Journal of Hazardous Materials 506 (2026) 141575

Contents lists available at ScienceDirect

Journal of Hazardous Materials

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

Effects of petroleum-based and biodegradable bio-based microplastics versus natural control particles on *Paramecium caudatum*, combined with *in situ* Raman spectroscopic detection

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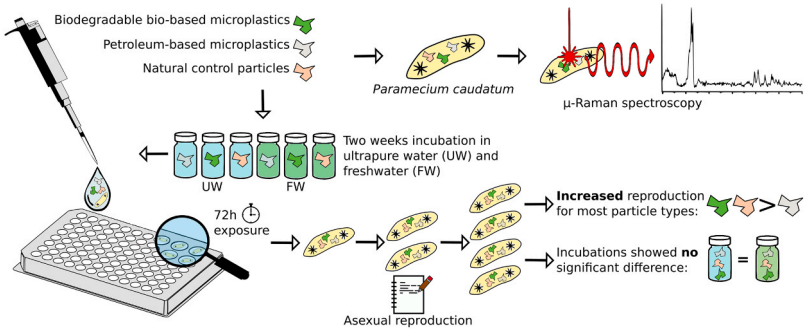
HIGHLIGHTS

- Particle identification within paramecia can be achieved using μ-Raman spectroscopy.
- Reproduction in *P. caudatum* increases with the presence of specific particles.
- Paramecia may try to digest biodegradable bio-based microplastics, as well as PET.
- Freshwater incubated particles have effects similar to pristine particles.

GRAPHICAL ABSTRACT



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https://doi.org/10.1016/j.jhazmat.2026.141575
Received 20 November 2025; Received in revised form 18 February 2026; Accepted 20 February 2026
Available online 22 February 2026

ARTICLE INFO

Keywords:
Microplastics
Ciliates
Raman Spectroscopy
Uptake
Biodegradable / bio-based plastics
Biofilms

ABSTRACT

The impacts of microplastics (MPs) are becoming increasingly concerning. Although many ecotoxicological studies have examined potential effects of MPs on organisms, most have tested only a limited range of pristine plastic types, which do not reflect the properties of environmentally conditioned plastics. This limits the extent to which the results can be applied to real-world situations. Additionally, understanding the ecological impact of MPs requires studies that begin at the lower levels of the food web. In freshwater ecosystems, unicellular ciliophora are a key part of these trophic levels. Studying the effects of MPs on this group is essential for understanding their overall impact on the ecosystem. This study aimed to address both issues by examining MP uptake and the impact of environmentally conditioned MPs on the ciliophora *Paramecium caudatum*. A 72-hour exposure was conducted using six petroleum-based (PB-) and four biodegradable bio-based (BB-) MP types at three concentrations, albeit higher than those found in the environment, along with three types of particle controls. All particles were incubated in ultrapure and freshwater to compare the effects of pristine versus environmentally conditioned MPs. Verification of particle uptake was performed with μ-Raman spectroscopy, confirming particle uptake without the need for fluorescent dyes, except for two control particles. The exposure experiments showed increased reproduction in all treatments with BB-MPs and control particles, except for one, whereas results for PB-MPs were inconsistent. No significant differences were observed between different particle incubation conditions. Our findings indicate that MP effects depend on plastic type, regardless of environmental conditioning, and that uptake by *P. caudatum* alters the Raman spectra of BB-MPs and PET particles.

1. Introduction

Over the past twenty years, it has become evident that environmental pollution caused by microplastics (MPs, 1–1000 μm, ISO 24187:2023, [1]) affects every part of the environment and the living organisms within it. As a result, the potential ecotoxicological effects of MP pollution have gained more attention, leading to increased research on how MPs impact different species. Originally focusing on marine species [2,3], studies now also include organisms from other environments [4,5], such as freshwater and soil ecosystems [6–10].

So far, most published studies have focused on pristine petroleum-based MPs (PB-MPs), such as polystyrene (PS), polyethylene (PE), polypropylene (PP), and polyvinylchloride (PVC) [11–14]. As a result, pollution caused by PB-MPs continues to raise public awareness. Consequently, the demand for seemingly safer alternatives, like bio-based and/or biodegradable (BB-) plastics such as polylactic acid (PLA), polyhydroxyalkanoates (PHAs), polybutylene succinate (PBS), polybutylene adipate terephthalate (PBAT), polycaprolactone (PCL), and starch-based plastics; see also Goel et al. [15], steadily increases in the market [16]. With the rising production of BB-plastics, these polymers are likely to significantly contribute to plastic waste and MP pollution in the future [17]. However, research on the effects of BB-MPs has received limited attention so far (e.g., [18–20]), and only a few studies report concerning results (e.g., [21-23]). Especially with the increase in BB-plastic production, more comparative research on the biological effects of PB- and BB-MPs is urgently needed.

To date, most studies on the effects of MPs have tested only a limited variety of PB-plastic types, mainly as pristine particles (e.g., [19, 24–26]). However, once MPs enter the environment, their surface quickly interacts with their surroundings. They are rapidly coated by a complex matrix of biomolecules from dissolved organic matter, humic and fulvic acids, extracellular polymeric substances (EPS), and inorganic materials such as minerals and ions, which together can form an eco-corona [27,28]. When particles are large enough, they can also be colonized by microbial communities, forming a biofilm known as the “plastisphere” [29]. All these factors can alter the behavior and toxicity of environmentally conditioned MPs and increase the likelihood of uptake by organisms and cells [30–32]. Therefore, exposing organisms in the laboratory to pristine MP particles that do not resemble the properties of environmentally conditioned MPs is unlikely to accurately reflect what occurs in the environment [33]. This limits the ability to extrapolate experimental results to real-world situations.

Regarding the studied biota, current laboratory investigations of MP effects on freshwater organisms mainly rely on various model species, such as the green algae *Acutodesmus obliquus* [34], *Chlorella vulgaris* [35], or animals like *Daphnia magna* [19,36,37], *Dreissena polymorpha* [38], *Lumbriculus variegatus* [39], *Gammarus fossarum* [40], *Cyprinus carpio* [11], or *Danio rerio* [41]. However, understanding the ecological impact of MPs requires more studies focusing on the lower levels of the food web. One group often overlooked are unicellular freshwater ciliophora [26,42–44]. Ciliophora are vital to aquatic ecosystems because they serve as a key link between primary producers and higher trophic levels [45,46]. While Ciliophora have been used as model organisms to study the effects of substances such as antibiotics [47], pesticides [45], and metals [48,49], comprehensive research on the effects of MP exposure on these organisms is still lacking. The few studies available on MP interactions with freshwater Ciliophora have used pristine PB-MP particles [26,42–44,50,51–58]. However, a detailed analysis of how different types of MPs, including both PB- and BB-MPs, as well as a comparison between pristine particles and environmentally conditioned MPs, is necessary for realistic risk assessments and for understanding potential ecosystem-level effects.

This study aimed to examine the potential effects of MPs on the freshwater ciliophora *Paramecium caudatum*. The organisms were exposed to various PB- and BB-MP fragments (all d_{50} ~20 μm). To assess environmentally relevant properties of MPs, we compared the effects of pristine MPs with those of environmentally conditioned MPs. To isolate the effects of particle presence alone, we included natural (non-plastic) control particles ([7,59], [60]) in additional treatments with cellulose fragments, kaolin (i.e., mineral particles), and mussel shell fragments (MS). Furthermore, our study aimed to confirm the uptake of all PB-, BB-MPs, and control particles within a single experimental setup without the need for fluorescence marking or fixation reagents, by developing an advanced μ-Raman spectroscopy method.

