Microplastics in the environment: Assessing the ingestion and effect of microplastics on freshwater rotifers in an environmental scenario

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Declaration of Authorship

I hereby declare that the thesis submitted is my own unaided work. All direct or indirect sources used are given as references. All contributions of co-authors are acknowledged.

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Abstract

Microplastics in the environments are estimated to increase in the near future due to increasing consumption of plastic product and also due to further fragmentation in small pieces. The fate and effects of MP once released into the freshwater environment are still scarcely studied, compared to the marine environment. In order to understand possible effect and interaction of MPs in freshwater environment, planktonic zooplankton organisms are very useful for their crucial trophic role. In particular freshwater rotifers are one of the most abundant organisms and they are the interface between primary producers and secondary consumers. The aim of my thesis was to investigate the ingestion and the effect of MPs in rotifers from a more natural scenario and to individuate processes such as the aggregation of MPs, the food dilution effect and the increasing concentrations of MPs that could influence the final outcome of MPs in the environment. In fact, in a near natural scenario MPs interaction with bacteria and algae, aggregations together with the size and concentration are considered drivers of ingestion and effect. The aggregation of MPs makes smaller MPs more available for rotifers and larger MPs less ingested. The negative effect caused by the ingestion of MPs was modulated by their size but also by the quantity and the quality of food that cause variable responses. In fact, rotifers in the environment are subjected to food limitation and the presence of MPs could exacerbate this condition and decrease the population and the reproduction input. Finally, in a scenario incorporating an entire zooplanktonic community, MPs were ingested by most individuals taking into account their feeding mode but also the concentration of MPs, which was found to be essential for the availability of MPs. This study highlights the importance to investigate MPs from a more environmental perspective, this in fact could provide an alternative and realistic view of effect of MPs in the ecosystem.

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Abbreviations

EPS	Extracellular polymeric substance
ER	Egg-ratio
ESD	Equivalent spherical diameter
MPs	Microplastics
MS	Microsphere
NPs	Nanoplastics
PA	Polyamide
PE	Polyethylene
PET	Polyethylene terephthalate
PMMA	Poly(methyl methacrylate)
PP	Polypropylene
PS	Polystyrene
PVA	Polyvinyl alcohol
PVC	Polyvinyl chloride
RNS	Reactive nitrogen species
ROS	reactive oxygen species
RS	reactive species
SPM	Suspended particulate matter
TEP	Transparent exopolymer particles

WC	Woods Hole Culture Medium

Summary

Their ubiquitous presence in the environment, their rapid rising output and persistence have made plastics pollution a major environmental problem. The increasing concentration of microplastics (MPs) due to further fragmentation is still underestimated in freshwater environment as well as the consequence at ecosystem level. This study analyses how natural factors such as presence MPs aggregated with bacteria and different algal food supply may alter the ingestion and effect of MP in rotifers under environmental conditions as opposed to laboratory conditions. Rotifers are planktonic organisms a negative effect on them could have consequence at higher trophic level. The ingestion rate of MPs alone, MPs in association with algal food of the same size and aggregated MPs was studied using a functional response approach in order to compare the parameters that determine the higher ingestion for one size rather than others, and for the aggregated MPs rather than the free one (Chapter 2). The impact of MPs in my study was investigated in relation to different food supply, such as the food concentration but also the food type. In addition, the exposure to different type of plastic as polyamide fragments and non- plastic items as silica beads was compared, in order to understand possible particles effect or negative response related to the type and shape of plastic. The species- specificity of the MPs effects is also taken into account, since two very similar species of brachionids were examined *Brachionus calyciflorus* and *Brachionus fernandoi.* The three different sizes of polystyrene MPs, polyamide fragments of different sizes, and silicate beads were tested in association with different concentrations and types of food. The purpose of this study (Chapter 3) was to find a relationship between the MPs concentrations and the quantity and quality of the food. In addition, the

two species were compared to test for possible divergence in response to MPs. Sublethal response as reproduction, population growth rate and survival were tested, reporting deleterious effects as decrease in population size and reproduction input, especially with MPs with the same size of the algal food provided. Proceding to a more complex and near-natural scenario a microcosm experiment was conducted and four different zooplankton groups composed by rotifers, cladocerans, adult and juvenile copepods and ostracods were exposed to three different MPs size (Chapter 4). The major drivers such as MPs concentration, size and feeding mode is taking into account in order to identify which group could be more susceptible to MPs ingestion when the whole community at the same time is exposed to different MPs size. MPs concentration and feeding mode more than size driven the ingestion of MPs ingestion. At high concentration more individuals ingested MPs, despite the size preference and the feeding mode, on the contrary at low concentration the MPs could be avoided or egested faster. This study highlights how environmental factors as well as the MPs concentration and feeding mode could play an important role in MPs study.

Zusammenfassung

Das allgegenwärtige Vorhandensein in der Umwelt, der rasche Anstieg und die Langlebigkeit haben Plastik zu einem großen Umweltproblem gemacht. Die zunehmende Konzentration von Plastik durch weitere Fragmentierung in Süßgewässern und die daraus resultierenden Konsequenzen auf Ökosystemebenen werden noch immer unterschätzt. Die Auswirkungen von MP auf planktonische Organismen wie den Rotatorien sind noch nicht hinreichend untersucht worden. Ein negativer Effekt könnte Folgen auf höhere trophische Ebenen haben. In dieser Studie wird untersucht, wie natürliche Faktoren die Aufnahme und Wirkung von Mikroplastik (MP) auf Rotatorien verändern können. Natürliche Faktoren, wie zum Beispiel durch Bakterien aggregierte MP, unterschiedliche Algennahrung, sowie verschiedene aquatische Tierarten, wurden genutzt, um Umweltbedingungen im Labor zu imitieren. Die Aufnahmerate von MP, von MP in Verbindung mit Algennahrung und von aggregiertem MP wurde untersucht (Kapitel 2). Durch die Aggregation von MP sind kleinere MP für Rotatorien besser verfügbar und größere MP werden weniger aufgenommen. Die Auswirkungen des MPs wurden in meiner Studie in Abhängigkeit von der Nahrungszufuhr, z. B. von der Algenkonzentration, aber auch von der Art der Algen untersucht. Darüber hinaus wurden Polyamidfragmente und Siliziumdioxidkügelchen verwendet, um mögliche Partikeleffekte oder negative Reaktionen im Zusammenhang mit der Art und Form des Plastiks zu verstehen. Zwei verwandte Brachyonidenarten wurden dabei untersucht. Die Wirkung von MP auf *B. calyciflorus* und *B. fernandoi* wurde in Kombination mit verschiedenen Konzentrationen und Arten von Futter getestet. Ziel dieser Studie (Kapitel 3) war es, eine Beziehung

zwischen den MP-Konzentrationen und der Qualität der Nahrung herzustellen. Darüber hinaus wurden die beiden Arten miteinander verglichen, um mögliche Unterschiede in der Reaktion auf MP festzustellen. Es wurden subletale Reaktionen, wie die Fortpflanzung, die Populationswachstumsrate und das Überleben untersucht, wobei eine schädliche Auswirkung in Form einer Verringerung der Populationsgröße durch geringere Fortpflanzung festgestellt wurde. In einem komplexeren und naturnahen Szenario, wurde ein Mikrokosmos-Experiment durchgeführt, bei dem vier verschiedene Zooplanktongruppen, bestehend aus Rotatorien, Cladoceren, Copepoden und Ostracoden, drei verschiedenen MP-Größen ausgesetzt wurden (Kapitel 4). Die wichtigsten Einflussfaktoren, wie die MP-Konzentration, die Größe und die Art der Nahrungsaufnahme, wurden berücksichtigt, um festzustellen, welche Gruppe anfälliger für die MP-Aufnahme ist. Die MP-Konzentration und die Art der Futteraufnahme beeinflussten die Aufnahme von MP stärker als die Größe. Bei hoher Konzentration nahmen trotz der Größenpräferenz und der Art der Futteraufnahme mehr Individuen das MP auf. Im Gegensatz dazu konnte die Aufnahme von MP bei niedriger Konzentration vermieden werden. Diese Studie verdeutlicht, dass Umweltfaktoren sowie die MP-Konzentration und die Art der Futteraufnahme eine wichtige Rolle bei der Untersuchung von MP spielen.

Chapter 1

General Introduction

1.1 Microplastic in the environment

Microplastics (MPs) are defined as plastics particles smaller than 5 mm (Koelmans et al., 2022). MPs have been found everywhere in the aquatic and terrestrial environment, from open oceans to remote alpine lakes (Lim, 2021). Recently MPs particles have been detected also in human blood (Leslie et al., 2022), lungs (Amato-Lourenço et al., 2021) and stool (Liebmann et al., 2018). Their increasing concentration and degradation is a crucial problem for the environment. Currently detected levels of MPs, although there are always limitations due to not sophisticated techniques that underestimates the smallest fraction (from 1 to 100 µm), continue to increase (Conkle et al., 2018; Lindeque et al., 2020; Way et al., 2022). Data on a global scale for plastic waste generation, collection and disposal are unreliable due to inconsistent reporting between countries or omitted values (Borrelle et al., 2020). On one side, plastic emission is underestimated and on the other side, measures taken to decrease plastic production are not sufficient (Edelson et al., 2021). The occurrence of MPs in marine systems was reported in 1972 and became a topic of interest for scientists in 2004 (Geyer, 2020). Since its invention, the concerns about plastic pollution increase exponentially as its production increased (see box 1).

Box 1: *Plastic: a curse in disguise*

All aspects of daily life involve the consumption of plastic and its low cost and long resistance has bring several benefits to public health. Over the last century have been produced several synthetic polymers sometimes made by natural substances like cellulose but more often were used petroleum and fossil fuels. The first synthetic polymer was invented in 1869 in order to substitute ivory, after that others semi natural materials were used to substitute natural substances as horn, linen and ivory (Geyer, 2020). This impressive discovery was considered a blessing for nature, as plastic could substitute any materials and protect nature for man exploitation. In 1907 was invented the first fully synthetic plastic and the plastics market start to grow (Geyer, 2020). During the World War II the production growth 300 % since most of the parachutes, ropes, body armor and more were made by plastics material. The surge in plastic production continued after the war. Plastics product replaced the majority of natural materials with better performance, for example in cars, packaging and furniture. With the increasing production, it grows also the accumulation of plastic debris. Plastic debris in the oceans was first observed during 70´ and it started to be considered a problem because of its long-lasting life (Ryan, 2015). Nowadays most plastic types are used for packaging applications and in smaller quantities for construction products (PlasticsEurope, 2021). The lifecycles of discarded plastics depend on the chemical nature of the material, the characteristics of the environment in which it was disposed and the degradation processes themselves. Currently the mass of plastic production is at about 450 million tons annually and set to double by 2045 (Bergmann et al., 2022). The total weight of the actual plastic mass with its diversity in terms of plastic type and chemicals it is already a challenge. In an ambitious scenario, researchers have estimated that between 20 and 53 Mt/year of plastic will be released to the aquatic ecosystem by 2030 (Bergmann et al., 2022) ...

... The accumulation of plastic in the environment exceed the natural and anthropological removal, this makes plastic a ”poorly reversible pollutant” (MacLeod et al., 2021). These pollutant accumulate to a level that cannot be readily reversed due to the impossibility to rapid reduce pollution levels below the threshold (MacLeod et al., 2021). In addition, the whole life cycle of plastic accounts for 4.5 % of our current greenhouse gas emissions (Bergmann et al., 2022).

Until few years ago the research field of MPs focused only on the ocean but rivers and lake has been found be a great source of MPs (van Emmerik and Schwarz, 2020). Lakes could act as reservoirs accumulating MPs, and rivers are also being considered, not only as a vehicle for the release of MPs into the marine and terrestrial environment, but as a host of MPs for decades, depending on the characteristics of river compartments (van Emmerik et al., 2022). Once released into the environment, MPs undergoes different processes: aggregation with other natural particles, which have very different compositions and characteristics (Wang et al., 2021) or be a surface for biofilm formation (Tu et al., 2020); they could degrade into even smaller pieces (Klein et al., 2018), sediment and be resuspended (Talbot and Chang, 2022; Li et al., 2022) and at last be ingested by aquatic organism (He et al., 2021). Over 750 between marine and freshwater species are known to have ingested or become entangled with MPs (Steer and Thompson, 2020; Rochman et al., 2013). At the beginning, the ecotoxicologist start running experiments with manufactured MPs because it was unknown which type of MPs were in the environment (Potthoff et al., 2017). Nowadays scientist have started to shift to more environmentally realistic conditions, in order to understand the potential impact, i.e., using different MPs type, shape as fragments, fibres and films (see box 2) , using biofilm coating which appear to make animals more prone to eat them (Polhill et al., 2022), testing lower concentrations and longer time exposure (Ockenden et al., 2021). In fact, the environmental impact is still poorly understood. Ecotoxicological studies has provided often conflicting results on the potential impact of MPs on organisms (Aljaibachi and Callaghan, 2018; Eltemsah and Bøhn, 2019; Jeong et al., 2016). Due to the intrinsic characteristic of MPs (see box 2) and the species used to test it (Jaikumar et al., 2019), the test results were sometimes extremely negative with a high mortality rate and sometimes completely neutral (Wang et al., 2019). Because of their small size MPs could be ingested by small aquatic organism as zooplankton (Scherer et al., 2018). The ingestion of MPs by freshwater zooplankton as cladocerans, copepods and rotifers are mostly related to an adverse effect (Yu et al., 2020). The decreased survival and reproduction, the behavioral alteration as impaired locomotion could have negative consequence at population and ecosystem level (Berlino et al., 2021). In fact, these organisms are crucially important for their role in the marine and freshwater food web, as they graze on primary producer and are source of nutrition for bigger consumer. In particular, the effect and the ingestion of MPs in freshwater rotifers has been understudied during these last years compared to cladoceran and copepods (Yu et al., 2020). Some rotifers species are one of the most abundant organisms in freshwater environments (Wallace and Snell, 2010), as filter feeders, their food size selection falls within the range of the smallest MPs found in the environment, particularly sizes smaller than 50 µm down to nanoplastics (NPs; <1 µm) (Mitrano et al., 2021). In fact, it is not new for freshwater rotifers to ingest and to be exposed to particles in the environment (DeMott, 1986), but the different characteristic of MPs could make them more available for these organisms (Koelmans et al., 2022). Labelled microsphere and inert particles have been used to test size food selection in planktonic rotifers (Kirk, 1988; Walz, 1995) but the effects of these particles have been understudied as well as the association with natural factor as bacteria and algal food. A few studies on MPs analysed the ingestion and sub-lethal response of rotifers exposed to plastic microspheres, in particular organisms such as *Brachionus* spp. were subjected to standardised laboratory tests (Won et al., 2017). These tests are usually designed to test chemical pollutants, applying short-term exposures and experiments on individuals and do not includethe presence of food. These experiments, although crucial for understanding effects at the cellular level, very often do not take into account natural factors such as biogenic aggregation of MPs, the presence of food of different quality and quantity and a potential species-specific effect on rotifers. The presence of these factors in the environment could indeed lead to different results than under laboratory conditions.

Since microplastics studies often lack of environmental reality (Weis and Palmquist, 2021), the presence of algal food and bacteria as well as a community approach would allow us to better understand the effect of MPs from an ecological point of view, in a context that mimics more natural conditions. Furthermore, the effect of MPs on aquatic organisms have been compared with those of natural particles, such as inert minerals, clay or silicate. Therefore, the aim of my work was to investigate if environmental factors such as bacteria and aggregation, different algal food and presence of multiple species influenced the interaction of MPs with freshwater rotifers in a more environmental relevant condition. Since the ingestion is the main way of interaction between aquatic organisms and MPs, I first examined the ingestion of MPs by freshwater rotifers under more environmentally relevant conditions: whether the ingestion of MPs is driven by size selectivity or is influenced by biotic factors such as the presence of algae or biofilms. Then I investigated the fitness response that follows ingestion of MPs. In this case I also examined whether more heterogeneous plastic size and type or even non-plastic particles as silica beads can lead to negative response in rotifers population. As additional environmental condition I wanted to consider the ingestion of MPs by a planktonic community. In fact, in the natural environment the organisms are part of a community formed by population of different species with different feeding modes, so it is important to know which group would be more affected by MPs ingestions and if the ingestion is driven more by MPs size or concentrations. In the next section of the "General Introduction" I will present the general pattern of MPs ingestion in freshwater zooplankton and the consequently effect at individual and population level. This will be followed by a section on rotifers ecology and available studies on MPs. In the last section of the "General Introduction" I will focus on MPs ingestion in a planktonic community, through a microcosm experimental set-up.

Box 2: *Microplastics characteristics: type, size and shape*

Microplastics (MPs) are pieces of plastic in a size range that goes from 5 mm to 1 µm (Rochman et al., 2019). They can occur in different shapes, sizes and type of polymers (Rochman et al., 2019). Depending on their manufactures and their fate in the environment, MPs are specifically designed as "primary microplastics": micrometer- sized particles deliberately manufactured for specific applications or products, such as pellets for industrial production (for example, for drink bottles) and microbeads (such as those used in personal care products) (Kasmuri et al., 2022). The so called "secondary microplastics" refers to particles formed from the fragmentation and breakdown of larger plastic debris (Kasmuri et al., 2022). The most abundant polymers in MPs are polyethylene (PE), polyethylene terephthalate (PET), polyamide (PA), polypropylene (PP), polystyrene (PS), polyvinyl alcohol (PVA) and polyvinyl chloride (PVC) (Strungaru et al., 2019). PS, in particular, is one of the most used plastic materials and it is expected to be found in most sediment and aquatic samples (PlasticsEurope, 2021). It is also one of the polymer types more found in sediments and water phases in German river, with a size lower than 200 µm (Klein et al., 2015). In the environment, each of these polymers has a certain range of densities, owing to differences in manufacture, crystallinity, additives, age and level of weathering and biofouling (Koelmans et al., 2022). Overall, the densities of MPs polymers found in nature range between roughly 0.8 and -3 g cm^{-3}, with a density of around 1 g cm^{-3} being most abundant (Koelmans et al., 2022).

The shape of MPs can vary greatly from one MPs to the others: beads have more regular and spherical shape, mostly found as primary MPs; fragments and fibers have spiky and irregular edge. Fragments, fibers and films are the most frequently reported MPs shape categories, since they can reach larger size (>300 µm) and are the most detected than beads (<300 µm) (Burns and Boxall, 2018). Larger MPs, in the size range of millimeter could also undergo further fragmentation and acquire more irregular shape (Enfrin et al., 2020). The intrinsic characteristic of MPs and their small size makes these materials difficult to remove from the environment (Padervand et al., 2020). Moreover, the actual concentration is still underestimated in the environment, due to technical limitation that destroy or modify the smallest fraction of MPs(Way et al., 2022; Tamminga et al., 2022).

1.2 Ingestion of microplastics by freshwater zooplankton

Once released into the environment, microplastics (MPs) can be ingested by a variety of organisms, particularly planktonic zooplankton seem to be more prone to ingest small MPs (1 to 50 µm) because of their feeding selectivity (Botterell et al., 2019). Small MPs are similar in size (1-20 µm) to primary producers such as unicellular algae or bacteria and organic matter (Wang et al., 2019). In particular, filter and suspension feeders organisms, such as rotifers and cladocers, are assumed to ingest MPs because they commonly feed on suspended particulate matter (SPM), microalgy and ingest a variety of sediment components (Scherer et al., 2018). In general, the minimum size of ingested particles is determined by the mesh size of the filter apparatus (Geller and Müller, 1981), and the maximum size is limited by the morphology of the mouthparts (Scherer et al., 2017). However, ingestion behavior cannot be generalized because of the difference in filter mesh size, such as in cladocera, and size-selective foraging, such as in rotifers and copepods (Scherer et al., 2018). For the latter, there is a species-specific degree of selectivity, as some of them feed directly on MP available in the size range of their food, while the more selective ones avoid ingesting MP(Scherer et al., 2017). Generalist eaters such as

Daphnia and herbivorous rotifers frequently ingest MP in laboratory experiments, while more specialized eaters such as cyclopoid and carnivorous rotifers are less likely to ingest it (Scherer et al., 2018). Although at high concentrations they can be ingested indirectly, through contaminated prey (He et al., 2021), or by mistake, through consumption of faecal pellets (Cole et al., 2016; Panti et al., 2015). Since the beginning of MPs investigation, zooplankton have been exposed to MPs in laboratory conditions (Phuong et al., 2016). It is still understudied the uptake and the ingestion of MPs in more natural conditions, in fact, in the environment MPs are always associated with environmental factor as algae and bacteria (Alimi et al., 2022; Lagarde et al., 2016). In a scenario that incorporate some of these factors, just few studies have tested the ingestion and the effect of MPs in common zooplankton species (Rozman and Kalčíková, 2022). The interaction of MPs with biotic factor as bacteria and algae with the formation of biofilm have also a crucial role in the uptake of MPs (Vroom et al., 2017). It is also likely that MPs in the environment are incorporated in hetero-aggregates, made of particulate matter, microbes and biopolymer (Michels et al., 2018). Moreover, extracellular polymeric substances (EPS), particularly transparent exopolymers particles (TEP), produced by microorganisms play a significant role in the formation of nano- and microplastic agglomerates (Cunha et al., 2019). For example, the colonization with different bacteria and algae could make the MPs more appetible for the planktonic organism and increase or decrease its nutritional value (Amariei et al., 2022). The biofilm formation will also change the buoyant properties of the plastic, making them sinking faster (Sooriyakumar et al., 2022). The aggregated MPs with particulate matter could be, depending on their size, more available for macro-feeders (fishes) or for bigger micro-feeders (planktonic crustacea) and also for benthic organisms (Porter et al., 2018). Quantify the ingestion of MPs in a more natural environment could give a real picture of what happen in the environment when MPs are released (Polhill et al., 2022; Xu et al., 2022). In general, for rotifers, feeding on algal food or particulate matter is a process that is quantified by the ingestion rate (Starkweather, 1980; Salt, 1987).

The same methods can be applied to MPs ingestion, in order to quantify the uptake of MPs for the organism such as rotifers. Ingestion or consumption rate is the ingestion per capita per time unit and it is fundamental to calculate the functional response (Starkweather, 1980). This describes the relationship between the number of prey or in this case of MPs particles consumed and their abundance (Pritchard et al., 2017). The presence of different sized algae or biogenic aggregation of MPs can influence the ingestion of MPs in the environment and can affect directly the response of the rotifers to MPs or indirectly affecting the normal feeding mode. For example, some organisms have been found more prone to ingest aggregated MPs particles then single MPs (Porter et al., 2018; Vroom et al., 2017), or take advantage of the biofilm formation on the aggregated particles (Amariei et al., 2022). In order to test the difference in the ingestion rate it is fundamental to calculate the parameter that describe a functional response to test difference between the ingestion of free-floating MPs and the aggregated ones.

1.2.1 Functional Response: an ecological tool lended to microplastics

Consumer -resource interaction is the key of ecology since functional response describe how the ingestion rate of a consumer changes with resource availability as a result of the consumer ´ s search for, capture and handling of resources(Jeschke et al., 2022). For this reason, functional response experiments have been used to investigate prey-predator theory as well as prey selectivity(Jeschke et al., 2022). This procedure could be useful to estimate the parameters governing the ingestion of MPs. In fact, uptake of MPs by biota may vary depending on MPs concentrations in the environment (Mbedzi et al., 2020). The concentration in the environment is highly variable spatiotemporally and impact of MPs uptake may vary due to their density-dependent effect (Mbedzi et al., 2020). There is still nowadays a lack of understanding of how ingestion rate responds to different MPs concentrations. Functional response used to quantify the natural consumer-resource interaction in many ecological fields can be useful for the quantification of direct MPs uptake by organisms. Three forms of functional responses are commonly described as: the density-independent linear Type I response; inversely density-dependent hyperbolic Type II response, where consumption rates are high at low densities, and; positively density dependent Type III response, which is sigmoidal due to low consumption rates at low densities (Jeschke et al., 2022; Hassell, 2020).

1.3 Effect of microplastics in freshwater zooplankton

The ultimate scope of assessing the ingestion and feeding type of freshwater organism is to evaluate the effect of MPs in aquatic filter feeders organism as they are especially prone to MPs exposure. The potential ecotoxicological effect of MPs has been studied in a wide range of aquatic organisms over the past few years (Yu et al., 2020).

Negative effects at individual level have been found but not in each organism tested, and sometimes contrasting results have been found on the same organisms (Rehse et al., 2016; Aljaibachi and Callaghan, 2018). Generally, the adverse effect consisting on increased oxygen consumption, inflammation, reduced lysosomal stability in the digestive gland, reduced antioxidant capacity, DNA damage, neurotoxicity, oxidative damage (see box 3), alteration of the genetic expression, ionic exchange and enzymatic activity (Koelmans et al., 2022). At individual and population level can cause lower reproduction success (Ogonowski et al., 2016), survival (Ziajahromi et al., 2018) and growth (Straub et al., 2017). Few studies indicated also indirect effect as changing in filtration capacity, swimming activity, oxidative defense, energy production and feeding habits (Koelmans et al., 2022; Xue et al., 2021). The negative effect on zooplankton given by MPs depended on several factors, as the size and shape of MPs (An et al., 2021; Schwarzer et al., 2022), as well as concentrations (Rehse et al., 2016) and MPs densities (Schwarzer et al., 2022). Additionally, the negative effect of MPs was always related to the presence of food and it exhibited a species-specific response (Yu et al., 2020; Aljaibachi and Callaghan, 2018). In fact, the presence of food particles alleviated the effect of MPs in acute and chronic test (see box 3) with cladocerans and rotifers (Horton et al., 2018; Xue et al., 2021). The negative effect could also be species specific as some species appear more sensitive than other, for example the cladocerans *Ceriodaphnia dubia* exhibited more negative response than *Daphnia* spp. (Jaikumar et al., 2018). Smaller sized MPs, in the size range of 1- 10 µm, and irregular shaped MPs (Schwarzer et al., 2022) are considered more toxic due to the ability to pass through tissue or be less efficiently egested (Botterell et al., 2019). For example, life history experiment with *D. magna* exposed to primary and secondary microplastic as well as kaolin shows high toxicity with secondary MPs (Ogonowski et al., 2016).

