Tissue translocation, multigenerational and population effects of microplastics in *Daphnia magna*

Dissertation

zur Erlangung des Doktorgrades

der Naturwissenschaften

vorgelegt beim Fachbereich Biowissenschaften

der Johann Wolfgang Goethe-Universität

in Frankfurt am Main

von

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aus Köln

Frankfurt (2021)

(D30)

Vom Fachbereich Biowissenschaften der Johann Wolfgang Goethe-Universität als Dissertation angenommen.

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"*Nearly everything is really interesting if you go into it deeply enough.*"
— Richard P. Feynman

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Abstract

The last century saw the widespread adoption of plastic materials throughout nearly every aspect of our lives. Plastics are synthetic polymers that are made up of monomer chains. The properties of the monomer in conjunction with chemical additives allow plastics to have a sheer endless variety of features and use cases. They are cheap, lightweight, and extremely durable. Plastic materials are often engineered for single-use and in conjunction with high production volumes and insufficient waste management and recycling across the globe, this leads to a large number of plastics entering the environment. Marine ecosystems are considered sinks. However, freshwater ecosystems as entry pathways are highly affected by plastic waste as well. Throughout the past decade, the impact of plastic waste on human and environmental health has received a lot of attention from the ecotoxicological community as well as the public. Small plastic fragments (< 1 mm called microplastics) are a large part of this emerging field of research. Within this, the water flea *Daphnia magna* is probably the most common organism that is used to assess microplastics toxicity. As a filter-feeding organism, it indiscriminately ingests particles from the water column and is thus highly susceptible to microplastics. For this thesis, we identified some gaps in the available data on the ecotoxicity of microplastics to daphnids. To illuminate some of those gaps the present thesis was aimed at five main aspects:

(1) Tissue translocation of spherical microplastics in *Daphnia magna*
(2) Investigation of the toxicity of irregularly shaped microplastics
(3) Multigenerational and population effects of microplastics
(4) Comparison of the toxicity of microplastics and natural particles
(5) Effects of particle-aging on microplastics toxicity

The thesis is comprised of three peer-reviewed articles and one so-far unpublished study as “additional results”. The first study was aimed at understanding tissue translocation of spherical microplastics to lipid storage droplets of daphnids. The crossing of biological membranes is discussed as a prerequisite to eliciting tissue damage and an inflammatory response. Previously, researchers reported the translocation of fluorescently labeled spherical microplastics to lipid storage droplets of daphnids, even though no plausible biological mechanism to explain this occurrence. Therefore, in order to learn more about this process and potentially

illuminate the mechanism we replicated the study. We were able to observe a fluorescence signal inside the lipid droplets only after increasing the exposure concentrations. Nonetheless, it appeared to be independent of particles. This led to the hypothesis, that the lipophilic fluorescent dye uncoupled from the particles and subsequently accumulated in lipid storage droplets. The hypothesis was further confirmed through an additional experiment with a silicone-based passive sampling device showing that the fluorescence occurred both independent of particles and digestive processes. Accordingly, we concluded that the reported findings were a microscopic artifact caused by the uncoupling of the dye from the particles. Therefore, a fluorescence signal alone is not a sufficient proxy to assume that particles have translocated. It needs to be coupled with additional methods to ensure that the observation is indeed caused by the translocation of particles.

It is still unclear whether the toxicity profile of microplastics is different from that of naturally occurring particles or if they are "just another particle", as there are innumerable amounts in the natural environment surrounding an organism. The goal of the second study was to compare the toxicity of irregularly shaped polystyrene microplastics to that of the natural particle kaolin. The environment is full of natural non-food particles that daphnids ingest more or less indiscriminately and therefore are well adapted to deal with. Daphnids have a short generation time and usually experience food limitation in nature. Therefore, short-term studies only looking at acute toxicity with *ad libitum* food availability are not representative of the exposure scenario in nature. For a more realistic scenario, we, therefore, used a four-generation multigenerational design under food limitation to investigate how effects translate from one generation to the next. We observed concentration-dependent effects of microplastics but not of natural particles on mortality, reproduction, and growth. Some of the effects increased from generation to generation, leading to the extinction of two treatment groups. Here, microplastics were more toxic than natural particles. At least part of this difference can be explained by physical properties leading to the quick sedimentation of the kaolin, while microplastics remained in the water column. Nonetheless, buoyancy and sedimentation would also affect exposure in the environment and are likely different for most microplastics than for most naturally occurring particle types.

The third study expanded on the multigenerational design to assess the effects of aging on microplastics toxicity. In the environment, microplastics are subjected to a

number of biotic and abiotic factors. Here, the formation of an eco-corona through the sorption of biological macromolecules is hypothesized to affect particle toxicity. That implies that the use of pristine, unaged microplastics is not representative of what organisms encounter in the field. We, therefore, simulated environmental aging by incubating irregularly shaped polystyrene microplastics in filtrated wastewater and compared their toxicity to that of particles incubated only in ultrapure water. Again, we used food limitation in a four-generation multigenerational design with daphnids. Overall, we observed a concentration-dependent effect of both wastewater and ultrapure water incubated particle types on mortality, reproduction, and growth. The wastewater-aged particles induced less mortality than the pristine particles. The generational effect was less significant than in the previous study. Extensive particle characterization could not conclusively show which of the particle properties led to the difference in toxicity of the aged vs. the pristine particles. We hypothesized that the sorption of dissolved organic carbon from the wastewater was the factor, but could not correlate that with electron microscopic observations, zeta-potential measurements, or other metrics. The lower toxicity of the microplastics compared to the previous study could be caused by the lack of nanoparticles caused by the incubation procedure, as nanoplastics are thought to have higher toxic potential. Overall, our results, in conjunction with literature show that particle aging affects microplastics toxicity and should be considered in study design in order not to overestimate particle toxicity.

The fourth study shifted the focus from an individual level to daphnid populations. Populations were exposed to mixtures of irregularly shaped polystyrene microplastics and the natural particle kieselguhr at a fixed concentration under food limitation. Population size, number of resting eggs, and size of the individuals in each population were recorded weekly. Population size increased early and peaked after two weeks, followed by a decline. The control populations and those exposed to a higher ratio of microplastics grew the largest, while higher kieselguhr concentrations led to smaller population sizes. Concurrently, the smaller populations produced more resting eggs, a clear sign of stress. Population structure (i.e., size of the individuals comprising the population) appeared unaffected by the particle exposure. This study showed that microplastics are not necessarily more toxic than natural particles. Kieselguhr has a pointy and sharp particle shape and known biocidic properties which might have played a role in the toxicity. Interestingly, the microplastics concentration was five

times higher than in the previous two experiments and yet produced limited toxicity. Here, particle aging could again play a role in the reduced toxicity. The frequency of medium exchange, and thus the introduction of pristine particles into the vessel, was only weekly, while it was thrice weekly for studies 2 + 3. Therefore, particles remained longer in the vessel and had more time to acquire an eco-corona from food particles, the daphnids themselves, or their feces.

Overall, the thesis could contribute significantly to the way microplastics ecotoxicity research is conducted with daphnids. We could show that the detection of fluorescence alone is not a valid proxy for tissue translocation of particles but must always be coupled with additional methods to identify the presence of particles in the target tissue. Multigenerational designs can be used to elicit effects not visible in short-term exposures of a single generation. Additionally, combining multiple generations with food limitations as additional stressor makes sense regarding potential effect mechanisms of microplastics towards daphnids. We could show that microplastics are not necessarily more toxic than natural particles, but that individual properties (density, shape, etc.) are important as well. We cannot extrapolate from one particle type to another and the literature provides evidence, that for other types of plastics the associated chemicals are a stronger driver of toxicity. Likewise, particle aging appears to modulate microplastics toxicity. This is in line with earlier reports on micro- and nanoplastics and engineered nanoparticles. Additionally, evaluation of our findings in the context of the dynamic energy budget theory indicates, that for the microplastics we used, a decrease in nutrient assimilation efficiency (i.e., reduced feeding rate, food dilution, regurgitation of boluses) in combination with increased metabolic cost (i.e., physical damage and increased movement) are likely the main mechanisms of toxicity. Therefore, considering the applicability of experimental data in *in silico* methods to extrapolate to other timeframes or concentrations is beneficial, not only to reduce labor and the dependence of animal tests.

Zusammenfassung

Im Laufe des letzten Jahrhunderts hat Plastik in nahezu jeden Aspekt unseres täglichen Lebens Einzug gehalten. Chemisch betrachtet ist Plastik der Überbegriff für synthetische Polymere, bestehend aus Ketten von Untereinheiten (Monomeren). Die Eigenschaften des Monomers in Kombination mit zugesetzten chemischen Additiven erlaubt eine fast grenzenlose Vielfalt und Anwendbarkeit von Plastikmaterialien. Die Erfindung und Verbreitung von Plastikmaterialien wurde maßgeblich durch den zweiten Weltkrieg vorangetrieben und stellt eine gesellschaftliche Revolution dar. Plastik ist leicht, preisgünstig, robust, und endlos formbar. Viele alltägliche Plastikprodukte sind für den einmaligen Gebrauch bestimmt und gelangen danach absichtlich oder unabsichtlich in die Umwelt, wo sie Jahre bis Jahrhunderte benötigen, um abgebaut zu werden. Teil dieser Problematik ist das ubiquitäre Auftreten partikulären Plastiks, das größtenteils aus der Verwitterung größerer Plastikstücke entsteht und als Mikroplastik (gemeinhin Partikel < 1 mm Größe) bezeichnet wird. Verwitterungsmechanismen sind sowohl chemisch-physikalischer Natur wie z.B. die Einwirkung von (ultravioletter) Strahlung, mechanischer Abrieb, Salzgehalte und Sauer bzw. basische Milieus. Zusätzlich kommt es nach Eintrag in die Umwelt zur Sorption biologischer Makromoleküle und den Bewuchs mit Mikroorganismen. Auch Fragmentierung durch biologische Prozesse (Darmpassage von Tieren) wurde bereits beschrieben.

Durch das breite Größenspektrum, in dem Mikroplastik auftritt, kann es Organismen aller biologischen Ebenen beeinflussen und ggf. von ihnen aufgenommen werden. Die Interaktion und Schadwirkungen von Mikroplastik auf verschiedene Organismen sind seit einigen Jahren Gegenstand vieler ökotoxikologischer Studien. Der wahrscheinlich meistuntersuchte Organismus ist hier der große Wasserfloh *Daphnia magna*, der als pelagischer Filtrierer Partikel bis zu einer Größe von 70-90 µm nicht-selektiv aus der Wasserphase aufnimmt. Die Untergrenze ist weniger klar definierbar, da hier weitere Prozesse über die Filtration hinaus zur Aufnahme von Partikeln führen können.

Allgemein werden zwei Hauptmechanismen für eine Schadwirkung durch Mikroplastik diskutiert: I) die Reduktion der Nahrungsverfügbarkeit, beispielsweise durch Verdünnung von Nahrungspartikeln mit Mikroplastik oder der reduzierten Nahrungsaufnahme oder II) eine mechanische Schädigung des Organismus.

Letzteres kann weiterhin unterschieden werden in interne und externe physische Schädigung. In einem Review-Artikel wurde die Aufnahme und der Transfer von Partikeln über Gewebegrenzen hinweg als Voraussetzung für das Auftreten von Entzündungsreaktionen und damit einhergehender Schadwirkung angenommen. Dieser Gewebetransfer von Mikroplastik wurde bereits in einer Vielzahl von Studien in aquatischen Invertebraten und Fischen untersucht. Im überwiegenden Teil dieser Studien wurden sphärische fluoreszenz-markierte Partikel genutzt, deren Gewebetransfer anhand eines Fluoreszenz-Signals im Zielgewebe nachgewiesen wurde. Biologisch betrachtet kommen drei Mechanismen in Frage, um einen Gewebetransfer zu bewirken, insgesamt ist der Transfer von für Partikel > 1 µm allerdings nicht plausibel. Da sie nur bedingt zwischen Futter- und nicht-Futter-Partikeln unterscheiden können, haben Daphnien mehrere Strategien und Adaptations-Mechanismen, um mit der Allgegenwärtigkeit nicht-verdaulicher Partikel umzugehen. Ihre Darminnenwand ist beispielsweise durch eine Chitin-basierte peritrophische Membran gegen mechanische Verletzungen geschützt, die gleichzeitig siebartig die Aufnahme größerer Partikel über den Darm verhindert. Unter anderem wegen der Existenz dieser Membran ist mindestens eine frühere Studie, die über Gewebetransfer von fluoreszierendem Mikroplastik in Daphnien aus dem Darm in Fettspeichergewebe berichtet hat, biologisch nicht plausibel.

Diese Thesis besteht aus drei in Fachzeitschriften veröffentlichten Papern (Schür et al. (2019; 2020; 2021), Annex 1-3), sowie einer unveröffentlichten Studie (Annex 4). Die Studien beschäftigen sich mit verschiedenen Aspekten der Interaktion und Effekten von Mikroplastik auf den großen Wasserfloh *Daphnia magna*. Übergeordnet wurden dabei fünf Ziele verfolgt, die teils übergreifend in mehreren Studien behandelt wurden:

(1) Gewebetransfer von sphärischem Mikroplastik.
(2) Untersuchung der Toxizität von irregulär geformten Mikroplastikpartikeln.
(3) Multigenerationale und Populations-Effekte von Mikroplastik.
(4) Vergleich der Toxizität von Mikroplastik und natürlichen Partikeln.
(5) Auswirkung von Partikel-Alterung auf die Mikroplastik-Toxizität.

Die erste Publikation (Schür et al. (2019), Annex 1) befasst sich mit dem Gewebetransfer von Mikro- und Nanoplastik-Partikeln in *Daphnia magna*. Durch die Reproduzierung einer früheren Studie wurde im Detail untersucht wie es zu einem

Transfer von Mikro- und Nanoplastik Partikeln in die Fettspeichergewebe von Daphnien kommen kann. Anfänglich konnten die Ergebnisse nicht reproduziert werden, weshalb eine zusätzliche Verfeinerung des Ansatzes durch Einbeziehung eines Probenbehandlungsprotokolls zur Verbesserung der Sichtbarkeit innerhalb der Tiere folgte. Erst nach zusätzlicher Erhöhung der Partikelkonzentration konnten Fluoreszenzsignale in den Fettspeichern der Daphnien nachgewiesen werden. Zumindest für die größeren der untersuchten Partikel (1 µm) war diese Fluoreszenz allerdings unabhängig von Partikeln. Daraus erwuchs die Hypothese, dass sich der lipophile Fluoreszenzfarbstoff von den Partikeln gelöst und in Fetttröpfchen akkumuliert hatte. Ein Transfer der Partikel selbst hat somit nicht stattgefunden. Dass das beobachtete Phänomen tatsächlich ein Artefakt ist, das aus der Lösung des Farbstoffes vom Partikel resultiert und dass dieser Prozess unabhängig von der Gegenwart des Tieres abläuft, konnte durch ein zusätzliches Experiment mit der Inkubation von einem Silikonstreifen als Absorptionsmedium zusammen mit einer Partikelsuspension nachgewiesen werden. Diese Erkenntnisse haben weitreichende Implikationen für die Erforschung von Gewebetransfer mittels Fluoreszenz. Unsere Ergebnisse zeigen, dass Fluoreszenz allein nicht das Vorhandensein von Partikeln beweist und immer mit weiteren Methoden kombiniert sein muss, um eindeutig zeigen zu können, dass es zum Übertritt von Partikeln in Gewebe kommt.

In der zweiten Studie (Schür et al. (2020), Annex 2) dieser Arbeit sollte untersucht werden, inwiefern sich das ökotoxikologische Profil von Mikroplastik und natürlich vorkommenden Partikeln unterscheidet. Aus der Literatur ist bekannt, dass Mikroplastik in Daphnien zu einer Reduktion der Nahrungsaufnahme führen kann. Effekte, die sich aus dem daraus resultierenden Energiedefizit ergeben wären wahrscheinlich bei einem Überangebot von Nahrung nicht detektierbar. Gleichzeitig sind Daphnien in der Natur vielfach einer schwankenden und defizitären Nahrungsverfügbarkeit ausgesetzt. Aus diesem Grund wurde im experimentellen Design eine Nahrungslimitation als zusätzlicher Stressor verwendet. Des Weiteren bezieht sich der überwiegende Teil der Literatur über Effekte von Mikroplastik in Daphnien auf kurze bis mittlere Expositions-Zeiträume und betrachtet in der Regel nur eine einzelne Generation. Da Daphnien allerdings eine Generationszeit von weniger als zwei Wochen haben und Mikroplastik über Jahrzehnte in der Umwelt verbleiben kann, kann angenommen werden, dass die Tiere nicht nur über ihre individuelle Lebensdauer hinweg, sondern über Generationen hinweg exponiert

werden. Um zu untersuchen, ob sich Mikroplastik-Effekte unter Nahrungslimitation von einer Generation zur anderen übertragen oder gar verstärken, wurde ein standardisiertes Testdesign zu einem vier Generationen umfassenden, multigenerationalen Szenario adaptiert. Darin wurden die Effekte von irregulär geformten Mikroplastik, das aus Kaffee-Becher-Deckeln hergestellt wurde, mit dem natürlichen Referenzpartikel Kaolin in Daphnien verglichen. In diesem Szenario stammte nach der ersten Generation die jeweilige Folgegeneration von Tieren ab, die ihre ganze Lebensdauer gegenüber den Partikeln exponiert gewesen waren. Zusätzlich wurde die Sensitivität der Nachkommen gegenüber einer Referenzchemikalie als Stellvertreter für die individuelle Fitness der Nachkommen untersucht. Im Experiment zeigten sich deutliche konzentrationsabhängige Effekte von Mikroplastik auf Überleben, Reproduktion und Wachstum der Tiere, während diese Effekte bei Kaolin nicht beobachtet wurden. Mit Hilfe der Multi-Generations-Studie konnte zudem gezeigt werden, dass sich die Effekte des Mikroplastiks teilweise von Generation zu Generation verstärkten, was bei zwei Behandlungsgruppen zum Aussterben führten. Die Sensitivität der Nachkommen gegenüber der Referenzsubstanz konnte jedoch nicht als aussagekräftiger Maßstab für die individuelle Fitness herangezogen werden. Insgesamt zeigte sich in der Studie, dass durch die Exposition der Daphnien über mehrere Generationen Effekte beobachtet werden konnten, die in kurzzeitigen Expositionsszenarien wohl verborgen geblieben wären. Mikroplastik war hier toxischer als der natürliche Partikeltyp, was aber zumindest teilweise auf physikalische Eigenschaften zurückzuführen ist. Der natürliche Partikeltyp sedimentierte schnell, während das Mikroplastik länger in der Wasserphase verblieb, in der sich Daphnien bevorzugt aufhalten.

Das Testdesign der zweiten Studie wurde in Studie 3 (Schür et al. (2021), Annex 3) verwendet, um den Einfluss von Partikel-Alterung zu untersuchen. In der Umwelt sind Plastikpartikel verschiedensten Einflüssen ausgesetzt. Diese können chemischer, physikalischer und biotischer Natur sein. Auch die Sorption verschiedener Chemikalien und biologischer Makromoleküle kann Teil des Alterungsprozesses sein und die Interaktion von Mikroplastik mit Organismen verändern. Um die Alterung von Mikroplastik zu simulieren, wurde irregulär geformtes Polystyrol-Mikroplastik in filtriertem Abwasser inkubiert, während ein anderer Teil in Ultrareinstwasser inkubiert wurde. Die Partikel wurden anschließend von der Flüssigkeit abgeschieden, resuspendiert und zur Exposition von Daphnien in einem ebenfalls vier Generationen

umfassenden Experiment eingesetzt. Hier zeigten sich, wie im vorherigen Experiment, konzentrationsabhängige Effekte auf Reproduktion, Wachstum und Überleben. Die Effektstärke war zwischen den beiden Partikeltypen vergleichbar, allerdings zeigten Behandlungsgruppen, die gegenüber dem Rohabwasser-gealterten Mikroplastik exponiert waren, eine geringere Mortalität. Ein Effekt auf die Größe von Nachkommen zum Zeitpunkt der Geburt konnte nicht beobachtet werden. Anhand von Wachstumskurven konnte gezeigt werden, dass eine verminderte Nahrungsverfügbarkeit der Elterntiere einen früheren Wachstumsschub der Nachkommen bewirkt, der sich aber nicht in der späteren Maximalgröße widerspiegelt. In einem intraspezifischen Wettbewerbskontext um begrenzte Nahrung würde das den entsprechenden Nachkommen einen kurzzeitigen Vorteil verschaffen. Sofern das Wachstum aber nicht in einer besseren Nahrungsverfügbarkeit münden würde, würde es die Tiere aber wahrscheinlich zusätzlich schwächen. Insgesamt waren der multigenerationale Effekt und die Effektstärke weniger ausgeprägt als in der vorherigen Studie. Dies könnte durch die Inkubation und Rückgewinnung der Partikel und die damit einhergehenden Filtrationsschritte erklärbar sein, durch die im Vergleich zur vorherigen Studie wahrscheinlich die Nanopartikelfraktion abgeschieden wurde. Eine umfassende Charakterisierung der beiden in dieser Studie verwendeten Partikeltypen zeigte in keinem der untersuchten Faktoren (Zeta-Potenzial, Biofilm-Bewuchs, Größen- und Massenverteilung, elektronenmikroskopische Untersuchung) einen Unterschied, der die Toxizität hätte erklären können. Visuell verblieb ein größerer Anteil der in Abwasser inkubierten Partikel in der Wasserphase, während die mit Ultrareinstwasser behandelten Partikel entweder auf der Oberfläche schwammen oder absanken. Die naheliegendste Erklärung, die sich mit Berichten aus der Literatur deckt, ist, dass gelöste biologische Makromoleküle aus dem Abwasser auf den Partikeln eine sogenannte Eco-Corona geformt haben, die dann die geringere Toxizität zur Folge hatte. Insgesamt konnte mit dieser Studie gezeigt werden, dass die Alterung von Partikeln in der Umwelt die Toxizität verringern kann. Im Umkehrschluss bedeutet dies, dass Studien, welche die Mikroplastik-Toxizität anhand von reinem Mikroplastik untersuchen ggf. die Toxizität im Freiland überschätzen können.

In der vierten Studie verschob sich der Fokus von individuellen Effekten auf die Populationsebene. Ziel war es hier erneut, Erkenntnisse über die Unterschiede im

toxikologischen Profil von Mikroplastik und natürlichen Partikeln gegenüber Daphnien zu sammeln. Daphnien-Populationen wurden gegenüber Mischungen aus irregulärem Polystyrol Mikroplastik und dem natürlichen Referenzpartikel Kieselgur (Diatomit) exponiert. Die Partikelzahl war dabei konstant, während die Anteile der Partikeltypen variiert wurde. Wöchentlich wurden die Anzahl und Größe aller Individuen, sowie die Zahl der Dauereier (ein deutliches Anzeichen für Stress) in den Populationen aufgenommen. Hier zeigte sich ein anfänglich starkes Populationswachstum, das seinen Höhepunkt nach zwei Wochen erreichte, gefolgt von einem steilen Rückgang der Populationsgröße. Die Behandlungsgruppen unterschieden sich vornehmlich in der Populationsgröße, welche mit zunehmendem Kieselgur-Anteil in den Partikel-Mischungen sank. Die Behandlungen, die ausschließlich bzw. vornehmlich gegenüber Mikroplastik exponiert waren, zeigten keine Abweichungen von der Kontrollgruppe. Dazu passend traten die meisten Dauereier in den Gruppen mit den höchsten Kieselgur-Anteilen auf. Effekte auf die Populations-Größenstruktur konnten nicht beobachtet werden. In dieser Studie zeigte sich, dass Mikroplastik nicht in jedem Kontext toxischer als natürliche Partikel ist. Kieselgur-Partikel sind elektronenmikroskopisch betrachtet sehr spitz und scharfkantig geformt und das Material besitzt biozide Wirksamkeit, was sicherlich zumindest einen Teil der Effekte erklärt. Unklar ist, warum identisches Mikroplastik bei fünffach höheren Konzentrationen verglichen mit den Studien 2 und 3 keine negativen Effekte zeigte. Auch hier könnte Partikel-Alterung eine Rolle gespielt haben und die unterschiedliche Effektstärke erklären. Die Partikel wurden nur wöchentlich und nicht drei Mal wöchentlich (wie in den Experimenten 2 und 3) erneuert und hatten daher länger Gelegenheit durch Interaktion von Futterpartikeln, den Daphnien selbst und deren Ausscheidungen eine Eco-Corona zu absorbieren. Auch verteilte sich die toxische Belastung auf zeitweise über 200 Individuen in der Population, verglichen mit nur einem einzelnen in den Studien 2 und 3.

Unsere Experimente konnten zeigen, dass ein Gewebetransfer von Mikroplastik für Partikel > 1 µm nicht plausibel ist und Fluoreszenz als Methode nicht alleinstehend angewandt werden sollte, um Gewebetransfer zu untersuchen. Weder Mikroplastik noch natürliche Partikel waren in unseren Experimenten durchgängig toxischer als der jeweils andere Partikeltyp. Hier können Ergebnisse, die mit einem Partikeltyp erhoben wurden, nicht auf andere übertragen werden, da die beeinflussenden Faktoren sehr divers sind und der Kontext der Exposition, der physiko-chemische

Eigenschaften der Partikel, sowie der Futterstatus der Tiere immer beachtet werden müssen. Partikel-Alterung zeigte sich als reduzierender Faktor für die Toxizität von Mikroplastik. Diese Erkenntnis sollte in die experimentelle Planung integriert werden, um der Unterschätzung der Schadwirkung vorzubeugen. Da Daphnien in der Natur einen Großteil des Jahres nahrungslimitiert sein können, erscheint es vielversprechend Experimente zur Partikel-Toxizität unter beschränkter Nahrungszufuhr durchzuführen, da sonst ebenfalls die Gefahr besteht die Schadwirkung zu unterschätzen. Die Betrachtung unsere Ergebnisse im Kontext der dynamic energy budget Theorie legt nahe, dass die Exposition mit Mikroplastik einerseits den Zugang zu Nährstoffen verringert (z.B. durch Verdünnung von Nahrungspartikeln, Wieder-Hochwürgen eines Bolus, Verringerung der Filtrationsrate) und andererseits den Energiebedarf des Tieres erhöht. Dies kann einerseits durch mechanische Verletzungen aber auch durch erhöhte körperliche Aktivität (z.B. durch verstärktes Reinigen der Filterarme von anhaftenden Partikeln) verursacht werden. Diese theoretischen Überlegungen decken sich mit Studien, die diese Mechanismen in Daphnien beschrieben haben. Die Ergebnisse deuten daher darauf hin, dass Mikroplastik in erster Linie in seiner partikulären Form zu negativen Effekten führt. Wie bereits dargestellt, ist es zum jetzigen Zeitpunkt jedoch nicht möglich, mit Polystyrol-Partikeln gewonnenen Erkenntnisse generell auf anderes Mikroplastik zu übertragen. Weitere Veröffentlichungen legen hier nahe, dass für andere Polymertypen (die z.B. auch nicht als Nahrungsmittelkontaktstoff verwendet werden) die chemische Komposition und Additive einen stärkeren Einfluss bei der Schadwirkung haben.

Nichtsdestotrotz sollten experimentelle Studien auf die Eigenschaften und Verhaltensweisen von Mikroplastik und die damit verbundenen Implikationen für das Expositionsszenario angepasst werden, beispielsweise durch Mehrgenerationen-Ansätze und Futterlimitierung. Auch sollte die Nutzbarkeit der generierten Daten zur Analyse mittels *in silico* Methoden beachtet werden, um durch Computer-Rechenleistung die Abhängigkeit von arbeitsintensiven Tierversuchen zu reduzieren.

List of Abbreviations

PE	Polyethylene
PP	Polypropylene
DEB	Dynamic energy budget
DOC	Dissolved organic carbon
EFSA	European Food Safety Authority
HFC	High-food control
ISO	International Organization for Standardization
LFC	Low-food control
MP	Microplastics
Mt	Metric tons
NOM	Natural organic matter
OECD	Organisation for Economic Co-operation and Development
PLA	Polylactic acid
PS	Polystyrene
PU	Polyurethane
PVC	Polyvinylchloride
TKTD	toxicokinetic/toxicodynamic
UV	Ultraviolet
wwMP	Wastewater-incubated microplastics (used in study 3)

Introduction

The plastics age

Since the industrial revolution, human activity has become a significant driver of global environmental change, outperforming natural processes in speed and severity. Such shifts in Earth's state are what divide geological eras (Lewis and Maslin 2015). Crutzen (2002) accordingly named the current geological epoch the "Anthropocene", with its beginning coinciding with the development of the steam engine roughly two centuries ago. The ascent of industrialization marks the point in time where human activity began impacting planetary systems to a degree that has the power to compromise their stability. In 2009, Rockström et al. established a framework of thresholds (planetary boundaries) for several Earth system processes associated with biophysical subsystems and processes (e.g., stratospheric ozone depletion, ocean acidification, global freshwater use, climate change, biodiversity loss). Crossing these thresholds is expected to force the earth outside of "*a safe operating space for humanity*", thus compromising life as we know it. Rockström et al. (2009) postulated that at least three Earth system processes are moving or have moved outside the stable Holocene state, with nine planetary boundaries at risk, including the one related to chemical pollution. In the context of planetary boundary threats, chemical pollution can be an umbrella term, but in order to quantify and assess the risk posed by it, chemicals and chemical mixtures need to be identified that are planetary boundary threats followed by an understanding of how they interact with the environment. To do so, Persson et al. (2013) postulated three criteria to categorize a chemical or mixture of chemicals as a potential planetary boundary threat: (I) The chemical or mixture of chemicals has a disruptive effect on a vital earth system process; (II) The disruptive effect is not discovered until it is or inevitably will become a problem on a planetary scale; (III) The effects of the pollutant in the environment cannot be readily reversed.

The pollution of the environment with plastics and the weathering of plastic have been recognized to result in the global exposure of the world's oceans to both plastic particles and plastic-associated chemicals (Jahnke et al. 2017). The authors concluded that currently weathering plastic debris meets at least two of the three conditions to be a planetary boundary thread defined by Persson et al. (2013) since the exposure is globally and not readily reversible due to plastic's high persistence (MacLeod et al. 2014). Accordingly, only our ignorance of the spread of the accompanying disruptive effect (or the lack thereof) of a vital Earth system process is

what stands in the way of categorizing it as a planetary boundary threat (Jahnke et al. 2017). In addition to being a valid threat plastic pollution is a symptom of a complex and intertwined set of problems in modern society.

Chemically, plastics are synthetic polymers consisting of chains of subunits (monomers). The type and properties of the monomer in combination with chemical additives give plastic materials their endless variety and applicability. Chemicals associated with plastics include intentionally added substances that are used to achieve material properties, such as color, toughness, flexibility, radiation resistance, and heat durability as well as unintentionally added substances that are impurities or side products of the manufacturing process (Groh et al. 2019). Polymers do occur naturally, such as cellulose and chitin, constituting plant cell walls and arthropod exoskeletons, respectively. However, these natural polymers are more limited in their usability. The first fully synthetic polymer was Bakelite, synthesized by Leo Baekeland in 1907 (Freinkel 2011), thus marking the beginning of the plastics age and with that the beginning of a major transformation in human society. Like many technical advancements, the development and widespread adoption of plastics was fueled by conflict. The attempt to substitute costly and limited “strategic materials” (e.g., aluminum, brass) with lightweight and cheap plastics increased innovation to such a degree that following the second world war anything could be made from plastics (Freinkel 2011).

Human culture and social evolution are largely driven by our ability to create and inherit narratives that drive our collective behavior (Harari 2015). The story behind plastic is no exception. It offered an escape from the archaic dependence on natural resources like timber and the human self-image of transcending nature by producing and shaping things with properties that could not be achieved from non-synthetic materials. In the process, plastic products granted a higher degree of perceived egalitarianism by making products that were considered luxury items available to a wider consumer base (examples here include billiard balls made from ivory and combs made from tortoiseshell; Freinkel, 2011). Economic interests in turn were also the driver behind the wide-ranging adoption of polyethylene (PE) and polypropylene (PP), since their respective feedstocks are byproducts of oil refinement, making the process more economically viable through the commercialization of formerly useless resources.

By the early 1970s, the production volume of plastic products surpassed that of steel (Freinkel 2011) through innovations and optimized manufacturing. With that came the spread throughout all aspects of everyday life, revolutionizing aviation, medicine, and packaging. The combination of low cost, durability, light weight, and endless moldability can be considered both a blessing and a curse. Today, pretty much every aspect of everyday life is based on plastics. The majority of plastic products are packaging materials, hence discarded after single use (PlasticsEurope 2020). Product design and the accompanying lifecycle considerations are largely a one-way street (McDonough and Braungart 2009) without considering the recovery and re-use of resources. This leads to the systemic lack of sustainable management and to wasting resources. As of 2015, only 9 % of the approximately 6300 million metric tons of plastic waste ever produced (out of 8300 Mt of virgin plastics ever produced) had been recycled, 12 % incinerated, and 79 % ended up in landfills or the natural environment (Geyer et al. 2017). Currently, the alternative to producing energy from plastic materials ("energy recovery", incineration) is to create products of lower quality ("down-cycling"), which is not a sustainable solution since new feedstock needs to be added and resources are irreversibly lost (McDonough and Braungart 2009). This is, to a certain degree, owed to the heterogeneity of plastic products and use cases on the market as well as the lack of transparency throughout the production process regarding feedstock material, and additive composition (Sattlegger et al. 2020). Downstream of plastic use functioning and efficient recycling systems on a global scale are the exception rather than the norm (Jambeck et al. 2015).

Data on waste management and discharge are lacking and difficult to collect, especially in developing countries with inadequate infrastructure and documentation. In their report in Science, Jambeck et al. (2015) estimated that in 2010 275 million metric tons of plastic waste was generated in 192 coastal countries of which 4.8 to 12.7 million entered the ocean. Their report concludes that a decrease in mismanaged waste could substantially reduce the inflow of plastic waste into the oceans. However, notwithstanding massive advances in waste management, waste discharge is going to rise in correlation with increasing plastic production (Borrelle et al. 2020). Concurrently, the long lifespan of plastic leads to its accumulation in the environment, with the ocean being a major sink (Woodall et al. 2014).

Freshwater systems as transport ways to the ocean are equally affected by the inflow of plastic waste. Inadequate end-of-life treatment of plastic waste as well as

wastewater treatment plant outlets and road and agricultural runoff result in the pollution of rivers with plastics. Plastic waste outnumbered fish larval biomass in the Austrian Danube river in the two years surveyed by Lechner et al.(2014). Estimates arrived at 1.15 to 2.41 million metric tons of plastic waste yearly entering the ocean with the top 20 polluting rivers accounting for 67 % of the global total (Lebreton et al. 2017). A study on Lake Garda showed the highest abundance of PS (45.6 %) and PE (43.1 %) with fragments originating from the breakdown of larger particles making up the majority of collected particles. Point sources, such as industrial areas contribute significantly to the abundance of industrial pellets, making plastic concentrations highly varying on a local scale. Accordingly, Dris et al. (2015) reviewed the literature on freshwater ecosystems and found variations along a factor of 1000 across the reviewed studies on plastic particle concentrations in lake shore and riverbank sediments. Notwithstanding the widespread contamination of freshwater systems with plastic debris, research efforts have neglected occurrence, fate, and impacts in these ecosystems (Dris et al. 2015).

As Freinkel (2011) put it: "*We take natural substances created over millions of years, fashion them into products designed for a few minutes' use, and then return them to the planet as litter that we've engineered to never go away*".

Plastic at every size: From macro to micro to nano

As long-lived and durable as plastics are during their useful life they slowly break down once they enter the environment when becoming exposed to many biotic and abiotic influences. Degradation depends on the chemical composition of each polymer type. Gewert et al. (2015) reviewed the pathways for the degradation of plastic polymers in the marine environment and concluded that polymers like PE, PP, polystyrene (PS), and polyvinylchloride (PVC) are likely degraded through abiotic factors before microbial degradation. More specifically, PE, PP, and PS are prone to photo-initiated oxidative degradation through the formation of carboxylic end-groups while PVC gets dechlorinated through ultraviolet (UV) light. Likewise, the passage through organisms' digestive systems can fragment particles (Dawson et al. 2018; Mateos-Cárdenas et al. 2020). The polymer fragments formed through these initial processes can then be biodegraded.

Not all microplastics (plastic particles < 1000 µm; Hartmann et al., 2019) are generated from the breakdown of larger pieces: a small minority is intentionally

produced in that small size range (Burns and Boxall 2018). These often spherical particles are used in cosmetics as defoliates or abrasives in dental hygiene products or are nurdles that are the raw material that is used in mold plastic products. While they are referred to as primary microplastics, microplastic particles that result from the breakdown of larger plastic pieces are called secondary microplastics. Here, a dispute whether tire wear particles should be considered secondary microplastics is ongoing (Sommer et al. 2018; Hüffer et al. 2019; Luo et al. 2021; Hartmann et al. 2019). The vast majority of particles reported in the environment are secondary microplastics, so the relevance of regulating primary microplastics has been discussed as well (Burton 2015).

Additives dictate the properties of individual plastic types as required by their use case and are thus an important factor for material integrity in the environment. Likewise, the chemicals released during degradation are not only monomers and oligomers but part of a "*diverse contaminant suite*" (Rochman et al. 2019). The released additives and their degradation products contribute to the chemical pollution originating from plastic material in the environment (Gewert et al. 2015). Chemicals from plastics have been shown to have toxicological relevance across biological levels (e.g., on daphnids and *in vitro*; Lithner, 2011; Zimmermann et al., 2019, 2020). Thus, the continuous growth in plastic production and inflow into the environment is cumulating in a substantial toxicity debt (Koelmans et al. 2017; Rillig et al. 2021).

The interplay of the abiotic degradation mechanisms described above with mechanical forces like abrasion not only leads to the release of monomers and oligomers but to the formation of small particles, for which Thompson et al. (2004) coined the term "micro-plastics" (MP). First reports of the occurrence of small plastic particles in the aquatic environment date back to the early 1970s (Carpenter and Smith 1972), shortly followed by reports of the ingestion of plastics by various fish species (Carpenter et al. 1972). A common vocabulary for the different particle sizes has emerged as a crucial basis for the communication between disciplines involved with research. While particle concentrations drive encounter rates with organisms and thus are the important metric for ecotoxicology, analytics is usually concerned with mass concentrations. The debate about what size ranges and stock materials qualify a particle as microplastic therefore is ongoing, with long-standing definitions encompassing everything below a diameter of 5 mm (Andrady 2011). Since this cutoff

is quite arbitrary, alternatives were discussed (Figure 1), with Hartmann et al. (2019) proposing a categorization framework with six (plus one optional) criteria for plastic debris. This framework includes the size ranges of nanoplastics at 1 to < 1000 nm, microplastics at 1 to < 1000 µm, mesoplastics at 1 to < 10 mm, and macroplastics at 1 cm and larger. This definition will be used throughout this thesis when specifically referring to nanoplastics, while microplastics will be used for particles < 1 mm of size, including nanoplastics.

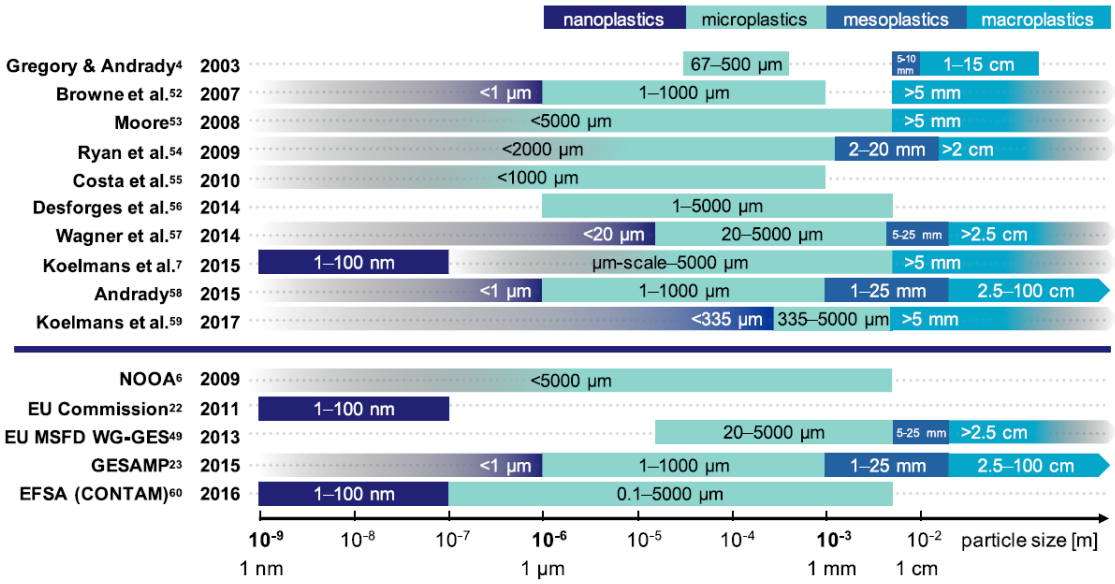

Figure 1: Examples of differences in the categorization of plastic debris according to size as applied (and/or defined) in scientific literature and in institutional reports. It should be noted that this does not represent an exhaustive overview of all used size classes. Figure from Hartmann et al. (2019).

The need for clearer definitions is driven by improvements in analytical capabilities (reviewed in Anger et al., 2018) and the desire for a better understanding of biota-particle interactions (reviewed for freshwater biota in Scherer et al., 2018). The latter is largely dictated by the particle size (Figure 2). Even though all microplastics appear more or less small to the human perception it is vastly different when put into a context with different biota. The ingestion of particles follows individual size preferences and is largely dependent on the feeding type, which also determines the degree of selectivity of a species for the size and type of particle (Scherer et al. 2018).

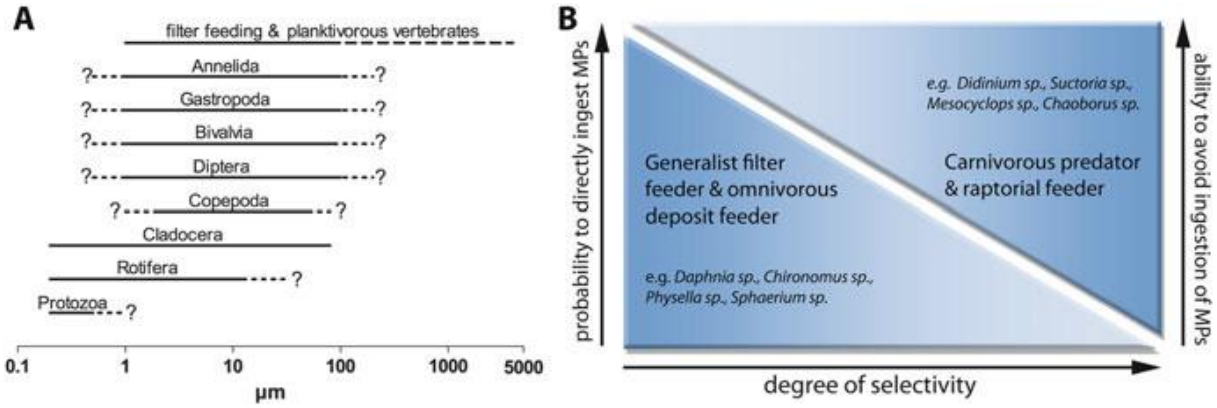

Figure 2: Estimated feeding size ranges on microplastic particles (a). Dotted lines and question marks indicate the lack of min to max limits based on ingested size classes. An increasing feeding selectivity decreases the probability to directly ingest microplastics (b). Figure from Scherer et al. (2018).

Microplastics and biota

Microplastics can interact with aquatic organisms of different trophic levels and sizes, ranging from primary producers, such as algae (Chae et al. 2018), to primary consumers, such as zooplankton (Ogonowski et al. 2016; Rist et al. 2017) to secondary consumers such as Osteichthyes (Y. Liu et al. 2019) and Cetaceans (Lusher et al. 2015). The fragmentation releases particles down to the nanometer range (Lambert and Wagner 2016a; Lambert and Wagner 2016b), thus creating particles that are available to all kinds of animals to ingest, and to some degree to cross biological membranes (Triebskorn et al. 2018).

Gouin et al. (2019) categorized the, according to them, plausible published effects of microplastics towards biota into two categories: (a) reduction of the ability of an organism to access nutrients leading to downstream effects on energy budget and (b) physical damage within or on to the surface of an organism leading to a variety of effects including stress, impacts of growth and reproduction, and mortality. This was later expanded to differentiate between internal and external physical damage as two independent mechanisms (de Ruijter et al. 2020). Interactions of biota with particles are largely dependent on the size-relation between the two. The ratio dictates whether a particle can only interact with the organismal surface, is readily ingestible, or can cross biological barriers into tissues.

A review paper not included in this thesis introduced the concept of tissue translocation as a prerequisite for inflammation in tissues and summarized the

literature available on the tissue translocation of micro- and nanoplastics in aquatic invertebrates and fish (Triebskorn et al. 2018). The transfer across biological membranes was reported in daphnids as early as 2009 (Rosenkranz et al. 2009). Likewise, several studies have reported the translocation of particles in other aquatic invertebrates as well as fish. Of the 62 particle types tested across 31 reviewed studies, 74 % were spherical. The dominant methods to investigate tissue translocation were fluorescence-based and usually relied on visual identification of a fluorescence signal, either in the whole specimen, in histological samples, or after digestion of the target tissue. Overall, the processes that could lead to the translocation of particles are only understood on a theoretical level and have not been observed *in vivo*. The review by Triebskorn et al. concludes that tissue translocation could likely happen through three mechanisms, is unlikely for particles smaller than those in the low micrometer scale, and is prone to false-positive and false-negative results.

Nutrition-related effects and the direct effects of inflammation and necrosis are mechanisms through which microplastics could harm organisms (de Ruijter et al. 2020). In the case of daphnids that could mean both, a "food dilution" (i.e., a mixture of food and non-food particles has a lower nutrient density compared to only food particles) and an increased energetic demands caused by behavioral adaptations to the presence of microplastics and other non-food particles. Inflammatory processes can also increase energetic demand to retain homeostasis. All this energetic drainage can manifest through effects on various endpoints commonly observed in ecotoxicity studies: mortality, reproduction, behavior, and growth.

Daphnia and microplastics

Animals from the genus *Daphnia* are likely the top species that to date have been used for microplastics effect studies. As of June 2021, PubMed lists 97 entries for the query "*(microplastic* OR nanoplastic*) AND (daphnia)*". The most common test species being the large waterflea *Daphnia magna* (used in this thesis) and the smaller species *Daphnia pulex*. The small crustaceans are found in most aquatic habitats and occupy a key position in aquatic ecosystems, both as primary consumers of algae and bacteria as well as important prey (Thorp et al. 2016). A parthenogenetic reproduction, short lifespan, and simple nutritional requirements have made daphnids a well-researched and widely-used model organism in general

ecotoxicology[1], evolutionary ecotoxicology (Jansen et al. 2011), and ecotoxicogenomics (Lee et al. 2019). Generally, daphnids as filter-feeding organisms indiscriminately ingest particles of up to 70-90 µm (Scherer et al. 2018), potentially making them susceptible to ingest microplastics as well as other non-food particles (Bilotta and Brazier 2008). The lower threshold of ingestion is unclear since, in addition to feeding, "*drinking*" and rectal water intake can lead to particle ingestion (Smirnov 2017). An abundance of data is available on acute toxicity of microplastics towards *Daphnia* species (≤ 96 h of exposure). Most studies used commercially available particles, often fluorescently labeled (Triebskorn et al. 2018). These are not representative of commonly found particle types in the environment. Thus, the use of fluorescently labeled particles does not translate well to environmental exposure scenarios (Burns and Boxall 2018).

The prevalence of studies with commercial and fluorescently labeled particles comes with potential pitfalls if not coupled with adequate controls to account for effects caused by the formulation (i.e., substance to ensure dispersion or prevent microorganism growth). For example, early microplastics studies were conducted without the awareness that the presence of sodium azide in microplastic suspensions, a biocidal agent to counter bacterial growth, by itself can affect the test organisms (Pikuda et al. 2018). Likewise, the presence of fluorescence was used as proof of particle presence, neglecting that the fluorescent dye could dissociate from the particle and cause these observations (Annex 1, Catarino et al. (2019)). Accordingly, the use of commercial particle formulations and the opacity about their constituents pose a problem to control and reproducibility. Nonetheless, standardized methods for the production and application of representative microplastics are currently lacking in the scientific literature.

