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Influence of Nano- and Small Microplastics on Ciliated Protozoan *Spirostomum ambiguum* (Müller, 1786) Ehrenberg, 1835

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Abstract: This study evaluated the uptake of secondary nano- and small microparticles by the protozoan *Spirostomum ambiguum*, comparing edible (baker's yeasts) and inedible (red latex) particles. Secondary nano- and microplastic particles were prepared from household materials made of four different polymers and served to the protozoans separately and as two-component mixtures in different proportions. The number and content of food vacuoles formed by the protozoan were analyzed using a digital microscope. The microscopic results showed that the protozoans ingested the secondary microplastic particles to a similar degree as the latex microspheres but to a lesser extent compared to the nutritional food—baker's yeasts. At the microplastic concentrations of 1000 and 10,000 particles mL^{-1}, no food vacuoles were observed inside the cells, which may be a finding of great ecological importance. In the protozoans served two-component mixtures, both microplastics and yeasts were found in the vacuoles formed by the organisms. The egestion of two-component vacuoles by the protozoans was slower than that of vacuoles containing a single component.

Keywords: microplastics in the environment; secondary microplastics; food vacuoles; ingestion

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1. Introduction

Due to their chemical stability and high resistance to degradation, plastics can be found in all ecosystems. In the last decade, several scientific reports have been published describing the fate and effects of microplastics in not only marine but also freshwater ecosystems [1–5]. Plastic particles that are used as raw materials or additives in personal care products, such as peelings and shower gels, are primary microplastics [5–8]. Secondary microplastics, on the other hand, are a more diverse group and include particles formed as a result of manufacturing processes in industries, laundry in households, and the photodegradation or mechanical grinding of larger pieces in the environment [6]. The term "nanoplastics" has not been defined yet, and studies have set the upper size limit of this class of particles at 1 or 0.1 µm [9]. The working group on Good Environmental Status has classified plastic particles based on their sizes as nanoplastics (1–1000 nm), small microplastics (20–1000 µm), and large microplastics (1–5 mm) [6]. Hartmann et al. recommended a similar division of particle sizes into nanoplastics (1–1000 nm) and microplastics (1–1000 µm) and proposed using the largest dimension as a classifier for the category [7]. When referring to biological systematics, particles with a size between 1 and 10 µm can cover both nanoplankton (2–20 µm) and picoplankton (0.2–2 µm). Particles of this size act as food for filter-feeding crustaceans, mussels, and protozoans [10]. The influence of microplastics on marine invertebrates, including shellfish, oysters, cnidarians, and crabs, has been investigated by numerous studies [1,11,12]. Crustaceans are a group of invertebrates most commonly used in studies of MP ingestion [13]. However, only a few have addressed the uptake of microplastics by freshwater organisms so far [6,14,15].

The uptake, accumulation, depuration, and toxic effects of microplastics on freshwater organisms were reviewed by Anbumani and Kakkar in an earlier study [6]. The ingestion of chemically inert microparticles can induce physical and biochemical effects, such as reduction of feeding performance, and decrease of selected enzyme activity [16]. Moreover, the various degradation products of additives and polymers may cause toxicity. The ingested micro- and nanoplastics can accumulate in organisms and be transferred along the food chain. A review by Shen et al. [5] showed that plastic particles could accumulate in fish tissues and even pass through the blood–brain barrier. The small size PS microspheres were ingested and accumulated more easily by the mussel *Mytilus coroscus* and could pass through the biological barriers [17]. Nanoparticles and microparticles differ in their toxic effects in different tissues. Yin et al. [8] presented those differences and indicated the need to undertake research on the unique toxicity mechanism of these two classes of particles.

Protozoans play an important role in freshwaters as primary consumers and active components of water and effluent purification systems [18]. They are also used as food by higher organisms and thus act as a link between bacteria and metazoans [19]. Since the mid-20th century, research works have reported that protozoans can ingest non-nutritional particles [19–23]. The literature presents much data on the uptake of nano- and microparticles by protozoans, based on the particle size [10,19,23] and based on whether the particles are edible or inedible [20,21,24]. In most of the studies, specially prepared particles, mainly spherical microspheres, were used for analyses [10,19,22,23,25–27]. However, in the natural environment, secondary microparticles of different shapes can be detected [28–30], and their uptake by protozoans may differ from that of primary microparticles.

The present study aimed to evaluate the uptake of secondary nano- and small microparticles in comparison to the round, regular-shaped edible (baker's yeasts, BY) and inedible (red latex, RL) particles by *Spirostomum ambiguum* protozoans. These secondary particles were prepared by grinding household plastics made of four different materials: polystyrene (PS), polyvinyl chloride (PVC), polyethylene terephthalate (PET), and phenolic resin (PhR). The particles were served to the protozoans both separately and as two-component mixtures in different proportions. The hypotheses of the study were as follows: (1) the ingestion of nano- and small microplastics and the formation of food vacuoles in protozoans are influenced by the concentration, shape, and chemical composition of the particles; (2) the extent of ingestion of secondary microplastics by protozoans is the same as that of inedible or edible particles of a regular shape. Significantly higher particles concentrations than those found in the environment were used due to the fact of performing analyses of microparticles uptake by *S. ambiguum* for the first time and the short observation time allocated.

2. Materials and Methods

2.1. Microparticles

Yeast (*Saccharomyces cerevisiae*) was used as nutritional particles. Ten milligrams of freeze-dried yeast were vortexed with 10 mL of Tyrode's solution. Spherical red latex beads (RL) of the same size (≈5 µm) were purchased from MicroBioTests (Gent, Belgium). They were used as reference, nontoxic, primary microparticles. Four types of colored secondary microplastics were prepared from household materials: PET from packed ketchup (red), PS from CD box (black), PVC from tap water pipes (white), and PhR from laboratory worktops (gray). The plastics were cut into small pieces and ground in liquid nitrogen using a cryomill (SPEX, type 6770; Metuchen, NJ, USA). Then, the resulting particles were sieved through a 100 µm steel mesh. The microplastic suspensions were prepared by mixing 2 g of particles with 500 mL of Tyrode's solution in glass bottles. They were vortexed using an ultrasonic bath (Polsonic, Type Sonic 6; Warsaw, Poland) for 20 min at 25 °C and stored in darkness at 25 °C. For preparing working microplastic suspensions, stock suspensions were shaken, dispersed in ultrasonic bath (20 min at 25 °C), and the correct amount of suspensions was transferred to Tyrode's solution. The working suspensions were freshly prepared before each analysis.

