

Uptake of plastic microbeads by ciliate *Paramecium aurelia*

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Abstract

Microplastics (MPs) are small fraction of plastics that are less than 5 mm in length. They are bountiful and widespread pollutants in the aquatic environment. A wide range of organisms which play an important role in the food web, ingest microplastic particles and transfer them to the higher trophic levels. In this work, ingestion of fluorescent polystyrene beads 2 µm of diameter by ciliated protozoa *Paramecium aurelia* in different concentrations and times of exposure was studied. We studied also the ingestion and clearance rate as well as formation of food vacuoles. The highest uptake of beads by ciliates reached 1047.2 ± 414.46 particles after 10 min of incubation. Food vacuoles formation reflected the ingestion rate of *P. aurelia*, which increased at higher beads concentration up to the 10th minute of incubation and decreased afterwards. On the contrary, the clearance rate persisted to be higher at low concentration. These findings showed that maximum capacity of microplastics ingestion by paramecia depended on beads concentration and on time of exposure.

Keywords: microplastics, particles, ingestion, ciliates, *Paramecium aurelia*

Introduction

Plastics as a synthetic or semi-synthetic organic materials due to their durability, ductility, lightness and wide range of applications are commonly used in variety of goods [1, 2]. Mass production of plastic has started in 1950 and increased rapidly from 2 million tonnes in 1950 up to about 381 million tonnes in 2015 showing an about 200 times growth in 65 years [2, 3].

Most of plastic are used on land however, plastic wastes that do not enter the loop of plastic waste management on land ultimately are released into rivers and coastal water areas and become a secondary sources of microplastics (MPs). Plastics can be released straight into the river with domestic sewage or they can unintentionally escape from wastewater treatment plants [4]. The impact of macro-plastics (size > 5 mm) on many organisms is well documented, both in freshwater and marine environments [5–7].

The size of microplastics less than 5 mm, enables their pervasive vertical distribution in the water column from surface to the benthic sediment, and makes them bioavailable to small water pelagic species, such e.g. zooplankton [8]. Evidence of ingestion by tiny organisms, has been confirmed both by laboratory and field research [9].

Plastics debris in different shapes and colours floats in the water column, sometimes mimic natural food [7] and are unintentionally consumed by crustaceans, molluscs, fishes, sea turtles [5, 7], and specifically critically endangered leatherbacks [6]. Moreover plastics in the offshore are consumed by Laysan albatross and Wedge-tailed shearwaters as they are detritus feeder birds [10]. However, in terms of size, smaller particles are more likely to be eaten by organisms easily.

Cole and co-workers [11], under laboratory experiments, have revealed that most planktonic Copepods ingested MPs in different size arrays (from 7.3 µm up to 30.6 µm), with 24-hour duration of exposure. The bioavailability of MPs, occurs also in the early stage of plankton's life cycle e.g. decapod's larvae ingested MPs size of 30 µm, when microplastic load was about 50 up to 200 microbeads per mL [12]. The availability of MPs in *Daphnia* and Mussel has effect on feeding activity of fishes and seals [13]. Overall, presence of MPs in aquatic organisms affects negatively their growth, development and lifespan [9]. Accumulation of particles in aquatic invertebrates can potentially lead to blockage of digestive system, thus making it possible to transfer MPs to higher trophic levels in terms of food web fate [14].

The presence of microplastics in aquatic environments, specifically in different water columns, led particularly to interactions between MPs and micro- and zooplankton. Those groups of organisms provide a link of energy transfer to the higher tro-

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phic levels [15]. Recent study by Cole et al. [11] showed, that zooplankton including copepods and decapods, could ingest MPs in different particle sizes. However, improvement of the research on MPs presence in different organisms should be carried out in order to understand their effect on the biology of the consumer [7, 11, 15]. Hence, the further research of microplastics in another taxa living in water as for example protists and small metazoa is needed, due to the fact that only a few studies addressed this issue [16]. Thus, the aim of this study is to (i) investigate the MPs ingestion on ciliated protozoa *Paramecium aurelia*, (ii) assess the clearance rate of labelled MPs in ciliates, (iii) examine the rate of ingestion of MPs in different times of exposure and concentrations and (iv) examine the formation rate of food vacuoles in paramecia.