Several effect mechanisms of microplastics have been hypothesized in daphnids. Immediate harm could be caused by clogging and damage to the gastrointestinal tract (Ogonowski et al. 2016). This would only follow the ingestion of particles, but ingestion is not a prerequisite for negative effects. At least two studies reported a reduction in feeding in the presence of microplastics (Ogonowski et al. 2016; Rist et al. 2017). Reduced nutrient uptake could then indirectly affect apical endpoints such

[1] This includes two test guidelines by the Organisation for Economic Co-operation and Development, OECD (OECD 2004; OECD 2012) and by the International Organization for Standardization, ISO (ISO 2018)

as mortality, growth, reproduction, and swimming behavior that are most prominently described in the literature but allow little to no insight into concrete underlying modes of action (Miracle and Ankley 2005). Interestingly, effects that are a result of reduced algal uptake, dilution of food particles by microplastics, or increased energetic demands (e.g., through increased movement of feeding appendages to clean them of particles) would likely be masked through an overabundance of food as is commonly provided in experiments. One example here is the OECD guideline for the *Daphnia magna* reproduction test (OECD 2012). Individual effects would then translate to the population level, for example through a decrease in fitness or growth and as a consequence competitiveness with other species, potentially affecting a whole ecosystem. Here, a discrepancy can be found in the design of studies on the effects of microplastics in daphnia: Daphnids have generation times of days to weeks and microplastics show a high persistence of decades to millennia. Therefore, exposure to microplastics in nature can be considered more or less continuous throughout an organism's lifetime. Here, multigenerational, and population-focused study designs could help to bridge the gap in time scales by exposing animals throughout their lifespan and for multiple generations. Nonetheless, they are generally scarce in microplastics ecotoxicology. Only a few studies such as Bosker et al. (2019) looked at population development under MP exposure. Martins and Guilhermino (2018), as well as Xu et al. (2020), performed experiments with multigenerational and long-term setups. Their findings underline that broadening the perspective beyond a single generation can yield insights on multigenerational effects as well as population recovery. The paucity regarding this kind of study design is likely due to the youthfulness of the research interest in microplastics in general and the laborious nature of this kind of study.

The mechanisms summarized by Gouin et al. (2019) are just as true for naturally occurring particles as they are for microplastics (Kirk 1991a; Bilotta and Brazier 2008; Robinson and Klaine 2008). Therefore, it is of fundamental importance to know whether microplastics possess unique properties that distinguish their toxicity profile from that of natural particles or whether they are “just another particle”. In accordance with the considerations of Gouin et al. (2019), I hypothesized that the impact of microplastics on daphnids follows multiple mechanisms and is likely related to the particulate nature more than properties unique to microplastics. This was addressed in two studies that are part of this dissertation (Annex 2 + 4).

Effects of particle aging

The fate of particles in the environment is largely determined by particle properties (Karami 2017). These can be altered through "aging" in the environment. In this case, aging can be understood as the deviation from original material properties through material degradation as well as the surface modification through the formation of eco-coronae and biofilms. Weathering occurs on timescales of days to years, whereas some processes affect some polymers more than others (Gewert et al. 2015). While the effects of UV radiation on plastics and the release of associated chemicals have been studied more widely (Bejgarn et al. 2015; Gewert et al. 2021; Klein et al. 2021; Gewert et al. 2018), the effects caused by the formation of an eco-/bio-corona[2] is not well understood. Corona acquisition can be distinguished from biofilm formation since for the former biological macromolecules sorb to the particle surface and for the latter microorganisms grow on the surface. Factoring in both corona and biofilm formation as pre-treatments for ecotoxicity testing of particles has been discussed as another advancement towards more realistic exposure scenarios, since the environment is never devoid of biological macromolecules, such as dissolved organic carbon (DOC) (Sobek et al. 2007) and microplastics are quickly colonized by microorganisms (Glaser 2020). Since both biofilm- and eco-corona-formation are possible mechanisms to modulate microplastics toxicity, paper 3 (Annex 3) evaluated if the sorption of DOC alters the toxicity of irregular microplastics.

[2] The terms are used interchangeably in literature.

How "realistic" is microplastics research?

Given the outlined shortcomings in the microplastics literature at the beginning of this thesis, this work aims to contribute to a better understanding of the factors that influence the toxicity of microplastics towards daphnids and improve upon the current approaches utilized to assess particle toxicity. The insights gained are related to:

- Use of fluorescent spherical microplastics to investigate tissue translocation without accounting for dye dissociation
- Lack of insights into multi-generational and population effects for organisms with short generation time
- Limited knowledge about the differences of microplastics and natural particles
- Ignorance about the effects of food limitation on microplastics toxicity
- Lack of knowledge about the effects of particle aging on toxicity

These points are addressed throughout the publications that constitute this thesis.

Aims and objectives of this thesis

The overarching aim of this thesis was to further the understanding of factors that influence the ecotoxicological potential of microplastics towards daphnids. Deficits are the mismatch between lack of realistic scenarios (use of unstably labeled fluorescent particles, short exposure durations, an overabundance of food, lack of natural reference material use, use of only pristine microplastics) and considerations about the special properties and behaviors elicited by microplastics in a test system. The steps towards a higher degree of realism were taken through four studies, three of which are published in peer-reviewed journals (Annex 1-3), and one is appended under "additional results" (Annex 4). Five main goals are covered within these studies about which we aimed to further our understanding: (1) Tissue translocation of spherical microplastics; (2) Toxicity of irregular microplastics; (3) Multigenerational and population effects of microplastics; (4) Comparison of the toxicity of microplastics and natural particles; and (5) Effects of aging on microplastics toxicity. These topics were sometimes covered in multiple studies to varying degrees (Figure 3).

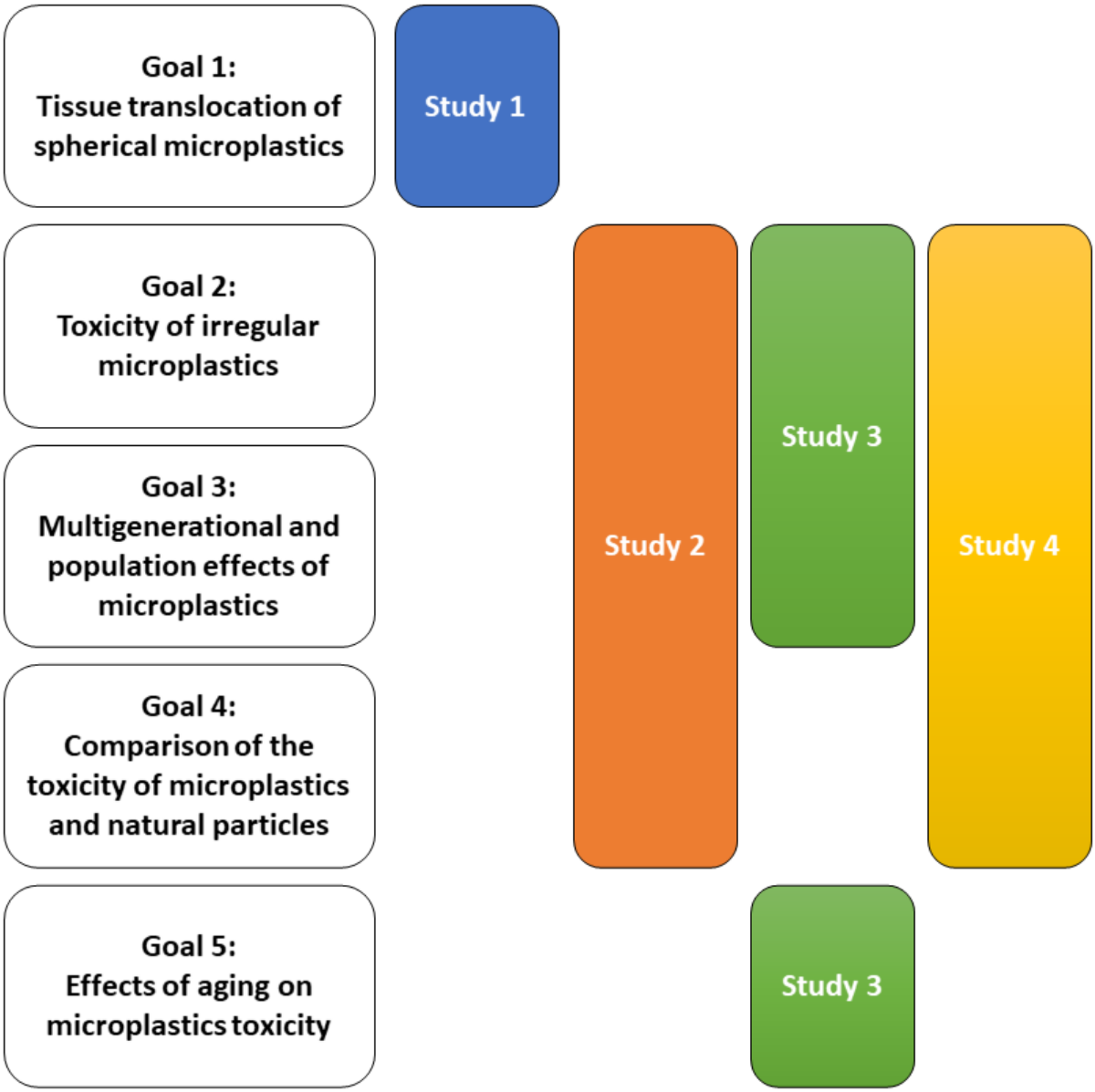

Figure 3: Overview of the main goals covered by the studies comprising this thesis (Annex 1-4).

The first study (Annex 1) was aimed at better understanding the mechanisms behind tissue translocation in *D. magna*. A previous study by Rosenkranz et al. (2009) had reported the tissue translocation of spherical, fluorescently labeled microplastics (20 and 1000 nm) to lipid storage droplets, indicated by a fluorescence signal in the tissue. Since this is not biologically plausible, we sought to gain a better understanding of the underlying biological mechanisms by replicating the study. At first, this was unsuccessful, even after the introduction of an additional specimen treatment protocol to improve visibility. Subsequently, we replicated their findings by using higher particle concentrations. Nonetheless, our observations questioned their conclusions and we conducted an additional experiment with a passive-sampling

approach. We were able to conclude that their findings were a microscopic artifact caused by the uncoupling of the fluorescent dye from the particles.

The goal of the second study (Annex 2) was to investigate whether the toxicity profile of irregular microplastics differs from that of natural reference particles. This was done through the adaptation of a standardized test guideline into a multigenerational design and adding food limitation as an additional stressor. Additionally, changes in sensitivity of the offspring of particle-exposed animals towards a reference chemical were measured as a proxy for individual fitness.

The third study (Annex 3) expanded on the previous multigenerational design to assess differences in toxicity between pristine irregular microplastics (incubated only in ultrapure water) and particles from the same batch that were incubated in filtrated wastewater. This was done to understand whether the toxicity of pristine microplastics differs from that of particles aged in the environment (e.g., through contact with biological macromolecules, such as DOC). Additionally, growth curves were used to investigate the effects of microplastics and food restriction on the adaptive strategies of daphnids.

Lastly, the fourth study (Annex 4) aimed to understand the effects of microplastics and natural particles on the population level. Daphnid population development and size structure under exposure to mixtures of microplastics and natural particles were recorded over 50 days. Here, food limitation again was used as an additional stressor to the populations. This study is included as additional results and is not published elsewhere.

As stated earlier, the goals of the studies overlapped to some degree, as studies 2-4 (Annex 2-4) all included irregular microplastics (Goal 2, Figure 3). In studies 2 and 3 we used a multigenerational design, while study 4 was conducted with the focus on the population level (Goal 3, Figure 3). Both studies 2 and 4 (Annex 2 + 4) compared the toxicity of microplastics to that of natural particles (kaolin in study 2; kieselguhr in study 4), while study 3 (Annex 3) compared the toxicity of aged and pristine microplastics. Here, evaluating the goals in different contexts allows for an evaluation between different scenarios. Throughout the thesis, measures to increase realism in microplastics ecotoxicology towards daphnids are critically evaluated and recommendations are given.

The following peer-reviewed publications are part of this dissertation:

A1 Schür C, Rist S, Baun A, Mayer P, Hartmann NB, Wagner M. 2019. When fluorescence is not a particle: the tissue translocation of microplastics in *Daphnia magna* seems an artifact. Environmental Toxicology and Chemistry.: 10.1002/etc.4436.

A2 Schür C, Zipp S, Thalau T, Wagner M. 2020. Microplastics but not natural particles induce multigenerational effects in *Daphnia magna*. Environmental Pollution. 260: 10.1016/j.envpol.2019.113904.

A3 Schür C, Weil C, Baum M, Wallraff J, Schreier M, Oehlmann J, Wagner M. 2021. Incubation in Wastewater Reduces the Multigenerational Effects of Microplastics in *Daphnia magna*. Environmental Science and Technology: 10.1021/acs.est.0c07911.

General Discussion

Summary of the main findings

The aim of this thesis is to critically reflect on methodological approaches currently employed in microplastics research and to offer guidance towards improved and more meaningful research. The first study of this thesis (Annex 1) questioned the biological plausibility of previous research that reported the translocation of fluorescent micro- and nanoplastics to lipid storage droplets in *D. magna* (Rosenkranz et al. 2009). The replication of the previous results using the original setup was unsuccessful, while higher particle concentrations led to comparable observations (i.e., fluorescence in lipid storage droplets and gut; Figures 2-4 in Annex 1). Nonetheless, fluorescent particles were clearly the origin of the fluorescence signal in the gut, while no particles were observable in the lipid droplets. This sparked the hypothesis that the reported tissue translocation was actually an artifact caused by the uncoupling of the fluorescent dye (fluorescein isothiocyanate) from the particles. The lipophilic dye subsequently transferred to the lipid droplets leading to the observed fluorescence there. An additional experiment with a novel passive sampling approach supported this notion. Particles were incubated with a silicone rubber strip under different conditions after which the rubber was fluorescent microscopically examined. It concurrently proved that the dye leaching occurred and is independent of the organism (i.e., not caused by digestive processes). Accordingly, the use of adequate controls instead of relying on fluorescence as a proxy for particle translocation is essential. Nonetheless, it is biologically plausible for nano-sized particles to penetrate biological membranes, even though analytical challenges for their detection remain.

The second study (Annex 2) compared effects of irregular microplastics with a natural reference material on individual daphnids. Herein we expanded the chronic toxicity test issued by the OECD (OECD 2012) to a four-generation multigenerational design. This was done to cover effects beyond a single generation and investigate how they affect the offspring, both immediately (as measured by acute sensitivity against a reference chemical) and through apical endpoints recorded during subsequent generations after chronic exposure. These experiments were performed under continued food limitation to more closely mimic environmental exposure scenarios. The results showed a concentration-dependent effect of the microplastics on survival and reproduction of exposed individuals, which to some extent increased over the generations. No effects were observed for the natural particles. The exposure to the

two highest microplastics concentrations (10,000 and 2,000 particles mL^{-1}) led to the extinction of the respective treatment groups, within one and four generations, respectively. These differences can, at least partly, be explained by physicochemical properties of the particles, such as density, and resulting behavior of the particles during exposure. Nonetheless, the study showed that, under the applied conditions, microplastics were more toxic than natural particles. Additionally, the multigenerational approach under food limitation illuminated effects that emerged only after several generations and led to the extinction of whole treatment groups, albeit at high particle concentrations. Therefore, the investigation of multiple exposed generations appears to be a legitimate approach to study this kind of emergent effect.

The third study (Annex 3) expanded on the idea of the multigenerational approach to investigate if pristine irregular plastic particles can be compared to particles that underwent environmental aging. This was done by using microplastics from the same batch that were either aged in filtrated wastewater or incubated in ultrapure water. Through the incubation in wastewater, we mimicked environmental aging through the acquisition of an eco-corona from the surrounding medium. We found that both particle types produced concentration- and time-dependent effects in daphnids on reproduction and growth but different effects on survival. Interestingly, even though the particles were prepared from the same raw material and in a similar manner as in the previous study (Annex 2), the toxicity was markedly lower. Extensive particle characterization in this chapter did not yield insights into specific properties that could explain the difference in toxic potential between aged and pristine microplastics. However, the sorption of DOC was hypothesized to mediate the toxicity.

The additional results (Annex 4) move the focus up to effects on the population level. Daphnid populations were exposed to a fixed particle concentration of mixtures of PS microplastics and the natural particle kieselguhr. Population size, size structure (i.e., animals were measured and assigned to size classes), and resting egg (*ephippiae*) formation were monitored weekly. The experiment ran for 50 days and we also used food limitation as an additional stressor. All populations grew in a comparable pattern with an initial growth phase followed by a decline in population size. Particle exposure affected both population size and resting egg formation. Effects were more severe when more kieselguhr was present in the particle mixture, while exposure to 100 % microplastics did not affect population growth or resting egg formation.

Ephippiae occurred in all populations, including controls, indicating that all conditions were stressful to the populations. Potential reasons here are a combination of food scarcity and population density. Population size structures appeared unaffected by particle exposure. Overall, in this experimental setup, natural particles had a greater effect on *Daphnia* populations than microplastics.

Overall, the studies comprising this thesis could show aspects that are critically understudied and to some degree ignored in the current body of knowledge on the ecotoxicity of microplastics towards daphnids. The presence of a fluorescence signal should not be used as the sole proxy for particle presence when studying tissue translocation due to the danger of leaching dye producing microscopic artifacts. Multigenerational and population experiments with food limitation can illuminate effects that would not be visible in short-term experiments. Effects of both microplastics and natural particles cannot be generalized for one particle type: In study 2 (Annex 2) microplastics were more toxic than natural particles, while in study 4 (Annex 4) the reverse was true. Particle aging, as occurs in the environment, reduced the effect of irregular microplastics on mortality, likely through the sorption of DOC from the medium. Trying to understand effects based on short-term experiments likely underestimates the toxicity while exposing daphnids to only pristine particles overestimates it. Here, the context of exposure is critical to understand particle-biota interactions.

Ingestion & tissue transfer

Tissue translocation is a prerequisite to mechanical effects on the tissue level. For a particle to elicit an acute inflammatory response in a tissue it must interact and damage it. To interact with a tissue beyond the immediate body surfaces (including the gastrointestinal tract), the particle first has to cross biological barriers and translocate inside the body (Figure 4). Translocation of particles to the lipid droplets or other regions inside the daphnid would have to follow a certain chain of events: 1) ingestion of particles, 2) passage across the peritrophic membrane, 3) transfer across the epithelium of the digestive tract, and 4) transport to the target tissue. For filter feeders, such as *Daphnia* species, active ingestion of particles depends on the morphology of the filter apparatus. More specifically, it depends on the distance of setae at the thoracic limbs, which ranges from 0.24 to 1.25 µm in *D. magna*. This distance changes during development and with food availability (Brendelberger and

Geller 1985; Lampert and Brendelberger 1996). Particles in the size range between 0.2 to 75 µm can be ingested (Scherer et al. 2018). Accordingly, particles < 200 nm are too small to actively ingest by filtration. However, several researchers observed the presence of smaller plastic particles in the gut of daphnids (Casado et al. 2013; Rist et al. 2017). Thus, other uptake mechanisms may contribute to nanoparticle uptake, including electrostatic attraction, active and passive "drinking", and uptake via the anus (Smirnov 2017).

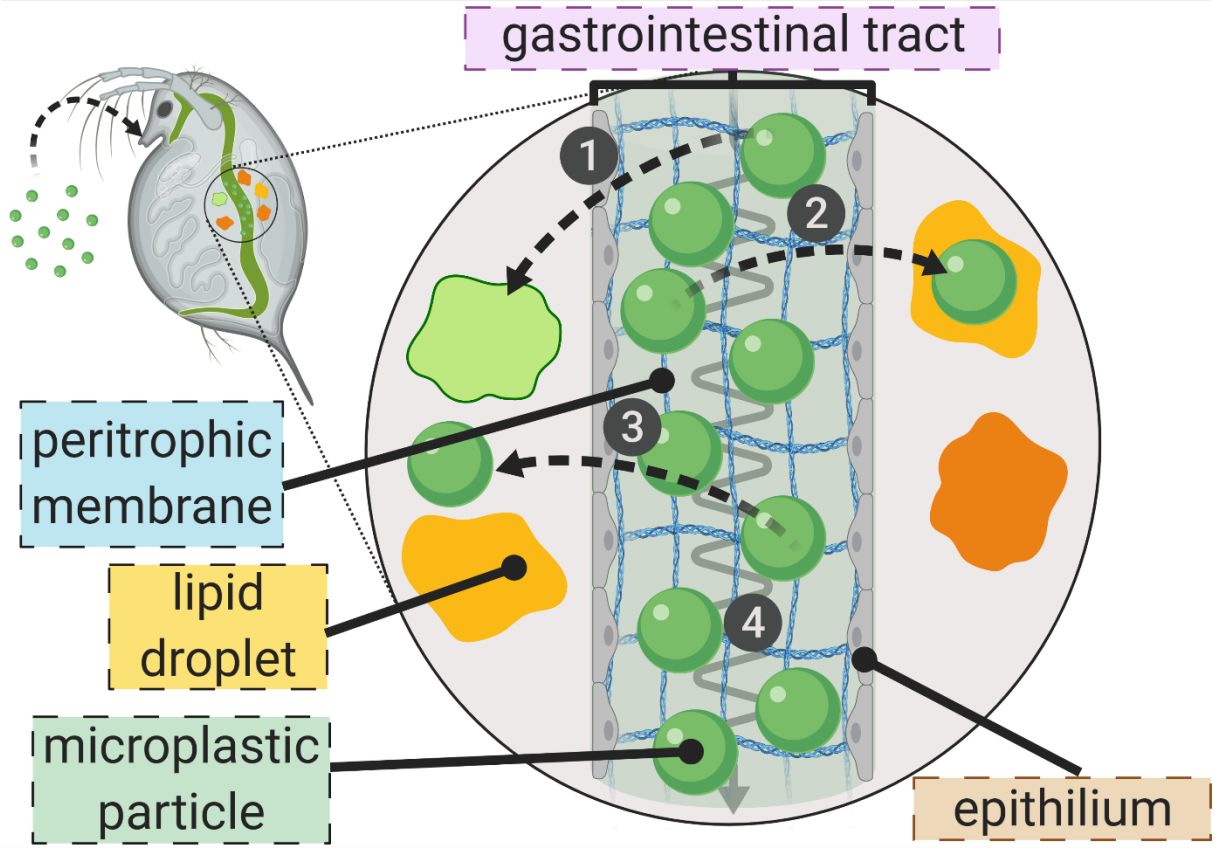

Figure 4: Potential pathways for microplastics ingested by daphnids: (1) the fluorescent dye can uncouple from the labeled particle and transfer to a different compartment, resulting in detection of a fluorescence signal in the tissue; (2) Particle translocates to lipid droplets resulting in the detection of a fluorescence signal in the tissue; (3) Particle crosses the epithelium (actively or passively) resulting in the detection of a fluorescence signal in the tissue ; (4)Particle does not cross the epithelium and passes through the intestinal tract without fluorescence being detected outside the intestines.

After ingestion, the food bolus is surrounded by the peritrophic membrane, a chitinous layer separating food and the midgut epithelium (Schultz and Kennedy 1976; Lehane 1997; Hansen and Peters 1998). The membrane reportedly is permeable for 130 nm but not for 327 nm latex beads (Hansen and Peters 1998). It thus prevents the uptake of larger particles via the epithelium through size exclusion. Once smaller particles have passed the peritrophic membrane and are in contact with the epithelium, cellular uptake depends on particle size, concentration, surface modification, and charge. The size of the smaller particles used in study 1 (20 nm, Annex 1) permits cellular uptake, and their negative surface charge enhances the attachment to cell surfaces (Zhu et al. 2013). Particles in the low nanometer range can passively cross membranes (Zhu et al. 2013). Currently, two additional mechanisms for the transfer of particles across epithelia are being discussed, namely 1) endocytosis, probably limited to particles < 1 μm, and 2) persorption limited to particles ≤ 150 μm (Wright and Kelly 2017). The latter has been reported in vertebrates but appears to be a rare occurrence (Volkheimer 1974). Importantly, an epithelial transfer can be facilitated by radiation and chemicals (Carr et al. 2012), tissue damage, and the loss of cells (Powell et al. 2010). The latter was observed by Mattsson et al. (2016) in the context of nanowire penetration of *Daphnia* epithelia.

Following uptake by epithelial cells, an active transport mechanism would need to transfer the particles to the lipid droplets. The lipid storage in Cladocerans is not well understood. Lipid droplets are scattered throughout the body but most prominently ventral to the gut. It has been postulated that fat globules and other particles are transported by blood cells and by minute drops (Smirnov 2017). However, these observations have been made more than a century ago. Thus, an updated understanding of the lipid assimilation, circulation, and storage in daphnids would help to interpret the potential tissue transfer of particles. Nonetheless, the peritrophic membrane remains the major morphological barrier preventing particles larger than 300 nm from entering the body of Cladocerans. Accordingly, based on current knowledge, reports on the tissue transfer of larger plastics are biologically implausible. Nonetheless, we cannot exclude the possibility that small nanoparticles can cross biological membranes, even though active transport mechanisms towards lipid droplets following the epithelial transfer are improbable.

Since the publication of paper 1 (Annex 1), tissue transfer and the pitfalls involved in its investigation have gained more attention (Gouin 2020). Catarino et al. (2019)

have arrived at similar conclusions to ours regarding the leaching of dye from commercial particles. They showed fluorescence in fatty tissues such as the yolk sack of zebrafish (*Danio rerio*) embryos after exposure to fluorescently labeled particles. Dialysis of particle suspensions before exposure eliminated the fluorescence in the tissue, leading to the conclusion that it was caused by dissolved dye. They have proposed the use of dialysis as a pre-treatment to clean particle suspensions before application in test systems. Other researchers demonstrated that dialysis should generally be used to avoid false-positive results in ecotoxicity studies with commercial microplastics caused by the additives. Here, nanoplastic formulations caused both *in vitro* and *in vivo* toxicity, while dialyzed particle suspensions produced no effects (Heinlaan et al. 2020). Both the cleanup step by Catarino et al. and the control via passive sampling used in paper 1 (Annex 1) were recently adopted by Xu et al. (2020) in a study on the effects of primary and secondary particles in *D. magna*. Other researchers have either treated their particles to remove potentially dissolved dye from the suspensions (Yu et al. 2020) or included additional methods (e.g., visual confirmation of particle presence) to enhance the validity of their observations (Mateos-Cárdenas et al. 2020; Zeytin et al. 2020). These improvements are immediately linked with the publication of our findings. Therefore, the findings presented in our study (Annex 1) have significantly impacted how research into tissue translocation and/or with fluorescently labeled particles is approached.

Microplastics vs. natural particles

The question of whether microplastics have unique properties (i.e. toxicological profile) that make them more harmful than naturally occurring particles has long been a point of uncertainty (Ogonowski et al. 2016; Scherer et al. 2018; Backhaus and Wagner 2019). The negative effects of suspended solids on biota are fairly well-studied because events like remobilization of sediments during floods or surface runoff happen frequently and can lead to a rapid input of suspended solids into an ecosystem. This then greatly affects the organisms in this system, potentially having grave ecological consequences. The magnitude of effects of suspended solids towards individual species depends on concentration, exposure duration, chemical composition, and particle size distribution (Bilotta and Brazier 2008). As Scherer et al. (2018) postulated: "*Only the simultaneous investigation and direct comparison of the toxicity of natural and polymeric particles will enable discovering specific MP* [microplastics]*-associated risks in the diversity of particulate matter. In the absence*

of this reference, adverse effects of MPs observed in the laboratory could be nothing but a representation of the (normal) biological response and physiological condition induced by natural particles. However, species in freshwater systems are adapted to naturally occurring particles, and it remains relatively unclear whether polymer particles act differently or have the potential to bypass protective adaptations."

Strategies to cope with non-food particles in the case of daphnids encompass a number of pathways, including morphological and behavioral adaptations. The peritrophic membrane shields the gut lining against mechanical damage (Lehane 1997). The shedding of the carapax during growth prevents particle accumulation (Auffan et al. 2013). They also include various behavioral mechanisms (Burns 1968a), such as temporary reduction in feeding rate (Ogonowski et al. 2016; Rist et al. 2017), and post-abdominal rejection of boluses (Kirk 1991a). These strategies are suitable for transient exposure durations to high concentrations of natural particles (e.g., caused by surface runoff into a water body (Robinson et al. 2010)). Feeding is reduced until particles have sedimented. Many microplastics are either neutrally buoyant (Karami 2017) or, if of lower density, can over time acquire a biofilm that renders them neutrally and negatively buoyant (Glaser 2020). Eco-corona (sorption of biological macromolecules) and biofilm-formation (adhesion of microorganisms), in turn, is affected by chemical properties, especially surface functionalization (Nasser et al. 2020). In conjunction with the material's long lifespan, this makes exposure to microplastics continuous throughout a daphnid's lifespan. Effects in one generation would then potentially affect their offspring, aggravating from generation to generation. To better understand if and how effects increase from one generation to the next was the purpose of using the multigenerational design in studies 2 and 3 of this thesis (Annex 2 + 3). We could conclude that effects can exacerbate from generation to generation. These findings were made at high particle concentrations (10,000 and 2,000 particles mL^{-1}). Nonetheless, they prove that short-term exposure will not necessarily illuminate the "real" toll microplastics exposure can take on an organism and its line of offspring.

Many factors influence the behavior of particles in the environment and their interactions with biota: surface morphology, surface charge, density, and size distribution of the particles, biofilm and eco-corona formation, and the release and sorption of molecules to the surface and/or polymer matrix of the particle. The variation of one of these factors at a time would allow identifying the factor

responsible for the potentially differing toxicity profile of microplastics compared to natural particles. Unfortunately, natural particles are as heterogenous as microplastics themselves. Thus, we can only draw limited conclusions from comparing two kinds of particles. In our experiments, we matched exposure concentrations according to size distributions. The size selection was limited by the available analytics device, which has a spatial detection limit of ≥ 2 µm. The first multigenerational study (Annex 2) found strong effects of high microplastics concentrations but no effect of kaolin as natural particles. In contrast, in the population experiment (Annex 4) the effect increased at higher ratios of kieselguhr in the microplastics-kieselguhr mixtures. In the first experiment, we observed a difference between the particles in the settling behavior (with kaolin sedimenting quickly and microplastics remaining in the water column). This observation is consistent with differences in material density. It thus might influence local exposure concentrations inside the vessel, even though daphnids can show directed movement along a food gradient (Neary et al. 1994). Likewise, kieselguhr should have similar settling behavior as kaolin, as they are of comparable density. Nonetheless, kieselguhr induced effects in study 4 (Annex 4), leading to the conclusion that sedimentation is not the only factor here.

In our studies comparing the effects of microplastics to natural particles (Annex 2 + 4), we matched particle concentrations based on a size range of 2 – 60 µm. Correspondingly, we cannot exclude effects caused by particles < 2 µm. Smaller particles are considered to be more toxic than large particles (Triebskorn et al. 2018). This is not reflected in our findings. The particle size distributions for study 2 (Figure S6 in Annex 2) show, that kaolin is comprised of mostly very small particles. Here, one could assume that the exponential trend towards smaller particles continues and that the majority of particles are < 2 µm. Nonetheless, we observed no toxicity by kaolin. In paper 3 (Annex 3), on the other hand, we observed reduced toxicity of the microplastics used compared to the same microplastics in paper 2 (Annex 2). As hypothesized in paper 3, this could be explained by a lack of nanoparticles due to the incubation and filtration process the particles underwent in study 3, but not in study 2. Once again, observations made for one particle type might not necessarily be true for another and particles would have matching size distributions to truly judge the effect of very small particles.

Theoretically, exposure duration was also equalized through the experimental setup but, differences in particle density and the behavior in suspension influence the interaction of animals with suspended particles (Annex 2; Karami, 2017). Since kaolin induced no effects (Annex 2), while kieselguhr did (Annex 4) generalizing between particle types is not possible. Likewise, Scherer et al. (2019) compared the effects of PVC particles to both kaolin and kieselguhr towards the larvae of the midge *Chironomus riparius*. The authors found a deleterious effect of both microplastics and kieselguhr, but a promoting effect of kaolin on the emergence of the larvae. Furthermore, they hypothesized that particle shape played a role in the differences since the sharper edges and more porous structure of kieselguhr could physically damage the organism. A key difference between their study and our findings is the feeding strategy of the organisms. Daphnids are primarily filter feeding in the water column while chironomid larvae are deposit feeders in close contact with the sediment (Thorp et al. 2016). Nonetheless, particle shape and mechanical damage as contributing factors would affect both species. The effects they observed were higher for PVC than kieselguhr when comparing mass-based concentrations. However, this was inversed when comparing numerical concentrations, as kieselguhr contained fewer particles per mass. Overall, the findings by Scherer et al. compliment ours insofar as they showed negative effects by kieselguhr and microplastics, but none by kaolin.

Not only natural particles are diverse in their properties and behavior. Microplastics have been described as a "*diverse contaminant suite*" (Rochman et al. 2019) and differ in properties as much as the material they originate from. This includes shape, crystallinity, and density, with the latter, for example, ranging from 0.85 for PP to 1.41 g cm^{-3} for polyoxymethylene (Lambert and Wagner 2018). As the microplastics used in this thesis were all made from PS, it is important to look beyond this material. Zimmermann et al. (2020), for example, compared the toxicity of irregular particles made from several "understudied" polymer types (namely, PVC, polyurethane (PU), and polylactic acid (PLA)) to kaolin in daphnids. They found that PVC most strongly affected reproductive output, while PLA had the strongest effect on survival. All microplastics induced higher effects than kaolin, even when comparing numerical concentrations and not only mass-based concentrations. This was interesting since the same mass of kaolin contained more particles than the microplastics. They tested the particles themselves, chemical extracts from the particles, the extracted (and thus

deemed free of extractable chemicals) microplastics, the extracts derived from this extraction, and so-called migrates (extracts using water as the accepting solvent over 21 d). Their findings suggest that the toxicity of PVC is caused by the chemicals, while for PLA and PU it was driven by the mere particulate nature. The shape did not appear to be the common driver of toxicity, as the particles produced by cryo-milling from different consumer products varied in shape but their roundness or sharpness could not be correlated to levels of toxicity. This underlines, like the findings of Scherer et al. (2019), that we cannot readily extrapolate from one kind of particle to others.

What this teaches us on a broader level is that, according to our findings, microplastics are not necessarily more toxic than natural particles or *vice versa*, but rather that it always depends on the context of exposure. Organism behavior in conjunction with particle properties will dictate encounter rates and the impact of particles. Additionally, I would argue that neutrally buoyant microplastics will likely lead to continuous exposure of pelagic organisms while sinking particles will more strongly influence benthic organisms. Likewise, toxicity could be caused by both chemical and physical factors and the properties of individual microplastics need to be considered. The degradation behavior of microplastics warrants further investigation to better understand if particles could potentially become more harmful through the development of sharper edges. The persistence of microplastics and environmental impacts can change particle properties (Annex 3, Jahnke et al. (2017)). Generally, it would make sense to design ecotoxicity studies with microplastics as closely to the materials, shape, and degradation state of what is encountered by organisms in their ecosystem. Currently, analytical constraints and the heterogenicity of sampling and analytical methods prevent us from achieving a comprehensive understanding of the state of particles across size ranges and properties in the environment. Unifactorial experimental designs (i.e., testing for the effect of isolated variables one at a time) are the compromise we are faced with.

Particle weathering

Paper 3 (Annex 3) of this thesis showed that conditioning in filtered wastewater reduced the toxicity of irregular microplastics towards daphnids, even though the mechanisms are not fully understood. We hypothesize that the acquisition of an eco-corona through contact with the animals and the longer retention of particles inside

the vessel played a role in the low toxicity of microplastics compared to kieselguhr in the population experiment (Annex 4) as well.

Plastic in the environment is subjected to different chemical and physical factors such as mechanical stress, (UV) radiation, changes in temperature, salinity, oxidizing conditions as well as interactions with biota. The latter includes colonization through microbes (Jahnke et al. 2017) as well as mechanical degradation from gut passage (Dawson et al. 2018; Mateos-Cárdenas et al. 2020). These processes can lead to the release of plastic-associated chemicals (Gewert et al. 2015). Likewise, chemicals from the environment can sorb to the particles. This scenario not only includes potentially harmful organic chemicals such as polycyclic aromatic hydrocarbons (Fries and Zarfl 2012; Mai et al. 2018) and other contaminants, but also the myriad of substances that naturally occur in water bodies. This natural organic matter (NOM) can be the degradation product of vegetation, algae, and bacterial biomass (Nasser et al. 2020). A large fraction can be dissolved and constitutes the DOC present in a system. A survey of 7,514 lakes from 6 continents found DOC concentrations to fall into the range of 0.1–332 mg L^{-1}. Particles entering this system will soon be coated with a so-called eco-corona. This coating can alter the behavior of particles in the medium as well as the interaction of particles with biota. Nanoparticles are particularly reactive through their large surface-to-volume ratio. They might acquire an eco-corona directly from the interaction with organismal tissue, damaging the organism in the process. A corona acquired before contact with an organism could reduce or neutralize the reactivity and thus mitigate the risk of damage, consequently reducing the toxic potential of particles. The reduction in reactivity likewise could reduce gut retention times and thus lower the effect on the feeding ability of daphnids. Natural organic matter is generally absent in standardized toxicity testing with daphnids since its presence can interfere with the bioavailability of organic pollutants. Consequently, when applying standardized guidelines to assess the toxicity of microplastics or engineered nanoparticles, eco-corona formation cannot occur, since most test media are mostly just salt formulations. The only potential source of NOM inside a test system is food or the test organism itself, as gut passage and organismal secretions of biomolecules can lead to the coating of particles. *In vitro* assays routinely include serum proteins as nourishment for the cells, thus automatically reducing the reactivity of nanoparticles (Nasser et al. 2020).

In study 4 (Annex 4) we observed a limited effect of irregular PS particles on *Daphnia* populations, while kieselguhr had a toxic effect. These findings differ from the toxic effects of similar microplastics in the multigenerational studies (Annex 2 + 3). Here, two key differences in study design could explain this discrepancy. The medium was renewed thrice weekly in the multigenerational studies and once weekly in the population study. With each exchange, pristine particles were introduced into the system. Therefore, in the population experiment, particles had more time to interact with food particles, organisms (of which there were a lot more than the singularly held individuals in the multigenerational experiments), and organismal excretions, leading to eco-corona-acquisition. We can then assume that part of particle toxicity came from acquiring an eco-corona from the daphnids themselves, while already corona-coated particles (i.e., from food and feces) were less reactive. The "reactionary debt" (i.e., matter required to coat all particles in a system with a corona, thus reducing their reactivity) would be divided among more individuals and their excretions in the case of the population experiment (Annex 4). This then would translate to an increased burden on the single individual in the multigenerational experiments. Gut passage might occur within minutes (Ogonowski et al. 2016; Scherer et al. 2017), leading to a high turnover of particles that had been inside a daphnid. Accordingly, this might explain the low microplastics toxicity in the population experiment vs. the multigenerational experiments. This is interesting since the exposure concentration in the population experiment (Annex 4) exceeded the highest in the multigenerational experiments by a factor of five. This warrants the assumption that, depending on exposure duration, concentration in the medium is less meaningful than a combination of particles per concentration and individual. Here, it must be pointed out that in the two multigenerational studies (Annex 2 + 3) we tracked endpoints of a specific individual, while we are ignorant about the individual fates of animals comprising the populations in study 4 (Annex 4). This

Several scientists have speculated about how aging can alter particle toxicity. One newer study by Motiei et al. (2021) was not previously discussed in paper 3 (Annex 3) and therefore is highlighted here. They incubated mixtures of microplastics and natural particles with bacterioplankton and compared their toxicity towards daphnids with pristine MP. They then used mass-specific DNA concentrations as a proxy for biofilm formation. The levels of biofilm formation on both the natural particles and the microplastics were comparable. They concluded that the biofilm, in

conjunction with aggregation behavior, is an influencing factor that reduced toxicity. This underlines the relevance of not only studying pristine particles since they never remain pristine for long. Likewise, as discussed in paper 3 (Annex 3), several other studies have shown that conditioning with biological macromolecules prior to exposure reduces particle toxicity (Fadare et al. 2019; Fadare et al. 2020; Ekvall et al. 2021; Jemec Kokalj et al. 2019; Giri and Mukherjee 2021). Jemec Kokalj et al. (2019) reported a reduction or no alteration of microplastics toxicity towards several species after incubation with river water or wastewater. Fadare et al. (2019; 2020) observed a reduction in acute toxicity to *D. magna* after microplastics had absorbed humic substances. Concurrently, dissolved organic matter mediated the toxicity of nanoplastics and silver ions to *D. magna* (Monikh et al. 2020). To my knowledge, no study has reported an increase in particle toxicity caused by the acquisition of an eco-corona.

Overall, it is likely that particle aging from to the acquisition of an eco-corona and biofilm formation will reduce particle toxicity compared to pristine particles. This implies that most toxicity studies with pristine microplastics overestimate microplastics toxicity if no pre-conditioning of particles has occurred. As argued by Nasser et al. (2020) pre-conditioning with NOM before exposure should be a part of ecotoxicity testing of nanomaterials (and consequently for micro- and nanoplastics). Our findings in paper 3 (Annex 3) of this thesis support this notion and are well aligned with the literature. Pre-conditioning of particles should generally be studied more and protocols need to be developed that mimic the aging process particles undergo in the environment. This step is crucial to make particle ecotoxicology more environmentally realistic and prevent an overestimation of toxicity through the use of only pristine particles.

Effects of food limitation

As stated earlier, daphnids possess adaptive strategies to particle exposure that include a reduction in feeding rate. This reduction in the presence of microplastics was between 18 and 30 % in two studies (Ogonowski et al. 2016; Rist et al. 2017). Likewise, the availability of food particles themselves influences the filtration rate

(Furuhagen et al. 2014). Concurrently, food dilution[3] has been discussed as a secondary mechanism affecting access to nutrients (Ogonowski et al. 2018; de Ruijter et al. 2020; Gouin et al. 2019). Our findings suggest that food limitation is an adequate stressor that in conjunction with microplastics exposure produces significant effects in daphnids. In their natural habitat, daphnids can experience food limitation for most of the year (Müller-Navarra and Lampert 1996). Poor nutritional status has been shown to usually increase toxicity (Heugens et al. 2001). Therefore, we hypothesized that an overabundance of food in a laboratory experiment would mask or buffer the effects of microplastics, leading to an underestimation of effects. The role of food availability and changing nutritional needs during an organism's lifecycle in standardized tests has been discussed before (Zimmer et al. 2012; Gomes et al. 2016; Betini et al. 2020). Food levels in the environment are never constant. Nonetheless, providing a fixed food quantity is necessary for the reproducibility and comparability of standardized tests. However, this approach may not be suitable for particle toxicity if the provided food level is too high. This appears to be the case for the OECD guideline 211 (OECD 2012), where a range of 0.1-0.2 mg carbon individual^{-1} day^{-1} is suggested to ensure the animals produce sufficient offspring. It has been shown in several studies, that food availability impacts the ingestion and toxicity of microplastics, nanoplastics, and engineered nanoparticles (Aljaibachi and Callaghan 2018; Y.-Y. Liu et al. 2019; Chae and An 2020; Kögel et al. 2020; Mueller et al. 2020; Motiei et al. 2021). This underlines that food plays a major role in the interaction of microplastics with daphnids, affecting behavior, ingestion, and nutritional content of the bolus for the animal. Food availability, therefore, is a crucial factor to consider in the planning and execution of microplastics ecotoxicology studies with daphnids to not underestimate the toxicity. Here, dynamic energy budget theory could provide useful insights into how metabolic processes in an organism are affected.

The context of dynamic energy budget theory

Dynamic energy budget (DEB) theory is a theoretical framework developed by Bas Kooijman that aims to describe metabolic processes on an individual level (Kooijman 2009). It makes certain assumptions about the prioritization of flows of a universally translatable energetic currency in an organism. These flows are related to the

[3] replacement of a fraction of nutritious food particles by non-nutritious natural particles or microplastics

assimilation of energy from food and the distribution of energy towards structural growth, maturation, and reproduction (Figure 5). It has been discussed as achieving coherence in biology by being consistent with thermodynamics, physics, chemistry, and evolution. It also is consistent with empirical data and can cover the individual life cycle from early development to death (Sousa et al. 2010). The model assumes that a fraction of energy is assimilated from a substrate (e.g., algae in the case of daphnids). The rest is converted to feces that are segregated. Energy is simplified as universally translatable between metabolic processes and enters a hypothetical reserve compartment (Figure 5).

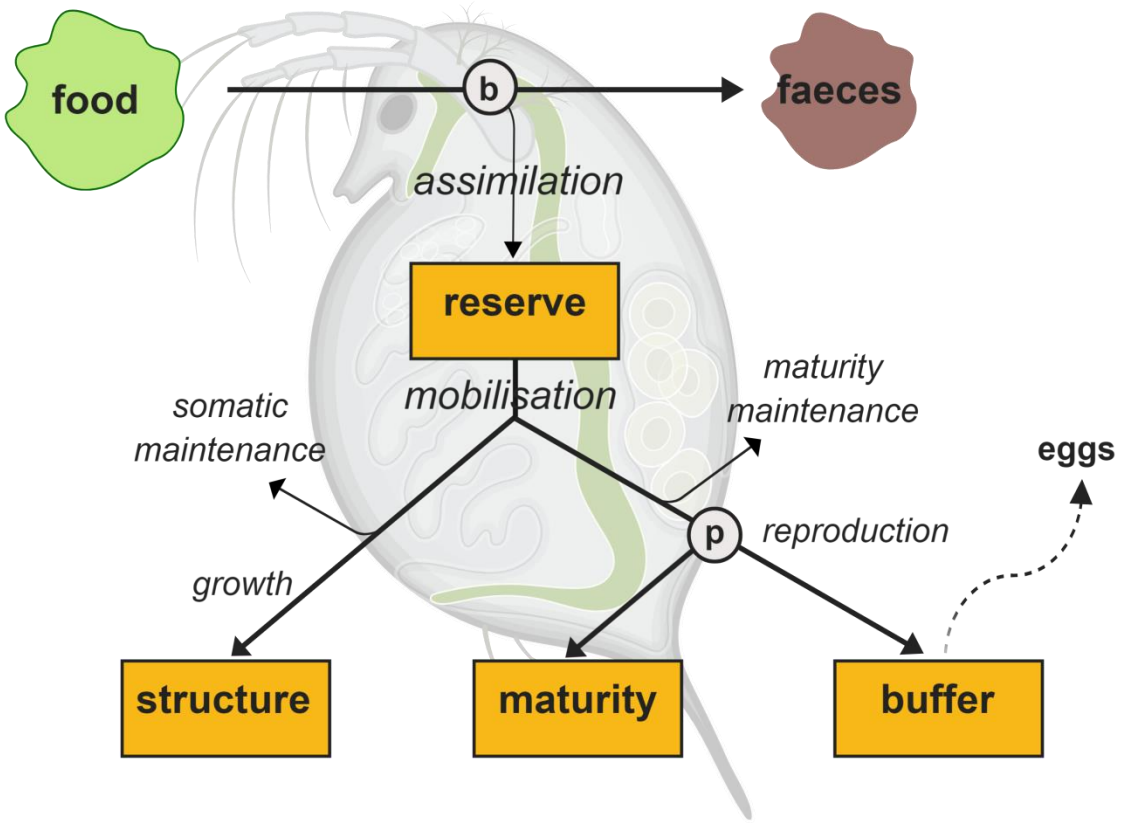

Figure 5: The dynamic energy budget model for metabolic processes; adapted from Jager and Zimmer (2012).

Energy is mobilized from that reserve and diverted into two channels: A fixed fraction (κ) is first distributed towards somatic maintenance. Broadly, somatic maintenance encompasses all metabolic demands that are not growth, maturation, or reproduction (e.g., detoxification, movement, and homeostasis). If sufficient energy is available to "pay" the cost for somatic maintenance then the surplus energy of the κ-fraction is used for somatic growth. The organism dies if insufficient energy is available to provide the energetic debt of somatic maintenance. For *Daphnia magna,* the κ

fraction is calculated to be around 58 % of the mobilized energy reserve (Kooijman and Gergs 2021). The 1-κ fraction is firstly used to achieve a mature (reproductive) state and after that to maintain that state. After this cost has been paid the remaining 1-κ fraction is diverted towards a reproduction buffer that then provides the energy to produce eggs or embryos. Accordingly, the energy reserve of offspring is correlated to the mother's energy reserve. Three life stages are differentiated in the DEB framework: Embryo, juvenile, and adult. Embryos do not assimilate energy; they sustain solely on the energy reserve provided by their mothers. This point in the model logic is consistent with several experimental observations in daphnids (Tessier et al. 1983). Juveniles assimilate energy from outside but do not yet reproduce. Adults have achieved a reproductive state and assimilate energy. The duration of each phase is species-specific as are parameters such as the ratio of κ and, consequently, 1-κ. For daphnids, reproduction begins roughly 5-11 days after release from the brood pouch (depending on conditions like food availability and temperature) and embryos are usually released every 2-3 days after that (Smirnov 2017).

DEB theory has come to be applied to ecotoxicology because toxicological effects can be considered disruptions of the energy flows. This is in line with the considerations by Gouin et al (2019) about potential pathways through which microplastics can affect biota. Mortality, for example, could be viewed as the failure to allocate sufficient energy to somatic maintenance, apart from being a stochastic event with the likelihood of death increasing with age (Jager 2017). Body size serves as a proxy for energy allocation towards growth. According to the model, growth takes a larger part of the mobilized energy over maturation or reproduction. Energy flows are directed towards maturation until the state of sexual maturity is reached. Thereafter, energy is directed to reproduction and maintenance of said maturation. This model could be used to derive insight into the mechanism through which the daphnids are influenced by the particles.

