer the latter for *Gammarus* spp. are conducted. Chapter 8 is an example of advanced studies for a deeper examination of particle effects in combination with other stressors like chemicals, which is a pursuing risk assessment.

9.3. Effects of plastic particles

In this thesis, the first two objectives were to establish a standardized bioassay for the river-dwelling *Gammarus roeseli* and to evaluate exposure scenarios for particles. From chapter 6 to 8, the conducted bioassays with *G. roeseli* and the particles did not only help to evaluate the procedures but also produced results regarding the possible effects. Hence, the third objective of the thesis, the question whether the particles impact *G. roeseli* or not was answered for natural sediment and silica micro and nanoparticles as well as for polyamide, polylactide, and polystyrene micro and nanoparticles. Regardless of the rather small bioassay in chapter 6 or the more extensive dietary exposure in chapter 7 or the conventional exposure via water in chapter 8: The particles, plastic or natural, did not induce any effect regarding the investigated endpoints to *G. roeseli*. Likewise, the carrier function of plastic particles was not only neglected compared to the overwhelming mass of natural particles but also refuted as instead the passive sampler function has been proven for polyamide with the PAH phenanthrene in chapter 8. The conclusion was, that the population of *G. roeseli* will not be affected by plastic particles in the current environmental concentrations.

These findings are in accordance with the literature. Studies with environmentally relevant plastic particle concentrations mostly confirm the absence of adverse effects of plastic (Cole and Galloway 2015; Green et al. 2019; Weber et al. 2018). This is extended in studies comparing natural particles with plastic particles in environmentally relevant concentrations (van Cauwenberghe et al. 2015; Weber et al. 2021a; Weber et al. 2021b). The conclusion was likewise that plastic particles are negligible nowadays compared to the concentration of natural particles in the environment (Triebskorn et al. 2019; Weber et al. 2021a). Only a few studies found effects induced by environmentally relevant concentrations of plastic particles (see review by Triebskorn et al. (2019)).

Apart from the overwhelming amount of studies with high plastic particle concentrations revealing effects on a wide range of aquatic species (Bhattacharya et al. 2010; Blarer and Burkhardt-Holm 2016; Booth et al. 2016; González-Pleiter et al. 2019; Green et al. 2019; Imhof and Laforsch 2016), plastic micro and nanoparticles do not seem to be of great hazard for the aquatic environment today (see risk

assessments by Adam et al. 2019; Burns and Boxall 2018). This thesis does contribute to this conclusion with two chapters (7 and 8) providing two different optimized scenarios for particle toxicity examination with various plastic and natural particles of different shapes, sizes, and concentrations.

9.4. Conclusion and Outlook

This thesis is a contribution to the ecotoxicological assessment of plastic pollution in the environment in three different ways.

First, a standardized and easy-to-conduct bioassay with *Gammarus* spp. is provided. This increases the reproducibility and comparability when the bioassay is applied as standard bioassay in the laboratories. Likewise, the test species diversity regarding the habitat is increased – in this case for river-dwelling organisms. By applying this bioassay, the knowledge of the toxicity of chemicals as well as particles can easily be extended and used for risk assessment in rivers. Therefore, the next step is to conduct a test battery with this bioassay in various laboratories to fill knowledge gaps.

Second, a new alternative exposure tool for particles (DECOTAB by Kampfraath et al. (2012)) has been intensively evaluated to overcome the challenges in aquatic particle exposure, including dispersion and distribution in the medium. Embedding the particles in the stable matrix allows a calculation of inner particle concentration which is closer to real exposure concentration than the nominal particle concentration in the beaker. In addition, this procedure does not require any organism samples to examine exposure concentration which may lower the number of used organisms as requested by REACH. In future investigations, this exposure pathway should be considered for dose-response-analyses with plotting the ingested particle concentration.

Third, the comprehensive exposure studies with plastic particles in current environmental concentrations with *Gammarus roeseli* revealed that neither shape (fragmented or spherical), plastic type (biodegradable or not), size (30 nm to 63 µm), nor concentration (lower ng to higher µg) might affect the population of *G. roeseli* in behavior and survival. Even the often-claimed assumption that plastics can act as carriers for chemicals in the aquatic environment has been refuted for plastic in combination with a hydrophobic chemical. Considering that studies without measured effects are often not published, these studies are a worthy contribution to the actual mass of effect studies as they add new insights into plastic toxicity.

Nevertheless, there are also many microplastic toxicity studies that reveal toxic effects from plastic in various ways, depending on one or more factors originating from the high variability of plastic properties. Underlined by the manifold proofs of starved and strangulated animals from macroplastic. Even if the risk assessments for recent

concentrations come to the conclusion that micro and nanoplastic are not hazardous for the environment today, there might be a tipping point the in future when the “high” concentrations are reached. Action in plastic handling is needed in any case.

10. References

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Publications

Bartonitz A, Anyanwu IN, Geist J, Imhof HK, Reichel J, Graßmann J, Drewes JE and Beggel S. 2020. Modulation of PAH toxicity on the freshwater organism *G. roeseli* by microparticles. *Environmental Pollution*, 260:113999. DOI: 10.1016/j.envpol.2020.113999.

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Oral presentations

Bartonitz A, Anyanwu IN, Geist J, Imhof HK, Reichel J, Graßmann J, Drewes JE and Beggel S. 2019. Modulation of PAH toxicty on freshwater organisms by microparticles 24. Jahrestagung des SETAC GLB, 4.9.-6.9.2019, Koblenz-Landau

Poster presentations

Bartonitz A, Anyanwu IN, Geist J, Imhof HK, Reichel J, Graßmann J, Drewes JE and Beggel S. 2019. Modulation of PAH toxicty on freshwater organisms by microparticles. 24. Jahrestagung des SETAC GLB, 4.9.-6.9.2019, Koblenz-Landau

Bartonitz A, Anyanwu IN, Geist J, Imhof HK, Reichel J, Graßmann J, Drewes JE and Beggel S. 2019. Modulation of PAH toxicty on freshwater organisms by microparticles MiWa-Abschlusskonferenz, 10.9.2019, Karlsruhe

Bartonitz A, Geist J, Imhof HK, Beggel S. 2020. Validation of a standardized particle toxicity testing via food
SETAC Europe 30th Annual Meeting, 3.5.-7.5.2020, virtual conference

Further publications not included in this thesis

Imhof HK, Al-Azzawi M, Bartonitz A, Beggel S, Drewes JE, Elsner M, vonder Esch E, Glas K, Ivleva NP, Kefer S, Knoop O, Langowski H-C, Miesbauer O, Reichel J, Schwaferts C, Weisser J, Geist JP. 2020. Der Eintrag von Mikroplastik in Lebensmittel und die Aquatische Umwelt und seine Folgen – aktuelle Fragen und Antworten. *Mitteilungen der Fachgruppe Umweltchemie und Ökotoxikologie (GDCh)*, 26:44-49

Reiber L, Knillmann S, Kaske O, Atencio LC, Bittner L, Albrecht JE, Götz A, Fahl AK, Beckers LM, Krauss M, Henkelmann B, Schramm KW, Inostroza PA, Schinkel L, Brauns M, Weitere M, Brack W, Liess M. 2021. Long-term effects of a catastrophic insecticide spill on stream invertebrates. Science of The Total Environment, 768:144456

11. Author contribution

Chapter 6 was a collective work, following authors contributed to it:

A. Götz: conceptualization, data curation, formal analysis, investigation, methodology, visualization, writing–original draft, review and editing; J. Geist: conceptualization, supervision, resources, writing–review and editing; H.K. Imhof and S. Beggel: conceptualization, formal analysis, methodology, supervision, writing–review and editing.