Materials and methods

Species, cultures and design of experiments

Paramecium aurelia

Culture of paramecia was grown at room temperature in a hay infusion medium in a jar 200 mL of volume stored at room temperature. Before the experiment, about 10 mL of mixed individuals were transferred into the 50 mL Pyrex glass beaker and then, 10 mL of spring water (Żywiec brand, Poland) was added. After 24 hours of acclimatization, paramecia were washed up using spring water in series and then sampled randomly for the experiments using a glass micropipette.

Microplastics particles

For experiments, commercial polystyrene fluorescent yellow-green latex beads (SIGMA: L4530-1ML) supplied by Sigma-Aldrich Poland were used. The type and diameter of the microbeads were selected so that they were clearly visible under fluorescence microscope and their size was similar to the size of the bacteria that *Paramecium* usually feeds on. The beads had 2 μm of diameter and were in 2.5% aqueous suspension counting 5.68×10^9 particles per 1 mL. The beads have fluorescence excitation and emission spectra similar to FITC with excitation waves maximum at 360 nm and emission maximum at 42 nm. The beads were stored in the fridge at 4°C in the dark. For experiments, the initial concentration of microbeads was diluted by pipetting 10 μl of them to 2 mL vial containing 240 μl of deionized water. The estimated concentration of microplastics was about 5.68×10^7 particles/250 μl . Thus, three different concentrations were made up by pipetting 1 μl , 3 μl , and 5 μl of diluted concentrations of MPs with an estimated concentration about 2.27×10^5 , 6.82×10^5 , and 11.36×10^5 particles respectively, which later is referred to low, medium and high concentration.

To assure that paramecia ingest MPs, we exposed them to fluorescent microplastic beads conducting the experiment in a 96-well tissue plate. The set of repetition consisted at least of 5 wells filled with 250 μl of spring water (Żywiec brand, Poland), one of three different concentrations of MPs, and 50 single cells of *Paramecium* randomly selected from ciliates culture. Ciliates were exposed for 5, 10, and 15 minutes to different concentrations of MPs and the microbeads uptake and the clear-