A recent scientific opinion paper requested by the European Food Safety Authority (EFSA) summarized the usefulness of three different toxicokinetic/toxicodynamic (TKTD) effect models (including DEBtox, a physiological model based on DEB theory) for regulatory risk assessment of pesticides for aquatic organisms (EFSA Panel on Plant Protection Products and their Residues et al. 2018). They concluded that even though the DEBtox modeling approach is currently limited to research applications, it shows great potential for future use in prospective environmental risk

assessment for pesticides. DEBtox can predict the response in sublethal endpoints (growth and reproduction) over different time scales.

In this thesis, the framework can be used independently of the application of a DEBtox model to gain mechanistic understandings. In the two multigenerational studies that are part of this thesis (Annex 2 + 3) we observed a delay of reproductive onset (Figure 6; data originally published as Figure S5 in Annex 2 (A) and Figure S8 in Annex 3 (B)) as well as effects on growth and reproduction.

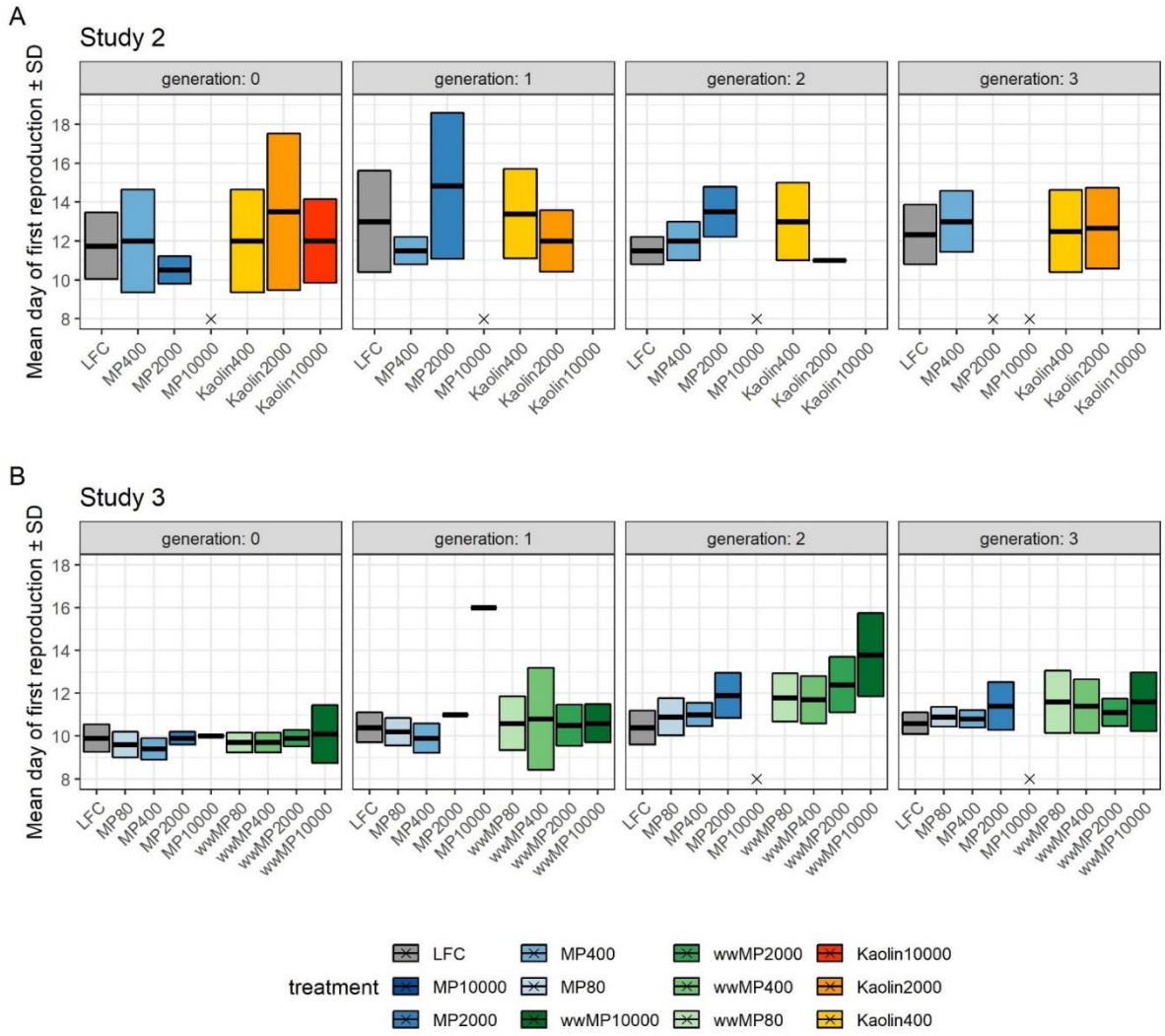

Figure 6: Mean day of first reproduction of the daphnids in study 2 (A) and 3 (B) (Annex 2 + 3). Crosses mark treatment groups that went extinct. Kaolin10000 was discontinued after the corresponding microplastics treatment had gone extinct. Data originally published in Schür et al. (2020; 2021).

This indicates potential effects on two mechanisms. Assimilation in the broader sense then encompasses food dilution, reduced absorption of nutrients from ingested food,

or a reduction in feeding rate as well as the removal of regurgitated boluses (DeMott 1986; Kirk 1991b). These decrease energy intake as "*supply-side effects*" (Calow 1991) (Figure 7, A). The other factor likely affected is somatic maintenance and can serve as the umbrella term for the general "cost of living" (Figure 7, B) (Bas Kooijman, personal communication, 2021). The higher maintenance cost could be caused by physical damage and increased movement[4]. This is opposed to hypothetical effects that would, for example, affect reproduction by increasing the cost per embryo that would manifest differently (Jager 2017).

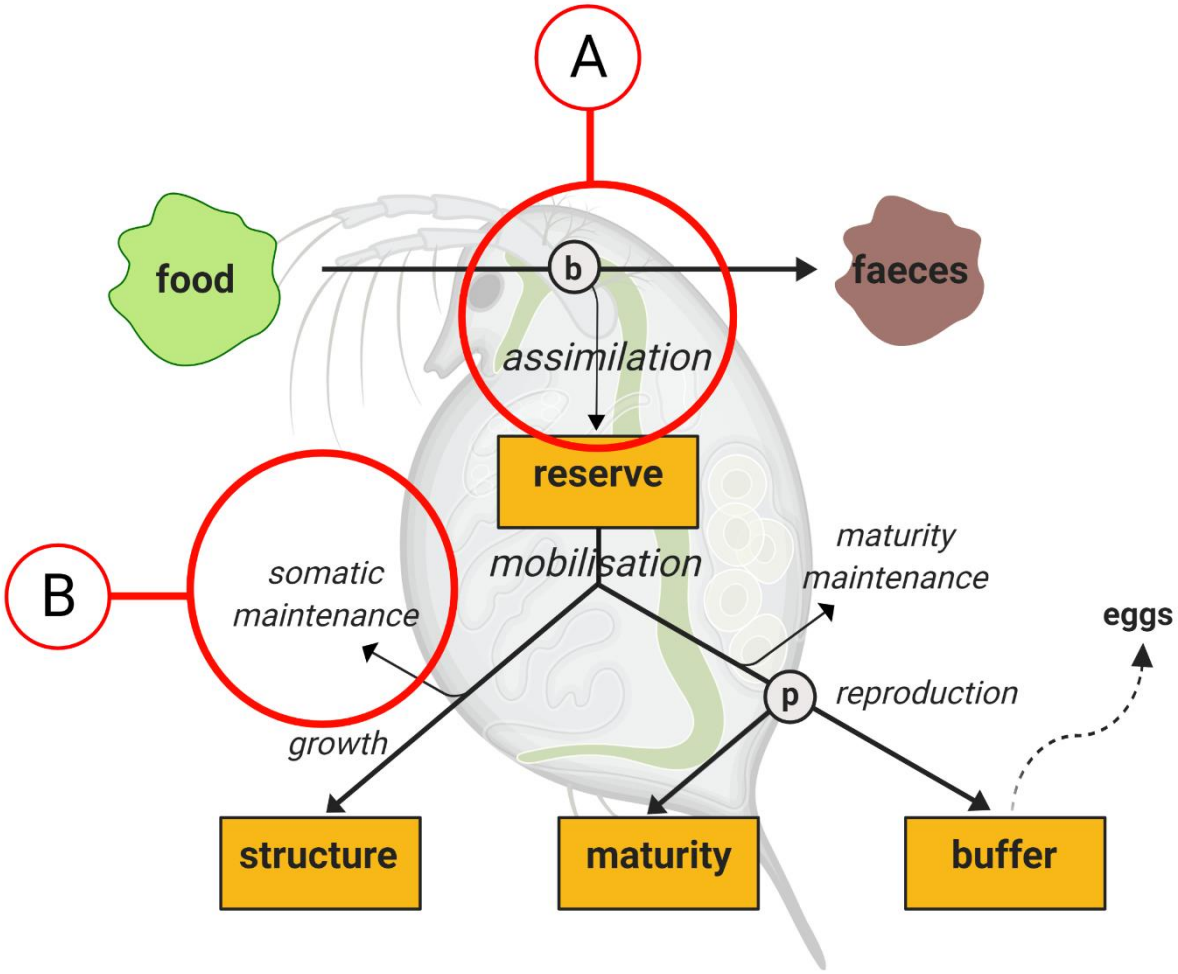

Figure 7: Possible effects of microplastics toxicity on energetic flows in the dynamic energy budget theory framework.

These effect mechanisms are therefore consistent across our findings, the literature, and the theoretical considerations in the DEB framework. Since most of these mechanisms could also be affected by natural particles that would lead to the

[4] upward flexion of the post-abdominal claw (Burns 1968a) to remove particles from the feeding appendages and the removal of regurgitated boluses.

conclusion that microplastics are “just another particle”, just producing different effects due to physico-chemical properties making them behave differently in laboratory studies. Still, it is unclear if they trigger effects at different concentrations than their natural counterparts.

To achieve a quantitative assessment of the effect magnitude parametrization of a DEBtox model with data on exposed and unexposed animals would be necessary. Usually, toxicokinetic models assume the internal concentration of a substance to be the prerequisite for a noxious effect (Kooijman 2009). Here, microplastics toxicity would be a special case, if behavioral changes would reduce the assimilation rate just by their presence in the surrounding medium and not after ingestion (Tjalling Jager, personal communication, 2020). Other studies showed that chemically-induced feeding rate depression negatively affects life-history traits (Agatz et al. 2013) and increases the population vulnerability of daphnids (Agatz et al. 2012). Therefore, this is a plausible mechanism to cause effects, even though it is not limited to microplastics exposure.

Body size is another factor that plays a role in feeding rate (McMahon 1965). The latter could play a role in particle availability since Burns (1968b) observed larger *D. magna* to ingest larger particles. In sum, this could relate to the mortality in our first multigenerational study (Annex 2) which occurred around or after day 10 of each generation. This correlates to reaching maturity (Figure 7 and Figure S5 in Annex 2) after the daphnids had undergone most of their structural growth (Duckworth et al. 2019). In that phase, energy allocation shifts from structural growth and maturation towards reproduction. Simultaneously, the larger body requires higher maintenance costs. In correlation to body size, the feeding rate is increased and a broader range of particles are available for ingestion. Consequently, the encounter rate for ingestible particles and the metabolic demand is increased (i.e., through more frequent rejection behavior). I hypothesized that there might be a size threshold after which the daphnids could interact with the majority of the particles while smaller animals were less affected. This idea was not reflected in the growth curves recorded in the second multigenerational study (Figure 5 in Annex 3). Here, mostly food levels of the parent generation and particle concentrations appeared to affect length development. The nutritional status of the parent generation then led to an early investment into more rapid growth that did not result in a larger body length at 21 d.

The use of our data in the context of DEB models and the production of data suitable for analysis using a DEB model approach is a very promising step towards unraveling the effects of microplastics in aquatic organisms. We could show that, according to DEB theory, a reduction in feeding rate, food dilution, and/or increased maintenance cost are plausible effect mechanisms for the animals in our multigenerational studies. This is consistent with both the adaptive mechanisms daphnids show towards particle exposure as reported in the literature. Further experiments to get a more detailed understanding of the interaction of food availability/food dilution and particle exposure are warranted. Here, matching different concentration metrics (i.e., surface area, volume, mass) of food and non-food particles against each other would make for a very interesting approach.

What is a more realistic scenario?

The publications comprising this thesis illuminated presumable shortcomings of current microplastics research and show how they impact the way the toxicity of microplastics towards daphnids is assessed. We showed that tissue translocation of microplastics beyond the nanometer scale in daphnids is implausible and an artifact of using fluorescently dyed microplastics. Reports on tissue translocation in the literature that are based on fluorescence as a proxy for particles should be met with skepticism. Hopefully, analytical methods will evolve to bridge this gap and better enable us to study tissue translocation and its effects. Here, metal-doped nanoparticles are a promising candidate in this line of research to investigate the fate and behavior of particles in the environment (Mitrano et al. 2019) and inside organisms (Redondo-Hasselerharm et al. 2021).

Nature is full of natural particles and daphnids appear to be fairly adapted to deal with that. Nonetheless, the physicochemical properties of microplastics are often different from natural particles and therefore can have differing effects. Here, context (i.e., frequency of medium renewal, agitation of the suspension, exposure duration, number of animals in the vessel) and the dose metrics are important to compare particles. Just like some natural particles were shown to elicit a higher toxic effect than others, the same will be true for different microplastics. We could not definitively conclude if microplastics had a different toxicological profile from natural particles.

By assuming that the acquisition of an eco-corona plays a role in toxicity (and its mediation), we need to make sure to better understand how the use of pristine or aged particles can change toxicity levels and what that means for our test designs. Our findings and the majority of the available literature suggest that aging will reduce particle toxicity, at least for nanoparticles through reducing reactivity. Using pristine irregular particles will likely underestimate the toxicity while aging through incubation with DOC (i.e., from humic acids, microorganisms, organismal exudates or homogenates, other dissolved proteins) is a relatively easy preparatory step that could be incorporated into ecotoxicity testing. Here, a better understanding of how different types of macromolecules interact with and affect particles is needed.

Every experimental design will be reductionist. *In vivo* tests for ecotoxicology are labor-intensive and limit the number of factors studied in a single experiment. Nonetheless, we were able to show that as a realistic stressor, food limitation is suitable to enhance the study of particle effects. The results also substantiated the idea that microplastics elicit, at least part of their effects, through a decrease in nutrient access and an increase in general metabolic cost (i.e., behavior, movement). More thought needs to be put into matching exposure to lifecycle duration. The continuous exposure of an individual can affect subsequent generation's fitness and indirectly lead to changes in growth or reproductive[5] strategy. If this strategy is unsuccessful that could increase the vulnerability of populations. The durations of our experiments were limited to 21 d, but ideally, full-lifecycle tests would be more insightful. A delay in reproduction could lead to a recorded decrease in reproduction, when really over the lifespan there was no difference in reproductive output but a shift of reproduction to a later period. Likewise, by expanding to full lifecycle multigenerational designs we could potentially illuminate a reduction in lifespan at lower particle concentrations. These data could then inform modeling approaches with reduced uncertainties. As we saw earlier, effects are usually a function of exposure concentration and time.

The level of biological organization at which an effect occurs matters just as well. This thesis looked mainly at apical endpoints in a single species. Just like nature is never devoid of non-food particles, there rarely is a single species or individual organism.

[5] We observed a shift to earlier growth in study 3 (Annex 3), but no measurable effect on neonate size at birth.

Apart from food-web studies (Chae et al. 2018; Hanslik et al. 2020), it would make sense to get a better understanding of how microplastics affect intra- and interspecies interactions. Inspiration can be found in Kirk et al. (1990; 1991b). They showed that kaolin particles could suppress Cladocera populations in favor of Rotifers gaining a competitive advantage. This shift was more distinct under food limitation. Conceivable approaches would also include something like mesocosm studies to observe shifts in plankton populations and the effects of natural weathering.

Likewise, a better mechanistical understanding of microplastics effects is needed. I was able to deduce through the use of DEB theory how energetic allocation in daphnids might be altered through exposure to microplastics. However, this was only based on the observation of apical endpoints. In other plastic types that are not food-contact materials, chemical additives may play a bigger role in the induction of effects. Further mechanistic discoveries could help to further distinguish the mode of actions for different plastic types and use cases. Here, mechanism-specific *in vitro* studies can be applied to plastic materials with a focus on associated chemicals. Zimmermann et al. (2019; 2020) lay the groundwork with the screening of a large number of plastic consumer products with *in vitro* methods for endocrine-specific assays. Alternatively, ecotoxicogenomics could help identify effects on the level of gene expression or epigenetics.

Generally, no one approach will be able to encompass the complexity of microplastics. So, as is common in ecotoxicology, we must consider the context of exposure and adapt our methods accordingly. The factors influencing microplastics-biota-interactions and how they affect organisms need to be better understood. Ashauer and Jager (2018) propose the path towards predictive ecotoxicology as clearly identifying data gaps and fundamental questions we need to answer. The generated data then enables *in silico* methods to extrapolate the data across different concentrations, time scales, and species to understand the drivers of toxicity. This needs to be coupled with monitoring data across a broader size range of particles to derive measures for risk. This could be summarized as a systems toxicology approach of integrating functional changes across biological levels of organization with classical toxicology (Sturla et al. 2014). Here, high computational capacities and cost-effective sequencing technologies enable the broad study of less-established endpoints. Examples here include the disruption of specific microbiomes (Fackelmann and Sommer 2019) through microplastics as well as ecotoxicogenomics approaches for

hypothesis generation and the discovery of toxicity mechanisms (Brinke and Buchinger 2016; Salibián 2017). These could complement other endpoints, which is why we chose to analyze the microbiome of selected daphnids from study 3 (Annex 3; Schür et al, in prep.).

Even though the effects we observed were at concentrations orders of magnitude beyond what is currently reported in nature, we can assume that microplastics contamination will rise in conjunction with global plastic production. Their persistence then could transform what we today deem unrealistically high concentrations into reality sooner or later.

Summary

- Fluorescence cannot be used as a proxy for translocated particles since the fluorescent dye can uncouple from the particles and accumulate in tissues.
- This artifact can lead to the misinterpretation of results, overestimating the prevalence of tissue translocation events.
- Passive sampling can be used to verify that the dye uncoupled from the particles. Dialysis can be used to remove dissolved dye and reduce its impact.
- Tissue translocation of particles to lipid droplets in daphnids is biologically implausible.
- Nutritional state and food availability affect endpoints routinely measured in *Daphnia* toxicity studies. Thus, they need consideration in the design and interpretation of experiments.
- The persistence of toxicants needs to be matched with the lifespan of the study organism. Short-lived organisms like daphnids will be exposed for their entire lifespan generation after generation and effects can exacerbate over time, as shown in this thesis.
- Both microplastics and natural particles can negatively affect daphnids and findings cannot be generalized from one particle type to another.
- Environmental aging like the acquisition of an eco-corona or biofilm likely reduces the reactivity of particles and thus the toxicity of particles.
- The evaluation of the multigenerational results in light of the dynamic energy budget framework hint towards a decrease in nutrient assimilation from food and an increase in metabolic demand as potential effect pathways for microplastics in daphnids.
- The context of exposure (e.g., duration, food availability) matters when studying microplastics ecotoxicology and should be considered in experimental design.
- Studies on subtle and understudied endpoints like microbiome dysbiosis, multi-species and competition experiments, and transcriptome analysis could help discover novel mechanistic pathways.
- When designing a study, the useability of data sets for *in silico* methods should be considered to allow extrapolation to other exposure conditions to achieve predictive ecotoxicology.

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Appendix

Annex 1: Paper 1

Title: When fluorescence is not a particle: the tissue translocation of microplastics in *Daphnia magna* seems an artifact

Journal: Environmental Toxicology and Chemistry

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Status: published

doi: 10.1002/etc.4436

WHEN FLUORESCENCE IS NOT A PARTICLE: THE TISSUE TRANSLOCATION OF MICROPLASTICS IN *DAPHNIA MAGNA* SEEMS AN ARTIFACT

Dye leaches from fluorescent particles

Abstract

Previous research reported the translocation of nano- and microplastics from the gastrointestinal tract to tissues in Daphnia magna, most prominently of fluorescent polystyrene (PS) beads to lipid droplets. For particles > 300 nm, such transfer is biologically implausible as the peritrophic membrane retains these in the daphnid gut. Thus, we aim at replicating the key study by Rosenkranz et al. (2009). We used confocal laser scanning microscopy to study the tissue transfer applying the original setup (neonates exposed to 20 and 1,000 nm PS beads at 2 µg L^{-1} for 4 and 24 h), the same setup with a fructose-based clearing, and a setup with a 1,000-fold higher concentration (2 mg L^{-1}). We used passive sampling to investigate whether the beads leach the fluorescent dye. While the 1,000 nm beads were visible in the gut at both exposure concentrations, the 20 nm beads were detectable at 2 mg L^{-1}, only. At this concentration, we observed fluorescence in lipid droplets in daphnids exposed to both particle types. However, this did not co-localize with the 1,000 nm beads which remained visible in the gut. We further confirmed the leaching of the fluorescent dye using a passive sampler, a method that can also be applied in future studies. In summary, we cannot replicate the original study but demonstrate that the fluorescence in the lipid droplets of *D. magna* results from leaching of the dye. Thus, the use of fluorescence as a surrogate for particles can lead to artifacts in uptake and translocation studies. This highlights the need to confirm the stability of the fluorescence label or to localize particles using alternative methods.

Keywords

nanoplastics, microbeads, microspheres, silicone, rubber, dye leaching

Introduction

Synthetic polymer particles in the nano- and micrometer size range, now referred to as nano- and microplastics (Hartmann et al. 2019), have been used for decades to study various biological processes, such as the feeding preferences of zooplankton (e.g., Burns, 1968), since they are often regarded inert. Depending on their size, small particles can pass through biological barriers and enter tissues (Wright et al. 2013). The subsequent physical damage is one of the adverse effects engineered nanoparticles or nano- and microplastics may have on aquatic organisms (Rist and Hartmann 2018). Therefore, investigating this phenomenon and the underlying mechanisms is highly relevant.

Previous research mostly focused on tissue translocation of small particles in mammals (Jani et al. 1989; Jani et al. 1990; Jani et al. 1992; Walczak et al. 2015) with a human health or drug delivery focus. With growing concerns about plastic pollution, this focus has shifted towards studying particle translocation in an ecotoxicological context. So far, more than 30 studies have investigated this in a range of species, most commonly in fish, crustaceans, and mollusks (Triebskorn et al. 2018). The majority of these studies reported a transfer of nano- and microplastics into tissues based on a visualization or measurement of fluorescence but without confirming that the fluorescent dye remained associated with the particles. It is well known from engineered nanoparticle research that dyes that are not covalently bound to the material can leach and cause artifacts (Kettiger et al. 2013; Rothen-Rutishauser et al. 2013). Within the analytical laboratory, reversible absorption of chemicals into and subsequent desorption from polymer beads and coatings is also well documented and massively exploited for Solid Phase Microextraction (SPME; Mayer et al., 2000), Solid Phase Extraction (SPE; Thurman and Mills 1998) and chromatographic separations on reverse phase high performance liquid chromatography (HPLC) columns (Davankov and Tsyurupa 2011). Here, it is well known that the release of absorbed chemicals can be triggered by the correct desorption medium and is affected by factors such as ionic strength and pH. The low pH in the daphnid intestinal tract (around pH 4.5; Smirnov (2017)), as compared to the medium, may thus be a relevant factor.

Rosenkranz et al. (2009) were the first to report the tissue transfer of nano- and microplastics in the freshwater cladoceran *Daphnia magna*. Due to a firm knowledge base and the wide use of this species as a standard test organism in ecotoxicology, it is now also widely used to study ingestion and toxicity of plastics (e.g., Ogonowski et al., 2016; Imhof et al., 2017; Rist et al., 2017; Scherer et al., 2017; Martins and Guilhermino, 2018). Rosenkranz et al. (2009) exposed *D. magna* for 30 min to 20 and 1,000 nm polystyrene (PS) beads (2 μg L^{-1}) and observed fluorescence in the lipid droplets in both adults and neonates. Transmission electron microscopy (TEM) seemed to confirm the presence of 1,000 nm beads in the lipid droplets but was inconclusive for the smaller particles. Based on this, the authors deduced that both particle sizes passed biological barriers and translocated from the digestive tract into the animals' tissue where they accumulated in lipid droplets. However, a biologically plausible mechanism for this observation is currently lacking.

A translocation of particles to the lipid droplets or other regions inside the daphnid body would have to follow a certain chain of events: ingestion of particles, passage across the peritrophic membrane, and transfer across the epithelium of the digestive tract, and transport to the target tissue. Importantly, the peritrophic membrane prevents a translocation of larger particles. Daphnids – like many other arthropods – produce this membrane in the foregut where it encloses the food pellets (with which it is excreted) to prevent mechanical injury and pathogen infiltration of the epithelium (Hansen and Peters 1998). The membrane consists of chitin microfibrils, polysaccharides and proteins (Georgi 1969) and is impermeable for particles ≥ 327 nm (Hansen and Peters 1998). Accordingly, a translocation of 1,000 nm microplastics in daphnids, as reported by Rosenkranz et al. (2009), seems biologically implausible and deserves re-assessment. Smaller particles, such as the 20 nm beads used in the same study, may pass the peritrophic membrane and can get in contact with the epithelium. A cellular uptake depends on particle size, concentration, surface modification, and charge and is plausible for 20 nm beads. The negative surface charge of the PS beads used by Rosenkranz et al. (2009) enhances the attachment to cell surfaces (Zhu et al. 2013). Particles can cross membranes passively (low nanometer range, Zhu et al. (2013)), via endocytosis (probably

limited to particles < 1 µm) or persorption (particles < 150 µm in mammals; Volkheimer, 1974; Wright and Kelly, 2017). Following uptake by epithelial cells, an active transport mechanism would need to transfer the particles to the lipid droplets which are scattered throughout the body but mostly located ventral to the gut. As the lipid storage in cladocerans is poorly understood, the potential mechanism of a transfer of particles to lipid droplets remains unknown. However, the peritrophic membrane will be the major morphological barrier preventing particles larger than 300 nm from entering the body of cladocerans. Accordingly, reports on the tissue transfer of larger plastics are biologically implausible based on current knowledge.

Therefore, the aim of this study is to replicate the findings of Rosenkranz et al. using (1) their original study design, (2) a sample preparation approach with improved sensitivity, (3) 1,000-fold higher exposure concentrations, and (4) a passive sampling experiment to investigate a potential leaching of the fluorescent dye from the PS beads.

Materials & Methods

Experimental design

We conducted four experiments to replicate the study of Rosenkranz et al. (2009) on tissue translocation of nano- and microplastics in *D. magna* and to further investigate this phenomenon. Experiment I aimed at replicating the original experimental design by Rosenkranz et al. (2009) as closely as possible. Since they reported tissue translocation for both neonates and adults at all exposure durations (0.5, 1, 2, 4, 6, 12, 24 h), we decided to focus on exposing neonates for 4 and 24 h, only. The exposure concentration was 2 µg L^{-1} for both particle types (20 and 1,000 nm) as in the original study. In experiment II, we applied the same exposure conditions as in Experiment I but used a fructose-based clearing method (SeeDB) to enhance the detection of particles in the daphnids. In experiment III, the particle concentrations were increased to 2 mg L^{-1} for each particle type, which is a 1,000-fold higher concentration than in the original study. Finally, Experiment IV was a passive sampling study with the PS beads used in Experiments I–III.

Plastic particles

The plastic particles were identical to the ones used by Rosenkranz et al. (2009), that is, 20 and 1,000 nm carboxylated PS beads (Fluospheres™) purchased from ThermoFisher. The beads were labelled with fluorescein isothiocyanate (FITC, λ_{Ex}: 505 nm, λ_{Em}: 515 nm). They were provided in a suspension containing 2 % (w/w) solids which was stored at 4 °C in the dark. Immediately before the experiments, the suspension was sonicated for 30 min (UR1, Retsch GmbH) and subsequently dispersed in U.S. EPA reconstituted hard water (Smith et al. 1997) in concentrations of 2 µg L^{-1} (Rosenkranz et al. 2009) or 2 mg L^{-1}.

Daphnid maintenance

The experiments were conducted with the water flea *D. magna* (clone from Birkendammen, Denmark) which was cultured in U.S. EPA reconstituted hard water in glass beakers. Cultures were kept at 20 °C and a light/dark cycle of 12:12 h. Every beaker contained twelve individuals in 800 mL

medium, fed daily with the green algae *Raphidocelis subcapitata* at a concentration of 2.5×10^5 cells mL^{-1}. The culture medium was renewed twice per week.

Exposure conditions

Before the experiments, *D. magna* neonates (< 24 h old) were starved for 24 h to reduce autofluorescence of ingested algae. In each experiment (Table 1), daphnids were exposed to the 20 nm and 1,000 nm particles for 4 h and 24 h. While a concentration of 2 μg L^{-1} was used in experiments I and II, the concentration was increased to 2 mg L^{-1} in experiment III. Negative controls not containing plastic particles were included in each experiment. The exposure was conducted in triplicates in 100 mL glass beakers containing 80 mL medium and five neonates each. The beakers were covered with glass lids to avoid evaporation and kept at 20 °C in the dark to prevent bleaching of the fluorescent particles. A total of 120 daphnids was exposed to 2 μg L^{-1} and 75 daphnids to 2 mg L^{-1} (Table 1). Additionally, the experiments included 30 and 15 control animals. In experiment I, specimens were preserved in 10 % formalin according to the method by Rosenkranz et al. (2009). To increase visibility, we used an adapted version of the fructose-based clearing method SeeDB (see below) in experiments II and III.

Fixation and tissue clearing protocol

We compared animals treated with 10 % formalin and an adapted version of SeeDB as a solvent-free clearing method (Ke et al. 2013). This procedure was chosen since previous experiments had shown that plastic particles dissolve when using solvent-based clearing methods (e.g., with benzyl alcohol/benzyl benzoate). Following the exposure, animals were rinsed twice by consecutive transfer to clean medium using a pipette. For the formalin fixation, five individuals per replicate were transferred to a glass vial containing 5 mL of a 10 % formalin solution and stored at 4 °C in the dark. For the SeeDB clearing, the specimens were preserved in glass vials with 5 mL of a 4 % para-formaldehyde (PFA) solution overnight. Subsequently, each individual was transferred into one well of a 96-well plate and transferred through a series of solutions with increasing fructose concentration (20, 40, 60, 80, and 100 % w/v). To reduce damage to the specimen due to repeated transfer the fructose solution was removed and replaced rather than transferring the specimen. For each step, 150 µL fructose solution were added to each well and the samples were kept at 4 °C in the dark for at least 4 h. Duration of each clearing step was reduced in comparison to the original protocol developed for whole mouse brains because of the smaller tissue size (Ke et al. 2013).

Confocal laser scanning microscopy

Samples were investigated using a Zeiss LSM780 (Carl Zeiss, Jena, Germany) confocal laser scanning microscope (CLSM) equipped with an argon laser (DPSS 561-10, λ_{Ex}: 488 nm, laser power: 0.025). We recorded two fluorescence channels: One to visualize the particles (λ_{Em}: 493–550 nm) and the other to visualize Nile Red stained (details see SI) lipid droplets in selected animals (λ_{Em}: 571–753 nm, details see SI) in addition to a brightfield image. The samples of each experiment were imaged using consistent settings with slight variations for the bright field digital gain, which did not affect the

fluorescence signals (details see SI). In addition to single images taken at 10× (Plan-Apochromat 10x/0.3 M27, pinhole diameter: 89.89 µm), 20× (Plan-Apochromat 20x/0.8 M27), and 40× (EC Plan-Neofluar 40x/0.75 Ph2 M27) magnifications, we recorded focus stacks for each animal (details see SI). Images were processed in FIJI 1.52i (Schindelin et al. 2012).

Leaching experiment

In experiment IV, we investigated the leaching of the fluorescence dye from the PS beads and subsequent transfer to a synthetic acceptor phase. Medical grade silicone rubber sheets (127 µm thickness, Technical Products Inc., Decatur, USA) were cut into strips of approximately 400 by 5,000 µm using a box cutter. The strips were placed in particle suspensions mimicking the conditions of experiment III for 24 h (2 mg L^{-1} of 1,000 nm beads in 80 mL U.S. EPA reconstituted hard water). Additionally, we also used a higher concentration of 200 mg L^{-1} over 24 h in a miniaturized setting (200 µL total volume, diluted with ultrapure water). All strips were rinsed in ultrapure water after the incubation, placed on an object slide and imaged using settings consistent with the daphnid images (λ_{Ex}: 488 nm, λ_{Em}: 493–550 nm, 10× magnification).

Results & Discussion

Replication of the Rosenkranz et al. study (experiment I)

In daphnids exposed to 20 nm PS beads, it was not possible to distinguish between control and exposed animals after 4 and 24 h (Figure 1A and B). Accordingly and in contrast to the findings of Rosenkranz et al., we did not detect fluorescence in daphnids exposed to the 20 nm particles at 2 µg L^{-1}. The 1,000 nm particles were clearly visible inside the digestive tract of most exposed animals (Figure 1 C, visible in 10 out of 15 animals after 4 h exposure, 12 out of 14 animals after 24 h). This is in line with previous studies, demonstrating that daphnids readily ingest nano- and microplastics (Jemec et al. 2016; Ogonowski et al. 2016; Rist et al. 2017; Scherer et al. 2017; Frydkjær et al. 2017; Canniff and Hoang 2018). We did not observe a difference regarding the amount of particles in animals exposed for 4 h compared to those exposed for 24 h. This is not surprising given the short gut retention time of microplastics in *D. magna* (Ogonowski et al. 2016; Scherer et al. 2017) that will result in a constant re-uptake of particles. A representative CLSM image of each specimen from experiment I is deposited on figshare (DOI: 10.6084/m9.figshare.7240469).

In their study, Rosenkranz et al. (2009) observed strong fluorescence of 1,000 nm beads in the digestive tract of all studied daphnids (further details in Rosenkranz, 2010). In contrast to our observations (Figure 1C), individual particles were not visible. This implies that in their study either higher ingestion, a lower CLSM resolution or a digital amplification of the fluorescence signal during CLSM prevented the imaging of individual particles. More importantly, Rosenkranz et al. reported strong fluorescence in the lipid droplets of daphnids exposed to 2 µg L^{-1} of both, 20 and 1,000 nm PS beads, and concluded that the particles had translocated and accumulated there. In contrast, we did not observe any fluorescence outside the digestive tract in the 60 specimens analyzed in experiment I. Thus, we were unable to reproduce the original findings of Rosenkranz et al. with identical experimental conditions and a large sample size.

The formalin-treated specimens were largely nontransparent (Figure 1A–C). At the same time, we observed a strong autofluorescence of the carapax in all animals from experiment I (Figure S1).

Sheehy and Ettershank (1988) investigated autofluorescence in *Daphnia carinata* and found regions with a blue and a green excitation, the latter being in a comparable wavelength range as the one used for green fluorescence in our and other studies. This includes the gut lumen and embryos in the brood pouch with blue green fluorescence. Additionally, they reported a post mortem increase in fluorescence. Accordingly, autofluorescence may be a confounding factor when studying the translocation of fluorescent particles that needs to be accounted for, for instance by imaging an adequate number of control animals. As Rosenkranz et al. (2009) did not provide images of such controls, it is impossible to evaluate whether autofluorescence interfered with their imaging analysis. Rosenkranz et al. partly account for that by using a quencher, however. Importantly, a number of CLSM settings can affect the (auto)fluorescence. While the authors kept the gain and offset stable, other settings (e.g., laser power, gain for each used channel, pinhole) remain unreported. Unless all images are recorded with consistent settings, a fluorescence signal of a tentative plastic particle may be nothing but the result of, for example, an elevated digital gain. Here, the full and transparent reporting of the controls and the imaging settings is essential.

Rosenkranz et al. provided additional TEM images that show dark structures in lipid droplets in both size classes. They acknowledge the presence of granular structures in both control animals and those treated with 20 nm particles. Thus, a translocation of the smaller beads based on TEM remains inconclusive. For the 1,000 nm beads, Rosenkranz et al. observed dark, oval structures with a diameter of about 2 µm. While the authors use that as major argument to support the idea of a tissue translocation, this is far from conclusive. For instance, in the TEM images provided in Rosenkranz (2010) the beads alone look somewhat distinct (spherical, 1,000 nm in diameter, fuzzy edges) from the structures observed in the lipid droplets. In any case, “visual” interpretation of TEM images may be prone to artifacts and misleading conclusions (Jensen et al. 2016) and the results by Rosenkranz et al. need to be followed up by in-depth TEM imaging.

Tissue translocation with improved animal transparency (experiment II)

In experiment II, 60 animals were exposed to PS beads under identical conditions as in experiment I. The specimens were cleared using the SeeDB method to increase their transmittance and, thus, the detection of fluorescent particles in the animals. The clearing improved the visibility (Figure 1D–F) compared to the original formalin fixation. However, this treatment reduced sample integrity, increasing the risk of damaging the specimens during handling. In accordance with experiment I, no fluorescence was observed in daphnids exposed to 20 nm beads (Figure 1E). After 4 and 24 h exposure, animals exposed to 1,000 nm beads had visible particles inside the gut (Figure 1F). No fluorescence in lipid droplets was observed in animals collected from either of the treatments. Therefore, an accumulation of nano- and microplastics in lipid droplets was not confirmed even when their detectability was improved.

Tissue translocation with a 1,000-fold higher concentration (experiment III)

Since no particle translocation was found in experiments I and II and the 20 nm beads were not detected at all under the experimental conditions used by Rosenkranz et al. (2009), we repeated the experiment with a 1,000-fold higher particle concentration (2 mg L^{-1}). The SeeDB clearing was applied based on the improvement on particle detection described above. In contrast to experiments I and II, fluorescence was observed in the lipid droplets and guts of animals exposed to both, 20 nm and 1,000 nm beads and after 4 h and 24 h exposure (Figure 2). Accordingly, the observation of Rosenkranz et al. can be replicated using a 1,000-fold higher concentration. Interestingly, the fluorescence in the lipid droplets quickly faded during CLSM imaging, indicating a quenching or photo-bleaching of the dye. The latter is common for fluorescent dyes such as FITC (Johnson et al. 1982). As in experiment II, the 1,000 nm particles were clearly visible as individual beads in the gut of the daphnids (Figure S2). In contrast to the fluorescence in the lipid droplets, the fluorescence of the 1,000 nm beads in the digestive system was stable throughout imaging (Figure 3). To follow up, we investigated regions of interest covering the gut and lipid droplets at higher magnifications. Here, we could clearly differentiate between the fluorescent beads in the gut and the fluorescence in the lipid droplets which did not co-localize with any particles (Figure 4). Therefore, the fluorescence observed

in the lipid droplets was not associated with the PS beads but probably caused by a leaching of the fluorescent dye. As FITC is lipophilic (estimated logK_{OW} of 4.69 according the U.S. EPA's EPI Suite (United States Environmental Protection Agency, Washington, DC, U.S.A. 2012) it is probable that the chemical dye, and not the dyed particles, translocates from the gut and accumulates in the lipid droplets. This would also be consistent with the rapid loss of fluorescence during imaging. If true, the fluorescence observed in lipid droplets by Rosenkranz et al. (2009) is not proof of a tissue translocation of nano- and microplastics but merely an artifact cause by a leaching of FITC from the PS beads.

Notably, while plastic particles were not detected in lipid droplets, 1,000 nm beads were observed in close proximity but outside the digestive tracts of some daphnids (Figure S2). However, this was rare and always in the context of a damaged gut which was probably caused by the sample handling. Brun et al. (2017) made a similar observation in daphnids exposed to 25 nm PS beads and attributed particles outside the gut to damages during sample preparation.

Dye leaching experiment (experiment IV)

To test the hypothesis that the fluorescent dye is leaching from the particles, we incubated medical grade silicone rubber strips over 24 h with 2 and 200 mg L^{-1} of 1,000 nm PS beads and subsequently imaged them using CLSM. After incubation with 2 mg L^{-1} particles, the strips emit a weak fluorescence (Figure 5 A and A') compared to the control strip. Some particles could not be washed off and adhered to the surface but are clearly visible. A strip incubated with 200 mg L^{-1} for the same period in ultrapure water exhibits a stronger fluorescence signal (Figure 5 C and C'), whereas the control strips did not (Figure 5 B and B'). These results indicate the transfer of FITC from the particles to a synthetic matrix with similar partitioning properties as the lipid droplets in Daphnia. The 5 mm silicone rubber strips have a much higher volume than the lipid droplets in Daphnia neonates. Additionally, the dye transfer will continue after exposure of the animals until the subsequent imaging. In our case the strips were imaged immediately after exposure, but the daphnids were stored before imaging. Therefore, the weaker fluorescence signal in strips incubated with 2 mg

L^{-1} PS beads is likely due to a larger acceptor volume and a shorter incubation time. Accordingly, the fluorescence in lipid storage droplets in daphnids caused by a leaching of the dye from 2 mg L^{-1} PS beads would be much stronger. This further supports that the fluorescence Rosenkranz et al. observed outside the digestive tract in fact might be an artifact caused by the leaching of the fluorescent dye. Interestingly, the shortcomings of using fluorescent dyes not covalently bound to particles have been discussed in the area of nanotoxicology (Kettiger et al. 2013; Rothen-Rutishauser et al. 2013). To avoid potential artifacts, future studies need to either demonstrate that the dye is not leaching under experimental or even digestive conditions (Gouliarmou et al. 2013). Alternatively, stably labeled particles e.g. with a metallic core (Mitrano et al. 2019) might be used or microscopic techniques that can certify the identity of the plastic particle by other means than fluorescence.

Are other studies affected?

Following Rosenkranz et al.'s publication, a number of studies have shown micrographs indicating fluorescence in daphnia lipid droplets, even though investigating tissue translocation was not necessarily their primary objective. When exposing *Daphnia galeata* to 5 mg L^{-1} of 51 nm green fluorescent PS beads, Cui et al. (2017) showed fluorescence inside embryos and lipid droplets, and concluded that there is a link to the observed toxicity. Brun et al. (2017) investigated the brood pouch of *D. magna* as a potential exposure pathway for embryos, a mechanism proposed by Rosenkranz et al. (2009). They did not observe a translocation of 25 nm PS beads (5 mg L^{-1}) to maternal lipid droplets but into embryos. As the brood pouch is continuously flushed with water (Seidl et al. 2002), an exposure of embryos via this mechanism is probable. However, the mechanism for a transfer of particles into the embryo remains unclear. Here, the limitations in CLSM might lead to misinterpretation because particles adhering to the chorion cannot easily be distinguished from ones inside the embryo (e.g., in case of low resolution or strong fluorescence scattering). Chae et al. (2018) presented fluorescing lipid droplets in *D. magna* fed with algae that had been exposed to 10 mg L^{-1} of 51 nm fluorescent PS particles. The authors localized fluorescence inside a daphnid through z-stack projections, an approach that has a limited spatial resolution due to a blurring and scattering of the

fluorescence signal across the z-axis. As none of the studies included controls for potential leaching of the fluorescent dye in vivo or during sample storage and imaging, it is not possible to evaluate whether it was indeed the fluorescent particles or just the fluorescent dye that translocated. Importantly, all studies used plastic particles with sizes <60 nm, making translocation biologically plausible (see introduction). However, the imaging and, thus, the unequivocal localization of nanoplastics in tissues remains a fundamental methodological challenge.

Of the 31 studies Triebskorn et al. (2018) reviewed regarding tissue translocation of nano- and microplastics in aquatic invertebrates and fish over 75 % used polystyrene, mostly as commercially available particles. Additionally, fluorescence was the most widely utilized method to evaluate tissue translocation. Thus, the occurrence of false positive results in other studies is plausible. However, as our experiments were performed with one type of particles only, it is not possible to generalize. The leaching and partitioning of dyes from other materials (e.g., different polymers) under other conditions (e.g., different media) remains to be investigated. Here, our passive sampling approach could be an effective screening method for the stability of dyes in commercial nano- and microplastics. Very recently, Catarino et al. (2019) published a study in zebrafish supporting the conclusion that dye leaching from 500 and 1,000 nm PS nanobeads causes artifacts. They suggested a dialysis step to remove uncoupled dye before performing toxicity studies. Dialysis was also recently presented as a method to account for biocidic additives to commercial particle suspensions (Pikuda et al. 2018). Taken together, these results highlight that fluorescent particles may not always be an appropriate surrogate for localizing nano- or microplastics in biological matrices.

Conclusion

The potential translocation of nano- and microplastics into animal tissues is toxicologically relevant as it may cause internal mechanical injury, inflammation, and bioaccumulation. Previous research has postulated a transfer of 20 and 1,000 nm PS beads from the gut to the lipid droplets of *D. magna*. Since the biological mechanism for this phenomenon is implausible, at least for the larger particles, the aim of the present study was to replicate these previous findings. When using the original experimental setup as well as a method with improved sensitivity, we did not observe a tissue translocation of 20 and 1,000 nm PS beads at an exposure concentration of 2 μg L^{-1}. When increasing the concentration by a factor of 1,000, fluorescence in lipid droplets was observed. However, this did not co-localize with the larger particles which remained in the gut lumen. This implies that the fluorescence in the daphnid tissue was caused by the partitioning of the fluorescent dye from the plastic particles to the lipid droplets, which we confirmed using passive sampling. Accordingly, studies reporting a tissue translocation of nano- and microplastics using fluorescence imaging only, are prone to artifacts and need to be interpreted with caution in the light of biological plausibility. Strategies to minimize the risk for dye leaching artifacts in future particle uptake and translocation studies include: (1) Dye leaching during the experiment might be reduced by pre-washing the particles, (2) the absence of leaching at experimental or digestive conditions might be confirmed by simple passive sampling experiments and (3) the observed fluorescence within the tissue should only be taken as an initial observation that cannot stand alone to proof particle uptake and translocation.

Supplemental Data

The Supplemental Data are available on the Wiley Online Library at doi: 10.1002/etc.xxxx

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Table 1: Design of the three experiments to study the tissue translocation of polystyrene nano- and microplastics in *Daphnia magna*.

Experiment	Plastic particles	Concentration	Exposure duration	Clearing	Replicates (individuals)
I (replication)	-	-	24 h	-	3 (15)
	20 nm	2 µg L^{-1}	4, 24 h	-	3 (15)
	1,000 nm	2 µg L^{-1}	4, 24 h	-	3 (15)
II (clearing)	-	-	24 h	SeeDB	3 (15)
	20 nm	2 µg L^{-1}	4, 24 h	SeeDB	3 (15)
	1,000 nm	2 µg L^{-1}	4, 24 h	SeeDB	3 (15)
III (higher concentration)	-	-	24 h	SeeDB	3 (15)
	20 nm	2 mg L^{-1}	4, 24 h	SeeDB	3 (15)
	1,000 nm	2 mg L^{-1}	4, 24 h	SeeDB	3 (15)

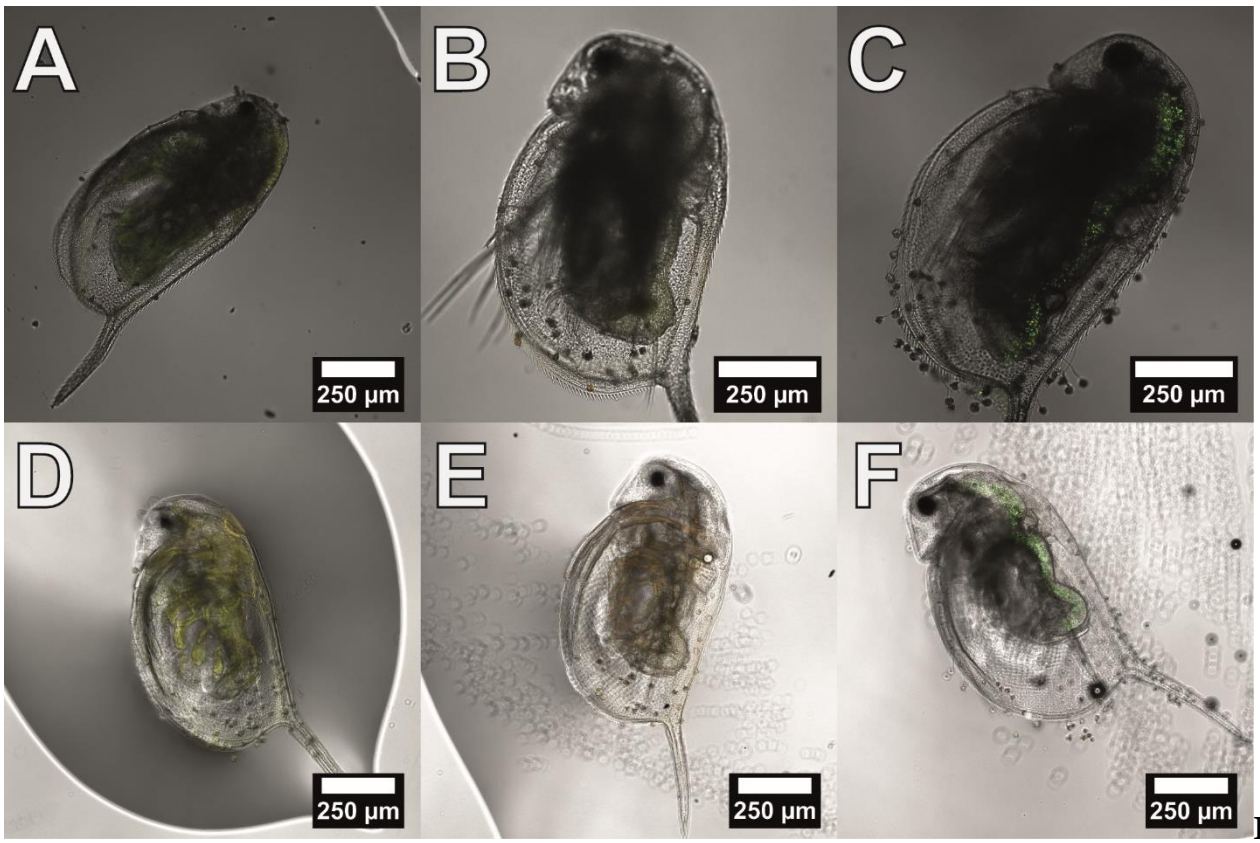

Figure 1: Representative images of fixated *Daphnia magna* neonates exposed to 2 µg L^{-1} fluorescent polystyrene particles using confocal laser scanning microscopy. A–C show specimens from experiment I that were treated identically to Rosenkranz et al.'s method (formalin fixation, experiment I). D–F represents daphnids treated with SeeDB clearing (experiment II). A+D: control animals, B+E: animals exposed for 24 h to 20 nm PS beads, C+F: animals exposed for 24 h to 1,000 nm PS beads. All images are single composite images extracted from a z-stack.