Chapter 7 was a collective work, following authors contributed to it:

A. Götz: conceptualization, data curation, formal analysis, investigation, methodology, visualization, writing - original draft, review and editing; S. Beggel: conceptualization, supervision, formal analysis, methodology, writing - review and editing; J. Geist: conceptualization, supervision, resources, writing - review and editing.

Chapter 8 was a collective work, following authors contributed to it:

Astrid Bartonitz: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Visualization, Writing - original draft, Writing - review & editing. Ihuoma N. Anyanwu: Conceptualization, Investigation, Methodology, Writing - review & editing. Juergen Geist: Conceptualization, Supervision, Resources, Writing - review & editing. Hannes K. Imhof: Conceptualization, Formal analysis, Supervision, Writing - review & editing. Julia Reichel: Methodology, Investigation, Writing - review & editing. Johanna Graßmann: Methodology, Supervision, Writing – review & editing. Joerg E. Drewes: Resources, Supervision, Writing - review & editing. Sebastian Beggel: Conceptualization, Formal analysis, Methodology, Supervision, Writing - review & editing.

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13. Appendix

13.1. Supplementary Information for chapter 5

13.1.1. Energy Reserves Measurement

Stability of the reagents

The measurement of the storage contents is colorimetric based, which implements a color change of the used reagent. The reagents are stable over some weeks (van Handel, 1985a, b), but it was of interest to find the accurate time when the reagents elapse and begin to falsify the results. Therefore, both reagents were tested on day one and seven after production. Both reagents were stored at 6 °C with exclusion of light as instructed by van Handel (1985a, b). Calibration curves were conducted with oil for lipid measurement and 1 mg/mL glucose solution for glucose/glycogen measurement.

For the anthron-reagent for glucose and glycogen measurement, results revealed a fast decline within one week (Figure 13.1). The calibration curve after storage of one week was flatter than the original, which lowers the resolution between different concentrations. Further, the optical density was increased for each concentration, from 0.1 to 1.3 in the lowest concentration and from 1.0 to 1.5 in the highest concentration. This implies increased measuring uncertainty because the slope is lowered which leads to smaller differences in the OD between high and low concentrations. Therefore, the identification of the amount of glucose/glycogen is more inexact. The consequence was to use up the reagent within three days and then always set up a new one for further samples.

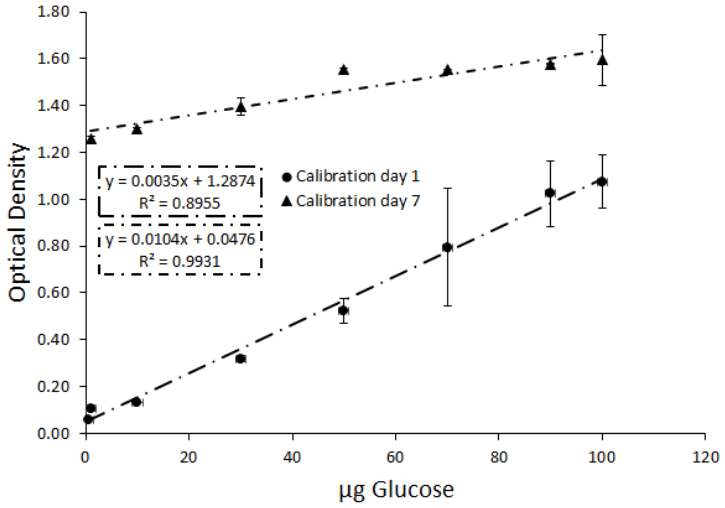


Figure 13.1: Changes in optical density for the calibration curve within one week of the anthron-reagent for glucose and glycogen measurement. The reagent was stored a 6 °C without light and measured after one and seven days. Samples were measured in triplicates and error bars represent standard deviation within the triplicates. Linear models were calculated with excel.

For the vanillin-phosphoric acid for lipid measurement, a shift of the intercept in the calibration curve of around -0.09 was detected within one week. Nevertheless, slope and measuring certainty were the same (Figure 13.2). The consequence was to either use up the reagent within three days like for the anthron-reagent or conduct another calibration curve after one week. As the anthron-reagent has to be used up within three days and then produced again, the decision was to handle the vanillin-phosphoric acid the same.

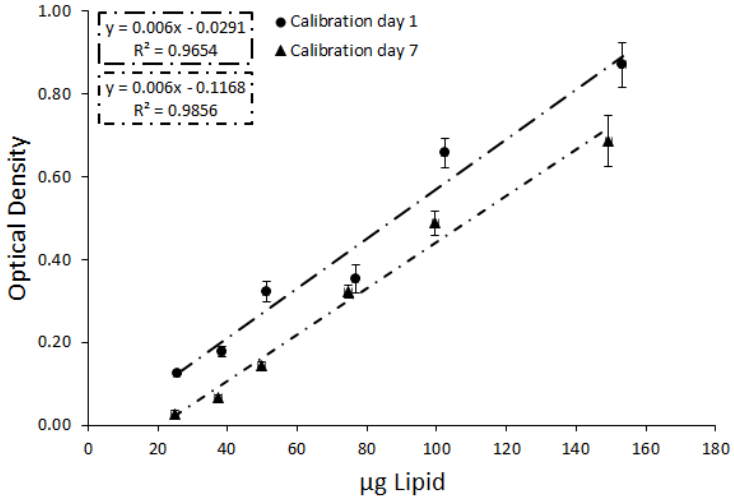


Figure 13.2: Changes in optical density for the calibration curve within one week of the vanillin-phosphoric acid for lipid measurement. The reagent was stored a 6 °C without light and measured after one and seven days. Samples were measured in triplicates and error bars represent standard deviation within the triplicates. Linear models were calculated with excel.

Reproducibility of the reagents

As the reagents must be used up fast, the next step was to test whether one calibration curve can be used for all new anthron-reagents and for the vanillin-phosphoric acid. If one calibration curve can be used, it is possible to save reagent and time. Therefore, for every new reagent a calibration was conducted and compared in slope and intercept to the other calibration curves. Reagent and calibration curves were made by three researchers at different dates to include systemic errors.