When studying the effect of MPs in freshwater organisms, it is important to distinguish two important aspects: physical and chemical effect. From the chemical point of view, plastic material is made by different polymer and additives (e.g., plasticizers, flame retardants, colorants) (Zimmermann et al., 2019). These compounds can leachate from plastic products and cause chemical toxicity (Zimmermann et al., 2021). Some of them are classified as endocrine disrupting chemicals adversely effecting life-cycle parameters of a broad range of species (Chen et al., 2019b). Besides additives, absorbed persistent organic pollutant has been found in MPs (Gateuille and Naffrechoux, 2022). The controversy arises when several laboratories' studies illustrate the capacity of MPs to modify adverse effect of chemical by affecting the bioavailability or acting as an additional stressor (Scherer et al., 2018). However, some other studies emphasize that MPs could be a minor influence as vectors since they are out-competed by natural occurring matter. In fact, desorbed chemicals might adsorb to food or sediment and decrease the potential relevance of MPs as a vector (Menéndez-Pedriza and Jaumot, 2020). It is important to notice that most of the studies on the vector hypothesis were made for marine species, the situation could be really different for freshwater environment. Freshwater compartment are exposed to a completely different and much larger spectrum of chemicals. This is because they receive a constant input of chemical from land-based sources (e.g., pesticides) and wastewater (Besseling et al., 2019). Besides the capacity of MPs to interact with toxic compound, some studies suggested that MPs can interfere with intra- and interspecies signaling (e.g., phero- and kairomones) (Rashid et al., 2020). The disturbance of this inter- and intraspecies communication can lead to an abnormal response in both signaler and receiver (Lürling and Scheffer, 2007; Trotter et al., 2019).

From the physical point of view MPs can be ingested and cause damage to the internal gut membrane, due to the passage, the aggregation and the accumulation in the stomach (Desforges et al., 2015). Due to the lack of nutrition, MP can be ingested and egested without providing nutritional benefit to the organism and may have an indirect effect diluting the food concentrations (Rauchschwalbe et al., 2021; Mueller et al., 2020). Reduced feeding related to the non selective ingestion and a decrease in fecundity concentration-dependent has been found in copepods, cladocerans and rotifers (Yu et al., 2020). Even thought their importance in freshwater ecosystem, the ingestion and effect of MPs in rotifers is less studied. Rotifera, in fact, is a phylum that comprehend more than 2000 species between freshwater and marine environment with very different features, moreover they have a crucial role in the food chain eating on algae and bacteria and feed themselves to larger zooplankton and fish larvae (Wallace and Snell, 2010).

Box 3: *Terms lended from ecotoxicology.*

Endpoint is the recorded observation coming from an in chemico method, an in vitro assay or an in vivo assay (Mouneyrac and Amiard-Triquet, 2013). The measurement of a biological effect (Mouneyrac and Amiard-Triquet, 2013). A large number of endpoints are used in regulatory assessments of chemicals.

Biomarkers was defined by Depledge (1994) as "a biochemical, cellular, physiological or behavioral variation that can be measured in tissue or body fluid samples or at the level of whole organisms that provides evidence of exposure to and/or effects of, one or more chemical pollutants (and/or radiations)" (Depledge and Fossi, 1994). Although biomarkers are sensitive tools in the evaluation of the health status of organisms, their lack of ecological relevance is frequently underlined and their use in ecological assessment questioned(Mouneyrac and Amiard-Triquet, 2013). Thus, some of the research in ecotoxicology currently focuses to fill the gap existing between sub-organismal and organismal responses to stress and effects occurring at higher levels of biological organization (e.g., population level) (ECO Update, 1994). In this sense, ecologically relevant biomarkers such as behavior, reproduction, growth, energy metabolism, lysosomal integrity, immunotoxicity, and genotoxicity biomarkers appear as promising candidates to improve ecological risk assessment and then to provide supports for environmental management and regulatory decisions.

Oxidative stress can be defined as a disturbed balance between the production of damaging reactive oxygen species (ROS) and reactive nitrogen species (RNS) and an organism capacity to deal with them. Increased reactive species (RS) production or reduced antioxidant defense cause oxidative damage of molecules (Prokić et al., 2019). Reactive species, as highly reactive molecules, interact with other molecules, adversely affecting very important biological molecules, such as DNA, proteins and lipids (Prokić et al., 2019). Progressive damage of biomolecules triggers an inflammatory response, cell and tissue damage and induce degenerative changes, which can result in cell senescence and death, organ dysfunction and loss and reduced organism performance (Prokić et al., 2019).

Acute and Chronic Test: Toxicity test measure lethal and sub-lethal effect, these effects are known as measurement endpoints: this are ecological attributes that may be adversely affected by exposure to site contaminants and that are readily measurable(ECO Update, 1994). Acute tests are short -term tests that measure the effects of exposure to relatively high concentration of chemicals(ECO Update, 1994). Lethality is usually the endpoint. Chronic test are longer-term tests that measure the effects of exposure to relatively lower, less toxic concentrations and the endpoint used are reproduction and growth and also lethality (ECO Update, 1994).

1.4 Rotifera

Rotifera is one phylum closely related to freshwater environment since they are numerical dominant in zooplankton community (Wallace and Snell, 2010). They exert a great grazing pressure on the small picoplankton and play a critical role in the microbial loop (Wallace and Snell, 2010). Their size range goes from 50 to 2000 µm in length. To identify rotifers, two features are important: the anterior end called corona, structure composed of cilia; and the mastax (Wallace and Snell, 2010). The corona is used for locomotion and to collect food (Wallace and Snell, 2010). The mastax is a muscular pharynx, which includes a complex set of jaws called trophi;

Figure 1.1: *Brachionus calyciflorus*. Photo from Claudia Drago

these are used from taxonomist to distinguish the species (Wallace and Snell, 2010). Two classes of rotifers are recognized: Paratoria and Eurotatoria (Wallace and Snell, 2010). The latter contained the sub-class Bdelloidea and Monogononta. The reproductive strategy is different for these two classes, for example Monogononta are intermittently sexual, producing males for a brief period of time in order to fertilize the embryos that became resting eggs and they can be also parthenogenetic (Wallace and Snell, 2010); Bdelloids instead are only parthenogenetic (Wallace and Snell, 2010). The Monogononta are the most studied group, in particular *Brachionus*; due to their ecological role and relatively easy maintenance they have been used for aquatic toxicity testing (Dahms et al., 2011). A variety of substances have been tested, such studies can show modified predator-prey interaction and community structure, as well as indirect effect like increasing threshold food levels (Won et al., 2017).

1.4.1 Population dynamics

In order to understand the cause of changes in population size and community structure it is crucial to quantify the relative contribution of mortality, reproduction and dispersal (Wallace and Snell, 2010). The major environmental factors affecting survival and reproduction are temperature, food quantity and quality, genotype and mode of reproduction (Wallace and Snell, 2010). Rotifers abundance is determined by food supply as a bottom-up forces and by predation as top-down forces shaping only temporal changes. Resource limitation, is always been experienced by organism in the natural environment and it is essential for competition interaction (Merriman and Kirk, 2000). Due to their feeding mode rotifers can be exposed in the environment to a huge algal biomass but if the nutritional quality is not adequate, they will experiment food limitation (Stelzer, 2001). Fluctuation in food levels is common in lakes and ponds and food levels can also decline rapidly with consumer increasing (Devetter and Sed'a, 2005). After experiment in the field that have measured and compared the food limitation in rotifers population in the wild, the overall conclusion was that rotifers are usually food-limited in the field (Ortega-Mayagoitia et al., 2011). This natural factor affecting and shaping populations dynamics in the environment is a crucial point to take into account when testing the effect at population level of MPs. MPs as inert particles, without any additional chemical leaching or adsorptions, could further dilute the food quantity and affecting the population growth rate. Experimentally the intensity of food limitation can be measured by the Δr, that express the intensity of food limitation trough the intrinsic growth rate of the population (Cordova et al., 2001). An important tool to predict and monitor the trend of population growth rate is exerted by the reproduction input as eggs (Sarma et al., 2005). The number of parthenogenetic eggs per number of females (ER) is an important indicator for predicting changes and oscillations in natural zooplankton populations and it can be an early warning system of stress in ecotoxicological studies (Sarma et al., 2005). The ER is directly related to the quantity of food availability; both food quality and food quantity have a great impact on rotifers survival and reproduction (Sarma et al., 2001). These measures used to study population dynamics (intensity of food restriction and egg ratio), are crucial since the effect of MPs could be related to decreased food intake and energy depletion. MPs studies often lack of environmental significance due to the investigation of endpoint (see box 3) that are difficult to scale up to effects in the environment (Burns and Boxall, 2018). From one side in fact there is the urgency to understand the ecological effect of MPs in aquatic ecosystem and on the other side more environmental relevant exposure scenario needs to be applied when studying MPs effect.

1.4.2 Microplastics studies on *Brachionus* spp.

Brachionus spp. is a genus of monogonont rotifers very abundant in marine and freshwater environment. Since they are easy to culture and maintain under laboratory conditions and due to their fast life cycle, *Brachionus* spp. are one of the most used organisms for ecotoxicological test (Dahms et al., 2011). Recently the effect of MPs on *Brachionus* spp., mostly marine species, has been investigated. Most of the studies have found an effect upon NPs exposure more than MPs, mostly marine species *B. plicatilis* or *B. koreanus* have been used and the test focused on individual level with acute and chronic toxicity test (Jeong et al., 2018; Venâncio et al., 2019; Morgana et al., 2019). These studies investigated the alteration of mortality (Morgana et al., 2019; Venâncio et al., 2019), reproduction (Jeong et al., 2016; Berber and Yurtsever, 2018), growth rate and antioxidant enzyme activity (Zheng et al., 2022). Different types and sizes of plastic has been used: PS (Jeong et al., 2016), PE(Xue et al., 2021), PMMA (Venâncio et al., 2019), the size range of MPs goes from 1 to 25 µm and for the NPs the range goes from 0.04 to 0.7 µm. The mostly used MPs was PS since it is the most found polymer type in marine and freshwater environments. Studies on MPs shows a high dose dependent effect causing reproduction decrease and involving the antioxidant enzyme activation (see table 1.1). The nutritional status acts as an important factor in the response to every toxicant compound; in particular this can modulate the immune and digestive response in rotifers exposed to MPs (Zheng et al., 2022). In another study it was shown that the algal food supply mitigated the negative effect on reproduction of MPs (Xue et al., 2021). Large MPs (6 µm) can also be egested faster than small MPs (1 µm) and NPs, this it seems influence the effect on individual level (Jeong et al., 2016). The effect of MP was investigated strictly from cellular and individual level without the introduction of any environmental factor. Food, for example, was always provided at saturated concentration and different biomarkers (see box 3) were analyzed (Liang et al., 2021). Although these studies are crucial to understand the mechanisms causing the negative effect on rotifers, an exposure more similar to the natural environment could lead to different results.

Table 1.1: Studies of MPs ingestion and effect in rotifers

Study	Endpoint	Type of Plastic	Size range (µm)	Species
(Setälä et al., 2014)	Microcosm experiment ingestion	PS	10	*Sincheta spp.*
(Jeong et al., 2016)	Growth, fecundity, life span	PS	0.05;0.5;6	*B. koreanus*
(Berber and Yurtsever, 2018)	LC50, Population growth	PE	10-22	*B. plicatilis*
(Morgana et al., 2019)	Mortality, swimming speed	PS	0.1	*B. plicatilis*
(Beiras et al., 2018)	Acute test MPs and chemical	PE	1 - 4	*B. plicatilis*
(Jeong et al., 2018)	Ingestion and accumulation; Xenobiotics defence; Oxidative stress	PS	0.05 ;0.5; 5	*B. koreanus*
(Berber, 2019)	Mortality	PS	0.05	*B. plicatilis*
(Saavedra et al., 2019)	EC50	PS	0.2	*B. calyciflorus*
(Sun et al., 2019)	Survival time, Reproduction, Body lenght	PS	0.07 ; 0.7 ; 7	*B. plicatilis*

Continued

Study	Endpoint	Type of Plastic	Size range (µm)	Species
(Venâncio et al., 2019)	Mortality	PMMA	0.04	*B. plicatilis*
(Drago et al., 2020)	Ingestion rate	PS	1; 3; 6	*B.calyciflorus*
(Alfonso et al., 2020)	Monitoring MPs concentration in a Lake	fibers	1000	*B. plicatilis*
(Fueser et al., 2020)	Microcosm experiment ingestion	PS	1	
(Drago and Weithoff, 2021)	Growth rate, reproduction, survival	PS, PA	1; 3; 6; 5-25	*B.calyciflorus*
(Jeong et al., 2021)	Reproduction, Growth with crude oil PAH exposure	PS	0.05	*B. koreanus*
(Kang et al., 2021)	MPs alleviation of As toxicity	PS	6; 0.05	*B. plicatilis*
(Liang et al., 2021)	Growth, reproduction, antioxidant enzyme	PS	1	*B. calyciflorus*

Continued

Study	Endpoint	Type of Plastic	Size range (µm)	Species
(Venâncio et al., 2021)	Fecundity, Lethality, population growth rate, generation time, feeding rate	PMMA	0.04	*B. plicatilis*
(Xue et al., 2021)	Fecundity, survival, swimming speed, enyime activity	PE	10- 21	*B. calyciflorus*
(Yoon et al., 2021)	MPs alleviation of endocrin-disruption potential	Latex beads	0.05	*B. koreanus*
(Zheng et al., 2022)	Immune and digestive enzime activity (Gene expression)	PS	5	*B. rotundiformis*
(Wang et al., 2022)	Total number of eggs, total number of offspring, first hatching time, life span, ingestion rate and body volume	PS	0.07	*B. plicatilis*

Continued

Study	Endpoint	Type of Plastic	Size range (µm)	Species
(Mao et al., 2022)	fecundity, time to first batch eggs, population growth rate, life span	PS	0.05; 0.1; 0.5	*B. plicatilis*

1.5 Availability and effect of microplastics at ecosystem level

The concern over the potential impact of MPs pollution on environmental heath is increased during the last years. Nevertheless, determining the ecological effects of MP is still challenging due to the different chemical and physical properties which vary with polymer chemistry, size range, shape, age, density (Rochman et al., 2019). These factor then influence the behaviour of MPs itself and the availability to the aquatic organisms (Botterell et al., 2019). Many studies have found negative effect of MPs ona single species experiments but less is known about the indirect effect that could disrupt key ecological interactions and functions (Ockenden et al., 2021). Moreover, if the negative effect is occurring at the base of the aquatic food web, changes in communities may disturb the productivity of an entire ecosystem (Ma et al., 2020). Even if MPs exposure has no adverse effects on one species, MPs could alter the interactions between species and have consequence for the trophic levels and cascading effects on ecological processes (Pan et al., 2022). The assessment of MP ingestion in the aquatic community is important to consider, as it is the first pathway of interaction between microplastics and organisms. Experiments introducing multiple species with different abundances may show an indirect effect of MPs such as prey-predator interaction (Sun et al., 2019) or food intake alteration (Cheng et al., 2020) or no effect (Redondo-Hasselerharm et al., 2020). The exposure to MPs and the ingestion in the environment occurs at the same time where multiple species competing for food and reproduction are present. Microcosm experiments, in this case, provides the opportunity of testing size preference, concentration dependency and moreover indicate which group of organisms are most at risk (Ockenden et al., 2021). Previous studies focused on the ingestion of MPs from a planktonic and benthic community indicating that benthic organisms were more prone to ingest and accumulate MPs, as for example Sätala and collegues show that all the taxa: copepods, cladocerans, rotifers, polychaeta and ciliate ingested MPs with higher accumulation of MPs in polychaeta (Setälä et al., 2014). In another study the size of MPs played an important role in the ingestion since the smaller size were more ingested than bigger size but more important was also the feeding mode (Silva et al., 2022). Most of the studies until now have concluded that the ingestion of MPs depended on particle concentration, feeding mode and encounter rate (Setälä et al., 2014; Rauchschwalbe et al., 2022). Moreover, some studies also emphasize that larger predator are more prone to ingest MPs when associate with small prey than consuming them directly (da Silva et al., 2022). In a microcosm experiment with benthic macroinvertebrate the biomass, abundances of the functional feeding groups and species richness and abundances was not affected by MPs (Stanković et al., 2022). In another study instead, there was an alteration in copepods abundances that lead to a slight shift in community composition (Rauchschwalbe et al., 2022). To understand the effect of MPs in freshwater environment it is crucial to continue investigating the interaction between multiple species through micro- or mesocosm experiments and when possible, utilize aquatic communities and factor that can mimic the natural environmental scenario.

1.6 Research question and objectives

The objective of the present work was to investigate the ingestion and effect of MPs in freshwater rotifers under more natural relevant conditions. In particular I investigate how biotic factor like different algal food, bacteria and aggregation can influence the ingestion of MPs. As a consequence of the ingestion the effect of MPs on populations dynamics was investigated. At last, in a microcosm experiment I examined the size -concentration availability of MPs ingestion for the whole planktonic community using different freshwater planktonic zooplankton species. Three research question were elaborated:

1. Is the ingestion of MPs by rotifers size dependent and is it influenced by the presence of algae and biogenic aggregations of MPs?

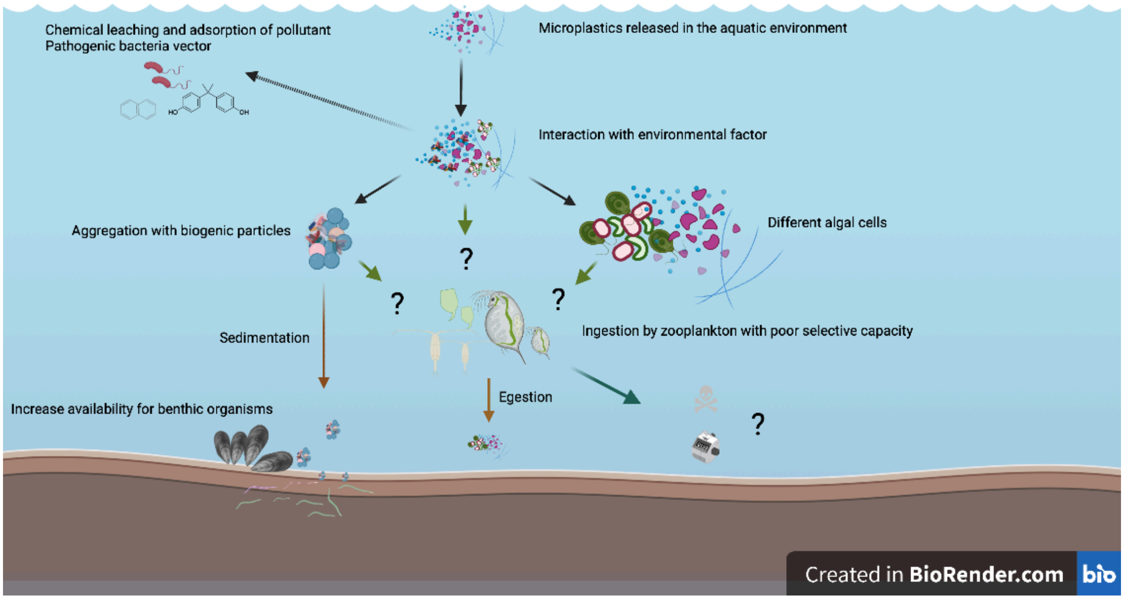

FIGURE 1.2: Graphical representation of the background knowledge and research questions of my thesis.

2. Do different ingested sizes and types of MPs and non-plastic items have an effect on fitness response of rotifers and how is this effect influenced by the food algal quantity and quality?

3. In a near-natural freshwater environment, does the ingestion of MPs by natural planktonic community depended on its size, concentration and on the feeding mode of the organisms?

1.7 Methodological approach

This cumulative dissertation consists of two peer-reviewed papers (Chapter 2 and 3) which have been published in Frontiers in Environmental Science and Toxics, respectively; and one manuscript in preparation for submission (Chapter 4). The 2^{nd} chapter of the dissertation regards the functional response and the ingestion of 1- , 3- and 6- µm polystyrene MPs beads by freshwater rotifers *Brachionus calyciflorus* under different conditions: MPs alone, MPs in association with algae and MPs aggregated with bacteria. The consequential study focuses on the fitness response as population growth rate, reproduction (egg-ratio) and survival, of two related rotifers species (*B. calyciflorus* and *B. fernandoi*) exposed to 1- , 3- and 6- µm polystyrene beads, 5 - 25 µm polyamide fragments and 3- µm silica beads in combination with two concentrations of one food species (*Monoraphidium minutum*) and a mix algal food (*M. minutum* and *Cryptomonas* sp.; Chapter 3). This is followed by a study where the ingestion of MPs by freshwater planktonic zooplankton is investigated in a near-natural condition. Through a microcosm experimental set-up it was possible to investigate the ingestion of 1- , 3- and 6- µm polystyrene beads in a planktonic community composed by 4 groups: rotifers, cladocerans, adults and juvenile copepods and ostracods. The percentage of organisms that ingested the MPs was examined in relation to the size and concentration of MPs and the feeding mode of the organism (Chapter 4). In the "General Discussion" (Chapter 5) the major findings and their implications for the ingestion and the effect of MPs in freshwater zooplankton are resumed. Further, knowledge gaps, additional research questions, and possible directions of future research are presented in chapter 6. All the practical work was conducted in the University of Potsdam and the author of the thesis was involved in the project MikroPlaTas (BMBF: 02WPL1448A) which gave the framework and motivation for the present work.

Chapter 2

Manuscript 1

Biogenic aggregatio of small microplastics alters their ingestion by a common freshwater micro-invertebrate

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Biogenic Aggregation of Small Microplastics Alters Their Ingestion by a Common Freshwater Micro-Invertebrate

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In recent years, increasing concerns have been raised about the environmental risk of microplastics in freshwater ecosystems. Small microplastics enter the water either directly or accumulate through disintegration of larger plastic particles. These particles might then be ingested by filter-feeding zooplankton, such as rotifers. Particles released into the water may also interact with the biota through the formation of aggregates, which might alter the uptake by zooplankton. In this study, we tested for size-specific aggregation of polystyrene microspheres and their ingestion by a common freshwater rotifer *Brachionus calyciflorus.* The ingestion of three sizes of polystyrene microspheres (MS) 1-, 3-, and 6-μm was investigated. Each MS size was tested in combination with three different treatments: MS as the sole food intake, MS in association with food algae and MS aggregated with biogenic matter. After 72 h incubation in pre-filtered natural river water, the majority of the 1-μm spheres occurred as aggregates. The larger the particles, the higher the relative number of single particles and the larger the aggregates. All particles were ingested by the rotifer following a Type-II functional response. The presence of algae did not influence the ingestion of the MS for all three sizes. The biogenic aggregation of microspheres led to a significant size-dependent alteration in their ingestion. Rotifers ingested more microspheres (MS) when exposed to aggregated 1- and 3-μm MS as compared to single spheres, whereas fewer aggregated 6-μm spheres were ingested. This indicates that the small particles when aggregated were in an effective size range for *Brachionus*, while the aggregated larger spheres became too large to be efficiently ingested. These observations provide the first evidence of a size- and aggregation-dependent feeding interaction between microplastics and rotifers. Microplastics when aggregated with biogenic particles in a natural environment can rapidly change their size-dependent availability. The aggregation properties of microplastics should be taken into account when performing experiments mimicking the natural environment.

Keywords: microplastics ingestion, *Brachionus calyciflorus*, aggregation, microplastics, polystyrene, functional response

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INTRODUCTION

Plastics have become a universal material due to their numerous properties. The mass production of plastics started in the 1950s at just one million tons per year. Nowadays, production has reached 335 million tons worldwide (Meng et al., 2020). Over 8 million tons of mostly single-use plastics enter the ocean each year (Jambeck et al., 2015), despite increasing recycling efforts and public awareness around the world. According to recent estimates, between 1.15 and 2.41 million tons of plastic are transported from rivers to the sea (Réu et al., 2019). In the past, research on the impact of plastic has focused on marine environments, with comparatively fewer studies conducted in freshwater habitats. Plastics are entering all ecosystems in all sizes, and large pieces disintegrate into smaller particles due to physical or chemical degradation. The resulting small particles below 5 mm are called secondary microplastics (Hartmann et al., 2019). In addition to these secondary microplastics, primary microplastics in the form of beads and pellets are manufactured to be used in personal care products and in industrial cleaning. Nanoplastics (size range from 1 to 1,000 nm) are frequently used in the cosmetics industry, though their use is decreasing (Strungaru et al., 2019). Particles from tire wear and shedding of microfibers from synthetic clothing also pollute aquatic ecosystems (Barboza et al., 2018). Unlike large plastic debris, microplastics are invisible to the naked eye and cannot be removed from the environment for recycling. The estimated number of microplastics smaller than 100 μm is still underestimated also in the marine environment (Lindeque et al., 2020).