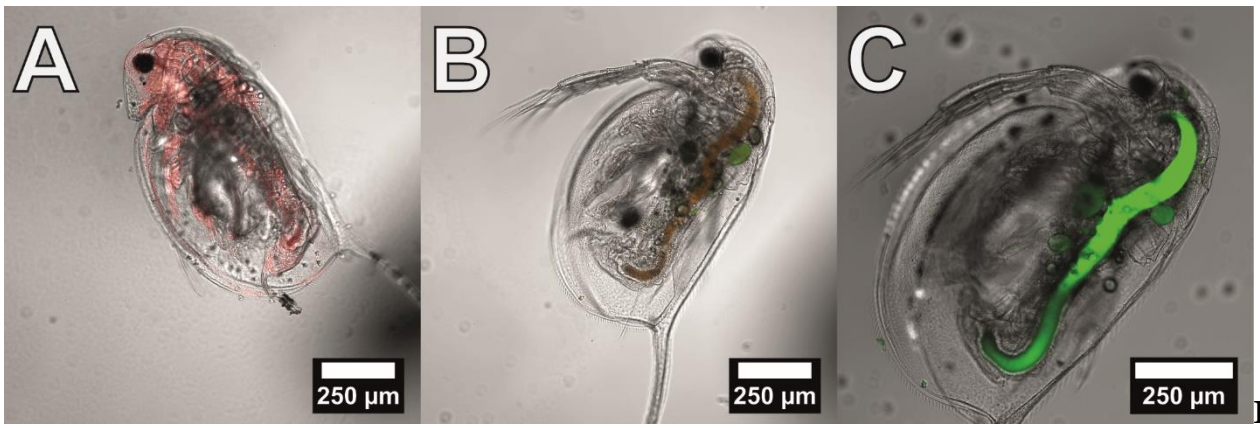

Figure 2: Representative images of SeeDB-cleared *Daphnia magna* neonates exposed to 2 mg L^{-1} fluorescent polystyrene particles using confocal laser scanning microscopy (experiment III). A: control animal, B: animal exposed for 24 h to 20 nm beads, C: animal exposed for 24 h to 1,000 nm beads. All images are single composite images extracted from a z-stack.

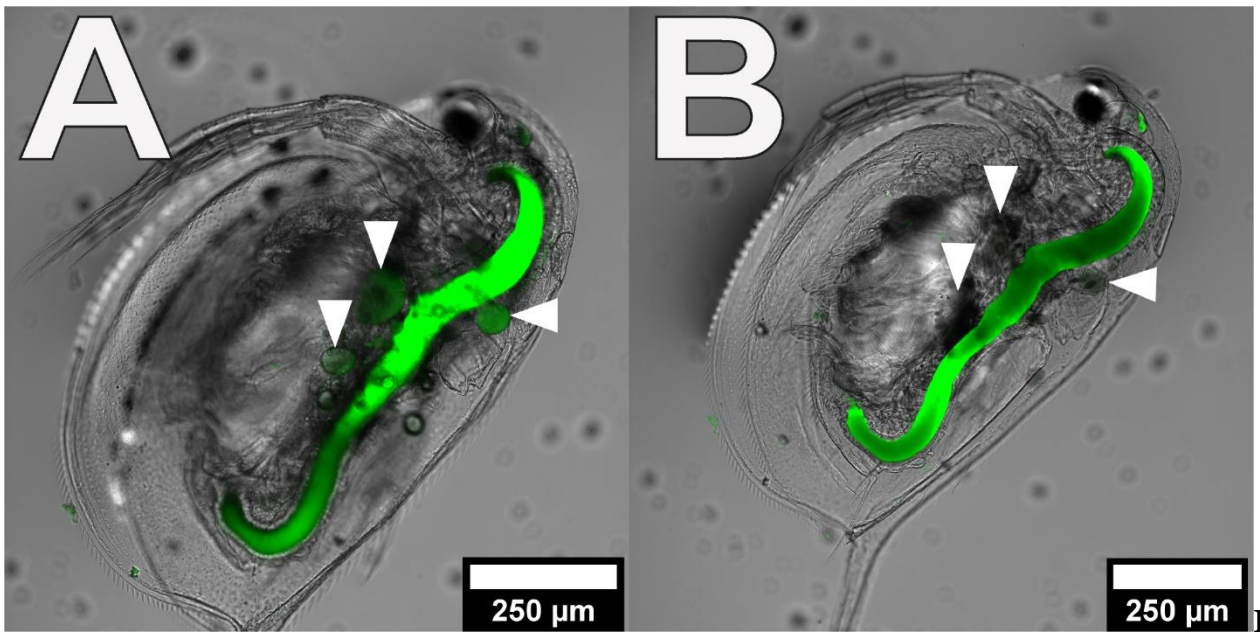

Figure 3: Identical *Daphnia magna* individual exposed to 1,000 nm PS particles for 24 h before (A) and after (B) 60 min of confocal laser scanning microscopy imaging. The fluorescence is clearly visible in the lipid droplets (white arrows) initially (A) but faded during investigation (B). The particle-associated fluorescence in the gut lumen did not change. Microscope imaging settings are identical for both micrographs except for the zoom factor (0.8 in A, 0.7 in B) and a 26.2 µm difference in the z position.

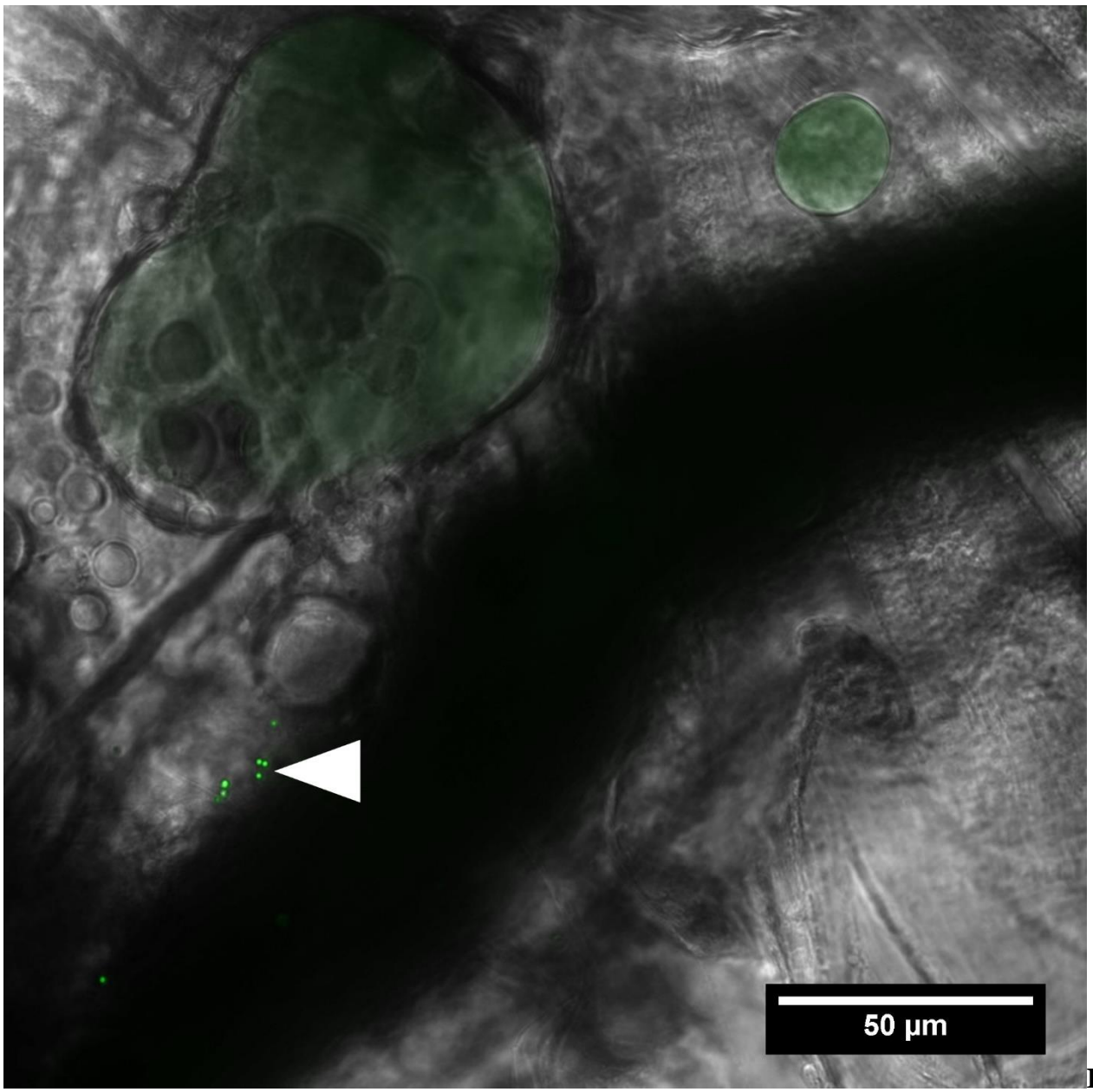

Figure 4: Localization of 1,000 nm PS beads in the lumen of the daphnid gut (white arrow) and the fluorescence in the lipid droplet. The fluorescence in the lipid droplets is not co-localized with the microplastics and quickly faded upon investigation.

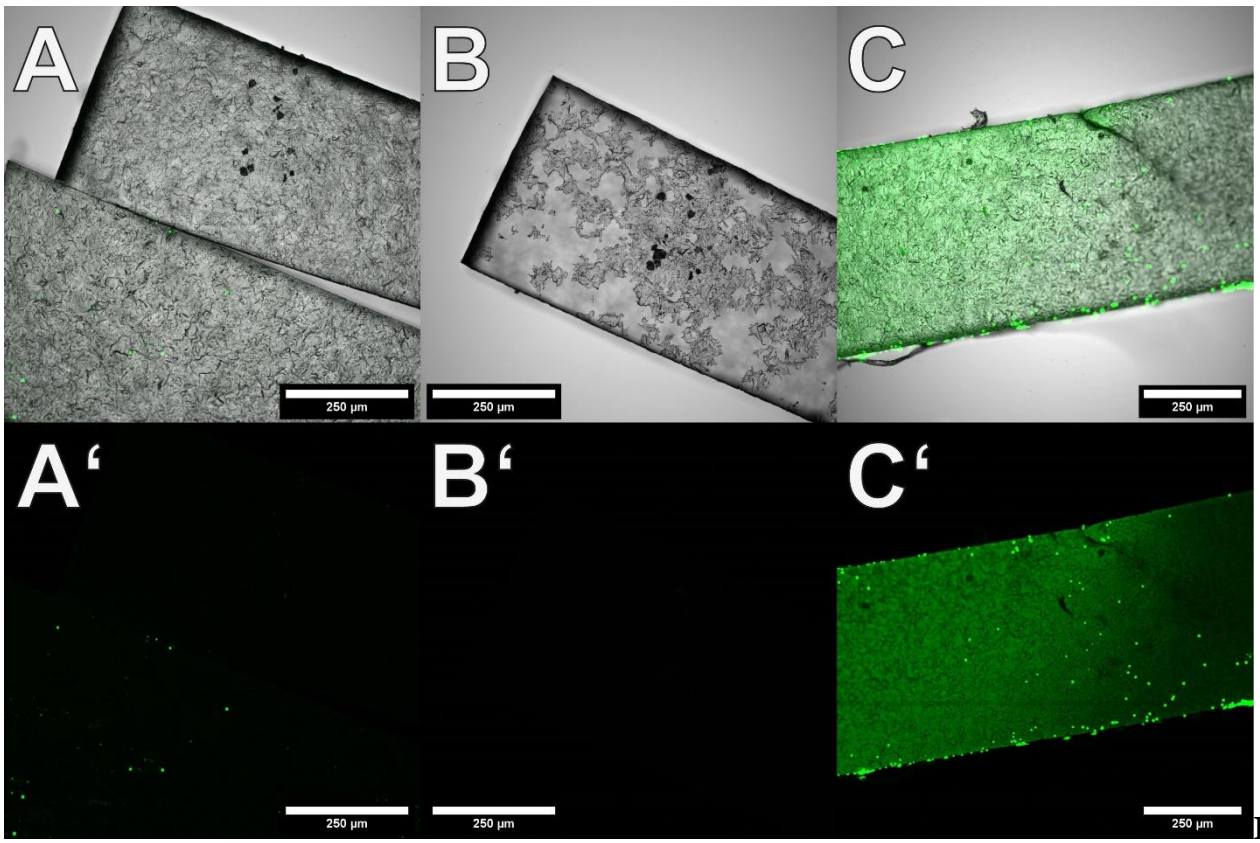

Figure 5: Transfer of fluorescent dye from 1,000 nm polystyrene bead suspensions (2 and 200 mg L^{-1}) to silicone rubber strips in fluorescence and brightfield channel (A, B, C) and fluorescence only (A', B', C'). A+A': The upper and the lower strips were incubated for 24 h in U.S. EPA reconstituted hard water without and with plastic particles, respectively; B+B': Control strip incubated for 24 h in ultrapure water; C+C': Strip incubated for 24 h in 200 mg L^{-1} bead suspension in ultrapure water.

Supplementary Material

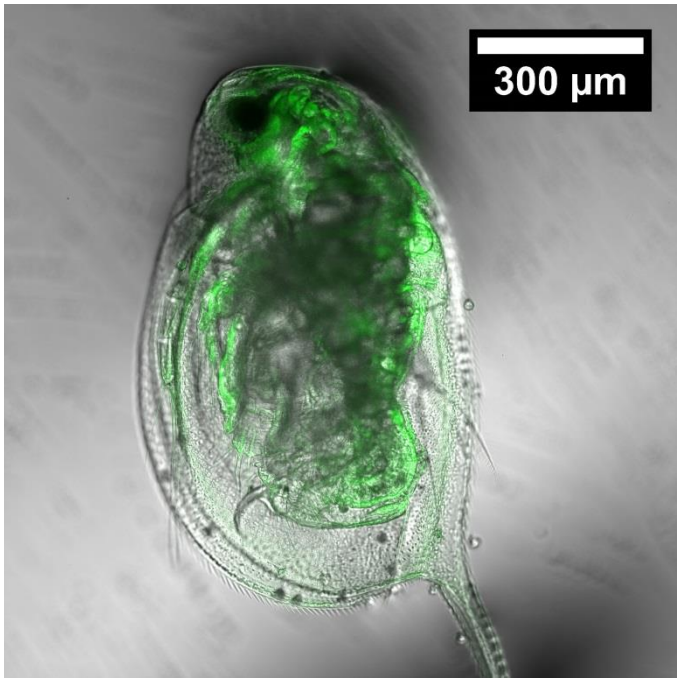

Figure S1: Control animal at high gain levels showing autofluorescence of the carapax.

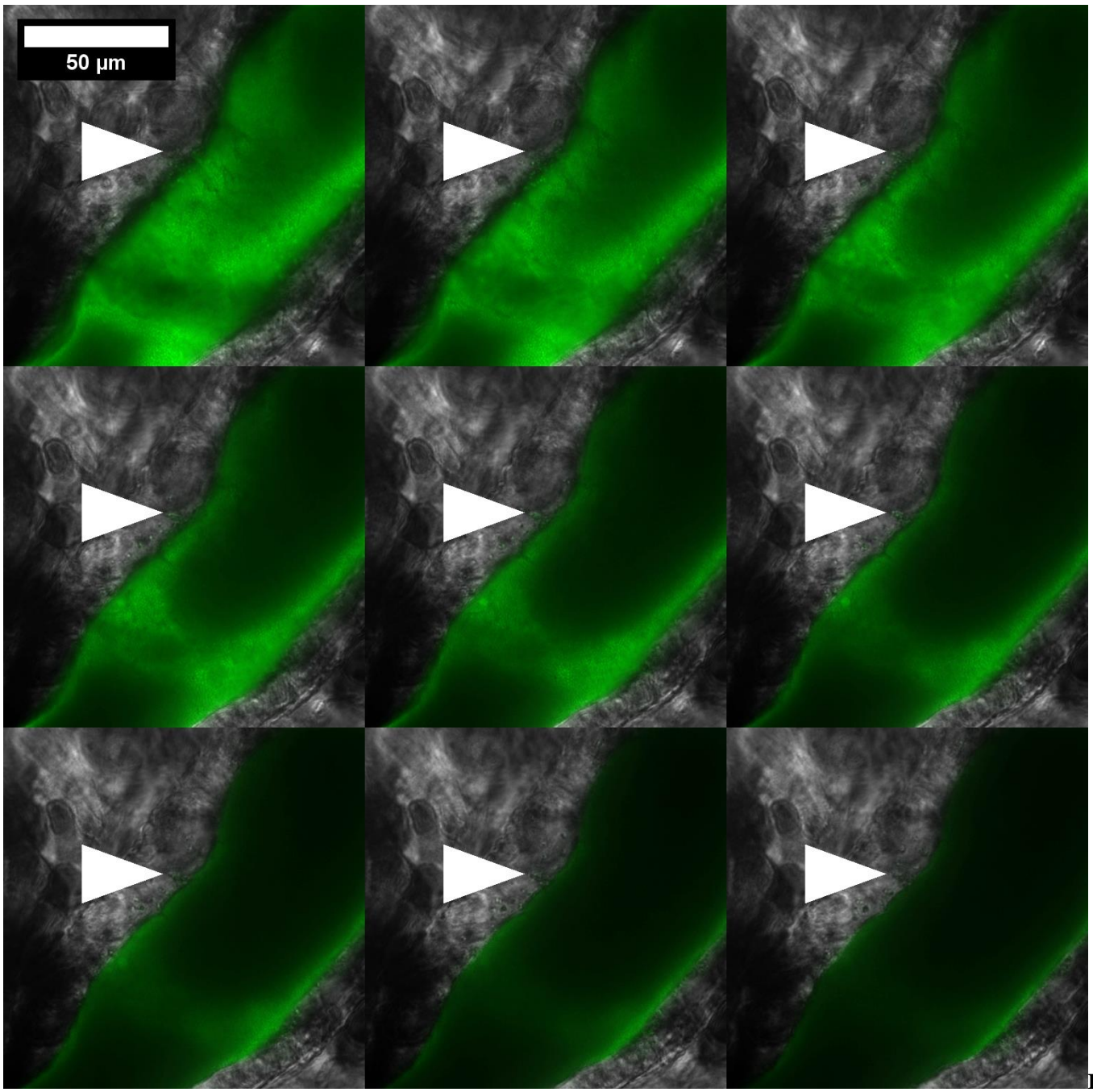

Figure S2: 1000 nm PS particles individually visible inside a daphnid's midgut after exposure for 24 h. The arrow indicates a rupture and the associated release of particles from the gut.

Table S1: Exposure scenario properties and respective animal identifier, exposure at 2 µg L^{-1}. The comment damaged indicates that a certain specimen was damaged during sample treatment or the microscopical procedure handling. Identifier refers to the image name available at figshare (doi: 10.6084/m9.figshare.7240469).

Treatment Formalin/ SeeDB	Particle (20 nm, 1000 nm, Control)	Exposure duration (4 h, 24 h)	Replicate number (1-3)	Specimen number (1-5)	Identifier	Comment
Formalin	20 nm	4 h	1	1	F-20-4-1-1	
Formalin	20 nm	4 h	1	2	F-20-4-1-2	
Formalin	20 nm	4 h	1	3	F-20-4-1-3	
Formalin	20 nm	4 h	1	4	F-20-4-1-4	
Formalin	20 nm	4 h	1	5	F-20-4-1-5	
Formalin	20 nm	4 h	2	1	F-20-4-2-1	
Formalin	20 nm	4 h	2	2	F-20-4-2-2	
Formalin	20 nm	4 h	2	3	F-20-4-2-3	
Formalin	20 nm	4 h	2	4	F-20-4-2-4	
Formalin	20 nm	4 h	2	5	F-20-4-2-5	
Formalin	20 nm	4 h	3	1	F-20-4-3-1	
Formalin	20 nm	4 h	3	2	F-20-4-3-2	
Formalin	20 nm	4 h	3	3	F-20-4-3-3	
Formalin	20 nm	4 h	3	4	F-20-4-3-4	
Formalin	20 nm	4 h	3	5	F-20-4-3-5	
Formalin	20 nm	24 h	1	1	F-20-24-1-1	
Formalin	20 nm	24 h	1	2	F-20-24-1-2	
Formalin	20 nm	24 h	1	3	F-20-24-1-3	
Formalin	20 nm	24 h	1	4	F-20-24-1-4	
Formalin	20 nm	24 h	1	5	F-20-24-1-5	*damaged*
Formalin	20 nm	24 h	2	1	F-20-24-2-1	
Formalin	20 nm	24 h	2	2	F-20-24-2-2	
Formalin	20 nm	24 h	2	3	F-20-24-2-3	
Formalin	20 nm	24 h	2	4	F-20-24-2-4	
Formalin	20 nm	24 h	2	5	F-20-24-2-5	
Formalin	20 nm	24 h	3	1	F-20-24-3-1	
Formalin	20 nm	24 h	3	2	F-20-24-3-2	
Formalin	20 nm	24 h	3	3	F-20-24-3-3	

Treatment Formalin/ SeeDB	Particle (20 nm, 1000 nm, Control)	Exposure duration (4 h, 24 h)	Replicate number (1-3)	Specimen number (1-5)	Identifier	Comment
Formalin	20 nm	24 h	3	4	F-20-24-3-4	
Formalin	20 nm	24 h	3	5	F-20-24-3-5	
Formalin	1000 nm	4 h	1	1	F-1-4-1-1	
Formalin	1000 nm	4 h	1	2	F-1-4-1-2	
Formalin	1000 nm	4 h	1	3	F-1-4-1-3	
Formalin	1000 nm	4 h	1	4	F-1-4-1-4	
Formalin	1000 nm	4 h	1	5	F-1-4-1-5	
Formalin	1000 nm	4 h	2	1	F-1-4-2-1	
Formalin	1000 nm	4 h	2	2	F-1-4-2-2	
Formalin	1000 nm	4 h	2	3	F-1-4-2-3	
Formalin	1000 nm	4 h	2	4	F-1-4-2-4	
Formalin	1000 nm	4 h	2	5	F-1-4-2-5	
Formalin	1000 nm	4 h	3	1	F-1-4-3-1	
Formalin	1000 nm	4 h	3	2	F-1-4-3-2	
Formalin	1000 nm	4 h	3	3	F-1-4-3-3	
Formalin	1000 nm	4 h	3	4	F-1-4-3-4	
Formalin	1000 nm	4 h	3	5	F-1-4-3-5	
Formalin	1000 nm	24 h	1	1	F-1-24-1-1	
Formalin	1000 nm	24 h	1	2	F-1-24-1-2	
Formalin	1000 nm	24 h	1	3	F-1-24-1-3	
Formalin	1000 nm	24 h	1	4	F-1-24-1-4	
Formalin	1000 nm	24 h	1	5	F-1-24-1-5	*damaged*
Formalin	1000 nm	24 h	2	1	F-1-24-2-1	
Formalin	1000 nm	24 h	2	2	F-1-24-2-2	
Formalin	1000 nm	24 h	2	3	F-1-24-2-3	
Formalin	1000 nm	24 h	2	4	F-1-24-2-4	
Formalin	1000 nm	24 h	2	5	F-1-24-2-5	
Formalin	1000 nm	24 h	3	1	F-1-24-3-1	
Formalin	1000 nm	24 h	3	2	F-1-24-3-2	
Formalin	1000 nm	24 h	3	3	F-1-24-3-3	
Formalin	1000 nm	24 h	3	4	F-1-24-3-4	

Treatment Formalin/ SeeDB	Particle (20 nm, 1000 nm, Control)	Exposure duration (4 h, 24 h)	Replicate number (1-3)	Specimen number (1-5)	Identifier	Comment
Formalin	1000 nm	24 h	3	5	F-1-24-3-5	
Formalin	Control	24 h	1	1	F-C-1-1	
Formalin	Control	24 h	1	2	F-C-1-2	
Formalin	Control	24 h	1	3	F-C-1-3	
Formalin	Control	24 h	1	4	F-C-1-4	
Formalin	Control	24 h	1	5	F-C-1-5	
Formalin	Control	24 h	2	1	F-C-2-1	
Formalin	Control	24 h	2	2	F-C-2-2	
Formalin	Control	24 h	2	3	F-C-2-3	
Formalin	Control	24 h	2	4	F-C-2-4	
Formalin	Control	24 h	2	5	F-C-2-5	
Formalin	Control	24 h	3	1	F-C-3-1	
Formalin	Control	24 h	3	2	F-C-3-2	
Formalin	Control	24 h	3	3	F-C-3-3	
Formalin	Control	24 h	3	4	F-C-3-4	
Formalin	Control	24 h	3	5	F-C-3-5	
SeeDB	20 nm	4 h	1	1	P-20-4-1-1	
SeeDB	20 nm	4 h	1	2	P-20-4-1-2	
SeeDB	20 nm	4 h	1	3	P-20-4-1-3	
SeeDB	20 nm	4 h	1	4	P-20-4-1-4	
SeeDB	20 nm	4 h	1	5	P-20-4-1-5	*damaged*
SeeDB	20 nm	4 h	2	1	P-20-4-2-1	
SeeDB	20 nm	4 h	2	2	P-20-4-2-2	
SeeDB	20 nm	4 h	2	3	P-20-4-2-3	
SeeDB	20 nm	4 h	2	4	P-20-4-2-4	
SeeDB	20 nm	4 h	2	5	P-20-4-2-5	
SeeDB	20 nm	4 h	3	1	P-20-4-3-1	
SeeDB	20 nm	4 h	3	2	P-20-4-3-2	
SeeDB	20 nm	4 h	3	3	P-20-4-3-3	
SeeDB	20 nm	4 h	3	4	P-20-4-3-4	
SeeDB	20 nm	4 h	3	5	P-20-4-3-5	*damag*

Treatment Formalin/ SeeDB	Particle (20 nm, 1000 nm, Control)	Exposure duration (4 h, 24 h)	Replicate number (1-3)	Specimen number (1-5)	Identifier	Comment
						ed
SeeDB	20 nm	24 h	1	1	P-20-24-1-1	
SeeDB	20 nm	24 h	1	2	P-20-24-1-2	
SeeDB	20 nm	24 h	1	3	P-20-24-1-3	
SeeDB	20 nm	24 h	1	4	P-20-24-1-4	*damaged*
SeeDB	20 nm	24 h	1	5	P-20-24-1-5	*damaged*
SeeDB	20 nm	24 h	2	1	P-20-24-2-1	
SeeDB	20 nm	24 h	2	2	P-20-24-2-2	
SeeDB	20 nm	24 h	2	3	P-20-24-2-3	
SeeDB	20 nm	24 h	2	4	P-20-24-2-4	
SeeDB	20 nm	24 h	2	5	P-20-24-2-5	
SeeDB	20 nm	24 h	3	1	P-20-24-3-1	
SeeDB	20 nm	24 h	3	2	P-20-24-3-2	
SeeDB	20 nm	24 h	3	3	P-20-24-3-3	
SeeDB	20 nm	24 h	3	4	P-20-24-3-4	
SeeDB	20 nm	24 h	3	5	P-20-24-3-5	*damaged*
SeeDB	1000 nm	4 h	1	1	P-1-4-1-1	
SeeDB	1000 nm	4 h	1	2	P-1-4-1-2	
SeeDB	1000 nm	4 h	1	3	P-1-4-1-3	*damaged*
SeeDB	1000 nm	4 h	1	4	P-1-4-1-4	*damaged*
SeeDB	1000 nm	4 h	1	5	P-1-4-1-5	*damaged*
SeeDB	1000 nm	4 h	2	1	P-1-4-2-1	
SeeDB	1000 nm	4 h	2	2	P-1-4-2-2	
SeeDB	1000 nm	4 h	2	3	P-1-4-2-3	
SeeDB	1000 nm	4 h	2	4	P-1-4-2-4	*damaged*

Treatment Formalin/ SeeDB	Particle (20 nm, 1000 nm, Control)	Exposure duration (4 h, 24 h)	Replicate number (1-3)	Specimen number (1-5)	Identifier	Comment
SeeDB	1000 nm	4 h	2	5	P-1-4-2-5	
SeeDB	1000 nm	4 h	3	1	P-1-4-3-1	
SeeDB	1000 nm	4 h	3	2	P-1-4-3-2	
SeeDB	1000 nm	4 h	3	3	P-1-4-3-3	*damaged*
SeeDB	1000 nm	4 h	3	4	P-1-4-3-4	
SeeDB	1000 nm	4 h	3	5	P-1-4-3-5	
SeeDB	1000 nm	24 h	1	1	P-1-24-1-1	
SeeDB	1000 nm	24 h	1	2	P-1-24-1-2	
SeeDB	1000 nm	24 h	1	3	P-1-24-1-3	
SeeDB	1000 nm	24 h	1	4	P-1-24-1-4	
SeeDB	1000 nm	24 h	1	5	P-1-24-1-5	
SeeDB	1000 nm	24 h	2	1	P-1-24-2-1	
SeeDB	1000 nm	24 h	2	2	P-1-24-2-2	
SeeDB	1000 nm	24 h	2	3	P-1-24-2-3	
SeeDB	1000 nm	24 h	2	4	P-1-24-2-4	*damaged*
SeeDB	1000 nm	24 h	2	5	P-1-24-2-5	
SeeDB	1000 nm	24 h	3	1	P-1-24-3-1	
SeeDB	1000 nm	24 h	3	2	P-1-24-3-2	
SeeDB	1000 nm	24 h	3	3	P-1-24-3-3	
SeeDB	1000 nm	24 h	3	4	P-1-24-3-4	
SeeDB	1000 nm	24 h	3	5	P-1-24-3-5	
SeeDB	Control	24 h	1	1	P-C-1-1	
SeeDB	Control	24 h	1	2	P-C-1-2	
SeeDB	Control	24 h	1	3	P-C-1-3	
SeeDB	Control	24 h	1	4	P-C-1-4	
SeeDB	Control	24 h	1	5	P-C-1-5	
SeeDB	Control	24 h	2	1	P-C-2-1	
SeeDB	Control	24 h	2	2	P-C-2-2	
SeeDB	Control	24 h	2	3	P-C-2-3	
SeeDB	Control	24 h	2	4	P-C-2-4	

Treatment Formalin/ SeeDB	Particle (20 nm, 1000 nm, Control)	Exposure duration (4 h, 24 h)	Replicate number (1-3)	Specimen number (1-5)	Identifier	Comment
SeeDB	Control	24 h	2	5	P-C-2-5	
SeeDB	Control	24 h	3	1	P-C-3-1	
SeeDB	Control	24 h	3	2	P-C-3-2	
SeeDB	Control	24 h	3	3	P-C-3-3	
SeeDB	Control	24 h	3	4	P-C-3-4	
SeeDB	Control	24 h	3	5	P-C-3-5	

Table S2: Exposure scenario properties and respective animal identifier, exposure at 2 mg L^{-1}. The comment damaged indicates that a certain specimen was damaged during sample treatment or the microscopical procedure handling. A number of animals were stained using Nile Red to improve visibility of lipid droplets. The protocol used was described previously by Jordão et al. (2015), they are marked with the comment "stained". Identifier refers to the image name available at figshare (DOI: 10.6084/m9.figshare.7240469).

Treatment	Particle (20 nm, 1000 nm, Control)	Exposure duration (4 h, 24 h)	Replicate number (1-3)	Specimen number (1-5)	Identifier	Comment
SeeDB	20 nm	4 h	1	1	P2-20-4-1-1	
SeeDB	20 nm	4 h	1	2	P2-20-4-1-2	
SeeDB	20 nm	4 h	1	3	P2-20-4-1-3	
SeeDB	20 nm	4 h	1	4	P2-20-4-1-4	
SeeDB	20 nm	4 h	1	5	P2-20-4-1-5	
SeeDB	20 nm	4 h	2	1	P2-20-4-2-1	
SeeDB	20 nm	4 h	2	2	P2-20-4-2-2	
SeeDB	20 nm	4 h	2	3	P2-20-4-2-3	
SeeDB	20 nm	4 h	2	4	P2-20-4-2-4	
SeeDB	20 nm	4 h	2	5	P2-20-4-2-5	
SeeDB	20 nm	4 h	3	1	P2-20-4-3-1	
SeeDB	20 nm	4 h	3	2	P2-20-4-3-2	
SeeDB	20 nm	4 h	3	3	P2-20-4-3-3	
SeeDB	20 nm	4 h	3	4	P2-20-4-3-4	
SeeDB	20 nm	4 h	3	5	P2-20-4-3-5	
SeeDB	20 nm	24 h	1	1	P2-20-24-1-1	
SeeDB	20 nm	24 h	1	2	P2-20-24-1-2	
SeeDB	20 nm	24 h	1	3	P2-20-24-1-3	
SeeDB	20 nm	24 h	1	4	P2-20-24-1-4	*damaged*
SeeDB	20 nm	24 h	1	5	P2-20-24-1-5	*damaged*
SeeDB	20 nm	24 h	2	1	P2-20-24-2-1	
SeeDB	20 nm	24 h	2	2	P2-20-24-2-2	*damaged*
SeeDB	20 nm	24 h	2	3	P2-20-24-2-3	
SeeDB	20 nm	24 h	2	4	P2-20-24-2-4	
SeeDB	20 nm	24 h	2	5	P2-20-24-2-5	*damaged*

Treatment	Particle (20 nm, 1000 nm, Control)	Exposure duration (4 h, 24 h)	Replicate number (1-3)	Specimen number (1-5)	Identifier	Comment
SeeDB	20 nm	24 h	3	1	P2-20-24-3-1	*stained*
SeeDB	20 nm	24 h	3	2	P2-20-24-3-2	*stained*
SeeDB	20 nm	24 h	3	3	P2-20-24-3-3	
SeeDB	20 nm	24 h	3	4	P2-20-24-3-4	*damaged*
SeeDB	20 nm	24 h	3	5	P2-20-24-3-5	*damaged*
SeeDB	1000 nm	4 h	1	1	P2-1-4-1-1	
SeeDB	1000 nm	4 h	1	2	P2-1-4-1-2	
SeeDB	1000 nm	4 h	1	3	P2-1-4-1-3	
SeeDB	1000 nm	4 h	1	4	P2-1-4-1-4	
SeeDB	1000 nm	4 h	1	5	P2-1-4-1-5	
SeeDB	1000 nm	4 h	2	1	P2-1-4-2-1	
SeeDB	1000 nm	4 h	2	2	P2-1-4-2-2	
SeeDB	1000 nm	4 h	2	3	P2-1-4-2-3	
SeeDB	1000 nm	4 h	2	4	P2-1-4-2-4	
SeeDB	1000 nm	4 h	2	5	P2-1-4-2-5	
SeeDB	1000 nm	4 h	3	1	P2-1-4-3-1	
SeeDB	1000 nm	4 h	3	2	P2-1-4-3-2	
SeeDB	1000 nm	4 h	3	3	P2-1-4-3-3	
SeeDB	1000 nm	4 h	3	4	P2-1-4-3-4	
SeeDB	1000 nm	4 h	3	5	P2-1-4-3-5	
SeeDB	1000 nm	24 h	1	1	P2-1-24-1-1	
SeeDB	1000 nm	24 h	1	2	P2-1-24-1-2	
SeeDB	1000 nm	24 h	1	3	P2-1-24-1-3	
SeeDB	1000 nm	24 h	1	4	P2-1-24-1-4	*damaged*
SeeDB	1000 nm	24 h	1	5	P2-1-24-1-5	*damaged*
SeeDB	1000 nm	24 h	2	1	P2-1-24-2-1	
SeeDB	1000 nm	24 h	2	2	P2-1-24-2-2	
SeeDB	1000 nm	24 h	2	3	P2-1-24-2-3	
SeeDB	1000 nm	24 h	2	4	P2-1-24-2-4	
SeeDB	1000 nm	24 h	2	5	P2-1-24-2-5	

Treatment	Particle (20 nm, 1000 nm, Control)	Exposure duration (4 h, 24 h)	Replicate number (1-3)	Specimen number (1-5)	Identifier	Comment
SeeDB	1000 nm	24 h	3	1	P2-1-24-3-1	*stained*
SeeDB	1000 nm	24 h	3	2	P2-1-24-3-2	*stained*
SeeDB	1000 nm	24 h	3	3	P2-1-24-3-3	
SeeDB	1000 nm	24 h	3	4	P2-1-24-3-4	*damaged*
SeeDB	1000 nm	24 h	3	5	P2-1-24-3-5	*damaged*
SeeDB	Control	-	1	1	P2-C-1-1	
SeeDB	Control	-	1	2	P2-C-1-2	
SeeDB	Control	-	1	3	P2-C-1-3	
SeeDB	Control	-	1	4	P2-C-1-4	
SeeDB	Control	-	1	5	P2-C-1-5	
SeeDB	Control	-	2	1	P2-C-2-1	
SeeDB	Control	-	2	2	P2-C-2-2	*damaged*
SeeDB	Control	-	2	3	P2-C-2-3	
SeeDB	Control	-	2	4	P2-C-2-4	*damaged*
SeeDB	Control	-	2	5	P2-C-2-5	
SeeDB	Control	-	3	1	P2-C-3-1	*stained*
SeeDB	Control	-	3	2	P2-C-3-2	*stained*
SeeDB	Control	-	3	3	P2-C-3-3	
SeeDB	Control	-	3	4	P2-C-3-4	
SeeDB	Control	-	3	5	P2-C-3-5	

Settings for confocal laserscanning microscopy

1: Exemplary imaging settings, extracted with FIJI (Schindelin et al. 2012) for an animal exposed to 20 nm particles for 4 h and subsequently treated with 10 % formalin.

Experiment|AcquisitionBlock|AcquisitionModeSetup|AcquisitionMode #1 = StackFocus
Experiment|AcquisitionBlock|AcquisitionModeSetup|BiDirectional #1 = false
Experiment|AcquisitionBlock|AcquisitionModeSetup|BiDirectionalZ #1 = false
Experiment|AcquisitionBlock|AcquisitionModeSetup|BitsPerSample #1 = 8
Experiment|AcquisitionBlock|AcquisitionModeSetup|CameraBinning #1 = 1
Experiment|AcquisitionBlock|AcquisitionModeSetup|CameraFrameHeight #1 = 1030
Experiment|AcquisitionBlock|AcquisitionModeSetup|CameraFrameOffsetX #1 = 0
Experiment|AcquisitionBlock|AcquisitionModeSetup|CameraFrameOffsetY #1 = 0
Experiment|AcquisitionBlock|AcquisitionModeSetup|CameraFrameWidth #1 = 1300
Experiment|AcquisitionBlock|AcquisitionModeSetup|CameraSuperSampling #1 = 0
Experiment|AcquisitionBlock|AcquisitionModeSetup|DimensionT #1 = 10
Experiment|AcquisitionBlock|AcquisitionModeSetup|DimensionX #1 = 2048
Experiment|AcquisitionBlock|AcquisitionModeSetup|DimensionY #1 = 2048
Experiment|AcquisitionBlock|AcquisitionModeSetup|DimensionZ #1 = 8
Experiment|AcquisitionBlock|AcquisitionModeSetup|FilterMethod #1 = Average
Experiment|AcquisitionBlock|AcquisitionModeSetup|FilterMode #1 = Line
Experiment|AcquisitionBlock|AcquisitionModeSetup|FilterSamplingNumber #1 = 16
Experiment|AcquisitionBlock|AcquisitionModeSetup|FitFramesizeToRoi #1 = false
Experiment|AcquisitionBlock|AcquisitionModeSetup|FocusStabilizer #1 = false
Experiment|AcquisitionBlock|AcquisitionModeSetup|HdrEnabled #1 = false
Experiment|AcquisitionBlock|AcquisitionModeSetup|HdrImagingMode #1 = 0
Experiment|AcquisitionBlock|AcquisitionModeSetup|HdrIntensity #1 = 1
Experiment|AcquisitionBlock|AcquisitionModeSetup|HdrNumFrames #1 = 1
Experiment|AcquisitionBlock|AcquisitionModeSetup|InterpolationY #1 = 1
Experiment|AcquisitionBlock|AcquisitionModeSetup|Objective #1 = Plan-Apochromat 10x/0.3 M27
Experiment|AcquisitionBlock|AcquisitionModeSetup|OffsetX #1 = 0
Experiment|AcquisitionBlock|AcquisitionModeSetup|OffsetY #1 = 0
Experiment|AcquisitionBlock|AcquisitionModeSetup|OffsetZ #1 = 0.00015779492514106573
Experiment|AcquisitionBlock|AcquisitionModeSetup|PixelPeriod #1 = 4.2307692307692309e-007
Experiment|AcquisitionBlock|AcquisitionModeSetup|PreScan #1 = false
Experiment|AcquisitionBlock|AcquisitionModeSetup|Rotation #1 = 0

Experiment|AcquisitionBlock|AcquisitionModeSetup|RtBinning #1 = 1
Experiment|AcquisitionBlock|AcquisitionModeSetup|RtFrameHeight #1 = 512
Experiment|AcquisitionBlock|AcquisitionModeSetup|RtFrameWidth #1 = 512
Experiment|AcquisitionBlock|AcquisitionModeSetup|RtLinePeriod #1 = 3.0000000000000001e-005
Experiment|AcquisitionBlock|AcquisitionModeSetup|RtOffsetX #1 = 0
Experiment|AcquisitionBlock|AcquisitionModeSetup|RtOffsetY #1 = 0
Experiment|AcquisitionBlock|AcquisitionModeSetup|RtRegionHeight #1 = 512
Experiment|AcquisitionBlock|AcquisitionModeSetup|RtRegionWidth #1 = 512
Experiment|AcquisitionBlock|AcquisitionModeSetup|RtSuperSampling #1 = 1
Experiment|AcquisitionBlock|AcquisitionModeSetup|RtZoom #1 = 1
Experiment|AcquisitionBlock|AcquisitionModeSetup|ScalingX #1 = 4.6125879858432777e-007
Experiment|AcquisitionBlock|AcquisitionModeSetup|ScalingY #1 = 4.6125879858432777e-007
Experiment|AcquisitionBlock|AcquisitionModeSetup|ScalingZ #1 = 2.2542132163009386e-005
Experiment|AcquisitionBlock|AcquisitionModeSetup|SimRotations #1 = 3
Experiment|AcquisitionBlock|AcquisitionModeSetup|TimeSeries #1 = false
Experiment|AcquisitionBlock|AcquisitionModeSetup|TrackMultiplexType #1 = Line
Experiment|AcquisitionBlock|AcquisitionModeSetup|UseRois #1 = false
Experiment|AcquisitionBlock|AcquisitionModeSetup|ZoomX #1 = 0.90000000000000002
Experiment|AcquisitionBlock|AcquisitionModeSetup|ZoomY #1 = 0.90000000000000002
Experiment|AcquisitionBlock|Laser|LaserName #1 = DPSS 561-10
Experiment|AcquisitionBlock|Laser|LaserName #2 = Argon
Experiment|AcquisitionBlock|Laser|LaserPower #1 = 0.02
Experiment|AcquisitionBlock|Laser|LaserPower #2 = 0.025000000000000001
Experiment|AcquisitionBlock|MultiTrackSetup|TrackSetup|Attenuator|ExcitationIntensity #1 = 0
Experiment|AcquisitionBlock|MultiTrackSetup|TrackSetup|Attenuator|Laser #1 = Argon
Experiment|AcquisitionBlock|MultiTrackSetup|TrackSetup|Attenuator|LaserSuppression #1 = false
Experiment|AcquisitionBlock|MultiTrackSetup|TrackSetup|Attenuator|Transmission #1 = 0.035000000000000003
Experiment|AcquisitionBlock|MultiTrackSetup|TrackSetup|Attenuator|Wavelength #1 = 4.8800000000000003e-007
Experiment|AcquisitionBlock|MultiTrackSetup|TrackSetup|BeamSplitter|BeamSplitterServoPosition #1 = 0
Experiment|AcquisitionBlock|MultiTrackSetup|TrackSetup|BeamSplitter|BeamSplitterServoPosition #2 = 0

Experiment|AcquisitionBlock|MultiTrackSetup|TrackSetup|BeamSplitter|BeamSplitterServoPositio
n #3 = 0
Experiment|AcquisitionBlock|MultiTrackSetup|TrackSetup|BeamSplitter|Filter #1 = MBS 488
Experiment|AcquisitionBlock|MultiTrackSetup|TrackSetup|BeamSplitter|Filter #2 = Plate
Experiment|AcquisitionBlock|MultiTrackSetup|TrackSetup|BeamSplitter|Filter #3 = Rear
Experiment|AcquisitionBlock|MultiTrackSetup|TrackSetup|BeamSplitter|Identifier #1 =
MainBeamSplitterDescanned1
Experiment|AcquisitionBlock|MultiTrackSetup|TrackSetup|BeamSplitter|Identifier #2 =
MainBeamSplitterDescanned2
Experiment|AcquisitionBlock|MultiTrackSetup|TrackSetup|BeamSplitter|Identifier #3 =
MainBeamSplitterNonDescanned
Experiment|AcquisitionBlock|MultiTrackSetup|TrackSetup|CameraIntegrationTime #1 =
4.2307692307692309e-007
Experiment|AcquisitionBlock|MultiTrackSetup|TrackSetup|CenterWavelength #1 =
5.5270582100000006e-007
Experiment|AcquisitionBlock|MultiTrackSetup|TrackSetup|CondensorAperture #1 = -1
Experiment|AcquisitionBlock|MultiTrackSetup|TrackSetup|CondensorFrontlensPosition #1 = -1
Experiment|AcquisitionBlock|MultiTrackSetup|TrackSetup|Detector|AmplifierGain #1 = 1
Experiment|AcquisitionBlock|MultiTrackSetup|TrackSetup|Detector|AmplifierGain #2 = 1
Experiment|AcquisitionBlock|MultiTrackSetup|TrackSetup|Detector|AmplifierGain #3 = 1
Experiment|AcquisitionBlock|MultiTrackSetup|TrackSetup|Detector|AmplifierOffset #1 = 0
Experiment|AcquisitionBlock|MultiTrackSetup|TrackSetup|Detector|AmplifierOffset #2 = 0
Experiment|AcquisitionBlock|MultiTrackSetup|TrackSetup|Detector|AmplifierOffset #3 = 0
Experiment|AcquisitionBlock|MultiTrackSetup|TrackSetup|Detector|Color #1 = #00FF00
Experiment|AcquisitionBlock|MultiTrackSetup|TrackSetup|Detector|Color #2 = #FF0000
Experiment|AcquisitionBlock|MultiTrackSetup|TrackSetup|Detector|Color #3 = #FFFFFF
Experiment|AcquisitionBlock|MultiTrackSetup|TrackSetup|Detector|DetectorIdentifier #1 =
DescannedPmt1
Experiment|AcquisitionBlock|MultiTrackSetup|TrackSetup|Detector|DetectorIdentifier #2 =
DescannedPmt2
Experiment|AcquisitionBlock|MultiTrackSetup|TrackSetup|Detector|DetectorIdentifier #3 =
NonDescannedTransmissionPmt1
Experiment|AcquisitionBlock|MultiTrackSetup|TrackSetup|Detector|DetectorMode #1 =
PhotonCounting