This method was developed based on previous studies that detected MPs in tissues and cells [61–64]. This approach enabled us to identify each type of plastic particle within the cells, thereby establishing an initial tool capable of creating a true dose-response curve for future research.

2. Materials and methods

2.1. Paramecium caudatum culture

The freshwater Ciliophora *P. caudatum* originated from a well-established in-house cultivation, which was originally purchased from Carolina Science GmbH (Berlin, Germany). The Ciliophora were kept in 500 mL Erlenmeyer Duran flasks (DURAN group, Wertheim, Germany) filled with Z medium [26,65] diluted 1:4 in ultrapure water (PURELAB Flex, ELGA LabWater, Veolia Water Technologies, Celle, Germany). The

culture was maintained in static conditions in dark cabinets and fed *ad libitum* with an infusion of dried celeriac root (*Apium graveolens*). Individuals for the experiments were randomly selected from cultivations at the logarithmic growth phase.

2.2. Pristine microplastics and control particles preparation

Fragments from ten different polymers and three natural control particles were selected for the experiments, all in sizes ~ 20 μm (d_{50}) (Figure SI 1–11). Six out of the ten MP types were chosen from petroleum-based polymers (PB-MP; Figures SI 1–6), while the remaining four were biodegradable-biobased (BB-MP; Figures SI 7–10). The PB-MPs selected for this experiment were Low-density Polyethylene (LDPE, Lupolen 1800 P, LyondellBasell Industries, Basell Polyolefine GmbH, Wesseling, Germany), Polystyrene (PS, PS 158 N, INEOS Styrolution Group, Frankfurt am Main, Germany), Polyamide (PA, PA66, Ultramid A27 E, BASF SE, Ludwigshafen, Germany), Polypropylene (PP, Moplen HP526J, LyondellBasell Industries, Basell Polyolefine GmbH, Wesseling, Germany), Poly(vinyl chloride) (PVC, S 3268, Vinnolit GmbH & Co. KG, Ismaning, Germany), and Poly(ethylene terephthalate) (PET, NEOGROUP, Klaipėda FEZ, Lithuania).

The BB-MPs selected for this experiment were Poly-3 (hydroxybutyrate) (P3HB, Biomer, Schwalbach, Germany), Poly (butylene succinate) (PBS, BioPBSFZ91PM, Mitsubishi Chemicals, Tokyo, Japan), and two variants of Poly(lactic acid) (PLA): Poly(D/L lactide acid) (PDLLA, NatureWorks, Nebraska, USA) and Poly(L lactide acid) (PLLA, TotalEnergies Corbion, Netherlands). These two variants differ in the stereospecificity of the lactide monomer and in their crystalline structures. PDLLA is amorphous, while PLLA has a crystalline structure. The MP fragments, bot BB- and PB-, were produced in-house by milling and sieving (by the Macromolecular Chemistry II, University of Bayreuth; CRC 1357 Microplastics, https://www.sfb-mikroplastik.uni-bayreuth.de/en/index.html and https://www.limnoplast-itn.eu/). A detailed procedure is reported in the SI.

As natural control particles, kaolin, cellulose, and ground mussel shells (MS), obtained from the quagga mussel (*Dreissena bugensis*), were included. The MS were prepared as previously described by Schwarzer et al. [66]. Kaolin fragments were purchased from Gebrüder Dorfner GmbH & Co. (Hirschau, Germany). Cellulose fragments were produced in-house from cellulose fibers for column chromatography (length 0.02–0.1 mm; Carl Roth GmbH + Co. KG, Karlsruhe, Germany, CAS No. 9004–34–6), using a Retsch CryoMill (Retsch GmbH, Haan, Germany) to obtain the desired size range (see Figure SI 11).

Size distributions of plastic particles and cellulose were determined using a combination of laser diffraction (LD) and dynamic image analysis (DIA; FLOWSYNC, Microtrac MRB, Montgomeryville and York, USA). The size distributions of the used MS and Kaolin particles are reported in Schwarzer et al. [66], and measurements were not repeated. All size distributions measured for this study are reported in SI Figures 1–11. For size distributions of MS and kaolin, see the supplementary information from Schwarzer et al. [66].

2.3. Microplastic and control particles incubation

All particles were incubated for two weeks in either freshwater (FW) coming from an artificial outdoor pond or ultrapure water (UW). The incubation was carried out as previously described by Ramsperger et al. [67] with a few adaptations. For each particle type, 20 mg of particle powder were incubated in 20 mL glass vials (autosampler vials; neoLab, Heidelberg, Germany). To maintain the natural composition of the incubation medium, the vials were centrifuged (2000 g, 20 min., room temperature) every three days, and the supernatant was replaced with the same volume of either FW or UW (MilliQ water). Such a procedure may affect biofilm formation but appears necessary to prevent particle aggregation and to keep biofilm formation under control [32]. Where necessary, Tween20 (0.001 %) was added to prevent particle aggregation (applied to PP and LDPE). The same procedure was applied to control medium vials without adding particles (one vial containing only UW, one containing only FW, both with and without Tween20).

2.4. Scanning electron microscopy analyses

The morphology and surface characteristics of the MP particles were analyzed using a scanning electron microscope (SEM, FEI Apreo Volumescope, Thermo Fisher Scientific, 5 kV, working distance 2 mm, T1 in-lens detector). For sample preparation, 100 μL of each particle suspension was pipetted onto a small piece of silicon wafer (approx. 1 cm) and left to sediment for 1 h. To preserve the biofilm, the particles were fixed using Karnovsky's fixative; 2 % PFA and 2.5 % glutaraldehyde in 1 × PBS), dehydrated in an increasing ethanol series (30, 50, 70, 80, and 90 % for 30 min each, 95 % and absolute ethanol for 1 h each), and dried in hexamethyldisilazane (HMDS) overnight. Then, the silicon wafers were placed on carbon conductive tabs (Ø 12 mm Plano GmbH, Wetzlar, Germany) and fixed to aluminum stubs (Ø 12 mm, Plano GmbH, Wetzlar, Germany). This procedure has been shown to preserve the biofilm structure on particle surfaces in previous studies [67], but alterations in surface structure cannot be ruled out. Samples were subsequently coated with a 4 nm-thick platinum layer (208HR sputter coater, Cressington, Watford, UK). It should be noted that a 4 nm platinum layer could alter nano-surface structures while it substantially improves sample conductivity [68]. The stubs were finally transferred into a desiccator and stored under vacuum until the micrographs were acquired.