Microplastic particles, particularly spheres, can be ingested by numerous zooplankton species (Kögel et al., 2020; Zheng et al., 2020). For example, rotifers, copepods, and cladocerans have the ability to ingest polystyrene microbeads ranging from 1.7 to 30.6 μm (Scherer et al., 2018). Typically, in these studies, microspheres are provided as defined and standardized food particles. Therefore, care is taken to avoid any clumps or aggregates and spheres are sonicated before use to assure that only singular particles are present. However, the plastic's surface serves as a physical substrate for microorganisms such as bacteria, fungi, algae and heterotrophic protists, which altogether form a complex biofilm. When the particles are in the size range of nanoparticles, they become enveloped within a biofilm (Martel et al., 2014; Ikuma et al., 2015; Summers et al., 2018). This conglomerate of plastics and biota is called "plastisphere" (Zettler et al., 2013; Kirstein et al., 2019; Amaral-Zettler et al., 2020). Moreover, extracellular polymeric substances (EPS), particularly transparent exopolymers particles (TEP), produced by microorganisms play a significant role in the formation of nano- and microplastic agglomerates (Cunha et al., 2019). These aggregated particles might be ingested by invertebrates having low feeding selectivity, such as filter-feeders (Scherer et al., 2017). The ingested plastic aggregates might then affect the fitness of the consumers (Vroom et al., 2017). Herbivorous rotifers are filter-feeding metazoans and their feeding behavior and low selectivity allows them to ingest small particles such as microplastics. In the freshwater food web, rotifers play a pivotal role in the energy transfer from primary producers to secondary consumers and potentially also transfer pollutants to higher trophic levels through ingestion and accumulation (Snell and Janssen, 1995). Several studies show that brachionid rotifers can ingest polystyrene microbeads with negative effects on their reproduction and growth rate (Juchelka and Snell, 1994; Baer et al., 2008; Jeong et al., 2016, 2018). Thus, these properties make rotifers very suitable animals for studying microplastics ingestion.

The aim of the present study was to investigate the ingestion of microplastics by the cosmopolitan freshwater planktonic rotifer *Brachionus calyciflorus*. We examined the ingestion of three sizes of polystyrene microspheres as single food particles, in association with a similar-sized food alga and as biogenic aggregated microspheres through bacteria and exopolymers particles. Moreover, we characterized the size of the aggregated microplastics and the number of singles MS.

We tested the following hypotheses: (i) the ingestion of microspheres is size-dependent and influenced by the presence of algal food, and (ii) the biogenic aggregation of microspheres influences their ingestion.

MATERIALS AND METHODS

Polystyrene Microsphere

As microplastic particles, we used polystyrene microspheres (MS) of three different diameters: 1.03, 3.06, and 5.73 μm; for convenience, we refer to them as 1, 3 and 6 MS (Polysciences, Inc. Fluoresbrite® YG Polystyrene Microspheres, USA) (see **Table 1**). A stock solution was prepared with deionized MilliQ water under sterile conditions to minimize bacterial growth. To keep the MS as singular particles, each stock solution was sonicated for 30 min and was mixed using a vortexer.

Organism

Stock cultures of all experimental organisms, algae and rotifers, were kept in glass flasks in a modified Woods Hole WC-medium (Guillard and Lorenzen, 1972) with regular substitution of the medium to sustain the continuous growth phase. Cultures were kept at 20°C in a light-dark cycle of 14:10 h and a light intensity of 35-μM photons s^{-1} m^{-2}. We used the herbivorous monogononta rotifer *Brachionus calyciflorus* s.s. Pallas 1776 [strain IGB (Paraskevopoulou et al., 2018)] as a generalist

TABLE 1 | Characteristics of the phytoplankton: *Synechococcus elongatus*, *Chlorella vulgaris,* and coccal green algae.

Phytoplankton	*S. elongatus*	*C. vulgaris*	Coccal green algae
Diameter (μm)	1.40	2.60	4.30
Shape	oblong	spherical	oblong
Polystyrene MS	1	3	6
Diameter (μm)	1.03	3.06	5.73
Shape	spherical	spherical	spherical

Note: for S. elongatus sizes were taken from Schälicke et al. (2019), for C. vulgaris and coccal green algae the size were taken prior the experiment. Characteristic of polystyrene microspheres from Fluoresbrite® YG Microspheres technical data sheet.

consumer. Stock cultures were maintained with the food alga *Monoraphidium minutum* (SAG 243-1, culture collection of algae, Göttingen, Germany). During the three days prior to the experiment, the rotifers were fed daily with 1×10^6 cells ml^{-1} to assure constant food saturation conditions (Fussmann et al., 2005). For the treatments with additional food algae, we used the phytoplankton species *Synechococcus elongatus* (SAG 89.79), *Chlorella vulgaris* (SAG 211-11b), and an unidentified coccal green alga grown under the same conditions as *M. minutum*.

Aggregation Experiment

To study the effect of a natural bacteria community on potential aggregation of the MS, we incubated the polystyrene MS in a pre-filtered water sample. In May, 2019 we took a natural water sample (1l) from the river Havel in the urban area of Potsdam (Germany). The sample was pre-filtered with a 30-μm mesh and afterwards filtered through a glass fiber filter (Whatman®, GF/C), retaining most microplankton and allowing bacteria to pass through. In order to avoid any differences among the MS sizes the experiment with the natural water was conducted contemporarily and on the same day. After incubating the MS in 3 ml for 72 h in a rocking shaker, the degree of aggregation was quantified in two steps. The concentration used for the experiment are shown in **Table 2** and they were the same used for the ingestion experiment. We first quantified the number of single particles (not aggregated) in a subsample for each concentration using a haemocytometer (Paul Marienfeld GmbH & Co., Germany) and a microscope (Zeiss Axioskop 2, Germany). Because we could not unambiguously determine the number of particles within one aggregate due to the formation of clumps, we filtered 1 ml subsample from each concentrations through 0.2-μm polycarbonate filters and stained them with DAPI (4′,6′-diamidino-2-phenylindole) and alcian blue. DAPI specifically binds to double-stranded DNA and polyphosphate (Zafiriou and Farrington, 1980), and with an aqueous solution of 0.02% alcian blue we could stain the polysaccharides contained in the transparent exopolymer particles (TEP) forming the aggregates. The stained polysaccharides were inspected microscopically on a glass slide, covered with immersion oil and a cover slip (Passow, 2002). From each sample, 30 pictures were randomly taken and the area of each aggregate was quantified using the open-source software ImageJ (Schneider et al., 2012). We quantified the aggregation for each particle size and concentration separately. We tested for differences in the frequency distribution (log2 scaled) among concentrations within each particle size using a test of homogeneity. We found no differences among the concentration in the 3 and 6 μm spheres ($\chi^2 = 9.7$ and 5.2, respectively; $p > 0.6$), but some differences within the 1 μm spheres with smaller aggregates at the lowest concentration ($\chi^2 = 19.9$, $p = 0.035$). We then pooled the data for each particle size and compared the median sizes among the different sizes. These were highly significantly different from each other (Kruskall-Wallis-test, $p = 0.005$).

TABLE 2 | Concentration and (bio) volume of phytoplankton (*Synechococcus elongatus*, *Chlorella vulgaris,* and coccal green algae) and polystyrene MS (1, 3, and 6 μm) used in the aggregation and ingestion experiments.

Concentration	*S. elongatus*	*C. vulgaris*	Coccal green algae
	Phytoplankton (cells/ml)		
1st	5.3×10^5	8.1×10^4	2.5×10^4
2nd	1.2×10^6	2.1×10^5	6.2×10^4
3rd	2.4×10^6	4.1×10^5	1.6×10^5
4th	4.8×10^6	8.2×10^5	8.1×10^5
5th	/	1.6×10^6	1.6×10^6
Concentration	**1**	**3**	**6**
	Polystyrene MS (p/ml)		
1st	1.3×10^6	5.0×10^4	7.7×10^3
2nd	3.0×10^6	1.3×10^5	1.9×10^4
3rd	6.0×10^6	2.5×10^5	5.0×10^4
4th	1.2×10^7	5.0×10^5	2.5×10^5
5th	/	1.0×10^6	5.0×10^5
Concentration	***S. elongatus* and 1 MS**	***C. vulgaris* and 3 MS**	**Coccal green algae and 6 MS**
	Biovolume (μm^3/ml)		
1st	7.6×10^5	7.5×10^5	7.6×10^5
2nd	1.7×10^6	1.9×10^6	1.9×10^6
3rd	3.4×10^6	3.8×10^6	4.9×10^6
4th	6.9×10^6	7.5×10^6	2.5×10^7
5th	/	1.5×10^7	4.9×10^7

Ingestion Experiment

We measured the ingestion rate in three treatments: microspheres as the sole food source (MS), microspheres in association with algae (MS + algae) and microspheres incubated with pre-filtered, natural water in the presence of bacteria (MS aggregated) (**Table 2**). For all three treatments we used several particle concentrations (**Table 2**), and for each particle concentration we had three replicates. From these data, the functional response was calculated; see below. Seventy-five randomly chosen adult rotifers from the prepared cultures were transferred into 3-ml particle suspension on a UVA-transparent polystyrene 12-well microtiter plate (Greiner Bio-One). After 2 min of exposure in a rocking shaker, the rotifers were washed with the medium, narcotized with carbonated water and preserved in Lugol's solution. The exposure time of 2 min takes the short gut passage time of <10 min and the practicability of the quantification of the ingested MS into account. At high concentrations, the particles form clumps in the animals' gut and cannot be quantified. The maximum particle concentration was chosen in order to cover the full range of the functional response until saturation (Mohr and Adrian, 2000; Fussmann et al., 2005; Seifert et al., 2014).

The number of ingested MS per individual rotifer was quantified using an epifluorescent microscope (Zeiss Axioskop 2, Germany). Between 30 and 36 rotifers per sample were transferred to a microscope slide and carefully squeezed under a coverslip until the MS were compressed into a single layer (Baer et al., 2008). The MS found in the gut and in the trophi of each rotifer were counted either directly or, in some cases, pictures of the gut were taken and the MS number was quantified with ImageJ. All experiments were run in triplicate. To study the effect of algae as an accompanying food source, algae of similar size were added for each size of MS. We used an additive design so that the addition of algae doubled the total volume of MS particles per treatment (**Table 2**). Prior to the addition, the cell number from stock cultures was quantified with a haemocytomer (Paul Marienfeld GmbH & Co., Germany) and the addition was adjusted to equalize the volume of the respective MS.

Statistical Analysis

The resource-dependent consumption of food items by a consumer can be described as functional response curves. The type I functional response has a linear relationship between ingestion and resource concentration until a saturation is reached and ingestion remains constant with increasing resource concentration. The type II functional response exhibits a saturation function where ingestion approaches asymptotically its maximum. The type III functional response has a sigmoidal shape with very low ingestion at low resource concentration. To fit and compare the consumer functional response, we followed the procedure of Pritchard et al. (2017). Type II and III can be characterized by:

$$N_e = N_0\left(1 - \exp\left(aN_0^q\left(hN_e - T\right)\right)\right) \tag{1}$$

N_0 is the initial resource concentration; T is the experimental time; a is the instantaneous resource attack rate of the consumer; h represents the time spent subjugating, ingesting and digesting the resource and q is the scaling exponent. Type II and III functional responses differ in their value for q: When $q = 0$ a type II functional response prevails, when $q > 0$ the sigmoidal type III prevails. The number of MS ingested during the experimental period of 2 min was expressed as MS ingested per hour.

For fitting the functional response, we used the r-package FRAIR v0.5.100 (Pritchard et al., 2017). Three steps were involved: (i) model selection, (ii) model fitting, and (iii) comparison of fit and coefficients. The model selection step was used to distinguish between Type-II (saturation function) and Type-III (sigmoidal) functional response. When the evidence for a Type-II response was positive, we fitted a linear functional response Type I and a functional response Type II and compared the models using the Akaike information criterion (AIC). After providing starting estimates and fixed values of the parameters, the model was optimized using maximum likelihood estimation (MLE) with the bbml package and the function ml2 (Bolker, 2008). The last step included comparisons of the fitted coefficients through two approaches: the delta or difference method of Juliano (2001), and non-parametric bootstrapping of the raw data. We compared the fitted coefficients for each MS size separately. The comparison of the fitted coefficients with the delta or difference methods of Juliano (2001) yielded the difference between two fitted coefficients. The functional responses were plotted with empirical approximations of 95% confidence intervals (CI) based on the bootstrapped model fits for the number of MS ingested per rotifer. The lack of overlap between the CIs of the model parameters was considered equivalent to a null hypothesis test (Pritchard et al., 2017). All statistical analyses were carried out using R 3.4.3 (R Core Team, 2017).

RESULTS

Aggregation Experiment

The incubation for 72 h with the natural bacteria community led to significant formations of aggregates that were size-specific. The percentage of single particles was the lowest in the smallest size: 1-μm MS 16 ± 1% (mean ± SE), and increased with the biggest sizes: 42 ± 3% (mean ± SE) for the 3-μm MS and 67 ± 8% (mean ± SE) for the 6-μm (**Figure 1A**). The vast majority of the 1-μm MS occurred in aggregates, whereas the majority of the 6-μm MS were single particles. The area of the aggregates also showed a size-specific pattern. The smaller the MS, the smaller the area of the aggregates: for the 1-μm, the area was 64 ± 16 μm^2 (mean ± SE), for the 3-μm 133 ± 18 μm^2 (mean ± SE), and 245 ± 33 μm^2 (mean ± SE) for the 6-μm spheres (**Figure 1B**). Staining with DAPI revealed the presence of bacteria in the size range of 0.5–2.0 μm (**Figures 2A,B**) and the alcian blue revealed that transparent exopolymer particles were involved in the formation of the aggregate (**Figures 2C–E**).

Functional Response

Ingestion Rate

Brachionus calyciflorus fed on all three tested sizes of polystyrene MS (**Figures 3A,B**), in all combinations: microplastics alone, microplastics in association with food algae and microplastics incubated with bacteria in natural water. For each size and treatment, the number of MS ingested by the rotifers increased with the increasing concentration of MS in suspension, reaching a plateau. The comparison with the different types of functional responses, based on the AIC (**Table 3**), revealed a Type-II functional response (**Figures 4A–C**). Subsequently, all functional response curves were fit by the Type-II functional response and the handling time and attack rate were calculated (**Table 4**).

With regard to the 1- and 3-μm MS, *B. calyciflorus* showed the highest ingestion of MS when these were aggregated with biogenic particles though this was less pronounced for the 3-μm MS (**Table 5**). We found lower ingestion when the MS were provided as the sole food source and in association with algae. The opposite pattern was found for the 6-μm MS, where the ingestion was higher when they were provided as the sole food source and lower when aggregated with biogenic particles (**Table 5**). The maximum number of ingested 1-μm MS occurred at the highest concentration of MS aggregated with biogenic particles, with 3155 MS h^{-1}. The 3-μm MS were ingested at a rate of 895 MS h^{-1} and the maximum ingestion occurred at the highest concentration of MS aggregated with biogenic particles. The maximum ingestion of 1483 MS h^{-1} occurred with the 6-μm MS at the highest concentration as single particles (**Table 5**).

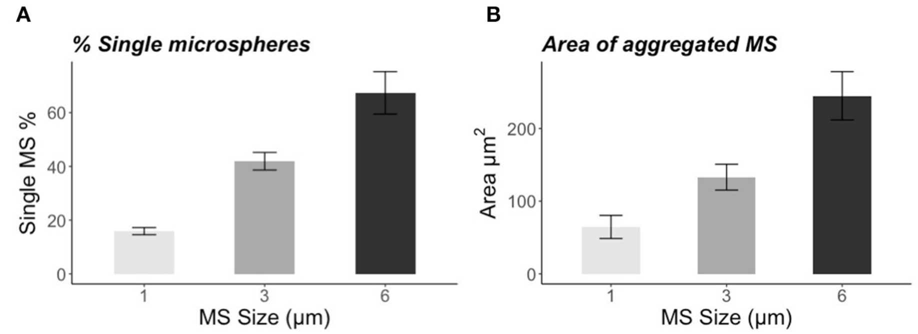

FIGURE 1 | (A) Percentage of single microspheres (1, 3, and 6 µm) found in the sample after 72 h of incubation with natural water and bacteria. **(B)** Area (µm^2) of aggregated microspheres (1, 3, and 6 µm) measured with ImageJ. The error bars represent SE.

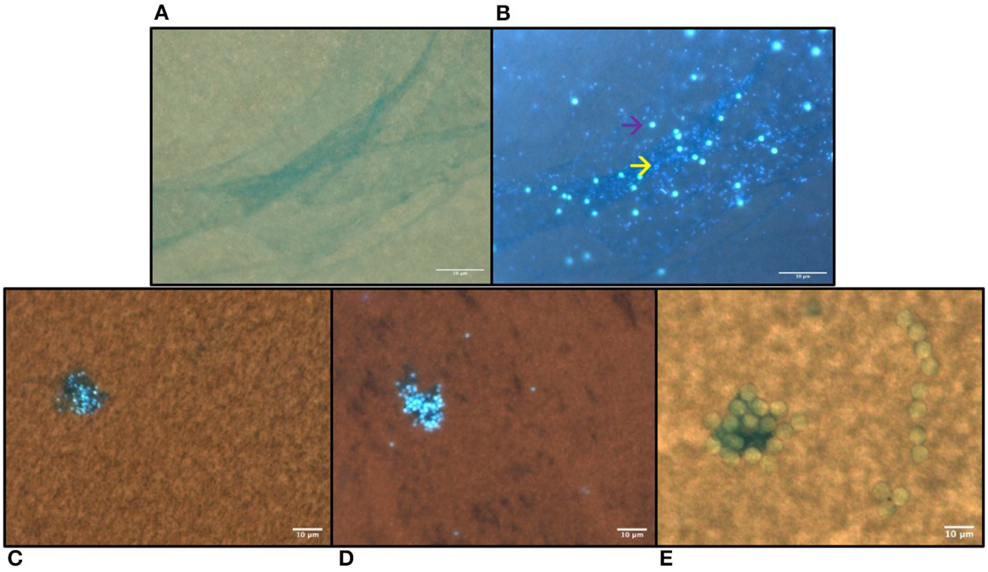

FIGURE 2 | Microplastic aggregation formed after 72 h of incubation in prefiltered natural freshwater stained with alcian blue and DAPI. **(A)** Transparent exopolymer particles (TEP) stained with alcian blue and DAPI in 1 µm MS sample visualized under bright light and **(B)** under UV-light, the yellow arrow indicates the bacteria and the purple arrow indicates the MS; **(C)** 1 µm, **(D)** 3 µm MS aggregation visualized with merged UV-light and bright light **(E)** 6 µm. The microspheres are visible also under the UV light **(B–D)**. The scale bar is 10 µm.

Attack Rate and Handling Time

The differences in the ingestion rates are reflected in the attack rate and the handling time: For the 1-µm MS, the attack rate of the aggregated particles increased by a factor of 6.65 compared to the singular particles (**Table 4**). This effect leveled off for the 3-µm MS with a factor of 1.57 and reversed for the 6-µm MS where the attack rate was 9 times lower for the aggregated MS (**Table 4**). The handling time differed much less between these two treatments.

DISCUSSION

The scope of this study was to investigate the ingestion of microplastics in the rotifer *B. calyciflorus* mediated by biogenic aggregation and in the presence of food algae. The three sizes of polystyrene MS (1, 3, and 6 µm) were ingested in all treatments. Our study has shown that the ingestion of the three sizes as the sole food, in association with algae or aggregated with biogenic particles followed the Holling's type II model and the ingestion can be influenced by the aggregation of MS.

Biogenic Aggregation of Microspheres

We found aggregations of the polystyrene microspheres (MS) in each concentration, after an incubation of 72 h in prefiltered natural freshwater. Staining these aggregates with DAPI and alcian blue revealed that they contained a community of bacteria and transparent exopolymer particles (TEP). The exopolymers are contained in the TEP encapsulated and trapped the microplastic particles to form an amorphous matrix. The presence and persistence of microplastics has been shown to

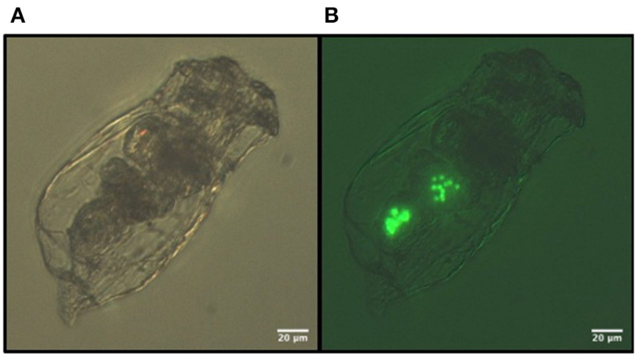

FIGURE 3 | Images of the 3 µm fluorescent polystyrene MS ingested by *B. calyciflorus*: **(A)** bright light, **(B)** fluorescent light.

TABLE 3 | The results of logistic regressions for the selection of type II are shown, together with the Akaike information criterion (AIC) values for fitted type II (see Equation 1 in the text) and type I functional response models for three sizes of MS (1, 3, and 6) and the treatments (MS, MS + algae, and MS aggregated).

		MS	MS + algae	MS aggregated
			1	
Logistic regression type II	**1st term**	-2.19×10^{-8}	-3.35×10^{-8}	-7.48×10^{-8}
	P	<0.001	<0.001	<0.001
AIC type II		**58504**	**79550**	**586746**
AIC type I		60509	84001	658551
			3	
Logistic regression type II	**1st term**	-9.40×10^{-8}	-4.80×10^{-8}	-1.01×10^{-8}
	P	<0.001	<0.001	<0.001
AIC type II		**163607**	**207505**	**182189**
AIC type I		207909	211959	226209
			6	
Logistic regression type II	**1st term**	-1.27×10^{-8}	-3.37×10^{-8}	-2.63×10^{-8}
	P	<0.001	<0.001	<0.001
AIC type II		**156904**	**65376**	**63665**
AIC type I		172702	113776	73698

The bold values indicates the AIC for the choosen model.

enhance the agglomeration of particulate matter and micro-algal cells (Kettner et al., 2019). Several studies have shown that bacteria and glycoproteins contribute to the formation of plastic aggregates. This confirms the high aggregation potential of microplastics to rapidly coagulate with biogenic particles, forming pronounced aggregates within a few days (Michels et al., 2018; Summers et al., 2018). Here, we provide further evidence on how fast the formation of aggregates can take place when the pristine plastic MS were exposed to prefiltered natural freshwater. The percentage of aggregated MS after 72 h of incubation with prefiltered natural water was size-specific: The smaller the particles, the higher the number of aggregated particles. In detail, the percentage of single particles was below 20% for the 1-µm spheres and the aggregates were smaller than those from larger spheres. This finding indicates that the small particles were easily trapped by the TEPs, although larger aggregates are not formed. On the contrary, a higher percentage of single MS 3 (>40%) and 6 µm (>60%) was found, but the aggregated MS were larger. This indicates that the larger particles were less efficiently captured by TEPs, but once they were caught, the aggregates grow larger. We did not find differences in the aggregation pattern within the different sphere sizes at different concentrations (except for a slightly higher share of small aggregates in the 1 µm MS at the lowest concentration); however, it should be taken into account that the absolute numbers of the tested particles differed among sizes because of size-specific differences in functional response curves (see **Table 1**).

The process of aggregation might change over the season due to different numbers and composition of bacteria, temperature and water chemistry, however, we believe that the process itself and the resulting pattern does not change much. In general, the aggregation of detritus and living organisms (bacteria, algae, fungi, and microzooplankton) is a common phenomenon in lakes and oceans, known as lake or marine snow (Grossart and Simon, 1993; Silver, 2015) and a substantial incorporation of plastics into these aggregates seems very likely. Another process that alters the properties of MP in the environment is due to aging and the association with colloids which modifies the particles' surface (Alimi et al., 2018). Thus, the environmental conditions together with the specific properties of the particles affects their aggregation behavior in aquatic environments (Wang et al., 2021).

It is still a matter of debate whether the microbial community associated with plastic is specific to that kind of substrate or to an unspecific community from the surrounding water (Amaral-Zettler et al., 2020). Either way, aggregate formation alters the properties of the plastic, leading to higher sedimentation, or altered ingestion by consumers (Besseling et al., 2017; Alimi et al., 2018; Summers et al., 2018).