Experiment|AcquisitionBlock|MultiTrackSetup|TrackSetup|Detector|DetectorMode #2 = PhotonCounting
Experiment|AcquisitionBlock|MultiTrackSetup|TrackSetup|Detector|DetectorMode #3 = PhotonCounting
Experiment|AcquisitionBlock|MultiTrackSetup|TrackSetup|Detector|DetectorWavelengthRange|WavelengthEnd #1 = 5.5000000000000003e-007
Experiment|AcquisitionBlock|MultiTrackSetup|TrackSetup|Detector|DetectorWavelengthRange|WavelengthEnd #2 = 7.5300000000000003e-007
Experiment|AcquisitionBlock|MultiTrackSetup|TrackSetup|Detector|DetectorWavelengthRange|WavelengthStart #1 = 4.9299999999999998e-007
Experiment|AcquisitionBlock|MultiTrackSetup|TrackSetup|Detector|DetectorWavelengthRange|WavelengthStart #2 = 5.7100000000000002e-007
Experiment|AcquisitionBlock|MultiTrackSetup|TrackSetup|Detector|DigitalGain #1 = 1
Experiment|AcquisitionBlock|MultiTrackSetup|TrackSetup|Detector|DigitalGain #2 = 1
Experiment|AcquisitionBlock|MultiTrackSetup|TrackSetup|Detector|DigitalGain #3 = 1
Experiment|AcquisitionBlock|MultiTrackSetup|TrackSetup|Detector|DigitalOffset #1 = 0
Experiment|AcquisitionBlock|MultiTrackSetup|TrackSetup|Detector|DigitalOffset #2 = 0
Experiment|AcquisitionBlock|MultiTrackSetup|TrackSetup|Detector|DigitalOffset #3 = 0
Experiment|AcquisitionBlock|MultiTrackSetup|TrackSetup|Detector|Dye #1 = Alexa Fluor 488
Experiment|AcquisitionBlock|MultiTrackSetup|TrackSetup|Detector|Dye #2 = Nile Red
Experiment|AcquisitionBlock|MultiTrackSetup|TrackSetup|Detector|Folder #1 = Alexa Fluor 488
Experiment|AcquisitionBlock|MultiTrackSetup|TrackSetup|Detector|Folder #2 = Nile Red
Experiment|AcquisitionBlock|MultiTrackSetup|TrackSetup|Detector|ImageChannelName #1 = Ch1
Experiment|AcquisitionBlock|MultiTrackSetup|TrackSetup|Detector|ImageChannelName #2 = Ch2
Experiment|AcquisitionBlock|MultiTrackSetup|TrackSetup|Detector|ImageChannelName #3 = TPMT
Experiment|AcquisitionBlock|MultiTrackSetup|TrackSetup|Detector|LaserSuppression #1 = true
Experiment|AcquisitionBlock|MultiTrackSetup|TrackSetup|Detector|LaserSuppression #2 = true
Experiment|AcquisitionBlock|MultiTrackSetup|TrackSetup|Detector|LaserSuppression #3 = true
Experiment|AcquisitionBlock|MultiTrackSetup|TrackSetup|Detector|Name #1 = PMT
Experiment|AcquisitionBlock|MultiTrackSetup|TrackSetup|Detector|Name #2 = PMT
Experiment|AcquisitionBlock|MultiTrackSetup|TrackSetup|Detector|Palette #1 = LsmDetectorPalette_0_0
Experiment|AcquisitionBlock|MultiTrackSetup|TrackSetup|Detector|Palette #2 = LsmDetectorPalette_0_1

Experiment|AcquisitionBlock|MultiTrackSetup|TrackSetup|Detector|Palette #3 = LsmDetectorPalette_0_2
Experiment|AcquisitionBlock|MultiTrackSetup|TrackSetup|Detector|PinholeDiameter #1 = 8.9897864999999983e-005
Experiment|AcquisitionBlock|MultiTrackSetup|TrackSetup|Detector|PinholeDiameter #2 = 8.9897864999999983e-005
Experiment|AcquisitionBlock|MultiTrackSetup|TrackSetup|Detector|PinholeDiameter #3 = 0
Experiment|AcquisitionBlock|MultiTrackSetup|TrackSetup|Detector|PureRatioSource #1 = false
Experiment|AcquisitionBlock|MultiTrackSetup|TrackSetup|Detector|PureRatioSource #2 = false
Experiment|AcquisitionBlock|MultiTrackSetup|TrackSetup|Detector|PureRatioSource #3 = false
Experiment|AcquisitionBlock|MultiTrackSetup|TrackSetup|Detector|SpectralScanChannels #1 = 32
Experiment|AcquisitionBlock|MultiTrackSetup|TrackSetup|Detector|SpectralScanChannels #2 = 32
Experiment|AcquisitionBlock|MultiTrackSetup|TrackSetup|Detector|SpectralScanChannels #3 = 32
Experiment|AcquisitionBlock|MultiTrackSetup|TrackSetup|Detector|Voltage #1 = 750
Experiment|AcquisitionBlock|MultiTrackSetup|TrackSetup|Detector|Voltage #2 = 750
Experiment|AcquisitionBlock|MultiTrackSetup|TrackSetup|Detector|Voltage #3 = 350
Experiment|AcquisitionBlock|MultiTrackSetup|TrackSetup|DeviceMode #1 = LSM_ChannelMode
Experiment|AcquisitionBlock|MultiTrackSetup|TrackSetup|FieldStopPosition #1 = -1
Experiment|AcquisitionBlock|MultiTrackSetup|TrackSetup|FilterTransmission #1 = -1
Experiment|AcquisitionBlock|MultiTrackSetup|TrackSetup|LaserSuppressionMode #1 = None
Experiment|AcquisitionBlock|MultiTrackSetup|TrackSetup|Name #1 = Track 1
Experiment|AcquisitionBlock|MultiTrackSetup|TrackSetup|ReflectedLightLampIntensity #1 = 0
Experiment|AcquisitionBlock|MultiTrackSetup|TrackSetup|SimGratingPeriod #1 = 0
Experiment|AcquisitionBlock|MultiTrackSetup|TrackSetup|TirfAngle #1 = 0
Experiment|AcquisitionBlock|MultiTrackSetup|TrackSetup|TransmittedLightLampIntensity #1 = 0
Experiment|AcquisitionBlock|MultiTrackSetup|TrackSetup|TubeLensPosition #1 = Lens LSM
Experiment|AcquisitionBlock|ZStackSetup|Extrapolate #1 = false
Experiment|AcquisitionBlock|ZStackSetup|Interpolation #1 = Cubic
Experiment|AcquisitionBlock|ZStackSetup|StackBrightnessCorrection #1 = false
Information|Image|Channel|AcquisitionMode #1 = LaserScanningConfocalMicroscopy
Information|Image|Channel|AcquisitionMode #2 = LaserScanningConfocalMicroscopy
Information|Image|Channel|AcquisitionMode #3 = LaserScanningConfocalMicroscopy
Information|Image|Channel|Attenuation #1 = 0.96499999999999997
Information|Image|Channel|Attenuation #2 = 0.96499999999999997
Information|Image|Channel|Attenuation #3 = 0.96499999999999997

Information|Image|Channel|Binning #1 = 1x1
Information|Image|Channel|Binning #2 = 1x1
Information|Image|Channel|Binning #3 = 1x1
Information|Image|Channel|ContrastMethod #1 = Fluorescence
Information|Image|Channel|ContrastMethod #2 = Fluorescence
Information|Image|Channel|ContrastMethod #3 = Fluorescence
Information|Image|Channel|DetectionWavelength|Ranges #1 = 493-550
Information|Image|Channel|DetectionWavelength|Ranges #2 = 571-753
Information|Image|Channel|Detector|Id #1 = Detector:0:0
Information|Image|Channel|Detector|Id #2 = Detector:0:1
Information|Image|Channel|Detector|Id #3 = Detector:0:2
Information|Image|Channel|DigitalGain #1 = 1
Information|Image|Channel|DigitalGain #2 = 1
Information|Image|Channel|DigitalGain #3 = 1
Information|Image|Channel|EmissionWavelength #1 = 521.5
Information|Image|Channel|EmissionWavelength #2 = 662
Information|Image|Channel|ExcitationWavelength #1 = 488.00000000000006
Information|Image|Channel|ExcitationWavelength #2 = 488.00000000000006
Information|Image|Channel|Fluor #1 = Alexa Fluor 488
Information|Image|Channel|Fluor #2 = Nile Red
Information|Image|Channel|Gain #1 = 750
Information|Image|Channel|Gain #2 = 750
Information|Image|Channel|Gain #3 = 350
Information|Image|Channel|Id #1 = 10431063541109012402321813928015980337 95
Information|Image|Channel|Id #2 = 40009751521097270794329309171728853314 55
Information|Image|Channel|Id #3 = 12484193471258603390198678749718302213 59
Information|Image|Channel|IlluminationType #1 = Epifluorescence
Information|Image|Channel|IlluminationType #2 = Epifluorescence
Information|Image|Channel|IlluminationType #3 = Epifluorescence
Information|Image|Channel|LaserScanInfo|Averaging #1 = 16
Information|Image|Channel|LaserScanInfo|Averaging #2 = 16
Information|Image|Channel|LaserScanInfo|Averaging #3 = 16
Information|Image|Channel|LaserScanInfo|FrameTime #1 = 66.544246153846146
Information|Image|Channel|LaserScanInfo|FrameTime #2 = 66.544246153846146
Information|Image|Channel|LaserScanInfo|FrameTime #3 = 66.544246153846146

Information|Image|Channel|LaserScanInfo|LineTime #1 = 3.00000000000000001e-005
Information|Image|Channel|LaserScanInfo|LineTime #2 = 3.00000000000000001e-005
Information|Image|Channel|LaserScanInfo|LineTime #3 = 3.00000000000000001e-005
Information|Image|Channel|LaserScanInfo|PixelTime #1 = 4.2307692307692309e-007
Information|Image|Channel|LaserScanInfo|PixelTime #2 = 4.2307692307692309e-007
Information|Image|Channel|LaserScanInfo|PixelTime #3 = 4.2307692307692309e-007
Information|Image|Channel|LaserScanInfo|SampleOffsetX #1 = 0
Information|Image|Channel|LaserScanInfo|SampleOffsetX #2 = 0
Information|Image|Channel|LaserScanInfo|SampleOffsetX #3 = 0
Information|Image|Channel|LaserScanInfo|SampleOffsetY #1 = 0
Information|Image|Channel|LaserScanInfo|SampleOffsetY #2 = 0
Information|Image|Channel|LaserScanInfo|SampleOffsetY #3 = 0
Information|Image|Channel|LaserScanInfo|SampleRotation #1 = 0
Information|Image|Channel|LaserScanInfo|SampleRotation #2 = 0
Information|Image|Channel|LaserScanInfo|SampleRotation #3 = 0
Information|Image|Channel|LaserScanInfo|ScanningMode #1 = LineSequential
Information|Image|Channel|LaserScanInfo|ScanningMode #2 = LineSequential
Information|Image|Channel|LaserScanInfo|ScanningMode #3 = LineSequential
Information|Image|Channel|LaserScanInfo|ZoomX #1 = 0.90000000000000002
Information|Image|Channel|LaserScanInfo|ZoomX #2 = 0.90000000000000002
Information|Image|Channel|LaserScanInfo|ZoomX #3 = 0.90000000000000002
Information|Image|Channel|LaserScanInfo|ZoomY #1 = 0.90000000000000002
Information|Image|Channel|LaserScanInfo|ZoomY #2 = 0.90000000000000002
Information|Image|Channel|LaserScanInfo|ZoomY #3 = 0.90000000000000002
Information|Image|Channel|LightSource|Id #1 = LightSource:1
Information|Image|Channel|LightSource|Id #2 = LightSource:1
Information|Image|Channel|LightSource|Id #3 = LightSource:1
Information|Image|Channel|Name #1 = Ch1
Information|Image|Channel|Name #2 = Ch2
Information|Image|Channel|Name #3 = T PMT
Information|Image|Channel|Offset #1 = 0
Information|Image|Channel|Offset #2 = 0
Information|Image|Channel|Offset #3 = 0
Information|Image|Channel|PhotonConversionFactor #1 = 207.47015408905679
Information|Image|Channel|PhotonConversionFactor #2 = 207.47015408905679

Information|Image|Channel|PhotonConversionFactor #3 = 207.47015408905679
Information|Image|Channel|PinholeSizeAiry #1 = 2.4405653194795907
Information|Image|Channel|PinholeSizeAiry #2 = 1.9225903536383786
Information|Image|Channel|Wavelength #1 = 488.00000000000006
Information|Image|Channel|Wavelength #2 = 488.00000000000006
Information|Image|Channel|Wavelength #3 = 488.00000000000006
Information|Image|ComponentBitCount #1 = 8
Information|Image|Medium #1 = Air
Information|Image|MicroscopeRef|Id #1 = Microscope:0
Information|Image|ObjectiveRef|Id #1 = Objective:0
Information|Image|OriginalScanData #1 = true
Information|Image|PixelType #1 = Gray8
Information|Image|RefractiveIndex #1 = 1
Information|Image|SizeC #1 = 3
Information|Image|SizeX #1 = 2048
Information|Image|SizeY #1 = 2048
Information|Image|SizeZ #1 = 8
Information|Image|S|Scene|Index #1 = 0
Information|Image|S|Scene|Position|X #1 = 8862.02
Information|Image|S|Scene|Position|Y #1 = -4597.4
Information|Image|S|Scene|Position|Z #1 = 1987.56
Information|Image|Track|ChannelRef|Id #1 = 104310635411090124023218139280159803379 5
Information|Image|Track|ChannelRef|Id #2 = 400097515210972707943293091717288533145 5
Information|Image|Track|ChannelRef|Id #3 = 124841934712586033901986787497183022135 9
Information|Image|Track|Id #1 = Track:0
Information|Image|T|BinaryList|AttachmentName #1 = TimeStamps
Information|Image|Z|Interval|Increment #1 = 22.542132163009388
Information|Image|Z|Interval|Start #1 = 0
Information|Image|Z|StartPosition #1 = 0
Information|Instrument|Detector|AmplificationGain #1 = 1
Information|Instrument|Detector|AmplificationGain #2 = 1
Information|Instrument|Detector|AmplificationGain #3 = 1
Information|Instrument|Detector|Gain #1 = 1
Information|Instrument|Detector|Gain #2 = 1
Information|Instrument|Detector|Gain #3 = 1

Information|Instrument|Detector|Id #1 = Detector:0:0
Information|Instrument|Detector|Id #2 = Detector:0:1
Information|Instrument|Detector|Id #3 = Detector:0:2
Information|Instrument|Detector|Type #1 = PMT
Information|Instrument|Detector|Type #2 = PMT
Information|Instrument|Detector|Zoom #1 = 1
Information|Instrument|Detector|Zoom #2 = 1
Information|Instrument|Detector|Zoom #3 = 1
Information|Instrument|Id #1 = Instrument:0
Information|Instrument|LightSource|Id #1 = LightSource:0
Information|Instrument|LightSource|Id #2 = LightSource:1
Information|Instrument|LightSource|LightSourceType|Laser|Wavelength #1 = 561.000000
Information|Instrument|LightSource|LightSourceType|Laser|Wavelength #2 = 458.000000
Information|Instrument|LightSource|Manufacturer|Model #1 = DPSS 561-10
Information|Instrument|LightSource|Manufacturer|Model #2 = Argon
Information|Instrument|LightSource|Power #1 = 20
Information|Instrument|LightSource|Power #2 = 25
Information|Instrument|Microscope|Id #1 = Microscope:0
Information|Instrument|Microscope|System #1 = LSM 780, AxioObserver
Information|Instrument|Objective|Id #1 = Objective:0
Information|Instrument|Objective|Immersion #1 = Air
Information|Instrument|Objective|LensNA #1 = 0.30000000000000004
Information|Instrument|Objective|Manufacturer|Model #1 = Plan-Apochromat 10x/0.3 M27
Information|Instrument|Objective|NominalMagnification #1 = 10
Information|User|DisplayName #1 = fcam

2: Exemplary imaging settings, extracted with FIJI (Schindelin et al. 2012) for an animal exposed to 20 nm particles for 24 h and subsequently treated with 4 % para-formaldehyde and SeeDB.

Experiment|AcquisitionBlock|AcquisitionModeSetup|AcquisitionMode #1 = StackFocus
Experiment|AcquisitionBlock|AcquisitionModeSetup|BiDirectional #1 = false
Experiment|AcquisitionBlock|AcquisitionModeSetup|BiDirectionalZ #1 = false
Experiment|AcquisitionBlock|AcquisitionModeSetup|BitsPerSample #1 = 8
Experiment|AcquisitionBlock|AcquisitionModeSetup|CameraBinning #1 = 1
Experiment|AcquisitionBlock|AcquisitionModeSetup|CameraFrameHeight #1 = 1030

Experiment|AcquisitionBlock|AcquisitionModeSetup|CameraFrameOffsetX #1 = 0
Experiment|AcquisitionBlock|AcquisitionModeSetup|CameraFrameOffsetY #1 = 0
Experiment|AcquisitionBlock|AcquisitionModeSetup|CameraFrameWidth #1 = 1300
Experiment|AcquisitionBlock|AcquisitionModeSetup|CameraSuperSampling #1 = 0
Experiment|AcquisitionBlock|AcquisitionModeSetup|DimensionT #1 = 10
Experiment|AcquisitionBlock|AcquisitionModeSetup|DimensionX #1 = 2048
Experiment|AcquisitionBlock|AcquisitionModeSetup|DimensionY #1 = 2048
Experiment|AcquisitionBlock|AcquisitionModeSetup|DimensionZ #1 = 11
Experiment|AcquisitionBlock|AcquisitionModeSetup|FilterMethod #1 = Average
Experiment|AcquisitionBlock|AcquisitionModeSetup|FilterMode #1 = Line
Experiment|AcquisitionBlock|AcquisitionModeSetup|FilterSamplingNumber #1 = 16
Experiment|AcquisitionBlock|AcquisitionModeSetup|FitFramesizeToRoi #1 = false
Experiment|AcquisitionBlock|AcquisitionModeSetup|FocusStabilizer #1 = false
Experiment|AcquisitionBlock|AcquisitionModeSetup|HdrEnabled #1 = false
Experiment|AcquisitionBlock|AcquisitionModeSetup|HdrImagingMode #1 = 0
Experiment|AcquisitionBlock|AcquisitionModeSetup|HdrIntensity #1 = 1
Experiment|AcquisitionBlock|AcquisitionModeSetup|HdrNumFrames #1 = 1
Experiment|AcquisitionBlock|AcquisitionModeSetup|InterpolationY #1 = 1
Experiment|AcquisitionBlock|AcquisitionModeSetup|Objective #1 = Plan-Apochromat 10x/0.3 M27
Experiment|AcquisitionBlock|AcquisitionModeSetup|OffsetX #1 = 0
Experiment|AcquisitionBlock|AcquisitionModeSetup|OffsetY #1 = 0
Experiment|AcquisitionBlock|AcquisitionModeSetup|OffsetZ #1 = 0.0002291230933333336
Experiment|AcquisitionBlock|AcquisitionModeSetup|PixelPeriod #1 = 4.2307692307692309e-007
Experiment|AcquisitionBlock|AcquisitionModeSetup|PreScan #1 = false
Experiment|AcquisitionBlock|AcquisitionModeSetup|Rotation #1 = 0
Experiment|AcquisitionBlock|AcquisitionModeSetup|RtBinning #1 = 1
Experiment|AcquisitionBlock|AcquisitionModeSetup|RtFrameHeight #1 = 512
Experiment|AcquisitionBlock|AcquisitionModeSetup|RtFrameWidth #1 = 512
Experiment|AcquisitionBlock|AcquisitionModeSetup|RtLinePeriod #1 = 3.0000000000000001e-005
Experiment|AcquisitionBlock|AcquisitionModeSetup|RtOffsetX #1 = 0
Experiment|AcquisitionBlock|AcquisitionModeSetup|RtOffsetY #1 = 0
Experiment|AcquisitionBlock|AcquisitionModeSetup|RtRegionHeight #1 = 512
Experiment|AcquisitionBlock|AcquisitionModeSetup|RtRegionWidth #1 = 512
Experiment|AcquisitionBlock|AcquisitionModeSetup|RtSuperSampling #1 = 1

Experiment|AcquisitionBlock|AcquisitionModeSetup|RtZoom #1 = 1
Experiment|AcquisitionBlock|AcquisitionModeSetup|ScalingX #1 = 6.9188819787649165e-007
Experiment|AcquisitionBlock|AcquisitionModeSetup|ScalingY #1 = 6.9188819787649165e-007
Experiment|AcquisitionBlock|AcquisitionModeSetup|ScalingZ #1 = 2.2912309333333329e-005
Experiment|AcquisitionBlock|AcquisitionModeSetup|SimRotations #1 = 3
Experiment|AcquisitionBlock|AcquisitionModeSetup|TimeSeries #1 = false
Experiment|AcquisitionBlock|AcquisitionModeSetup|TrackMultiplexType #1 = Line
Experiment|AcquisitionBlock|AcquisitionModeSetup|UseRois #1 = false
Experiment|AcquisitionBlock|AcquisitionModeSetup|ZoomX #1 = 0.59999999999999998
Experiment|AcquisitionBlock|AcquisitionModeSetup|ZoomY #1 = 0.59999999999999998
Experiment|AcquisitionBlock|Laser|LaserName #1 = Argon
Experiment|AcquisitionBlock|Laser|LaserPower #1 = 0.025000000000000001
Experiment|AcquisitionBlock|MultiTrackSetup|TrackSetup|Attenuator|ExcitationIntensity #1 = 0
Experiment|AcquisitionBlock|MultiTrackSetup|TrackSetup|Attenuator|Laser #1 = Argon
Experiment|AcquisitionBlock|MultiTrackSetup|TrackSetup|Attenuator|LaserSuppression #1 = false
Experiment|AcquisitionBlock|MultiTrackSetup|TrackSetup|Attenuator|Transmission #1 = 0.035000000000000003
Experiment|AcquisitionBlock|MultiTrackSetup|TrackSetup|Attenuator|Wavelength #1 = 4.8800000000000003e-007
Experiment|AcquisitionBlock|MultiTrackSetup|TrackSetup|BeamSplitter|BeamSplitterServoPosition #1 = 0
Experiment|AcquisitionBlock|MultiTrackSetup|TrackSetup|BeamSplitter|BeamSplitterServoPosition #2 = 0
Experiment|AcquisitionBlock|MultiTrackSetup|TrackSetup|BeamSplitter|BeamSplitterServoPosition #3 = 0
Experiment|AcquisitionBlock|MultiTrackSetup|TrackSetup|BeamSplitter|Filter #1 = MBS 488
Experiment|AcquisitionBlock|MultiTrackSetup|TrackSetup|BeamSplitter|Filter #2 = Plate
Experiment|AcquisitionBlock|MultiTrackSetup|TrackSetup|BeamSplitter|Filter #3 = Rear
Experiment|AcquisitionBlock|MultiTrackSetup|TrackSetup|BeamSplitter|Identifier #1 = MainBeamSplitterDescanned1
Experiment|AcquisitionBlock|MultiTrackSetup|TrackSetup|BeamSplitter|Identifier #2 = MainBeamSplitterDescanned2
Experiment|AcquisitionBlock|MultiTrackSetup|TrackSetup|BeamSplitter|Identifier #3 = MainBeamSplitterNonDescanned

Experiment|AcquisitionBlock|MultiTrackSetup|TrackSetup|CameraIntegrationTime #1 = 4.2307692307692309e-007
Experiment|AcquisitionBlock|MultiTrackSetup|TrackSetup|CenterWavelength #1 = 5.5270582100000006e-007
Experiment|AcquisitionBlock|MultiTrackSetup|TrackSetup|CondensorAperture #1 = -1
Experiment|AcquisitionBlock|MultiTrackSetup|TrackSetup|CondensorFrontlensPosition #1 = -1
Experiment|AcquisitionBlock|MultiTrackSetup|TrackSetup|Detector|AmplifierGain #1 = 1
Experiment|AcquisitionBlock|MultiTrackSetup|TrackSetup|Detector|AmplifierGain #2 = 1
Experiment|AcquisitionBlock|MultiTrackSetup|TrackSetup|Detector|AmplifierGain #3 = 1
Experiment|AcquisitionBlock|MultiTrackSetup|TrackSetup|Detector|AmplifierOffset #1 = 0
Experiment|AcquisitionBlock|MultiTrackSetup|TrackSetup|Detector|AmplifierOffset #2 = 0
Experiment|AcquisitionBlock|MultiTrackSetup|TrackSetup|Detector|AmplifierOffset #3 = 0
Experiment|AcquisitionBlock|MultiTrackSetup|TrackSetup|Detector|Color #1 = #00FF00
Experiment|AcquisitionBlock|MultiTrackSetup|TrackSetup|Detector|Color #2 = #FF0000
Experiment|AcquisitionBlock|MultiTrackSetup|TrackSetup|Detector|Color #3 = #FFFFFF
Experiment|AcquisitionBlock|MultiTrackSetup|TrackSetup|Detector|DetectorIdentifier #1 = DescannedPmt1
Experiment|AcquisitionBlock|MultiTrackSetup|TrackSetup|Detector|DetectorIdentifier #2 = DescannedPmt2
Experiment|AcquisitionBlock|MultiTrackSetup|TrackSetup|Detector|DetectorIdentifier #3 = NonDescannedTransmissionPmt1
Experiment|AcquisitionBlock|MultiTrackSetup|TrackSetup|Detector|DetectorMode #1 = PhotonCounting
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Experiment|AcquisitionBlock|MultiTrackSetup|TrackSetup|Detector|DetectorMode #3 = PhotonCounting
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Localization of lipid droplets with Nile Red

To localize lipid droplets, we stained selected daphnids from experiment III with the fluorescent dye Nile Red using an adapted version of the protocol described by Jordão et al. (2015). Staining was carried out for 60 min using a 1.5 µM Nile Red solution in acetone followed by rinsing the animals twice in deionized water and then transferring them back to their respective fructose solution.

In addition to specimen preparation with SeeDB selected animals were stained with Nile Red to properly identify the lipid droplets as was earlier done by Brun et al. (2017). Utilization of two excitation wavelengths in theory enables imaging of both the stained lipid droplets as well as the fluorescent particles. The staining resulted in fluorescence in the lipid droplets. Staining was inconsistent across different structures and resulted in varying degrees of fluorescence. Depending on the compartment there were differences in the fluorescence signal in the presence of plastic particles that could not always clearly be associated with either Nile Red or the fluorescent particles. As mentioned earlier the specimen treatment with SeeDB reduced the specimen integrity which often led to deformation of the animal during the staining process and the subsequent transfer back into the storage solution or onto the microscope slide. Due to these difficulties the combination of Nile Red staining with cleared specimens does not appear to be a scalable approach to investigate fluorescent particles and lipid droplets. The staining protocol is better applied to living animals.

Annex 2: Paper 2

Title: Microplastics but not natural particles induce multigenerational effects in *Daphnia magna*

Journal: Environmental Pollution

Contributors Christoph Schür (CS), Sebastian Zipp (SZ), Tobias Thalau (TT), Martin Wagner (MW)

Status: published

doi: 10.1016/j.envpol.2019.113904

Environmental Pollution 260 (2020) 113904

Contents lists available at ScienceDirect

Environmental Pollution

journal homepage: www.elsevier.com/locate/envpol

Microplastics but not natural particles induce multigenerational effects in *Daphnia magna*☆

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ARTICLE INFO

Article history:
Received 18 September 2019
Received in revised form
16 December 2019
Accepted 30 December 2019
Available online 31 December 2019

ABSTRACT

Several studies have investigated the effects of nano- and microplastics on daphnids as key freshwater species. However, while information is abundant on the acute toxicity of plastic beads, little is known regarding the multigenerational effects of irregular microplastics. In addition, a comparison of microplastics to naturally occurring particles is missing. Therefore, we investigated the effects of irregular, secondary polystyrene microplastics (<63 μm) and kaolin as natural reference particle on the survival, reproduction, and growth of *Daphnia magna* over four generations under food-limited conditions. Additionally, we tested the sensitivity of the neonates in each generation to a reference compound as a proxy for offspring fitness. Exposure to high concentrations of microplastics (10,000 and 2000 particles mL^{-1}) reduced daphnid survival, resulting in extinction within one and four generations, respectively. Microplastics also affected reproduction and growth. Importantly, an exposure to kaolin at similar concentrations did not induce negative effects. The sensitivity of neonates to potassium dichromate was not affected by maternal exposure to particles. Taken together, our study demonstrates that irregular PS particles are more toxic than natural kaolin in daphnids exposed over multiple generations under food limitation. Thus, our work builds towards more realistic exposure scenarios needed to better understand the impacts of microplastics on zooplankton.

1. Introduction

Human activity distinctly impacts Earth system processes with potentially disastrous consequences, such as climate change and the loss of biodiversity (Rockström et al., 2009). Persson et al. (2013) and MacLeod et al. (2014) discussed the potential of chemical pollution to also compromise a "safe operating space for humanity" and defined three criteria for a pollutant to be considered a planetary boundary threat: (I) The chemical or mixture of chemicals has a disruptive effect on a vital Earth system process, (II) the disruptive effect is not discovered until it is, or inevitably will become, a problem at a planetary scale, (III) the effect of the pollutant in the environment cannot be readily reversed. The occurrence of large amounts of plastic debris in natural environments already is ubiquitous in scale (criterion II) and irreversible (III). Therefore, it is of interest to better understand its effect on earth processes, especially potential adverse effects and underlying mechanisms of plastics on biological systems at different trophic levels (Jahnke et al., 2017).

Of special concern in this context are small plastic particles formed during the degradation of larger debris, so-called secondary microplastics (MP). These can be ingested by zooplankton, which as primary consumer has a key role in aquatic food webs. Cladocera, such as Daphnia species, are model organisms for ecotoxicology and there is no shortage of studies investigating the effects of nano- and microplastics on daphnids (e.g., Ogonowski et al. (2016); Rist et al. (2017)). However, with some exceptions (e.g., Bosker et al. (2019); Jaikumar et al. (2019)), most studies report acute toxicity using high concentrations of commercially available spherical nano- and microplastics and short exposure durations. While irregular MP are predominant in the environment (Burns and Boxall, 2018), the literature provides limited insight into their chronic toxicity. In addition, a multitude of other factors will modulate biota-particle interactions, such as feeding type, inter- and intra-species competition, biofilm formation, hydrodynamics,

☆ This paper has been recommended for acceptance by Sarah Harmon.
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https://doi.org/10.1016/j.envpol.2019.113904
0269-7491/

and presence of other particles (Scherer et al., 2018). Again, these are currently poorly understood.

Three other aspects are important when looking at the body of knowledge: First, short-lived biota will be exposed to MP over multiple generations and so far only one study addresses this (Martins and Guilhermino, 2018). Second, both MP (Ogonowski et al., 2016; Rist et al., 2017; Scherer et al., 2017) and inorganic natural particles (Kirk, 1991a) are readily ingested by daphnids without providing nutrition. Thus, it is reasonable to assume that high food levels in an experiment, such as those recommended by the OECD guideline 211 (OECD, 2012), can mask potential effects occurring through food dilution or increased energy expenditure. Third, as natural particulate matter is abundant in aquatic ecosystems, the toxicity of MP needs to be compared to naturally occurring particles to investigate whether the former are indeed more toxic than the latter (Ogonowski et al., 2018; Backhaus and Wagner, 2019). Here, kaolin clay as a representative for natural suspended particulate matter can adversely affect cladocerans (Kirk and Gilbert, 1990; Kirk, 1992; Robinson et al., 2010).

To address these aspects, we conducted a four-generation experiment in which we exposed *Daphnia magna* to irregular polystyrene MP and kaolin as a natural reference particle at three concentrations (400, 2000 and 10,000 particles mL^{-1}) under food-limited conditions.

2. Materials & methods

2.1. Particle preparation

Polystyrene (PS) lids of coffee-to-go cups obtained from a local bakery were used to produce irregular MP for this study. They were cut into small pieces, frozen in liquid nitrogen and then ground in a ball mill (Retsch MM400, Retsch GmbH, Germany) at 30 Hz for 30 s. The process of freezing and grinding was repeated 2–4 times to produce sufficient amounts of MP. The resulting powder was sieved to ≤63 μm using a sediment shaker (Retsch AS 200 basic, Retsch GmbH, Germany). While all MP used in this study was prepared in one batch, the preparation of multiple batches using identical conditions resulted in MP with similar properties (data not shown). The procedure is, thus, reproducible. Kaolin (~$Al_2Si_2O_5(OH)_4$, CAS 1332-58-7, Merck, Darmstadt, Germany) was sieved as described above. We characterized the density of the PS MP as described in the Supplementary materials (Table S1).

Prior to use, MP and kaolin were suspended in M4 medium (1 g L^{-1}) and shaken for 48 h. The concentrations and particle size distributions of the stock suspensions were determined using a Coulter counter (Multisizer 3, Beckman Coulter, Germany; orifice tube with 100 μm aperture diameter for a particle size range of 2.0–60 μm) and adjusted to the desired concentrations by dilution in M4 medium. For that, the particle suspensions were continuously stirred, 0.5–1 mL taken from the middle of the water column and transferred immediately to the Coulter counter beaker which was also stirred continuously. Scanning electron microscope images of both particle types were taken using a Hitachi S-4500 scanning electron microscope (Supplementary Material, Fig. S1). The suspension was continuously stirred during transfer to the test vessels.

2.2. Daphnia culture

Ten *Daphnia magna* individuals were cultured in 1 L of Elendt M4 medium (OECD, 2012) at 20 °C with a 16:8 light:dark cycle. Daphnids were fed with green algae (*Desmodesmus subspicatus*) thrice a week supplying 0.15 mg carbon per individual per day (mgC $daphnid^{-1}$ d^{-1}). The medium was completely renewed once a week.

2.3. Multigenerational study

The multigenerational study basically consisted of four consecutive semi-static reproduction experiments (21 d, OECD guideline 211) similar to the design used by Völker et al. (2013). The decision to investigate four generations represents a compromise between maximizing the number of generations and feasibility. We used food limitation as additional stressor because we hypothesized that high food levels recommended by standard guidelines will mask the effects of a particle exposure. This accounts for the fact that food is rarely in ample supply in the real world and that the additional ingestion of particles without nutritional value will exacerbate the effect of a food limitation.

Specimens for the first generation (F0, <24 h old neonates) were taken from the daphnid culture (see above). The offspring of the previous experiment were then transferred to the next experiment (i.e., generation) and treated identical to its parents. For this, neonates (<24 h old) from the 3rd brood of each treatment were pooled, and 20 individuals randomly picked for the next generation (F2 animals of the PS2000 treatment produced 16 neonates, only). Each treatment consisted of 20 daphnids that were held individually in 100 mL glass beaker containing 50 mL Elendt M4 medium. Animals were fed daily with *D. subspicatus*. The medium was exchanged completely thrice a week by transferring the daphnids to new vessels. The exposure duration was 21 d for the first three generations. In the fourth generation, we extended the duration to 26 d because the reproduction in the high-food control was delayed.

The daphnids in the high-food control (HFC) without particles were fed 0.15 mgC $daphnid^{-1}$ d^{-1} according to the OECD guideline (OECD, 2012). To induce food limitation, the animals in all other treatment groups were fed a lower food level (0.05 mgC $daphnid^{-1}$ d^{-1}). We selected this feeding regime on the basis of a pilot experiment which had shown that feeding with 0.05 mgC $daphnid^{-1}$ d^{-1} results in a significant reduction of reproduction while still meeting the OECD validity criterion for reproductive output (Fig. S2). Concurrently, survival was not affected by the low food level. The low-food treatments included another negative control group without particles (low-food control, LFC) and groups exposed to 400, 2000 and 10,000 particles mL^{-1} MP or kaolin.

The particle concentrations in the exposure vessels were determined throughout the experiment with a Coulter counter using separate beakers that were prepared identically to the ones in the experiment but did not contain daphnids and algae. Parallel to the water exchanges, we collected 0.5–1 mL water from the middle of the water column from these beakers after light agitation and determined the particle concentrations using Coulter counting (see Supplementary material for details). The results indicate that the nominal and actual particle concentrations match reasonably well (Table S2). We, thus, report the results using the nominal concentrations.

Each day, we recorded the mortality of adult daphnids (immobility for 15 s after agitation (OECD, 2004)) and their reproductive output (neonates per female). Neonates were removed and pooled to create the next generation (3rd brood) or perform acute toxicity tests with potassium dichromate (4th brood). At the end of each experiment, the surviving adults were preserved in 70 % ethanol. Their size was determined using a stereo microscope (Olympus SZ61, Olympus GmbH, Germany) and the software Diskus (version 4.50.1458) by measuring the distance between the center of the eye and the base of the apical spinus (Ogonowski et al., 2016).

Neonates from the fourth brood of each generation were

exposed to 0.302–2.5 mg L^{-1} potassium dichromate (CAS 7778-50-9, ≥99.0 %, Sigma-Aldrich, Steinheim, Germany) following the OECD guideline 202 for Daphnia Acute Immobilization Tests (OECD, 2004). Each potassium dichromate concentration was tested in four replicates with five daphnids each. For treatments with low reproduction, we reduced the number of replicates to account for limited offspring availability (see Table S3). We derived the median lethal concentrations (LC_{50}) in each test to compare the neonates' sensitivity across treatments. Since the LC_{50} range considered valid by OECD (0.6–2.1 mg L^{-1}) is very broad, we established the baseline LC_{50} for our daphnid culture in three independent acute tests with neonates raised under culturing conditions, that is, high food levels (see Fig. S3).

2.4. Data analysis

The data was analyzed using GraphPad Prism (Version 5.04 for Windows, GraphPad Software, La Jolla, California, USA) with two-way ANOVA with Bonferroni post-hoc test and RStudio 1.1.463 (RStudio Team, 2016) with R Version 3.5.2 (R Core Team, 2018) with the tidyverse package (Wickham, 2017). Life-history parameters were analyzed using two-way ANOVA with Bonferroni multiple comparisons test of each treatment and generation against the corresponding generation in the LFC treatment group. LC_{50} values for the potassium dichromate sensitivity were estimated using linear regression (max. likelihood) in ToxRat Professional 3.0.0 (ToxRat Solutions, Alsdorf, Germany).

3. Results

3.1. Survival

The food limitation we used in the multigenerational study did not affect the survival of the *D. magna*. Over four generations, the survival of daphnids in the low- and high-food control groups was ≥80 % (Fig. 1, Fig. S4, Table S4). This is in accordance with the validity criterion according to the OECD guideline 211 (OECD, 2012). In the treatment groups with the highest PS concentration (10,000 particles mL^{-1}), mortality continuously increased after four days of exposure in the first generation (Fig. 1). This resulted in an almost complete extinction of daphnids with one surviving individual after 21 d. In contrast, all animals survived when exposed to 10,000 particles mL^{-1} kaolin as natural reference particle. In the treatments with medium concentrations, 79 % of animals exposed to 2000 PS particles mL^{-1} survived the first generation. The survival then declined further in the second and third generations (55 and 35 %), followed by a complete extinction in the fourth generation. In contrast, survival in the corresponding kaolin treatment was 95–100 % throughout all generations. At the lowest concentration tested (400 particles mL^{-1}), survival of animals in the PS and kaolin treatment was ≥75 and ≥90 % throughout the multigenerational study.

3.2. Reproduction

As expected, food limitation reduced the reproductive output in all treatment groups compared to the high-food control (Fig. 2). The mean number of neonates per surviving female was between 29 and 39 % lower across generations in the low-food compared to the high-food control (Table S4). The reproductive output of animals from the PS groups (F0 and F1) and the kaolin groups (F0, F2, F3) was similar to the low-food control (<5 % difference). Interestingly, we observed a marked decrease in reproduction from the first to the second generation across all groups. In the subsequent generations, the reproduction of the animals in the high and low-food control, and the kaolin treatments recovered to the initial level. In contrast, the reproductive output of daphnids exposed to PS remained >10 % (PS4000) or > 20 % (PS2000) lower that in the corresponding low-food control. This reduction is statistically significant for animals exposed to 2000 PS particles mL^{-1} in F2 ($p < 0.05$) (two-way ANOVA with Bonferroni multiple comparisons test, generation: $F(3, 351) = 37.27$, treatment: $F(4, 351) = 60.49$, interaction: $F(12, 351) = 1.714$).

According to the OECD (OECD, 2012), a single reproduction test with food provided at 0.1–0.2 mgC $daphnid^{-1}$ d^{-1} should yield at least 60 offspring per surviving control animal to be valid. This was achieved in the first and third generation but not in the second and fourth generation of daphnids in the high-food control. In the latter, reproduction was delayed ($p < 0.01$, Fig. 1, Fig. S5). Thus, we extended the 21-d period in the fourth generation up to 26 days for the HFC group in order to obtain neonates from the fourth brood for acute toxicity testing. When the fourth brood was included, F3 control animals met the validity criterion. With regard to the timing of reproduction, all other treatment groups reproduced consistently without significant differences regarding the day of the first brood (two-way ANOVA with Bonferroni multiple comparisons test: Generation: $F(3, 351) = 6.798$, treatment: $F(4; 351) = 1.112$, interaction: $F(12, 351) = 4.403$; Fig. S5).

3.3. Growth

The level of food affected the size of adult *D. magna* after 21 d (26 d in F3 for HFC and Kaolin400) as animals from the high-food control were larger than daphnids kept at food-limited conditions in all except the fourth generation (Fig. 3). The median size of animals held at low-food conditions in the first generation was 3.99 ± 0.41 mm, while the control animals held at high food levels were 4.59 ± 0.52, 4.39 ± 0.18 mm, and 4.45 ± 17 mm in the first three generations followed by a decrease to 4.04 ± 0.29 mm in the fourth generation. The animals in the HFC control group were consistently larger than the ones in the LFC control group ($p < 0.001$), except for the last generation (two-way ANOVA with Bonferroni multiple comparisons test, generation: $F(3, 351) = 19.29$, treatment: $F(4, 351) = 45.58$, interaction: $F(12, 351) = 1.321$). The body size of daphnids from all PS treatment groups slightly decreased over the subsequent generations. This trend was more distinct in the PS than in the kaolin treatments. This difference is statistically significant for animals exposed to 2000 PS particles mL^{-1} in generation 1 ($p < 0.001$) but not for the other treatment groups or generations.

3.4. Neonate sensitivity to potassium dichromate

We investigated the sensitivity of neonates from the fourth brood to potassium dichromate to assess offspring fitness as a result of the parents' exposure. The tested concentrations covered the lethal concentrations for 50 % of the animals (LC_{50}) established in three experiments with neonates of parents from our laboratory culture (Fig. S3). The LC_{50}s were inside that sensitivity range in the first-generation offspring from all treatment groups (Fig. 4, grey areas). The acute toxicity in neonates from parents from the high-food control remained stable over the consecutive generations. In contrast, the offspring from parents that received lower food levels were more sensitive in F1 and F2. Compared to that, fourth generation neonates were less sensitive to potassium dichromate. Here, neonates from parents exposed to 2000 particles mL^{-1} kaolin had the highest LC_{50}.

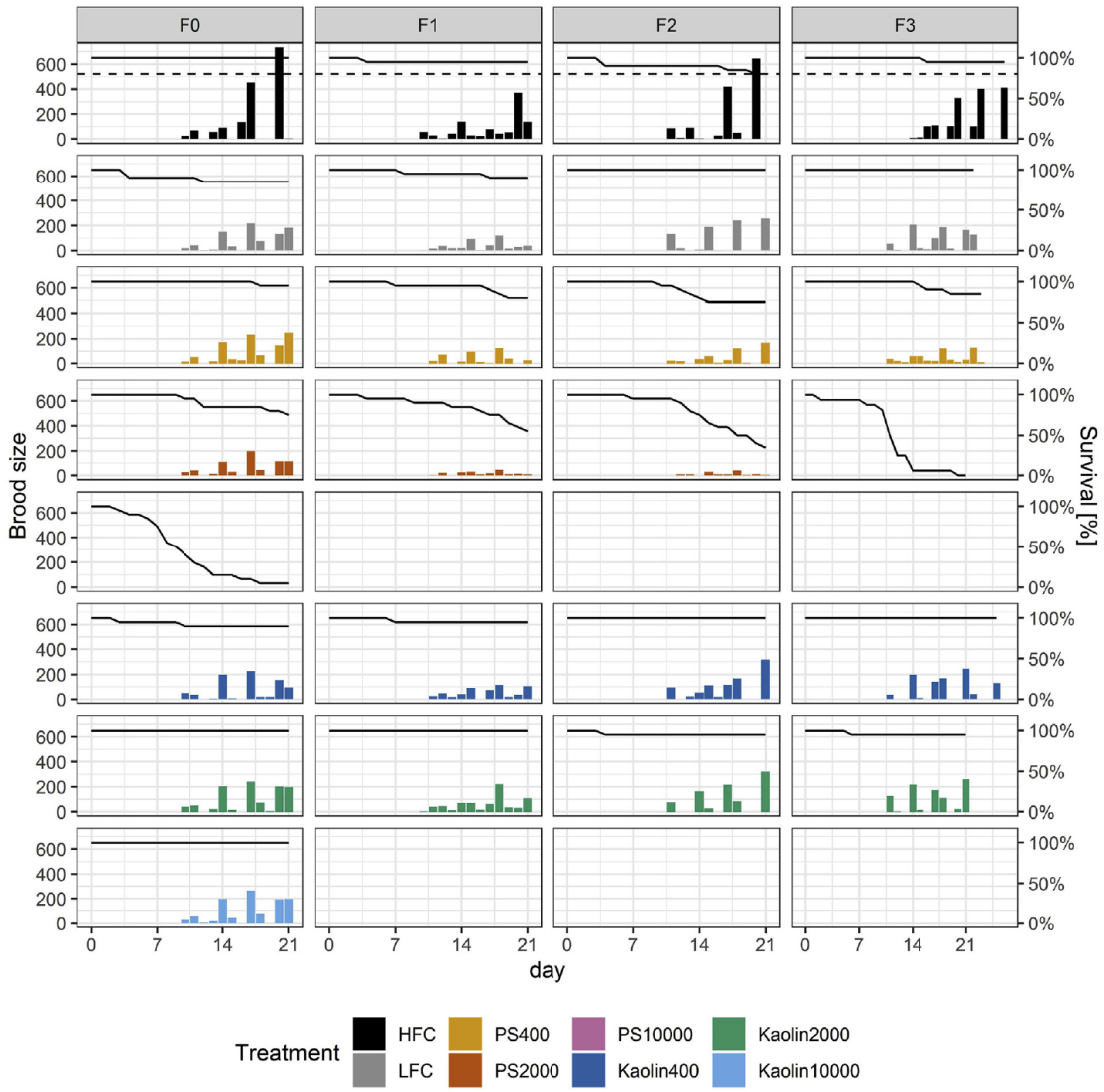

Fig. 1. Survival and reproduction of *D. magna* exposed to polystyrene (PS) microplastics and kaolin over the course of four generations. The animals were exposed to 400, 2000 and 10,000 particles mL^{-1} of PS microplastics or kaolin. Relative survival data (black lines) is plotted on the right axis, total reproductive output per day (bars) is plotted on the left axis. HFC = high-food control, LFC = low-food control. The treatment groups PS10000 (extinct) and Kaolin10000 were discontinued after the first generation (F0).

4. Discussion

There is no shortage of studies that investigate MP effects on daphnids. Until autumn 2018, 14 studies were available on *D. magna*, alone (Triebskorn et al., 2018). However, the vast majority of these reports provide acute toxicity data and/or tested commercially available plastic beads. While this may be a valid point of departure for investigating the toxicity of MP, the knowledge gains are limited because acute toxicity is a result of short-term exposure to very high concentrations and spherical MP are rare in the environment. Unsurprisingly, current MP research has, thus, been criticized for its lack of "realism" (Burns and Boxall, 2018).

To move forward to more relevant exposure scenarios, we investigated the multigenerational effects of irregular MP in comparison to naturally occurring kaolin particles under food-limited conditions. We consider these conditions more realistic because (1) short-lived species are usually exposed to a stressor for more than one generation, (2) plastic fragments resemble the degraded, secondary MP common in aquatic ecosystems, and (3) ample food supply is the exception not the rule. In addition, comparing the toxicity of plastic and non-plastic particles is important to investigate whether MP are indeed more toxic than the natural particulate matter ubiquitous in aquatic environments (Scherer et al. (2017); Backhaus and Wagner (2019)).

4.1. Polystyrene microplastics induce multigenerational effects in D. magna

Over the course of four generations, MP exposure caused overt mortality in *D. magna*, resulting in extinction during the first (10,000 particles mL^{-1}) and fourth generation (2000 particles

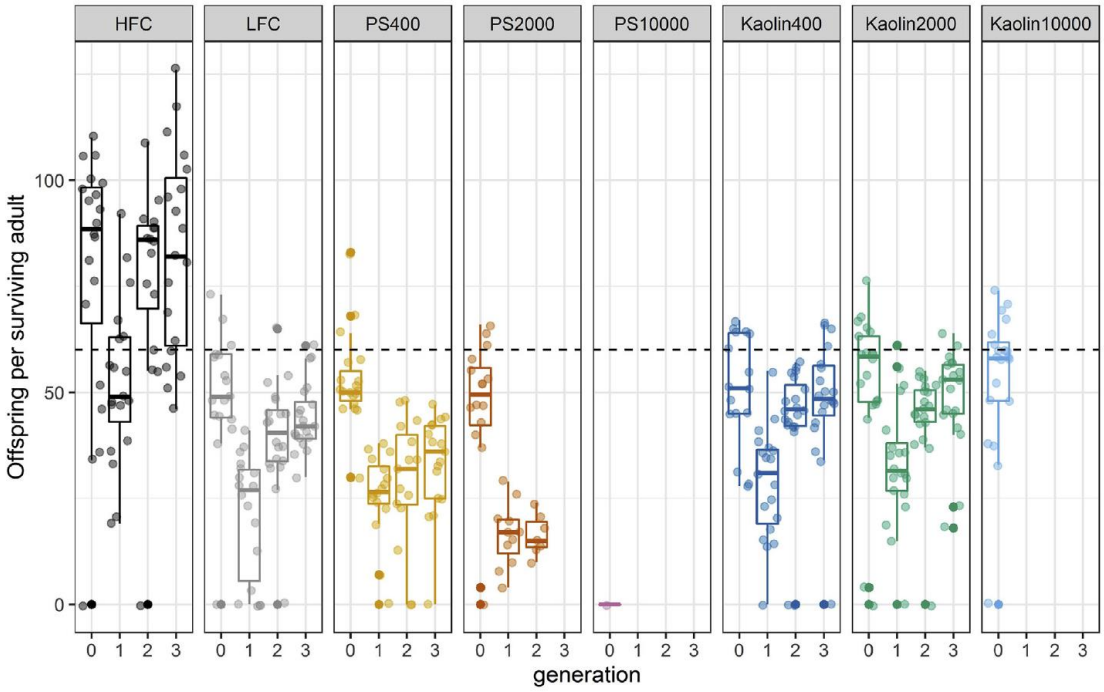

Fig. 2. Offspring produced per surviving *D. magna* exposed to polystyrene (PS) microplastics and kaolin over the course of four generations. The animals were exposed to 400, 2000 and 10,000 particles mL^{-1} of PS microplastics or kaolin. HFC = high-food control, LFC = low-food control. The treatment groups PS10000 (extinct) and Kaolin10000 were discontinued after the first generation (F0).