2.2. Microscopic Imaging

A Keyence VHX 7000 digital microscope (KEYENCE International, Mechelen, Belgium) was used for both observing protozoan food vacuoles and obtaining microparticle measurements. A dedicated image analysis software was used for observation, counting, and measurement of particles.

The concentrations of the particle suspensions were determined using a Bürker counting chamber. The particle size histograms were compiled using the measurements of at least 3000 particles.

2.3. Protozoan S. ambiguum

The ciliated protozoan *S. ambiguum* has been cultured in the Department of Environmental Health Sciences, Medical University of Warsaw, Poland, for over 40 years based on a previously described procedure [31]. The organisms were maintained on a bacterized medium with oatmeal as a nutrient source. To eliminate the residues from the culture, the protozoans were rinsed twice with freshly prepared Tyrode's solution before the tests and incubated in a fresh medium for at least 1 h. The medium used for incubation consisted of a minimum amount of inorganic components that are needed for the survival of the protozoans for up to 8 d [31].

The number of food vacuoles in individual ciliates was counted with the Keyence digital microscope. Briefly, 10–20 protozoans were transferred to Tyrode's solution containing 0.4 mM nickel nitrate. The background suspension remaining in the subsample was removed by transferring the immobilized cells to fresh Tyrode's solution. Finally, the protozoans were placed on a microscopic slide and covered with a coverslip. Images were taken within 30 min. For each time-course sample, 15 *S. ambiguum* protozoans were assessed under a 200× magnification. To evaluate the contents of food vacuoles, some cells were observed under a 500× magnification.

2.4. Ingestion Studies

Three time-course experiments were performed in the study. In the first experiment, which aimed to determine particle ingestion by *S. ambiguum*, the cells were incubated with different concentrations (10^3, 10^4, 10^5, and 10^6 particles mL^{-1}) of the tested suspensions. The tested concentration of the plastic particles used in the study was in the range of optimal food suspensions for the growth of the protozoan. However, this concentration was at least 4 orders of magnitude higher than those found in surface waters [30]. The number and content of food vacuoles in the cells were determined after incubation for 2 and 24 h at 25 °C. In the second experiment, which aimed to determine food preferences, *S. ambiguum* was first fed with different kinds of microplastics for 2 h, and then BY or RL suspension of the same concentration as microplastics was added. The particle number of each component of the suspension was identical (5×10^5 particles mL^{-1}). One-component suspensions with the same particle concentration constituted the controls. The number of vacuoles was determined after 4, 24, and 48 h of incubation at 25 °C. In the third experiment, the protozoans were served the mixtures of microplastics and BY in three different proportions (1:3; 1:1, and 3:1) based on the number of particles per mL. The total concentration of all components in the mixture was 10^6 particles mL^{-1}, which implies that in a 1:3 mixture, the concentrations of BY and MP were 2.5×10^5 and 7.5×10^5 particles mL^{-1}, respectively, and in a 3:1 mixture, they were 7.5×10^5 and 2.5×10^5 particles mL^{-1}, respectively. The number of vacuoles was determined only after 24 h of incubation at 25 °C in this experiment.

2.5. Data Treatment

Data were analyzed for distribution normality and outliers (Grubbs' test). Analysis of the data was done with the Statistica software and Microsoft Excel with Analysis ToolPak. A two-sample t-test assuming unequal variances was used to evaluate statistical significance

of the differences between the experimental groups. Differences were considered significant at $p < 0.05$.

3. Results

3.1. Particles

Figure 1 presents the particle size distribution by number of the tested suspensions, and Figure 2 presents the microphotographs of the tested particles. BY and RL were found to be homogenous in shape (Figure 2a,b) and dimension (4.8 ± 0.2 and 4.8 ± 0.7 µm, respectively) (Figure 1a,b). One-third of the BY particles were larger than 6 µm in size. This was mainly due to the presence of dividing cells and small aggregates containing two to four cells. RL did not mostly form aggregates.