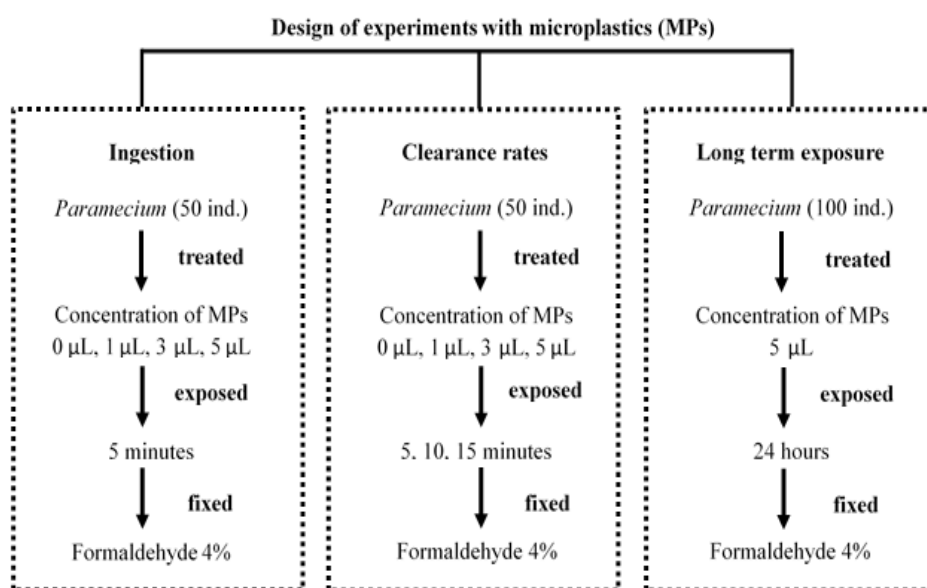


Figure 1. An overall schematic of experiments conducted in this study

ance rate was studied. Then after elapsed time of exposition the sample was fixed with a drop of formaldehyde 4%. Each time 50 single individuals of *Paramecium* from 5 wells with different MPs concentration were taken using thin glass micropipette. The individual cell was treated as repetition for further microscopic analysis. To measure the ingestion in long-term exposure, 100 individuals of randomly selected *Paramecium* were exposed to MPs for 24 hours in high concentration ($5 \mu\text{l} = 2.27 \times 10^5$ particles/ $250 \mu\text{l}$) in five wells. At the end of the experiment, ciliates were fixed by adding a drop of formalin. Then sample was analysed using Nikon Eclipse E-80 fluorescence microscopy and ingestion and long-term exposure were ascertained by viewing about 50 individuals at 200 up to 400 times magnifications. The image of 10 randomly selected specimen was taken by using Nikon camera DS-Ri2 DO accompanied by NIH computer software. The images were then used for the analysis of uptake rate, clearance rate, and food vacuole formations using ImageJ software.

Clearance rate

In order to measure the clearance rate (C) per individual in each experiment at different concentrations and time the calculation described by Peters [17] was followed:

$$C = B / (S \times Gp)$$

in which B was beads counted inside the *Paramecium* cell; S was concentration of bead particles; and Gp was gut passage time that refers to time of cells exposure to the bead suspension in the experiment.

Image analysis

For analysis of the amount of particles ingested by *Paramecium*, ImageJ software was used to reads pixels as units of the image size which were then converted to μm . Thus, the unit of ImageJ was set the same as pixel used in NIH software followed as,

- 200 times magnification: 1 pixel on ImageJ = $0.37 \mu\text{m}$
- 400 times magnification: 1 pixel on ImageJ = $0.18 \mu\text{m}$.

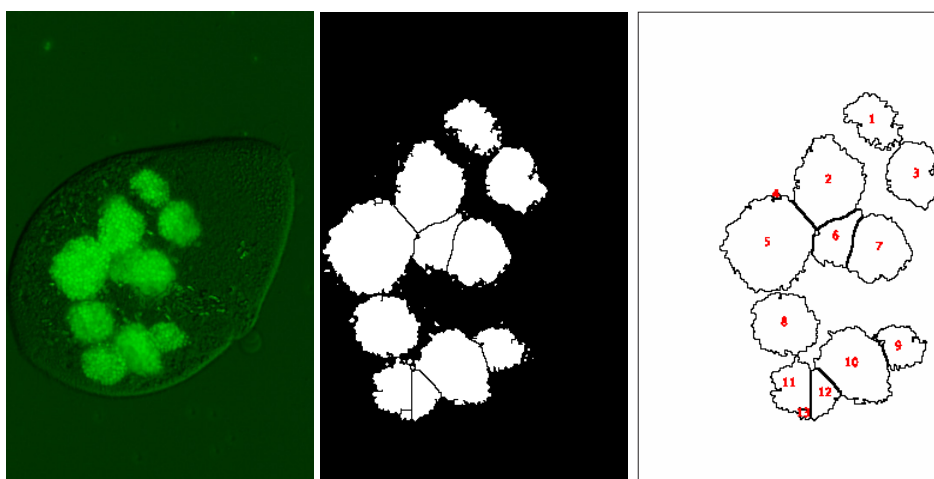


Figure 2. Consecutive steps of the food vacuoles' analysis using ImageJ software

To assure the amount of particles eaten by ciliates, the images were adjusted to a specific colour using a threshold. To calculate the amount of MPs, it was assumed that the microplastics were round on two-dimensional visualization (Fig. 2).

In the next step the area of single microplastic bead was measured and the amount of MPs ingested by single *Paramecium* was determined.

Statistical analysis

To compare the ingestion rates of ciliates at different microbeads concentrations the data were analysed using Statistica 13.3.

Regression analysis was done by Microsoft Excel confirmed with Statistica, in order to get regression of clearance rate of tested paramecia. The graphs were produced by Origin 2016 software.