For both reagents, the calibration curves varied in slope and intercept. The slope of four calibration curves made with anthron-reagent for glucose/glycogen measurement was between 0.006 to 0.010 whereas the intercept was in a range of 0.0246 and 0.1085 (Figure 13.3). Same is true for the calibration curves made with vanillin-phosphoric acid for lipid measurement. The slope of three calibration curves varied from 0.004 and 0.007 and the intercept was between 0.0291 and 0.1071 (Figure 13.4).

Therefore, a calibration curve was necessary for every new reagent to ensure precise measurement.

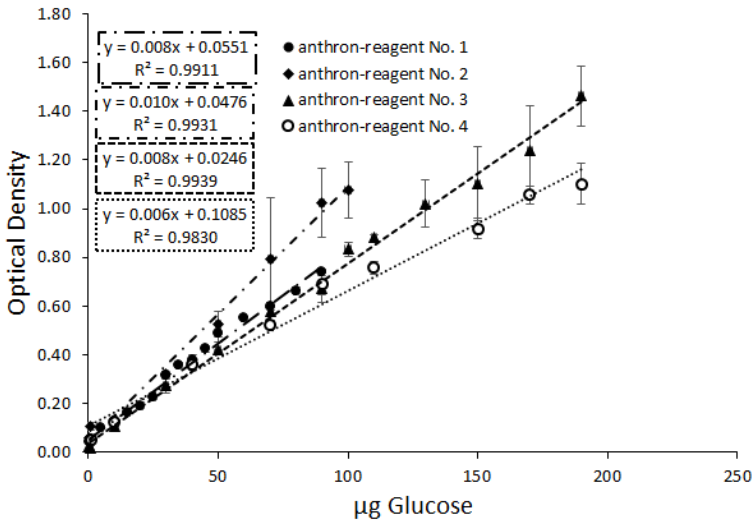


Figure 13.3: Changes in optical density for the calibration curve of four anthron-reagents. The reagents were made by three researchers at different days. Measurement occurred one day after production. Storage was at 6 °C with exclusion of light.

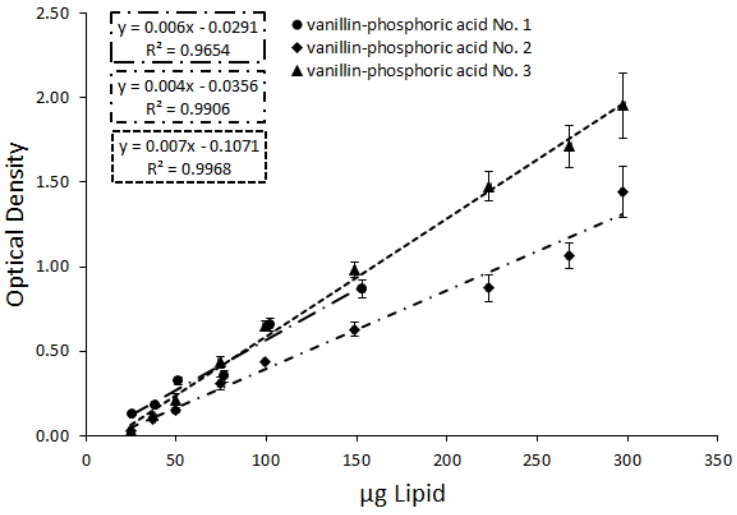


Figure 13.4: Changes in optical density for the calibration curve of three vanillin-phosphoric acids. The reagents were made by three researchers at different days. Measurement occurred one day after production. Storage was at 6 °C with exclusion of light.

Comparison of two wavelengths

The measuring certainty decreases with increasing optical density. Sometimes samples are above the range and it is recommended to measure at another wavelength of 490 nm for high-concentrated lipid-solutions (original is 525 nm) and 555 nm for high-concentrated glucose- and glycogen-solutions (original is 625 nm) (van Handel 1985a, 1985b). This is meant to result in 50 % lower optical density for high-concentrated samples and therefore in the optimal range for measurement. To validate this approach, calibration curves for both reagents at each recommended wavelength were measured at once.

Measuring different glucose concentrations with anthron-reagent at 555 nm revealed a 14 % reduction in optical density for the highest concentration of 90 µg glucose. Nevertheless, with decreasing concentration, the reduction of the optical density decreases, too. 45 µg glucose resulted in only 5 % lowered optical density. The reduction for all concentrations was in mean 7 ± 5 %. The slope of the calibration curve changed from 0.007 at 625 nm to 0.005 at 555 nm (Figure 13.5). Further, the standard deviation was increased in the high concentrations for the alternative wavelength.

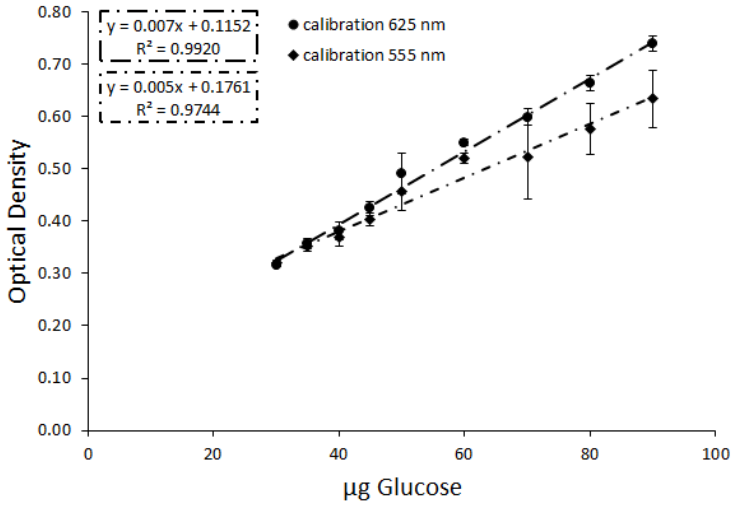


Figure 13.5: Changes in optical density for the calibration curve for glucose/glycogen measurement based on different wavelengths. Second wavelength of 555 nm is recommended when the measurement at 625 nm resulted in optical density above the range for measuring certainty. The samples were measured in triplicates at both wavelengths one day after reagent production.

A varying reduction in optical density can be observed for the lipid measurement with vanillin-phosphoric acid (Figure 13.6). Measuring with 490 nm instead of 525 nm resulted in 23 % reduction for the highest concentration of 149 µg lipid. At 75 µg lipid, reduction was 32 %. In mean the optical density was decreased around 26 ± 17 %. The slope of the calibration curve changed from 0.006 at 525 nm to 0.004 at 490 nm.