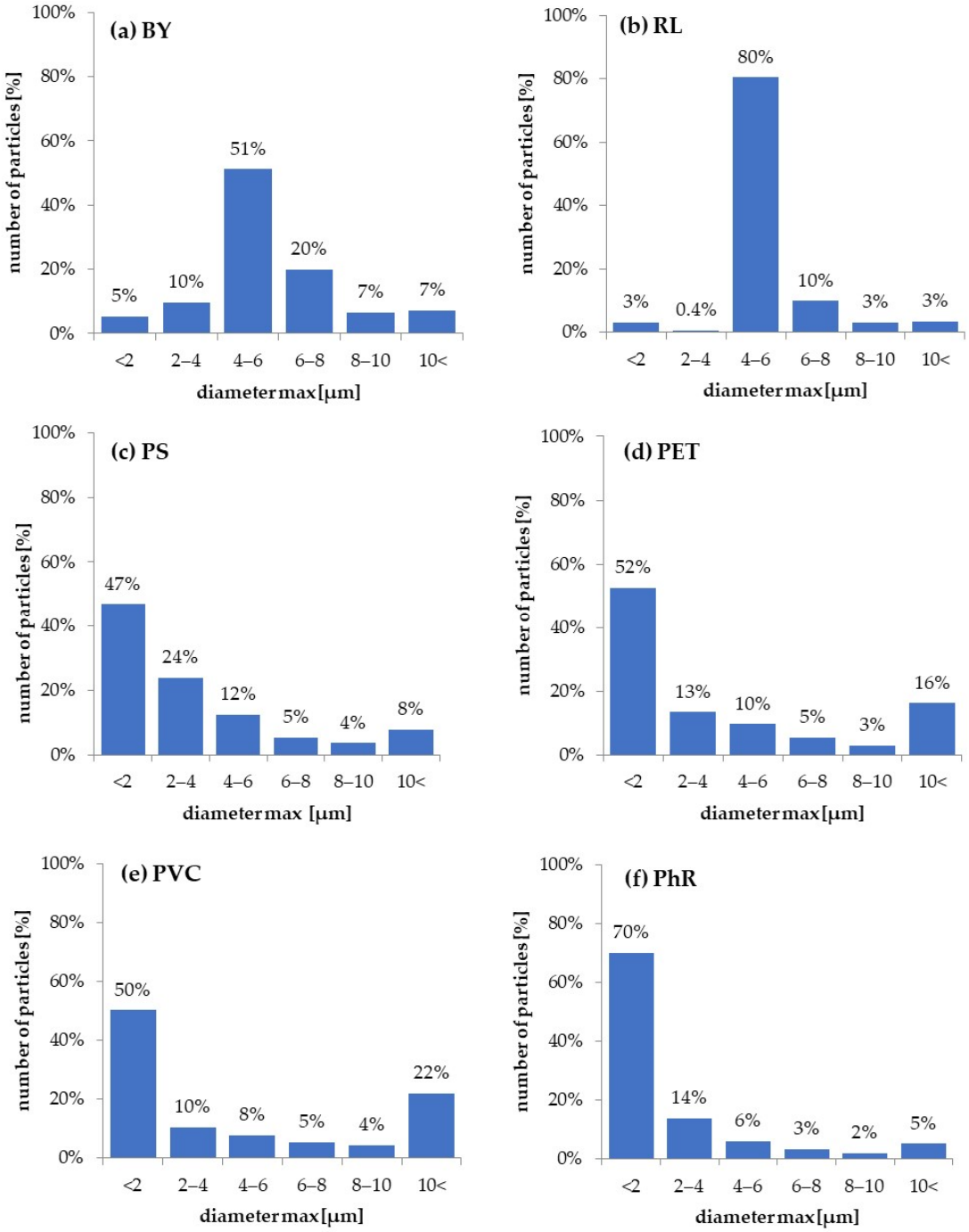

Figure 1. Size distribution of nano and small microparticles in the tested suspensions: (**a**) BY; (**b**) RL; (**c**) PS; (**d**) PET; (**e**) PVC; (**f**) PhR.

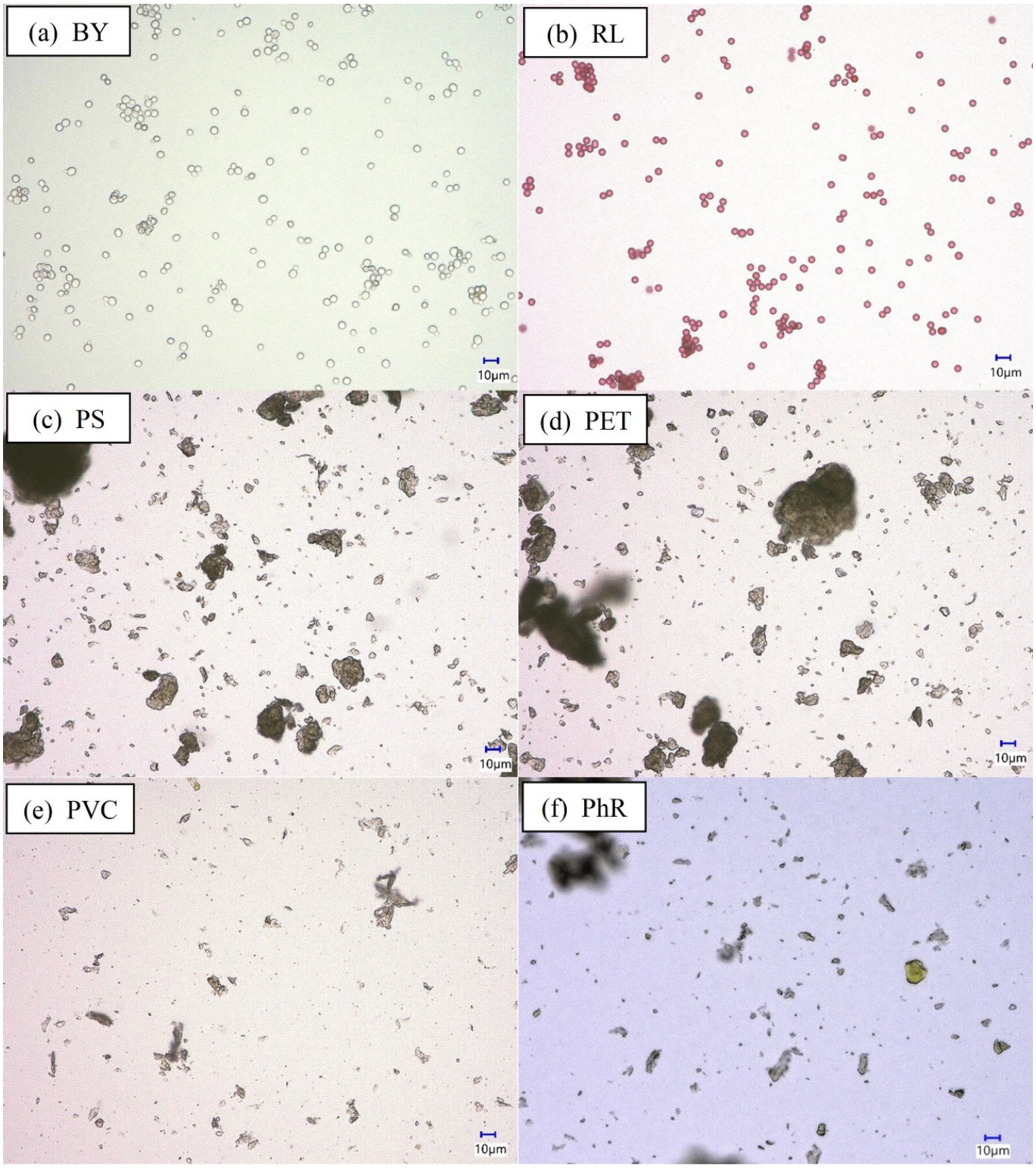

Figure 2. Microphotographs of the used microparticles: (**a**) BY; (**b**) RL; (**c**) PS; (**d**) PET; (**e**) PVC; (**f**) PhR.