Results

Microplastics (MPs) ingestion by Paramecium aurelia

Fluorescent labelled microplastic particles $2 \mu\text{m}$ in diameter, were perceptible in *Paramecium* food vacuoles in different concentration and exposure time. The whole batch of tested individ-

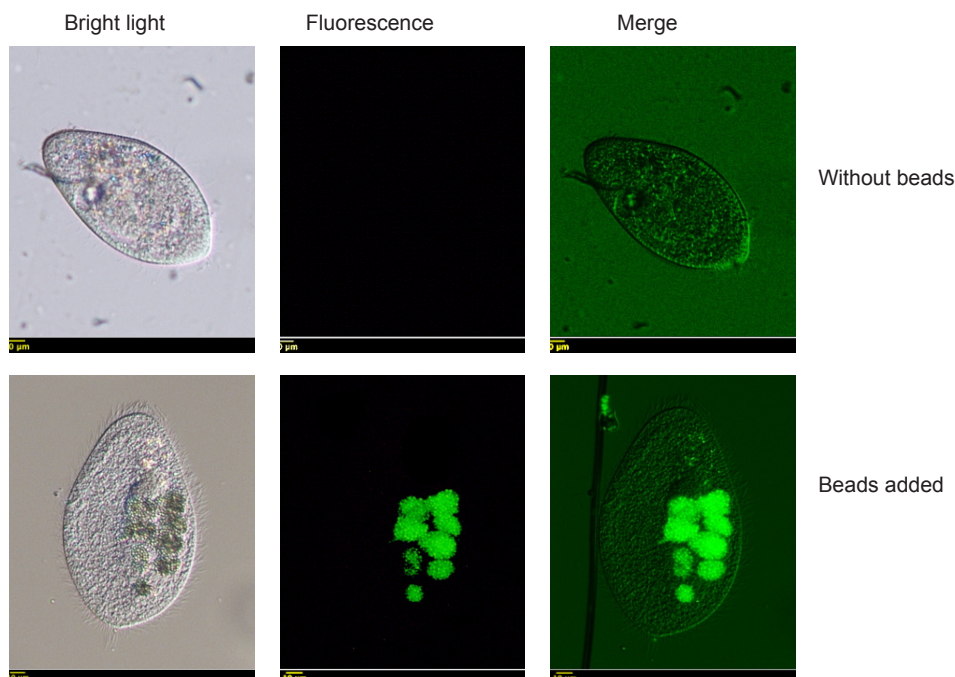


Figure 3. Fluorescently labelled microplastic beads engulfed by ciliate *Paramecium aurelia* visible under microscope in bright field, epifluorescence and both techniques merged

uals exhibited positive ingestion of MPs (Fig. 3). The number of microbeads ingested by single *Paramecium* varied, ranging from an average 172.1 ± 189.46 up to 1047.3 ± 414.46 particles.

Ingestion of microbeads by *Paramecium* depended on the concentration of beads and time exposure (Fig. 4) and differed significantly ($p < 0.001$). The number of microbeads ingested by ciliates hit its peak value in medium concentration treatment, following 10 minutes of exposure, whilst reached its lowest value, in low concentration treatment, after 5 minutes of exposure. Starting from exposure for 5 minutes, ingestion of microbeads by ciliates in low and medium concentrations treatment was not significantly different, by average 172.1 ± 189.46 and 234.6 ± 240.45 , respectively.

Meanwhile, following a high MPs' concentration treatment, the ingestion was higher, reaching ca. 581.9 ± 327.76 ($p < 0.001$). Following 10 minutes of incubation, the ingestion of particles in the treatment with low and medium concentrations, increased significantly ($p < 0.001$) and accounted for ca. 678.1 ± 356.97 and 1047.2 ± 414.46 of ingested beads, respectively. In turn the increase in number of ingested particles was detectable following exposure to high MPs' concentration, however it was not significant ($p = 0.993$).

The treatment at low, medium and high MPs concentration after 15 minutes incubation showed, that there was no significant difference within and the ingestion reached an average ca. 798.1 ± 324.10 ; 875.2 ± 372.90 ; and 752.5 ± 294.00 , respective-

ly. In long time exposure experiment, it was noted that the ingestion of beads after 24 h was statistically significant ($p < 0.001$) at low, medium and high concentrations, reaching 335.2 ± 387.33 , 314.2 ± 324.08 and 323.4 ± 334.48 , respectively.