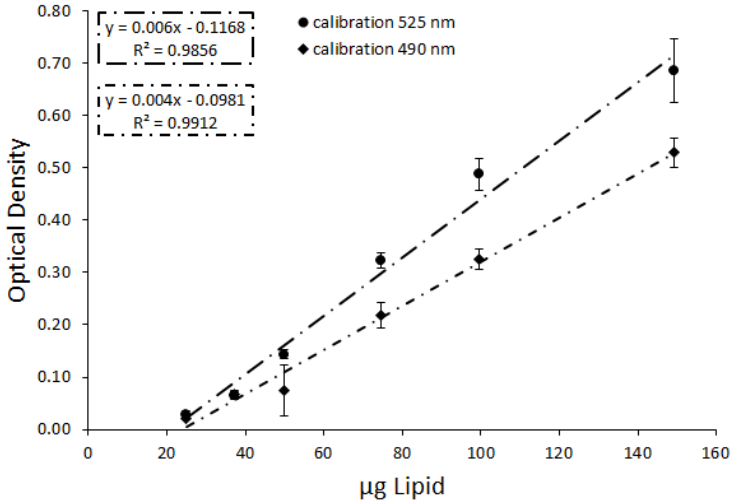


Figure 13.6: Changes in optical density for the calibration curve for lipid measurement based on different wavelengths. Second wavelength of 490 nm is recommended when the measurement at 525 nm resulted in optical density above the range for measuring certainty. The samples were measured in triplicates at both wavelengths one day after reagent production.

Measurement of both reagents at different wavelengths revealed a decreased optical density. It can be assumed that the relative reduction in optical density increases with

increasing concentrations above the tested range. Nevertheless, reduction did not reach 50 % and it is likely that the difference in both wavelengths is not sufficiently significant to be a possible alternative. Especially for glucose and glycogen measurement, the standard deviation was increased and can result in more incorrect values than measurement with the original wavelength.

Comparison of dilution

Another recommended alternative to improve measurement of high-concentrated solutions was dilution (van Handel 1985a, 1985b). Therefore, a calibration curve for both reagents was conducted followed by dilution of the samples with the reagent in 1:5 ratio and measured again.

The anthron-reagent used for glucose/glycogen measurement revealed a clear reduction of optical density when samples were diluted. The optical density of the highest concentration decreased from 0.58 ± 0.02 to 0.13 ± 0.01 and in the lowest concentrations. This is a reduction for all concentrations of around 80 ± 2 % (Figure 13.7). The slope changed from 0.007 to 0.001 so the calibration curve with the diluted samples was very flat.

When the samples measured with vanillin-phosphoric acid were diluted, reduction of optical density was around 72 ± 1 %. In the highest concentration the value decreased from 1.96 ± 0.19 to 0.54 ± 0.04 and in the lowest concentration from 0.65 ± 0.03 to 0.16 ± 0.01. The slope became flatter and changed from 0.007 to 0.002 (Figure 13.8).

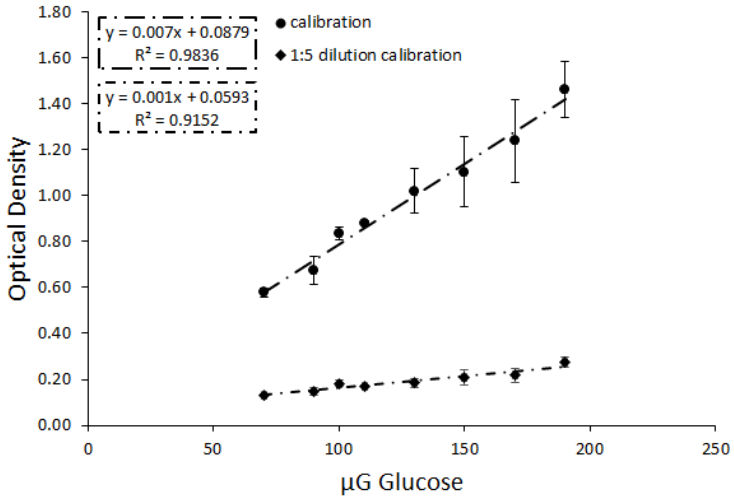


Figure 13.7: Changes in optical density for the calibration curve for glucose/glycogen measurement after dilution of the samples. Dilution is recommended when the measurement resulted in optical density above the range for measuring certainty. The samples were measured in triplicates one day after reagent production.

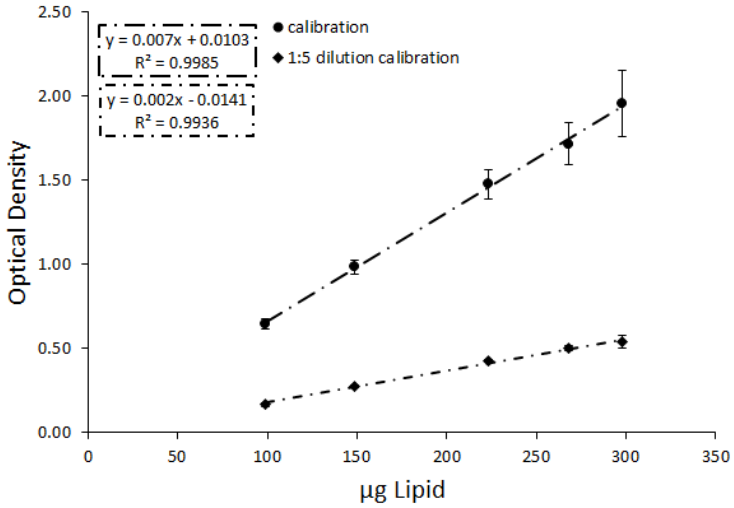


Figure 13.8: Changes in optical density for the calibration curve for lipid measurement after dilution of the samples. Dilution is recommended when the measurement resulted in optical density above the range for measuring certainty. The samples were measured in triplicates one day after reagent production.

For measurements with one of the reagents, the reduction was very high with around 70 and 80 %. This indicates that dilution of the samples is an acceptable alternative when samples are high concentrated. Nevertheless, the dilution of the samples resulted in flat calibration curves. This strongly lowers the resolution and can result in incorrect values. The effect of sample dilution is again and more clearly shown in Figure 13.9, which shows the results of various samples before and after 1:5 dilution. For all three storage substances, the dilution of the samples resulted in nearly same optical density although the concentrations were clearly different before.

