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Interactions between polystyrene microplastics and marine phytoplankton lead to species-specific hetero-aggregation

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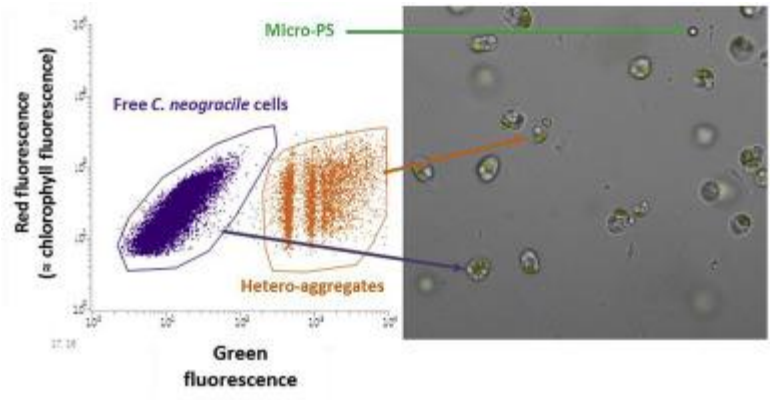
Abstract :

To understand the fate and impacts of microplastics (MP) in the marine ecosystems, it is essential to investigate their interactions with phytoplankton as these may affect MP bioavailability to marine organisms as well as their fate in the water column. However, the behaviour of MP with marine phytoplanktonic cells remains little studied and thus unpredictable. The present study assessed the potential for phytoplankton cells to form hetero-aggregates with small micro-polystyrene (micro-PS) particles depending on microalgal species and physiological status. A prymnesiophycea, Tisochrysis lutea, a dinoflagellate, Heterocapsa triquetra, and a diatom, Chaetoceros neogracile, were exposed to micro-PS (2 µm diameter; 3.96 µg L−1) during their growth culture cycles. Micro-PS were quantified using an innovative flow-cytometry approach, which allowed the monitoring of the micro-PS repartition in microalgal cultures and the distinction between free suspended micro-PS and hetero-aggregates of micro-PS and microalgae. Hetero-aggregation was observed for C. neogracile during the stationary growth phase. The highest levels of micro-PS were "lost" from solution, sticking to flasks, with T. lutea and H. triquetra cultures. This loss of micro-PS sticking to the flask walls increased with the age of the culture for both species. No effects of micro-PS were observed on microalgal physiology in terms of growth and chlorophyll fluorescence. Overall, these results highlight the potential for single phytoplankton cells and residual organic matter to interact with microplastics, and thus potentially influence their distribution and bioavailability in experimental systems and the water column.

Graphical abstrac



Highlights

► *Chaetoceros neogracile* formed hetero-aggregates with micro-polystyrene. ► Hetero-aggregation of *C. neogracile* increased with culture age. ► Micro-polystyrene did not affect growth, morphology or fluorescence of free algal cells. ► Microplastic distribution and bioavailability may differ among species and experimental systems. ► Flow cytometry and 3D microscopy are good tools for studying MP distribution in water.

Keywords : Polystyrene, Microplastic, Aggregates, Microalgae, Marine, Phytoplankton

Introduction

Microplastics (MP), defined as plastic particles below 5 mm diameter (Arthur et al., 2009), constitute an emerging threat in marine ecosystems due to the overall quantity of plastic debris entering the oceans every year. Indeed, Jambeck et al. (2015) estimated that 4.8 to 12.7 million metric tons of plastic waste entered the world's oceans in 2010, with a steady increase expected in the following years. In addition, the ubiquitous nature of these microparticles has been shown to lead to their accumulation in oceans over the last decades (Eriksen et al., 2014; Woodall et al., 2014). According to Van Sebille et al. (2015), up to 51.2×10^{12} MP particles are presently floating in marine environments worldwide.

Ingestion of MP by marine organisms, via direct uptake of free MP (Cole et al., 2011; Rochman et al., 2016; Sussarellu et al., 2016) or through consumption of contaminated preys (Farrell and Nelson, 2013), has been demonstrated in laboratory studies. Numerous field studies have also shown the presence of MP in fishes (Boerger et al., 2010; Lusher et al., 2013; Rummel et al., 2016; Sanchez et al., 2014), crustaceans (Murray and Cowie, 2011) and a wide range of filter feeders including bivalves (Van Cauwenberghe and Janssen, 2014), polychaetes (Van Cauwenberghe et al., 2015) and whales (Besseling et al., 2015). Such findings are of great concern, as physical and toxicological impacts have been observed both in cases of MP ingestion (for review see Rochman et al. (2016); Wright et al. (2013); for recent studies see Green (2016); Jemec et al. (2016); Paul-Pont et al. (2016); Sussarellu et al. (2016)) and food chain transfer arising from predator/prey interactions (Farrell and Nelson, 2013; Setälä et al., 2014). Nevertheless, it is still not clear how MP enter the marine food web. While free MP can be taken up directly by organisms (e.g. from mussels to crabs (Farrell and Nelson, 2013) or from beach hoppers to ray-finned fish (Tosetto et al., 2017)), they could also be ingested via vectors. For instance, (Ward and Kach, 2009) experimentally demonstrated a facilitated transfer of nanoplastics (NP) to filter feeders through marine aggregates.

In the marine environment, MP can drift alone in the water column, but are also likely to interact with the surrounding marine plankton (Cole et al., 2016, 2013; Lagarde et al., 2016; Long et al., 2015; Setälä et al., 2014). For instance MP and NP can be efficiently incorporated in marine phytoplankton aggregates, which modify MP settlement rates in the water column (Long et al., 2015) and favour ingestion of NP by suspension-feeding bivalves (Ward and Kach, 2009). However, the behaviour of MP with phytoplanktonic aggregates and single cells has been little studied. The interactions of MP with phytoplankton have only been reported at very high concentrations, ranging from 25 mg to 2 g L^{-1}. Studies on freshwater microalgae demonstrated significant interactions and rapid formation of hetero-aggregates when microalgae were exposed to 20 nm nano-polystyrene at 80–800 mg L^{-1} (Bhattacharya et al., 2010) and 400–1000 µm MP (polypropylene and high-density polyethylene) at 1

g L^{-1} (Lagarde et al., 2016). The only report of aggregation with a marine species was observed between the diatom *Skeletonema costatum* and micro-polyvinyl chloride (micro-PVC at 50–2000 mg L^{-1}) (Zhang et al., 2017). Significant impact on growth rate was reported for the marine flagellate *Dunaliella tertiolecta* exposed to 250 mg L^{-1} of 0.05 µm micro-polystyrene (micro-PS) (Sjollema et al., 2016), but no hetero-aggregation was observed.