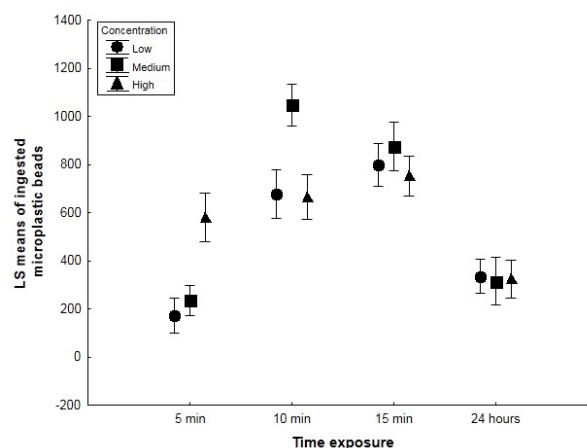


Figure 4. Comparison of microplastic beads ingested by *Paramecium aurelia* in different concentrations and time exposure. Differences in interaction effect of both concentration and time of exposure were statistically significant ($F_{(6, 658)} = 13.664$, $p < 0.001$)

Those numbers indicated that varying microbeads concentrations used for this particular treatment had no significant statistical relevance ($p = 1.000$ for all comparison). The not-quantitatively analysed observation during the experiments showed, that

ingested by *Paramecium* MPs were not metabolised and later egested in a form of compact balls full of particles.

Clearance rate of *Paramecium aurelia*

Comparison of clearance rate in different MPs concentration and incubation times, showed significant difference in both microbeads concentration and time exposures (ANOVA, $p < 0.001$) (Fig. 5). The highest clearance rate occurred in treatment with low beads concentration, whereas the lowest was observed in treatment with their high concentration. The initial clearance rate after 5 min. of the incubation was highest at a low concentration, reaching ca. $1.52 \times 10^{-4} \pm 1.67 \times 10^{-4}$ and followed by high and medium concentrations, which both did not differ significantly ($p = 0.850$). A noticeable increase was observed after 10 minutes of incubation for treatments in low and medium beads' concentrations and reached ca. $2.99 \times 10^{-4} \pm 1.57 \times 10^{-4}$ and $1.54 \times 10^{-4} \pm 0.61 \times 10^{-4}$, respectively. After 15 minutes of the exposure, the clearance rate perceived decrement at both low and medium concentrations to $2.34 \times 10^{-4} \pm 0.95 \times 10^{-4}$ and $0.86 \times 10^{-4} \pm 0.36$, respectively. The decrement continued along with increased time exposure for up to 24 hours.

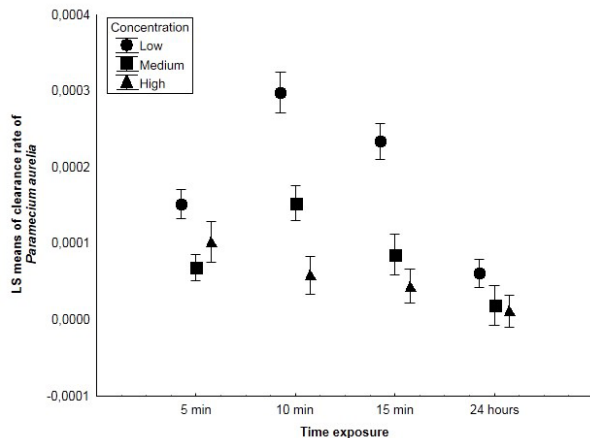


Figure 5. Clearance rate of *Paramecium aurelia* for various time exposure and different microbeads concentrations. Comparison analysis of clearance rate according to time exposure and concentration variation was significantly different ($F_{(6, 658)} = 17.978, p < 0.01$)