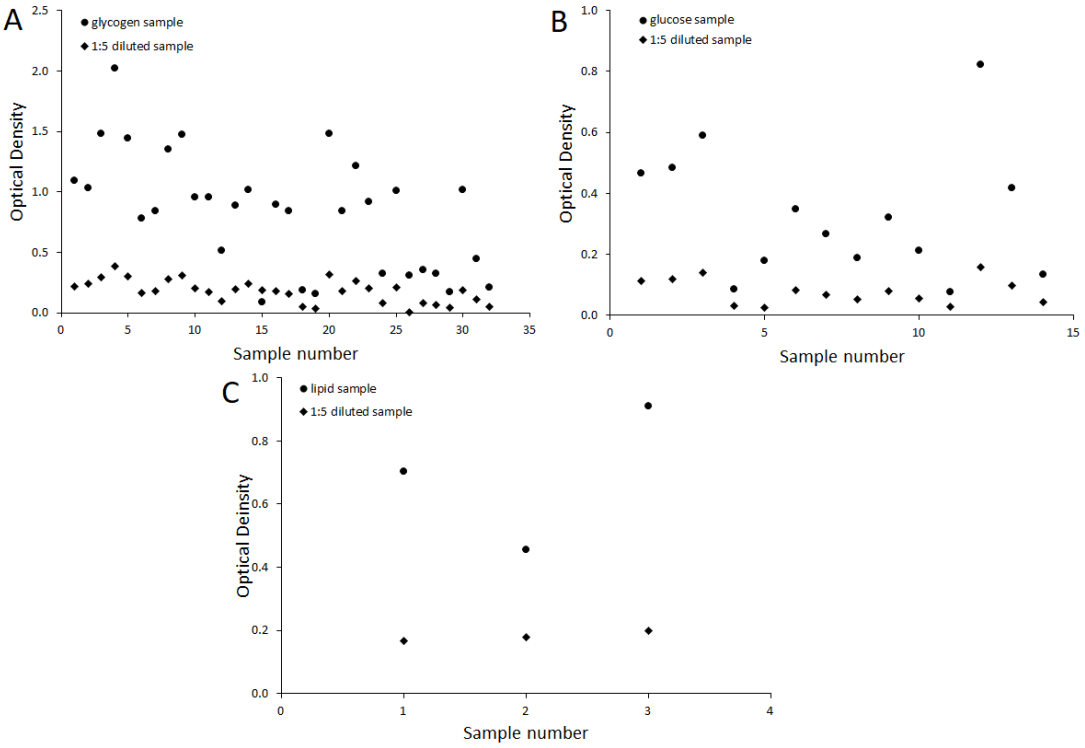


Figure 13.9: Results of samples for all three storage substances before and after 1:5 dilution with the corresponding reagent. A) is the measurement of the solutions of glycogen samples, B) is the measurement of the solutions of glucose samples and C) is the measurement of the solutions of lipid samples.

Another consideration was that the diluted solutions need to react again with the added reagent. Therefore, 50 µg and 150 µg glucose concentrations were measured, diluted and measured, and heated for 17 min and measured again. The heating did very slightly increase the optical density for booth concentrations, but the slope was the same as after dilution (Figure 13.10). As consequence of the decreased resolution, the dilution method for high-concentrated samples was neglected.

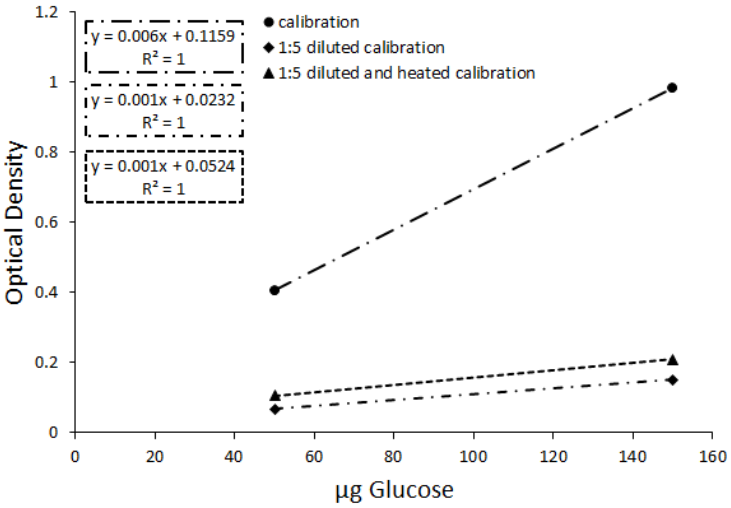


Figure 13.10: Changes in optical density for two glucose concentrations before and after dilution and heating. Dilution is recommended when the measurement resulted in optical density above the range for measuring certainty. The samples were measured in triplicates one day after reagent production.

13.1.2. Examination of suspension preparation for (plastic) particles

The first methods were chosen as they require no solvents or surfactants that might change the outcomes of the study. As the most basic approach, particles were given with a spoon to the water and quickly visibly agglomerated on the surface of the artificial water. Subsequently, the particles were tried to be brought under the surface by either sonication, mixing with the agitator, or pre-wetting. All three procedures led to the same distribution of the microparticles; agglomerating on the water surface.

Another approach was to pre-wet the particles in the water. The four hours or one day pre-wetted particles raised and accumulated on the surface when pipetted from the treated stock suspension into the medium as before without any preparation.

As the particles agglomerated in each approach, solutions with solvents were examined. Crystalline cetyl alcohol was used to decrease the surface tension. The aim was to bring the particles through the surface and prevent agglomeration. The cetyl alcohol slightly lowered the agglomeration, but also with this chemical, the particles raised to the surface and agglomerated.

The surfactants lecithin and bovine serum albumin led to the partly effective suspension of the particles in the medium with and without preparing a stock suspension. After application of the particles, no agglomeration on the surface was observed but particles were also not detectable in the medium as the surfactants made the medium muddy/cloudy. Another negative effect was that the surfactants foamed, which led to particles attached to the test vessel surface above the foam independent of whether a stock suspension of surfactant and particles was used or not. In addition, lecithin, which is also used as a nutritional supplement, lowered the feeding activity of *Gammarus roeseli* on the offered food, and the toxicity of a chemical was also lowered when added to the medium-lecithin-mixture.

The last approach was with solvents. Suspensions made with methanol or ethanol worked best when stock suspensions were prepared. When particles in the stock suspension of pure ethanol or methanol were added to the medium, particles slowly sank to the bottom of the beaker. In this approach, it is necessary to pipet directly into the medium because some particles also stick to the surface when the tip is above the surface. This approach was chosen as the particles remained in the medium and allowed an aqueous exposition to *Gammarus roeseli*, but it must be kept in mind that

the particles did not remain homogeneous distributed in the water column like a chemical.

13.2. Supplementary information for chapter 6

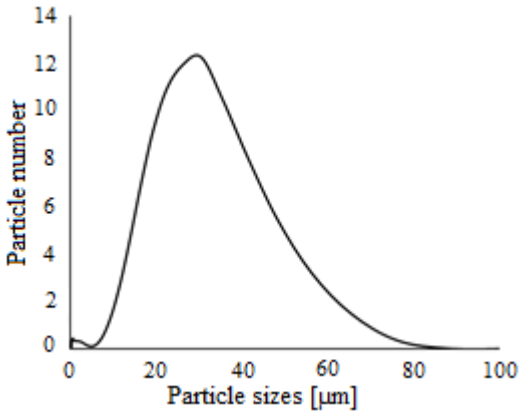


Figure 13.11: Particle sizes of 10 - 65 µm red fluorescent polystyrene microparticles in stock-suspension after centrifugation. Particles were produced by centrifugal milling (0 - 125 µm, Ultra Centrifugal Mill Type ZM 200), suspended in ethanol and centrifuged according to Correia and Loeschner (2018). Size distribution was verified using a Mastersizer S longbed.