From recent literature (Maes et al., 2017; Sgier et al., 2016; Shim et al., 2016), tools based on fluorescence analysis, such as Flow cytometry (FCM), appear to be a relevant approach for rapid and robust detection and analysis of microplastics. FCM is a routine method that allows the analysis of various particle types within a flux. This technique enables the quantification and the characterization of particle parameters through measurements of light scattering and fluorescence (natural or following staining) after excitation by a laser beam. FCM has already been widely used for the study of microorganisms, including marine phytoplankton (Estrada et al., 2004; Lelong et al., 2011b; Pomati et al., 2011). FCM has the advantage of quickly and simultaneously analysing several parameters on large quantities of suspended cells. Moreover, FCM can be combined with several extensions such as camera or cell sorter, which allows the detection of microplastics (Sgier et al., 2016).

The aim of the present study is to investigate the potential for marine phytoplankton cells to aggregate with micro-PS depending on phytoplankton species and physiological state under experimental conditions. The present study focused on small MP (2-µm yellow-green fluorescent polystyrene microspheres; micro-PS). Polystyrene is one of the three most commonly used plastic polymers worldwide (alongside polyethylene and polypropylene), and is frequently found among microplastics sampled at sea (Barnes et al., 2009; Browne et al., 2010; Hidalgo-ruz et al., 2012). Three different marine phytoplankton species from different taxonomic groups were tested. The species were selected based on their predominance in marine phytoplankton communities and because of their common inclusion in bivalve diets (Dalsgaard et al., 2003; Malviya et al., 2016; Robert et al., 2004). The diatom *Chaetoceros neogracile* is a non-motile cell (width = 4 µm, length = 7 µm) encased in siliceous valves known as frustules covered with an organic coating (Hecky et al., 1973). The prymnesiophyceae *Tisochrysis lutea* is a small motile cell (width = 5 µm, length = 6 µm) covered by a dense layer of thin organic scales (Bendif et al., 2013). The dinoflagellate *Heterocapsa triquetra* is a motile cell (diameter = 16 µm, length = 23 µm) encased in a theca covered by an external plasmic membrane (Dodge and Crawford, 1970). The partitioning (aggregation and attachment to algae) of micro-PS was evaluated over an entire culture cycle, from seeding to stationary growth phase for each algal species. The potential toxic effects of MP on microalgal physiology was investigated through the analysis of growth rates and chlorophyll auto-fluorescence, which can be used as a proxy

of photosynthetic efficiency (Lelong et al., 2011a). All analyses (cell and particle counts, formation of hetero-aggregates, algal viability and photosynthetic activity) were performed by flow cytometry.

Materials and methods

Algal culture

Two species of algae, *Chaetoceros neogracile* (5.3 µm) and *Tisochrysis lutea* (4.5 µm) were obtained from the Scottish Marine Institute. The dinoflagellate *Heterocapsa triquetra* (18 µm) (strain HT99PZ - Ehrenberg, 1840) was isolated in the Penzé river (bay of Morlaix, France). Non-axenic cultures were grown in autoclaved F/2 medium (Guillard, 1975) made with filtered (0.22 µm) natural seawater. F/2 medium was enriched with silica (1.07 10^{-4} M) for the diatom *C. neogracile*. Cultures were performed in 250 mL glassware balloon flasks filled with 100 mL of medium maintained at 16°C under a 12/12 hour photoperiod with 92 ± 13 µmol photons m^{-2} s^{-1}.

Microplastic exposure

Pristine 2-µm polystyrene beads (micro-PS) (yellow-green fluorescent, density of 1.05 g mL^{-1}, smooth and uncharged, solution in deionised water containing 0.1% Tween 20) obtained from Polysciences Inc. were used in this experiment. Exposures were performed in glass flasks to minimize losses caused by plastic attraction to the flask walls. Micro-PS were added to the medium at the beginning of the experiment just after microalgal inoculation (9 10^5 micro-PS mL^{-1}, corresponding to 3.96 µg L^{-1}). It is noteworthy that the same experiment was also performed using a concentration of micro-PS ten times higher (9 10^6 micro-PS mL^{-1}, corresponding to 39.6 µg L^{-1}) than the concentration presented here. Data from this second experiment are not discussed in the paper as they give identical results, but they are available in supplementary tables 1 to 7. A good dispersion of the micro-PS in the culture media was confirmed in a preliminary experiment. Two sets of controls were set up: non-exposed microalgae cultured without addition of micro-PS, and micro-PS maintained in clean F/2 medium without any algae. Cultures and control flasks were set up in triplicate and were sampled 6 to 8 times from early exponential phase to stationary growth phase for flow cytometric and microscopic analyses. Micro-PS and microalgae were kept in suspension and homogenized by gently stirring the flasks every day and prior to sampling. Micro-PS detection and count were performed on fresh samples, while algal cell concentrations and chlorophyll fluorescence intensity were measured on fixed samples (glutaraldehyde, 0.3% final concentration), frozen in liquid nitrogen and stored at -80°C. Growth rates (day^{-1}) were estimated according to the following equation:

$$\mu = \frac{\mathrm{Ln}(C_2/C_1)}{T_2 - T_1}$$

C_1 and C_2 being the cell concentrations (cell mL^{-1}) at T_1 and T_2 (days) corresponding to the beginning and the end of the exponential growth phase, respectively.