Food vacuole formation in *Paramecium aurelia*

Food vacuole formation count in *Paramecium* was influenced by time of exposure and concentration of microbeads (Fig. 6), and was significantly different ($p < 0.001$). The highest number of formed food vacuoles was ca. 9.9 ± 2.49 units, following 15 minutes of exposure to high concentration of microbeads content. It was found that after 5 minutes of exposure at both low

and medium concentration treatment the result did not differ significantly and reached between 3.1 ± 1.5 and 4.2 ± 2.72 , respectively. Meanwhile, at high concentration the result was perceived significantly higher than others, peaking at 6.8 ± 1.85 . Food vacuole formation count increased significantly ($p < 0.001$) after 10 minutes of exposure, for both low and medium microbeads concentration treatment, reaching the average of 7.2 ± 2.25 and 9.8 ± 2.54 , respectively, whereas at high concentration, its rate remained flat ($p = 1.000$). Fixation after 15 minutes proved that the formation of food vacuoles at medium microbeads concentration decreased slightly to 8.5 ± 2.36 , whereas, on contrary it increased at both low ($p = 0.071$) and high ($p < 0.001$) microbeads concentrations to 9.1 ± 2.65 and 9.9 ± 2.49 respectively. A long time exposure experiment showed, that food vacuole formation declined significantly for all concentration treatment ($p < 0.001$, for all comparison with prior time exposure). Variation of the concentrations did not present any significant difference ($p > 0.900$) for all levels of MPs' concentration treatments, where the average reached was 6.5 ± 3.16 ; 6.0 ± 3.87 ; and 5.8 ± 2.99 , respectively.

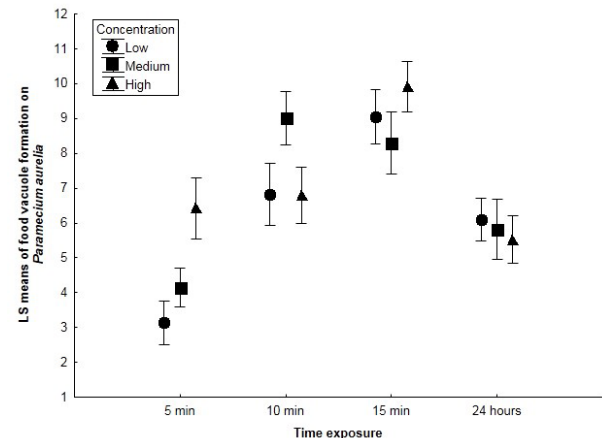
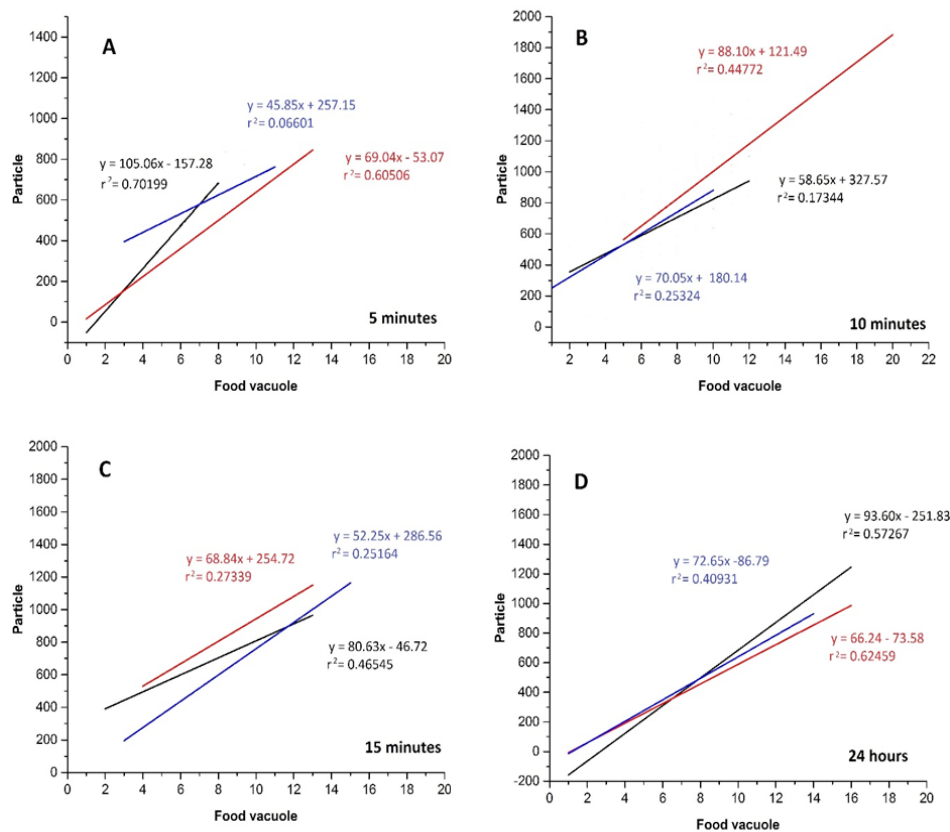