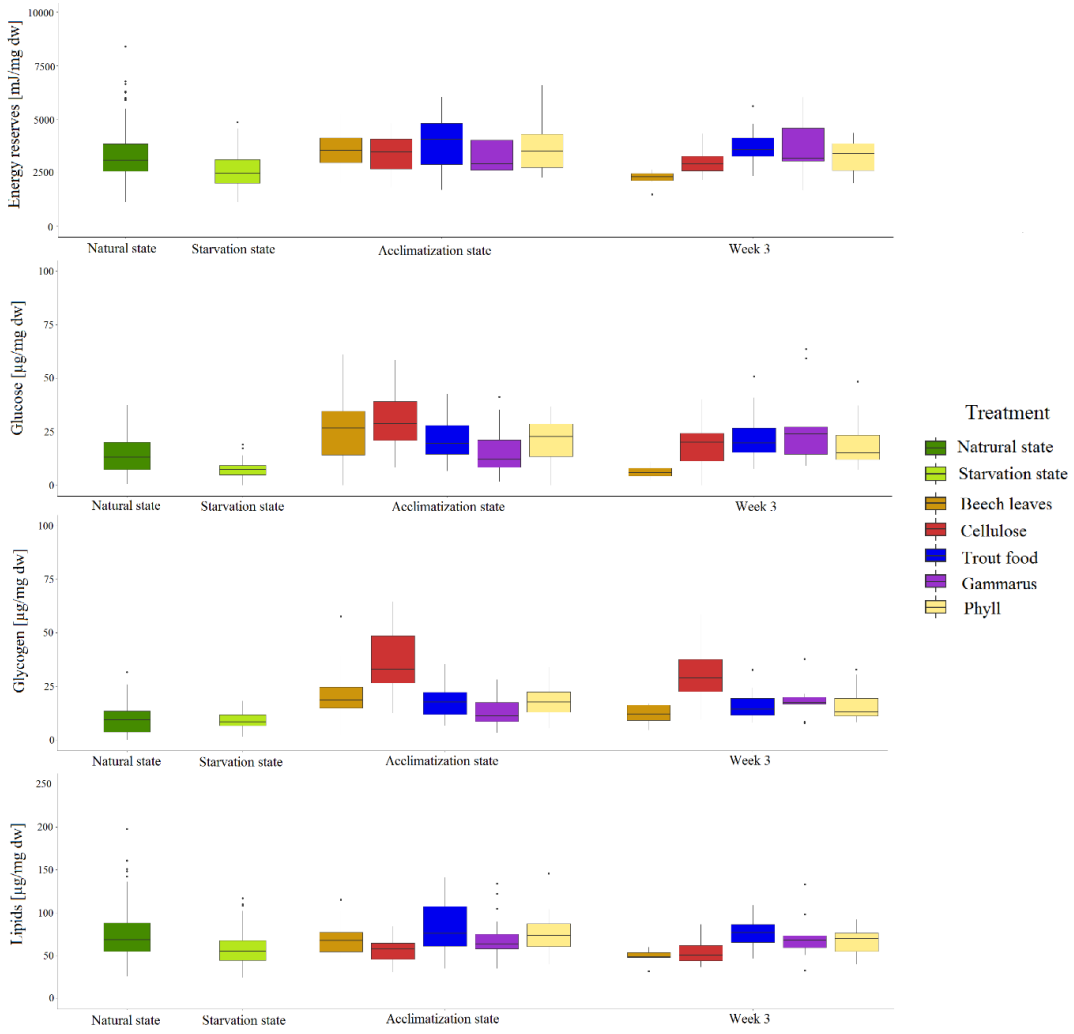


Figure 13.12: Energy reserves and storage substances of natural state (freshly caught gammarids, -7 days), starvation state (gammarids starved for 16 days), acclimatization state (0 days) and gammarids after three weeks exposure with five different DECOTAB formulations. Energy reserves were calculated from the storage substances with the following energy values, lipid: 39,500 mJ/mg, glucose and glycogen: 17,500 mJ/mg.