Flow cytometry (FCM)

Microalgal cells and micro-PS were counted using a FACSCalibur flow cytometer (Becton Dickinson, San Diego, CA, USA) equipped with a blue laser (excitation 488 nm). All particles analysed by FCM were characterized according to their forward scatter (FSC), side scatter (SSC) and red (FL3; red emission filter long pass, 670 nm) or green fluorescence intensity (FL1; green emission filter band pass, 530/30 nm). Microalgal populations were identified according to their high level of FSC, high level of FL3, which is related to chlorophyll autofluorescence (Galbraith et al., 1988; Hariskos et al., 2015; Sosik et al., 1989) and low level of FL1 (Figures 1A and 1G).

Micro-PS and micro-PS homo-aggregates were identified by their lower levels of FSC and FL3 and their high levels of FL1 related to their green fluorescence (Figures 1D and 1E). Hetero-aggregates were identified by their high levels of FSC, FL3 and FL1 (Figures 1G and 1H). Neither algal debris nor micro-PS homo-aggregates disturbed the FCM analysis as they exhibited different features in the FCM measurements: lower levels of FSC, SSC and FL3 for microalgal debris and higher level of FL1 but still low level of FL3 for micro-PS homo-aggregates.

Data were analysed using Cellquest software and concentrations of microalgal cells or micro-PS were calculated (cell mL^{-1}) from the number of events per unit time and the estimate of the FacsCalibur flow rate measured according to Marie et al. (1999). By measuring the FL1 fluorescence intensity it was possible to determine single micro-PS and groups of 2, 3 or more micro-PS, either in hetero-aggregates (Figure 1I) or in micro-PS homo-aggregates. Concentrations of micro-PS were thus corrected according to the number of beads in micro-PS aggregates.

Micro-PS partitioning in the glass flasks was defined as (i) free suspended beads, (ii) hetero-aggregates constituted by micro-PS and microalgal cells and (iii) microbeads adsorbed to the glassware (on the flask walls). The amount of adsorbed beads was estimated by subtracting the number of micro-PS present in the media (as free suspended beads, micro-PS homo-aggregates or hetero-aggregates) at the time of sampling from the number of micro-PS present in the glass flasks (measured at the start of the incubation).

3D Fluorescent Microscopy

As it was impossible to tell by flow cytometry whether the beads were present inside or outside the microalgal cells, microscope observations were performed to confirm the adhesion and/or engulfment of the micro-PS to/by algae. Both the attachment of micro-PS to algal cells and phagotrophic events were observed using a Zeiss Axio Observer Z1 inverted fluorescence microscope coupled to a 3D Vivatome unit. Fluorescent micro-PS could be viewed using GFP filters (Excitation 494/20 nm, Emission 536/40 nm) and chloroplasts were visible using DsRed filters (Excitation 575/25 nm, Emission 628/40 nm). Z-stack was used to visualize internal beads with Axovision Rel. 4.8 software.

Statistics

Statistical analyses of growth rates and physiological parameters (Fl3 auto-fluorescence, FSC and SSC) were performed using R software (R Foundation for Statistical Computing, Vienna, 2011) with a level of statistical significance at p-value < 0.05. Differences in growth rate and chlorophyll fluorescence intensity among the different experimental conditions were assessed with non-parametric Kruskal-Wallis tests. Results are expressed as mean value ± standard error (SE).

Results

1. Micro-PS distribution in the cultures

In the control flasks containing only micro-PS, the micro-PS concentration suspended in the water decreased over time (Figure 2A) as the micro-PS adsorbed to the glassware. The percentage of suspended micro-PS was 91 ± 4% (mean ± SE) after 24 h, and then progressively decreased down to 47 ± 7% at day 28 and increased back to 66 ± 5% at day 35. Within the suspended micro-PS fraction, it is worth noting that the amounts of micro-PS homo-aggregates increased over time in both the control and the exposed treatment flasks. Bacterial aggregates (Figure 3A) were detected by microscopic observations in all the flasks, including the control flask, with some micro-PS trapped within these aggregates (Figure 3B).

In *C. neogracile* flasks, 81 ± 3% of the micro-PS were in suspension and no hetero-aggregates were formed at day 1 (Figure 2B). By the mid-exponential growth phase (day 13), the percentage of suspended micro-PS increased to 96 ± 1% (Figure 2B), these were free and neither bound to nor inside the diatoms. At the end of the exponential phase (day 20), micro-PS were predominantly free in suspension (83 ± 3%), and in low proportions in the form of hetero-aggregates (2 ± 1%) (Figures

1H, 1I and 3C) and adsorbed to the glassware (14 ± 3%) (Figure 2B). Finally, in stationary growth phase (day 29), the proportion of hetero-aggregates consisting of diatoms and micro-PS reached 19 ± 6%.

In *T. lutea* flasks (Figure 2C), no data is available for days 1 and 2 due to sampling issues. On day 3, no hetero-aggregates were observed and 79 ± 4% of the micro-PS appeared in suspension as free beads or homo-aggregates. The fraction of micro-PS stuck to the glassware increased throughout the growth cycle up to 75 ± 4% at the end of the exponential growth phase (day 14), while 22 ± 4% of the remaining micro-PS were suspended and only 3 ± 1% were detected as hetero-aggregates. During the stationary growth phase, no hetero-aggregates were observed, most micro-PS were adsorbed to the glassware (97 ± 1%), while the remaining 3 ± 1% were in suspension.

In *H. triquetra* cultures (Fig. 2D), 22 ± 2% of the micro-PS were adsorbed to the glassware and 78 ± 2% remained free in suspension at day 1. The proportion of micro-PS free in suspension decreased steadily over time reaching 15 ± 2% in the stationary growth phase (day 24). Although they could not be adequately quantified by FCM throughout the growth cycle, occasional cases of phagotrophy of micro-PS (Figure 3D), aggregation of micro-PS within cell lysis products (Figure 3E), and hetero-aggregation (Figure 3F) were observed through microscopic observations on *H. triquetra.*

2. Impact of micro-PS on microalgal physiology

No significant effect of micro-PS exposure was detected on the growth rate of any of the three species at any time during the experiment. *C. neogracile*, *H. triquetra* and *T. lutea* reached the stationary growth phase after 22, 23 and 15 days, respectively. Mean growth rates were higher for *T. lutea* with 0.450 ± 0.004 $days^{-1}$ (mean ± SE) in controls and 0.440 ± 0.005 $days^{-1}$ for the micro-PS treatment. Mean growth rate of *C. neogracile* was 0.227 ± 0.004 $days^{-1}$ for the control and 0.227 ± 0.011 $days^{-1}$ when exposed to micro-PS. *H. triquetra* had the lowest growth rate, with 0.206 ± 0.006 $days^{-1}$ in the control and 0.198 ± 0.005 $days^{-1}$ with micro-PS.