Ingestion of Microplastics Particles as the Sole Food Source

The ingestion of the MS (1, 3, and 6 µm), even if considered below the optimal size of feeding efficiency (Rothhaupt, 1990a), showed a Type-II functional response model. Previous studies demonstrated that the highest feeding efficiency for *B. calyciflorus* and closely related *Brachionus* species is in the range of a 3.5- to ∼10-µm equivalent spherical diameter (ESD) (Vadstein, 1993; Baer et al., 2008) or even higher (Pagano, 2008). We found a Type-II functional response for the 1-µm MS, considered in the similar size range of (large) bacteria or small algae. In general, very small particles were ingested with lower efficiency, but the presence of aggregated small particles can increase the ingestion efficiency. As in Rothhaupt (1990b), the larger MS (6 µm) are preferably ingested in terms of biovolume than the smaller MS (1 and 3 µm), as the attack rate is higher. Comparing the ingestion rate of 3-µm MS as a sole food source from our study

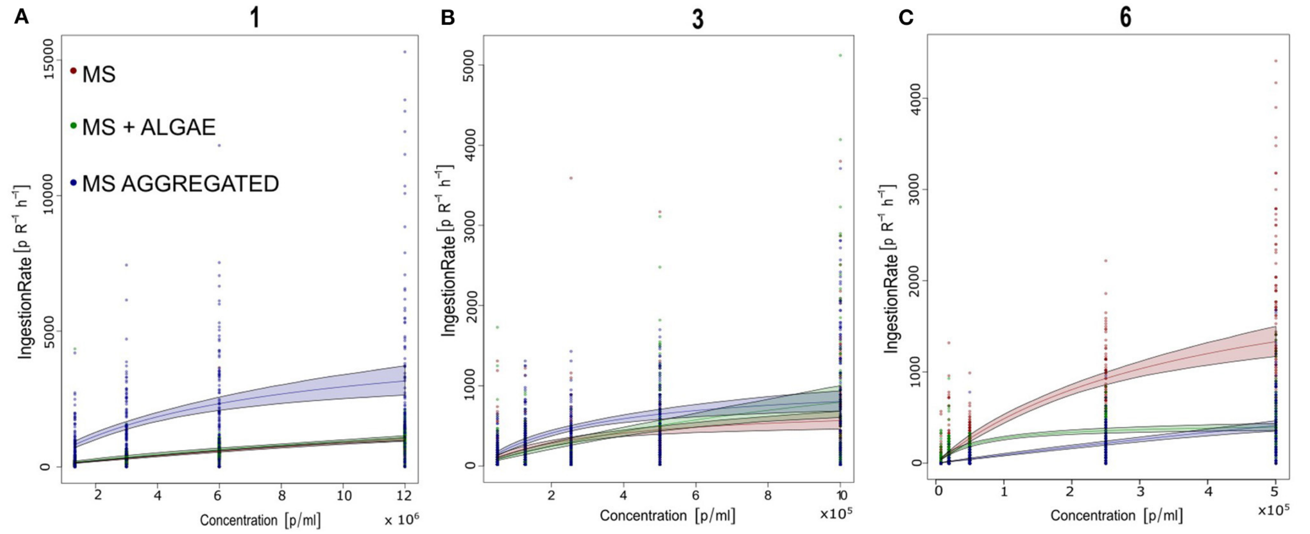

FIGURE 4 | Number of polystyrene microspheres ingested by *Brachionus calyciflorus* (IGB) within 1h at offered microsphere densities of 10^3-10^7 particles / ml. Data from all 3 replicates are shown. The curves represent the best-fitting functional response model (Rogers Type II functional response). Parameter estimated for each treatment and size are given in **Tables 3**, **4**. **(A)** 1 µm; **(B)** 3 µm; and **(C)** 6 µm.

TABLE 4 | Parameter estimates from type II (see Equation 1 in the text) functional responses for three sizes of MS (1, 3, and 6) and the treatments (MS, MS + algae, MS aggregated).

		MS	**MS + algae**	**MS aggregated**
			1	
Type II	Attack rate	1.11×10^{-4} $\pm 5.90 \times 10^{-7}$ ($p < 0.001$)	1.36×10^{-4} $\pm 7.41 \times 10^{-7}$ ($p < 0.001$)	$7.38\ x10^{-4}$ $\pm 2.54\ x1\ 0^{-6}$ ($p < 0.001$)
	Handling time	2.33×10^{-4} $\pm 5.67 \times 10^{-6}$ ($p < 0.001$)	3.35×10^{-4} $\pm 5.02 \times 10^{-6}$ ($p < 0.001$)	2.03×10^{-4} $\pm 7.70 \times 10^{-7}$ ($p < 0.001$)
			3	
Type II	Attack rate	20.62×10^{-4} $\pm 1.63 \times 10^{-5}$ ($p < 0.001$)	13.06×10^{-4} $\pm 8.13 \times 10^{-6}$ ($p < 0.001$)	31.36×10^{-4} $\pm 2.08 \times 10^{-5}$ ($p < 0.001$)
	Handling time	13.44×10^{-4} $\pm 9.54 \times 10^{-5}$ ($p < 0.001$)	5.24×10^{-4} $\pm 7.89 \times 10^{-6}$ ($p < 0.001$)	9.56×10^{-4} $\pm 5.28 \times 10^{-6}$ ($p < 0.001$)
			6	
Type II	Attack rate	61.57×10^{-4} $\pm 3.39 \times 10^{-5}$ ($p < 0.001$)	72.84×10^{-4} $\pm 6.61 \times 10^{-5}$ ($p < 0.001$)	9.46×10^{-4} $\pm 9.17 \times 10^{-6}$ ($p < 0.001$)
	Handling time	4.24×10^{-4} $\pm 3.03 \times 10^{-6}$ ($p < 0.001$)	22.51×10^{-4} $\pm 1.09 \times 10^{-5}$ ($p < 0.001$)	3.06×10^{-4} $\pm 2.58 \times 10^{-5}$ ($p < 0.001$)

Data are the original maximum likelihood estimates ± SE and the p-values.

with the ingestion of the similar-sized food alga *Monoraphidium minutum,* we found a similar maximum ingestion rate as in Fussmann et al. (2005), using the same algal and rotifer strains but applying the radioisotope method. The highest ingestion based on biovolume was found for the 6-µm MS. The volume of one 6-µm MS is eight times larger than one 3 µm in size, and 216 times larger than a 1-µm MS. Thus, the total microplastics uptake of 1-µm MS was lower than for the larger-sized MS. However, toxicity does not necessarily correlate with the total amount of ingested plastic. Mueller et al. (2020), found for freshwater nematodes that the toxicity increased with the surface area-to-volume ratio of the applied microspheres. The absolute

TABLE 5 | The highest ingestion of microspheres per rotifers per hours.

	MS	MS+ algae	MS aggregated
		Maximum ingestion per hours (mean ± SE)	
1	1014 ± 34	1066 ± 44	3155 ± 330
3	639 ± 60	752 ± 107	895 ± 78
6	1483 ± 89	439 ± 27	415 ± 34

The ingested microspheres for each rotifer are multiplied per hours and expressed as mean of all three replicates ± SE.

concentration of microplastics in this study were quite high. However, for this size fraction of 1–6 μm, no reliable data on the distribution and abundance in the field are available and it is suggested that with increasing fragmentation of larger particles the concentration of such small particles strongly increases. Most field studies about microplastics are limited by the sampling methodology and the respective detection limit of the devices that were used (Besseling et al., 2019). A commonly used lower limit of mesh size lies between 300 and 800 μm. Applying smaller mesh sizes would retain a broader fraction of MPs (Wiggin and Holland, 2019) but would be more difficult to handle. A huge amount of microplastics enters the environment via the discharge from waste water treatment plants. Whereas particles larger than 10 μm are relatively efficiently removed, smaller particles likely enter the environment in higher rates (Chen et al., 2018). Once these particles are released to the environment, they might accumulate through sedimentation as aggregates in regions with low flow velocity, for example in reservoirs. Resuspension of plastics from the sediment might then makes it available again for the biota (Besseling et al., 2019).

Ingestion of MS Together With Food Algae

Rotifers are often regarded as unselective filter-feeders; however, some degree of selectivity has been found (Starkweather, 1980). In experiments with flavored polystyrene spheres (Demott, 1986), it was found that *B. calyciflorus* fed preferentially on 12-μm spheres, but did not discriminate against those with adsorbed algal flavors (Snell, 1998). Similarly, large daphnids exhibit no taste discrimination for small beads and smaller daphnids show some degree of taste and acute size discrimination. Contrarily, calanoid copepods can evaluate the resource quality in small and large particles and discriminate accordingly (Scherer et al., 2017). Thus, non-discriminating filter feeders are expected to take up more MP than raptorial feeders which might lead to reduced food intake and population growth and supports selective feeders (Setälä et al., 2016). In our experiment, we added the algae to the tested MS concentrations, leading to an increase in the total number of available particles. Thus, with algae added to low microplastics concentrations when the functional response curve is nearly linear, the uptake of the spheres is not necessarily reduced. Only at high particle concentrations, is the uptake of plastic particles expected to decrease due to algal additions.

Ingestion of MS Aggregated With Biogenic Particles

The biogenic formation of aggregates within 72 h specifically altered the ingestion of smaller and larger microplastics particles.

It is known from previous studies that the diet of *B. calyciflorus* includes not only algae but also bacteria to some extent. Bacteria may be utilized as food (Raatz et al., 2018) and, moreover, it seems likely that *Brachionus* can also ingest larger detrital particles and bacterial aggregates, deriving nutritional benefit from those cells as well (Starkweather et al., 1979).

The difference between the parameters' estimation when the MS are associated with biogenic particles shows us that the ingestion of MS is influenced by their size. Despite the size selectivity of the rotifers *B. calyciflorus*, the presence of aggregated MS increases the feeding efficiency of particles considered below the optimal size, such as 1 and 3 μm, by making them more available when aggregated; ingestion of otherwise edible MS (6 μm) can be prevented through aggregation. These results are reflected in the differences in the calculated attack rates between the experiments with and without aggregation. When the MS were ingested, it was not possible to recognize whether they were ingested as singular or aggregated particles. Nevertheless, the reduction in the attack rate of the 6-μm MS when aggregates were present indicates that the large particles were inedible and interfered with the ingestion of the well-edible singular MS. The high attack rate for the aggregated 1-μm MS indicates that the aggregates were of a well-edible size. However, technically, the effective number of particles was lower and the attack on one aggregate represents the attack on all MS within this aggregate. Zhao et al. (2018) found that the aggregation of small MP (0.5–1 μm) and nanoplastics (30 and 100 nm) facilitated the uptake from mussels. Besides the increase in particle size through aggregation, changes in surface topography and density were found. Once incorporated into aggregates, several transformations can occur, including an increase in the effective particle size and change in surface topography and density as a result of the physical and biological processes in the aggregate microcosm (Zhao et al., 2018).

Once a food item was captured, the calculated handling time varied only little among these two treatments. Thus, the similar handling times of singular MS and aggregated MS indicates that the handling time increases proportionately to the number of MS within the aggregate. This means that the "effective" handling time of an aggregate with 10 MS can be 10 times longer than the handling time of one singular MS in order to end up at the same calculated handling time. Overall, the ingestion and the effect on life history and survival can differ depending on whether microplastics are provided in a pristine state or aged and/or in natural water.

We did not test for toxicity; however, the toxicity of polystyrene nanoplastics to the rotifer *Brachionus plicatilis* was lower when the particles were provided in natural sea water compared to reconstituted sea water (Manfra et al., 2017). A potential reason for that was the interplay between surface charge, aggregation and salt. In a study on marine copepods, the aging of plastics promoted their uptake by marine zooplankton (Vroom et al., 2017). Even the type of plastic on which a natural biofilm developed influenced the food quality of the biofilm for a freshwater snail (Vosshage et al., 2018). These results underline the importance of experiments under near-natural conditions to better estimate the effect of microplastics on the biota and to complement standardized toxicological tests.

CONCLUSION

Our results demonstrate that the aggregation of MS accelerates the ingestion of smaller MS particles and prevents the ingestion of the largest ones in the freshwater rotifer *B. calyciflorus*. The aggregation potential of microplastics has to be considered in order to recreate the environmental interaction between microplastics and aquatic organisms. The aggregation processes, together with degradation processes, are the cause of physical and chemical alteration of pristine microplastics. These two processes might alter the response of the aquatic organism to microplastics in laboratory and natural environments. In particular, non-selective filter feeders such as crustaceans and rotifers that feed mainly size-specific (Burns, 1968; Geller, 1981; Bern, 1990; Brendelberger, 1991; Baer et al., 2008; Scherer et al., 2017) are affected by aggregation processes. Consequently, the variation in the MS size range might lead to an increased interaction between the smallest particles and aquatic consumers. To test for the response of aquatic organisms to microplastics, the increased or decreased ingestion of microplastics is fundamental to take into account. In order to fill this gap, further studies are needed on the direct and indirect effects of aggregated microplastics on the life cycles of aquatic consumers.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

CD, JP, and GW designed the experiment. CD conducted the experiment and performed the analysis of the data. CD and GW wrote the manuscript. All authors discussed the results and provided extensive comments on the manuscript concerning the analysis, interpretation, and writing. All authors approved the final version and have accepted accountability for all aspects of the work.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fenvs.2020.574274/full#supplementary-material

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Chapter 3

Manuscript 2

Variable fitness response of two rotifers species exposed to microplastics particles: the role of food quantity and quality

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Variable Fitness Response of Two Rotifer Species Exposed to Microplastics Particles: The Role of Food Quantity and Quality

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Abstract: Plastic pollution is an increasing environmental problem, but a comprehensive understanding of its effect in the environment is still missing. The wide variety of size, shape, and polymer composition of plastics impedes an adequate risk assessment. We investigated the effect of differently sized polystyrene beads (1-, 3-, 6-µm; PS) and polyamide fragments (5–25 µm, PA) and non-plastics items such as silica beads (3-µm, SiO_2) on the population growth, reproduction (egg ratio), and survival of two common aquatic micro invertebrates: the rotifer species *Brachionus calyciflorus* and *Brachionus fernandoi*. The MPs were combined with food quantity, limiting and saturating food concentration, and with food of different quality. We found variable fitness responses with a significant effect of 3-µm PS on the population growth rate in both rotifer species with respect to food quantity. An interaction between the food quality and the MPs treatments was found in the reproduction of *B. calyciflorus*. PA and SiO_2 beads had no effect on fitness response. This study provides further evidence of the indirect effect of MPs in planktonic rotifers and the importance of testing different environmental conditions that could influence the effect of MPs.

Keywords: microplastics; population growth rate; polystyrene; polyamide; silica beads; fitness response; rotifers; *Brachionus fernandoi*; *Brachionus calyciflorus*; egg ratio

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1. Introduction

Plastic pollution is continuously increasing and without effective control, it will become more and more serious in the future. Currently, about 60 to 80% of the litter material in the environment is plastic [1].Plastic litter has a broad size, ranging from large plastic fishing nets and fragments of containers to very small particles in the millimeter or micrometer range and down to nanoparticles below 1 µm. Microplastics (MPs) have been found virtually everywhere in both terrestrial and aquatic ecosystems such as rivers, lakes, and oceans [2,3]. Plastics can enter aquatic systems from waste water treatment plants [4], through surface runoff [5–7], or from being deposited through the air [8]. Many studies have reported that microplastics harm a wide variety of aquatic organisms: the ingestion of large amounts of microplastics by aquatic organisms can reduce energy reserves and can affect growth and reproduction, which consequently increases the mortality of, for example, crustaceans [9], fish, mollusca, anellida[10]. The uptake of MPs from even smaller zooplankton can make them more available to larger taxa [11]. However, evidence supporting a quantitative risk assessment for microplastics is still missing due to a lack of method standardization and result ambiguity [12].A study from Sun et al. [13] showed that small-sized microplastics (0.07 µm; 0.05 µm) decreased rotifer survival and reproduction, whereas large-sized microplastics (0.7 and 7 µm) had no effect on rotifer life history traits. In contrast, Xue et al., [14] showed that larger microplastics (10–22 µm), in association with the algal food of similar size, suppressed the reproduction of rotifer, and this negative effect could be alleviated by increasing the food supply. Similar discrepancies have been found in studies conducted with the microcrustacean *Daphnia* [15,16]. Such discrepancies can result from different experimental set-ups, different shapes and types of plastics, and their relationship with food availability or food-size selection. Because of the shapes, size, and polymer composition of microplastics, there is still a necessity to better understand the effect of microplastics on aquatic organisms. Representative forms of microplastics in the environment are fragments and fibers, while microspheres are found less often [17,18]. Fragments and fibers accounted for 60% of all types of MPs, even in remote areas such as Lake Hovsgol in Mongolia [19]. One relevant component of shape is "spikiness". It was shown that spiky particles (e.g., filaments) and irregularly shaped particles (e.g., fragments) had showed a greater potential to harm animals than smooth particles such as spheres did, because spiky particles are more difficult to egest than smooth particles [20].

Rotifers are a widely distributed group of zooplankton that is present in all types of freshwater and brackish water bodies. They play an important role in aquatic food webs at the interface between primary producers and secondary consumers. As filter feeding organisms, rotifers have a very limited capability for food particle selection. Thus, rotifers cannot avoid the ingestion of plastic particles while they are feeding on natural food, such as algae. Therefore, rotifers are good model organisms for the study of and to understand how microplastic pollution influences aquatic ecosystems. Since field populations of rotifers are often resource limited [21–24], resource availability and natural fluctuation of algal growth should also be taken into account when estimating the risk of plastic pollution. We tested two closely related rotifers species, which were previously considered as one species, *Brachionus calyciflorus* and *Brachionus fernandoi*. These two species, even though they have a very similar morphology, exhibit different ecology and life history traits [25–27].

We used 1-, 3-, 6-, µm polystyrene beads (PS) because they are commonly used in toxicological studies of other organisms [28,29]. In addition, we used polyamide nylon fragments (PA) that were 5–25 µm in length because they are relevant in the field. As a non-plastic control, we used silica beads (SiO_2) (3 µm), and as the positive control, we used a treatment without artificial particles (only food algae). The different artificial beads were offered together with food algae at limiting and saturating food concentrations [30]. Moreover, the effects of the different microplastics were tested in *B. calyciflorus* in association with a different algal diet of *Monoraphidium minutum* and *Cryptomonas* sp., which is considered to be a high-quality food that can be ingested by rotifers [31,32].

The aim of this study was to quantify and compare the effect of differently sized and shaped particles made of different materials. We hypothesized that (1) the ingested beads could induce a decrease in the growth rate and reproduction of brachionids, acting as non-nutritional particles and that (2) the effect of microplastics is influenced by the food quantity and food quality.

2. Materials and Methods

2.1. Cultivation of Organisms

We used two species of pelagic rotifers, *Brachionus calyciflorus* s.s. (strain USA) and *B. fernandoi* (strain A10; [26]). Rotifers were raised in six well microtiter plates with sterile and vitamin-supplemented Woods Hole Culture Medium (WC) with saturating densities of *Monoraphidium minutum* (SAG 243-1, Culture Collection of Algae, University of Göttingen, Germany; ESD = 3.5 µm) as food. The phytoplankton species *Cryptomonas* sp. (Culture collection Göttingen, strain SAG-26-80; ESD = 5.9 µm [33]) was used as additional food in the food quality experiments [26]. Cultures were kept at 20 °C in a light–dark cycle of 14:10 h and at a light intensity of 35 µM photon s^{-1} m^{-2} photosynthetic active radiation (300–700 nm). Prior to the experiment, the rotifers were sieved through a mesh (30 µm) and were rinsed with sterile culture medium in order to separate them from their food. The carbon content was determined by an elemental analyzer (Euro EA 3000, HEKAtech Gmbh, Wegberg, Germany).

2.2. Microplastics

We used polystyrene microspheres (PS) of three different diameters as the microplastic beads in this study: 1.03, 3.06, and 5.73 µm (Polysciences, Inc. Fluoresbrite® YG Polystyrene Microspheres, Warrington, USA); for convenience, we refer to them as 1-, 3- and 6-PS. A stock solution was prepared with deionized MilliQ water under sterile conditions to minimize bacterial growth. To keep the beads as singular particles, each stock solution was sonicated for 30 min and was mixed using a vortexer. Stock suspensions of silica (SiO_2) beads in the size of 3.0 (cat. #SiO_2-F-3.0) were purchased from microParticles GmbH (Berlin, Germany). The stock solution was prepared using the same methods as the one prepared for the PS beads. Nylon fragments (5–25 µm) were prepared by size fractionating polyamide nylon-6 powder (nylon, PA) (Goodfellow; AM306010) with 25 µm cellulose filter (Whatman® qualitative filter paper, Grade 4) and 5 µm nylon mesh under a laminar flow hood. Prior to use, the microplastics were exposed to UV-light for 20 min to avoid bacterial contamination. For quantification, the fragments were suspended in ultrapure water and were analyzed with an electronic particle counter (CASY Schärfe System GmbH, Reutlingen, Germany) to assess the concentration and the total volume; moreover, a subsample was inspected using microscope, and the stock concentration and size range was assessed (Figure S2). The PS microbeads, the silica beads, and the PA fragments used in the present study have been previously used in numerous studies determining the effect and the ingestion of microplastics in pelagic and benthic organisms [28,29,34,35].

2.3. Experimental Procedure

For the population growth experiments, the two rotifer species fed on two carbon concentrations (0.5 mg C L^{-1}, "Limiting food concentration" LF and 2 mg C L^{-1} "Saturating food concentration" HF, Table S1) of *M. minutum* in combination with 1, 3, 6 PS beads, three types of SiO_2 beads, and 2 mg/L PA fragments with four replicates (Table S2). In this study, we used the same total amount of plastic (or silica) material, i.e., smaller particles were provided in higher numbers than larger particles.

In the second experiment, only the rotifer species *B.calyciflorus* was fed with a mix of algae species: *M. minutum* and *Cryptomonas* sp. Two carbon concentrations (0.5 "LF" and 2 mg C L^{-1} "HF") were used. Both algal species were supplied in 0.25 mg C L^{-1} for LF and 1 mg C L^{-1} for HF, respectively. *B. fernandoi* was not exposed to the mixture of algal food because it became mictic, i.e., it switched to sexual reproduction when fed with the mixed diet.

The experiment was conducted in 6-well microtiter plates at 20 °C in the dark to avoid additional algal growth. In the beginning, 10 individuals were randomly chosen from the stock culture and were pipetted into each well filled with 10 mL of the respective food suspension. At intervals of 24 h, the animals (live and dead) and their eggs were counted in each well. When the populations increased, 10 live individuals were randomly picked and transferred into new wells daily, receiving fresh food suspensions. In a case where less than 10 individuals survived, all of the remaining animals were transferred. The experiment lasted for 10 days (there was the exception of one replicate from *B. fernandoi* at low food concentration that got lost). Microtiter plates were placed on a rocker (Bio-Rad, Double Rocker, Labnet International Inc., Woodbridge, NJ, USA) to reduce the particle sedimentation. For each replicate the intrinsic growth rate (r), the egg ratio (m; eggs/female), and the survival (l) per day (t) were calculated on a daily basis using the following equations [36–38]:

$$r = ln(N_t) - ln(N_{t-1}) \tag{1}$$

$$m = \frac{H_t}{N_t} \tag{2}$$

$$l = 1 - \frac{D_t}{N_{t-1}} \tag{3}$$

where $N_{(t-1)}$ is the initial number of individuals and where N_t, H_t, and D_t are the final numbers of individuals, total eggs, and dead, respectively, on consecutive experimental days. The population growth rate (d^{-1}) of each replicate as well as reproduction (eggs $ind^{-1}\ d^{-1}$) and the probability of survival (d^{-1}) were calculated by averaging r, m, or l of consecutive experimental days.

2.4. Statistical Analysis

To compare the results from different experiments, we used the intensity of growth rate reduction (Δr) relative to the control group. The intensity of the growth rate reduction (Δr) was expressed as the difference in the per capita population growth rates with and without microbeads; a measure often used in food limitation experiments follows [21,23,24,39,40]:

$$\Delta r = r_c - r_s \tag{4}$$

where r_c is the per capita population growth rate in the experiment without microbeads (control), and r_s is the growth rate with the microbeads. A statistically significant growth reduction was present if the 95% confidence limits did not include zero and if the confidence intervals did not overlap. The effect of plastics and the interaction of food quantity, food quality, and plastics on the egg ratio and percentage of survival was analyzed using three-way ANOVAs and a pairwise comparison (Emmeans test) grouped by food against the reference group "control" with Bonferroni adjustment. The egg ratio was square-root transformed, and the percentage of survival was Yeo–Johnson transformed (lambda = 4.99) with the R-package "bestNormalize". Normality was assessed graphically using QQ-plot, and the homogeneity of variances was assessed using Levene's test. All of the statistical analyses were performed, and graphs were generated using R software (version 1.1.383).

3. Results

3.1. Effect of the MP Beads on Population Growth Rate

Brachionus calyciflorus and *B. fernandoi* experienced significant population growth rate reductions when exposed to the PS beads (Figure S5). Otherwise, there were no significant growth rate reductions in the treatments using PA fragments and silica beads (Figures S1, S3, and S4 showing ingested polymers).

In detail, we found a significant growth rate reduction when *B. calyciflorus* was only fed on the *M. minutum* algae with the 1-µm PS beads (Δr = 0.14; CI = 0.061) and 3- (Δr = 0.16; CI = 0.079) at the saturating food concentration. For the limiting food concentration, we found significant growth reductions with the 3- (Δr = 0.31; CI = 0.072) and 6-µm beads (Δr = 0.19; CI = 0.067). Contrarily, when a mixed algal diet was provided to *B. calyciflorus*, no growth rate reduction was found at the saturating food concentration, and the rotifers showed a significant decrease in growth rate for the limiting food concentration for particles that were 3 µm in size (PS: Δr = 0.25; CI = 0.171; silicate Δr = 0.14; CI = 0.103). In a similar manner, *B. fernandoi* exhibited no growth rate reductions at the saturating food concentrations, and only exhibited reductions when exposed to the limiting food concentration and to the 3-µm PS beads (Δr = 0.20; CI = 0.071), where we found a significant decrease in growth rate (Figure 1).

3.2. Effect of the MP Beads on Reproduction

Brachionus calyciflorus and *B. fernandoi* responded similarly regarding the production of eggs per individual (F_{1137} = 1.3, p = 0.26; Table 1 and Figure 2).