The secondary plastic particles prepared in this study were heterogenous in both size (Figure 1c–f) and shape (Figure 2c–f). Most particles by numbers, especially PhR, were smaller than 2 μm in size. On the other hand, the size of 22% and 16% of the PVC and PET microplastics exceeded 10 μm. This difference in both the shape and size of microparticles enabled the assessment of the real impact that the nano- and small microparticles occurring in the natural environment can have on protozoans.

3.2. Uptake of Particles by S. ambiguum

3.2.1. Influence of Particle Concentration

To analyze whether the concentration of particles had any influence on their uptake by *S. ambiguum*, the protozoans were transferred to suspensions containing microparticles in different concentrations (10^3, 10^4, 10^5, and 10^6 particles mL^{-1}). The number of formed food vacuoles and their content were assessed with the Keyence VHX 7000 digital microscope after 2 and 24 h of incubation. It was observed that the protozoans ingested the particles and formed food vacuoles in all kinds of suspensions, but the extent of ingestion and vacuole formation varied (Table 1). After 2 h of incubation, the number of food vacuoles formed in the microsphere suspensions did not differ between nutritional (BY) and non-nutritive (RL) suspensions. The number of vacuoles increased with increasing concentration of microspheres but only up to 10^5 particles mL^{-1}. The protozoans showed the least preference for PET and PhR particles, as after 2 h of incubation, food vacuoles were observed only in the most concentrated suspensions, with their number varying from 0 to 2.

Table 1. Number of vacuoles formed by the protozoan *S. ambiguum* in suspensions with different microparticle concentrations after 2 h and 24 h of incubation.

		No [1]	10^3 Particles mL^{-1}			10^4 Particles mL^{-1}			10^5 Particles mL^{-1}			10^6 Particles mL^{-1}		
			AVE ± SD	MIN	MAX	AVE ± SD	MIN	MAX	AVE ± SD	MIN	MAX	AVE ± SD	MIN	MAX
2 h	BY	13–19	1.1 ± 1.0	0	3	7.3 ± 2.3	4	11	9.9 ± 2.4	5	14	13.3 ± 3.9	6	22
	RL	13–17	3.1 ± 1.9	1	7	6.5 ± 3.1	2	11	10.0 ± 3.5	5	15	10.8 ± 2.9	5	17
	PS	14–18	1.1 ± 1.4	0	4	0.3 ± 0.6 [2]	0	2	5.5 ± 2.0 [2]	2	9	7.1 ± 2.0 [2]	3	10
	PET	12–17	0	0	0	0	0	0	0	0	0	1.1 ± 1.0 [2]	0	2
	PVC	13–18	0	0	0	0	0	0	1.2 ± 0.7 [2]	0	2	3.3 ± 1.5 [2]	1	6
	PhR	13–17	0	0	0	0	0	0	0	0	0	0.9 ± 1.0 [2]	0	2
24 h	BY	12–20	0.5 ± 0.9	0	2	8.8 ± 3.2	4	16	12.9 ± 3.1	9	19	28.0 ± 3.5	24	30
	RL	13–20	0	0	0	0.8 ± 0.8 [2]	0	2	4.2 ± 1.8 [2]	2	7	14.7 ± 2.4 [2]	12	19
	PS	13–17	0	0	0	0	0	0	8.2 ± 2.1 [2]	6	12	9.2 ± 2.2 [2]	6	14
	PET	13–18	0	0	0	0	0	0	3.7 ± 1.7 [2]	2	8	14.1 ± 4.7 [2]	6	20
	PVC	14–17	0	0	0	0	0	0	6.2 ± 2.2 [2]	3	11	7.0 ± 1.4 [2]	5	10
	PhR	13–19	0	0	0	1.7 ± 0.9 [2]	0	3	2.0 ± 0.8 [2]	1	3	14.6 ± 2.8 [2]	11	20

[1]—the number of protozoans tested; [2]—significantly different ($p < 0.05$) from the control (BY).

After 24 h, it was observed that the number of vacuoles in the most concentrated BY suspension was doubled. The vacuoles were also larger and contained several dozen yeast cells (Figure 3a). Similarly, in the two most concentrated PET, PVC, and PhR suspensions, the number of vacuoles was increased significantly compared to that observed after 2 h. By contrast, in the samples containing 10^3 and 10^4 particles mL^{-1}, no or only single vacuoles were observed even after 24 h.

In most of the tested suspensions, the protozoans formed round vacuoles, which contained from several to several dozens of particles (Figure 3). The size of vacuoles slightly increased with increasing particle concentration and time of incubation. One exception was PS, in the case of which round vacuoles were observed only in suspensions with concentrations up to 10^5 particles mL^{-1}, while in those containing 10^6 particles mL^{-1}, the vacuoles were of elongated shape (Figure 4). This may indicate the formation of the phagosome at regular intervals, regardless of the amount of material deposited at the bottom of the oral cavity.