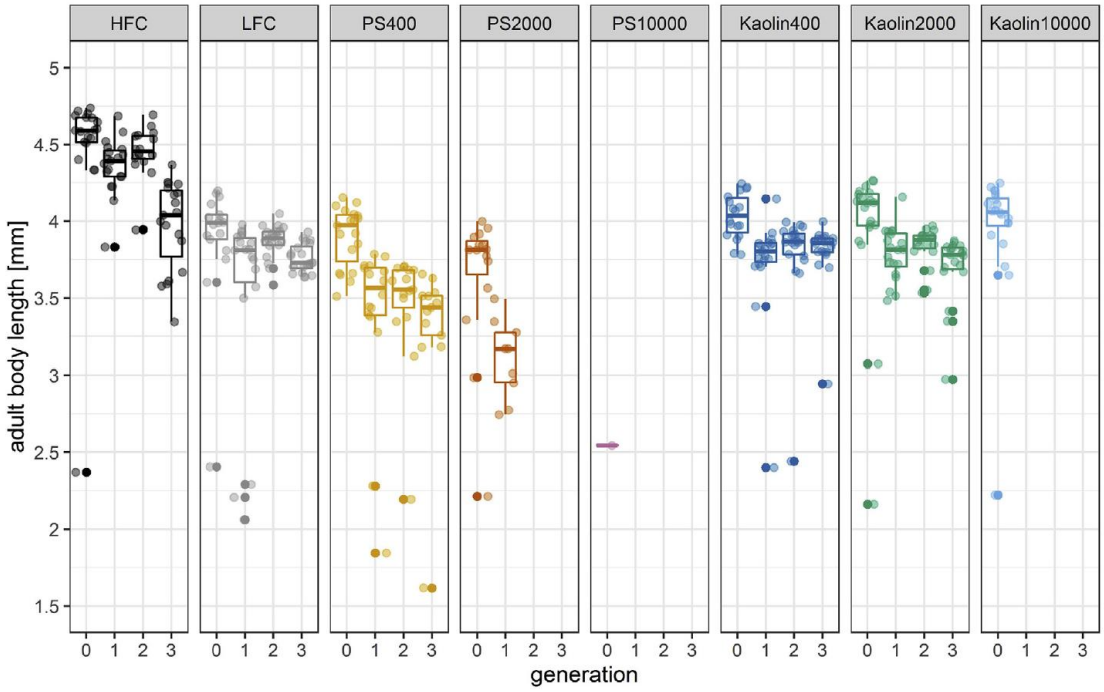

Fig. 3. Size of adult *D. magna* individuals exposed to polystyrene (PS) microplastics and kaolin over the course of four generations. The animals were exposed to 400, 2000 and 10,000 particles mL^{-1} of PS microplastics or kaolin. HFC = high-food control, LFC = low-food control. The treatment groups PS10000 (extinct) and Kaolin10000 were discontinued after the first generation (F0). The size was determined at the end of each generation.

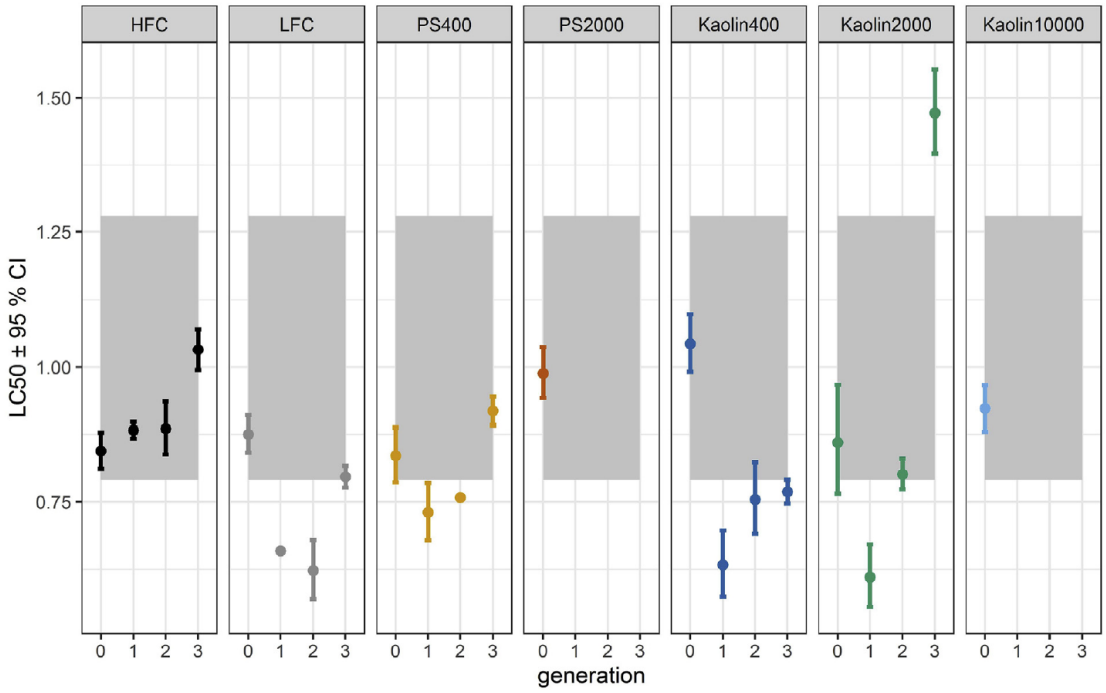

Fig. 4. Acute toxicity of potassium dichromate (LC_{50}) in offspring of *D. magna* exposed to polystyrene (PS) microplastics and kaolin over the course of four generations. HFC = high-food control, LFC = low-food control. Data is missing in treatment groups which did not produce a sufficient number of neonates.

mL^{-1}). An exposure to corresponding concentrations of kaolin did not. Exposure to MP also reduced the reproduction and growth of daphnids.

So far, only one other study investigated the multigenerational effects of MP in daphnids (Martins and Guilhermino, 2018). Here, *D. magna* exposed to fluorescent plastic spheres (1–5 μm, unknown polymer) at a concentration of approximately 18,300 particles mL^{-1} (calculated from the information given in the paper for 2 μm beads) went extinct within two generations. This is very similar to our results, even though their experiment was conducted at a higher food level (0.322 compared to our 0.05 mgC $individual^{-1}$ day^{-1}) and higher particle concentrations. The more severe toxicity we observed at the highest MP concentration (10,000 particles mL^{-1}) suggests that either the irregular PS MP are more toxic than spherical MP and/or that food limitation increases the sensitivity of daphnids to MP. The latter hypothesis is supported by previous studies showing that food limitation amplifies the toxicity of MP in *D. magna* (Aljaibachi and Callaghan, 2018). However, to quantify the impact of food limitation in our study, a full factorial design would have been required.

Investigating the population-level effects of PS microbeads (1–5 μm) in *D. magna*, Bosker et al. (2019) exposed daphnid populations at their carrying capacity (limited by food) over 21 d. The authors reported a significant decrease of the total population size and biomass. Exposure to MP concentrations similar to ours (1000 and 10,000 particles mL^{-1}) reduced the number of adults but not of neonates. This is somewhat in line with our findings in such that adult survival was more affected than reproduction. Contrary to Bosker et al. (2019), we observed a decrease in daphnid size. This could be related to differences in population density which resulted in a smaller body size compared to our study. Nonetheless, the experimental approaches are hard to compare since they held a number of individuals at a population-limiting food level (food availability per individual changing throughout the population growth) while we provided a constant food amount per individual. Overall, the few available studies on multigenerational effects as well as on food limitation imply that the impacts of MP may be overlooked in standard toxicity testing, even when using a well-established model such as *D. magna*.

4.2. Natural kaolin does not affect daphnids over four generations

In contrast to PS MP, kaolin as natural reference particle did not cause multigenerational effects in our study. So far, few studies directly compare the toxicity of MP to that of naturally occurring particles. In line with our findings, Ogonowski et al. (2016) reported that exposure to irregular polyethylene MP reduced the survival and reproduction of *D. magna* whereas kaolin did not. In the shore crab *Carcinus maenas*, PS microspheres caused a transient change in oxygen consumption whereas "natural sediment" did not (Watts et al., 2016). Casado et al. (2013) and Straub et al. (2017) compared the effects of different nano- and microplastics to silica particles and generally found little to no effect of the latter both *in vitro* and *in vivo*. Thus, and despite the limited evidence available, MP appear to be more toxic than natural particles.

However, suspended solids can have negative effects on aquatic biota (Bilotta and Brazier, 2008) irrespective of their natural or anthropogenic origin. The former are, thus, not necessarily more benign than the latter. Accordingly, a range of studies reported effects of kaolin on the feeding rate, growth, reproduction, and population dynamics in daphnid species (Kirk and Gilbert, 1990; Kirk, 1991b; Kirk, 1991a; Kirk, 1992; Robinson et al., 2010; Maisanaba et al., 2015). However, suspended solids in surface waters are diverse regarding their physicochemical properties. Thus,

one major challenge is to find appropriate reference materials matching the properties of MP as close as possible (Scherer et al., 2018).

4.3. Why are microplastics more toxic than kaolin?

The observation that PS MP are more toxic than kaolin can be explained by differences in their physicochemical properties. Regarding their irregular shape and surface structure, both particles are very similar (Fig. S1). However, kaolin particles are smaller than PS particles, with the major size fraction being <10 μm in diameter (Fig. S6). Compared to that, the size distribution of PS particles is more evenly spread between 2 and 50 μm. Another obvious difference is that kaolin (2.6 g cm^{-3}) is much denser than PS (0.96–1.05 g cm^{-3}). Accordingly, the higher toxicity of PS MP in our study might be related to its larger size and lower density. However, although not verified experimentally in this study, there is a range of other relevant properties, most importantly the surface charge and chemical composition.

While differences in size and density may be directly linked to the adverse effects of PS MP in our study, they will also dictate the exposure of the animal to the particles. Particle size is a relevant factor that determines the particle uptake by aquatic biota (Scherer et al., 2018). *D. magna* readily ingests MP < 90 μm (Scherer et al., 2017). The morphology of the filter apparatus determines the lower size limit (200 nm; Brendelberger (1991)). Additionally, processes like drinking and rectal water uptake can lead to the ingestion of very small particles (Smirnov 2017). Thus, the major size fraction of both particle types would be ingestible by *D. magna*. More importantly, the denser kaolin has a higher sinking velocity than PS. This results in a rapid sedimentation of kaolin while most PS particles remained buoyant (Table S5). Thus, the PS exposure was rather continuous while each medium exchange led to a pulsed exposure to kaolin. As daphnids mainly feed from the water column, PS might have been more available than kaolin. Therefore, we hypothesize that differences in particle behavior resulted in different exposures that in turn affected the outcomes we observed. To test this, uptake studies would be needed which are complicated by the lack of analytical techniques to quantify very small, non-fluorescent particles in biota.

There are multiple ways to account for particle fate in MP studies. To compensate for differences in sedimentation, continuous agitation can be used to keep particles in suspension irrespective of density and shape (Frydkjær et al., 2017; Gerdes et al., 2018). However, particle exposure in natural systems will always depend on the properties of the suspended solids. Daphnids will move in the water column along food gradients (Neary et al., 1994) and, thus, also feed on sedimented material. Simply characterizing the particle fate in the water column of the system, therefore, will not fully cover the interaction of particles with the animal. Therefore, future toxicity studies should quantify the fraction that is available to the animals (e.g., concentrations in the water column) as well as the uptake of MP and reference particles to enable a better comparison of their effects.

While investigating the chemicals leaching from the MP used here is beyond the scope of our study, they may contribute to the observed toxicity. Recently, Zimmermann et al. (2019) demonstrated that one out of four PS consumer products contain chemicals inducing *in vitro* toxicity. As this included baseline toxicity in the Microtox assay which is correlated with adverse effects in *D. magna* (Calleja et al., 1986), it is possible that compounds leaching from the MP in our study had an effect. In contrast, Lithner et al. (2009) did not observe acute toxicity when exposing *D. magna* to leachates from a disposable PS drinking cup. The same was true for *Nitocra spinipes* exposed to leachates from UV radiated PS drinking cups (Bejgarn et al. (2015). Regardless these differences, PS will leach, amongst others, its monomer styrene which is toxic in daphnids at high concentrations (Cushman et al., 1997). Accordingly, the contribution of chemicals leaching from PS MP remains to be investigated in future work.

4.4. Potential mechanisms

The underlying idea of our multigenerational study is that the effect of a treatment propagates from parents to offspring and, thus, exacerbates over generations. We challenged daphnids by limiting their food supply and expected this to reduce the fitness of the neonates forming consecutive generations (Tessier and Consolatti, 1989). This is because daphnids supply their offspring with energy reserves in relation to their own energy reserve (Tessier et al., 1983).

We observed a decline of reproduction, body size and an increase of neonate sensitivity to potassium dichromate from the first to the second generation of *D. magna* held under food-limited conditions. This is an indicator for the potential depletion of maternal energy reserves, since the animals in the first generation were derived from a culture held at higher food concentrations. In addition, a change in population density might have contributed as well as the parents of the first generation were held at 10 individuals L^{-1} whereas their offspring were held at 0.05 individuals L^{-1} during the experiments. In any case, the multigenerational effects of the food limitation are probably mediated via a reduced energy transfer from mothers to offspring.

Generally, a hormetic response and phenotypic selection could have taken place as well. The results generally question the suitability of potassium dichromate to assess offspring fitness, since we did not observe a correlation between LC_{50} values and other endpoints. Pieters and Liess (2006) for example observed changes in fenvalerate sensitivity of offspring to adults held at different food levels. Therefore, other chemicals might be a better indicator for effects produced by particle exposure under food limitation. This could be a promising field for future studies to explore as this generally appears to be a sensible approach.

The effects of the food limitation on reproduction and growth in our study were exacerbated by the exposure to PS MP. A reduction in feeding rate, food dilution and increased rejection behavior can decrease the intake of nutrients. Daphnids reduce feeding upon exposure to both natural particles (Kirk, 1991a) as well as MP (Ogonowski et al., 2016; Rist et al., 2017). Additionally, non-nutritious particles increase rejection behavior and, thus, further decrease the food intake (Kirk, 1991a). Importantly, food dilution through non-nutritious particles will also decrease the energy intake at the same energy expenditure. Particle exposures can also increase the energetic cost caused by additional cleaning and rejection behavior (Richman and Dodson, 1983). Apart from these energetic effects, MP might affect the physiology of the digestive system. Unfortunately, little is known about the digestive processes in daphnids (Smirnov 2017).

Taken together, there are a number of plausible mechanisms by which MP ingestion can affect the energy budget of daphnids. However, because direct toxicity will also affect the energy intake and expenditure (e.g., for detoxication) it remains to be demonstrated which mechanism is dominant for MP.

5. Conclusions

In this multigenerational study, we exposed *D. magna* over four generations to PS MP and kaolin as a natural reference particle. Importantly, daphnids were held at food-limited conditions which reduced reproduction and growth but did not affect survival. MP

negatively affected all life-history endpoint except time to maturation, while kaolin did not. The toxicity of MP was most pronounced regarding the daphnids' survival, increased over the generations and resulted in the extinction of animals in the first (10,000 particles mL^{-1}) and fourth generation (2000 particles mL^{-1}). This highlights that MP have multigenerational effects probably caused by a decrease of maternal fitness and nutritional status. The absence of toxicity of kaolin can at least partially be attributed to a lower bioavailability resulting from sedimentation. While it remains challenging to find appropriate reference particles that closely match the physicochemical properties of MP, our study demonstrates that irregular PS particles are more toxic than natural kaolin. Thus, our work builds towards more realistic exposure scenarios that cover irregular MP and natural reference materials as well as food limitation and multigenerational effects.

Declaration of interest

The authors declare no conflict of interest.

CRediT authorship contribution statement

Christoph Schür: Conceptualization, Methodology, Investigation, Formal analysis, Visualization, Writing - original draft, Writing - review & editing. **Sebastian Zipp:** Investigation, Writing - review & editing. **Tobias Thalau:** Investigation, Writing - review & editing. **Martin Wagner:** Funding acquisition, Conceptualization, Methodology, Writing - original draft, Writing - review & editing.

Acknowledgements

This work was supported by the German Federal Ministry for Education and Research [02WRS1378I]. The authors thank Niklas Döring (Goethe University) for his assistance with the scanning electron microscope and Lisa Zimmermann (Goethe University) and Lucian Iordachescu (Aalborg University) for assistance with FTIR analysis. The graphical abstract was created with BioRender.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2019.113904.

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Supplementary Material

Material and particle characterization

We confirmed the polymer type of the plastic material used in this study (Figure S7), its density (Table S1) and provide scanning electron microscopy images (Figure S1) and particle size distributions (Figure S6) of both particle types.

Fourier-transform infrared spectroscopy (FTIR) was performed on a PerkinElmer Spectrum Two instrument in ATR mode (4000–400 cm^{-1}). The FTIR spectrum of the coffee cup lid (Figure S7) was compared to a spectral library using the siMPle software (Version 1.0.0., http://www.simple-plastics.eu) and matched "Polystyrene.67" with a score of 61.2 %. Thus, the coffee cup lids consisted of polystyrene.

We estimated the density of the polystyrene by punching out 20 circles from the flat area of two coffee cup lids. We determined their size (Figure S8, Table S1, Olympus SZX7, Olympus cellSens Standard 2.2) and weight (Table S1) and calculated the area and volume of the circles to derive the density which was 0.81 ± 0.06 g cm^{-3}. This is likely an underestimation since measuring the thickness of the material under the binocular produced some variability (± 7.2 % relative standard deviation (RSD)) which translates to a RSD of 7.8 % in the density. Puncturing the material did not produce perfect edges, which increased uncertainty regarding material diameter and thickness. Since according to the literature PS has a density of 0.96–1.05 g cm^{-3} (Lambert and Wagner 2018), we can assume that the particles will be more or less neutrally buoyant in the water column.

In the exposure vessels with the high MP concentration (2000 and 10000 particles mL^{-1}) some particles floated on the surface shortly after application, while the majority remained buoyant in the water column. The suspensions of both particle types were shaken in medium for at least 48 h prior to application in the test. This markedly improved stability of the PS suspensions, whereas no change was visible for kaolin particles. The ratio of floating and suspended particles was not determined. As discussed in the main manuscript, kaolin sedimented rather quickly.

Table S5 provides theoretical sinking velocities for particles with different densities and sizes. The particle size distributions are available in Figure S6.

To investigate the particle shapes and surface characteristics of the PS MP and kaolin we recoded micrographs at 300× and 1500× magnification using a Hitachi S-4500 scanning electron microscope (Figure S1). While the particle types differ in size (kaolin particles are smaller than PS MP, their irregular shape and rough surface is similar.

Herrington et al (1992) provide information on the surface charge of kaolin.

Table S1: Determination of the density of the polystyrene coffee cup lids used to make microplastics. Flat areas from the center part of the lid were punch holed (n=20). These circles were then measured in diameter, thickness, and weight from which the surface area, volume and density were calculated.

Replicate	Measured diameter [mm]	Measured thickness [mm]	Measured weight [mg]	Calculated surface area [mm^2]	Calculated volume [mm^3]	Calculated density [$g\ cm^{-3}$]
1	5.37	0.230	7.21	22.67	5.22	0.72
2	5.39	0.234	7.19	22.83	5.35	0.74
3	5.39	0.221	7.30	22.80	5.03	0.69
4	5.37	0.258	7.50	22.67	5.84	0.78
5	5.52	0.241	7.03	23.93	5.77	0.82
6	5.38	0.264	7.12	22.70	6.00	0.84
7	5.47	0.245	7.46	23.52	5.75	0.77
8	5.50	0.246	7.12	23.74	5.84	0.82
9	5.34	0.256	7.22	22.39	5.73	0.79
10	5.35	0.273	7.31	22.48	6.14	0.84
11	5.34	0.264	7.07	22.42	5.92	0.84
12	5.32	0.249	7.31	22.24	5.53	0.76
13	5.31	0.280	7.05	22.18	6.20	0.88
14	5.33	0.281	7.44	22.33	6.28	0.84
15	5.41	0.267	7.19	22.98	6.14	0.85
16	5.34	0.234	6.99	22.39	5.24	0.75
17	5.34	0.279	7.22	22.42	6.26	0.87
18	5.36	0.284	7.16	22.58	6.40	0.89
19	5.32	0.256	7.07	22.24	5.69	0.80
20	5.30	0.280	6.38	22.08	6.18	0.97
Mean	**5.37**	**0.257**	**7.17**	**22.68**	**5.83**	**0.81**
SD	**0.059**	**0.019**	**0.230**	**0.500**	**0.380**	**0.060**
RSD [%]	**1.1**	**7.3**	**3.1**	**2.2**	**6.6**	**7.8**

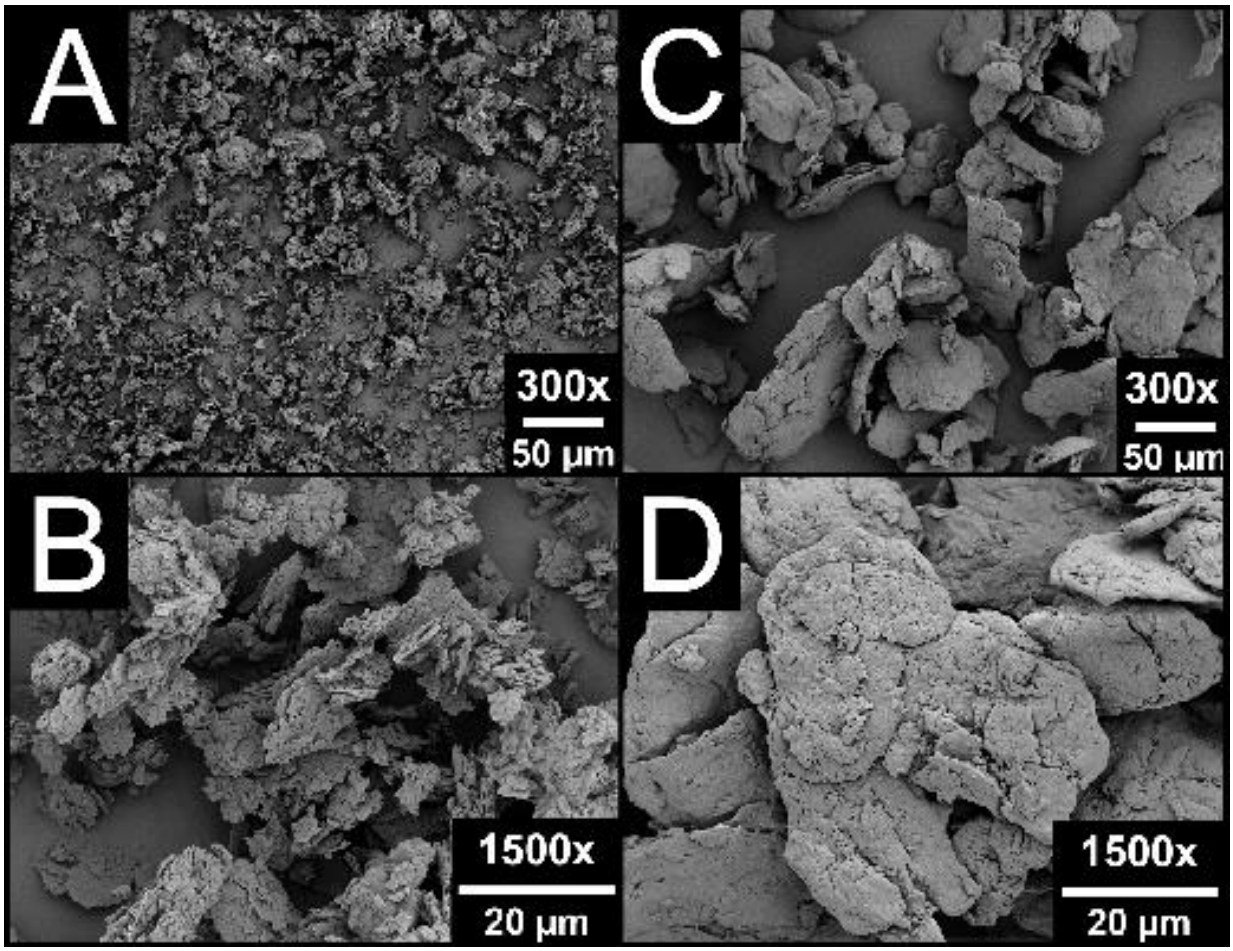

Figure S8: Scanning electron microscope images of the kaolin (A+B) and polystyrene (C+D) particles used in this study at 300× (A+C) and 1500× (C+D) magnification.

Reproduction at different food levels

Since we aimed at conducting the multigenerational experiment using low-food conditions, we performed a Daphnia reproduction test (OECD 2012) with different food levels (0.01, 0.025, 0.05, 0.075, 0.1, and 0.2 mgC daphnid^{-1} day^{-1}). Feeding with 0.1 and 0.2 mg C individual^{-1} d^{-1} are the conditions recommended in the OECD guideline 211 (OECD 2012). 0.05 mgC daphnid^{-1} d^{-1} was the lowest food level that still resulted in reproductive output that met the validity criteria of the OECD 211 guideline (60 neonates per surviving adult) and was, therefore, selected as the low food level to be used in the multigenerational experiment (Figure S2).

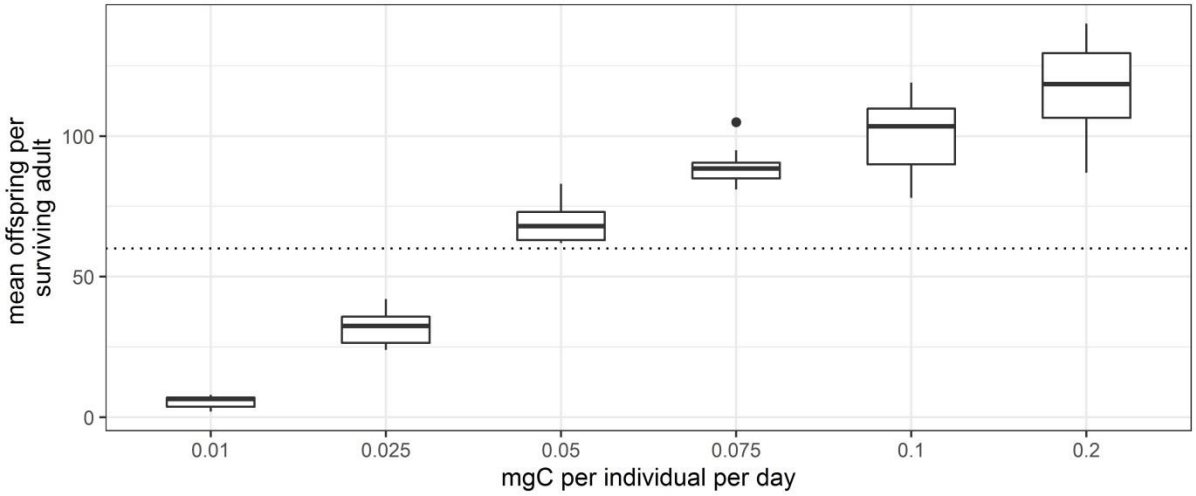

Figure S9: Reproductive output of *D. magna* individuals during 21 d under different feeding regimens. Animals were held individually and fed 0.01, 0.025, 0.05, 0.075, 0.1, or 0.2 mg carbon individual^{-1} d^{-1}. The two levels of 0.1 and 0.2 mg C individual^{-1} d^{-1} are the feeding conditions proposed in the OECD guideline 211 (OECD 2012). The dotted line indicates the OECD 211 validity criterion of minimum produced offspring per surviving adult animal over 21 d.

Table S2: Nominal and measured particle concentrations. For each treatment, at least three vessels were prepared identical to the ones used to expose daphnids but did not contain algae and animal.

Treatment	Nominal concentration (particles mL^{-1})	Mean measured concentration ± SD (particles mL^{-1})	N (replicates with 3 technical replicates each)
PS400	400	787 ± 53.7	3
PS2000	2000	2005 ± 262.5	3
PS10000	10000	12234 ± 2214	4
Kaolin400	400	1776 ± 829.9	4
Kaolin2000	2000	3928 ± 309.2	4
Kaolin10000	10000	10195 ± 1793.8	3

Table S3: Results of the acute toxicity tests with potassium dichromate conducted with the 4th brood of each treatment per generation, given as 50 % lethal concentration (LC_{50}) values with the range of the 95 % confidence interval. n.a. = not analyzed

Treatment	Generation	N (animals/replicate)	LC_{50}	95 % Confidence Interval
HFC	0	4 (5)	0.844	0.812–0.878
	1	4 (5)	0.882	0.867–0.898
	2	4 (5)	0.885	0.838–0.935
	3	4 (5)	1.032	0.994–1.070
LFC	0	4 (5)	0.875	0.842–0.911
	1	4 (5)	0.659	(very wide)
	2	4 (5)	0.623	0.570–0.680
	3	3 (5)	0.797	0.777–0.817
PS400	0	4 (5)	0.836	0.786–0.888
	1	3 (5)	0.731	0.679–0.785
	2	3 (5)	0.758	(very wide)
	3	3 (5)	0.918	0.892–0.944
PS2000	0	4 (5)	0.988	0.942–1.037
	1–3	n.a. because of low reproduction		
PS10000	0	n.a. because of low reproduction		
	1–3	n.a. because of extinction		
Kaolin400	0	4 (5)	1.043	0.991–1.098
	1	4 (5)	0.633	0.575–0.698
	2	4 (5)	0.755	0.691–0.824
	3	3 (5)	0.769	0.747–0.791
Kaolin2000	0	4 (5)	0.860	0.765–0.968
	1	4 (5)	0.611	0.556–0.672
	2	4 (5)	0.801	0.773–0.830
	3	4 (5)	1.472	1.396–1.553
Kaolin10000	0	4 (5)	0.923	0.880–0.967
	1–3	n.a.		

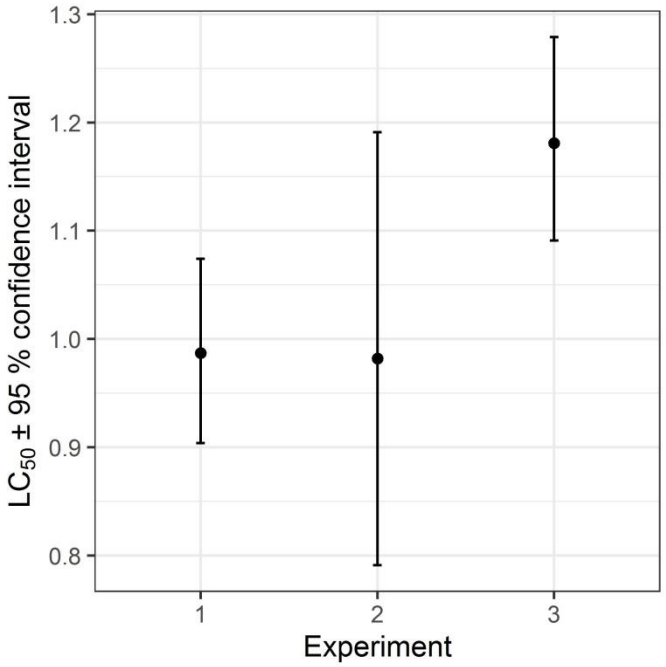

Figure S10: Lethal concentrations for 50 % (LC_{50}) of neonate *D. magna* in three independent acute toxicity tests with potassium dichromate, performed with neonates from a laboratory culture maintained at high food levels (0.15 mg carbon individual^{-1} d^{-1}).

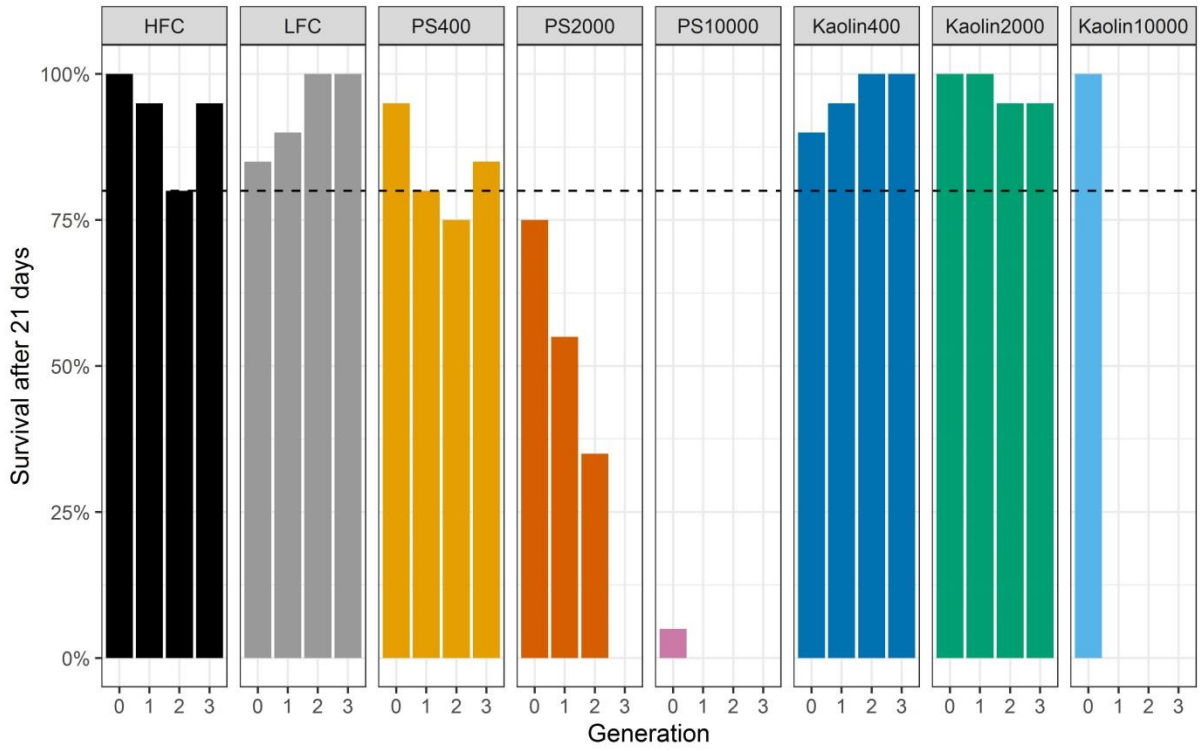

Figure S11: Survival of *Daphnia magna* at the end of each generation and exposure **to high food levels (HFC), low food levels (LFC) and three concentrations (400, 2000, and 10000 particles mL^{-1}) each of polystyrene (PS) microplastics and kaolin.** The dotted line indicates the OECD 211 validity criterion (80 % survival in the controls).

Table S4: Daphnid survival at the end of each generation, mean first day of reproduction, mean offspring per surviving adult, and median size per surviving adult.

Treatment	Generation	Survival after 21 d [%]	Mean day of first reproduction ± SD	Mean offspring per surviving adult ± SD	Median size per surviving adult ± SD [µm]
HFC	0	100	11.33 ± 1.53	78.2 ± 29.6	4592 ± 521
HFC	1	95	11.50 ± 1.29	52.5 ± 19.0	4391 ± 183
HFC	2	80	11.50 ± 0.71	76.4 ± 25.1	4454 ± 168
HFC	3	95	14.80 ± 1.92	82.9 ± 24.1	4039 ± 288
LFC	0	85	11.75 ± 1.71	49.5 ± 15.8	3989 ± 410
LFC	1	90	13.00 ± 2.61	21.1 ± 14.6	3812 ± 634
LFC	2	100	11.50 ± 0.71	39.6 ± 13.1	3890 ± 109
LFC	3	100	12.33 ± 1.53	44.0 ± 8.13	3728 ± 93
PS400	0	95	12.00 ± 2.65	52.8 ± 10.7	3973 ± 195
PS400	1	80	11.50 ± 0.71	25.8 ± 10.3	3566 ± 545
PS400	2	75	12.00 ± 1.00	28.8 ± 15.2	3554 ± 385
PS400	3	85	13.00 ± 1.58	33.0 ± 11.8	3438 ± 525
PS2000	0	75	10.50 ± 0.71	45.3 ± 18.8	3814 ± 472

Treatment	Generation	Survival after 21 d [%]	Mean day of first reproduction ± SD	Mean offspring per surviving adult ± SD	Median size per surviving adult ± SD [µm]
PS2000	1	55	14.83 ± 3.76	16.4 ± 7.42	3169 ± 254
PS2000	2	35	13.50 ± 1.29	16.4 ± 4.86	n.a.[a]
PS2000	3	0	-	-	-
PS10000	0	5	-	-	2545
Kaolin400	0	90	12.00 ± 2.65	50.7 ± 13.2	4033 ± 146
Kaolin400	1	95	13.40 ± 2.30	28.6 ± 13.1	3802 ± 348
Kaolin400	2	100	13.00 ± 2.00	43.0 ± 15.6	3867 ± 331
Kaolin400	3	100	12.50 ± 2.12	48.1 ± 14.5	3858 ± 212
Kaolin2000	0	100	13.50 ± 4.04	52.0 ± 19.0	4120 ± 493
Kaolin2000	1	100	12.00 ± 1.58	33.4 ± 14.3	3814 ± 168
Kaolin2000	2	95	11.00 ± 0.00	44.2 ± 11.9	3878 ± 123
Kaolin2000	3	95	12.67 ± 2.08	48.6 ± 11.9	3781 ± 225
Kaolin10000	0	100	12.00 ± 2.16	53.7 ± 16.8	4060 ± 437

[a] not analyzed because the animals were accidently discarded before size measurements

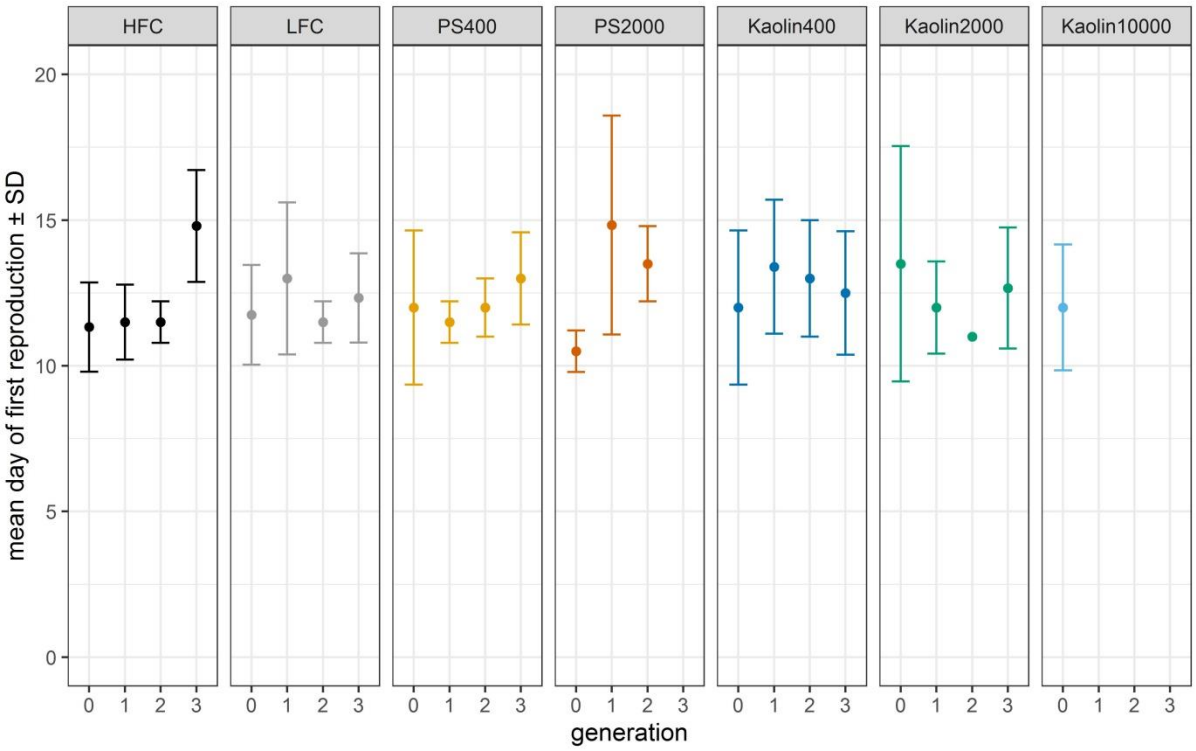

Figure S12: Mean day of first reproduction of *Daphnia magna* after 21 days exposure to high food levels (HFC), low food levels (LFC) and three concentrations (400, 2000, and 10000 particles mL^{-1}) each of polystyrene (PS) microplastics and kaolin. The Kaolin10000 treatment group was discontinued after the extinction of the PS10000 treatment group in the first generation (F0).

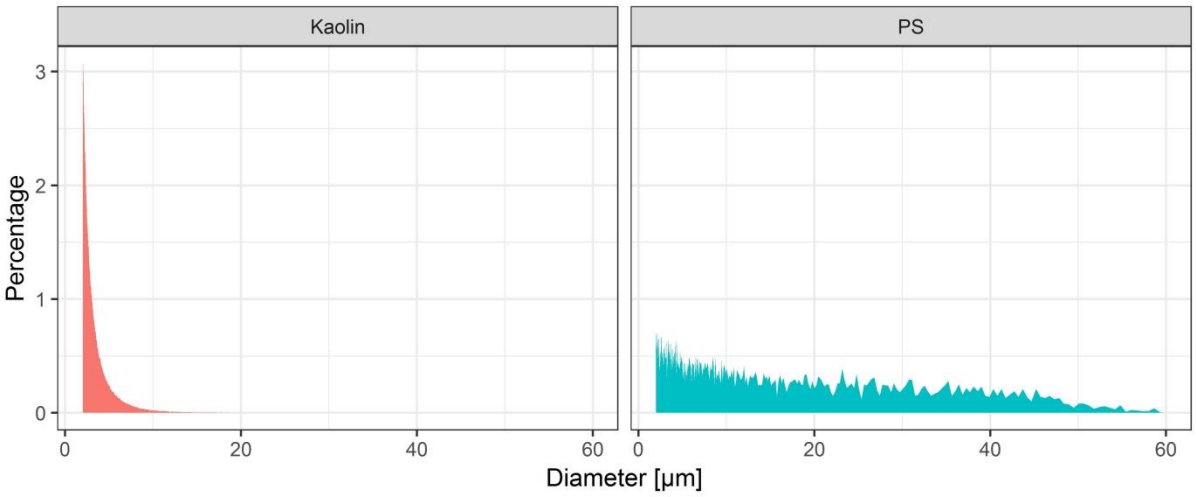

Figure S13: Particle size distributions for kaolin and PS particles. The size distribution was derived from 1 g L^{-1} stock suspensions in M4 medium using a Beckman Coulter Multisizer 3.

Table S5: Theoretical sinking velocities for hypothetical spherical particles made of polystyrene or kaolin.

Material type	Assumed density [g cm^{-3}]	Hypothetical particle diameter [µm]	Calculated sinking velocity [cm h^{-1}]
Kaolin	2.6	1	0.33
		10	32.9
Polystyrene	0.95/1.05	1	-0.0098/0.0098
		10	-0.9826/0.9826

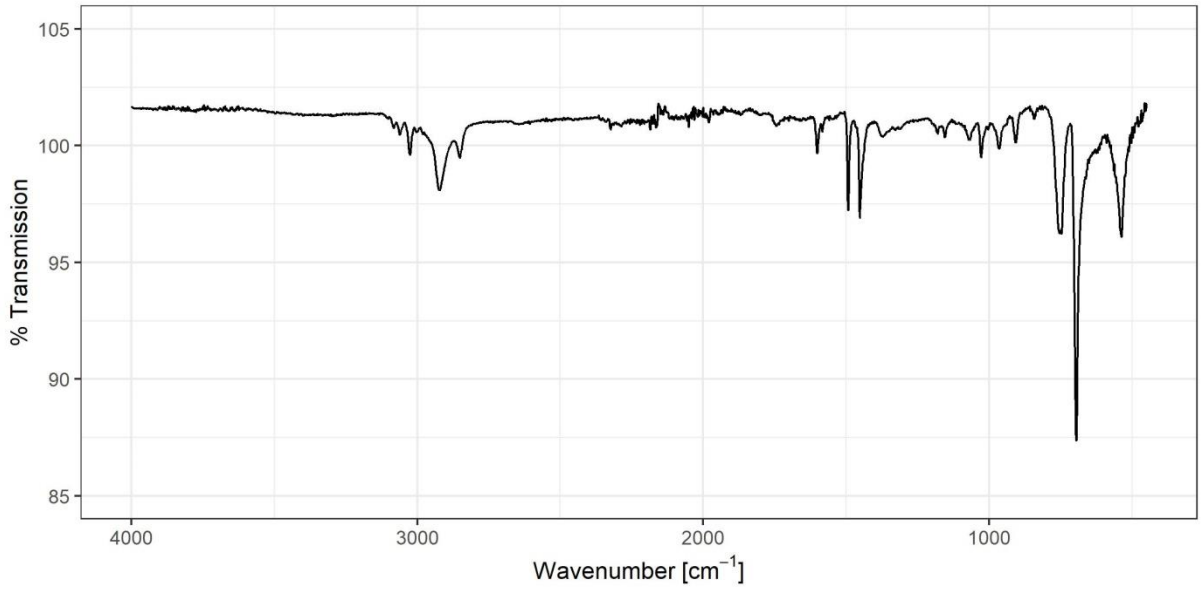

Figure S14: Fourier-transform infrared spectrum of the coffee cup lid material used to produce the irregular microplastics particles used throughout the study. The spectrum confirms that the plastic is polystyrene.

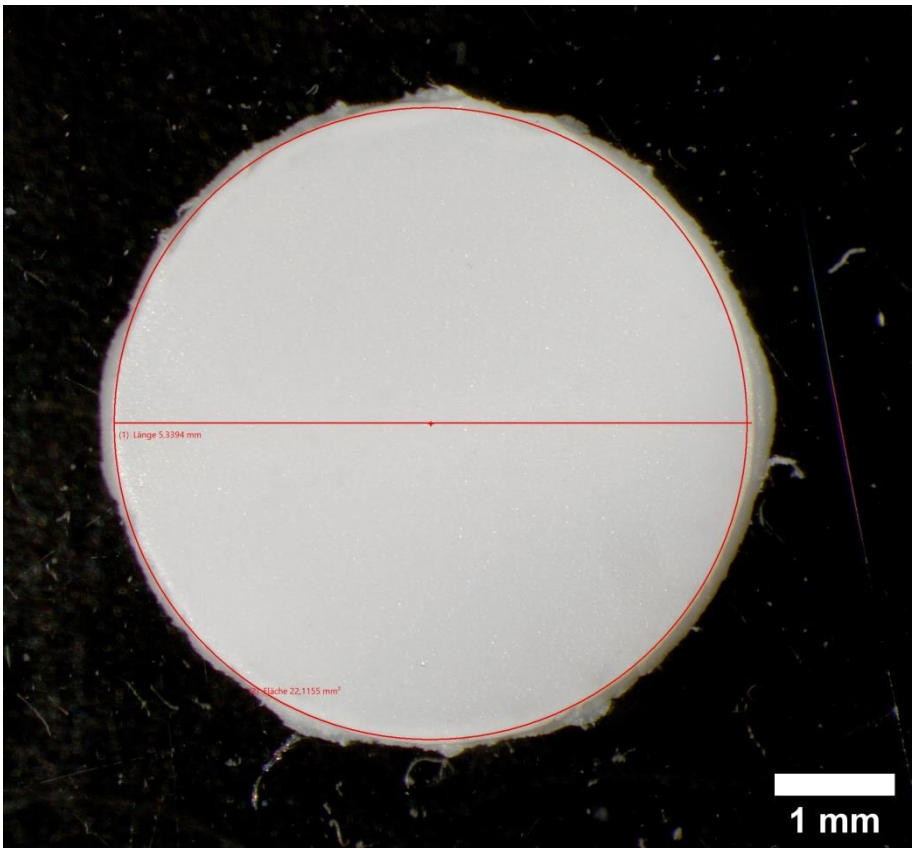

Figure S15: Example of a plastic circle from a coffee cup lid (replicate 9) used to determine the density. Flat areas from the center part of the lid were punch holed (n=20). The circular pieces were then measured in diameter, thickness, and weight from which the surface area, volume and density was calculated.