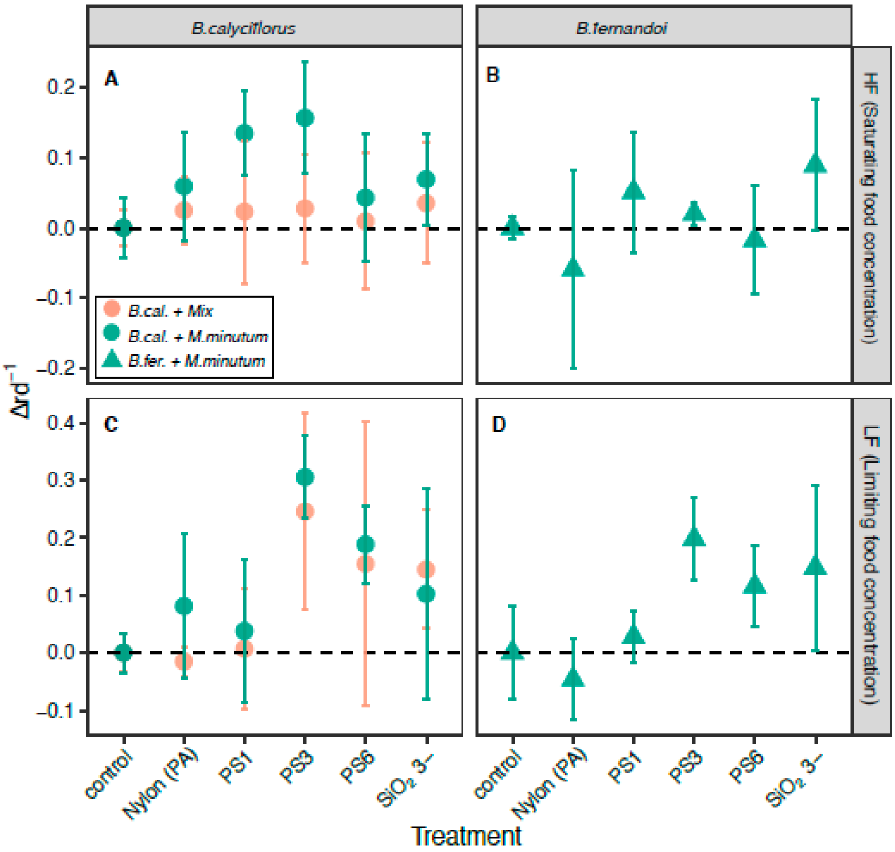

Figure 1. Intensity of food reduction ($\Delta r \pm$ 95% confidence interval (CI)) of the rotifer *B. calyciflorus* and *B. fernandoi* at high and low food concentrations; (**A**–**C**) the red circles refer to the experiment with *B. calyciflorus* and the mixed algal diet (*M. minutum* and *Cryptomonas* sp.), and the green circles refers to the experiment with *B. calyciflorus* and one algal species (*M. minutum*); (**B**–**D**) the green triangle refers to *B. fernandoi*.

Table 1. Results of three-way ANOVAs using square-root transformed data on the egg ratio and Yeo–Johnson transformed data on survival (lambda = 4.99) for the two rotifer species (*Brachionus calyciflorus* and *Brachionus fernandoi*) and the two algal diets (*Monoraphidium minutum*; *Monoraphidium minutum* + *Cryptomonas* sp.). The two species were provided with two quantities (0.5 and 2.0 mg C L^{-1}) of *Monoraphidium minutum*. *B. calyciflorus* was provided with the same food quantities of a mixture of *Monoraphidium minutum* and *Cryptomonas* sp. as food.

	Egg-Ratio			**Probability of Survival**		
Independent variables	**Df**	**F-Value**	***p*-Value**	**Df**	**F-Value**	***p*-Value**
Alg	1137	125.5	<0.0001	1137	0.4	0.534
food	1137	997.0	<0.0001	1137	28.6	<0.0001
food × Alg	1137	33.5	<0.0001	1137	2.8	0.099
food × Treatment	5137	1.0	0.422	5137	3.9	<0.01
Specie	1137	1.3	0.258	1137	20.2	<0.0001
Specie × food	1137	16.6	<0.0001	1137	2.4	0.126
Specie × food × Treatment	5137	1.5	0.190	5137	0.6	0.699
Specie × Treatment	5137	0.3	0.907	5137	3.3	<0.01
Treatment	5137	20.3	<0.0001	5137	5.6	<0.001
Treatment × Alg	5137	4.2	<0.01	5137	3.2	<0.01

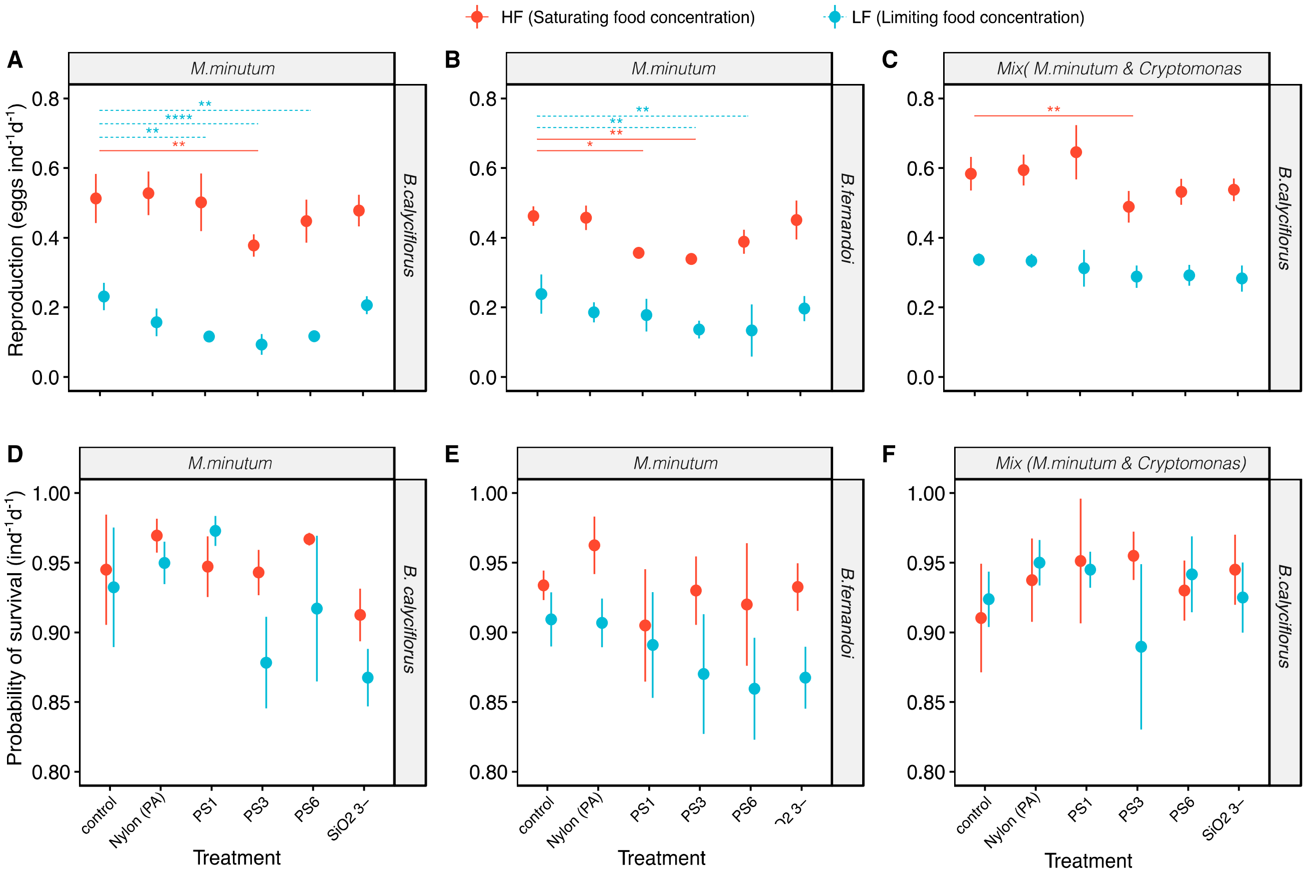

Figure 2. A−B−C egg ratio of *B. calyciflorus* and *B. fernandoi* exposed to the microbeads (mean ± SD); (**A**) egg ratio from *B. calyciflorus* fed on one algal species (*M. minutum*), with a statistically significant difference between the control group and the microbead treatment group; (**B**) egg ratio from *B. fernandoi* fed on one algal species (*M. minutum*), with a statistically significant difference between the control group and the microbead treatment group; (**C**) egg ratio from *B. calyciflorus* fed on mix algal diet (*M. minutum* and *Cryptomonas* sp.), with a statistically significant difference between the control group and the microbead treatment group; D−E−F percentage of survival of *B. calyciflorus* and *B. fernandoi* exposed to the microbeads (mean ± SD); (**D**) survival of *B. calyciflorus* fed on one algal species (*M. minutum*), with a statistically significant difference between the control group and the microbead treatment group; (**E**) survival from *B. fernandoi* feeding on one algal specie (*M. minutum*); (**F**) survival from *B. calyciflorus* fed on mix algal diet (*M. minutum* and *Cryptomonas* sp.), with a statistically significant difference between the control group and the microbead treatment group.

The egg productions were affected by the food concentration (F_{1137} = 997.0, $p < 0.0001$; Table 1), the different algal diets (F_{1137} = 125.5, $p < 0.0001$; Table 1), and the plastic treatments (F_{5137} = 20.3, $p < 0.0001$; Table 1). Moreover, the effect of the food concentrations on the egg ratio differed between the two rotifer species (F_{1137} = 16.6, $p < 0.0001$; Table 1) and between the two algal diets within the same species (F_{1137} = 33.5, $p < 0.0001$; Table 1). Regarding the effect of the plastic treatments, in general, we did not find significant changes after limiting the saturating food concentration (F_{5137} = 1.0, $p = 0.42$; Table 1); on the contrary, the effect varied between the two algal diets (F_{5137} = 4.23, $p < 0.01$; Table 1). The rotifers responded differently depending on the plastic treatments, but no significantly different effect was found between the control group and the rotifers exposed to PA fragments and silica beads. A reduction in egg production was mostly found with the 3-µm PS beads, with the exception of the experiment with *B. calyciflorus* when limiting then food concentration in the mixed algal diet. *B. calyciflorus* was more vulnerable to a decrease in the egg ratio when fed on a monoculture diet and with PS beads when the food concentration was limited (LF: PS1, $p < 0.01$; PS3, $p < 0.0001$; PS6, $p < 0.01$; Table S3), and a minor vulnerability was also shown with the saturating food concentration (HF: PS3, $p < 0.01$; Table S3). When the mixed algal diet was provided, *B. calyciflorus* exhibited a less pronounced decrease in the egg ratio, with the only significant reduction only being seen with the 3-µm PS beads (HF: PS3, $p < 0.01$; Table S3). Similarly, *B. fernandoi* showed an eggs ratio reduction with PS beads at the saturating (HF: PS1, $p < 0.05$; PS3, $p < 0.01$; Table S3) and limiting food concentrations (LF: PS3, $p < 0.01$; PS6, $p < 0.01$; Table S3).

3.3. Effect of the MP Beads on Survival

The probability of survival was affected by the food quantity ($F_{1137} = 28.6$, $p < 0.0001$; Table 1) and plastic treatments ($F_{5137} = 5.6$, $p < 0.001$; Table 1) and differed between the two species ($F_{1137} = 20.2$, $p < 0.0001$; Table 1). The effect of the beads changed depending on the food concentration ($F_{5137} = 3.9$, $p < 0.01$; Table 1), on the algal diet ($F_{5137} = 3.2$, $p < 0.01$; Table 1), and on the species ($F_{5137} = 3.3$, $p < 0.01$; Table 1). Nevertheless, for the two species and the different algal diets, no significant differences were found between the control group and the beads.

4. Discussion

The aim of this research was to investigate and compare the effects of different sizes and types of microbeads and the role of food quantity and quality in a freshwater rotifer population. In this study, we highlighted the decrease of the population growth rate and reproduction (egg ratio) of two freshwater rotifer species, *Brachionus calyciflorus* and *Brachionus fernandoi*, in response to exposure to PS beads at the limiting food concentration. Moreover, *B. calyciflorus* exhibited reduced fitness when exposed to MPs with a single algal food species at the saturating food concentration. In contrast, the (PA) nylon fragments and the silicate beads had no effect on the population growth rate, egg ratio, and survival.

4.1. The Role of Food Quantity and Food Quality on Microplastics Effect

Our experiments showed that the population growth rates of the two rotifers species and with both algal diets were more affected at the limiting food concentration with the presence of the 3-µm PS beads. Only *B. calyciflorus* showed a reduction in the population growth rate at a high food concentration with the monoculture algal diet. In fact, the population growth rate of *B. calyciflorus* did not decline when a mixed algal diet was provided at the saturating food concentration; similarly, *B. fernandoi* only exhibited a reduced population growth rate at the limiting food concentration. In addition, the growth rate reduction was less pronounced in *B. calyciflorus* with the mixed algal diet than it was with the monoculture algal diet (Figure S1). The egg production was also mostly affected mostly by the PS beads; the effect of the microplastics, if present, was not influenced by the different food concentration but instead depended more on the algal diet provided to the rotifers. For instance, *B. calyciflorus* and *B. fernandoi* showed a reduced egg ratio at the limiting and saturating food concentrations, with different intensities, but when a mix algal diet was provided, *B. calyciflorus* only exhibited a reduced egg ratio with the 3-µm PS beads at the saturating food concentration and had no effect at the limiting food concentration. For *B. calyciflorus* at the limiting food concentration, we found an inverse relation between the population growth rate and the number of eggs produced, where the number of individuals decreased but not the number of eggs; in contrast, at the saturating food concentration, the number of eggs per individual declined, but not the number of individuals. Although the population growth rate and egg ratio are expected to be linked to each other, they do not match perfectly. On the one hand, at low food levels, animals can increase their life span at the expense of reproduction. In our experimental set up, this led to a lower growth rate reduction but to a strong decline in the egg ratio. On the other hand, at the maximal growth rates, a high number of not yet reproducing juveniles are part of the population, leading to sub-maximal egg ratios. Our findings are in accordance with Korez et al., [41] where a marine isopod was not affected by microplastics when they received a sufficient amount of food with a high nutritional quality. A surplus in the microplastics at a low food concentration caused a significant reduction in food uptake and digestive enzyme activities. One likely explanation for the decrease in egg ratio in rotifers that is connected to microbeads exposure, is the food dilution effects, which have been found in nematodes and crustacea [12,29]. Microbeads, which are mostly of the same size of the supplied food, interfere with normal food ingestion, and in addition, the particles act as a non-food item, providing no energy resource. Thus, the microbeads occupy space in the digestive tract, decreasing the available space for algal food. A similar study on cladocerans determined that chronic exposure to PS beads led to a reduction in the number of offspring, which could be explained by the downregulation of several digestive enzymes that can interfere with the animal´s nutrient supply and that can affect their fitness [42].

Food quality may be more important in the explanation of the variation in zooplankton fitness than food quantity [43]. The food quality acts on consumer physiology through morphological traits such as the shape as well as the nutritional value. This is evident for organisms such as rotifers, who strongly depend on dietary nutrient supply. A decrease in food supply may lead to a shift in energy allocation and less available energy, resulting in a decrease fitness response [44–46]. Our findings indicate no differences between the two species in terms of the egg ratio, but as in previous studies, the food quantity influenced the reproduction differently [38]. Previous studies demonstrated the importance of food quality effects on the population growth rate, fecundity, and survival [47] as well as the differences in the life history traits between *B. calyciflorus* and *B. fernandoi* feeding on different algal foods [38]. Divergence in other life history traits were found [27] between *B. fernandoi* and *B. calyciflorus* by Zhang et al. since *B. fernandoi* invests less in sexual reproduction and has a higher population growth rate than the others brachionids. In addition, *B. calyciflorus* has a higher heat tolerance than *B. fernandoi* [26].These findings support the finding that *B. fernandoi* and *B. calyciflorus* differ in their ecology and react to stressors in a different way.

4.2. Size Particles Effect

The population growth rate and reproduction of the two rotifer species was significantly reduced when exposed to 3-µm PS beads. The size of the 3-µm PS beads is close to the size of the food alga and is at the lower end of the efficiently used food-size spectrum in *Brachionus* species [48–51]. This can explain why an effect was only found for the 3- and 6-µm beads. Our results are in accordance with Xue et al., [14], who showed that the reproduction of rotifers was suppressed when they were exposed to polyethylene microbeads (10–20 µm) along with algal food of a similar size. In our experiment, the survival percentage was not affected by the presence of microbeads, even when exposed to 3-µm PS, which had the strongest negative fitness response.

Different results were found by testing very small, nano-sized PS particles (37 nm, 0.07 µm) in marine brachionids, where the population growth rate decreased by more than 50%. On the contrary, large-sized PS beads had no effect on the population growth rate and reproduction [13]. The different results could be related to the different feeding efficiencies of the rotifer species. Furthermore, the nano-sized plastic beads mostly interfered at the cellular level. Micro- to medium-sized particles, similar to those in the present study, and particles that are up to 20 µm in size might interfere with the feeding and may dilute the food; in addition, large particles seem to have no effect on micro-zooplankton because they are non-edible food for them [48–51].

4.3. Silica and (PA) Nylon Microbeads

No effect on the fitness response was found when the rotifers were exposed to silica beads and polyamide fragments. The concentration and the specific density of the material play an important role in the uptake of particles in rotifers and could be a likely explanation for our findings. In fact, silica beads and the polyamide (PA) have a higher specific weight and a higher sinking velocity than PS. To prevent sedimentation, we applied agitation, but the ingestion process itself might have been affected by the weight. One may speculate that heavy particles are difficult to ingest. In the natural environments, animals are exposed to particles along with other suspended solids. A number of studies found no negative effects on the fitness of rotifers when they were exposed to suspended clay, whereas cladocerans were affected by clay particles [52,53]. Although rotifers and cladocerans are typical filter feeders, rotifers can feed more selectively, and they were able to avoid ingesting clay particles [52,53]. These results suggest that rotifers might be less affected by plastic pollution than cladocerans. Studying the effect of irregularly shaped MPs, *D. magna* was more affected by MPs than by mineral particles of a similar size, potentially leading to extinction within one and four generations [44,54,55]. A mechanism counteracting the ingestion of fragments is aggregation, which leads to particle sizes that are unable to be digested [20,49]. Until now, no general conclusion can be drawn as to which factors drive the ingestion and impact the size, shape, weight, and type of plastics on animals: Klein et al. [56] have recently found that the ingestion of beads and fragments in freshwater shrimp was more influenced by the size of the particles than by their shape, whereas the ingestion was not influenced by the presence of the food. Copepods, instead, ingest more fragments than beads or fibers [57]. Marine off-shore zooplankton ingested more fragments than the ones close to the urban coast [58]. These findings suggest a strong particle type and a species-specific role.

4.4. Ecological Relevance

A crucial issue in the research on plastic pollution is that the detection of particles becomes more and more difficult with decreasing size. At the moment, there is no method available that can reliably quantify microplastics in the size range used in this study in natural water samples with algae, bacteria, and detritus. The concentration of the smallest MPs size (<10 µm) cannot be estimated at present, but from modelling studies, it is likely that the number of MPs in the environment increases when the size decreases [59]. For instance, the number of particles in marine environment and freshwater sediment has been underestimated due to technical limitation [60,61]. At the time of the study, the concentrations of microbeads were, most likely, higher than the ones in the field; however, with increasing production and fragmentation, the amount of small microplastics will increase continuously.

Typically, laboratory conditions are chosen to match the needs of the test species as well as possible. In contrast, in the field, environmental conditions are highly variable over time and are often suboptimal in terms of temperature or food supply. In particular, food supply can vary strongly from low to high and vice versa over the course of mere days [62]. Under such suboptimal conditions, when animals are already stressed, the effects of pollutants can be stronger than they would be under ideal conditions, as demonstrated in the present study. Furthermore, the PS beads used for the experiment do not contain plasticizer or additives since they are used for standard tests. In fact, the polymer type and the chemicals that they contain can contribute to the toxicity of microplastics, creating an additional stress [63]. Indeed, one single plastic product can contain hundreds of chemicals [64]. These include additives such as antioxidants, flame retardants, plasticizers, and colorants as well as residual monomers and oligomers and side products of polymerization and compounds and impurities [65]. Once taken up, these plastic chemicals can have negative impacts. For instance, aqueous leachates from epoxy resin or PVC plastic products can induce acute toxicity [66] and alter life history traits [67] in *Daphnia magna*. Still, studies on the contribution of plastic chemicals to microplastic toxicity are scarce. Studies testing for the combined effects of more than two factors are generally rare [68]. In a study with *Daphnia*, Hiltunen et al. [69] tested for temperature, food quality, and microplastics. Using lower plastic concentrations, as was also the case in our study, they found that decreased food quality had the biggest effect on life history, and the low plastic concentrations had no effect. In another study, increasing the food quantity disproportionately reduced the uptake of MP, and no effect on *Daphnia* life history was found [70]. However, some results only become apparent after long-term exposure [71]. Combining these results, food quantity and quality have a strong impact on consumer life history that can be enhanced by high microplastic pollution.

5. Conclusions

Our study reveals that the negative effect of microplastics on a common freshwater invertebrate depends on the environmental conditions, which in this study, were food quality and quantity. This is one reason for the differing results in microplastic research and requires more attention in terms of plastic risk assessment. In addition, although standardized toxicological tests provide useful information on the toxic potential of pollutants, more realistic studies with various environmental conditions are needed to obtain deeper and more comprehensive insights on the problem of plastic pollution.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/toxics9110305/s1, Figure S1: Population growth rate, Figure S2: Size range distribution of PA nylon beads, Figures S3 and S4: PA beads ingested by *B. calyciflorus*, Figure S5A,B: PS beads ingested by *B. calyciflorus*. Table S1: Concentration of food algae, Table S2: Concentration of microbeads, Table S3: Results from the Emmeans' test.

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Chapter 4

Manuscript 3

Feeding type and microplastic particle concentration drives the ingestibility of polystyrene spheres in a natural zooplankton community

in preparation

Claudia Drago, Guntram Weithoff

Abstract

The mismanagement of plastic waste in recent decades led to global plastic pollution and severe environmental problems. Much of the plastic waste in the environment degrades into small pieces forming microplastics. Thus, an increasing number of microplastics in the environment is forecasted and the ingestion of such microplastics by zooplankton might cause serious problems. Using a microcosm experiment including rotifers, cladocerans, copepods and ostracods, we tested the availability of three sizes of microplastics of 1-, 3- and 6- µm at very high (10^6 particles/ml) and low (10^3 particles/ml) concentrations for a natural planktonic community. This allowed us the directly compare ingestion by different species from distant taxonomical groups. At low concentrations, 40 % of the cladocerans ingested MPs, a higher percentage than in all other groups suggesting that the unselective filter-feeding mode facilitates the ingestion. At high concentrations, more than 70 % of the organisms ingested MPs with moderate differences among groups. These high percentages show that at high numbers of plastic particles even raptorial feeders cannot prevent plastic ingestion. The size of the MPs had no effect on the ingestibility. We showed with a near-natural experiment that both, feeding type and particle concentration substantially affects microplastic ingestion by zooplankton.

4.1 Introduction

A global production of 330 million tons of plastics per year has been estimated for the last decades [1]. The overuse and mismanagement of plastics has led to a severe environmental problem [2]. Large plastics can degrade over time or break down during use and disposal [3] resulting in different sizes and shapes; in addition, microplastics is intentionally produced and added to products (abrasive granules in cosmetics) [4]. The focus on microplastics (MPs) in freshwater environments is relatively recent, and it plays an important role in transport and accumulation [5]. The increasing concentration of microplastics [6,7] and the recent underestimation of the number of MPs in the environment, especially for the smaller sizes, is a source of concern for the aquatic scientific community [8]. Once in the environment, microplastics interact with planktonic and benthic organisms; primary producers such as algae and consumers such as crustaceans or rotifers [9]. Planktonic microorganisms are the basis of aquatic food webs, through the herbivore food chain and the microbial loop. The feeding mode and the interaction between planktonic communities make these organisms more susceptible to small MPs. Numerous studies have focused on the ingestion of MP by planktonic zooplankton under controlled laboratory conditions in single-species experiments. Studies addressing the effect of microplastics on more than one taxon in near-natural planktonic communities are scarce [10]. Ingestion has been studied for several taxa and shown to depend on particle concentration, feeding strategies and encounter rate but only a few studies have considered microcosm or mesocosm experiments. Fueser et al. [11] studied MP ingestion in a natural microbenthic community and found a relationship between the feeding mode of nematode species and MP ingestion. A study by da Silva [10] tested the ingestion of different MP sizes in a planktonic community, smaller MPs were ingested more than larger MPs and ciliates and protists contributed more than meso-zooplankton in MPs ingestion. Contrasting results were found with a benthic community, where no effect on composition was found after 3 months of exposure and some effects were found in oligochaete abundances after 15 months [12,13]. The bioavailability of MPs for planktonic organisms is still unclear as MPs can also take different shape, degrade or aggregate in the environment[14], sediment [15] or be resuspended [16]. The aim of this research was to study the bioavailability of MPs in a planktonic community, we want to examine the percentage of organisms that would ingest three sizes of MPs: 1, 3 and 6 µm polystyrene beads at extremely high or at environmentally relevant concentrations[17]. We also want to compare the availability of the sizes and the difference among the groups.

4.2 Methods

4.2.1 Microplastics

As microplastic particles, we used fluorescently labelled polystyrene microbeads (MPs) of three different diameters: 1.03, 3.06, and 5.73 µm (Polysciences, Inc. Fluoresbrite® YG Polystyrene Microspheres, USA, for convenience, we refer to them as 1, 3 and 6 PS). A stock solution was prepared with deionized MilliQ water under sterile conditions to minimize bacterial growth. To keep the MPs as singular particles, each stock solution was sonicated for 30 minutes and was mixed using a vortexer prior to use.