No differences were observed in microalgal chlorophyll content (as estimated by autofluorescence intensity detected on the FL3 detector of the flow-cytometer), forward scatter or side scatter between controls and micro-PS exposed cultures for the three tested species (Tables 1, 2 and 3).

Discussion

Hetero-aggregation with micro-PS is dependent on species and culture physiological stage

Hetero-aggregation between MP and *C. neogracile* is in agreement with field observations that showed plastic particles associated with diatoms, including the pelagic genus *Chaetoceros* (Reisser et al., 2014; Zettler et al., 2013). Our results tend to show that exposures of *C. neogracile* to micro-PS resulted in hetero-aggregation when cells reach the stationary growth phase. The release by phytoplankton of extracellular polysaccharides (EPS) with sticky properties is common for phytoplankton species and can induce aggregation. The genus *Chaetoceros* is particularly known to produce high amount of TEP (Transparent exopolymeric particles) resulting from EPS aggregation (Kiørboe and Hansen, 1993; Li et al., 2016; Passow et al., 1994). Moreover, EPS excretion with increasing stickiness is often associated with nutrient depletion, which occurs at the end of culture (Joiris et al., 1982; Kiørboe et al., 1990; Logan and Alldredge, 1989). Since EPS/TEP production was not measured here, further studies are needed to confirm the hypothesis linking cell physiology, EPS and the formation of hetero-aggregates with micro-PS.

In the current study, neither the micro-PS stock solution nor the algal cultures were axenic. Bacteria associated with the micro-PS stock solution or algal cultures had likely grown in experimental cultures and controls. Bacteria can also produce exopolysaccharides with sticky properties (Bhaskar et al., 2005; Passow, 2002b). Moreover, bacteria and algae can be associated to produce EPS, TEPs, and promote aggregation (Alldredge et al., 1993; Passow, 2002a,b). Thus it is reasonable to assume that bacterial exudates or debris may also have participated in the homo- and hetero-aggregation of micro-PS and/or their adsorption to glassware. In addition to bacterial and algal exudate, lysis of algal cells during the growth cycle may have released some partially dissolved organic molecules that possibly acted as glue. This was observed in the *H. triquetra* cultures, where micro-PS seemed to be trapped in the lysis products of dying cells. This suggests that the decrease in free suspended micro-PS, even after re-suspension by stirring, is associated with some organic binding of micro-PS to the glassware.

Phagotrophy describes the process by which unicellular organisms derive their food by engulfing and digesting other cells. As far as we know, this is the first report of MP phagotrophy by phytoplankton, even though phagotrophy of micro-PS was only rarely observed in this experiment. The dinoflagellate *H. triquetra* is a mixotrophic algal species able to phagocytose bacteria and small algae (Legrand et al., 1998). Such observations indicate that mixotrophy could be another pathway for MPs to enter the food web and is therefore a subject that deserves to be studied further.

Micro-PS exposure had no effect on algal physiology

Micro-PS did not affect algal growth or the physiological parameters measured by flow cytometry under our experimental conditions (3.96 µg L^{-1}; for the higher concentration of 39.6 µg L^{-1} see results

in supplementary tables 4 to 7). This is in agreement with previous studies that explored the effect of MP, tested at similar or higher concentrations, on the health of different microalgal species (Davarpanah and Guilhermino, 2015; Lagarde et al., 2016; Sjollema et al., 2016). Due to confounding factors of MP dose and size in laboratory experiments, conflicting results on MP toxicity exist in the literature. Zhang et al. (2017) found a negative impact of micro-PVC (average 1 µm) on *Skeletonema costatum* growth and chlorophyll fluorescence at high concentration (50 mg L^{-1}). They hypothesized that blockage of alveoles and cell surface physical damage were responsible for the decrease in microalgal growth. Similarly, Sjollema et al. (2016) highlighted an impact of micro-PS on the growth rate but not on photosynthetic efficiency for the marine flagellate *Dunaliella tertiolecta*, but only at very high exposure concentrations (250 mg L^{-1}) with small particle size (0.05 µm). For larger microplastics (> 400 µm) of different polymer type (polypropylene and high density polyethylene), no deleterious effects were observed on the freshwater microalgae *Chlamydomas reinhardtii*, even at high concentration (1 g L^{-1}) (Lagarde et al., 2016). However, growth and photosynthesis appeared affected in the freshwater algae *Scenedesmus* sp. at 1 g L^{-1} (Besseling et al., 2014) and *Chlorella* sp. at 1.8 mg L^{-1} (Bhattacharya et al., 2010) exposed to polystyrene nanoparticles (< 70 nm). Indeed, particle size may be a crucial parameter for toxicity. Nanoplastics may be more likely than MP to interact with algal cell membranes by, for instance, inducing shading, or blocking microalgal pores or gas exchanges (Bhattacharya et al., 2010). In addition, a molecular simulation study by Rossi and Monticelli (2014) predicted that PS nanoparticles could permeate lipid membranes. This would severely affect the activity of membrane proteins and enhance absorption of NP compared to MP.