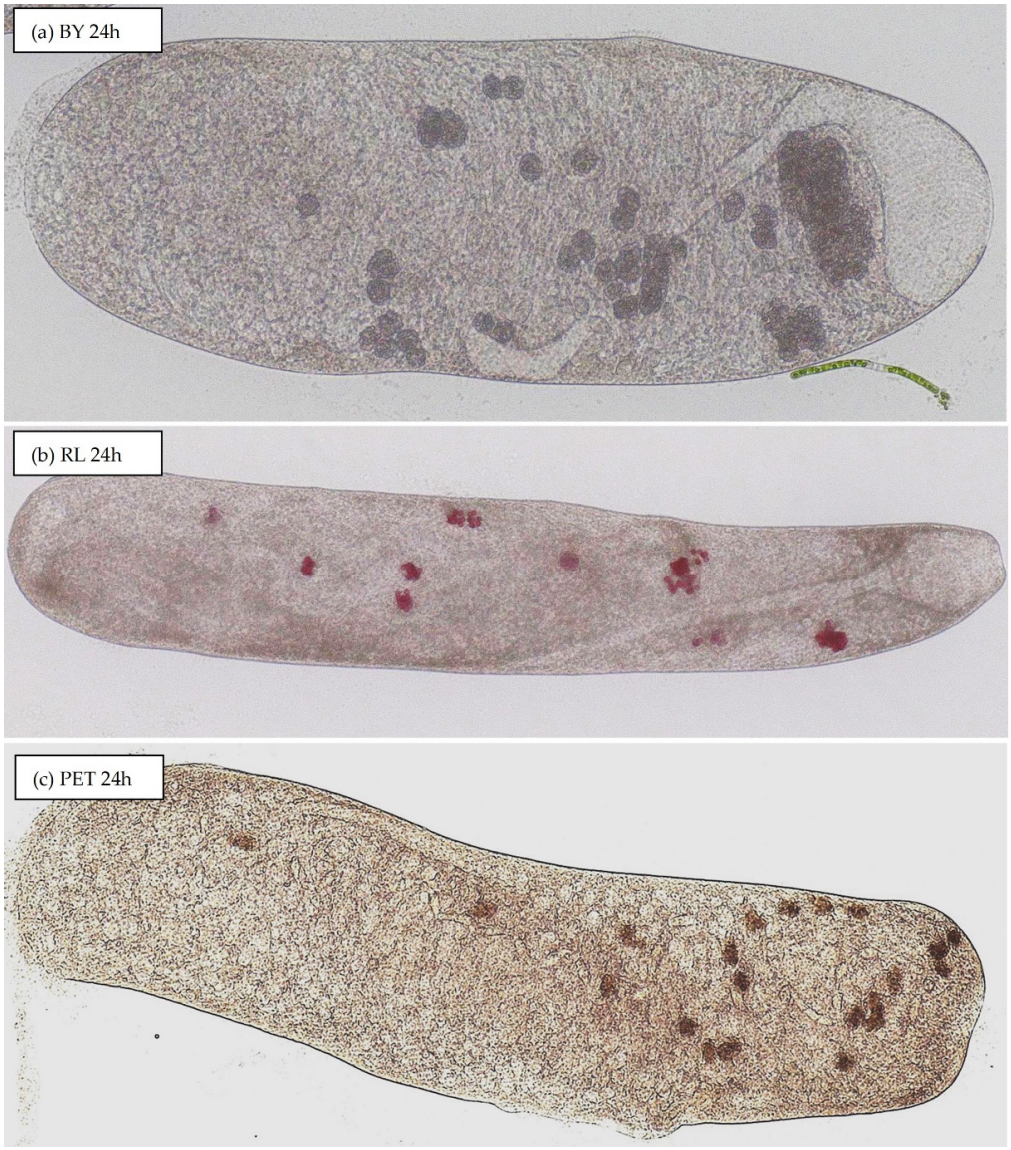

Figure 3. Food vacuoles formed in *S. ambiguum* in (**a**) BY, (**b**) RL, and (**c**) PET suspensions after 24 h.

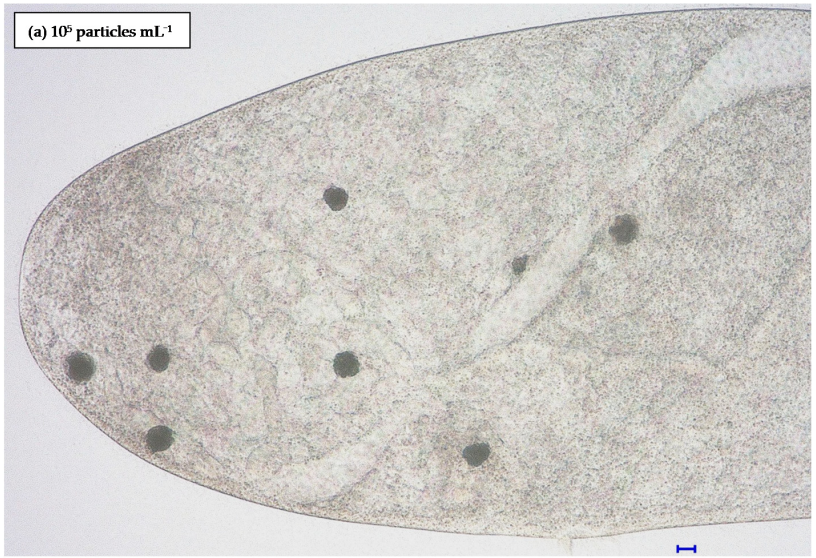

Figure 4. *Cont.*

Figure 4. Food vacuoles formed in *S. ambiguum* in the PS suspension with a concentration of (**a**) 10^5 and (**b**) 10^6 particles mL^{-1}. Blue bar represents 10 μm.

3.2.2. Material Composition

With respect to the material composition of the secondary microparticles, it was observed that after 24 h of incubation, the protozoans formed the least number of vacuoles in PVC and PS suspensions, although the PS particles were the most frequently ingested secondary microplastics after 2 h (Table 1).

Compared to 2 h of incubation, a significant decrease in the number of vacuoles was noted in the low-concentrated RL samples after 24 h (Table 1). Moreover, in the most concentrated RL suspension, only half the number of vacuoles were present in *S. ambiguum* compared to the BY sample. On the other hand, the number of vacuoles significantly increased in suspensions containing secondary microplastics. This can be explained as follows: first, due to the formation of vacuoles with irregular-shaped microparticles by protozoans, it takes longer to reach their maximum number (up to 20–30). Second, compared to edible yeasts (BY) and irregular-shaped inedible particles, spherical inedible particles (RL) may be eliminated faster by protozoans. However, a more detailed analysis of the processes of microplastic ingestion and egestion by *S. ambiguum* is required.

3.2.3. Binary Mixtures of Spherical Particles with Secondary Microplastics

In the second experiment, two sets of two-component mixtures were served to protozoans: BY with microplastics (MP) and RL with secondary microplastics. The protozoans were placed in the MP suspensions for 2 h; then, BY or RL suspensions were added. The number of vacuoles was counted after 4, 24, and 48 h of incubation at 25 °C (Table 2). An additional two sets of BY–MP mixtures were prepared with different ratios of BY and MP as 1:3, 1:1, and 3:1. For these mixtures, the number of vacuoles was determined only after 24 h of incubation at 25 °C (Table 3). The aim of these experiments was to check whether protozoans ingest different types of particles selectively or microplastics, which are particles differing in composition, shape, and size, are ingested at the same rate as nutritious food (BY).