4.2.2 Experimental procedure

Circa 50 litre of a natural water containing a zooplankton community was collected from an urban lake (Heiligensee, Potsdam, Germany). The community was concentrated by sieving it through 30 µm mesh and acclimated to laboratory conditions in particle-free lake water supplemented with algal food (*Monoraphidium minutum* and *Chlamydomonas noctigama*). Prior to the experiment, 200 ml of the stock was sieved again to remove algal food and resuspended with particle-free lake water. For each replicate, 10 ml were transferred in to glass jars and the MPs were added in the target concentration. To reach an equilibrium between ingestion and egestion, the experiment was run for 24 h and to avoid sedimentation, a rocker shaker plate was used. We used two concentrations of MPs for each size: 10^3 and 10^6 p ml-1 and ran 3 replicates for each treatment. The organisms were fixed with 90% ethanol and preserved in the fridge at 8 C until microscopical inspection. The samples were examined using a fluorescent microscope (Zeiss, Axioskop, Germany) and the presence of ingested beads was recorded. Zooplankton was identified to the genus level (except for copepods), and if necessary Calcofluor White Stain (Sigma-Aldrich Chemie GmbH, Germany), was used aiding rotifer identification by staining the trophi.

4.2.3 Statistical analysis

The percentage of individuals that ingested beads was calculated per each replicate and mean and standard error was taken into account. Kruskal-Wallis test was first applied to investigate differences in the percentage of individuals with ingested beads among the beads size (1-, 3-, 6- µm) and between the concentrations (high and low). The difference among the group was investigated by Kruskal-Wallis test and when significant, Wilcoxon-Mann-Whitney test was performed with Bonferroni correction. The same analysis was performed separately for the genus *Keratella* spp. and *Thrichocerca* spp. and for the class calanoida and cyclopoida. The analysis was carried out with RStudio [18].

4.3 Results

In total 1740 individual animals were analysed (Tab.S1). For rotifers and cladocerans 17 genera were identified, copepods were divided into calanoida, cyclopoida and nauplii. Polystyrene beads of all three sizes were ingested by all groups at high concentration (Fig. 4.1). The percentage of animals having ingested beads was similar among the different size treatments(Kruskal-Wallis test: $p > 0.05$, Tab.S2), however, the number of animals that had ingested plastics was higher at high bead concentrations (Kruskal-Wallis test: Cladocera $p < 0.05$; Copepoda $p < 0.001$; Nauplii $p < 0.001$; Ostracoda $p < 0.01$; Rotifera $p < 0.001$; Tab.S3) In detail, a difference between high and low concentrations of beads was found for all groups (Cladocera, Copepoda, Nauplii, Ostracoda, Rotifera; Tab.S3). The percentage of animals that ingested MPs was not different among the groups (Kruskal-Wallis test: $p > 0.05$; Tab.S4).

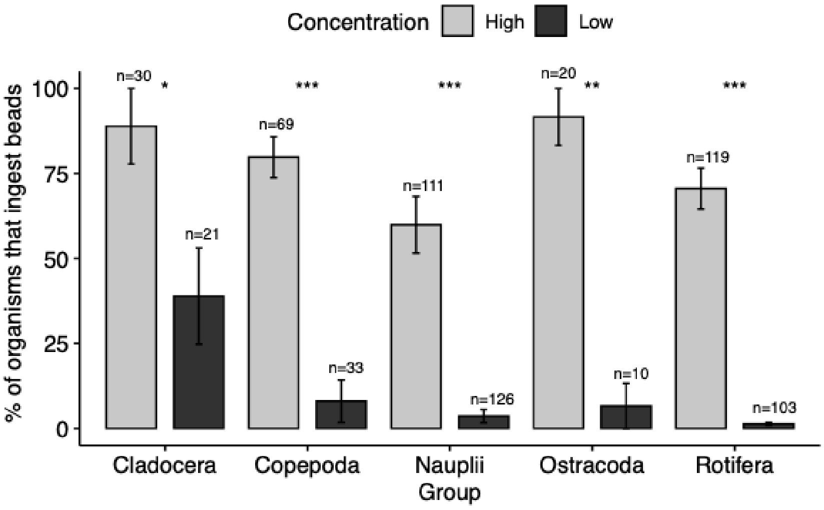

Figure 4.1: Percentage of individuals that ingest the MPs (mean ±se). The three beads' sizes are pooled and the stars represent significant difference between high and low concentration of beads. N represent the total number of organisms. *Keratella* spp and *Trichocerca* spp are not included.

4.3.1 Rotifera

In total 1320 rotifers from 12 genera were identified in our experiment; the abundance varies from more than 100 specimens (*Keratella* spp.) to one specimen (*Kellikottia* spp.) per sample (Fig. 4.2)

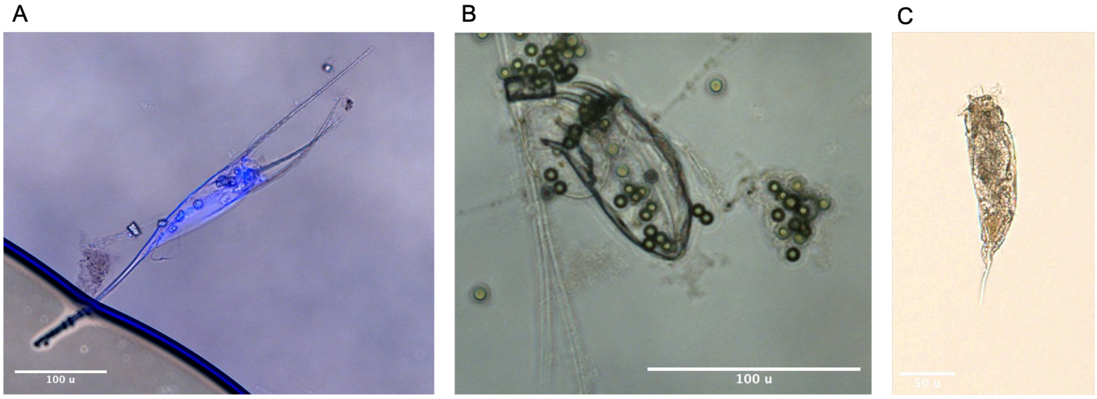

Figure 4.2: Rotifers identified in the experimental sample (A: *Kellikottia* spp. fluorescent UV light; B: *Keratella* spp. with ingested 3 µm PS beads; *Trichocerca* spp. bright field). Photo from Claudia Drago

The majority ingested all three sizes of beads. The results of the two most abundant species, *Trichocerca* and *Keratella* spp., were analyzed separately (Fig 4.3A). We found a higher percentage of animals having ingested beads at higher concentrations but no difference among the sizes. *Keratella* and *Trichocerca* did not differ between each other at different sizes (Tab.S5 ; S6).

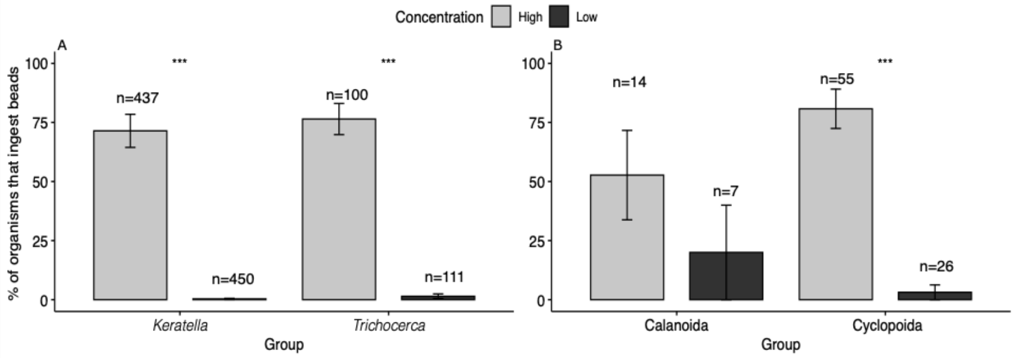

FIGURE 4.3: Percentage of animals (A: *Keratella* spp. and *Trichocerca* spp.; B: calanoida and cyclopoida) that ingested the beads (mean±se). The three beads size are pooled together and the stars represents the significant difference between high and low MPs concentration.

4.3.2 Cladocera

In total 51 specimens from 5 genera were identified; the abundance of each genus varied among the samples. Among all groups, Cladocera exhibited the highest percentage of organism that ingested beads at low concentrations (Fig. 4.1 and 4.4).

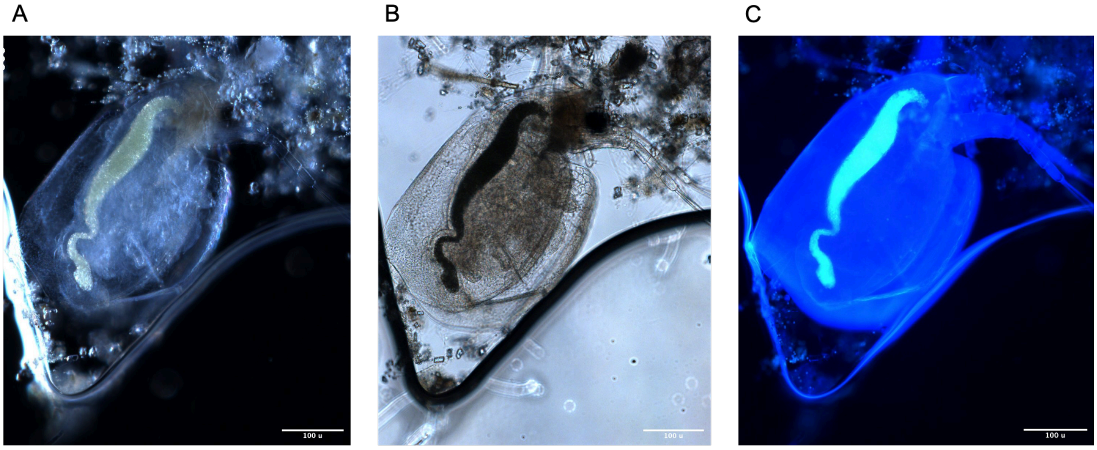

FIGURE 4.4: Cladocerans identified in the experimental sample, *Ceriodaphnia* spp. (A: under dark field light; B: bright field light; C: UV fluorescent light). The gut of the organism appears full of 3 µm PS beads. Photo from Claudia Drago

4.3.3 Copepoda

The copepoda were only classified as calanoida, cyclopoida and nauplii, in total 21 calanoida, 111 cyclopoida and 237 nauplii were identified. There was no difference in the percentage of organism that ingested the beads between the two classes, for calanoida there was no difference even for high and low beads concentration (Kruskal-Wallis test; p=0.085; Tab.S8; Fig. 4.3B, 4.5).

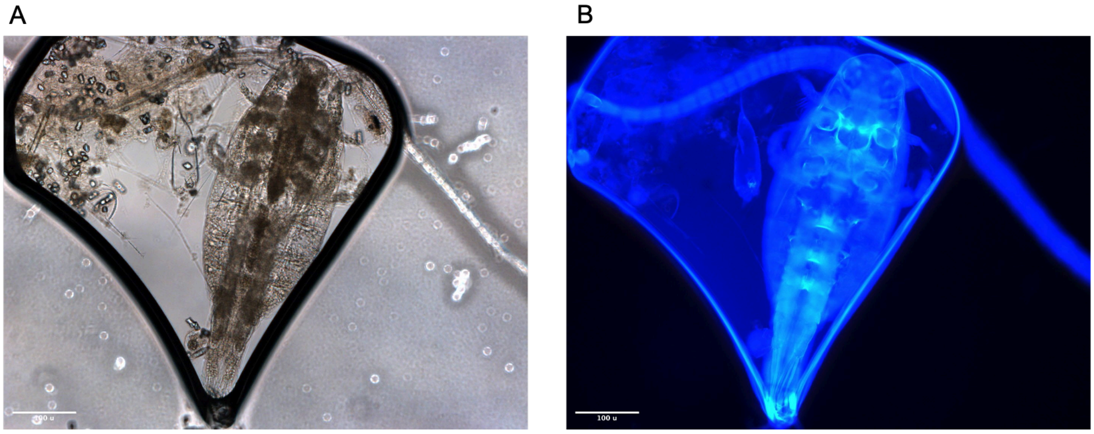

Figure 4.5: Copepoda, Cyclopoida identified in the experimental sample (A: bright field light; B: UV fluorescent light. On the left side of the copepod, it is possible to see *Trichocerca*. Photo from Claudia Drago

The percentage of animals that ingest the beads decrease significantly at low beads concentration for cyclopoida (Kruskal-Wallis test; p <0.001; Tab.S7).

4.4 Discussion

The aim of the study was to quantify the availability of different microplastics size in a planktonic zooplankton community at near-natural conditions. With a community composed of 4 zooplankton groups: rotifers, the most abundant, followed by copepods nauplii, cladocera, adult copepods and ostracods; we quantified the percentage of individuals who ingested MPs under the same experimental conditions, this can be useful to estimate the effect on the different groups. The beads size range (1-, 3-, 6- µm) was ingested by all groups and by almost all organisms

(Table S1). Although each group expresses its own food preference, this size range is within the ingestion range of all the groups considered. In fact, several rotifers and cladocerans feed efficiently on 1- , 3- and 6- µm spheres [19–21]. From field work studies and laboratories experience, several calanoids were able to ingest different size range: in the field from 21 to 280 µm and in the lab from 0.05-, 0.5- to 6 µm beads [9,22,23]. A recent study [10] shows that 0.5-, 1- and 3- µm MPs were consumed by all zooplankton taxa, from protist to cladocerans but at different rate. In general, for filter feeders the minimum ingested particle size is determined by the mesh size of the filtering apparatus and the maximum size by the morphology of mouthparts [24]. Because of the uncountable number of MPs inside some organisms we didn´t consider the rate of consumptions but only the availability of the plastic per each animal. To investigate the difference among the groups we pooled the results of the three beads size, knowing that similar proportion of organism ingested the three beads size. A large number of animals ingested beads at high concentration: More than 70 % of the individuals ingested the MPs, at low concentration, the percentage did not exceed 40 %. The highest percentage was found in ostracods, followed by cladocerans, copepods and rotifers at high concentration. Planktonic filter feeders are more prone to ingest particles in the water column, but at low concentration some of them as rotifers and copepods are more selective and they could avoid the ingestion of MPs. The feeding type and the selectivity of the consumer together with size, shape and concentration of MPs influence their ingestion. In a study on a benthic community [11] a similar result was found for copepods and chironomid larvae with high percentages of ingestion at high concentrations of beads. In a coastal brackish (Baltic Sea) study nauplii, cladocerans and rotifers ingested MPs at high concentration and only a few rotifers were ingesting MPs also at low concentration [25,26]. Increasing the beads concentrations increase the proportions of organism that will ingest the beads and lead to a high encounter rate resulting in an increased feeding rate[24] as in our study. At low concentration of beads, only the cladocerans exhibited a percentage of ingestions that exceed 40 %, even if not significant these results could be explained with the feeding mode of cladocerans. Cladocerans as filter feeders use specialized filtering structures to strain suspended particles, they might be more prone to ingest MPs as they ingest a variety of seston components[27]. On the contrary copepods use filtering and raptorial type strategies and they are able to manipulate particles and have high food selectivity, however, filter feeders in this group may not distinguish small particles or consume microplastic indirectly as it happened at high beads concentration[28]. Young stages of copepods exploit resources according to their body size, especially in terms of minimum particle size [28]. Raptorial feeders (cyclopoid copepods and some rotifers) are more prone to select a specific algal size or phytoflagellates and high concentrations of smaller particles do not interfere with the ingestion of their preferred algal food [29,30]. Studies on *Keratella* spp. indicate that the morphology of the feeding apparatus (corona and mastax) facilitate the collection of different shape and size of particles including bacteria and detritus [29]. *Trichocerca* spp, for example, feed on small suspended particles and filamentous algae in the water[31]. These rotifers could avoid the ingestion and accumulation of MPs at low concentration. Also, ostracods eat alga and detritus and they are able to feed in the interface between sediment and water[32]. Incubating the microcosm for 24h we considered the different ingestion-egestion times of groups trying to not overlap with the reingestion of the beads. In addition, we did not take into account the effect on community composition and the trophic transfer of MPs through the food chain. As planktonic zooplankton are the basic food for carnivore invertebrate and fish, the ingestion of the MPs could be transported to higher trophic level and have an indirect effect on those[26]. The ingestion of microplastics could make the chemicals, contained[33] or absorbed[34] during their residence in the aquatic system, in the plastic more available for the organisms. Few studies have investigated the effect of MPs on freshwater community, for example, studies on benthic macroinvertebrate community composition have found a slight change in community structure[35]. On a long term (15 months) the abundances of oligochaetes was affected but not in a shorter experiment (3 months) [12,13]. Due to technical limitations the quantification of MPs in the environment is still underestimated, but future trends argue that the concentration in the environment will increase in the next decades[6,8]. A strong effort is needed to decrease the introduction of plastics into the environment[38]. For example, in marine environment the plastics will continue to degrade and the abundance will increase as the size will decrease [8,36]. As the concentration increase, more and more species could be susceptible to ingest MPs. Our experiment demonstrates that high percentage of a planktonic community ingest MPs in different size range at extremely high concentrations. At more environmentally relevant concentration just few organisms ingest MPs depending more on their feeding type. Microcosm experiment are important to understand the bioavailability and the mechanism that the whole community adopt when MPs are in the environment. Additional studies are needed to understand how the planktonic community will react with higher exposure time and different MPs.

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Chapter 5

General Discussion

Microplastics (MPs) once released into the environment can aggregate and be ingested by aquatic organisms. Depending on their size, ingested MPs cause a decrease in organisms' fitness, e.g decrease in reproduction and growth rate. This negative effect, could be influenced by the presence of different food quantity and quality. In a multiple species scenario with different zooplankton groups, MPs at high concentration can be ingested easily by the majority of aquatic organisms either because of their feeding mode or by mistake. This dissertation elucidates with laboratory experiments how some environmental factors such as MPs aggregation, food quantity and quality and MPs characteristics such as size and concentration can influence the ingestion and the effect of MPs in freshwater zooplankton. In particular, special attention was given to rotifers, an important phylum in the freshwater environment and little studied from the perspective of MPs.

- Chapter 2 supports the hypothesis that small MPs (1- and 3- µm) when aggregated with bacteria are more ingested than single MPs accordingly to their size-range, in contrast larger MPs (6- µm) are less ingested when aggregated.
- Chapter 3 indicates that the food quantity and quality provided to the rotifers influence the effect of MPs in two freshwater rotifers with slightly differences between species.

- Chapter 4 shows that the concentration of MPs and the different feeding mode of aquatic organisms are the major drivers of MPs ingestion in a freshwater planktonic community.

These results highlighted the crucial role of environmental factors in MPs´ studies. Ingestion of MPs is governed not only by dietary size selectivity, but also by environmental variables that occur in nature such as the presence of food and MPs aggregation. Aggregation makes smaller MPs (1 µm), which are normally ingested less efficiently, more available to rotifers. In contrast, large MPs (6 µm) that usually are more efficiently ingested, once aggregated became less uptaken (Chapter 2). Ingestion is followed by the effect at population level: size selectivity drives the ingestion and the presence of different algal species and abundances drives the negative effect of MPs. These diluting the food, cause reduced reproduction and a consequent decrease in population size. In a different scenario, that incorporates multiple zooplankton species, the concentration of MPs plays a key role along with the feeding mode of planktonic organisms. Ingestion is influenced by the feeding mode at low concentrations, thus with organisms such as cladocerans, more likely to ingest MPs and raptorial zooplankton more likely to avoid ingesting MPs. At high concentrations almost all organisms can ingest or come into contact with MPs presumably by mistake or indirect ingestion. Suggestions for further research directives are provided in the chapter “Conclusion and Outlook” which might help elucidate the fate of MPs in freshwater planktonic community.

5.1 Size, concentration and aggregation influence the ingestion of MPs by freshwater planktonic rotifers

5.1.1 Size and concentration

The ingestion of MPs by freshwater organism is one important path of interaction of MPs in the environment, depending on the size range, MPs could be ingested by

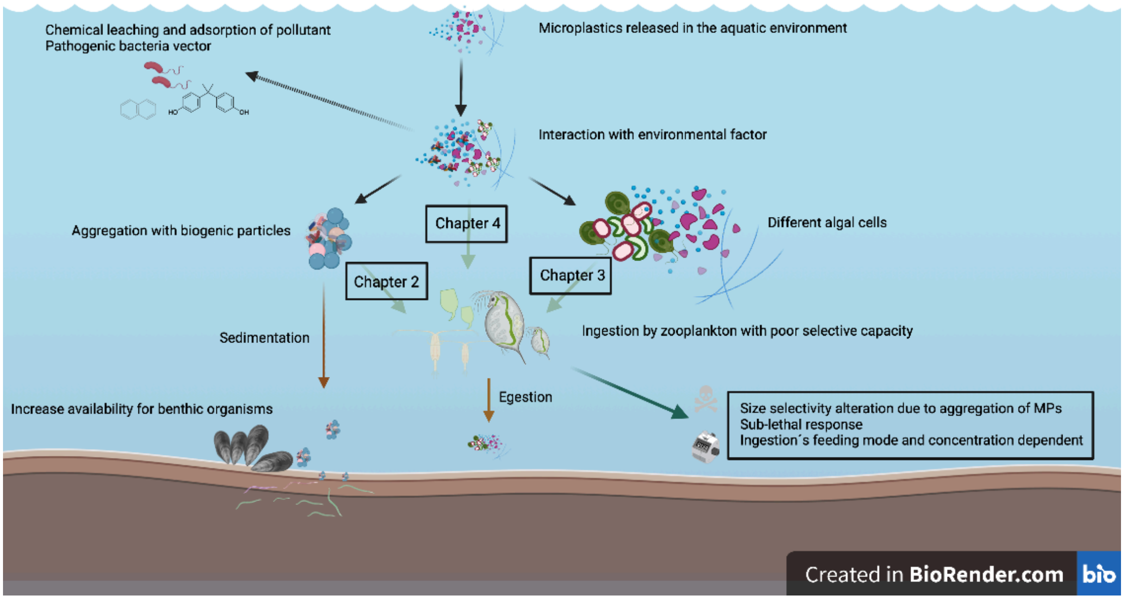

FIGURE 5.1: Graphical representation of the major findings of my thesis.

different organisms. The ingestion rate is a useful tool to quantify MPs burden and uptake, especially for rotifers. These, in fact, are considered together with cladocerans and copepods more prone to ingest small suspended particles and algal food on the same size range of small MPs. In chapter 2 was shown as the ingestion of the MPs (1, 3, and 6 µm), even if their size is considered below the optimal size of feeding efficiency (Rothhaupt, 1990b,a), followed a Type-II functional response model. The type II functional response refers to the ability of rotifers to eat large quantity of MPs at low concentration and reach a saturation level at high concentration. For particles smaller than 4 µm rotifers have a low filtering capacity and it is possible for rotifers to select actively their food depending on their size (Hansen et al., 1997). Previous studies demonstrated that the highest feeding efficiency for *B. calyciflorus* and closely related *Brachionus* spp. is in the range of a 3.5- to 10-µm equivalent spherical diameter (ESD) (Vadstein et al., 1993; Baer et al., 2008) as seen in my study at chapter 2 or even higher (Pagano, 2008). In fact, also the polyamide fragments in a size range from 5 to 25 µm were also easily ingested by *B. calyciflorus* and *B. fernandoi*, confirming the capacity of brachionids to eat larger beads also if not in their size preference (Chapter 3). In general, very small particles (1µm) were ingested with lower efficiency, but the presence of aggregated small MPs increases

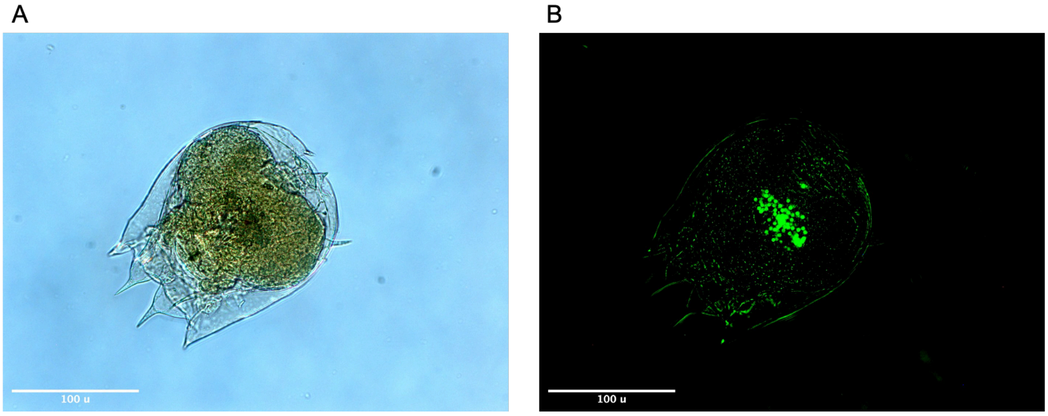

FIGURE 5.2: *Brachionus* spp. ingesting 3 µm PS beads (A: bright field light; B: fluorescent field light). Photo from Claudia Drago

the ingestion efficiency as shown in Chapter 2.

As in Rothhaupt (Rothhaupt, 1990a), the larger PS MPs (6 µm) were preferably ingested in terms of biovolume than the smaller PS MPs (1 and 3 µm) (Fig. 5.2), comparing attack rate and handling time of the three bead sizes. These findings could be useful to estimated which size of MPs could be more dangerous for particular aquatic organism and identify which algal food size would be neglected during the normal feeding. Not only the size selectivity is an important factor in the uptake but also the concentrations of MPs. The higher concentration of MPs leads to a higher ingestion rate, these results are also true when MPs were provided to the whole zooplankton community (Chapter 4). In chapter 4 a large number of animals ingested MPs at high concentration: more than 70 % of the individuals ingested the MPs; at low concentration, the percentage did not exceed 40%. At high MPs concentration not only the ingestion rate is expected to rise as in the experiment with *B. calyciflorus* (Chapter 2) but also the percentage of animals that will encounter plastics may increase. Planktonic filter feeders are more prone to ingest particles in the water column, but at low concentration some of them as predatory rotifers and copepods are more selective and they could avoid the ingestion of MPs (Wilson, 1973; Donaghay and Small, 1979). Increasing the beads concentrations increase the proportions of organism that will ingest the beads and lead to a high encounter rate resulting in an increased feeding rate (Scherer et al., 2017) as in chapter 2 and

4. Regarding the concentration in the environment, the actual data on MPs in the aquatic environment underestimate the number of MPs in the size range used in my study (Lindeque et al., 2020; Way et al., 2022) at chapter 2 and 4. Moreover, the increasing degradation of bigger plastics items to small MPs in the environment could raise the risk for aquatic organism to ingest MPs. As the size of MPs decrease the concentration in the environment will increase, making MPs more available for a wide range of organisms (Mattsson et al., 2021).