2.5. Raman spectroscopic analyses

2.5.1. Instrument specifications

All Raman spectroscopic measurements were performed using a micro-Raman spectrometer (WITec Alpha 300 RA+, Ulm, Germany) equipped with a UHTS 300 spectrometer and a back-illuminated Andor Newton 970 EMCCD camera. A frequency-doubled Nd-YAG laser with a wavelength of 532 nm was used as the excitation source. The exciting laser radiation was coupled to a Zeiss microscope through a wavelength-specific single-mode optical fiber. The laser beam was focused onto the sample by means of a 50 × long working distance [numerical aperture (NA) = 0.7, lateral resolution ca. 500 nm] and 100 × (NA = 0.9, lateral resolution ca. 300 nm) Zeiss objective. The focal length of the spectrometer is 300 mm, and it is equipped with a diffraction grating with a groove density of 600 lines per millimeter, providing a spectral resolution of ~3–4 cm^{-1}. The laser power used was approximately 5–15 mW at the fiber for all measurements. Raman scattered light was detected by a Peltier-cooled complementary metal oxide semiconductor–based CCD with a sensor size of 1600 × 200 px. The instrument was operated by the integrated Witec Control Five software (version 5). All spectra were acquired in the 3600–500 cm^{-1} spectral range. The acquisition was mainly carried out using the Raman imaging mode, in which each cell was imaged entirely at a specific lateral resolution. The acquired data were preprocessed using instrument-integrated software that allowed compensating for cosmic radiation and other background signals. Each Raman Image required an acquisition time of ~ 20 min under the 50x magnification and comprised a data set constituting a total of approximately 12,500 individual spectra. The Raman images were processed using the Witec integrated Project software (version 5.3) and were subjected to k-means cluster analysis coupled with spectral demixing to visualize the spatial distribution of different components within the Raman images (MP signature and *Paramecium* cell component signature).

Each cell component spectrum represents a combination of different elements, such as proteins, lipids, carbohydrates, and secondary metabolites (e.g., carotenoids). The used algorithm identified a spectrum as "Component 1" based on how different it was from the spatially averaged spectrum obtained from the image. This means that each cell component had distinct combinations of biomolecules, along with

consistent variation in the intensity of a specific peak. Finally, a component with a signature resembling a polymer spectrum, along with residual biochemical signatures, was detected as another individual component.

This process enabled the separation of spectral data by grouping them according to similarities within the dataset and minimizing spectral variance within each cluster [69]. The application of k-means has been previously shown for hyperspectral images of MPs analysis [70] and even nanoparticles in cells [71]. The advantage of this method for data analysis is that no prior knowledge of the data set analyzed is necessary, whereas other applied algorithms have to be trained for the specific detection [62] of, e.g., polymers like in random forests [72,73]. This implies that k-means may also help to identify patterns in a sample that trained algorithms may not recognize [74]. This may become relevant when analyzing particle uptake in environmental samples.

2.5.2. Sample preparation

For Raman analysis of MP uptake by paramecia, cells were exposed overnight to 100,000 particles mL^{-1} of the pristine MPs of each treatment, respectively. After the exposure, the cells were transferred onto a glass slide in 10 μL of the exposure medium. Glass fragments from crushed cover slides were placed on the microscopy slide as spacers to prevent the cells from being pressed, and a cover slide (18 ×18 mm) was placed on top. The so-prepared microscopic slides were sealed with transparent nail polish (trend it up nail polish, dm-drogerie markt GmbH + Co. KG, Germany) and, once the cells stopped moving, were analyzed using a Raman microscope.

2.6. 72-hour exposure

Individuals of *P. caudatum* were exposed in static conditions to three different concentrations of the selected particles (500, 5000, and 50,000 particles mL^{-1}). These concentrations were selected to cover a wide range, which could potentially reveal any concentration-dependent effects. However, it should be noted that all concentrations are higher than those reported in freshwater environments [75]. Each treatment was replicated fifteen times and randomly distributed in 96-well plates (Eppendorf Group, Hamburg, Germany). The well plates were maintained in dark boxes and placed on an orbital shaker (KS125 basic IKA LABORTECHNIK, IKA Werke GmbH & Co. KG, Staufen, Germany) at 1.5 mot min^{-1} to reduce particle settling and promote particle uptake. Each well initially contained one individual *P. caudatum* cell in 250 μL of the treatment suspension in Z medium, with 2 $mgCL^{-1}$ of the algae *Acutodesmus obliquus*. A detergent-free incubation control (same as particle treatment but without added particles) and a Tween20 control (same as particle treatment without particles but with added Tween20 0.001 %) were also included in the experiments. The chosen concentration of Tween20 was kept low enough to prevent detachment of any biofilm, which could occur at higher concentrations (>0.1–1 %) of the surfactant due to disruption of interfacial and extracellular matrix interactions [76]. Concentrations below 0.01 % were not reported to inhibit biofilm formation [76]. To minimize dilution effects from the incubation medium, all stock suspensions were previously diluted in Z medium before being added to the exposure wells. The organisms were then checked at three time points every 24 h (24, 48, and 72 h) using a stereo microscope (Leica MS5, Leica Microsystems, Wetzlar, Germany), and the number of living/free-moving paramecia was recorded.

2.7. Statistical analyses

Asexual reproduction of paramecia exposed to MPs and natural control particles was analyzed by counting the number of paramecia in the wells after 24, 48, and 72 h of exposure. In a first analysis, we wanted to know whether the different particle treatments changed the rate of binary fission compared to the controls. To this end, we fit a generalized linear model with Conway-Maxwell-Poisson distributed error term and log link to the number of paramecia, including particle type (control, Tween control, cellulose, kaolin, MS, PLLA, PDLLA, PBS, P3HB, PA, PET, PS, PVC, LDPE and PP), incubation media (FW and UW), and interaction of particle type and incubation media and time nested in particle type as fixed factors (glmmTMB package version 1.1.12; [77]). Residuals were checked with the package DHARMa version 0.4.7 [78] with simulation-based diagnostics to assess model fit and dispersion. The overall effects of the predictors and interaction terms were evaluated via a type II ANOVA calculated by the *Anova()* function from the package *car* version 3.1–3 [79]. Estimated marginal means for the different particle types and custom pairwise comparisons between particle types and controls were conducted using the package *emmeans* version 1.11.2–8 [80]. For the comparison of particle treatments with controls, emmeans calculated marginal means for mean exposure times (i.e., 48 h) and mean particle concentrations. LDPE and PP responses were compared to the particle-free Tween control, because Tween was used in these two MP treatments as a detergent to get the particles into suspension. All other polymer particles and the natural control particles were compared to the particle and detergent-free control. P-values for pairwise comparisons were corrected for multiple comparisons based on Benjamini and Hochberg [81]. To test for particle type differences in concentration dependency and reproduction over time, we fit a second model to the data, excluding all control data. In this second model, particle type, concentration, time, incubation media, and the interaction of the latter three with particle type were included as fixed effects. Again, a Conway-Maxwell-Poisson error distribution with logit link was used, adding an additional dispersion parameter (quasi-poisson). Estimated marginal means were again derived from the model using *emmeans*. All analyses were done in R version 4.5.1 (R Core Team 2022). Data preparation was done with the help of the *dplyr* package version 1.1.4 [82].

3. Results

3.1. MP characterization and biofilm formation

After exposure to FW for 2 weeks, we observed structures typical of biofilm formation on the surface of the MP particles (e.g., PDLLA and PET) and natural control particles (e.g., MS). These structures were not visible on particles incubated in UW (Fig. 1). Structures included amorphous shapes, which likely consisted of biomolecules and remnants of organisms present in the freshwater. Additionally, cells of various shapes and sizes were present on the particles' surface, likely representing both prokaryotic and eukaryotic organisms. In samples prepared from freshwater without particles, we observed various prokaryotic and eukaryotic organisms, such as *Vorticella spp*. (Figure SI 13). Overall, the SEM images show that upon environmental exposure, the MP particles developed a biofilm, consistent with earlier results [67,83,84]. For SEM images of all particles and overview images, see Figures SI 12 & 13.