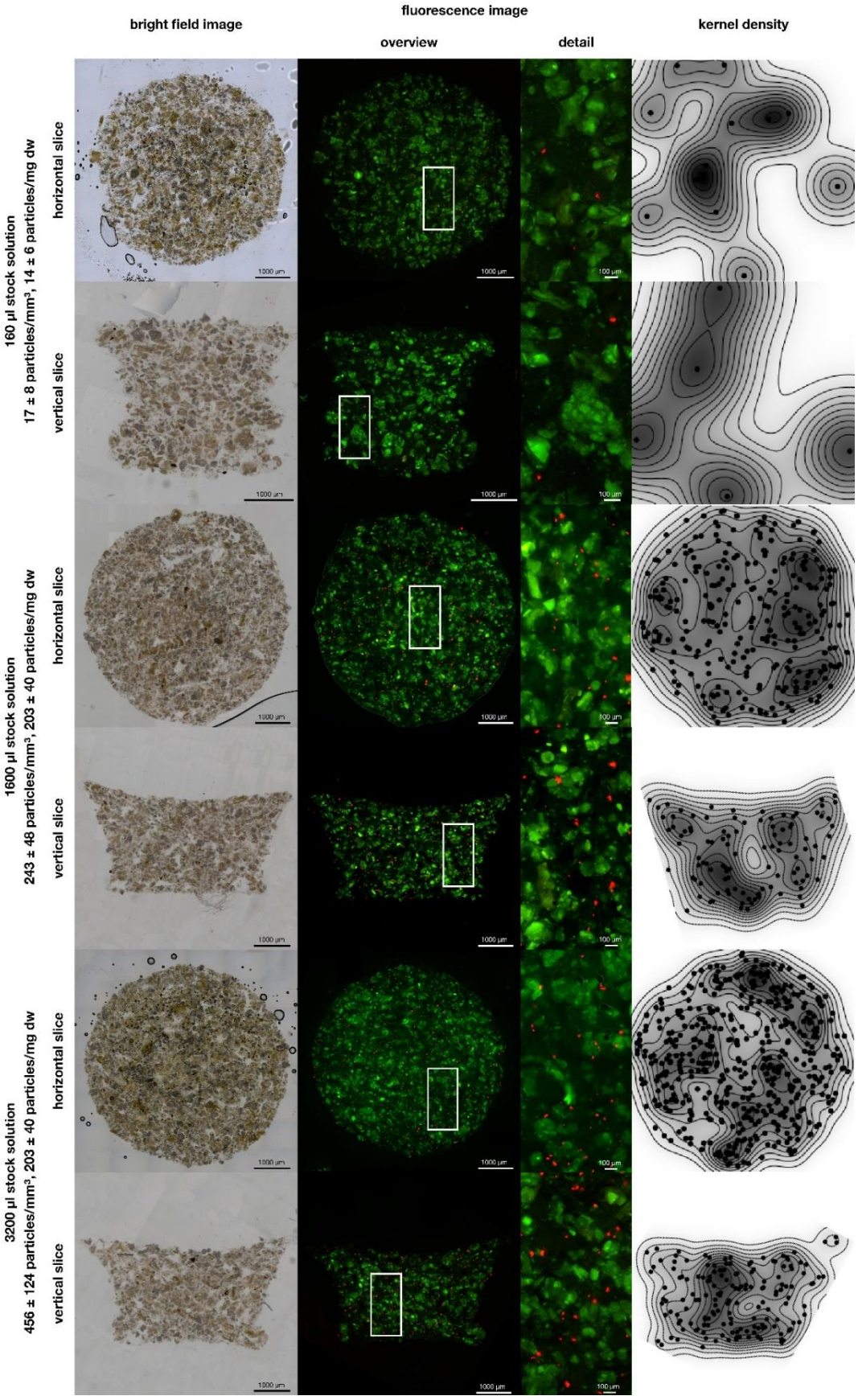
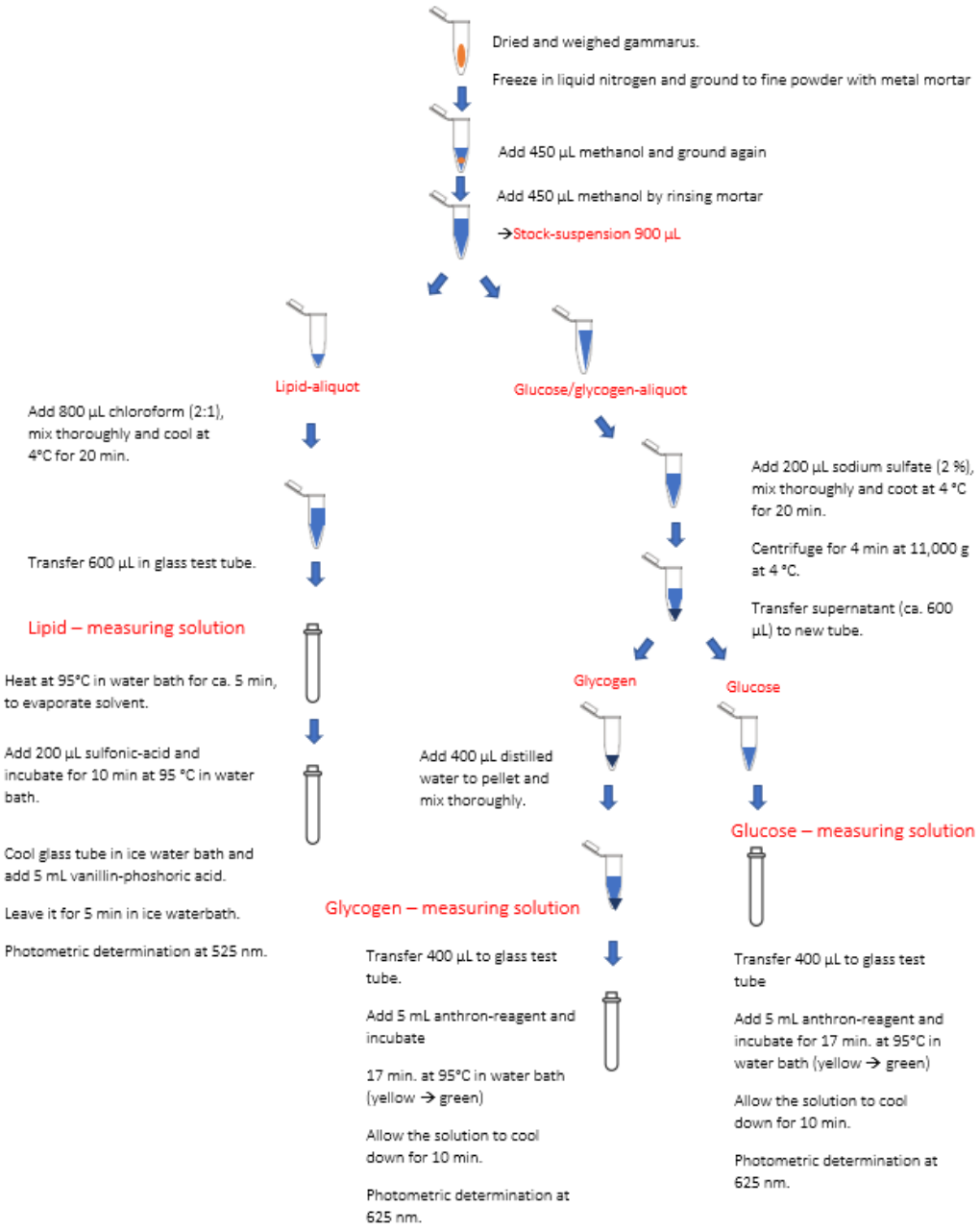


Figure 13.13: Brightfield image, fluorescence microscopy image and kernel density plot of 50 µm vertical and horizontal sections of DECOTABs loaded with 160, 1600 and 3200 µL stock-suspension of red fluorescent particles with 10 - 65 µm. Red fluorescent particles were hand marked and counted using FIJI.

Flowchart for lipid and glucose/glycogen determination for *Gammarus roeseli*



13.3. Supplementary Information for chapter 7

Table 13.1: Mean mass eaten by the gammarids of each treatment in the first and second week and the calculated theoretical ingested particles per day and gammarid.

Treatment	Particle conc. [ng/mg]	Time [w]	Mean Mass eaten [µg/ga*d]	Mean particles ingested [ng/ga*d]
Co.0.0	0	1	184.55 ± 68.74	0.00
H.0.0	0	1	0 ± 0	0.00
PLA.2000.H	419	1	256.94 ± 44.05	107.66
PLA.2000.L	4.19	1	213.25 ± 75.06	0.89
PLA.500.H	419	1	221.16 ± 51.63	92.67
PLA.500.L	4.19	1	178.40 ± 63.01	0.75
PS.100.H	431	1	280.71 ± 71.49	120.99
PS.100.L	4.31	1	335.23 ± 140.89	1.44
PS.1000.H	431	1	201.82 ± 52.14	86.98
PS.1000.L	4.31	1	221.74 ± 48.82	0.96
PS.30.H	431	1	163.74 ± 64.30	70.57
PS.30.L	4.31	1	220.64 ± 52.86	0.95
PS.500.H	431	1	250.63 ± 57.49	108.02
PS.500.L	4.31	1	291.47 ± 161.66	1.26
Silica.100.H	838	1	284.33 ± 75.59	238.27
Silica.100.L	8.38	1	287.45 ± 116.38	2.41
Silica.1000.H	838	1	270.97 ± 51.21	227.07
Silica.1000.L	8.38	1	205.75 ± 101.62	1.72
Silica.30.H	838	1	247.63 ± 87.77	207.51
Silica.30.L	8.38	1	280.85 ± 58.99	2.35
Silica.500.H	838	1	141.53 ± 39.32	118.61
Silica.500.L	8.38	1	207.05 ± 58.12	1.74
Co.0.0	0	2	237.56 ± 78.60	0.00
H.0.0	0	2	0 ± 0	0.00
PLA.2000.H	419	2	622.24 ± 563.33	260.72
PLA.2000.L	4.19	2	435.94 ± 432.81	1.83
PLA.500.H	419	2	245.23 ± 106.27	102.75
PLA.500.L	4.19	2	245.61 ± 84.59	1.03
PS.100.H	431	2	280.48 ± 105.20	120.89
PS.100.L	4.31	2	391.32 ± 175.81	1.69
PS.1000.H	431	2	231.77 ± 85.05	99.89
PS.1000.L	4.31	2	526.31 ± 421.35	2.27
PS.30.H	431	2	285.85 ± 188.36	123.20
PS.30.L	4.31	2	319.13 ± 108.99	1.38
PS.500.H	431	2	1164.08 ± 1638.68	501.72
PS.500.L	4.31	2	443.06 ± 345.27	1.91
Silica.100.H	838	2	384.71 ± 172.35	322.39
Silica.100.L	8.38	2	259.18 ± 80.42	2.17
Silica.1000.H	838	2	327.70 ± 103.35	274.62
Silica.1000.L	8.38	2	482.35 ± 585.51	4.04
Silica.30.H	838	2	405.05 ± 593.68	339.43