Regarding one-component samples, after 4 h, only half of the number of vacuoles were found in protozoans in the MP suspensions compared to the BY suspensions. The number of vacuoles did not change after 24 h of incubation, except for PVC suspensions, in which it decreased further to half. When the incubation time was extended to 48 h, a significant decrease in the number of vacuoles was noted in BY suspensions, while the vacuoles were completely egested in the other samples (Table 2).

Table 2. Number of vacuoles formed by the protozoan *S. ambiguum* in different microparticle suspensions made of individual components and binary mixtures (1:1).

	No [1]	4 h			24 h			48 h		
		AVE ± SD	MIN	MAX	AVE ± SD	MIN	MAX	AVE ± SD	MIN	MAX
BY	16–20	22.8 ± 4.2	9	28	22.4 ± 6.5	11	35	6.9 ± 5.1	0	17
RL	15–18	14.1 ± 4.3 [2]	7	22	15.1 ± 3.2 [2]	11	21	0	0	0
PS	10–12	12.6 ± 3.3 [2]	8	20	16.9 ± 2.8 [2]	14	23	0	0	0
PET	12–16	10.4 ± 2.8 [2]	7	16	15.4 ± 3.4 [2]	10	22	0	0	0
PVC	11–16	13.4 ± 5.4 [2]	4	23	6.2 ± 2.0 [2]	4	11	0	0	0
PhR	13–16	13.7 ± 2.7 [2]	9	19	13.7 ± 5.5 [2]	5	24	0	0	0
BY + RL	15–17	26.8 ± 7.1	17	39	19.9 ± 2.8	15	25	8.8 ± 5.5	0	15
BY + PS	13–27	24.4 ± 7.6	12	39	20.9 ± 4.4	14	31	8.1 ± 3.9	1	14
BY + PET	13–16	25.2 ± 6.9	15	36	20.8 ± 4.6	12	29	7.4 ± 3.4	0	12
BY + PVC	11–16	11.9 ± 3.1 [2]	7	19	14.7 ± 5.6 [2]	7	28	4.6 ± 1.7 [2]	3	8
BY + PhR	9–16	22.7 ± 7.5	7	36	26.3 ± 4.0	17	34	6.0 ± 3.2	1	12
RL + PS	14–15	11.5 ± 3.5 [2]	6	19	17.3 ± 6.3 [2]	1	25	4.9 ± 1.9	2	9
RL + PVC	15–16	6.4 ± 2.5 [2]	2	11	10.3 ± 4.4 [2]	4	24	0	0	0
RL + PhR	13–16	15.6 ± 3.9	10	24	16.3 ± 2.6	12	22	0	0	0

[1]—the number of protozoans; [2]—significantly different ($p < 0.05$) from the control (BY).

Table 3. Number of vacuoles formed by the protozoan *S. ambiguum* after 24 h in binary MP + BY mixtures having different proportions of components.

	No [1]	BY:MP = 1:3			BY:MP = 1:1			BY:MP = 3:1		
		AVE ± SD	MIN	MAX	AVE ± SD	MIN	MAX	AVE ± SD	MIN	MAX
BY + RL	16–17	20.4 ± 3.1	16	27	19.9 ± 2.8	15	25	23.3 ± 3.7	16	28
BY + PS	14–27	21.0 ± 3.7	15	28	20.9 ± 4.4	14	31	20.6 ± 3.7	12	27
BY + PET	13–16	21.6 ± 3.3	17	29	20.8 ± 4.6	12	29	23.7 ± 5.1	10	32
BY + PVC	11–17	8.0 ± 3.3 [2]	4	17	14.7 ± 5.6 [2]	7	28	24.5 ± 4.7	17	32
BY + PhR	13–16	22.5 ± 4.4	14	34	26.3 ± 4.0	17	34	27.3 ± 4.5	21	37

[1]—the number of protozoans; [2]—significantly different ($p < 0.05$) from the control (BY).

In the two-component samples, the addition of BY induced the formation of food vacuoles in protozoans. After 4 and 24 h of incubation, the number of food vacuoles inside the cells was found to be the same as in the BY suspension (Table 2). The only exception was PVC suspension, in which vacuole formation was significantly inhibited. The addition of RL to the MP suspensions did not stimulate the ingestion of MP, as the number of vacuoles formed in the RL + MP mixtures was similar to that in the samples containing a single component (RL or MP). As indicated above, the only exception was PVC suspension, in which RL ingestion was inhibited. The food vacuoles that formed in the mixtures contained both components of the mixtures (Figure 5). In all mixtures (except for BY + PVC), the addition of BY stimulated the formation of at least 20 vacuoles regardless of the BY:MP ratio (Table 3). An increase in the share of PVC in the binary mixture with BY caused a reduction in the intake of particles from 24.5 (BY:PVC = 3:1) to 14.7 (BY:PVC = 1:1) and 8.0 (BY:PVC = 1:3).