3.2. Particle uptake

Particle uptake was confirmed using Raman spectroscopy. All particle types were detected within the cells, except for cellulose and kaolin. Raman images acquired from individual cells were reconstructed from the k-means clustering analysis to visualize particle uptake. Fig. 2 shows the uptake of LDPE MP particles. The clustering analysis reveals the presence of an average of 5–6 clusters (color-coded spectra) corresponding to the LDPE particles, different cell components, and the medium in which the cells were suspended. The same approach was applied to visualize the uptake of the other particles (Figures SI 14–16).

The uptake of fluorescent PS MPs was also confirmed via Raman spectroscopy (Figure SI 17) and fluorescence microscopy (Figure SI 18; for a detailed method description, see SI and [26]). Raman Imaging data also revealed a change in the spectral profiles of the BB-MP particles within *P. caudatum* cells when compared to the spectra obtained from

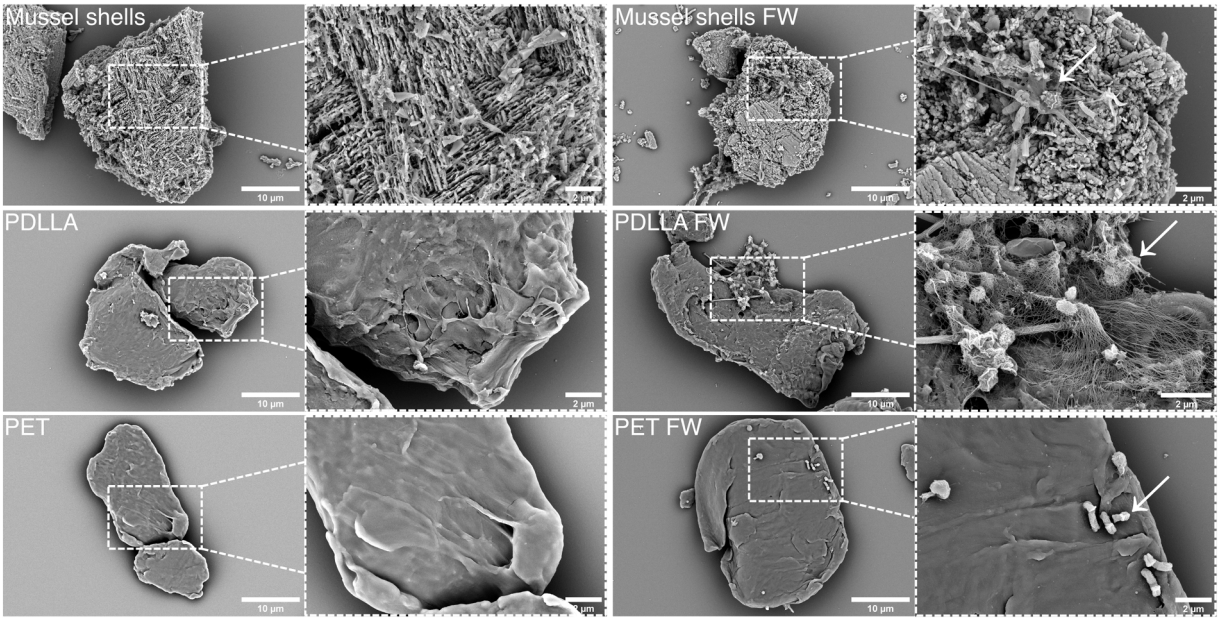

Fig. 1. SEM images of selected incubated particles (Mussel shells – MS, PDLLA – BB-MP, and PET – PB-MP). The two left columns (left: particle overview; right: close-up of the same particle) show the UW-incubated particles, while the two right columns (left: particle overview; right: close-up of the same particle) show the freshwater (FW) ones. After incubation in FW for 2 weeks, all MP particles and natural control particles developed a biofilm. The biofilms included organisms of various sizes, likely comprising both prokaryotic and eukaryotic species (indicated by white arrows in the 4th column). Furthermore, the particles were covered with amorphous structures, which likely consisted of biomolecules and remnants of freshwater organisms. For SEM images of all particles, see Figures SI 12 & 13.

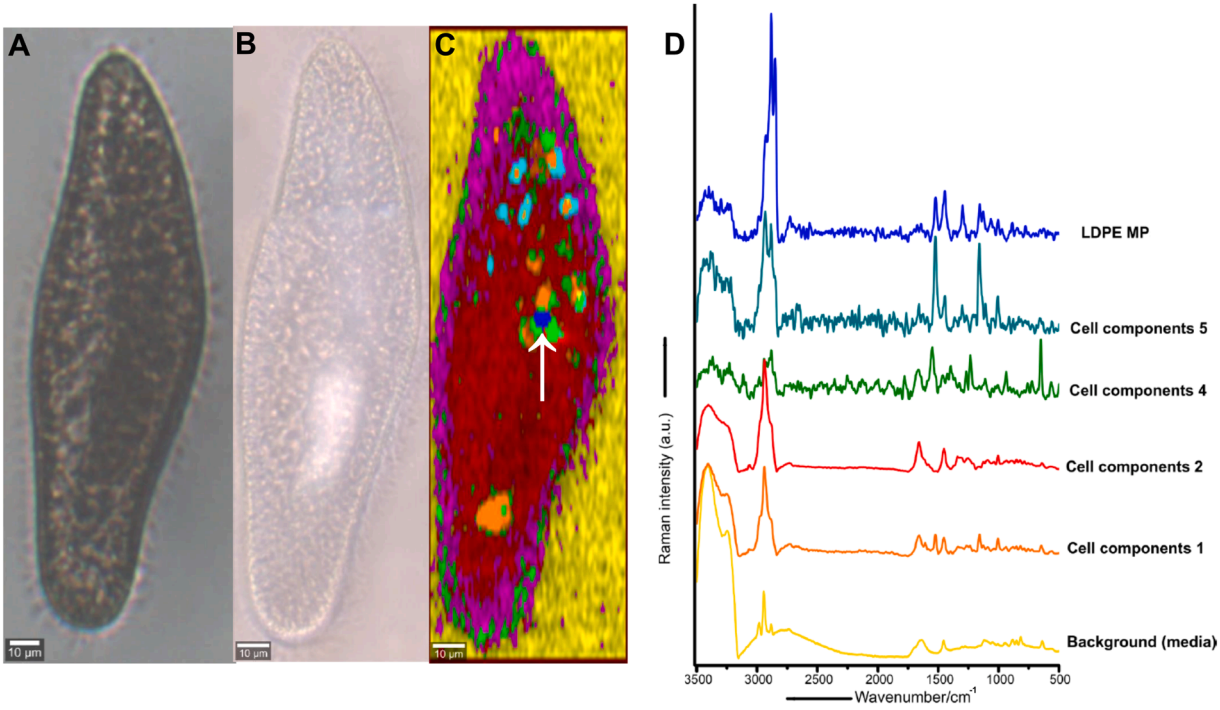

Fig. 2. Analysis of particle uptake by Raman Imaging; A) Bright-field microscopic image of the paramecium, taken at 10x magnification; B) Bright-field microscopic stitched image of the paramecium, single tiles were taken at 50x magnification, using the objective of the Raman microscope; C) Raman image reconstructed from the k-means cluster analysis and spectral demixing depicting the different components (LDPE MP, background and cell components 1–5) color coded according to the spectra shown in (D) respectively. White arrow = LDPE MP; D) Mean Raman spectra of different cellular components separated by the data analysis, along with the spectrum for LDPE MP used for particle depiction, color-coded in blue, and the mean spectra of the background representing the media in which the paramecia were reared.

the same pristine BB-MP particles (Fig. 3; for reference spectra of every particle type, see Figures SI 19–22).