Figure 6. Comparison of food vacuole formation of *Paramecium aurelia* according to time exposure at different MPs concentrations ($F_{(6, 628)} = 13.639, p < 0.001$)

Figure 7 shows that a positive correlation between food vacuole formation and the amount of MPs ingested by *Paramecium* occurred ($p < 0.001$). It was revealed that the higher number of food vacuoles formed was a result of ingested number of the microplastic beads. The variances in food vacuole formation and amounts of beads ingested by individual depended on different levels of microbeads concentration and exposure time.



Note: Concentrations: black line—low; red line—medium; blue line—high. Time exposure: A—5 minutes; B—10 minutes; C—15 minutes; D—24 hours.

Figure 7. Correlation between food vacuole formation and the amount of ingested MPs by *Paramecium aurelia* in different concentrations and time exposure

Discussion

Ciliate *Paramecium aurelia* is a filter-feeder species which ingest plastic microbeads during continuous filtration process relied on microplastic concentrations and time exposure. Experiments showed, that in the first 5 minutes of incubation in solutions with plastic microbeads all individuals of *Paramecium* took up the particles readily in all treatments. Moreover as the preliminary experiments not included in this study showed, that this kind of chosen microbeads and their densities were not visibly toxic or harmful for the ciliates even in long time cultures. Therefore the survival test of ciliates was not considered to be necessary to perform. As a result, the number of food vacuoles formed, reflected the amount of MPs ingested and showed positive correlation with volume of microbeads ingested. The intensity of food vacuoles formation varied and depended on the microparticles concentration and time of the incubation. The number of food vacuoles increased along with increasing beads concentration up to 10th min. of incubation, then decreased and appeared to no longer depend on varying beads concentrations.

A similar result was reported by Fok and co-workers [18] in *Paramecium multimicronucleatum* fed with 0.26 μm fluorescent beads where number of food vacuoles increased along with increasing particle concentration. It was also confirmed that the formation of food vacuoles was strongly influenced by time, and after 30 min. of treatment the cumulative amount of food vacuoles became stagnant [18, 19]. Food vacuole formation in ciliates, including *Paramecium*, is sensitive to external environmental changes like temperature, pH, size and concentration of particles in the medium. Furthermore, Ramoino [20] described that in *Paramecium primaurelia* under constant conditions, food vacuole formation rate increased at different life span clones. This was not studied in case of tested strain of *Paramecium*, however, we noticed, that the time required for food vacuoles to form was shorter in solutions with high MPs' concentrations. This observation was similar to those of Ramoino [20] which showed that the number of food vacuoles reflected the amount of particles ingested by *Paramecium*.

Ciliates, in particular *P. aurelia* showed different responses in terms of MPs' uptake. We observed that the higher was

the concentration of the MPs, the more particles were ingested. However, such outcome depended also on the length of time of incubation with the beads. In our experiment, the longer incubation time did not reflect on higher microplastic particles ingestion. Our experiment showed, that the ingestion of particles by paramecia increased and differed for varying MPs' concentrations in line with time up to 10 min. then remained stagnant and afterwards decreased. This leads to the conclusion, that after first 10 min. of the experiment, different concentrations of microplastic particles have no any visible effect on the MPs' ingestion.

This observation complied with the model presented by Peters [17] showing that the feeding rate of planktonic organisms linearly increased with increasing food concentration until it reached a point where the feeding rate became stagnant, even though the food concentration was further increasing. In addition, an experiment on *Paramecium caudatum* fed with three different kinds of bacteria added with density of 10000 bacteria per mL showed, that the consumption rate decreased gradually following the increase in incubation time until it has reached the maximum point [21].