5.1.2 Aggregation of MPs and ingestion

In the environment, MPs can aggregate with particulate matter, bacteria and microalgal cells (Michels et al., 2018). This, within a few days together with glycoproteins, contribute to the formation of hetero-aggregates (Summers et al., 2018; Hossain et al., 2019). Moreover, MPs can be also find in the environment as homo-aggregates, constitutes only by plastics particles (Rogers et al., 2020). In the experiment at Chapter 2 it is shown how different the MPs were ingested when they were aggregated or free-floating. The aggregation of MPs within a 72h experiment (Chapter 2) altered the ingestion of smaller and larger MPs. The paramenter estimation differed between the free floating MPs and the MPs aggregated with biogenic particles (Chapter 2)(Fig. 5.3).

Despite the size selectivity of the rotifers *B. calyciflorus*, the presence of aggregated MPs increases the feeding efficiency of particles considered below the optimal size, such as 1- and 3-µm, by making them more available when aggregated; ingestion of otherwise edible MPs (6- µm) can be prevented through aggregation. These results support the hypothesis that organisms in the environment come into contact with or ingest MPs that have undergone alterations, such as aggregation. Therefore, the uptake of MPs may be decreased or increased depending on environmental characteristics.

In general, the aggregation of detritus and living organisms (bacteria, algae, fungi, and microzooplankton) is a common phenomenon in lakes and oceans, known as

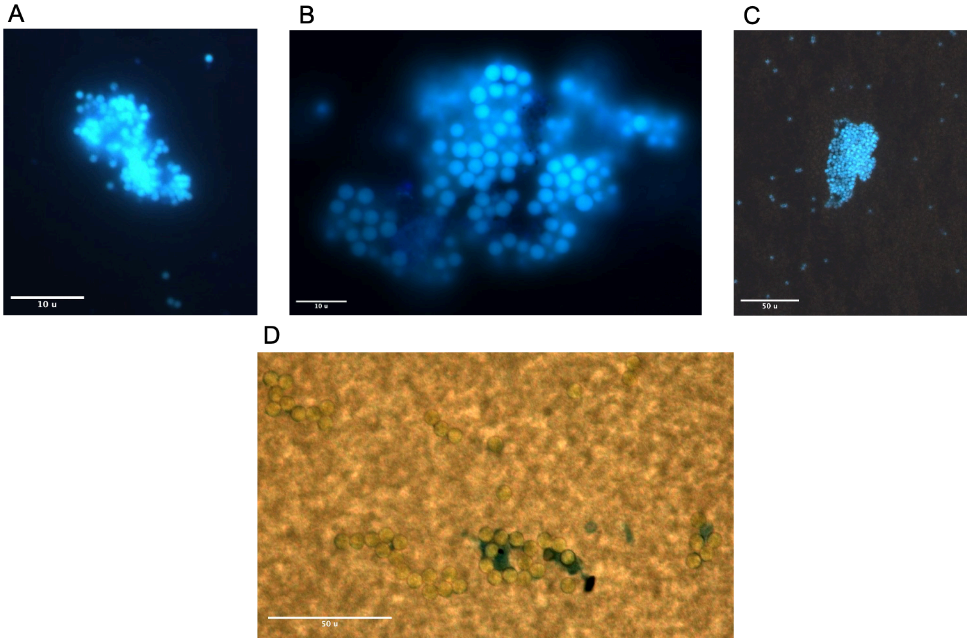

FIGURE 5.3: PS MPs aggregated with TEP and bacteria and beads (A: 1 µm PS beads UV fluorenscent light; B: 6 µm PS beads; C: 3 µm beads; D: 6 µm beads under bright field light with TEP). Photo from Claudia Drago

lake or marine snow (Grossart and Simon, 1993; Alldredge et al., 1998) and a substantial incorporation of plastics into these aggregates seems very likely. Thus, the environmental conditions together with the specific properties of the particles affects their aggregation in aquatic environments (Wang et al., 2021). In chapter 2, *B. calyciflorus* increased the ingestion rate of the small 1 µm MPs when aggregated, in accordance with study on mussels and copepods, where also the sinking rate of the fecal pellets was altered (Cole et al., 2016; Welden and Cowie, 2016; Zhao et al., 2018). The formation of MP aggregates can alter the properties of the plastic, leading to higher sedimentation, e.g. in the study from Porter (Porter et al., 2018) where the aggregation of MPs of different type and shape incorporate in marine snow increase their sinking velocity but decrease the sinking velocity of the marine snow incorporating low-density plastics. However, the aggregation of larger MPs could lead to a fast sedimentation of MPs on the aquatic soil, making even low density MPs more available for the ingestion by benthic organisms as for example, mussels, worms and nematodes (Porter et al., 2018; Vroom et al., 2017) then for the planktonic one (Besseling et al., 2017). Besides, the biofilm can change the sinking velocity (Leiser et al., 2020), the size distribution of MPs aggregations (Semcesen and Wells, 2021) and more important can improve the nutritional appearance (Vroom et al., 2017) and quality of MPs (Polhill et al., 2022).

In addition, the association of MPs with colloid and with specific microbial community "Plastisphere" contributes to the dispersion of the microbial community in the water column and moreover also potentially pathogenic genus as *Vibrio* spp. may be dispersed over long distance (Amaral-Zettler et al., 2020). The size of the aggregation can also be related to the toxicity: altering the surface area which is a key factor that determine the transport of the released additives and the adsorbed contaminants (Zocchi and Sommaruga, 2019). Compared to large aggregates, small aggregates of MPs with larger surface areas and more reactive sites may release more contaminants and degrade faster (Chen et al., 2019a). Little is still known about the distribution of aggregates along the water column and how much of MPs are in aggregated or free-floating state (Wang et al., 2021). Moreover, the estimation of the nutritional quality of the aggregates and the overlap with feeding areas of aquatic consumer are still lacking.

5.2 Food supply and heterogeneity of plastic influence the effect of MP

5.2.1 Food supply

The inability of rotifers and in particular of *Brachionus* spp.to distinguish between food and MPs lead them to ingest MPs. In experiments with flavored polystyrene spheres (DeMott, 1986), it was found that *B. calyciflorus* did not discriminate against those with adsorbed algal flavors. Nevertheless, in the experiment at chapter 2, the attack rate parameter did not vary between the algal and single MPs beads treatment. Thus, this result cannot support the hypothesis that *B. calyciflorus* was not able to distinguish between algae and MPs due to the similar ingestion rate of MPs. Even though, a decrease in algal food uptake was found in a study on *D. magna*, these organisms ingested less algal food due to the presence of MPs (Ogonowski et al., 2016). Additionally, nematodes exposed to smaller MPs reduce the ingestion of bacteria instead the larger particles affect the feeding indirectly with unpaired pumping rates (Rauchschwalbe et al., 2021). In chapter 3 the reduced fitness of two brachionids species was reported mainly at limiting food concentration, when MPs size was similar to the size of the algal food provided. With monoculture algal diet *B. calyciflorus* and *B. fernandoi* showed a reduction in the population growth rate at limiting food concentration. Instead, when mix algal diet was provided, *B. calyciflorus* exhibited normal population growth rate. *B. calyciflorus* and *B. fernandoi* showed a reduced egg ratio at the limiting and saturating monoculture food concentrations, with different intensities, but when a mix algal diet was provided, *B. calyciflorus* only exhibited a reduced egg ratio with the 3- µm PS beads at the saturating food concentration and had no effect at the limiting food concentration, indicating that the MPs effect depended more on the algal diet provided to the rotifers. Food quality and quantity may be important in the explanation of the variation in zooplankton fitness (Müller-Navarra et al., 2000). The presence of food supply is considered one crucial factor modulating the effect of MPs in zooplankton. This is evident for organisms such as rotifers, who strongly depend on dietary nutrient supply Schälicke et al. (2019). A decrease in food supply may lead to a shift in energy allocation and less available energy, resulting in a decrease fitness response (Ogonowski et al., 2016; Guilhermino et al., 2021).

Testing low food and high food concentration can be advantageous to understand the effect of MPs in the environment (Pawlak et al., 2022), since rotifers population in the wild often experiences changing algal diet, food limitation and period of starvation. A similar study on rotifers found that larger MPs (10–22 µm), in association with the algal food of similar size, suppressed the reproduction of rotifers, this negative effect could be alleviated by increasing the food supply (Xue et al., 2021). Also others organisms responded in a similar way, for example marine isopods and nematodes (Korez et al., 2019). These organisms were not affected by MPs when they received a sufficient amount of food with a high nutritional quality. A surplus in the MPs at a low food concentration caused a significant reduction in food uptake and digestive enzyme activities. In nematodes, instead it was found that the presence of non-nutritional items in the size range of food stimulated a pharyngeal pumping useful for screening the food. However, when high food concentration was provided, the ingestion of non-nutritional particles as MPs could not be avoided (Fueser et al., 2020; Rauchschwalbe et al., 2021). One likely explanation for the decrease in fitness in rotifers is the food dilution effect, which has been reported or nematodes and crustacea (Amariei et al., 2022; Rauchschwalbe et al., 2021; Mueller et al., 2020). MPs , which are mostly of the same size of the supplied food, interfere with normal food ingestion, and in addition, the particles act as a non-food item, providing no energy resource. Thus, the MPs occupy space in the digestive tract, decreasing the available space for algal food, depleting the energy reserve useful for growth and reproduction (Schür et al., 2020).

5.2.2 Natural particles and irregular shaped MPs

In a more natural scenario MPs are not the only particles in the environment and the MPs have different shape and polymer type (Ogonowski et al., 2018). Thus, is crucial also to compare and test these two categories. In my study, silica beads and irregular shaped polyamide MPs had no effect on rotifers fitness (Chapter 3). Some studies, comparing the effect of suspended natural particles on cladocerans and rotifers, find out that rotifers were less affected then cladocerans (Kirk and Gilbert, 1990; Kirk, 1991). In daphnids, kaolin particles and primary MPs were not toxic as irregular shaped MPs (Ogonowski et al., 2016). In general, studies comparing MPs with natural particles (for instance, red clay, kaolin or natural sediment) under controlled settings show that the adverse effect induced by MPs occur at lower concentrations than for natural particles. Similarly, a study on mussels found out that the clearance rate was more affected by the ingestion of MPs then natural particles (Harris and Carrington, 2020). Regarding the sub lethal effect, some studies found less impact of silica beads than MPs in nematodes (Mueller et al., 2020; Rauchschwalbe et al., 2021). In the experimental design at Chapter 3 the main reason why silicate was not affecting the fitness response may be explained by higher density than MPs and fast sedimentations, moderate shaking probably could resuspend the plastics beads easier than the silica ones. In fact, previous study on *B. calyciflorus* has investigated the effect of suspended particles in a turbulent environment and they found out that the presence of silica beads as suspended particles decreases the ingestion rate of algal food (Miquelis et al., 1998). Comparing the characteristic of MPs and natural particles it is important to consider the intrinsic MPs characteristics. Low and high density, high persistence, wide size range and variable shape make MPs unique particles. Some natural particles can have similar shape and size but they have higher density, some others that have similar densities are less persistent than MPs (Koelmans et al., 2022). However, under turbulent mixing condition, small high-density plastics and natural particles probably would be easily resuspended. In my study, even if slightly turbulence was provided, polyamide plastics exhibit no negative effect; likely due to less uptake because of their larger size and higher polymer densities than PS. Contrarily, for cladocerans, irregular shaped MPs are more toxic than sphere and also the egestion of them seems more problematic (Frydkjær et al., 2017; Jaikumar et al., 2018). The discrepancy in results could depends on the MPs characteristic as size and shape, but more important seems to be connected to specie-specificity of the negative effects. Thus, it is crucial to take into account and identify traits that make species more susceptible to MPs.

5.2.3 Species-specific effect

Zooplankton have different feeding modes and size selectivity, so it is important to consider different species to understand which organisms may be most susceptible to MPs. Therefore, species-specificity became also a crucial factor. My findings indicate no differences between the two species of *Brachionus* in terms of the egg production, but *B. fernandoi* showed no population growth rate reduction at saturating food concentration indicating a better resistance to MPs (Chapter 3). The hypothesis that *B. fernandoi* and *B. calyciflorus* differ in their ecology and react to stressors in a different way is supported by the divergence in other life history traits, found by Zhang (Zhang et al., 2019; Paraskevopoulou et al., 2020) since *B. fernandoi* invests less in sexual reproduction and has a higher population growth rate than the others brachionids. Microplastics studies should then focus more on the specie -specificity of MPs effect, since also similar species could react in a different way to the same stressor. Depending of on their feeding habits similar species of i.e sea urchin (Suckling, 2021), copepods (Koski et al., 2021), cladocerans (Jaikumar et al., 2018) exhibit contrasting results and were more or less negatively affected by MPs ingestion (Zebrowski et al., 2022).

5.3 Availability of MPs depends on concentration and feeding mode of the organism

Microplastics resistance to complete degradation and further fragmentation make this material a pollutant like no other in the environment, thus MPs could reach high concentration in the future. In Chapter 4, the three sizes of PS MPs were tested on a zooplankton community that incorporated cladocerans, copepods, ostracods and rotifers (Fig 5.4) . The percentage of animals that ingested MP didn't change with the size of the beads provided. Although each group express its own size preference the particular size range used in my study (1- 3- 6- µm) was within the ingestion range of all the groups considered (Rothhaupt, 1990b; Wilson, 1973; Bogdan and Gilbert, 1984; Yu et al., 2020). In fact, several rotifers, cladocerans and calanoids feed efficiently on 1- , 3- and 6- µm beads in lab experiment (Jeong et al., 2016; Cole et al., 2013). From field work it is known that several calanoids were able to ingest different size range: from 21 to 280 µm (Botterell et al., 2019). Similarly, a recent study (da Silva et al., 2022) shows that 0.5-, 1- and 3- µm MPs were consumed by all zooplankton taxa, from protist to cladocerans but at different rates. The

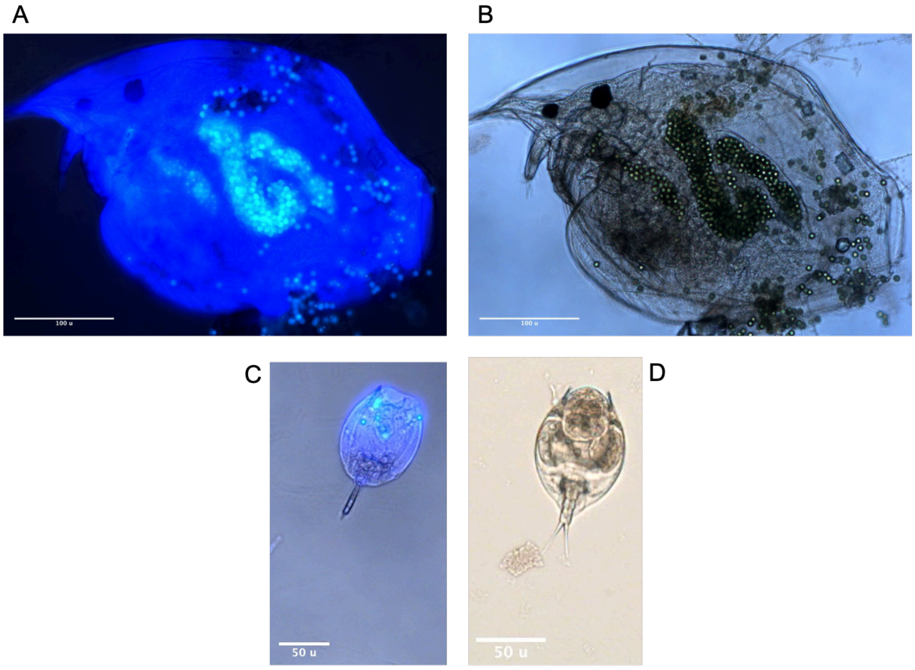

FIGURE 5.4: Organisms identified from the experimental sample (A: *Alonella* spp. with 6 µm PS beads UV fluorenscent light; B: *Alonella* spp. 6 µm PS beads bright field light; C: *Lecane* spp.; D: *Lepadella* spp.). Photo from Claudia Drago

majority of organism in each groups ingested MPs at very high concentrations. At more environmentally relevant and therefore lower MP concentrations just some organisms, e.g. cladocerans, reveal higher MPs ingestion. A reason is provided by the feeding mode and the larger body size of the cladocerans. Cladocerans as filter feeders use specialized filtering structures to strain suspended particles, they might be more prone to ingest MPs as they ingest a variety of seston components (Scherer et al., 2017).

On the contrary copepods use filtering and raptorial type strategies and they are able to manipulate particles and have a high food selectivity, however, filter feeders in this group may not distinguish small particles or consume microplastic indirectly as it happened at high beads concentration (Hansen et al., 1997). At low concentration raptorial copepods and rotifers species as *Keratella* and *Trichocerca* spp. could avoid the ingestion of MPs. These findings are in accordance with others studies on ingestion in aquatic community: in a benthic community (Fueser et al., 2020) high percentage of ingestion was found in copepods and chironomid larvae when high concentration of beads was provided. In a coastal brackish (Baltic Sea) study nauplii, cladocerans and rotifers ingested MPs at high concentration and only a few rotifers were ingesting MPs also at low concentration (Setälä et al., 2014, 2018). Another study on benthic macroinvertebrate also found no correlation between the size and the accumulation of MPs and that MPs ingestion depended mostly on the feeding habits, in that study in fact deposit feeders were found with higher percentage of ingestion (Stanković et al., 2022). The higher concentration of beads in combination with the feeding mode of the aquatic organism drove the uptake of MPs. The concentration used in the three studies (Chapter 2, 3 and 4) are extremely high in comparison to the values found in the environment. Nevertheless, these concentrations may be reached in the future or may currently be underestimated due to technical limitations or might be found in particular hot-spots, due to weather condition: extreme as hurricane or floods (Stanton et al., 2020).

5.3.1 Consequences at ecosystem level

Primary producers and consumers have a crucial role in freshwater ecosystem, providing important ecosystem services. Zooplankton in particular as rotifers, copepod and cladocera are the key of trophic transfer, preventing eutrophication, providing bioindication and supplying fatty acids to secondary consumers. Thus, maintaining the overall ecological balance, structure and function of an ecosystem. A negative effect on this functional groups could be responsible for reduced flora and fauna biomass, productivity and carbon sequestrations. Few studies have tested the effect of MPs on ecological functions. A study investigating the community structure of phytoplankton found out that at high concentration MPs induced changes in community composition and highlight the potential risk for food webs and ecosystem functioning (Hitchcock, 2022). Moreover, in general shifting in community equilibria could affect prey selectivity by predator and subsequently competition among prey population growth. The ubiquity of MPs could also potentially lead to losses in ecosystem productivity as for example the growth of algae has been shown to be inhibited (Zheng et al., 2021), causing reduced biomass, the presence of MPs in fecal pellets could lower the efficiency of carbon sequestration in the ocean (Cole et al., 2016). In a mesocosm study with algae, cladocera and larval fly as predator a long-term exposure to PE microplastics reduced the stability and persistence of the grazer population via increased predation risk and reduced reproductive capacity for grazer species (Pan et al., 2022). MPs then are able to affect the trophic cascade strength and stability of plankton ecosystems via behavior-mediated indirect interactions, suggesting that microplastics have more extensive impacts on aquatic ecosystems (Pan et al., 2022). Another microcosm study showed that elevated MPs concentration could have a low impact directly the zooplankton population dynamics, but can influence morphological changing in larval stage of chironomids and transfer to terrestrial habitats (Yıldız et al., 2022). Increasing concentration of MP and its negative effect on zooplankton, could lead to loss of resilience in shallow lake that would change the entire food web (Kong and Koelmans, 2019). For example, decrease in population as for example of the lungworms would suppress the diversity of sediments habitat, indirectly affecting fish and birds (Troost et al., 2018). MPs can endanger the entire plankton ecosystem simple affecting individual behavior, even if they don't have significant effect on the survival of the given test species, this highlights the importance of community-level studies to reveal specific risk in plankton ecosystem.

Chapter 6

Conclusion and outlook

Aquatic organisms, in particular planktonic unselective filter feeders are more prone than other organism to ingest MPs in the environment (Scherer et al., 2017). In particular freshwater brachionids are expected to ingest MPs particles according to their food size selectivity (Vadstein et al., 1993; Rothhaupt, 1990a; Hansen et al., 1997). The size selectivity is species-specific, depending on the morphology of the mouth part and the gathering apparatus (Scherer et al., 2017). Small MPs, in the size range 1 - 300 µm in the environment are currently underestimated, due to technical limitation (Lv et al., 2021). It is likely that MPs once in the environment will aggregate by themselves or with natural particles, forming aggregate that will float or sediment fast depending on the nature of the aggregate and on the density of the polymer (Leiser et al., 2020; Michels et al., 2018; Summers et al., 2018). The rapid colonization and aggregation of MPs by microalga and microbes might affect the buoyancy and the ingestion of the aggregates (Porter et al., 2018; Leiser et al., 2021). For example, the biofilm formation led to a major ingestion from aquatic organism (Vroom et al., 2017) due to higher appetibility or because of larger volume (Fabra et al., 2021). For brachionids the presence of MPs aggregation in the environment could be very different from exposure in the laboratory where MPs are often offered as single particles. The formation of aggregate and biofilm could lead to a higher ingestion or higher availability of MPs to aquatic organism, however the feeding mode of aquatic organism has to be considered, since for example non selective rotifers as brachionus will ingest more of the smallest MPs avoiding the larger size. As the presence of bacteria and aggregation can affect ingestion, the presence of different types of food at different concentrations influence the effect at population level (Wright et al., 2013; Colomer et al., 2019). In fact, the decreased fitness of *Brachionus* spp. is modulated by the size similarity of MPs and algal food. Since only the physical aspect of MPs was considered in my study, the negative effect on rotifers is strongly associated with food dilution. It is known that the different quantity and quality of food algae could alleviate the effect of MPs in rotifers (Yoon et al., 2021). Not only the different food can provide more or less resistance to the dilution effect of MPs, but also the different rotifers species could react in a different way. Either because their life history traits are different as it happens for *B. calyciflorus* and *B.fernandoi*, or due to their feeding mode as for example for cladocerans and raptorial rotifers (Scherer et al., 2017). Although the feeding modes of the pelagic zooplankton is different, with extremely high concentration of MPs, these organisms will ingest MPs accidentally or indirectly(Geng et al., 2021). This possibility is known already for raptorial copepods that usually tend to avoid and strictly select their food but in the case of MPs they could not avoid the ingestion (Botterell et al., 2020; Coppock et al., 2019; Cole et al., 2019). The different types of MPs and shape could also influence the ingestion and the effect since for example the fragment used in my study were easily ingested by the rotifers but do not exhibit any negative effect. These findings are in contrast with previous study on different planktonic organism that exhibit higher toxicity with irregular shaped MPs (Ogonowski et al., 2016). Also, to compare the effect of MPs with natural particles as silicate beads reveal that MPs were more deleterious in accordance with previous studies (Schür et al., 2020; Scherer et al., 2020). MPs in the environment behave differently than in laboratories studies, the presence of factor as bacteria, aggregation, food supply can influence the ingestion and effects of MPs. A negative effect on primary consumer as the zooplankton group could have consequences in species abundances and shifting community (Kong and Koelmans, 2019). Investigating microplastics in an environmental context may vary current knowledge on the subject, as aggregated microplastics may be more or less ingested in the environment depending on their formation, and the effect of microplastics per se is greatly influenced by the presence of algae as well as the higher or lower concentration and the feeding mode of the different zooplankton group influence the availability of MPs. Although this study contributes to understand the ingestion and the effect of MPs in freshwater rotifers from a more natural and ecological perspective, several new issues (which occurred trough this study) have not been addressed yet:

- The biofilm formation and the aggregation of MPs could also lead to a different effect on the fitness of rotifers; as for example different biofilm formations from different aquatic environments could be more or less dangerous or even provide more nutritional values to the rotifers. Another aspect that has not been evaluated in rotifers is the effect of MPs biofilm on chemical cues as kairomones. In fact, some rotifers species when stimulated with kairomones predators adopt trait defense that led to morphological changes along with the generations (Gilbert, 1999). The presence of MPs even if not ingested directly by the rotifers could interfere with the chemical communications. Previous studies on *Daphnia magna* and a predator exhibit an alteration of trait response when MPs were present (Trotter et al., 2019).
- It has been found that the response to MPs is species-specific (Suckling, 2021). It would be useful to individuate the rotifers traits that could be more susceptible to MPs effect, as for example rotifers that invest more in survival and less in reproduction and vice versa. Moreover, to focus on rotifers species that are less studied but more abundant in freshwater environment could give a more exhaustive view of the possible effect at ecosystem level.
- Different endpoints could be used to test the effect of MPs as for example behavioral alteration (swimming and feeding) and transgenerational studies to have a complete view of the response to MPs exposure.

- Testing the chemical effect of MPs on rotifers. In fact, the behavior of MPs in freshwater environments could differ due to the higher presence of pollutant in freshwater rivers and lakes and the higher closeness with wastewater treatment plants, the adsorption of chemical from the environment and the chemical leaching from plastics particles due to degradation could harm a wide range of aquatic organisms.