Specifically, the changes in PBS MP compared to its reference show a baseline increase and a general broadening from 3500 to 500 cm^{-1}, indicating oxidation and fluorescence from organic residue attachment. The reduced sharpness and intensity in the C═O stretching

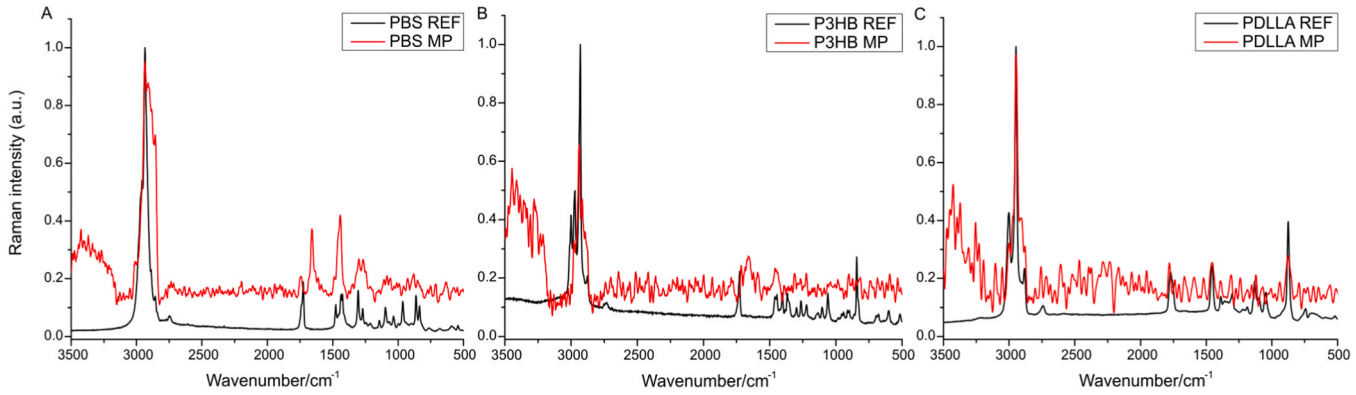

Fig. 3. Changes in the μ-Raman spectra of BB-MP particles after uptake by *P. caudatum*, compared to their references. Reference spectra are shown in black, and the altered spectra of the MP particle inside *P. caudatum* are shown in red. (A) PBS; (B) P3HB; (C) PDLLA.

(~1735 cm^{-1}) ester bond region suggest hydrolysis of ester linkages and partial depolymerization. A decrease in intensity of the backbone region (~1100–1200 cm^{-1}) involving C–O–C and C–C vibrational modes provides evidence of chain scission and backbone degradation. The changes in PDLLA MP in comparison to its reference show changes in the regions ~2950–3000 cm^{-1} C–H stretching region (CH_3, CH, CH_2), with notably higher intensity and slight broadening, indicating chain scission leading to more terminal groups or partial oxidation. Additionally, the decrease or shift in intensity of the ~1760–1750 cm^{-1} C═O stretching (ester group) in PDLLA MP suggests ester bond cleavage or hydrolysis of the PDLLA backbone. Changes in P3HB MP, compared to its reference, include increased baseline and noise. A broad elevation across the spectrum suggests possible fluorescence from organic residues. The reduced sharpness of the C═O peak (~1725 cm^{-1}) and slight broadening imply ester bond hydrolysis or cleavage of carbonyl groups. The decreased intensity in the C–O–C region (1050–1150 cm^{-1}) indicates chain scission and reduced polymer integrity. The loss of a well-defined peak at ~850 cm^{-1} points to material fragmentation. Finally, the broadened CH stretching (~2950 cm^{-1}) region, which is less defined, reflects local chain disorder and altered hydrogen environments resulting from degradation.

3.3. *Effects of MPs on asexual reproduction of Paramecium caudatum*

Overall, asexual reproduction (binary fission) in particle treatments was higher than in the corresponding particle-free controls, but the strength of these effects differed among different particle types (Fig. 4; Conway-Maxwell Poisson regression, Wald chi-square test: χ^2_{14} = 791.23, $p < 2.2e\text{-}16$). All BB-MPs and three out of six PB-MPs (PET, LDPE, and PP) increased binary fission compared to the corresponding particle-free control ($p < 0.05$ for all comparisons; for full results and values for marginal mean estimates see supplementary tables S1 and S2). However, LDPE and PP increased binary fission only when incubated in FW ($z_{ratio\_LDPE} = 3.43$, $p_{LDPE} = 0.001$, $z_{ratio\_PP} = 2.51$, $p_{PP} = 0.02$), but not when incubated in UW ($z_{ratio\_LDPE} = 1.72$, $p_{LDPE} = 0.13$, $z_{ratio\_PP} = 0.29$, $p_{PP} = 0.77$). Among the natural control particles, binary fission was significantly higher in treatments with cellulose (FW: $z_{ratio} = 6.18$, $p < 0.0001$, UW: $z_{ratio} = 5.26$, $p < 0.0001$) and kaolin (FW: $z_{ratio} = 6.05$, $p < 0.0001$, UW: $z_{ratio} = 5.91$, $p < 0.0001$), while the number of cells in treatments with MS fragments did not differ significantly from the control (FW: $z_{ratio} = 1.35$, $p = 0.25$, UW: $z_{ratio} = 0.52$, $p = 0.71$). Tween20-exposed individuals had reproduction rates similar to those of individuals exposed to the Tween-free control, revealing no adverse effects of this surfactant (see SI for discussion on Tween20).

In our detailed comparison of effects on reproduction in response to different particle types (excluding controls), we found that the number of paramecia increased over time (Conway-Maxwell Poisson regression, Wald chi-square test: $\chi^2_2 = 785.20$, $p < 2.2e\text{-}16$) and this increase

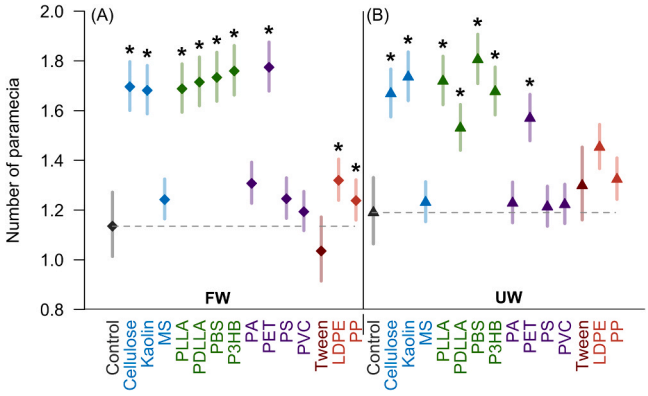

Fig. 4. Estimated marginal means (and 95 % confidence intervals) of numbers of *P. caudatum* in an experiment comparing the effects of different MP particles with natural control particles and blank controls. (A) Particles incubated for two weeks in natural freshwater (FW). (B) Particles incubated for two weeks in ultrapure water (UW). LDPE (low-density polyethylene) and PP (polypropylene) treatments contained Tween20 and are compared to the Tween control (Tween). All other particles are compared to the detergent-free control (Control). Significant differences are indicated by asterisks ($p < 0.05$). Blue: natural control particles, green: BB-MPs, purple: PB-MPs, red: PB-MPs with Tween20.