Annex 3: Paper 3

Title: Incubation in Wastewater Reduces the Multigenerational Effects of Microplastics in *Daphnia magna*

Journal: Environmental Science and Technology

Contributors: Christoph Schür (CS), Carolin Weil (CW), Marlene Baum (MB), Jonas Wallraff (JW), Michael Schreier (MS), Jörg Oehlmann (JO), Martin Wagner (MW)

Status: published

doi: 10.1021/acs.est.0c07911

Environmental Science & Technology

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Article

Incubation in Wastewater Reduces the Multigenerational Effects of Microplastics in *Daphnia magna*

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Cite This: https://dx.doi.org/10.1021/acs.est.0c07911

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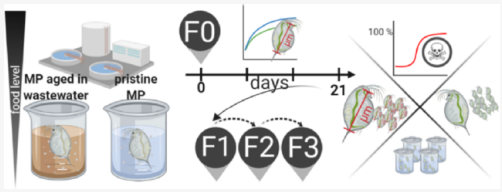
ABSTRACT: The aging of microplastics in the environment changes their physicochemical properties. While this may affect their toxicity, comparative data on the effects of aged compared to pristine microplastics are scarce. One of those aging processes is the sorption of chemicals, which has mainly been studied for individual pollutants present in marine ecosystems. To investigate how the sorption of a complex mixture of freshwater pollutants affects the toxicity of microplastics, we incubated irregular polystyrene particles (≤63 μm) in either wastewater or ultrapure water. We exposed *Daphnia magna* to these aged microplastics and their pristine counterparts (80, 400, 2000, and 10,000 particles mL^{-1}) over four generations using food limitation as an additional, environmentally realistic stressor. Both particle types affect the survival, reproduction, adult and neonate body lengths, and growth. An exposure to pristine microplastics results in the extinction of the third generation of daphnids. In contrast, wastewater-incubated particles induced a lower mortality. The incubation with wastewater does not change the microplastics' size, surface charge, and structure. Consistent with the literature, we assume that the adsorption of dissolved organic matter is a key aging process reducing the toxicity of microplastics. Consequently, toxicity testing using pristine microplastics may overestimate the effects of plastic particles in nature.



INTRODUCTION

Small plastic particles (microplastics, MPs) are ubiquitous in the aquatic environment,[1] where they can interact with and affect a large number of biota.[2] MPs undergo transformation processes in the environment driven by chemical, physical, and biological processes.[3] This "aging" greatly affects the behavior and fate of MPs in the aquatic environment.[4] Nonetheless, many ecotoxicological studies investigate the effects of pristine and/or spherical MPs, even though they are not very representative of MPs in the environment. Previous research indicates that aging and the presence of natural organic matter alter the toxicity of engineered nanoparticles.[5,6] It is currently unclear how this applies to MPs, but it is likely that aging also modulates the MP toxicity.

MPs may undergo a number of different aging processes that can affect their behavior and fate in the environment: biofilm formation can alter the particle density and surface chemistry,[7] and chemicals can adsorb or absorb to the particles that then may act as vectors and increase the chemical exposure of biota ("Trojan horse" or "vector effect"[8]). Likewise, natural organic matter is abundant in the aquatic environment and can also adsorb to plastic particles, forming a corona, and alter their surface chemistry and behavior.[9] All these processes will probably affect MP−biota interactions but are currently largely unaccounted for in the ecotoxicological research.

Previous studies have relatively consistently shown that particle toxicity changes in the presence of or after treatment with dissolved organic matter,[10] humic acids,[11] and wastewater.[5,12] The latter is a relevant scenario because even though wastewater treatment plants effectively remove MP, they discharge large amounts of effluents that can constitute a significant fraction of the water in smaller water bodies.[13] Whether and how the adsorption of the complex chemical mixture present in wastewater affects the toxicity of MPs remains unclear to date. In addition, previous studies cover the acute toxicity of aged spherical microplastics, only. Thus, the chronic, long-term effects of an exposure to pristine versus aged, irregular MPs commonly found in nature remain unknown.

Accordingly, the aim of this study is to compare the long-term effects of irregular polystyrene MPs after incubation in either filtered raw wastewater or ultrapure water on *Daphnia magna* over four generations. This multigenerational setup allows investigating effects beyond a single daphnid lifecycle.

Received: November 23, 2020
Revised: January 13, 2021
Accepted: January 21, 2021

Moreover, the experiment was conducted under food limitation because this more closely mimics environmental conditions. We used this experimental design in a previous study and demonstrated that MPs are more toxic than natural particles.[14] This approach was combined with experiments monitoring the individual growth of daphnids in relation to their maternal food availability and MP exposure. We hypothesized that the toxicity of wastewater-incubated MP increases in case the sorption of wastewater-borne pollutants dominates (vector effect). Alternatively, we assume the toxicity to decrease if the adsorption of dissolved organic matter or biofilm formation is the driving factor.

MATERIALS AND METHODS

Particle Preparation and Characterization. We obtained polystyrene (PS) coffee-to-go-cup lids from a local bakery to produce the MPs as described previously.[14] In brief, the lids were cut into small pieces, frozen in liquid nitrogen, and then ground in a swing mill (Retsch MM400, Retsch, Germany). The resulting powder was sieved to ≤63 μm (Retsch AS 200 basic, Retsch, Germany) and characterized as described in Schür et al.[14]

The PS MPs we used in this study were either incubated in wastewater (wastewater-incubated, wwMP) or in ultrapure water (pristine, MP). To produce the former, we sampled the influent of the wastewater treatment plant Bad Homburg vor der Höhe (size class IV, Germany). The influent is raw wastewater that is treated with a bar screen, only. The 24 h composite sample consisted of 1 L of wastewater sampled every 2 h between March 31st and April 1st, 2019, 8 am (pH: 7.4; electric conductivity: 934 μS cm^{-1}). No rainfall was recorded in the 5 days prior to sampling.

The wastewater was filtered using a 0.2 μm filter (Rapid Flow system, Nalgene) directly after sampling to remove suspended solids and microbes. The MP was then incubated in the filtrate at 1 g L^{-1} for 38 h at 4 °C. After that, we recovered the incubated MP by vacuum filtration using a 0.2 μm filter. The particles were then frozen at −80 °C and lyophilized to remove the residual water (Alpha 1−4 LSC plus, Martin Christ, Germany). The pristine MPs were treated identically except that they were incubated in ultrapure water instead of wastewater. Both particle types were stored at −80 °C to minimize the degradation of potentially sorbed chemicals and the particles themselves.

The concentrations and particle size distributions (2−60 μm, Figure S1) of the stock suspensions were determined using a Coulter counter (100 μm aperture, Multisizer 3, Beckman Coulter, Germany; measurements in filtrated (<0.2 μm) 0.98% NaCl solution, electric conductivity:17.03 mS cm^{-1}, pH: 7.36). The correlation between the nominal and measured exposure concentrations via this method is described in Schür et al.[14] The zeta potentials of the particles (Figure S2) were determined after suspension in M4 medium using a Zetasizer Nano ZS (red laser, 4 mW, 632.8 nm). Both particle types were imaged using scanning electron microscopy (SEM) after freeze-drying (Figure 1). For that, 20 μL of each suspension was transferred to the sample holder, dried under a heat lamp, sputtered with gold, and imaged in a Hitachi S-4500 system. We performed an experiment to investigate the biofilm formation and changes in the surface structure of the particles over 9 days in M4 medium (conductivity: 703 μS cm^{-1}, pH: 6.94) using SEM (for details, see Supporting Information and Figure S5). The Fourier transform infrared spectroscopy (ATR-FTIR) spectra (FTIR Spectrum Two, PerkinElmer; LiTa03 detector, range: 4000−450 cm^{-1}) of the raw material before and after grinding and sieving and the two particle powders after incubation and freeze-drying (MP and wwMP) are given in the Supporting Information (Figures S3 and S4). The spectral data is available at figshare under doi 10.6084/m9.figshare.12311495. Additional information on the material and particle characterization can be found in Schür et al.[14]

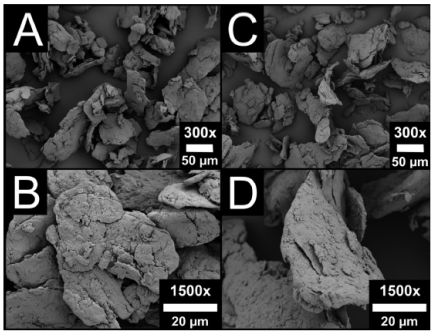


Figure 1. Scanning electron microscopy (SEM) images of the polystyrene microplastics used in this study at two magnifications: 300× (A + C) and 1500× (B + C) of particles incubated in ultrapure water (A + B) and wastewater (C + D).

Daphnia Culture. Ten *D. magna* individuals were cultured in 1 L of Elendt M4 medium[15] at 20 °C with a 16:8 h light:dark cycle. The daphnids were fed with the algae *Desmodesmus subspicatus* thrice a week at 0.2 mg carbon per individual per day (mgC $daphnid^{-1}$ d^{-1}). The medium was fully renewed once a week.

Multigenerational Experiment. The multigenerational experiment basically consisted of four consecutive semi-static reproduction experiments (21 days, OECD guideline 221), similar to the design used in Schür et al.[14] Each generation included two control groups held at different food levels and treatments with four concentrations per particle type (pristine MP and wastewater-incubated wwMP). The specimens for the first generation (F0, <24 h old neonates) were taken from the daphnid culture (see above). The offspring of this experiment was transferred to the next experiment (i.e., generation) and treated identical to its parents. For this, <24 h old neonates from the third brood of each treatment were pooled, and 20 individuals were randomly picked for the next generation with the exception of animals of the MP10000 group (seven neonates in F1 and 16 neonates in F2 constituted the following generations for this treatment group).

The animals in each treatment were held individually in a 100 mL glass beaker containing 50 mL of Elendt M4 medium[15] that was fully exchanged thrice weekly by transferring the parent animal to a new vessel. Animals were fed daily with *D. subspicatus* with daphnids in the high food control (HFC, negative control without MPs) receiving 0.2 mgC $daphnid^{-1}$ d^{-1} according to the Organisation for Economic Co-operation and Development (OECD) guideline.[15] The animals in all other treatment groups were fed a lower food level of 0.05 mgC $daphnid^{-1}$ d^{-1} to induce food limitation that decreases the reproduction but not survival.[14] The low-food treatments included another negative control group without particles (low food control, LFC).

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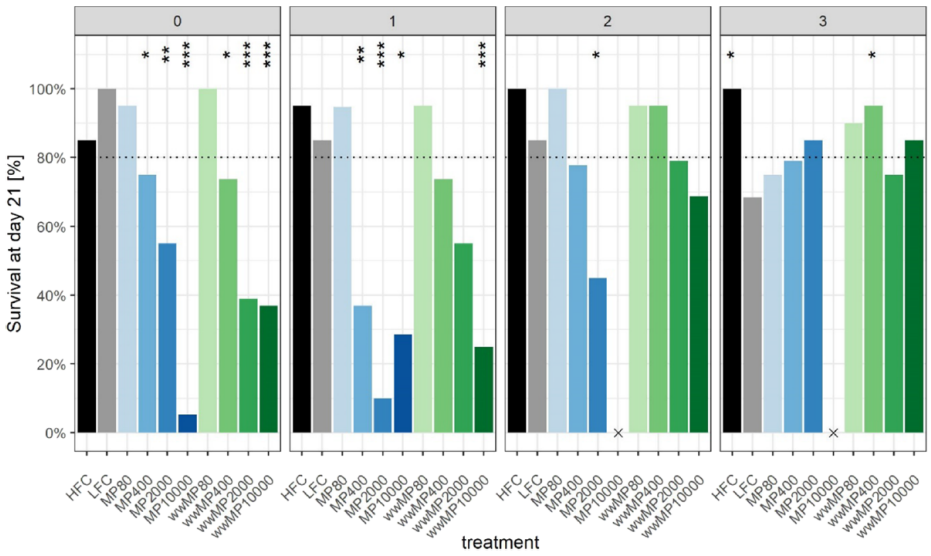

Figure 2. Survival of *D. magna* exposed to pristine microplastics (MP) and wastewater-incubated microplastics (wwMP) over the four generations. The animals were held at high food levels (high food control, HFC), low food levels (low food control, LFC), and low food levels in combination with 80, 400, 2000, and 10,000 MP or wwMP mL^{-1}. The MP10000 treatment group went extinct in F2 (crosses). The Fisher's exact test was against the corresponding LFC (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$).

The daphnids were exposed to 80, 400, 2000, and 10,000 particles mL^{-1} of pristine or wastewater-incubated MPs over the course of four generations. Each week, we prepared a fresh stock suspension by suspending the respective MP powder in M4 medium for 48 h on an orbital shaker. The stock suspensions were then transferred to new test vessels with each of the three weekly water exchanges (resulting in a total use period of 9 days), and parent animals were carefully added using a pipette.

We recorded the mortality (15 s immobility after agitation[16]) and their reproductive output (neonates per female) daily. The neonates were removed and discarded (first and second broods), pooled to create the next generation (third brood), or transferred to 70% ethanol for the size determination (fourth brood). The parent animals were photographed at the end of each generation to determine their length (center of the eye to the base of the apical spinus).

Growth Experiments. To investigate the effects of the maternal diet on the growth curve during the MP exposure, we conducted two experiments in which we exposed daphnids over 21 days to MPs and wwMPs and monitored their individual growth thrice a week. The first experiment was carried out with 10 neonates per group taken from the *Daphnia* culture and is therefore equivalent to the F0 generation of the multigenerational experiment. For the second experiment, a separate culture was reared at low food levels (0.05 mgC $individual^{-1}$ day^{-1}) for 16 days (equivalent to F0 in the multigenerational experiment). Neonates (<24 h old) from these two cultures were exposed to MPs as described above. The number of replicates was increased to 15 and 20 in the treatment groups exposed to 2000 and 10,000 particles mL^{-1}, respectively, to account for the high mortality in the prior experiments. During each water exchange (thrice weekly, on days 0, 2, 5, 7, 9, 12, 14, 16, 19, and 21), each individual's body length was measured from the center of the eye to the base of the apical spinus.

Data Analysis. The data analysis was carried out using R 3.6.1[17] with RStudio 1.2.1335[18] and the tidyverse package version 1.2.1.[19] The survival data were analyzed using Fisher's exact tests in R. All other data were analyzed using two-way ANOVA with Bonferroni multiple comparison tests in GraphPad Prism (version 5.04 for Windows, GraphPad Software, La Jolla, California, USA). The treatments were compared against the LFC group from the corresponding generation. The growth data were fitted using a von Bertalanffy growth function with bootstrapped confidence intervals according to Ogle[20] using the R packages FSA[21] and car.[22] The details, code, and parameters are provided in the Supporting Information. The raw data and model outputs are available at figshare under doi 10.6084/m9.figshare.12311495.

Boxplots were created with the geom_boxplot() function of ggplot2[23] and followed the basic boxplot of McGill (1978).[24] Additional R packages used for the analysis and visualization include readxl[25] and patchwork.[26]

The MP10000 treatment was excluded from statistical analyses because the low survival, reproduction, and extinction in F2 resulted in a small sample size. The animals that died throughout the experiment were counted toward mortality but not toward the other endpoints. The animals that died from handling were completely excluded from all the analyses.

RESULTS

Particle Characterization. The size distributions of the pristine and wastewater-incubated MPs are very similar (Figure S1). The mean zeta potentials of the particles incubated in ultrapure water and wastewater are −10.02 ± 0.93 and −10.96 ± 2.09 mV, respectively (n = 10 each, Figure S2). The SEM images of the MPs after lyophilization show no obvious differences in the surface morphology (Figure 1). Likewise, the surface morphology is not altered by incubation in M4 medium, and there is no apparent biofilm formation over the

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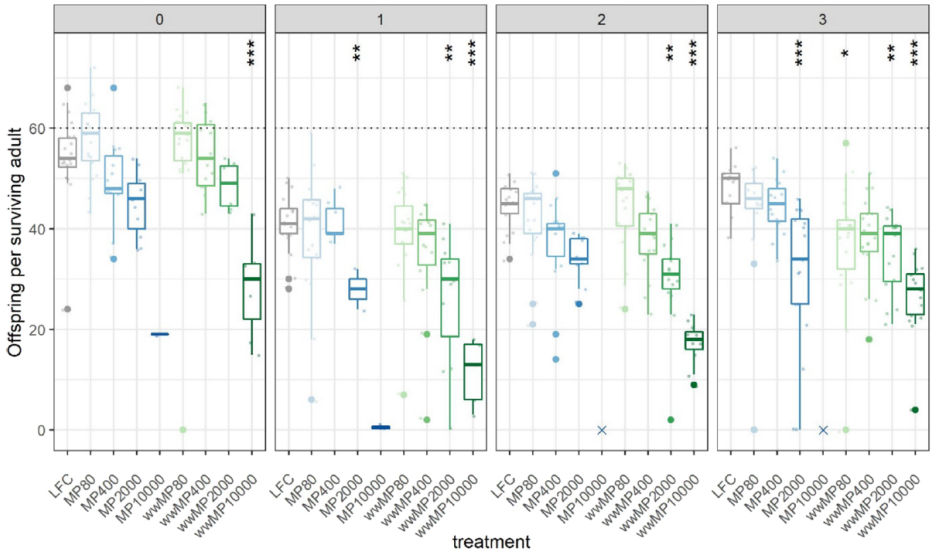

Figure 3. Reproduction of *D. magna* exposed to pristine microplastics (MP) and wastewater-incubated microplastics (wwMP) over the four generations. The animals were held at high food levels (high food control, HFC, Figure S7), low food levels (low food control, LFC), and low food levels in combination with 80, 400, 2000, and 10,000 MP or wwMP mL^{-1}. The MP10000 treatment group went extinct in F2 (crosses). Two-way ANOVA with Bonferroni multiple comparison tests against the corresponding LFC (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$).

maximum time the MP suspensions were used in the experiments (9 and 2 days of suspension on an orbital shaker followed by a 7 day use period, Figure S6). The FTIR spectra are similar for the two MP types (Figures S3 and S4). The particle behavior after the application in the exposure vessels differed between the particle types: soon after mixing of the stock suspensions in M4 medium, the pristine MPs either floated or sedimented to a larger degree than the wastewater-incubated MPs that remained in the water column more consistently (Figure S5).

Survival. The survival of daphnids was affected by the exposure to MPs in concert with food limitation, but not by food limitation alone (Figure 2). The mortality was <20% in the control groups except in the final generation for the LFC. Thus, the experiment was valid according to the OECD guideline.[15] Overall, the survival of daphnids exposed to ≥400 particles mL^{-1} of either particle type was lower than that of the control groups. Interestingly, the survival in these treatment groups increased over the generations, and mortality was lower in the animals exposed to wastewater-incubated particles.

Both MP types had a concentration-dependent impact on survival in F0−F2 but not in F3 (Figure 2, Table S1). The daphnids exposed to 80 particles mL^{-1} of both particle types were unaffected with a maximum mortality of 25% in the fourth generation of the MP80 treatment group ($p > 0.05$). The survival of animals exposed to 400 and 2000 MP mL^{-1} (MP400/MP2000) decreased from F0 to F1 and increased to approximately 80% in the consecutive generations. Only two animals survived in the F1 generation of the MP2000 treatment ($p < 0.001$ compared to F1 of the LFC group), so this can be considered a bottle-neck event. However, these two animals produced sufficient offsprings to start F2. Only 5% of *D. magna* survived when exposed to 10,000 MP mL^{-1} (MP10000, $p < 0.001$) in the first generation. They produced only seven neonates forming F1 followed by the extinction of that treatment group in F2 ($p < 0.05$).

Mortality was less pronounced in animals exposed to wastewater-incubated MPs. Again, an exposure to 80 wwMP mL^{-1} did not affect the survival ($p > 0.05$). In the wwMP400 treatment group, the survival rates were 74% in F0 ($p < 0.05$) and 74% in F1 ($p > 0.05$), followed by two generations in which 95% of the daphnids survived ($p < 0.05$ and $p > 0.05$, respectively). The lowest survival was observed in the first generation of the wwMP2000 treatment group (39%, $p < 0.001$) followed by 55, 79, and 75% (all $p > 0.05$), respectively. In contrast to the treatment with pristine MPs, the daphnids exposed to 10,000 wwMP mL^{-1} survived all the four generations with the lowest survival in F1 (25%, $p < 0.001$) followed by 37% in F0 ($p < 0.001$) and 69 and 85% in F2 and F3 (both $p > 0.05$), respectively.

Reproduction. The limitation of food supply and the exposure to MPs affected the reproduction of *D. magna* (Figure S7, Table S1). The mean numbers of offsprings per surviving adult in the first generation were 155 ± 48.6 and 54.2 ± 8.79 in the HFC and LFC, respectively. Again, the experiment was valid according to the OECD validity criterion for reproduction (>60 neonates per surviving adult in the HFC[15]). The daphnids in the HFC group produced significantly more offsprings over the course of each generation compared to the LFC group ($p < 0.001$, two-way ANOVA with Bonferroni multiple comparison tests, generation: $F(3, 663) = 84$, treatment: $F(8, 663) = 314$, interaction: $F(24, 663) = 12.74$).

The reproductive output of daphnids from all the treatment groups decreased from the first to the second generation (Figure 3). For the HFC, this trend continued for F1 (130.2 ± 19.0 neonates per surviving adult) and F2 (84.3 ± 15.2) followed by a slight increase to 88.3 ± 26.1 neonates per surviving adult in F3. Under food limitation, the mean reproductive output remained below 60 for all other treatments (LFC, MP/wwMP80−10,000). The reproduction was significantly lower compared to the LFC of the

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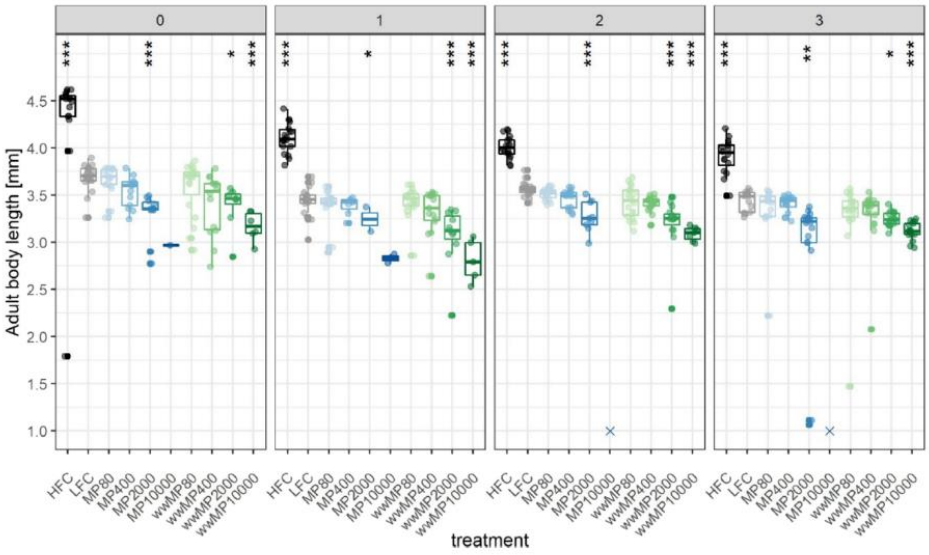

Figure 4. Body length of an adult *D. magna* exposed to pristine microplastics (MP) and wastewater-incubated microplastics (wwMP) over the four generations. The animals were held at high food levels (high food control, HFC), low food levels (low food control, LFC), and low food levels in combination with 80, 400, 2000, and 10,000 MP or wwMP mL^{-1}. The MP10000 treatment group went extinct in F2 (crosses). Two-way ANOVA with Bonferroni multiple comparison tests against the corresponding LFC ($*p < 0.05$, $**p < 0.01$, and $***p < 0.001$).

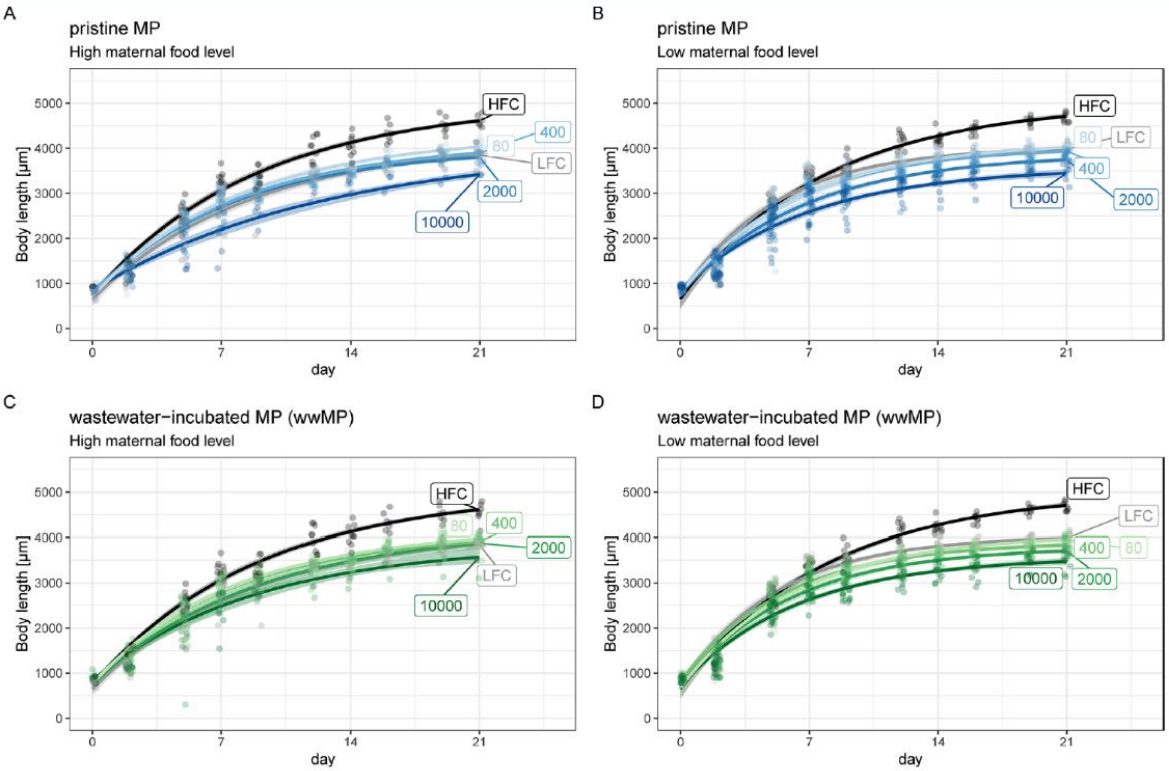

Figure 5. Body length development of daphnids originating from parents fed with high (A + C) or low (B + D) food levels. The offspring was unexposed (black and gray, HFC/LFC) or exposed to polystyrene microplastics (80, 400, 2000, and 10,000 particles mL^{-1}) incubated in wastewater (wwMP, C + D, green) and ultrapure water (MP, A + B, blue) prior to exposure.

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corresponding generation for the daphnids from the treatments MP2000 (F1 and F3: $p < 0.01$), wwMP2000 (F1−F3: $p < 0.01$), and wwMP10000 (F0−F3: $p < 0.001$).

An exposure to MP affected the timing of reproduction in the third and the fourth generations. In comparison to the animals from the LFC from the corresponding generation, the day of the first reproduction (Figures S8 and S9) was significantly delayed in the daphnids exposed to pristine MPs (MP2000, F2 and F3: $p < 0.05$). The MP10000 treatment was excluded from the statistical analysis due to few data points and subsequent extinction. Compared to the LFC, the reproduction was delayed in all treatment groups with wastewater-incubated MPs (wwMP80−10,000, F2 and F3: $p < 0.05$) with the exception of wwMP2000 in the F3 generation.

Body Length. The reduced food supply and MP exposure significantly affected the body length of adults after 21 days (Figure 4, Table S1). The adult daphnids were significantly larger in the HFC than in the corresponding LFC ($p < 0.001$, two-way ANOVA with Bonferroni multiple comparison tests, generation: $F(3, 663) = 36.23$, treatment: $F(8, 663) = 104.4$, interaction: $F(24, 663) = 2.04$). The *D. magna* species exposed to pristine and wastewater-incubated MPs at concentrations of 2000 particles mL^{-1} or higher were significantly smaller compared to the animals from the corresponding LFC ($p < 0.05$).

The body length of the neonates of the fourth brood was also affected by food limitation and an exposure to MP (see detailed results in the Supporting Information, Figure S10 and Table S2).

Growth Curve Experiments. As a follow up, we conducted two experiments to investigate the body length development of individuals held at conditions similar to the multigenerational study with high and low maternal food levels. This is representative for the daphnids in F0 (parents from the high food culture) and F1−F3 of the multigenerational experiment (parents from the low food level F0).

For the animals in the control groups provided with high amounts of food, the food status of the parental generation played no role with regard to the length development (Figure S11). Both growth curves mostly overlap. In the daphnids with low maternal food levels receiving low food levels, we observed a higher initial growth but a similar terminal length after 21 days compared to animals originating from a high food culture. This indicates an immediate effect of maternal food limitation coupled with food limitation on the growth. The daphnids fed with high food levels grew very similarly independent of whether their parents had received high or low food quantities. In contrast, animals fed low food levels grew faster when their parents had been starved compared to the offspring of parents that had received high food levels. After 21 days, the maternal food status did not affect the length of animals from the HFC or LFC.

The animals with high maternal food levels that were exposed to 10,000 particles mL^{-1} pristine MPs had an observable effect on the length development (Figure 5). However, we observed a high mortality in this group that caused the poor data coverage beyond day 10. This effect was less pronounced for the daphnids exposed to the same concentration of wastewater-incubated MPs. The lower concentrations produced no apparent effects on the length development notwithstanding the particle type.

When we exposed individuals originating from a culture with low maternal food levels to MPs, the length development was reduced in a concentration-dependent manner for both particle types starting from 2000 particles mL^{-1}. Similar to the LFC group originating from the low-food culture, we observed a higher initial growth preceding a longer phase of low growth after this initial spurt. Overall, low maternal food status coupled with food limitation of the offspring led to a higher initial growth, while high food availability led to a higher growth irrespective of maternal food status.

DISCUSSION

We compared the multigenerational effects of irregular MPs that were incubated in wastewater or ultrapure water over the four generations of *D. magna* held under food limitation. We found that the exposure to wastewater-incubated MPs resulted in a lower mortality than pristine MPs. The toxicity of the two particle types did not differ significantly for other life history parameters.

Multigenerational Effects. In a previous study, we used a similar design to compare the effects of PS MPs to natural clay particles.[14] We demonstrated that MPs affected the life history of *D. magna* with increasing effects over the generations while kaolin did not. A number of general patterns are consistent across the two studies, most notably the change in median body size from the first to the second generation, which we hypothesized to be due to the changes in the population density. Although the PS MP treatment group in Schür et al. (2020) is very similar to the one with the pristine MP used here, we observed differences between both studies. For example, the daphnids exposed to 10,000 MP mL^{-1} went extinct in F0 in the previous and in F1 in the present study. The animals exposed to 2000 MP particles mL^{-1} did not survive F3,[14] whereas in this study, they recovered from the high mortality in F0 and F1 and survived throughout all the four generations. Overall, the lower toxicity observed in the present study could be due to the removal of the particle fraction <0.2 μm and of chemicals by filtration and the shorter use period of the stock suspensions (maximum of one week) resulting in a lower fragmentation and leaching of chemicals. In the present study, both would reduce the load of nanoplastics and chemicals in the experiment, potentially decreasing the toxicity.

A comparison with two other studies investigating the multigenerational effects of plastic particles in daphnids highlights the impact of food limitation. In *D. magna* exposed to 20 nm PS beads (50 mg L^{-1}, ca. 1.1×10^{13} particles mL^{-1}) in F0 followed by a two-generation recovery, the reproduction but not the growth was negatively affected.[27] In *D. pulex*, the exposure to 71 nm PS beads (1 μg L^{-1}, ca. 5×10^{6} particles mL^{-1}) over three generations induced a higher reproduction and a lower growth in F2 daphnids.[28] While these two studies are not directly comparable to ours (beads vs fragments, nano- vs microplastics, etc.), we observed much stronger multigenerational effects under food limitation. This highlights that an environmentally more realistic scenario in which food is scarce might exacerbate the toxicity of plastic particles.

Toxicity of Aged Microplastics. The question of whether and how aging affects the MP toxicity is the key to better understand their environmental risks.[4] However, only few toxicity studies address this question so far.[29] Here, the aging during wastewater treatment is particularly interesting as wastewater is considered a major point source of MPs.[30] In previous studies, an incubation of plastic particles with dissolved organic matter (e.g., humic acid, wastewater, and

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river water) either reduced their toxicity to microalgae,[31,32] fairy shrimps, and rotifers[33] or did not induce effects different from pristine MPs in duckweeds and zebrafishes.[12,34] This implies that the aging of plastic particles reduces or does not change their toxicity in a range of species.

These findings are similar for daphnids: after the adsorption of humic substances, nanoplastics and MPs had a lower acute toxicity in *D. magna*.[9,11] Weathering polyethylene MPs from a facial scrub in a landfill leachate as well as the spring, river, and wastewater did not increase the acute toxicity in daphnids compared to pristine MPs.[12,34] Recently, Monikh et al.[10] found that dissolved organic matter mitigated the acute toxicity of nanoplastics and silver ions in *D. magna*. Whereas these are all short-term studies, our findings add to this by demonstrating that aged MPs also have a lower effect on the survival in a chronic and multigenerational exposure scenario. While the evidence for a lower toxicity of plastic particles after incubation with dissolved organic matter seems very consistent, this may not be a general pattern. Nasser and Lynch[35] instead observed a higher mortality when they exposed *D. magna* to functionalized nanoplastics coated with an ecocorona from daphnid biomolecules. The conditioned nanoplastics had a longer gut retention time, which can be the reason for the higher toxicity. This indicates that different types of coronae (dissolved organic matter vs biomolecules) can change the toxicity in very different directions during the aging processes.

Importantly, studies with engineered nanomaterials have already addressed similar questions.[36] Two studies with titanium dioxide and silver nanomaterials recently applied a multigenerational design similar to ours and showed that nanomaterials aged in class V lowland water or in model wastewater treatment plants have a lower overall toxicity in daphnids than pristine nanomaterials.[5,6] The consistency with our results points toward a common process by which aging reduces the toxicity of plastic and engineered particles alike. If correct, this opens up opportunities for a read-across approach for comparing the hazards of (aged) synthetic particles.

Overall, most available studies on aging of microplastics and nanomaterials are in accordance with our results. Even though the knowledge on the long-term, multigenerational effects of aged particles is still limited, there is a general trend toward an unaltered or lower toxicity to daphnids after aging alone or in the presence of dissolved organic matter.

Causes for a Lower Toxicity of Wastewater-Incubated Microplastics. When designing this study, we considered three processes that could alter the toxicity of wastewater-incubated compared to pristine MPs: the sorption and consecutive desorption of wastewater-borne chemicals that would increase toxicity as well as the sorption of dissolved organic matter and biofilm formation that would result in lower effects.

The vector hypothesis states that the sorption of chemicals to MPs and their subsequent desorption after ingestion by an animal increases the exposure to these chemicals and thus, exacerbates the toxicity of MPs. Our results do not support this idea as the MPs incubated for 38 h in wastewater were less toxic than pristine MPs with regard to mortality and similarly toxic regarding the other endpoints. Because wastewater contains hundreds to thousands of so-called micropollutants, we did not investigate the ad-, ab-, or desorption of the specific chemicals. However, previous research has shown that polyacrylic beads efficiently remove the toxicity (as a sum parameter for chemicals) from raw wastewater within 2−6 h.[37] While PS fragments and polymer-specific properties will affect sorption,[38] the sorption of a mixture of wastewater-borne compounds to MPs in our study is very probable. Accordingly, the lack of a higher toxicity of wastewater-incubated MPs can be attributed to a low desorption of chemicals (even though we used a clean medium), a low partitioning of chemicals to daphnids, and/or a low toxicity of the desorbed chemicals in daphnids.

Dissolved organic matter, a complex mixture of high molecular-weight compounds such as humic substances, proteins, and free amino acids, is abundant in aquatic ecosystems.[39] It adsorbs to MPs (e.g., Abdurahman et al.[40]) and can form an ecocorona that is thought to change the toxicity of MPs.[41] Based on previous research with PS MPs and humic acids,[42,43] we expected that the adsorption of dissolved organic matter from wastewater to MP would result in a shift of their zeta potentials. Contrary to our expectation, the zeta potentials of the pristine and wastewater-incubated PS MPs were similar. Whereas this may imply that the adsorption of dissolved organic matter from wastewater to MP is negligible, a more recent study using fluorescence spectroscopy demonstrated that humic acid readily adsorbs to PS MPs while the zeta potentials remained unchanged.[11] In a follow up study, incubating MPs with various humic substances did not change the zeta potential either.[9] Accordingly, it is reasonable to assume that dissolved organic matter adsorbed to MPs in our study. This is further supported by the observation that an incubation in wastewater improved the stability of the MP suspension.

The third process that can influence the interaction of MPs with the daphnids is biofilm formation during the incubation and/or the experiment. This could affect the toxicity in two ways: the biofilm could provide additional nutrition relieving the stress of food limitation and/or change the surface structure of the MP that reduces the mechanical damage caused by irregular MPs. The SEM micrographs of the MPs taken after the incubation in wastewater (Figure 1) and throughout the use period in M4 medium (Figure S6) show no evidence of biofilm formation, thus, refuting biofilm formation as a relevant factor.

Accordingly, the differences in behavior and toxicity of the pristine and wastewater-incubated MPs were unrelated to the physicochemical properties we analyzed (particle size, surface charge and structure, and biofilm formation). Based on the better dispersion of wastewater-incubated MPs, we believe that the adsorption of dissolved organic matter may be a key factor modulating the toxicity. As this may not affect zeta potentials, other techniques, such as fluorescence spectroscopy, might be better suited for characterizing dissolved organic matter on MPs.

Interestingly, the more stable dispersion of wastewater-incubated MP results in a higher bioavailability of aged MPs and thus, also a higher exposure of daphnids. While we did not quantify the uptake of both MP types, it is worth noting that this resulted in a lower mortality. In addition, it is interesting to find out why the two particle types differed markedly in their effect on the survival but not the other endpoints. That implies two independent modes of action of the MPs of which the one influencing mortality is buffered by the incubation in wastewater, while the one affecting the other endpoints is not.

https://dx.doi.org/10.1021/acs.est.0c07911
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■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.est.0c07911.

Particle size and calculated mass distributions for the microplastics used in this study (Figure S1); zeta potentials of the microplastics used in this study (Figure S2); FTIR spectra of the raw material before and after grinding (Figure S3); FTIR spectra of the microplastics used in this study (Figure S4); photograph of stock suspensions of microplastics suspended in M4 medium that were previously incubated in ultrapure water and wastewater (Figure S5); scanning electron microscopy images of microplastics incubated in ultrapure water and wastewater after suspension in M4 medium for 0, 2, 7, and 9 days (Figure S6); extension of Figure 3 showing the reproduction of *D. magna* control animals at two different food levels over four generations (Figure S7); timing of the first reproduction of the daphnids exposed to microplastics over four generation (Figure S8); reproductive frequency of the daphnids exposed to microplastics over four generations (Figure S9); body lengths of neonates of the fourth brood of *D. magna* exposed to microplastics over four generations (Figure S10); growth curves for daphnids in the two growth experiments over 21 days originating from cultures held at either high or low food levels without added particles (Figure S11); data for the endpoints survival, reproduction, and size of an adult *D. magna* exposed to microplastics over four generations (Table S1); neonate body length and sample sizes of the fourth brood of *D. magna* exposed to microplastics over four generations (Table S2); and R-code used to produce the predictions underlying the growth curves (PDF)

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Author Contributions

Conceptualization, methodology, investigation, formal analysis, visualization, and writing of the original draft including review and editing were done by C.S.; investigation and writing (review and editing) were done by C.W., M.B., J.W., and M.S.; funding acquisition, project administration, supervision, and writing (review and editing) were done by J.O.; and funding acquisition, conceptualization, methodology, writing of original draft, and writing of review and editing were done by M.W.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This study was supported by the German Federal Ministry for Education and Research (02WRS1378I and 03F0789D). We would like to thank Niklas Döring (Goethe University Frankfurt) for the assistance with SEM imaging, Kristina Klein (Goethe University Frankfurt) for the assistance with FTIR measurements, and Oliver Clemens and Stephan Wollstadt (TU Darmstadt) for the zeta potential analyses. The graphical abstract was created with BioRender.

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https://dx.doi.org/10.1021/acs.est.0c07911
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Supplementary Material

Supporting Information

Incubation in wastewater reduces the multigenerational effects of microplastics in *Daphnia magna*

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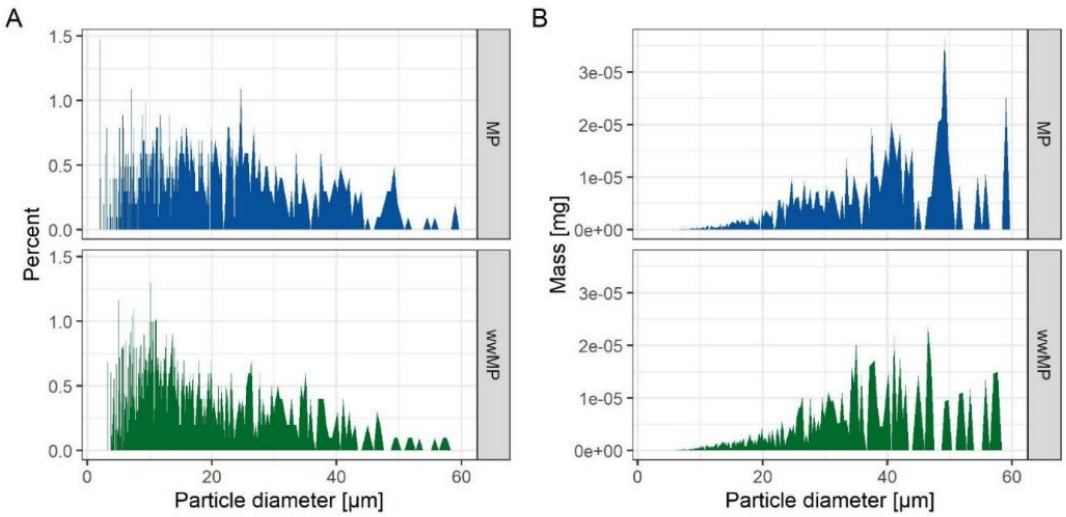
Summary: 16 pages, 11 figures, 2 tables

17 **Particle characterization**

18 **Size and mass distribution**



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20 **Figure S1: Particle size and mass distributions for the pristine (blue, top) and**

21 **wastewater-incubated microplastics (green, below) used in this study**. Particle size

22 distributions (A) were measured using a Multisizer 3 (Beckman Coulter). Each distribution is

23 the average of four to five independent replicates each. The distribution in combination with

24 assumed particle densities of 1.05 g cm^{-3} and sperical shape were used to calculate theoretical

25 mass distributions (B).

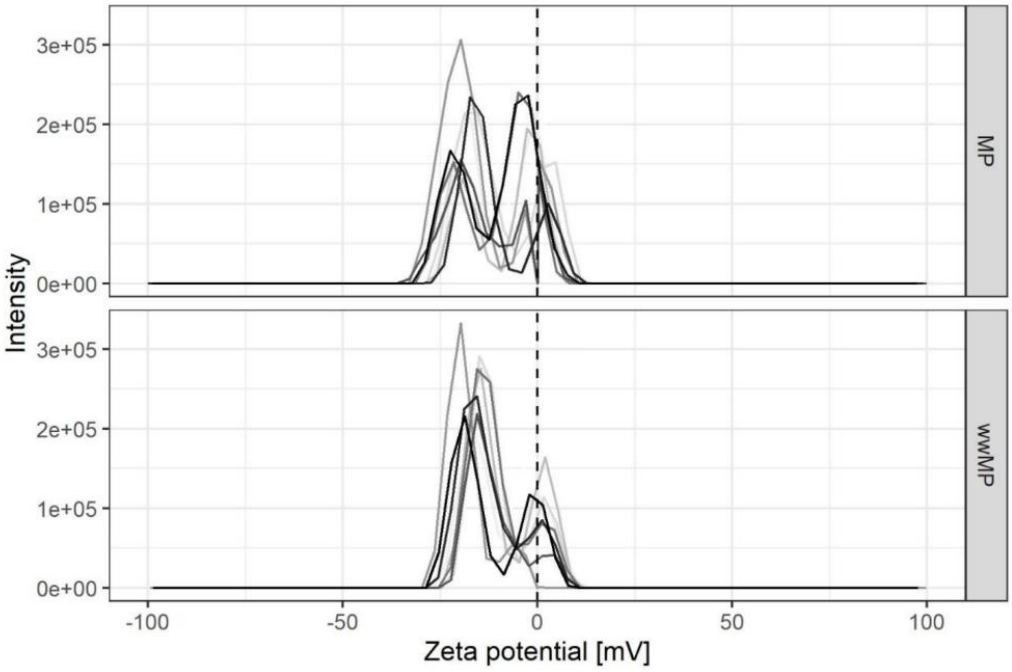


Figure S2: Zeta potential of pristine (MP, top) and wastewater-incubated microplastics (wwMP, bottom, n = 10).

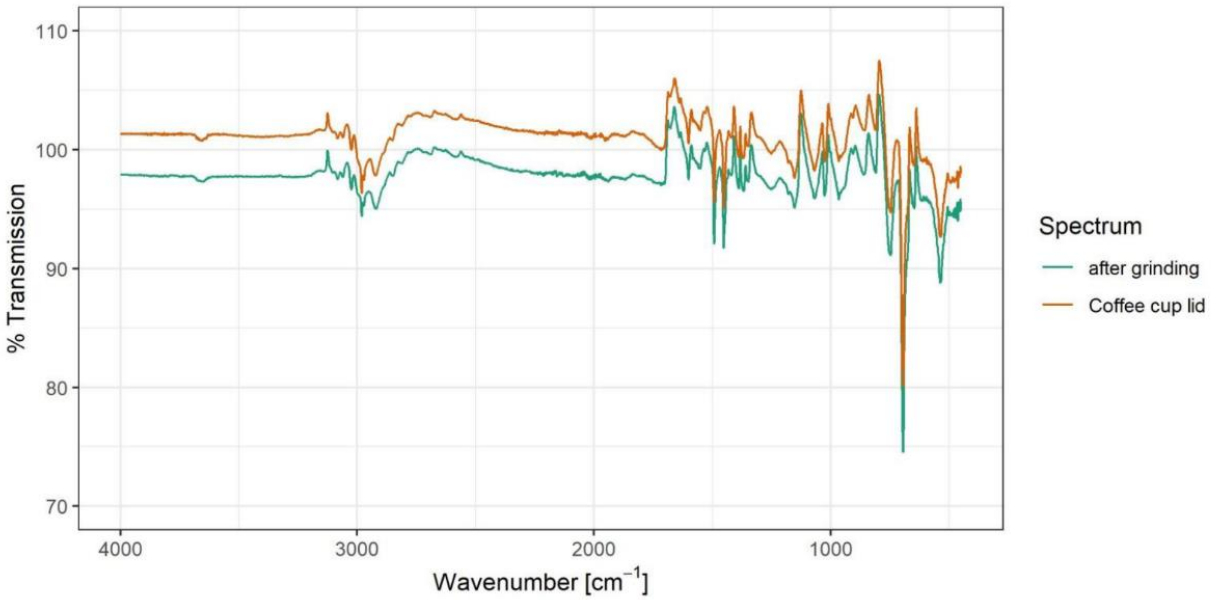


Figure S3: Fourier-transform infrared spectroscopy (FTIR) spectra of the coffee-to-go-cup lids that were the raw material for the microplastics in this study before and after grinding.

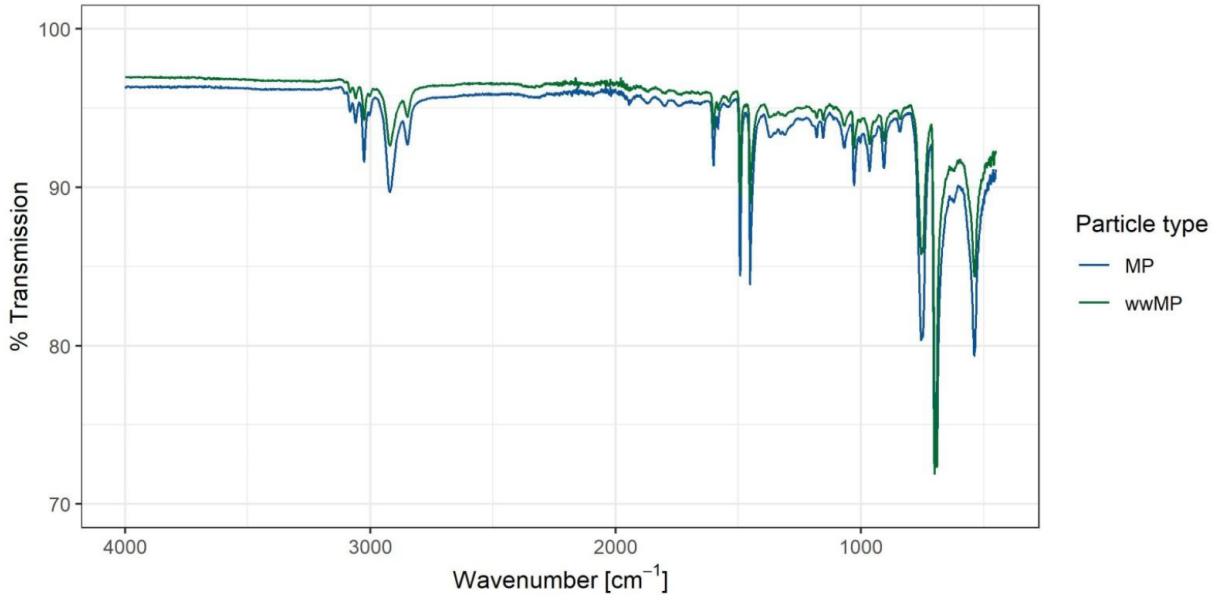


Figure S4: Fourier-transform infrared spectroscopy (FTIR) spectra of the pristine (MP) and wastewater-incubated microplastics (wwMP).