- Microcosm experiment can be a useful tool to understand the effect on community composition and the consequence at ecosystem level. For example, the decrease in reproduction could promote the abundance of different species at the expense of the species more vulnerable. This effect may lead to a shift in community composition without drastic effect on individual level.

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I would especially like to thank my supervisor Guntram for supporting and motivating me over the past four years with his patience and understanding and his helpfulness despite the hardships of the past two years. I would also like to thank Katrin for her good advice and optimism during the meetings.

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Appendix A

Supplementary materials for Chapter 3

Manuscript 2

"Variable fitness response of two rotifers species exposed to microplastics particles: the role of food quantity and quality"

Supplementary Material for the manuscript "Variable Fitness Response of Two Rotifer Species Exposed to Microplastics Particles: The Role of Food Quantity and Quality".

Table S1. Concentration of food alga used for the experiments at saturating food concentration and limiting food concentration. The concentrations are expressed in cell/ml and correspond to 2 mgCL^{-1}.

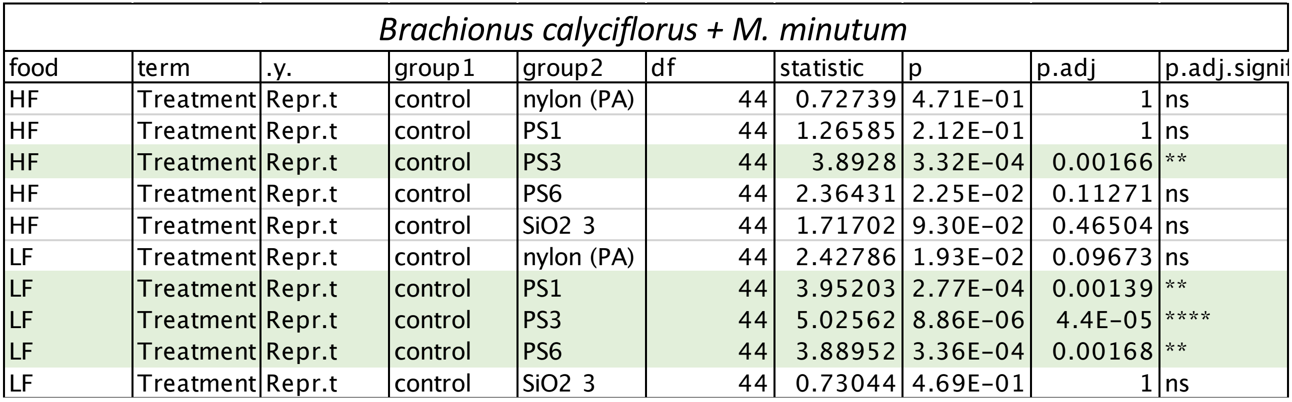
Saturating food concentration (HF) (cell/ml)		
B. calyciflorus & *B. fernandoi*	*B. calyciflorus*	
M. minutum	*M.minutum*	*Cryptomonas*
2.07 x 10^5	1.04 x 10^5	9.98 x 10^3

Limiting food concentration (LF) (cell/ml)		
B. calyciflorus & *B. fernandoi*	*B. calyciflorus*	
M. minutum	*M.minutum*	*Cryptomonas*
5.18 x 10^4	2.59 x 10^4	2.50 x 10^3

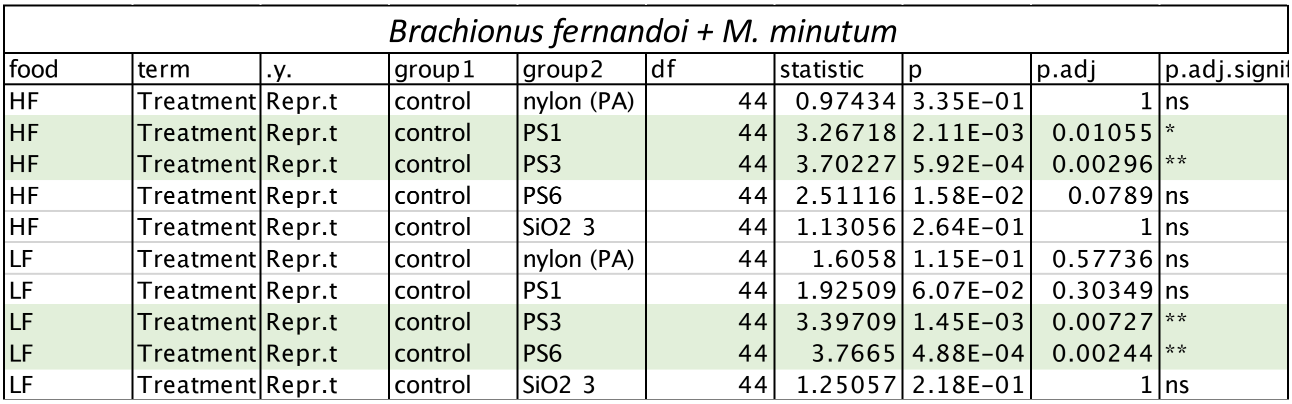
Table S2. Concentration of the microbeads used in the experiments. The concentrations are expressed as number of plastics per ml and correspond to 2 mg/L

Plastic (p/ml)				**Silicate**
PS			PA	SiO_2
1 µm	3 µm	6 µm	5 µm - 25 µm	3µm
3.33 x 10^6	1.27 x 10^5	1.93 x 10^4	1.00 x 10^3	7.96 x 10^4

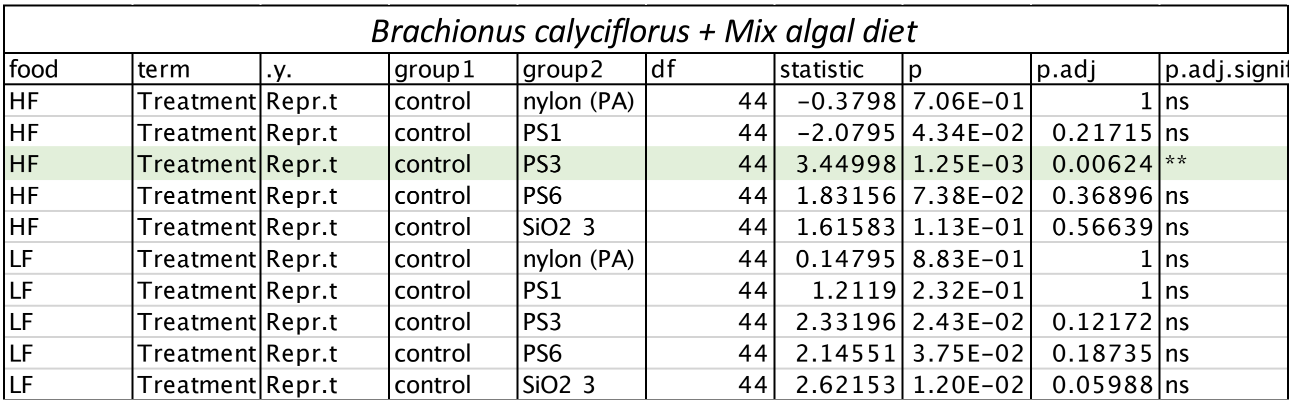
Table S3. Results from the pairwise comparisons (Emmeans test) relative to the egg ratio (Repr.t) of *B.calyciflorus* + one algal species, *B. fernandoi* + one algal species and *B.calyciflorus* + mix algal diet. The egg ratio was square root transformed and grouped by food against the reference group control. P values were adjusted with Bonferroni and significance is expressed as $p<0.05$.



Brachionus calyciflorus + M. minutum									
food	term	.y.	group1	group2	df	statistic	p	p.adj	p.adj.signi
HF	Treatment	Repr.t	control	nylon (PA)	44	0.72739	4.71E-01	1	ns
HF	Treatment	Repr.t	control	PS1	44	1.26585	2.12E-01	1	ns
HF	Treatment	Repr.t	control	PS3	44	3.8928	3.32E-04	0.00166	**
HF	Treatment	Repr.t	control	PS6	44	2.36431	2.25E-02	0.11271	ns
HF	Treatment	Repr.t	control	SiO2 3	44	1.71702	9.30E-02	0.46504	ns
LF	Treatment	Repr.t	control	nylon (PA)	44	2.42786	1.93E-02	0.09673	ns
LF	Treatment	Repr.t	control	PS1	44	3.95203	2.77E-04	0.00139	**
LF	Treatment	Repr.t	control	PS3	44	5.02562	8.86E-06	4.4E-05	****
LF	Treatment	Repr.t	control	PS6	44	3.88952	3.36E-04	0.00168	**
LF	Treatment	Repr.t	control	SiO2 3	44	0.73044	4.69E-01	1	ns



Brachionus fernandoi + M. minutum									
food	term	.y.	group1	group2	df	statistic	p	p.adj	p.adj.signi
HF	Treatment	Repr.t	control	nylon (PA)	44	0.97434	3.35E-01	1	ns
HF	Treatment	Repr.t	control	PS1	44	3.26718	2.11E-03	0.01055	*
HF	Treatment	Repr.t	control	PS3	44	3.70227	5.92E-04	0.00296	**
HF	Treatment	Repr.t	control	PS6	44	2.51116	1.58E-02	0.0789	ns
HF	Treatment	Repr.t	control	SiO2 3	44	1.13056	2.64E-01	1	ns
LF	Treatment	Repr.t	control	nylon (PA)	44	1.6058	1.15E-01	0.57736	ns
LF	Treatment	Repr.t	control	PS1	44	1.92509	6.07E-02	0.30349	ns
LF	Treatment	Repr.t	control	PS3	44	3.39709	1.45E-03	0.00727	**
LF	Treatment	Repr.t	control	PS6	44	3.7665	4.88E-04	0.00244	**
LF	Treatment	Repr.t	control	SiO2 3	44	1.25057	2.18E-01	1	ns



Brachionus calyciflorus + Mix algal diet									
food	term	.y.	group1	group2	df	statistic	p	p.adj	p.adj.signi
HF	Treatment	Repr.t	control	nylon (PA)	44	-0.3798	7.06E-01	1	ns
HF	Treatment	Repr.t	control	PS1	44	-2.0795	4.34E-02	0.21715	ns
HF	Treatment	Repr.t	control	PS3	44	3.44998	1.25E-03	0.00624	**
HF	Treatment	Repr.t	control	PS6	44	1.83156	7.38E-02	0.36896	ns
HF	Treatment	Repr.t	control	SiO2 3	44	1.61583	1.13E-01	0.56639	ns
LF	Treatment	Repr.t	control	nylon (PA)	44	0.14795	8.83E-01	1	ns
LF	Treatment	Repr.t	control	PS1	44	1.2119	2.32E-01	1	ns
LF	Treatment	Repr.t	control	PS3	44	2.33196	2.43E-02	0.12172	ns
LF	Treatment	Repr.t	control	PS6	44	2.14551	3.75E-02	0.18735	ns
LF	Treatment	Repr.t	control	SiO2 3	44	2.62153	1.20E-02	0.05988	ns

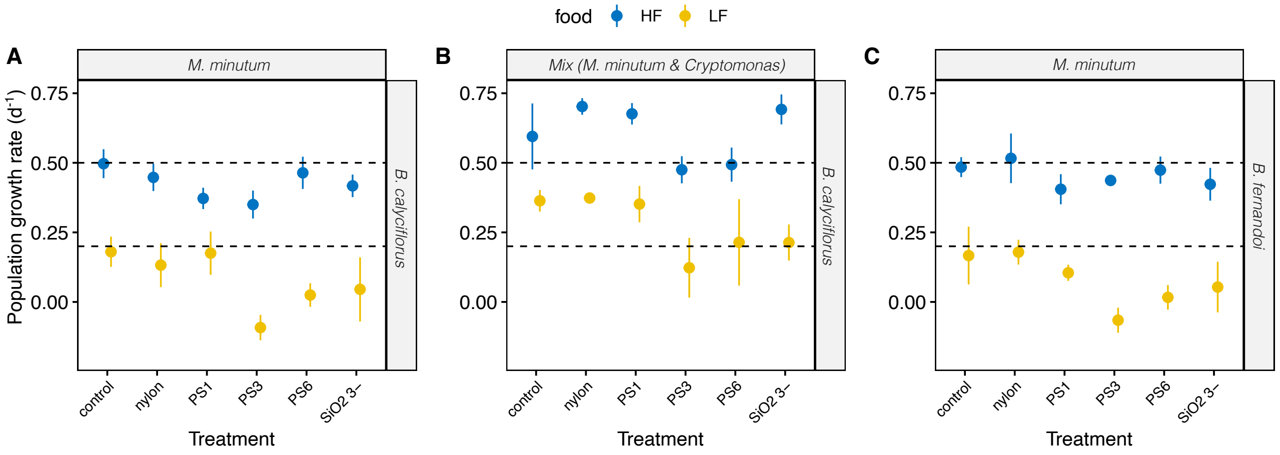


Figure S1. Population growth rate (mean ± SD) of *B. calyciflorus* (a) and *B. fernandoi* (c)with one algal species and mix algal diet for *B.calyciflorus* (b) . Blue dots represent the population growth rate at saturating food concentration (HF) and the yellow dots are the population growth rate at limiting food concentration (LF).

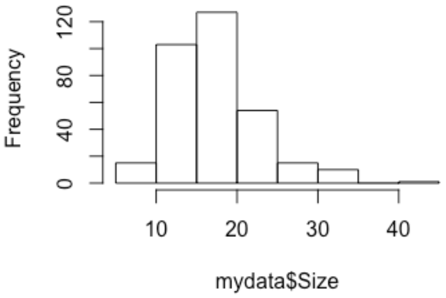


Figure S2. Size range distribution of the PA Nylon beads.

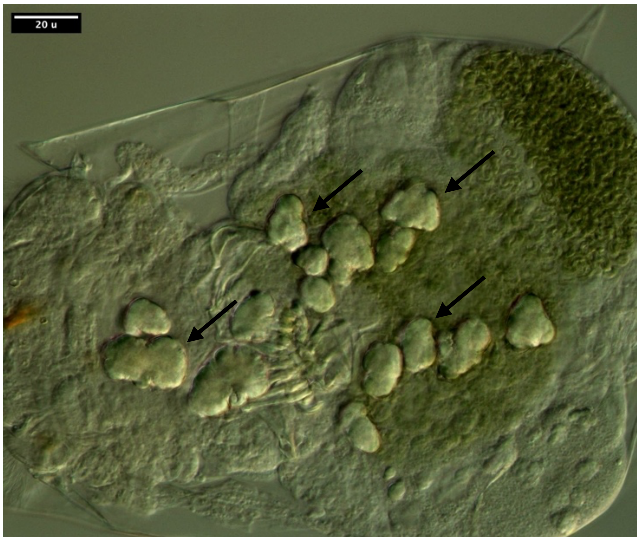


Figure S3. PA Nylon beads ingested by *B.calyciflorus,* the beads are indicated with the black arrows.

Figure S4. PA Nylon beads ingested by *B.calyciflorus* fixed with Lugol, the PA beads are indicated with the arrow.

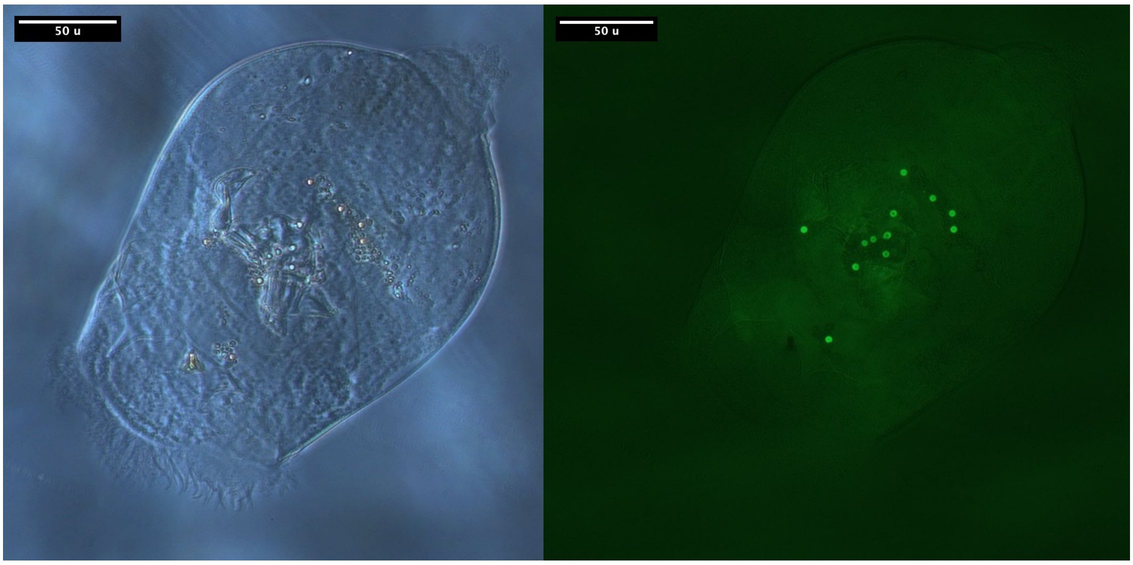


Figure S5. PS beads (3µm) ingested by *B.calyciflorus*

Appendix B

Supplementary materials for Chapter 4

Manuscript 3

"Feeding type and microplastic particle concentration drives the ingestibility of polystyrene spheres in a natural zooplankton community"

Supplementary Materials for the manuscript "Feeding type and microplastic particle concentration drives the ingestibility of polystyrene spheres in a natural zooplankton community"

Table S1. Number of individuals with genera and class in the experiment with their respective number of individuals ingesting the three beads' sizes at high and low beads concentration. The three replicates per each concentration and the two concentrations (high and low) are pooled together.

Group	Genus / Class	1		3		6	
		Number of organisms that ingest beads	Total	Number of organisms that ingest beads	Total	Number of organisms that ingest beads	Total
Rotifera	*Keratella*	119	279	84	279	97	329
	Keratella quadrata	1	5	0	5	4	5
	Trichocerca	33	78	23	61	25	72
	Lecane	2	17	3	11	7	19
	Lepadella	9	14	0	1	3	11
	Colurella	2	6	1	6	0	7
	Sincheta	4	13	3	11	5	17
	Cephalodella	5	7	5	11	2	5
	Kellikottia	0	1	0	0	1	2
	Monommata	7	9	2	9	5	13
	Polyarthra	0	1	0	0	0	0
	Bdelloids	0	0	0	2	0	1
	Trichotria	4	5	0	2	2	6
Cladocera	*Alona*	0	2	0	0	4	4
	Alonella	6	6	1	1	1	1
	Bosmina	0	1	0	1	0	0
	Pleuroxus	0	0	1	1	1	1
	Ceriodaphnia	8	9	9	10	9	14
Copepoda	*Calanoida*	6	7	1	2	5	12
	Cyclopoida	12	25	15	21	15	35
	Nauplii	16	73	30	74	22	90
Ostracoda		7	8	6	8	6	14

Table S2. Kruskal-Wallis test for the three sizes of MPs per each group at high and low concentrations of MPs

Kruskal-Wallis test for the size 1-3-6							
Concentration	Group	y.	n	statistic	df	p	method
High	Cladocera	Perc	9	2	2	0.368	Kruskal-Wallis
Low	Cladocera	Perc	9	1.859327	2	0.395	Kruskal-Wallis
High	Copepoda	Perc	9	2.440678	2	0.295	Kruskal-Wallis
Low	Copepoda	Perc	8	0.809524	2	0.667	Kruskal-Wallis
High	Nauplii	Perc	9	2.19209	2	0.334	Kruskal-Wallis
Low	Nauplii	Perc	8	4.229167	2	0.121	Kruskal-Wallis
High	Ostracoda	Perc	8	1.666667	2	0.435	Kruskal-Wallis
Low	Ostracoda	Perc	5	0.666667	2	0.717	Kruskal-Wallis
High	Rotifera	Perc	9	3.2	2	0.202	Kruskal-Wallis
Low	Rotifera	Perc	9	1.911504	2	0.385	Kruskal-Wallis

Table S3. Kruskal-Wallis test to test the difference between high and low MPs concentration in each group.

Kruskal-Wallis test for Concentration						
Group	.y.	n	statistic	df	p	method
Cladocera	Perc	18	6.266582	1	**0.0123**	Kruskal-Wallis
Copepoda	Perc	17	12.23893	1	**0.000468**	Kruskal-Wallis
Nauplii	Perc	17	12.33249	1	**0.000445**	Kruskal-Wallis
Ostracoda	Perc	13	9.986364	1	**0.00158**	Kruskal-Wallis
Rotifera	Perc	18	12.88254	1	**0.000332**	Kruskal-Wallis

Table S4. Wilcoxon test with Bonferroni correction for the differences between group at high and low MPs concentrations, the three beads' sizes are pooled together.

Concentration	.y.	group1	group2	n1	n2	statistic	p	p.adj	p.adj.signif
High	Perc	Cladocera	Copepoda	9	9	64	0.026	0.154	ns
High	Perc	Cladocera	Nauplii	9	9	64	0.026	0.154	ns
High	Perc	Cladocera	Rotifera	9	9	68	0.011	0.065	ns
High	Perc	Copepoda	Nauplii	9	9	59	0.11	0.66	ns
High	Perc	Copepoda	Rotifera	9	9	52.5	0.308	1	ns
High	Perc	Nauplii	Rotifera	9	9	26	0.215	1	ns
Low	Perc	Cladocera	Copepoda	9	8	50.5	0.131	0.786	ns
Low	Perc	Cladocera	Nauplii	9	8	50	0.159	0.954	ns
Low	Perc	Cladocera	Rotifera	9	9	51	0.362	1	ns
Low	Perc	Copepoda	Nauplii	8	8	31	0.949	1	ns
Low	Perc	Copepoda	Rotifera	8	9	27	0.375	1	ns
Low	Perc	Nauplii	Rotifera	8	9	34.5	0.919	1	ns
High	Perc	Cladocera	Copepoda	9	9	64	0.026	0.154	ns
High	Perc	Cladocera	Nauplii	9	9	64	0.026	0.154	ns

High	Perc	Cladocera	Rotifera	9	9	68	0.011	0.065	ns
High	Perc	Copepoda	Nauplii	9	9	59	0.11	0.66	ns
High	Perc	Copepoda	Rotifera	9	9	52.5	0.308	1	ns
High	Perc	Nauplii	Rotifera	9	9	26	0.215	1	ns
Low	Perc	Cladocera	Copepoda	9	8	50.5	0.131	0.786	ns
Low	Perc	Cladocera	Nauplii	9	8	50	0.159	0.954	ns

Table S5. Kruskal-Wallis test to test differences between high and low concentration in *Keratella* spp. and *Trichocerca* spp.

Group	.y.	n	statistic	df	p	method
Keratella	Perc	18	13.57393	1	**0.000229**	Kruskal-Wallis
Trichocerca	Perc	18	13.60373	1	**0.000226**	Kruskal-Wallis

Table S6. Kruskal-Wallis test to test differences between keratella and trichocerca at high and low concentration, the size of MPs is pooled together.

Group	.y.	group1	group2	n1	n2	statistic	p	p.adj	p.adj.signif
High	Perc	Keratella	Trichocerca	9	9	0.707513	0.479248	0.479248	ns
Low	Perc	Keratella	Trichocerca	9	9	0.242483	0.808406	0.808406	ns

Table S7. Kruskal-Wallis test to test differences between high and low concentration in Calanoida and Cyclopoida

Group	.y.	n	statistic	df	p	method
Calanoida	Perc	12	2.957983	1	0.0855	Kruskal-Wallis
Cyclopoida	Perc	17	13.12601	1	**0.000291**	Kruskal-Wallis

Table S8. Kruskal-Wallis test to test differences between Calanoida and Cyclopoida at high and low MPs concentrations.

Concentration	.y.	n	statistic	df	p	method
High	Perc	16	0.889299	1	0.346	Kruskal-Wallis
Low	Perc	13	0.216667	1	0.642	Kruskal-Wallis

Supplementary methods

Organisms

For the microcosm the organisms were collected from the urban lake Heiligeensee, Potsdam, Germany. The sampling was conducted in September 2021. The water sample was filtered on site with 30 μm mesh and they were acclimated to laboratory condition in the dark for one week and filtered (0.8 μm mesh) water lake and mix algal food (*Monoraphidium minutum* and *Chlamidomonas noctigama*) was provided.

The taxa presented in the water sample were checked prior the experiment to assure homogeneous replicates and ≥ 150 organisms per replicate were used for the experiment.

Supplementary results

Ingestion of MPs in the rotifera and cladocerans group

In total 12 genus and 1320 rotifers were identified in our experiment; the abundance varies from more than 100 specimens (*Keratella* spp.) to one specimen (*Kellikottia* spp.) per sample.

For example, *Cephalodella* spp. ingested all three sizes at high concentrations as well as *Trichocerca*, *Keratella*, *Sincheta* spp. Some genera were less abundant as for example *Kellikottia* spp., *Lecane* spp., *Lepadella* and they had variable ingestion of the beads, i.e. *Colurella* spp. ingested just 1 and 3 μm beads at high concentration or *Trichotria* spp. that ingest 1 and 6 μm beads.

Five genera of the cladocerans were identified and the abundance varies among the sizes. For example, *Ceriodaphnia* was present in every treatment and ingested the three sizes, *Alona* ingested easily 6 μm but not the 1 μm, *Alonella* instead ingested the three beads size. For *Bosmina* and *Pleuroxus* few specimens where found and they had variable ingestions. The most abundant genus was *Ceriodaphnia* with 33 specimens and the majority of them ingested the beads at high and low beads concentration.

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