Bioavailability and distribution of microplastics must be measured in laboratory experiments

Our results demonstrated that MP behaviour under the conditions of this experimental study, here using phytoplankton cultures, cannot be predicted and requires rigorous measurement. It was clearly shown that micro-PS may adsorb to glassware, form homo-aggregates and hetero-aggregates with phytoplankton cells, residual organic matter and/or bacteria exudates. These results underline the need to quantify the bioavailability and distribution of MPs in the experimental systems. Furthermore, MP distribution in different media (e.g. suspended, floating, adsorbed to experimental containers, trapped in organic aggregates, or adsorbed on or ingested by organisms) must be assessed to obtain accurate values of the actual MP concentration to which the organisms are exposed, as it is commonly done for other pollutant studies. For instance, in Sussarellu et al. (2016), a supplementary tank without animals was set up for each treatment to evaluate micro-PS sinking or sticking to the plastic (polymethylmetacrylate, commonly known as Plexiglas) tank walls. Estimation of exposure concentration in the water surrounding the animals was half the nominal mass concentration. This also applies to other polymer types, especially polyethylene, which has a lower

density than seawater and positive buoyancy, which may prevent homogeneous exposure. During laboratory experiments, the particles may remain at the water surface, i.e. unavailable to marine organisms such as filter feeders (Von Moos et al., 2012). To avoid this, Green (2016) reduced the buoyancy of neutral MP (high density polyethylene and polylactic acid) by mixing them with cultures of *Isochrysis galbana* 3 days prior to exposure. This made them bioavailable to flat oysters *Ostrea edulis*. Overall, the variety of MP occurring in the environment is more diverse (polymer size, type, shape and concentration) than the spherical MP commonly used in laboratory experiments and this should be adequately addressed in future studies (Huvet et al., 2016). Considered together, these findings show that experiments should be designed carefully, specifically for MP, in order to avoid difficulties/artefacts caused by the intrinsic properties of these particles.

Future directions for field studies

Observations of micro-PS adsorbing to suspended cells of *C. neogracile* and bacterial aggregates suggests that suspended particulate organic matter may influence MP buoyancy and settling in the water column (and *vice versa*), as already suggested for phytoplankton aggregates (Long et al., 2015) and zooplankton faecal pellets (Cole et al., 2016). If hetero-aggregation occurs in marine environments (yet to be proven), this may contribute to the incorporation of MP in phytoplankton aggregates (marine snow) as it is forming, in addition to their direct incorporation in phytoplankton aggregates once these are already formed and settling to the bottom (Long et al., 2015). This is of particular concern as aggregation was shown to enhance ingestion of 0.1 µm nano-PS by bivalves (Ward and Kach, 2009). Altogether these results support the hypothesis that the phytoplankton compartment is a potential sink and vector for MP trophic transfer in marine food webs.

Although this study has highlighted a mechanism, the ecological relevance of such laboratory observations is likely low, as they are far from reflecting the complexity of the marine environment (relatively static conditions, small volumes, high algal cell concentrations, one microalgal species, one plastic type, size, dose, etc.). For instance, hetero-aggregation appears dependent on both plastic composition (Lagarde et al., 2016) and microalgal species, it is thus likely to vary with changes in phytoplankton communities. Further studies moving towards more realistic scenarios (e.g. natural plankton communities or mesocosm experiments) are required to evaluate the ecological relevance of phytoplankton/MP hetero-aggregation. To make this possible, technological developments are required to improve MP sampling at sea, especially the fraction consisting of small MP, which may be associated with suspended organic and inorganic materials. For instance, sediment traps should be used at sea to collect marine snow and faecal pellets, potentially containing associated MP. Relevant concentrations experiments should also be favoured (Lenz et al., 2016), however this requires urgent

methodological development for quantifying the smallest microplastics (<50 µm) in marine ecosystems (Huvet et al., 2016). For instance, the mass concentration of 3.96 µg L^{-1} used in the present study was lower than microplastic concentrations measured in various ecosystems (see Supplementary Table 1 in Sussarellu et al. (2016)) even though strict comparison between >330µm and 2 µm fraction are hard to make.

Flow cytometry and 3D microscopy are useful tools for investigating MP distribution in water

Flow cytometry enabled us to investigate the distribution of fluorescent micro-PS within the phytoplankton cultures, quantifying the proportion and the state of micro-PS in suspension as free beads, homo-aggregates and hetero-aggregates made of micro-PS and microalgae. The quantification limit of the flow cytometer used in the present study (FACSCalibur FCM) allowed the measurement of micro-PS even at low concentrations, i.e. down to 500 micro-PS mL^{-1} (meaning a mass of 2 ng L^{-1}) for an analysis of 1 minute. It is noteworthy that this quantification threshold could also be decreased by increasing either flow rate or processing time. While flow cytometry could provide a fast and quantitative method to analyse micro-PS concentration and distribution, we consider that visual confirmations are required to ensure a proper interpretation of cytograms. In our case, use of 3D microscopy allowed micro-PS adsorbed to the cell surface to be distinguished from those phagocytized by microalgae. Considering the recent isolation of microplastics from freshwater samples by FCM (Sgier et al., 2016) as well as technical advances in staining of non-fluorescent microplastic particles for detection and quantification purposes (Maes et al., 2017; Shim et al., 2016), flow cytometry represents a promising tool for efficiently detecting, quantifying and even isolating (when coupled to a sorter (Sgier et al., 2016)) the fraction of small microplastics (<200 µm) that remain largely unknown in marine environments (Huvet et al., 2016). In addition, the use of dynamic imaging particle analysis by FlowCam, which automatically captures digital images of particles as they are carried in a fluid stream and thus combines the quantitative power of flow cytometry with microscopy tools, could lead to great advances in the field of microplastic research.

Conclusion

The interactions and impacts of 2 µm micro-PS (3.96 µg L^{-1}) on marine phytoplankton was experimentally assessed on 3 microalgal species using flow cytometry. While no effects of micro-PS exposure was observed on algal growth and fluorescence, distribution of micro-PS in algal cultures appeared dependent on the species and the physiological state of the algae. Hetero-aggregation was exclusively observed with the diatom *C. neogracile* in stationary phase, probably in relation with increase in cell stickiness, EPS production and/or bacterial aggregates that changed with the age of the culture. This study also provided some methodological recommendations to properly assess the

particle distribution and bioavailability when conducting microplastics laboratory experiments. Finally, the present work highlighted the use of flow cytometry as a promising tool to quantify and characterize small microplastics (<200 μm) in seawater.