Silica.30.L	8.38	2	337.45 ± 89.34	2.83
Silica.500.H	838	2	236.55 ± 150.65	198.22
Silica.500.L	8.38	2	241.90 ± 93.44	2.03

Rawdata are open access in Dryad Database. DOI:10.5061/dryad.547d7wmbj. Calculation tools are available from the corresponding author (geist@tum.de).

13.4. Supplementary Information for chapter 8

Table 13.2: Statistical data for mortality models in the acute toxicity test for 24 h and 48 h.

Exposure	Time [h]	Model	AIC	dF	Function + R^2
Phenanthrene	24	LL2.5	197.9103	6	
		LL2.5[1]	195.6294	5	
		LL.3	193.9107	4	
		LL.3[1]	192.6419	3	
		LL.3[1,2,3]	192.6292	2	y=0+(100-0)/(1+exp(-12*(log(x)-log(471.9437))))
		LL.4	194.5661	5	R^2=0.9602
		LL.4[1]	194.0368	4	
		LL.5	196.5660	6	
		LL.5[1]	195.5772	5	
		W1.4	194.5667	5	
		W2.4	194.5662	5	
Phenanthrene	48	LL2.5	194.9930	6	
		LL2.5[1]	193.0016	5	
		LL.3	191.5302	4	
		LL.3[1,2]	191.0888	3	y=0+(100-0)/(1+exp(-3.272*(log(x)-log(441.074))))
		LL.4	191.7750	5	R^2=0.9930
		LL.4[1]	189.8102	4	
		LL.5	192.6136	6	
		LL.5[1]	191.6209	5	
		W1.4	191.7865	5	
		W2.4	191.6913	5	

[1] with fixed maximum to 100, [2] chosen model, [3] with fixed slope to -12

Table 13.3: Statistical data for mortality models in the sublethal toxicity test for 24 h and 48 h.

Exposure	Time [h]	Model	AIC	dF	Function + R^2
Phenanthrene	24	LL2.5	265.4658	6	
		LL2.5[1]	281.2266	5	
		LL.3	261.4770	4	
		LL.3[1,2]	260.0910	3	y=0+(100-0)/(1+exp(-0.9163*(log(x)-
		LL.4	263.4765	5	log(2514.1324))))
		LL.4[1]	262.0687	4	R^2=0.8868
		LL.5	265.4708	6	
		LL.5[1]	263.8288	5	
		W1.4	263.4606	5	
		W2.4	263.4812	5	
Phenanthrene	48	LL2.5	287.6022	6	
		LL2.5[1]	285.9377	5	
		LL.3	283.6348	4	
		LL.3[1,2]	281.9314	3	y=0+(100-0)/(1+exp(-5.496*(log(x)-
		LL.4	284.2637	5	log(444.630))))
		LL.4[1]	282.2561	4	R^2=0.9920
		LL.5	286.2561	6	
		LL.5[1]	284.2555	5	
		W1.4	285.2552	5	
		W2.4	284.2686	5	
Phenanthrene + Polyamide	24	LL2.5	556.5272	6	
		LL2.5[1]	554.5367	5	
		LL.3	556.8194	4	
		LL.3[1]	557.5723	3	
		LL.4	557.6840	5	y=1.7449+(1819.1632-1.7449)*exp(-exp(-
		LL.4[1]	559.9413	4	0.7201*(log(x)-log(5392.9638))))
		LL.5	561.9874	6	R^2=0.8699
		LL.5[1]	561.3624	5	
		W1.4[2]	553.4068	5	
		W2.4	562.1432	5	
Phenanthrene + Polyamide	48	LL2.5	580.8632	6	
		LL2.5[1,2]	578.2640	5	y=0.007282+(100-0.007282)/((1+exp(-
		LL.3	591.7329	4	10.792979*(log(x)-6.617577))))^0.583187)
		LL.3[1]	594.5381	3	R^2=0.5976
		LL.4	594.4638	5	
		LL.4[1]	596.3873	4	
		LL.5	596.0914	6	
		LL.5[1]	597.9086	5	
		W1.4	583.4833	5	
		W2.4	596.6392	5	
Phenanthrene + Sediment	24	LL2.5	580.6719	6	
		LL2.5[1,2]	578.5549	5	y=0.002405+(100-0.002405)/((1+exp(-
		LL.3	585.5026	4	9.543490*(log(x)-6.668061)))^0.627698)
		LL.3[1]	587.3274	3	R^2=0.8329
		LL.4	586.2232	5	
		LL.4[1]	589.2411	4	
		LL.5	589.2849	6	
		LL.5[1]	591.2876	5	
		W1.4	579.0043	5	
		W2.4	590.0956	5	
Phenanthrene + Sediment	48	LL2.5	640.6879	6	
		LL2.5[1,2]	637.9042	5	y=0.00371+(100-0.00371)/((1+exp(-
		LL.3	650.1816	4	22.80440*(log(x)-6.47131)))^0.56676)
		LL.3[1]	655.2934	3	R^2=0.9201
		LL.4	652.6535	5	
		LL.4[1]	657.7234	4	
		LL.5	656.8026	6	
		LL.5[1]	659.0511	5	
		W1.4	639.8003	5	
		W2.4	657.8964	5	

[1] with fixed maximum to 100, [2] chosen model

Table 13.4: Statistical data for velocity models in the sublethal toxicity test for 24 h and 48 h.

Exposure	Time [h]	R^2	Adjusted R^2	Resid Stand Err	Function
Phenanthrene	24	0.246	0.3421	0.4249 on 165 dF	y=0.977054-0.001522*x
Phenanthrene	48	0.2542	0.2489	0.5726 on 142 dF	y=1.32611-0.002486*x
Phenanthrene + Polyamide	24	0.3575	0.3541	0.6097 on 191 dF	y=1.575892-0.002455*x
Phenanthrene + Polyamide	48	0.3152	0.3114	0.6515 on 181 dF	y=1.825603-0.002455*x
Phenanthrene + Sediment	24	0.3794	0.3761	0.5909 on 192 dF	y=1.55076-0.002434*x
Phenanthrene + Sediment	48	0.2177	0.2132	0.6908 on 175 dF	y=1.753712-0.002224*x