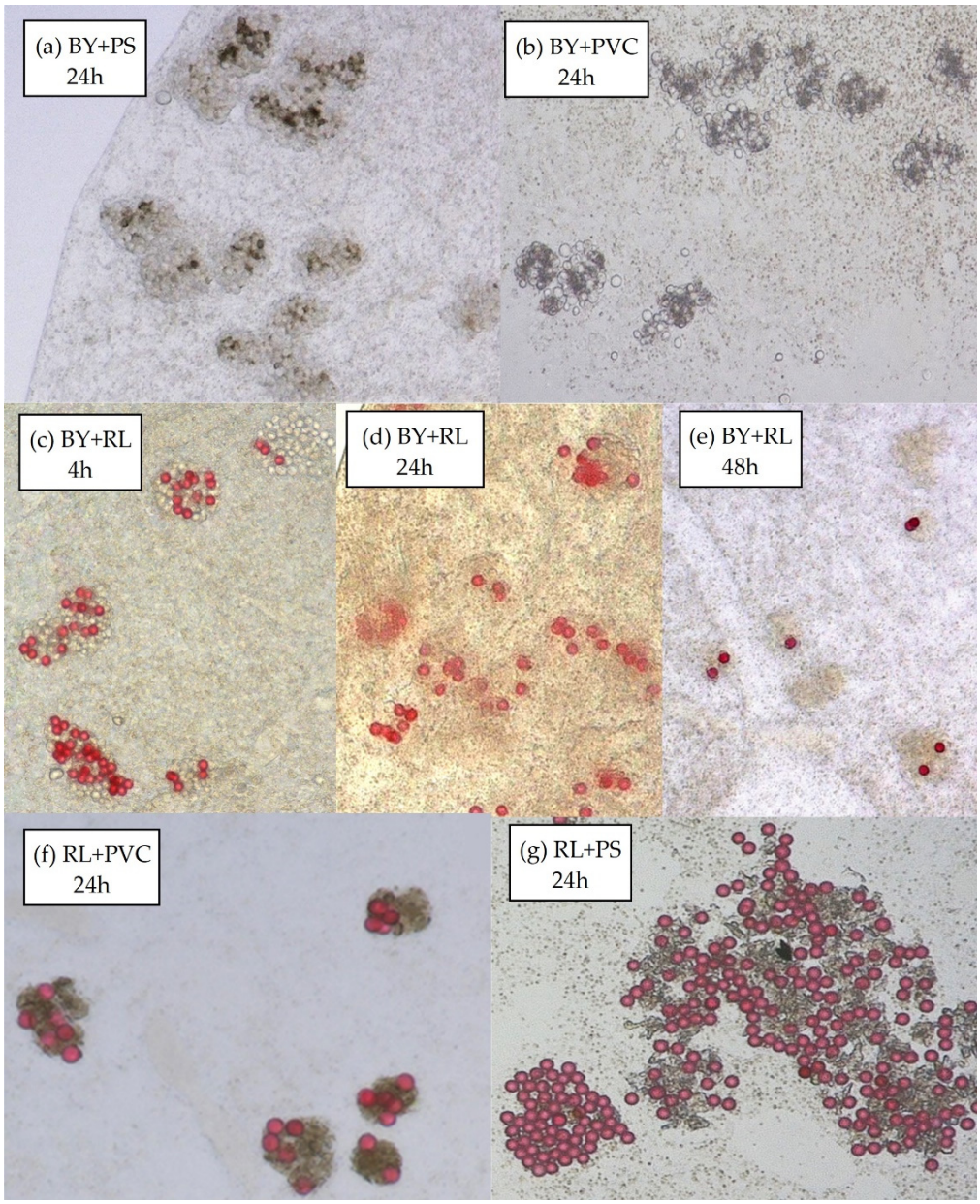

Figure 5. Food vacuoles with the mixture of particles: (**a**) BY + PS after 24 h; (**b**) BY + PVC after 24 h; (**c**) BY + RL after 4 h; (**d**) BY + RL after 24 h; (**e**) BY + RL after 48 h; (**f**) RL + PS after 24 h; (**g**) RL + PVC after 24 h.

Extending the incubation time to 48 h induced the egestion of MP by the protozoans in almost all samples. However, in the suspensions containing the edible component (BY), vacuoles with MP and digested yeast residues were still observed after 48 h (Figure 5e).

4. Discussion

This study assessed the effect of secondary nano- and microplastics on filter-feeding protozoans using secondary nano- and small microplastics prepared from household materials. Studies on microparticle uptake conducted so far have only used microspheres with a defined shape and size distribution. These particles could serve as an equivalent of primary plastics [32]. Polymers, by definition, are chemically inert molecules. However, microplastics made by grinding products can contain substances of low molecular weight such as monomers, dyes, plasticizers, and degradation products [33], which are capable of affecting the biological activity of protozoans. In the present study, no acute toxic effects

were observed in *S. ambiguum* protozoans in the conducted experiments. The available literature has no publications on the toxic effects of secondary microplastics made of various materials on protozoans. A study showed that highly concentrated (>2 g L^{-1}) leachates from PVC and epoxy resins had toxic effects on *Daphnia magna* as the leachates contained hydrophobic organic compounds [34]. In another study, Renzi et al. [35] observed high mortality and increased immobilization of *D. magna* in PVC suspensions. However, this effect was assumed to be caused by physical interactions of the particles with the crustaceans. A study using PS microbeads showed that they did not cause toxicity to *D. magna* when the organisms were incubated for only 2 d, but increased mortality was observed after 5 d of incubation [36]. PS microbeads did not cause mortality to *Artemia salina* but caused a delay of I and II instars at 10^3 and 10^4 particles mL^{-1} [37]. The same levels of PE microbeads were toxic to a rotifer *Brachionus calyciflorus*, decreasing the swimming linear speed and a net reproductive rate [38]. Much lower concentrations of secondary PVC microparticles, close to the levels occurring in the surface waters, negatively affected blue mussel *Mytilus* spp. but only after a long time of exposure [16].

The protozoan *S. ambiguum* ingests all kinds of microplastics. Studies performed with an aim of assessing particle uptake by protozoans usually used specially synthesized microparticles [19,23,25], but in the aquatic environment, particles of various sizes and shapes occur [6,28,30,39]. Additionally, particles suspended in natural waters are made of different materials, which mainly include polyethylene, polypropylene, and PET, as well as PS and PVC [6,39]. The present study used specially synthesized latex microspheres that are very similar to yeast cells, and four secondary nano- and microparticles of different shapes and sizes, made of PS, PET, PVC, and PhR. To our best knowledge, no study has investigated the uptake of secondary nano- and small microplastics by protozoans. Fenchel [19] studied suspension feeding in different ciliated protozoans and found different size spectra for particles, which were retained and ingested. Juchelka and Snell [25] assessed the sublethal toxicity of several toxicants on medium-sized ciliates *Paramecium aurelia* using 2 µm fluorescein-labeled latex microspheres. The authors observed the formation of food vacuoles with microbeads after 30 min of incubation, whereas in the present study, food vacuoles with BY and RL were formed slowly by the ciliate *S. ambiguum* with the maximum number of vacuoles found after 4 h.