Particle surface characterization

After incubation in ultrapure water (MP) and filtered raw wastewater effluent (wwMP) the two particle batches were recovered through filtration followed by lyophilization. To create stock suspensions for the daphnids experiments, these two particle types were suspended in M4 medium. Behavior of the two types of MP differed markedly with the pristine MP microplastics were more stably dispersed and remained in the water column more consistently (Figure S5).

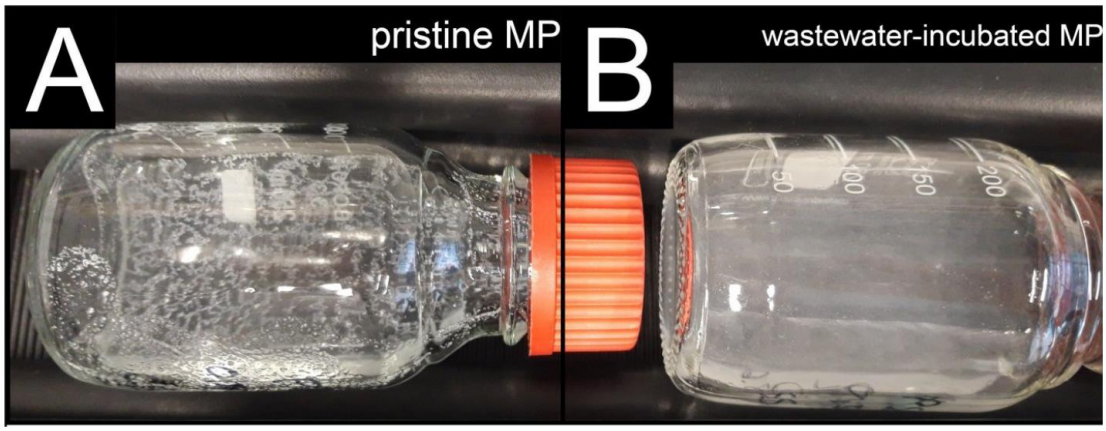
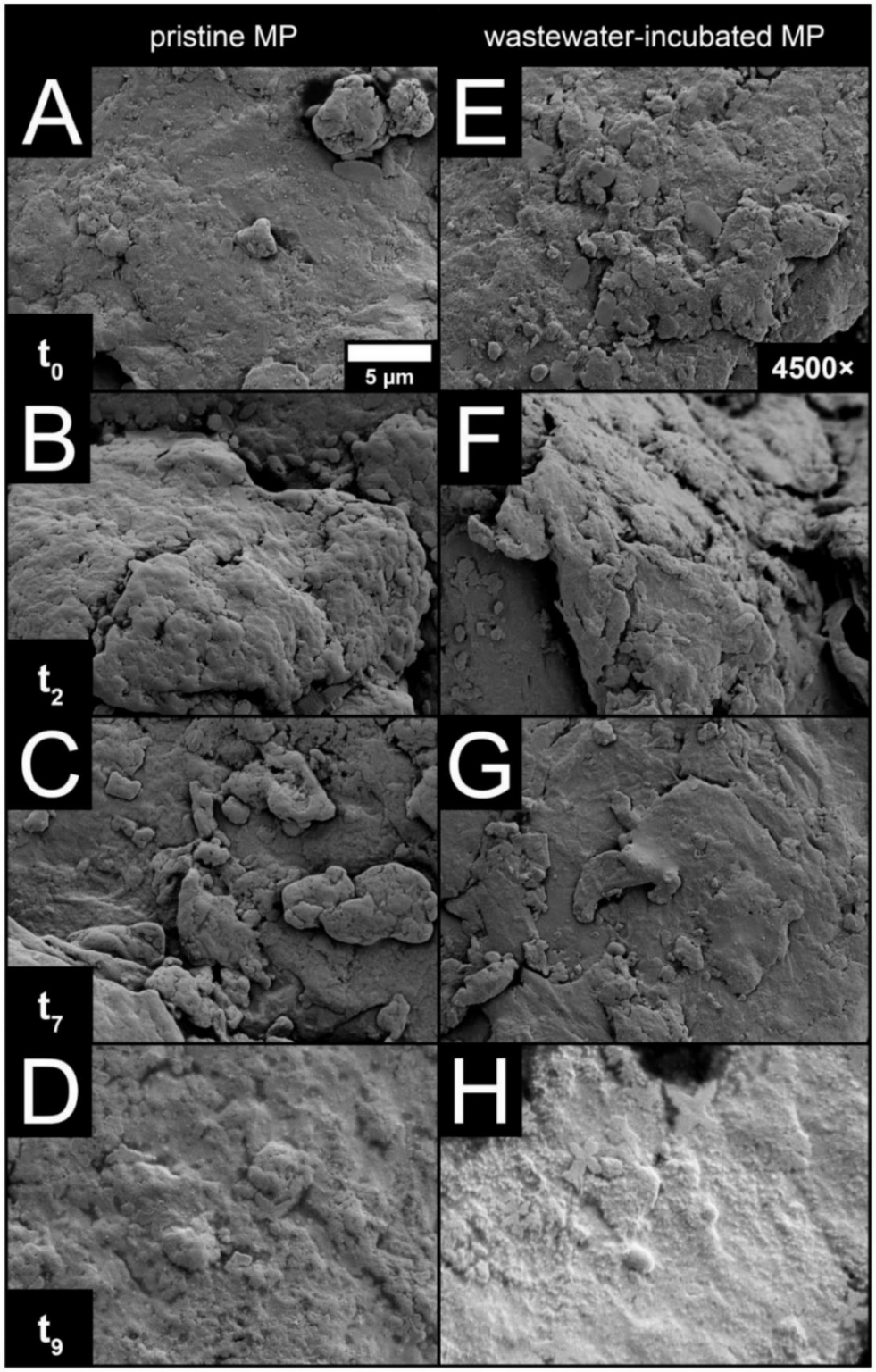


Figure S5: Stock suspensions of polystyrene MP particles in M4 medium that were previously incubated in ultrapure water (A, MP) and wastewater (B, wwMP) after 48 h on an orbital shaker. The photograph was taken immediately after stopping the shaker.

To investigate if this difference in behavior was related to changes in surface structure or biofilm formation, potentially as a result of sorbed dissolved organic matter, we incubated both types of MP identically to the particles used in the toxicity experiments and imaged them using scanning electron microscopy (SEM) immediately after suspension in M4 medium (Figure S6, A+E) and after 48 h of incubation under continuous shaking at room temperature (Figure S6, B+F). Henceforth, the suspensions were stored at 4 °C and imaged after another 5 (Figure S6, C+G) and 7 d (Figure S6, D+H), the latter being the maximum use duration of each stock suspension during the multigenerational experiment.



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60 **Figure S6: Scanning electron microscopy images of microplastics incubated in ultrapure**

61 **water (A-D) and wastewater (E-H) after suspension in M4 medium for 0, 2, 7, and 9 d.**

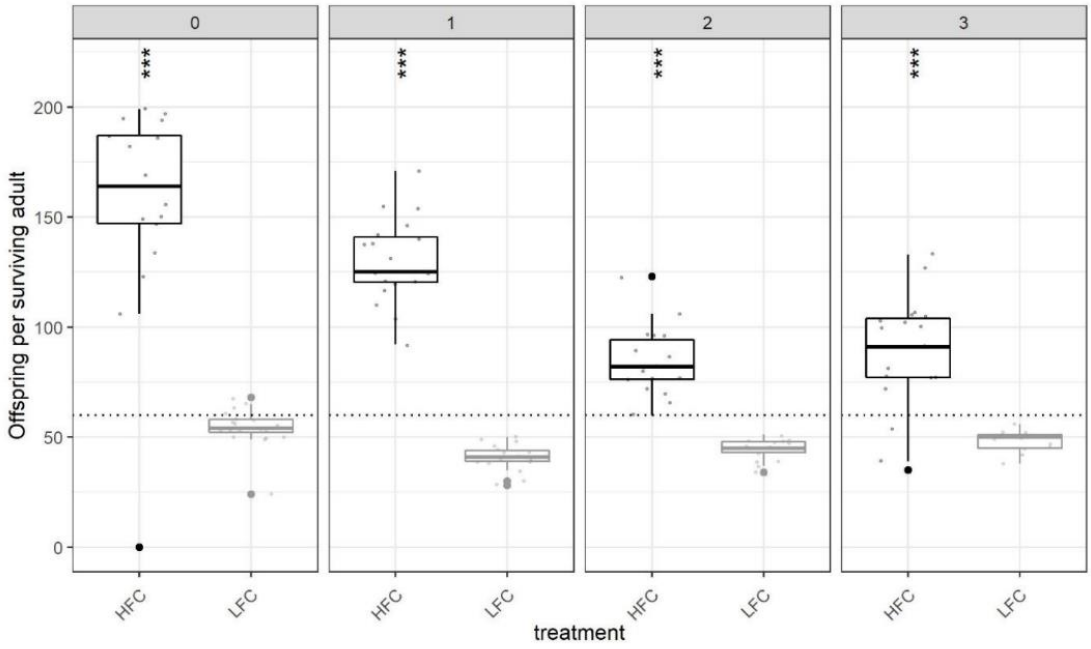
62 **Table S1: Survival, reproduction, and size of *D. magna* exposed to pristine (MP) and**
63 **wastewater-incubated microplastics (wwMP) over four generations.** The animals were
64 held at high food levels (high food control, HFC), low food levels (low food control, LFC),
65 and low food levels in combination with 80, 400, 2000, and 10000 MP or wwMP mL^{-1}.

Treatment and generation	N	Survival after 21 d [%]	Day of first repro-duction*	Offspring per surviving adult*	Size per surviving adult [µm]**
HFC F0	20	85	9.7 ± 0.79	155.2 ± 48.6	4518 ± 670
HFC F1	20	95	9 ± 0	130.2 ± 19.0	4089 ± 155
HFC F2	18	100	10.5 ± 1.2	84.3 ± 15.2	4001.5 ± 117
HFC F3	19	100	10.7 ± 0.73	88.3 ± 26.1	3947 ± 185
LFC F0	20	100	9.9 ± 0.64	54.2 ± 8.79	3700 ± 140
LFC F1	20	85	10.4 ± 0.7	40.8 ± 6.04	3451 ± 158
LFC F2	20	85	10.4 ± 0.8	44.4 ± 4.61	3559 ± 81
LFC F3	19	68	10.6 ± 0.51	48.2 ± 4.76	3485 ± 108
MP80 F0	20	95	9.6 ± 0.6	57.8 ± 7.39	3698 ± 153
MP80 F1	19	95	10.2 ± 0.65	38.5 ± 12.45	3425.5 ± 181
MP80 F2	19	100	10.9 ± 0.88	42.3 ± 8.1	3521 ± 61
MP80 F3	20	75	10.9 ± 0.47	42.9 ± 12.96	3432 ± 325
MP400 F0	20	75	9.4 ± 0.51	48.8 ± 9.01	3600 ± 162
MP400 F1	19	37	9.9 ± 0.69	41.4 ± 3.99	3423 ± 99
MP400 F2	18	78	11 ± 0.55	37.3 ± 10.05	3492 ± 102
MP400 F3	19	79	10.8 ± 0.41	44.6 ± 5.45	3438.5 ± 92
MP2000 F0	20	55	9.9 ± 0.3	44.8 ± 6.34	3351 ± 234
MP2000 F1	20	10	11 ± 0	28 ± 5.66	3243 ± 185

Treatment and generation	N	Survival after 21 d [%]	Day of first repro-duction*	Offspring per surviving adult*	Size per surviving adult [µm]**
MP2000 F2	20	45	11.9 ± 1.05	34 ± 4.9	3252 ± 164
MP2000 F3	20	85	11.4 ± 1.12	30.5 ± 14.91	3218 ± 708
MP10000 F0	19	5	10 ± 0	19 ± 0	2964 ± 0
MP10000 F1	7	29	16 ± 0	0.5 ± 0.71	2826.5 ± 68
MP10000 F2	-	-	-	-	-
MP10000 F3	-	-	-	-	-
wwMP80 F0	19	100	9.7 ± 0.46	55.4 ± 14.16	3703 ± 283
wwMP80 F1	20	95	10.6 ± 1.26	39.1 ± 9.87	3465 ± 163
wwMP80 F2	20	95	11.8 ± 1.13	44.3 ± 8.47	3440 ± 167
wwMP80 F3	20	90	11.6 ± 1.46	37 ± 12.61	3360.5 ± 465
wwMP400 F0	19	74	9.7 ± 0.47	54.6 ± 6.94	3535.5 ± 328
wwMP400 F1	19	74	10.8 ± 2.39	34.8 ± 11.86	3362 ± 240
wwMP400 F2	20	95	11.7 ± 1.1	37.9 ± 6.7	3435 ± 78
wwMP400 F3	20	95	11.4 ± 1.26	38.3 ± 8.19	3390 ± 320
wwMP2000 F0	18	39	9.9 ± 0.38	48.6 ± 4.58	3459.5 ± 237
wwMP2000 F2	19	79	12.4 ± 1.3	29.8 ± 8.97	3252 ± 273
wwMP2000 F3	20	75	11.1 ± 0.64	35 ± 7.86	3240 ± 79
wwMP10000 F0	19	37	10.1 ± 1.35	28.3 ± 9.74	3168 ± 157
wwMP10000 F1	20	25	10.6 ± 0.89	11.4 ± 6.66	2791 ± 224
wwMP10000 F2	16	69	13.8 ± 1.94	17.3 ± 4.27	3099 ± 74
wwMP10000 F3	20	85	11.6 ± 1.37	26.6 ± 7.33	3117 ± 91

66 * mean ± standard deviation, ** median ± SD

67 **Reproduction**



68

69 **Figure S7: Reproduction of *D. magna* control animals over four generations.** The animals
70 were held at high food levels (high food control, HFC) and low food levels (low food control,
71 LFC). Two-way ANOVA with Bonferroni multiple comparison tests against the
72 corresponding LFC (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

73

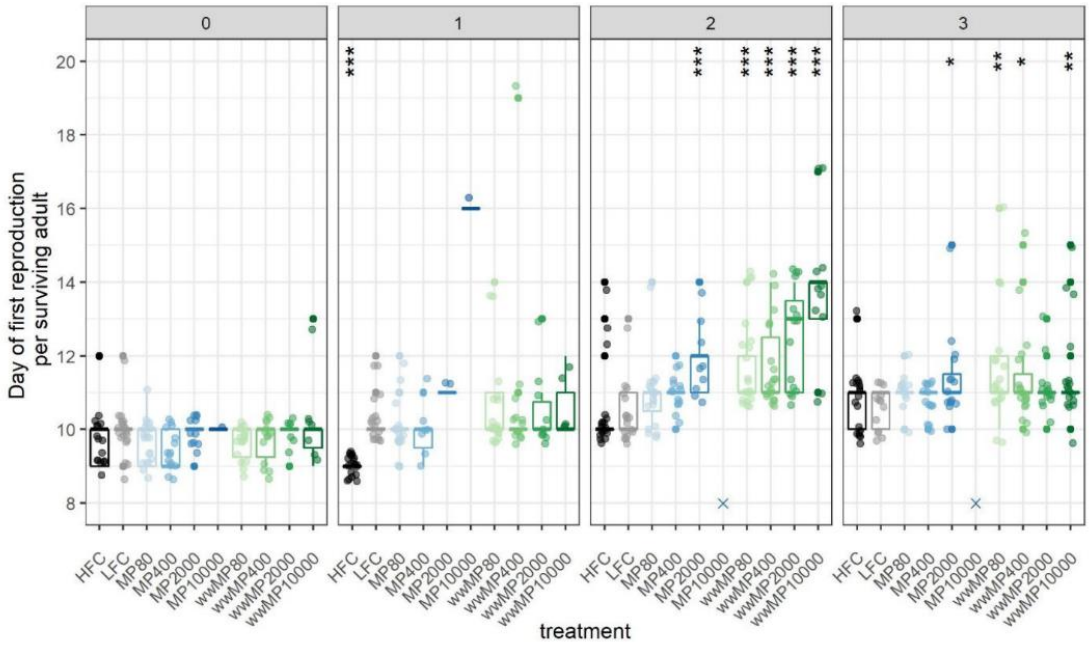


Figure S8: Timing of first reproduction of *D. magna* exposed to pristine (MP) and wastewater-incubated microplastics (wwMP) over four generations. The animals were held at high food levels (high food control, HFC), low food levels (low food control, LFC), and low food levels in combination with 80, 400, 2000, and 10000 MP or wwMP mL^{-1}. The MP10000 treatment group went extinct in F2 (crosses). Two-way ANOVA with Bonferroni multiple comparisons test against the corresponding LFC (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

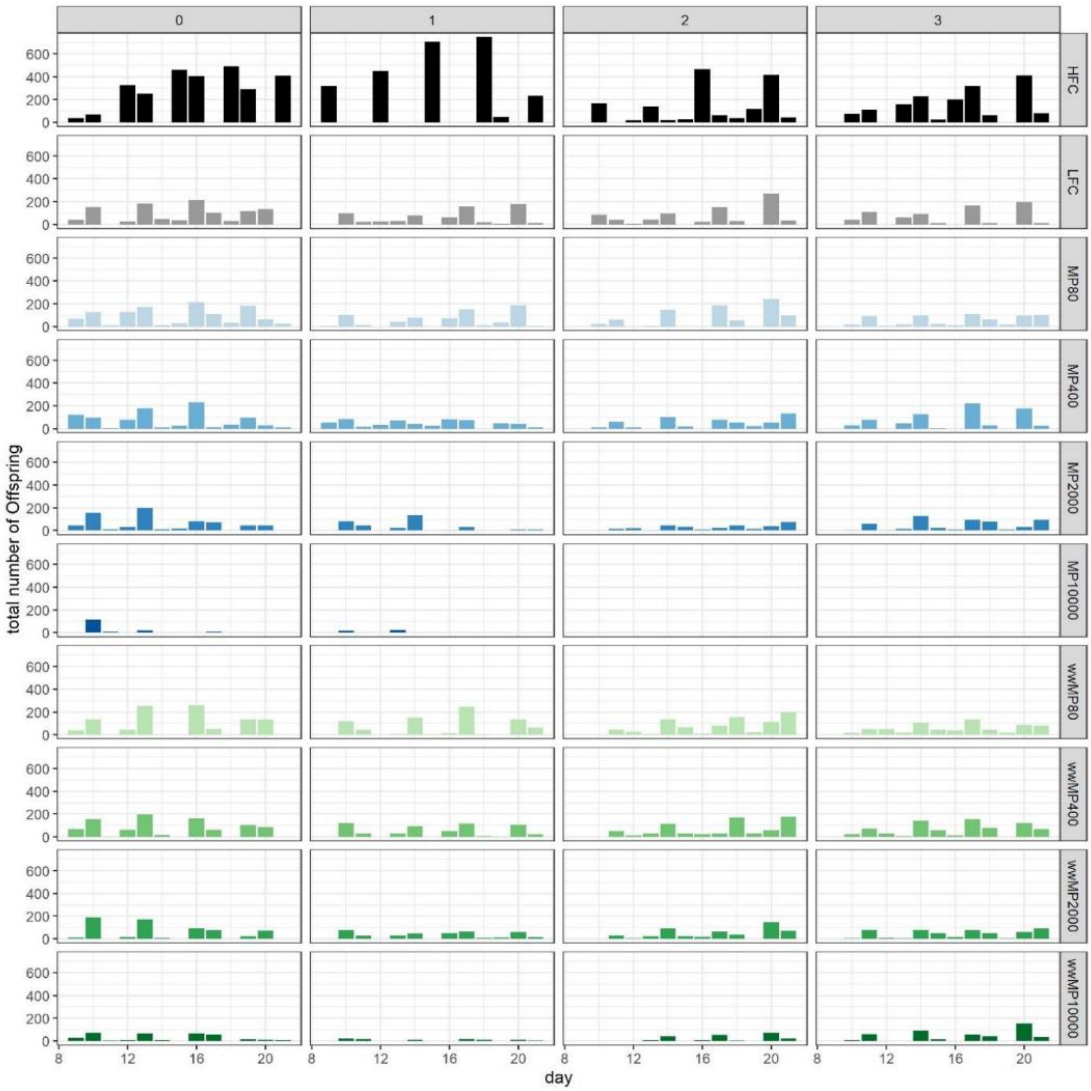


Figure S9: Reproductive frequency of *D. magna* exposed to pristine (MP) and wastewater-incubated microplastics (wwMP) over four generations. The animals were held at high food levels (high food control, HFC), low food levels (low food control, LFC), and low food levels in combination with 80, 400, 2000, and 10000 MP or wwMP mL^{-1}. The MP10000 treatment group went extinct in F2.

Neonate size

The body length of 4th brood neonates was significantly affected by the food supply as well as exposure to MP (Figure S10, Table S2). Neonates fed with high food levels (HFC) were significantly small than animals from the LFC in F0 ($p<0.001$) and F1 ($p<0.01$). Over the generations, neonate size decreased in the LFC but not the HFC. Neonates exposed to MP mostly had a lower median size in the second compared to the first generation followed by no significant change from the second to the third generation. For the last generation, we then often observe a slight increase in body length. Significant differences compared to the LFC group are mostly observed in the first and/or last generation, likely due to the relatively large changes the neonate size in the LFC group covers between the first and last generation. Generally, no clear pattern related to MP exposure and the particle type could be observed.

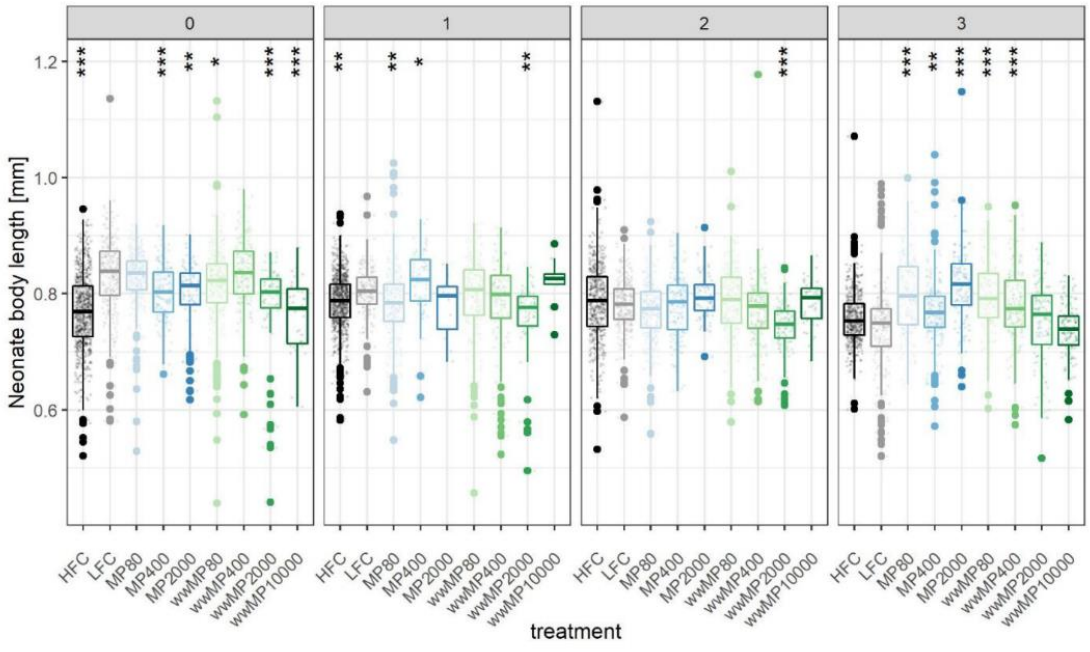


Figure S10: Body length of neonates of the 4th brood of *D. magna* exposed to pristine (MP) and wastewater-incubated microplastics (wwMP) over four generations. The parent animals were held under high food levels (high food control, HFC), low food levels (low food

105 control, LFC), and low food levels in combination with different concentrations of
106 polystyrene microplastics (80, 400, 2000, and 10000 particles mL^{-1}). The MP10000 did not
107 produce offspring in the 4^{th} brood and is therefore excluded from the graph. Two-way
108 ANOVA with Bonferroni multiple comparisons test against the corresponding LFC treatment
109 group (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). Sample sizes are available in Table S2.

110

111 **Table S2: Neonate body length of the 4th brood of *D. magna* exposed to pristine (MP)**
112 **and wastewater-incubated microplastics (wwMP) over four generations.** Parents were
113 held at high food levels (high food control, HFC), low food levels (low food control, LFC),
114 and low food levels in combination with different concentrations of polystyrene microplastics
115 (80, 400, 2000, and 10000 particles mL^{-1}).

Treatment and generation	N	Median body length ± standard deviation[mm]	Level of significance[a]
HFC F0	748	0.769 ± 0.064	< 0.001
HFC F1	779	0.788 ± 0.047	< 0.01
HFC F2	480	0.788 ± 0.065	> 0.05
HFC F3	463	0.753 ± 0.045	> 0.05
LFC F0	256	0.839 ± 0.069	-
LFC F1	193	0.804 ± 0.042	-
LFC F2	259	0.782 ± 0.046	-
LFC F3	193	0.749 ± 0.079	-
MP80 F0	289	0.835 ± 0.049	> 0.05
MP80 F1	230	0.785 ± 0.068	< 0.01
MP80 F2	290	0.774 ± 0.05	> 0.05
MP80 F3	176	0.797 ± 0.065	< 0.001
MP400 F0	160	0.803 ± 0.046	< 0.001
MP400 F1	76	0.825 ± 0.053	< 0.05
MP400 F2	186	0.786 ± 0.054	> 0.05
MP400 F3	210	0.768 ± 0.06	< 0.01
MP2000 F0	89	0.814 ± 0.064	< 0.01
MP2000 F1	13	0.796 ± 0.052	> 0.05

Treatment and generation	N	Median body length ± standard deviation[mm]	Level of significance[a]
MP2000 F2	72	0.793 ± 0.038	> 0.05
MP2000 F3	119	0.816 ± 0.067	< 0.001
wwMP80 F0	257	0.823 ± 0.072	< 0.05
wwMP80 F1	179	0.807 ± 0.071	> 0.05
wwMP80 F2	226	0.79 ± 0.059	> 0.05
wwMP80 F3	136	0.792 ± 0.06	< 0.001
wwMP400 F0	183	0.836 ± 0.06	> 0.05
wwMP400 F1	123	0.799 ± 0.079	> 0.05
wwMP400 F2	191	0.779 ± 0.058	> 0.05
wwMP400 F3	175	0.774 ± 0.066	< 0.001
wwMP2000 F0	90	0.803 ± 0.085	< 0.001
wwMP2000 F1	74	0.777 ± 0.063	< 0.01
wwMP2000 F2	114	0.748 ± 0.042	< 0.001
wwMP2000 F3	146	0.765 ± 0.072	> 0.05
wwMP10000 F0	34	0.775 ± 0.063	< 0.001
wwMP10000 F1	9	0.826 ± 0.046	> 0.05
wwMP10000 F2	45	0.793 ± 0.038	> 0.05
wwMP10000 F3	142	0.739 ± 0.045	> 0.05

116 [a] Two-way ANOVA with Bonferroni multiple comparisons test against the LFC group.

Growth experiments

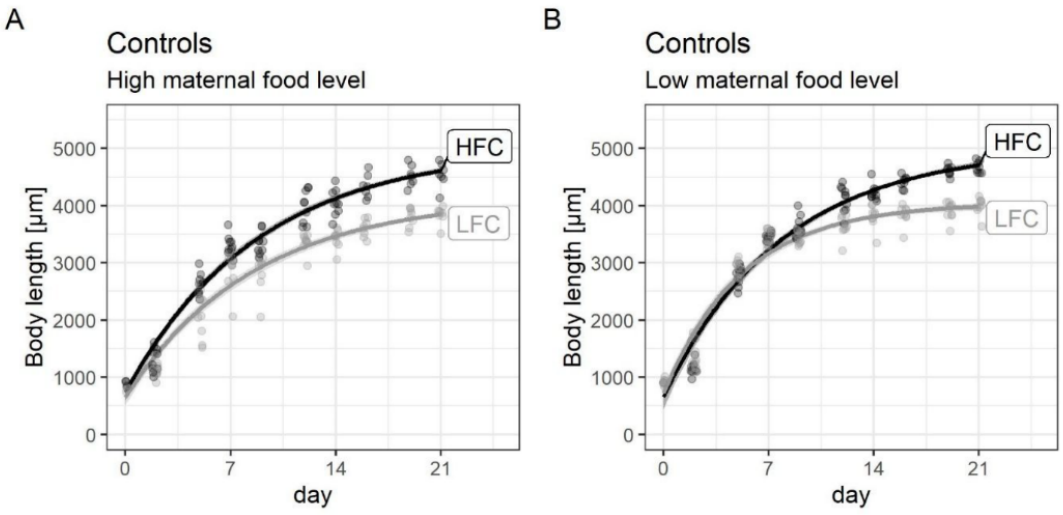


Figure S11: Growth curves for daphnids in the two growth experiments over 21 days originating from cultures held at either high (A) or low (B) food levels.

Von Bertalanffy Growth Function

The growth functions were calculated in R with RStudio and the FSA package (Ogle et al. 2019).

$$E[L \mid t] = L_{\infty}\left(1 - e^{-K(t - t_0)}\right)$$

Where, according to Ogle et al. (2019),

- $E[L \mid t] =$ expected/average length at time/age t,
- L_{∞} = asymptotic mean length,
- K = exponential rate of approach to L_{∞},

- t_0 = the theoretical age when length = 0 (a modeling artifact).

To ensure comparability we kept the range of days to calculate the initial predictions at -1 and 30 with a step size of 0.1.

R-code used to produce the predictions:

```
library(tidyverse)
library(FishR)

vb <- vbFuns(param="Typical")
f.starts <- vbStarts(length~day,data=data_filtered)
f.fit <- nls(length~vb(day,Linf,K,t0),data=data_filtered,start=f.starts)
f.boot1 <- Boot(f.fit)
days <- seq(-1,30,by=0.1)
predict2 <- function(x) predict(x,data.frame(day=days))
f.boot2 <- Boot(f.fit,f=predict2)
preds1 <- data.frame(days,
          predict(f.fit,data.frame(day=days)),
          confint(f.boot2),
          treatment=trinput)
names(preds1) <- c("day","fit","LCI","UCI","treatment")
preds2 <- filter(preds1,day>=daysum$minday,day<=daysum$maxday)
```

The output of the predictions (preds1, predictions not reduced to the actually observed day-range but between day -1 and 30) is available at figshare under doi 10.6084/m9.figshare.12311495.

Annex 4: Additional results

Title: Effects of microplastics and kieselguhr mixtures on population development in *Daphnia magna*

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These findings are not published elsewhere.

Aims

In the environment, microplastics are never the only non-food particles organisms interact with. Therefore it is not reasonable to assess their impacts in the sterile environment that is an experimental setup devoid of other particles. Likewise, daphnids are rarely alone but rather, as r-strategists, form large, often short-lived populations. Population growth rates are high, but quickly reach a carrying capacity limited by space and/or food. Accordingly, we performed a population experiment with *Daphnia magna* in which populations with a defined age structure and size were continuously exposed to mixtures of microplastics and the natural particle kieselguhr under constant food levels. The aim of this study was to compare the effects of microplastics to natural particles and their mixtures on the population level.

Materials & Methods

The initial daphnid populations consisted of 3 adults (2 weeks old), 5 juveniles (1 week old), and 8 neonates (< 72 h old) held in 1 L glass vessels containing 900 mL Elendt M4 medium (OECD 2012). Each population was kept for 50 days and fed a constant ration of 0.5 mgC d^{-1} of the green algae *Desmodesmus subspicatus*. Treatment groups were exposed to a total of 50,000 particles mL^{-1} of varying ratios of irregularly shaped polystyrene microplastics and kieselguhr (n = 3; Table 1, Figure 1)

Table 1: Ratios and absolute particle concentrations of microplastics and kieselguhr in the treatment groups of the population experiment.

Treatment	**Microplastics**		**Kieselguhr**	
	%	*Particles mL^{-1}*	*%*	*Particles mL^{-1}*
Control	0	0	0	0
MP100	100	50,000	0	0
MP80	80	40,000	20	10,000
MP60	60	30,000	40	20,000
MP50	50	25,000	50	25,000
MP40	40	20,000	60	30,000
MP20	20	10,000	80	40,000
MP0	0	0	100	50,000

Populations were feed thrice weekly and medium was exchanged approximately weekly on days 7, 14, 21, 28, 37, 42, and 50. On each medium exchange, populations were sieved, transferred to an hourglass, and photographed. ImageJ (Schneider et al. 2012) was used to then quantify living animals and the number of resting eggs (Figure 2). Individual body lengths were measured from the center of the eye to the base of the apical spinus (Ogonowski et al. 2016).

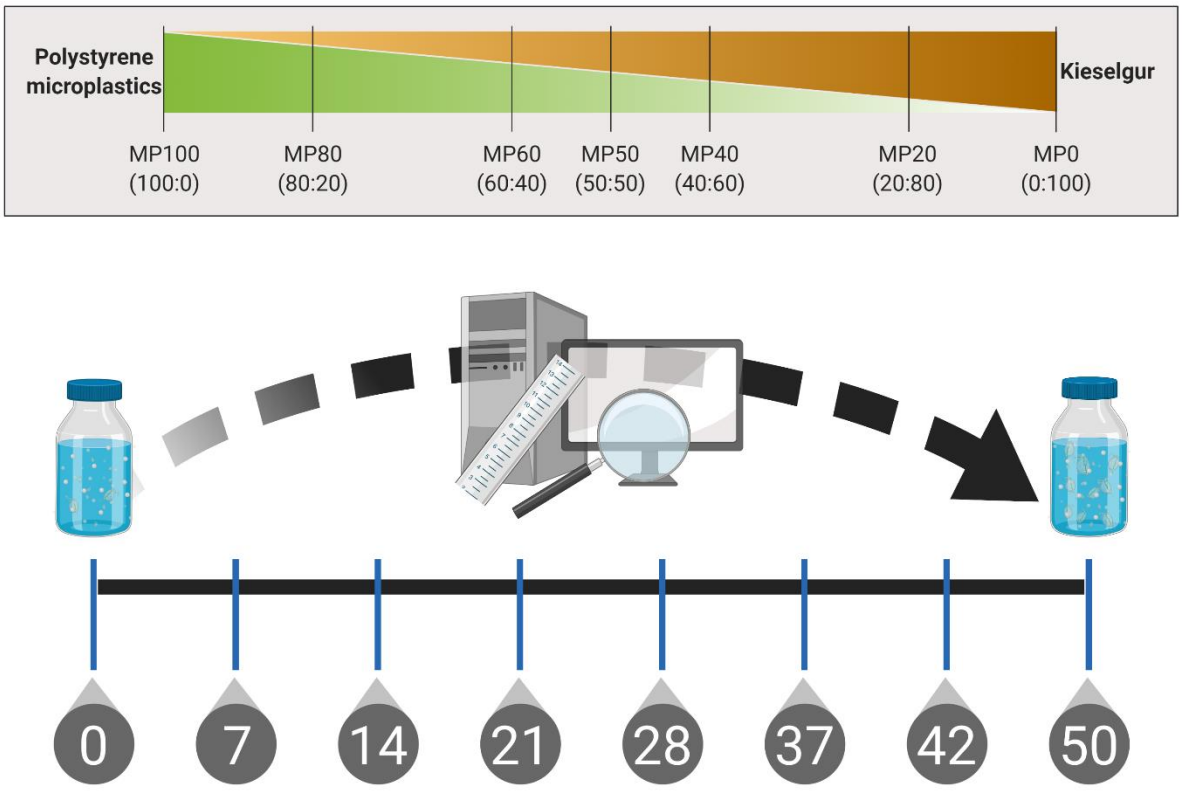

Figure 1: Experimental setup for the population experiment with *D. magna*. Defined initial populations were exposed to different ratios of kieselguhr and irregular microplastics under food limitation.

Results & Discussion

Overall, the experiment included three main endpoints: absolute population size, demography, and the number of resting eggs (*ephippiae*) per population. Resting eggs can be seen as a stress indicator to both high population density and low food levels (Smirnov 2017). All populations, both exposed to particles and of the control group, showed rapid initial growth (Figure 2), likely because the available food was sufficient to sustain the small populations in the beginning. Population sizes peaked after 14 days and reached their lowest recorded size on day 50. We observed a concentration-dependent effect in the particle treatments with the MP100 treatment population sizes (100 % microplastics, 0 % natural particles) being close to the control populations and population sizes decreasing with increasing ratios of kieselguhr in the mixture. Resting egg formation occurred in all populations after day 14 but to varying degrees. Here, we observed a similar but small concentration-dependent trend: control and MP100 populations have the lowest number of resting eggs while animals in the MP40-MP0 treatments produced around 100 *ephippiae*.

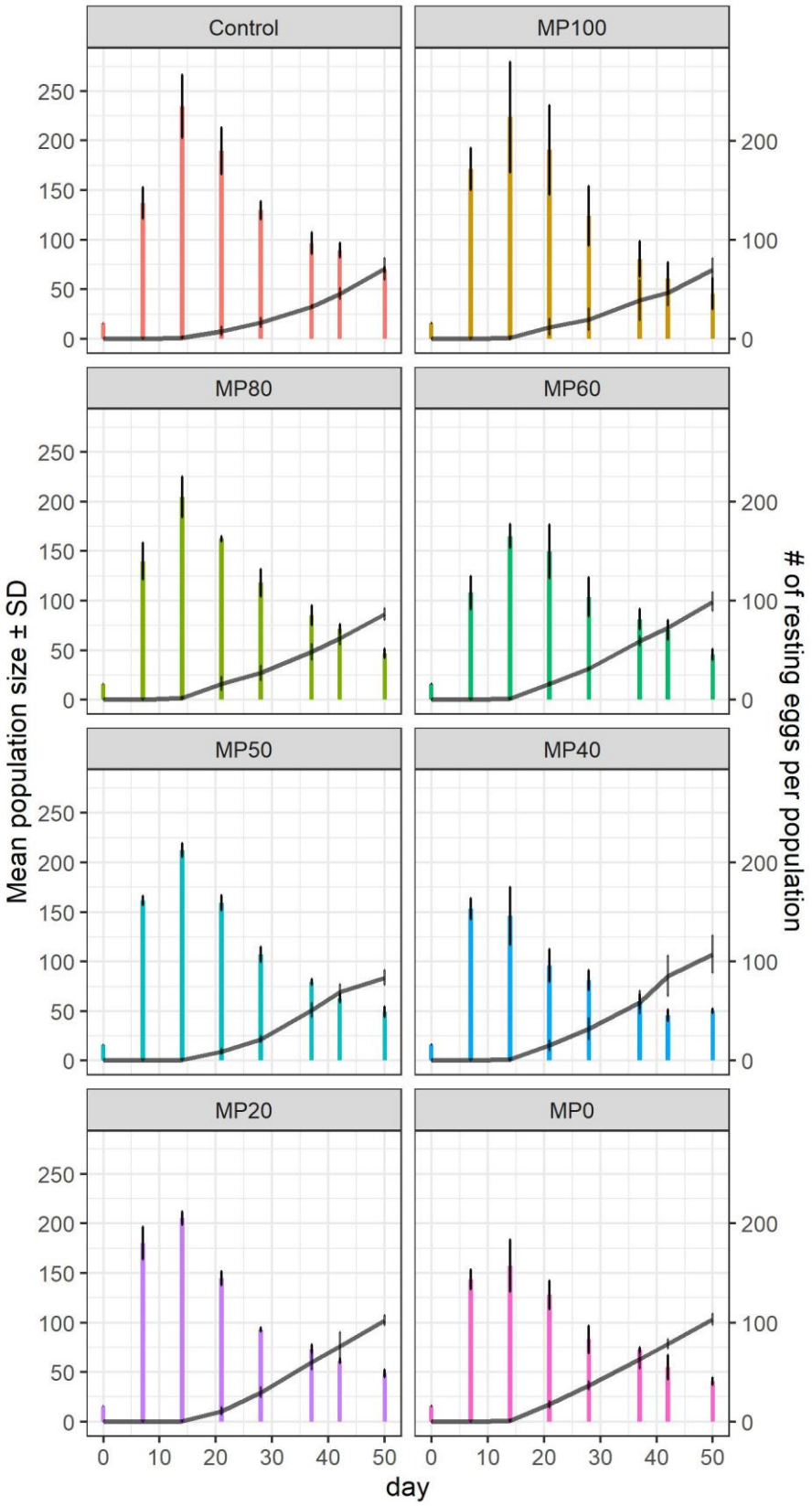

Figure 2: Population sizes and *ephippiae* occurrence in *D. magna* populations exposed to mixtures of polystyrene MP and kieselguhr.

Animals that comprised initial populations came from a culture that was fed *ad libitum* and therefore potentially possess a certain energetic reserve protecting them against starvation (Tessier et al. 1983). Even though this observation is consistent with the theory of dynamic energy budget theory (see General Discussion), evidence by Gliwicz and Guisande (1992) exists that contradicts this. They found that the reproductive strategies of clones reared at high and low food levels differed. Clones under high food levels produced more but smaller offspring that were not resistant against starvation. Mothers held at low food levels produced smaller clutches of larger offspring that were more resistant against starvation. Here, the differences are likely related to a lack of normalization to body size in the latter study. Peak population sizes at day 14 likely mark the moment where the maternal reserve inherited from the culture fed *ad libitum* is depleted while the population reaches a density limit for the volume available in the vessel. The latter is supported by the observation that both the control populations and the MP100 populations reach similar peak population sizes, while their terminal sizes differ. We can deduce from the data that the polystyrene MP have a limited effect on population growth and resting egg formation, while kieselguhr has a clear concentration-dependent effect.

The body length of each individual in a population was measured weekly and used to describe body size distributions among populations (Figure 3). Body lengths were categorized into four size classes (Class 1: ≤ 1599 µm; Class 2: 1600–2699 µm; Class 3: 2700–3799 µm; Class 4: > 3800 µm) roughly relating to the developmental stages neonate, juvenile, adult, and large adult. The initial population growth is driven by the production of neonates (Class 1). This dominance of small animals persists longer in control populations and populations exposed to higher ratios of MP compared to kieselguhr before “graduating” to adulthood. This largely relates to the growth duration of daphnids born in the initial population growth spurt before reaching holding capacity/food limitation. Overall, effects on population composition are minor and consistent with the other endpoints.

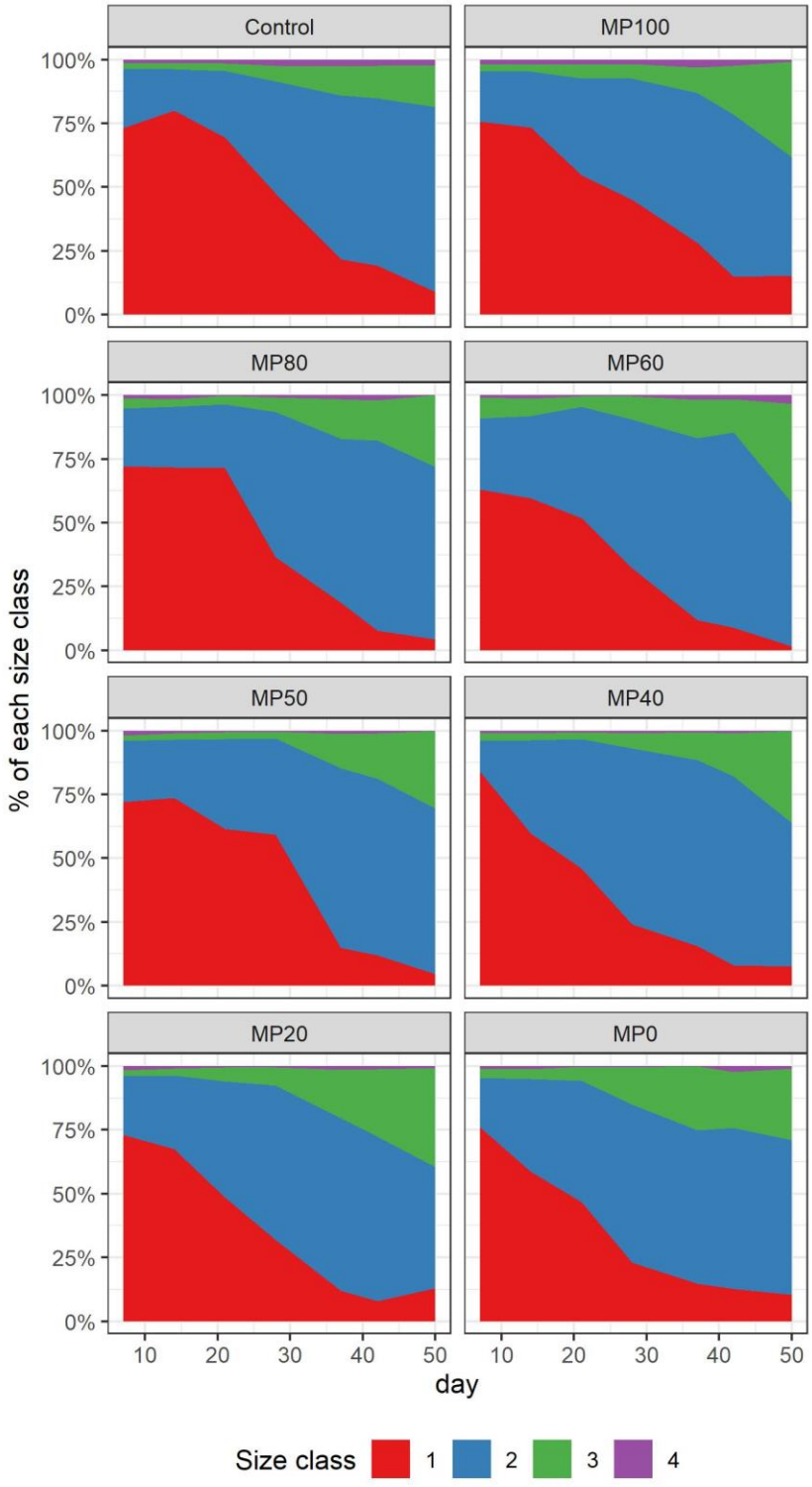

Figure 3: Size class distribution of body lengths in *D. magna* populations exposed to mixtures of polystyrene MP and kieselguhr. Class 1: ≤ 1599 µm, Class 2: 1600–2699 µm, Class 3: 2700–3799 µm, Class 4: > 3800 µm.

Overall, in this experimental setup MP-kieselguhr mixtures with higher ratios of kieselguhr showed to be detrimental for population development and promoted *ephippiae* formation in food-limited *D. magna* populations. Populations exposed to

50,000 particles mL^{-1} of polystyrene MP did not develop differently from control populations. Population effects are critically under-researched regarding MP. To our knowledge, only Bosker et al. (2019) investigated the effects of MP on the population development of daphnids and found a significant decline in population biomass due to MP exposure. Their general approach was different from ours as they first let populations grow to holding capacity before introducing particle stress. They saw no effect on size structure or *ephippiae* occurrence.

Kieselguhr has known biocidic, insecticidic, and acaricidic properties (European Food Safety Authority (EFSA) et al. 2020) and has earlier been used as natural reference material in MP studies (Scherer et al. 2019). In these studies, kieselguhr was toxic in larvae of *Chironomus riparius*, but less than PVC particles. Kaolin was tested as another naturally occurring particle and actually had positive effects on the animals. These shape-specific properties might qualify kieselguhr as an appropriate surrogate for naturally occurring non-food particles to help identify effects caused primarily by the particle shape. Nonetheless, pure kieselguhr will likely be more aggressive than a mixture of different suspended solids, as would occur in the environment.

The study showed that some natural particles can be more toxic than a mixture of natural particles and microplastics or microplastics by themselves. Therefore, transferring findings on one particle type to another is not easily possible, as study 2 (Annex 2) showed toxicity by the microplastics but not the natural particle kaolin. The fact that the particles remained in the medium for longer than in the multigenerational studies (Annex 2 + 3) and had more daphnids to interact with could have led to particle aging through eco-corona-formation from the animals, food, and microorganisms in the vessel. This could then potentially decrease toxicity, as was observed in study 3 (Annex 3). These points are further addressed in the general discussion.