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Figure 1. Cytograms of *Chaetoceros neogracile* culture (A, B and C), micro-PS solution (D, E and F) and *C. neogracile* culture exposed to micro-PS (G, H and I). The total microalgal population is illustrated on dot plots A, D and G, representing red fluorescence (Fl3-Height) vs. forward scatter (FSC-Height). Free *C. neogracile* cells (purple blue region), residual free micro-PS (green region) and *C. neogracile*/micro-PS hetero-aggregates (orange region) can be distinguished on dot plots B, E and H, representing red fluorescence (Fl3-Height) vs. green fluorescence (FL1-Height). Free algal cells (purple line) and hetero-aggregates (orange line) can also be distinguished on histograms C, F and I, representing the counts vs. green fluorescence (Fl1-Height). The entire free micro-PS population was visible and quantified with other flow cytometry settings (data not shown).

Figure 2. Microplastic partitioning over a growth cycle expressed as the percentage of total micro-PS. A: Control flask, B: *Chaetoceros neogracile,* C: *Heterocapsa triquetra,* D: *Tisochrysis lutea.* Solid green lines represent the microalgal concentration (cells mL^{-1}); dot-dashed yellow lines represent the percentage of free suspended micro-PS; dotted blue lines represent the percentage of micro-PS adsorbed to the glassware and long dash red lines the percentage of hetero-aggregates. Error bars represent the standard error (n = 3).

Figure 3. A: Micrograph of a bacterial aggregate after Sybr Green staining (1X final concentration); bacteria can be seen in green (without micro-PS). B: Micrograph of micro-PS (green) free and trapped in bacterial aggregate (not SybrGreen stained). Both microbial aggregates and micro-PS are visible in green. C: Micrograph of micro-PS and *Chaetoceros neogracile* as free particles and hetero-aggregates, arrows indicate micro-PS. D: Orthogonal view from 3-Dimensional micrograph (x40) of a *H. triquetra* cell that has phagocytosed micro-PS. Phagocytosed micro-PS (in green) trapped between chloroplasts (in red). The central picture shows a reconstruction of 16 stacked fluorescence images (thickness = 7.5 µm). The flanking pictures show cross-sections compiled along the z-axis (left box) and x-axis (upper box). For 3D visualisation, please see the supplementary files. E: Micrograph of lysed cell of *H. triquetra* with micro-PS (green) trapped in lysis products. F: Maximum intensity projection of a *H. triquetra*/micro-PS heteroaggregate stained with calcofluor (1% final concentration). A *H. triquetra* cell is visible in blue under Dapi filters (Excitation 406/15nm, Emission 457/50 nm). Bacterial nuclei (picture A) and micro-PS (pictures B, D, E and F) are visible in green under GFP filters (Excitation 494/20nm, Emission 536/40 nm). Chlorophyll (pictures D and E) is visible in red under DsRed filters (Excitation 575/25nm, Emission 628/40 nm).