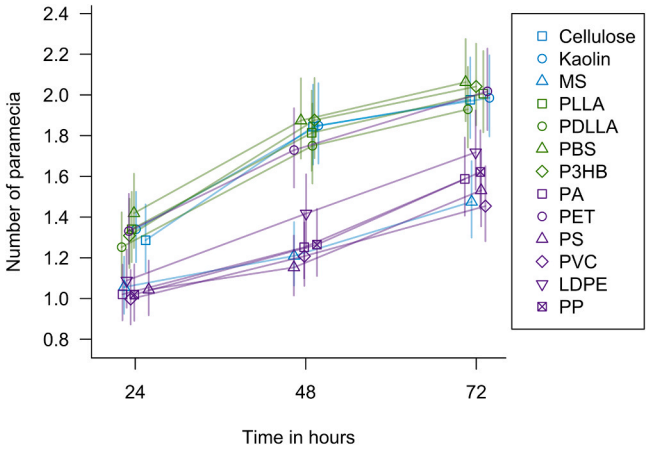

Fig. 5. Asexual reproduction of individuals of *P. caudatum* over time in response to different MPs (green: BB-MPs, purple: PB-MPs) and natural control particles (blue). Estimated marginal means are shown with 95 % confidence intervals.

differed among different particle types (interaction time x particle type: $\chi^2_{24} = 39.58$, $p < 0.02$; Fig. 5).

Asexual reproduction also differed across particle concentrations, but no clear dose-dependent pattern (i.e., no consistent increase in effect size with increasing MP concentration) was observed ($\chi^2_2 = 7.44$, $p = 0.024$; Fig. 6). In addition, the response across the three particle concentrations differed among different particle types (interaction concentration x particle type: $\chi^2_{24} = 89.33$, $p < 1.9e\text{-}9$).

Overall, asexual reproduction did not differ significantly between particles incubated in FW and particles incubated in UW ($\chi^2_1 = 1.03$, $p = 0.31$; Fig. 7), but the direction of effect differed among different particle types (interaction incubation x particle type: $\chi^2_{12} = 28.70$, $p = 0.004$).

4. Discussion

This study aimed to assess the potential effects of pristine MPs (PB- and BB-MPs) and natural control particles on the reproduction of *P. caudatum*, compared to the same particles after environmental conditioning. To determine particle uptake and potential alterations of MPs after ingestion, MPs were identified inside the organisms using Raman spectroscopy.

4.1. Particle identification using μ-Raman imaging

Using μ-Raman imaging, most particles were identified within *P. caudatum*, and their signals could be distinguished from those of the surrounding biological material. Our analysis showed the uptake of all MP types to which the paramecia were exposed, as well as that of the natural control particle MS. The lack of identification of kaolin and cellulose could have had several reasons. First, cellulose may not have been detected during Raman analysis because the spectra were too similar to those of the cellular components, preventing a clear distinction of the cellulose signal from the surrounding cytoplasm. Thus, the Raman spectra of *Paramecium* cells and cellulose show notable similarities, particularly in peaks related to carbohydrate structures, as both contain significant amounts of polysaccharides [85,86]. Furthermore, Raman scattering intensity can vary significantly with polarization and orientation, especially in anisotropic polymers and crystalline materials such as the cellulose particles used in this study. As a result, randomly oriented particles may produce weak or inconsistent spectral signatures, making identification difficult [87]. Additionally, weak signals may be obscured by fluorescence from surrounding biological material excited by the laser, resulting in a signal several orders of magnitude stronger

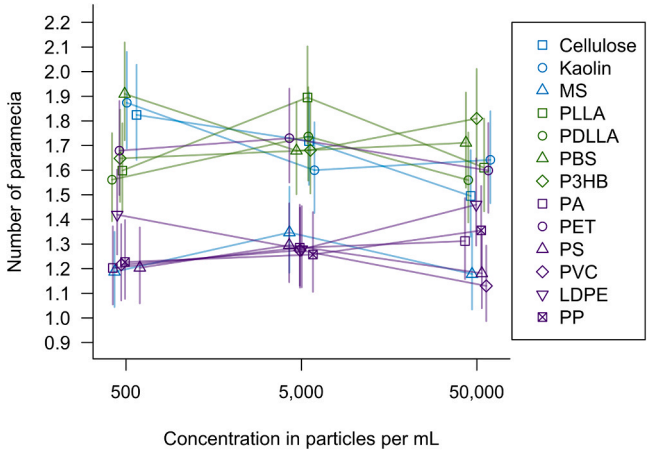

Fig. 6. Asexual reproduction of individuals of *P. caudatum* in response to different concentrations of MPs (green: BB-MPs, purple: PB-MPs) and natural control particles (blue). Estimated marginal means are shown with 95 % confidence intervals.

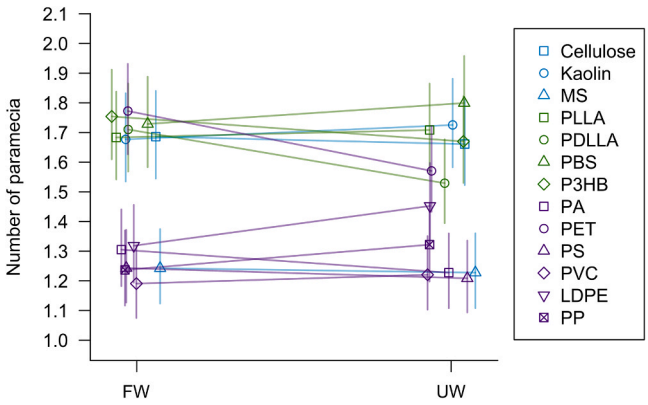

Fig. 7. Asexual reproduction of individuals of *P. caudatum* in response to MP particles (green: BB-MPs, purple: PB-MPs) and natural control particles (blue) incubated in either natural FW or UW prior to their use in exposure bioassays. Estimated marginal means are shown with 95 % confidence intervals.

than that of the target particles, which makes their identification nearly impossible [88]. To complicate matters further, cellulose particles may have been digested during the cyclosis process.

Some protists, such as those found in the gastrointestinal tract of ruminants [89] or termites [90], are known to be capable of digesting cellulose. Although this has not yet been demonstrated experimentally, cellulose digestion is also likely to occur in *P. caudatum*, as it has already been shown for other Ciliophora, such as *Diploplastron affine* [91]. Also, Ciliophora feed on various algae and phytoplankton that are small enough to be ingested and whose cell walls are partly composed of cellulose [52,92]. Given that paramecia lack any hardened structures to crush these algae, as is the case in other phytoplankton-feeding organisms [93], it can be inferred that they can break down the algal cell walls chemically [94]. However, due to the limited literature on this topic, further research is necessary to clarify this aspect.

Other factors that may have contributed to the lack of particle detection for kaolin and cellulose include their different material densities and agglomeration tendencies [60]. For instance, kaolin has a higher density (~2.6 g cm^{-3}; [66]) than the MP particles used in this study (lowest density: LDPE ~0.9 g cm^{-3}; highest density: PET ~1.4 g cm^{-3}; [95]), potentially leading to increased settling of particles at the bottom of the wells despite the gentle shaking throughout the exposure. This could have reduced the availability of kaolin particles to the paramecia, as the experiment was conducted under static conditions, meaning the exposure medium was not exchanged during the exposure period. However, MS, whose uptake was confirmed by the Raman analyses (Fig. SI 14), has a similarly high density (~2.9 g cm^{-3}; [66]) as kaolin. Additionally, cellulose has a density (~1.3 g cm^{-3}; [66]) comparable to that of MPs, and cellulose particles were also not identified in our Raman analyses. Thus, density alone cannot explain why we have not detected kaolin and cellulose particles inside the cells.