Although *Paramecium* exhibited preference for a particular food size [4, 22], it did not show the ability to select either nutritive or non-nutritive foods [23]. This finding was early noticed by Bragg [24], who studied *Paramecium trichium* and *P. caudatum*. He found, that both investigated species were limited when selecting between nutritive and non-nutritive food particles. Whilst, it did not point out directly to our tested organism, considering the high number of MPs ingested by *P. aurelia*, it gave us a hint that this particular species appears to have no ability to distinguish among nutritive and non-nutritive particles.

During the experiment we also measured the clearance rate of *Paramecium*, showing that it was high even in low microplastic particles concentrations. In the low concentration of particles the clearance rate increased along with the incubation time to the maximum incubation point up to 10 min. The clearance rate, however, decrease gradually with time of incubation at high concentration.

Obtained results fitted to the model described by Peters [17], that the clearance rate of planktonic organisms decreased gradually after reaching maximum point even though the concentrations of food increased. Furthermore, Fenchel [22] discovered, that the clearance rate of ciliates decreased, when they feed on large particles and different species have a different preferred food size which could be ingested. Used in our experiment microbeads of 2 μm in diameter had a dimension of typical bacteria and therefore were in optimal size for that species of *Paramecium*.

In the long term incubation experiment, it was shown that microplastic particles concentration had no effect on the amount of particles ingested, neither on the food vacuole formation process, nor on the clearance rate of *Paramecium*. Additionally, our investigation revealed, that under these conditions the number of MPs ingested plummeted. It is presented in our study that the clearance rate plunged at the lowest point, similarly to the amount of created food vacuoles which experienced significant decrease. It is also evident, that particles ingestion and clearance rate increased over the incubation time and reached the peak at some point prior to decline. This long term treatment suggests, that this was closely related to the egestion process. Cole and coworker [11] explained that the egestion process in filter-feeders organisms occurred in a matter of hours. Moreover, it was described that the egestion of microplastic particles did not cause damage to the organism since there was no toxic effect in the population exposed to MPs for one or two days [23]. They also showed, that in ciliate *Tetrahymena* sp. microplastic particles were released outside in the form of compact microplastic balls of food vacuole size [23]. Similar to those results, we did not observe the negative short-term impact of engulfed MPs on ciliates. However, other results showed that in case of small metazoan e.g. rotifers fed on nanoparticles less than 1 μm , their growth rate depressed [25]. Furthermore, MPs in environment can also decrease fecundity, lifespan, reproduction time and body size, while increasing oxidative stress and antioxidant enzyme [26]. Microparticles ingestion by *Paramecium* seems to not cause harm effects, since ciliates could egest the particles after some time [19]. As a problem of microplastics waste becomes more and more widespread all over the world, further studies concerning the fate of plastic particles in the environment and their effect on biocenosis are necessary.

Conclusions

Results obtained in our experiments showed that *Paramecium aurelia* readily ingested microplastic particles 2 μm in diameter. The ingestion of MPs was governed by beads concentration and length of incubation times. Food vacuole formation in ciliates depicted the amount of MPs ingested. Not quantitatively studied observations showed that ingested microplastic particles by *Paramecium* were later egested in a form of compact balls full of ingested particles. Further studies are necessary to show related aspects of MPs occurrence in natural environment and their effect on microorganisms and whole biocenosis.

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Author Contributions

Conceptualization, Janusz Fyda; methodology, Janusz Fyda; software, Failasuf Aulia Nugroho; validation, Failasuf Aulia Nugroho and Janusz Fyda; formal analysis, Failasuf Aulia Nugroho and Janusz Fyda; investigation, Failasuf Aulia Nugroho; resources, Failasuf Aulia Nugroho; data curation, Failasuf Aulia Nugroho and Janusz Fyda; writing—original draft preparation, Failasuf Aulia Nugroho; writing—review and editing, Janusz Fyda; visualization, Failasuf Aulia Nugroho and Janusz Fyda; supervision, Janusz Fyda; project administration, Janusz Fyda; funding acquisition, Janusz Fyda.

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