Figure 1

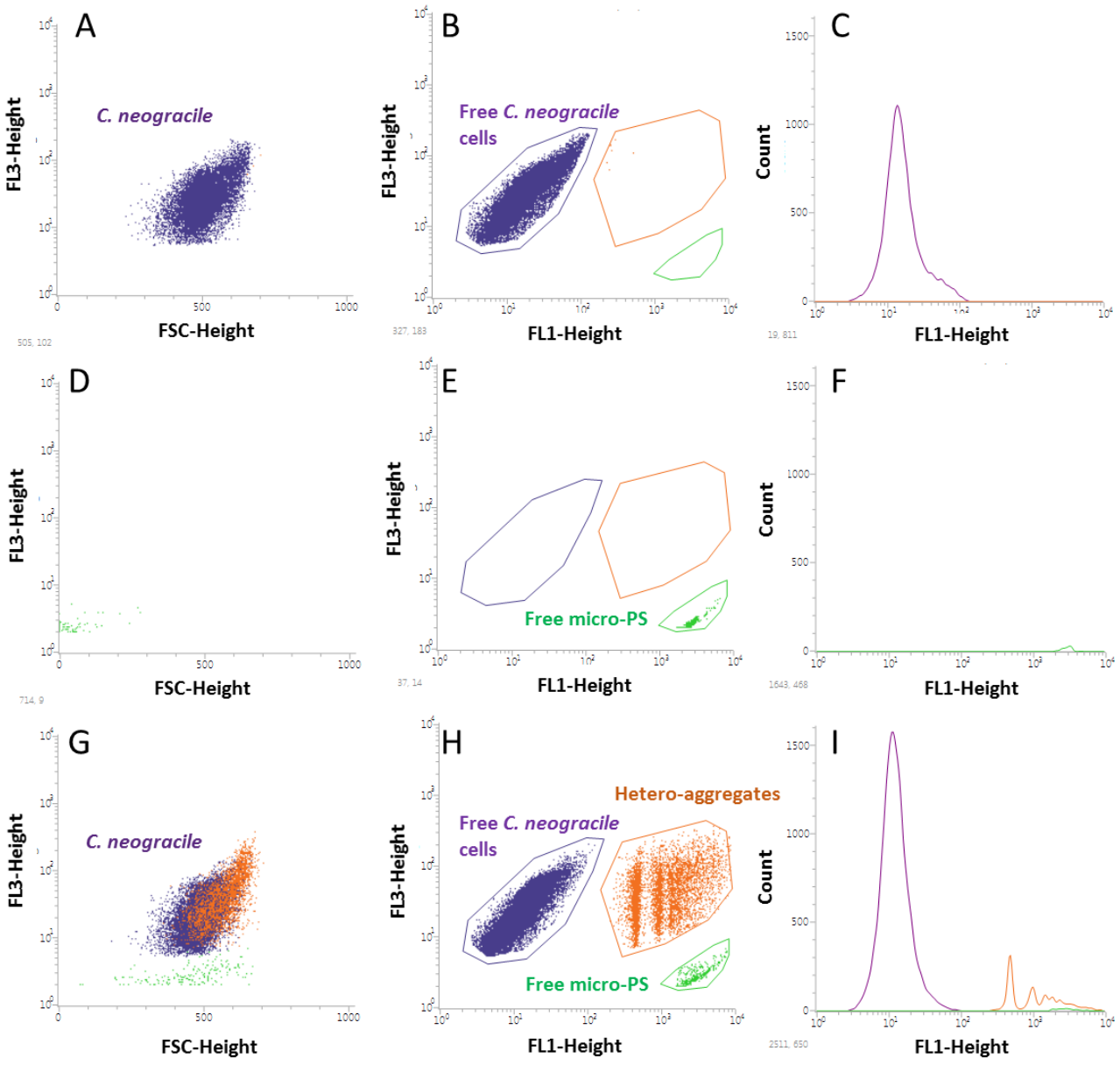

A
C. neogracile
FL3-Height
FSC-Height
505, 102
B
Free C. neogracile cells
FL3-Height
FL1-Height
327, 183
C
Count
FL1-Height
19, 811
D
FL3-Height
FSC-Height
714, 9
E
Free micro-PS
FL3-Height
FL1-Height
37, 14
F
Count
FL1-Height
1643, 468
G
C. neogracile
FL3-Height
FSC-Height
H
Hetero-aggregates
Free C. neogracile cells
Free micro-PS
FL3-Height
FL1-Height
I
Count
FL1-Height
2511, 650

Figure 2

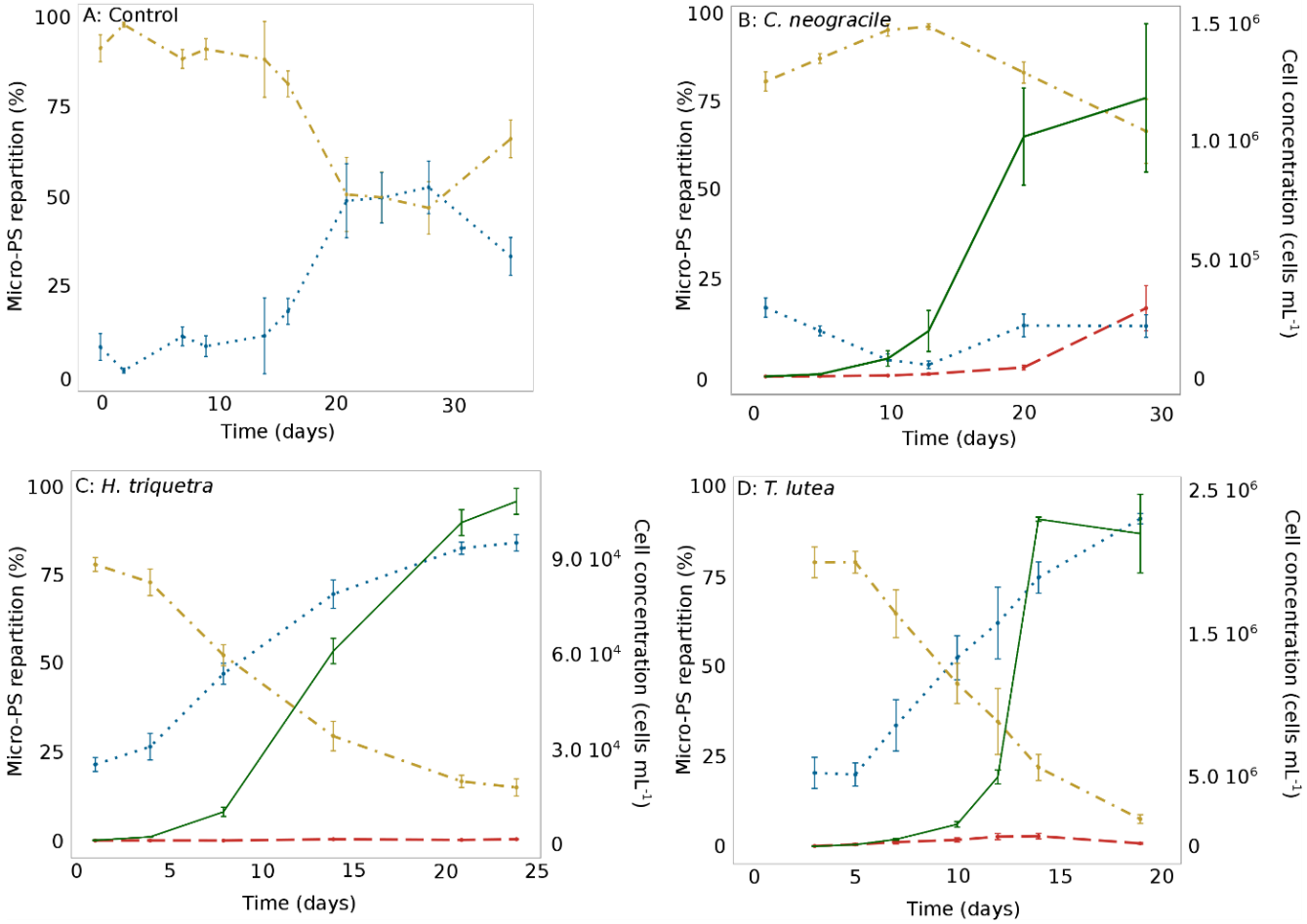
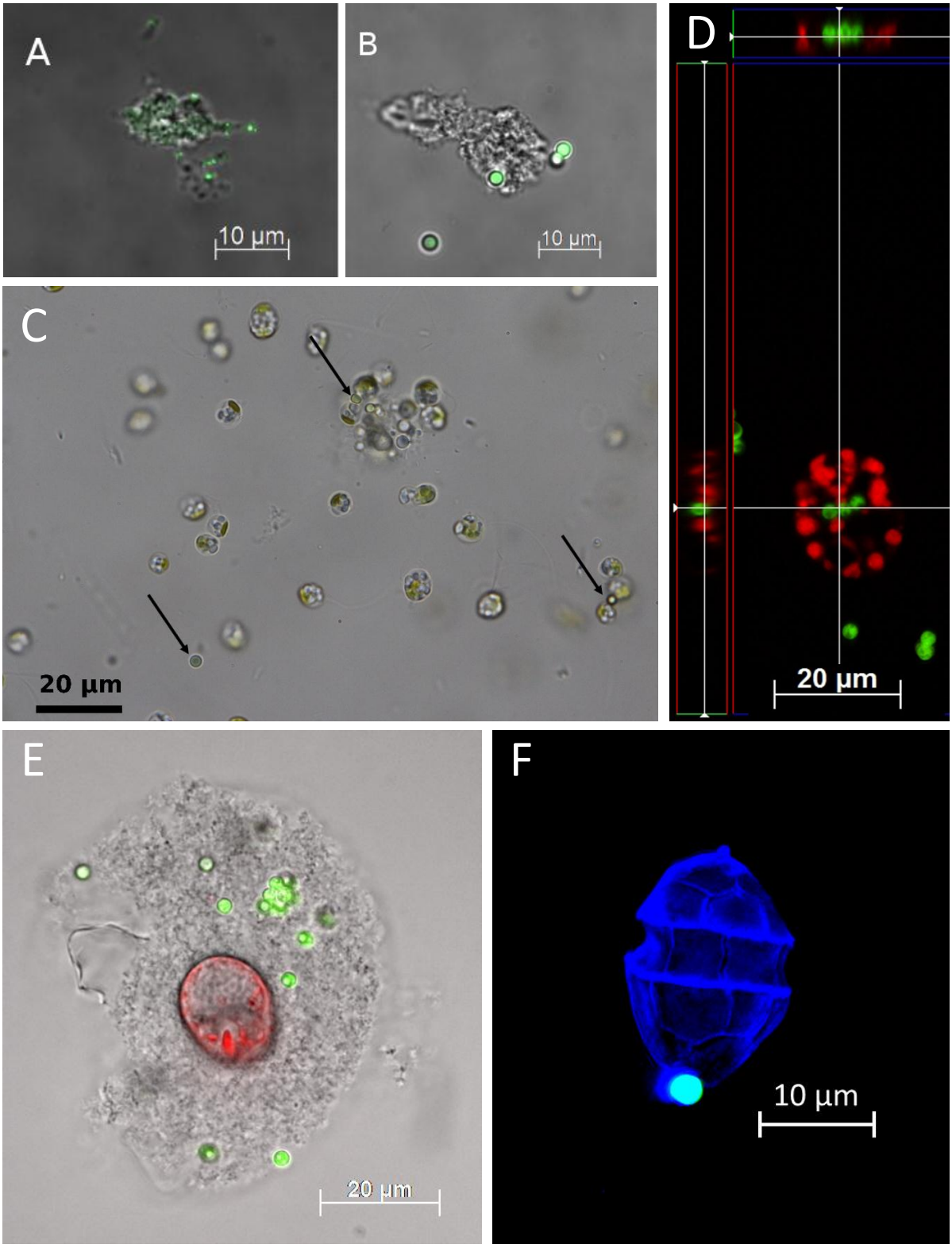