Suspension feeders feed on food particles that are freely suspended in water. They are capable of unique morphological adaptations to concentrate the particles [19]. The distance between cilia of the undulating membrane, which acts as a sieve for particles, indicates the minimum size of particles that can be retained by the protozoans. On the other hand, the mouth size of protozoans determines the maximum particle size. The minimum and maximum particle size values have not yet been determined for *S. ambiguum*. However, Fenchel [19] reported that spirotrich ciliates cannot retain particles smaller than 1–2 µm, while the biggest tested protozoan (*Paramecium caudatum*) can ingest particles with a size of up to 6 µm. Although *S. ambiguum* (≈2 mm) is much larger than *P. caudatum* (≈0.054 mm), it formed equal numbers of food vacuoles with microdiamonds having a diameter of 1–7 µm [21]. In the case of larger particles, the number of vacuoles formed by both protozoans was lower. The protozoan *S. ambiguum* feeds on bacteria in culture; however, it preferably feeds on BY with a cell diameter of ≈5 µm (Figure 3a). Thus, in this study, BY and RL microspheres of similar diameters were used as control nutritional and non-nutritive particles, respectively. The analysis of the content of vacuoles by microscopic examination revealed that *S. ambiguum* ingested secondary microplastics with a size of up to 10 µm; however, most of the particles ingested by the organisms were much smaller (Figure 5).

The results of the study confirmed Fenchel's [19] hypothesis on the constant feeding rate of ciliates and that only a limited number of vacuoles are formed in a given time. In the case of *S. ambiguum*, the maximum number of vacuoles formed was around 30 in the BY suspension with 10^5 BY particles mL^{-1}, but further increase in the food concentration did not cause any increase in intake. A study on *Tetrahymena pyriformis* showed that vacuole

formation was higher in the first 40 min of feeding, and then, the intake remained constant for 5 h, which indicates the balance between intake and egestion [26].

Two hypotheses have been put forth to explain the mechanism of food vacuole formation: (1) mechanical stimulation of cytostome by the food particles and (2) activation of chemoreceptors by low-molecular-weight compounds. Filter-feeding ciliates show less selectivity for particles than other protozoans. However, they have receptors that can be activated by selected compounds—for example, those present in a protease–peptone extract [26]. Railkin [21] hypothesized about the threshold concentration of microparticle suspensions needed to induce vacuole formation. Subthreshold stimulation of the ciliate cytostome cannot be an effective trigger for the process of food vacuole formation. The present study showed that in low-concentrated suspensions ($<10^5$ particles mL^{-1}) containing irregular-shaped microspheres, *S. ambiguum* did not form food vacuoles, whereas in the microbead suspensions, the vacuoles were formed, regardless of whether the particles were edible (BY) or inedible (RL) (Table 1). These results support the hypothesis of the mechanical stimulation of cytostome by particles of a specific, round shape.

Railkin [20] investigated food intake by protozoans depending on the proportion of edible (yeast) to inedible particles (coal). As observed in the present study, the author found that both *S. ambiguum* and *P. caudatum* could not distinguish between edible and inedible particles. He also reported that depending on the type of inedible ingredient present in the mixture, the rate of food vacuole formation differed, although all the tested ingredients were nontoxic. Dürichen et al. [26] observed that *T. pyriformis* did not show any food preferences and took up both bacteria and non-nutritive microspheres to the same extent. Albano et al. [37] found that the presence of PS microbeads significantly reduced microalgal ingestion by *A. salina*, which consequently led to a reduction in the body length and a developmental delay.

The egestion of inedible particles by protozoans has not been widely studied thus far. In this study, plastic microparticles were found to be quickly egested from the cells, unless they were collected together with edible particles. The crustacean *D. magna* was shown to rapidly ingest inedible microparticles but egest regular-shaped, round particles much faster than irregular-shaped microplastics [40]. Cole et al. [41] observed that microplastic-laden fecal pellets were egested by the crustacean *Calanus helgolandicus* within hours, in contrast to individual plastic microbeads, which remained in the body for up to 7 d. The authors also found that high concentrations of microbeads decreased the ingestion of algae by crustacea. Prolonged gut retention times and gut blockage may be harmful to crustaceans. Similarly, the prolonged presence of microparticles may affect the retention of the cell membrane in protozoans, which requires detailed investigation.

Ecological Consequences

Plastic nano- and small microparticles are ubiquitous in the environment. Determining their concentration in water is challenging and necessitates the application of complicated procedures and advanced equipment [29,30,42]. Due to technical reasons, only particles larger than 40–100 µm have been detected in monitoring programs, while freshwaters are mainly dominated by smaller particles. Dubaish et al. [30] using a 40 µm mesh recorded up to 1770 and 650 L^{-1} plastic granules and fibers in the Jade system in the southern North Sea. The results of the project "MiWa—Microplastics in the Water Cycle" showed that 96% of particles in the Elbe River were sized below 20 µm, and their concentration ranged up to 9 $\times 10^2$ particles L^{-1}. This is only a negligible fraction of all edible and inedible particles suspended in river water, whose concentration may be as high as 10^8 particles L^{-1} [42]. Filter feeders can adapt well to feed on particles of different nutritional values [32]. The present study indicated that only PVC particles affected the uptake of BY by *S. ambiguum*, and that the uptake of BY was affected only when the concentration of PVC particles was higher than its concentration. A high concentration of PE microplastics (10^3–10^4 pieces mL^{-1}) reduced the *B. calyciflorus* reproductive rate, and similarly to our findings, this effect was alleviated by high food concentration [38]. However, the concentration of plastic nano-

and microparticles applied in our study was several orders of magnitude higher than that found in surface waters [30]. These findings suggest that not-aged secondary microplastics may not have any negative short-time effects on ciliated protozoans in the natural environment. Further studies are necessary to simulate a real-live scenario taking into account contamination levels similar to environmental as well as significantly longer exposure times of the test organisms [16].

However, microplastics are subject to aging in the environment and can adsorb both bacterial biofilm and toxic substances on their surface. Hence, further research is needed to explore the interaction of microplastics with protozoa, for which *S. ambiguum* can be an excellent test organism.

5. Conclusions

The protozoan *S. ambiguum* is a favorable ciliate for research on microparticle uptake due to its large size enabling microscopic observations and long retention time of particles in the cells.

In this study, the protozoans took up the secondary microplastic particles and formed food vacuoles only after the threshold concentration of particles was exceeded. This threshold concentration was much higher than the levels of plastic nano- and microparticles detected in natural environment. This indicates that the probability of harmful effects of microplastics on protozoans in natural waters is very low.

It was also found that the presence of food reduced the negative effects of PVC on vacuole formation by *S. ambiguum*.

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