Another factor to consider when detecting kaolin is its smaller size compared to other particles. Kaolin had a d_{50} of less than 2 μm [66], which may have made it more difficult to identify within the cells. Similar challenges in detecting small particles have been reported for the identification of small PS MPs in *Eisenia fetida* by Kniese et al. [62]. Since 50 % of all kaolin particles were close to the detection limit of the Raman instrument used (~1–2 μm), and considering the surrounding cellular components, particle detection and identification may have been compromised in the exposed individuals. However, this does not apply to cellulose, which had larger particles ($d_{50} = 26.07$ μm; Figure SI 11) and should not have limited Raman identification. Additionally, other particles used in this study had a size distribution similar to that of cellulose (Figures SI 1–10) and were identified within the exposed organisms (Figures SI 14–17). Therefore, it remains unclear why kaolin and cellulose uptake and/or identification were absent.

Besides kaolin and cellulose, all other particles were identified in *P. caudatum*. Interestingly, our analyses revealed that the Raman spectral profiles of the BB-MP fragments inside the cells differed from those of the raw material. This occurs because the chemistry of BB-MPs tends to change due to factors such as hydrolysis and chain scission resulting from processes during cyclosis within the organisms (e.g., oxidative stress and enzymatic digestion), which can break chemical bonds. Hence, in such cases, new Raman peaks may appear, and existing peaks may exhibit reduced intensity and a shift in the Raman spectra. During cyclosis, vacuoles are acidified to support the digestion of food particles [96], thereby providing conditions for, e.g., acid phosphatase activity [97–99]. Therefore, the onset of an acidic environment within the vacuole after particle uptake might have altered the Raman spectra of the BB-MPs in our measurements, while the spectra of the PB-MP particles remained stable. This is supported by studies reporting that both weathering and acidic environments can break chemical bonds and lead to the adsorption of biomolecules from the vacuole onto the particle. If this occurs within the vacuole due to acidity, it can alter the Raman spectral profiles of the BB-MPs [100–103]. This may explain the differences observed in the spectral profiles of the ingested BB-MPs compared to their references.

Although not all particle types were identified within the Ciliophora in the present study, detecting all MP particles inside the cells using μ-Raman spectroscopy represents a first step toward developing dose-response models. This means that with the method established here, quantifying MP uptake could be feasible in future experiments with *P. caudatum* or other unicellular eukaryotes. Such data could then help determine realistic effect concentrations and establish a threshold dose-response relationship, providing clear implications for MP risk and hazard assessment [104].

However, it should be noted that applying μ-Raman spectroscopy in this way presents certain challenges for researchers. Some of these will be discussed below. (1) Depth-dependent signal attenuation: Raman signals weaken as they penetrate deeper into the cell due to scattering and absorption, which may cause an underestimation of particles located at greater depths of the sample. (2) High-resolution mapping is time-consuming: Raman imaging requires scanning point by point, making it slow for large areas or many cells. (3) Lack of linear correlation: Raman signal intensity does not always have a direct linear relationship with MP mass or volume because of differences in particle size, shape, and orientation. (4) Analyzing statistically meaningful numbers of paramecia can be tedious due to slow imaging speeds, complicating population-level quantification, which is essential in dose-response studies. (5) Smaller particles (such as nanoplastics) may generate weaker signals, and irregular shapes can scatter light unpredictably, impacting detection efficiency. (6) Surface modifications or coatings: Environmental weathering or biofouling of MPs can change Raman signatures, making identification more difficult. (7) Particle agglomeration inside cells can also pose a major obstacle to quantification [105–108].

4.2. Particle effects on asexual reproduction

We observed significant effects of the different particles tested, related to particle type and concentration, but independent of biofilm presence. The only treatment differing from this pattern was UW-incubated PET. The overall pattern was an increase in reproduction among paramecia exposed to particles. Specifically, both BB-MPs and PET-MPs had a significant positive impact on reproduction, regardless of environmental conditioning. PB-MPs, however, showed binary fission rates similar to those of the respective controls, except for PET.

An increase in reproduction, particularly upon exposure to BB-MPs, may suggest that paramecia try to digest these particles to use them as a carbon source. This was also hypothesized for, e.g., terrestrial earthworms, where similar positive effects of BB-MPs (PLLA and PCL) on the reproduction of *Eisenia fetida* were observed [109]. Additionally, degradation of biodegradable PLGA by marine microbial plankton (including Ciliophoran organisms) was also implied in a recent study [110]. Similarly, it may be possible that *P. caudatum* can digest these plastics, as suggested by alterations in their Raman spectral profiles. Increased reproduction was also noted in individuals exposed to PET. Despite PET being a non-biodegradable polymer, several microorganisms and enzymes capable of degrading it have been previously isolated [111]. Hence, as with the BB-MPs, albeit speculative, our results could suggest that *P. caudatum* may also try to utilize PET as a carbon source. This aspect, however, would require further investigation in future studies, using, for example, ^{13}C-labelled polymers [109] or amino acid stable isotope fingerprinting [112] to track the carbon's fate. Therefore, because there is no experimental evidence proving that these organisms can digest these plastics, the idea of using these particles, especially non-degradable PET, as a carbon source remains speculative.

Another important factor that may explain the increased reproduction in the particle treatments in our experiments is that some unicellular eukaryotes, such as *Tetrahymena*, multiply much faster when particulates are present in the medium [113]. Thus, higher particle concentrations (including non-nutritional PS particles; Rasmussen and Modeweg 1973) increase filtration rates [114] and, consequently, stimulate the formation of food vacuoles. In this context, an increased number of food vacuoles was observed to lead to increased growth rates [115]. Generally, Ciliophorans are adapted to particle-rich environments, which supports sustained population performance in such settings [114,116]. This, along with possible digestive processes, could explain our findings of increased reproductive rates in most particle treatments, even though no studies specifically report this phenomenon in *Paramecium*. However, if particle-stimulated growth were the only reason for the positive outcomes in this study, it should have been observed across all experimental treatments, but this was not the case. In fact, the test organisms were exposed to similar concentrations of the other particle types (i.e., PVC, PS, MS, and LDPE and PP in the UW treatments), and none of these showed such an increase in reproduction. Thus, the positive effects of reproductive stimulation might have been suppressed by other factors that could not be disentangled in the present study (e.g., leaching chemicals from the plastic particles), suggesting a negative effect of these MP types on asexual reproduction.

As no significant difference was observed between most FW- and UW-incubated particles, we could assume that the present biofilm does not contribute significantly to the food demand of the paramecia, hence, the positive effect observed in terms of reproduction probably comes from the particles themselves. Nevertheless, as previously stated, not all MPs induced this positive effect. Therefore, either some MPs, but not all, could serve as a carbon source for the exposed paramecia, or some MPs might induce negative effects, negating the positive effect observed with particle presence alone.

The lack of significant differences in incubation type on the reproduction rates of *P. caudatum* likely also reflects the non-selective feeding behavior of these organisms [51,117]. The feeding strategy of *Paramecium* sp. involves indiscriminate suspension feeding, which might explain why particle surface characteristics, including the presence of a biofilm, do not influence uptake. However, more detailed research is necessary to examine how particle surface characteristics affect uptake by *Paramecium* sp. and their potential impacts. This is because, even if uptake remains unaffected, particles coated with a biofilm may exhibit enhanced sorption capabilities for pollutants, increasing their hazard potential [33,118]. Therefore, although our study did not observe differences in reproduction with the presence of a biofilm, pollutants adsorbed by the biofilm on particles in the environment could still cause significant adverse effects on the biota.

Similar to our study, positive effects of MP exposure on the population growth of the Ciliophora *Blepharisma japonicum* were also observed for polystyrene (PS) microbeads [50]. In their study, the authors hypothesized that hormesis might explain this observation. This phenomenon describes a positive response to low concentrations of a

contaminant or a naturally occurring element (e.g., suspended minerals), while higher doses may produce the opposite effect [119,120]. In MP ecotoxicology research, hormesis has been suggested for various aquatic organisms, including *Ceriodaphnia dubia*, *Eriocheir sinensis*, and *Amphibalanus amphitrite* [121]. Therefore, we cannot rule out the possibility that this could also apply to *P. caudatum*, which might explain the positive effects observed in our study. However, the wide range of concentrations used in our study (500–50,000 particles mL^{-1}) showed no dose-response pattern, which would be expected if hormesis were the cause.

To rule out that the observed effects in this study were solely due to general particle exposure, three natural control particles were included in the experimental design. Considering these particles, independently of the environmental conditioning, a significant increase in reproduction was observed when *P. caudatum* was exposed to cellulose and kaolin. In contrast, those exposed to MS had a reproduction rate similar to that of the particle-free control. This finding indicates that MS was the most suitable particle control, as it was intended to be on the same level as the particle-free control, and supports the hypothesis that the positive effect on the reproductive outcome is particle type dependent. Additionally, MS was the only particle control for which uptake was proven (see paragraph 4.1). While an increase in reproduction by cellulose particles may be explained by their digestion (see paragraph 4.1), the increase in reproduction by kaolin particles was unexpected. As discussed above, the presence of particles alone could have already been advantageous enough to produce this result, although it could also be attributed to hormesis. Nonetheless, kaolin is ubiquitous in freshwater ecosystems, so unicellular organisms in the environment are generally exposed to fluctuating concentrations of this material, and positive effects of kaolin in unicellular eukaryotes have already been found in other studies [122].

These previous studies suggest that, in natural environments, the combination of high feeding capacity and omnivorous strategy typical of oligotrich unicellular eukaryotes leads to the uptake of large amounts of non-nutritive particles, such as clays. Therefore, to remain competitive with other bacterivorous organisms, they adapted to high concentrations of mineral particles that might otherwise disrupt their feeding activity. Boenigk et al. (2004) hypothesized that the observed increase in cell proliferation in the presence of mineral particles might be due to organic nutrients bound to the particles' surfaces, thereby providing a higher energy input.

4.3. Ecological relevance

Given that this study was conducted using relatively high concentrations (500–50,000 particles mL^{-1}), which exceed MP concentrations found in the environment (e.g., [75,123,124]), translating the findings to real-world scenarios proves challenging. However, particulates, both natural and anthropogenic, are common in aquatic environments [125] and are likely to exceed the concentrations used in this study. This is important to consider, as one of the main findings of the present study was the reproductive stimulation of *P. caudatum* by the presence of particles. Therefore, this finding is relevant in environments with high suspended particle concentrations, thereby enhancing the study's overall ecological significance. On the other hand, to accurately assess MP effects on *P. caudatum*, additional studies using environmentally relevant concentrations are necessary. The present study represents a first step towards that goal, establishing a promising method for particle quantification using μ-Raman spectroscopy and a convenient approach for testing MP toxicity in *P. caudatum* and other single-celled organisms.

5. Conclusion

This study demonstrated that particle uptake occurs in *P. caudatum* across various types of plastics, including PB- and BB-plastics, as well as natural control particles. Additionally, our results showed that environmental conditioning of particles prior to testing did not affect the asexual reproduction rates of *P. caudatum*. The effects of particle exposure on reproduction were positive, indicating that the presence of most particle types in this study stimulated reproduction and that paramecia may try to digest and potentially use some plastics as a carbon source. However, further research is necessary to understand the mechanisms behind this seemingly beneficial effect. This becomes even more important given the vital role Ciliophora play in ecosystems. For example, longer exposure experiments with individual cells and populations should be conducted to better assess the impacts of PB- and BB-MPs on *P. caudatum*. Moreover, to provide clearer insights for risk and hazard assessments of MP exposure in *P. caudatum*, subsequent experiments involving various plastic types, e.g., to establish a clear dose-response curve, would be helpful. Future studies should include more realistic, environmentally relevant concentrations. The ones selected in this study, in fact, although appropriate for investigating concentration-dependent effects, do not reflect a natural contamination scenario. The Raman spectroscopic method developed in this study qualitatively confirms particle uptake and could also quantify it, reducing the need for potentially toxic fluorescent dyes and fixation chemicals in Ciliophora MP ecotoxicology. However, to quantify particle uptake comprehensively, future studies would need to improve this method by developing workflows that enable higher sample throughput.

Environmental implication

Ciliophora exhibit a wide range of functional diversity and are therefore key components of aquatic food webs. Since they feed on particulates, they may be affected by microplastics. This study compared the effects of bio-based biodegradable microplastics and petroleum-based ones, with and without biofilm, on the reproduction of *Paramecium caudatum*. Results indicated that bio-based biodegradable microplastics, along with PET, promoted reproduction. Furthermore, μ-Raman spectroscopy enables the identification of microplastics within the cytoplasm and the detection of spectral changes of particles after uptake. This could help establish realistic dose-response curves in ecotoxicological studies using unicellular organisms.

CRediT authorship contribution statement

Sven Ritschar: Writing – review & editing, Formal analysis. **Marvin Kiene:** Writing – review & editing, Formal analysis. **Vinay K. Bangalore Narayana:** Writing – review & editing, Validation, Methodology, Investigation, Formal analysis, Data curation, Visualization. **Simon Wieland:** Writing – review & editing, Visualization, Methodology, Investigation. **Michael Schwarzer:** Writing – review & editing, Writing – original draft, Visualization, Validation, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Simona Mondellini:** Writing – review & editing, Writing – original draft, Visualization, Validation, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Magdalena M. Mair:** Writing – review & editing, Validation, Formal analysis, Data curation. **Christian Laforsch:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Funding acquisition, Conceptualization. **Holger Kress:** Writing – review & editing, Project administration, Funding acquisition. **Martin G.J. Löder:** Writing – review & editing, Validation, Supervision, Project administration. **Julian Brehm:** Writing – review & editing, Formal analysis.

Funding

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 860720 and from the Deutsche Forschungsgemeinschaft (DFG, German Research Foundation) – Project Number 391977956 – SFB

1357.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

Prof. Dr. Seema Agarwal, Dr. Daniel Wagner, and Dipannita Ghosh (Macromolecular Chemistry II, University of Bayreuth) are acknowledged for providing the plastic fragments and for measurements of size distributions within the collaborative framework of the CRC 1357 (Project Z01) and LimnoPlast (EU H2020 grant agreement No 860720). Mechthild Kredler and Hannah Ganzleben are acknowledged for organism husbandry. Prof. Dr. Thomas Scheibel and Dr. Hendrik Bargel are acknowledged for their support with the scanning electron microscope. The SEM was funded by the Deutsche Forschungsgemeinschaft (DFG GZ: INST 91/366–1 FUGG and INST 91/427–1 FUGG). We sincerely thank the Keylab "Synthesis and Molecular Characterization" at the University of Bayreuth for providing the infrastructure for the Raman measurements.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jhazmat.2026.141575.

Data Availability

Data will be made available on request.

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