A: Control
B: C. neogracile
C: H. triquetra
D: T. lutea
Micro-PS repartition (%)
Cell concentration (cells mL-1)
Time (days)
100
75
50
25
0
1.5 10^6
1.0 10^6
5.0 10^5
9.0 10^4
6.0 10^4
3.0 10^4
2.5 10^6
5.0 10^6
0
5
10
15
20
25
30

Figure 3



Interactions between polystyrene microplastics and marine phytoplankton lead to species-specific hetero-aggregation

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Supplementary files

Supplementary table 1:

C. neogracile						
	90 000 micro-PS mL^{-1}			900 000 micro-PS mL^{-1}		
Days	**Free micro-PS**	**Hetero-aggregates**	**Flask walls**	**Free micro-PS**	**Hetero-aggregates**	**Flask walls**
1	81.0 ± 2.7	0.0 ± 0.0	18.9 ± 2.6	84.5 ± 3.1	0.0 ± 0.0	15.5 ± 3.1
5	87.4 ± 1.3	0.1 ± 0.3	12.5 ± 1.3	90.2 ± 1.9	0.1 ± 0.0	9.7 ± 1.9
10	95.2 ± 1.7	0.3 ± 0.1	4.5 ± 1.7	96.9 ± 2.0	0.3 ± 0.1	2.8 ± 2.0
13	96.1 ± 0.8	0.7 ± 0.3	3.2 ± 1.1	97.1 ± 1.8	0.7 ± 0.2	2.2 ± 1.9
20	83.5 ± 2.9	2.5 ± 0.7	14.0 ± 3.1	88.0 ± 2.0	2.5 ± 0.3	9.5 ± 2.3
29	67.4 ± 8.8	18.8 ± 6.2	13.9 ± 3.1	75.6 ± 1.7	7.3 ± 3.4	17.1 ± 5.0

Supplementary table 2:

H. triquetra						
	90 000 micro-PS mL^{-1}			900 000 micro-PS mL^{-1}		
Days	**Free micro-PS**	**Hetero-aggregates**	**Flask walls**	**Free micro-PS**	**Hetero-aggregates**	**Flask walls**
1	78.4 ± 2.2	0.0 ± 0.0	22.9 ± 2.2	53.9 ± 1.3	0.0 ± 0.0	46.1 ± 1.3
4	73.3 ± 3.8	0.0 ± 0.0	26.7 ± 3.8	48.9 ± 0.4	0.0 ± 0.0	51.1 ± 0.4
8	52.6 ± 3.0	0.0 ± 0.0	47.4 ± 3.0	39.0 ± 2.5	0.1 ± 0.0	61.0 ± 2.5
14	29.7 ± 4.2	0.4 ± 0.2	70.0 ± 4.0	22.9 ± 2.1	0.5 ± 0.2	76.7 ± 2.0
21	16.8 ± 1.7	0.2 ± 0.0	83.0 ± 1.7	14.2 ± 2.8	0.1 ± 0.0	85.7 ± 2.8
24	15.1 ± 2.4	0.4 ± 0.1	84.5 ± 2.3	15.1 ± 1.4	0.1 ± 0.0	84.8 ± 1.4

Supplementary table 3:

T. lutea						
	90 000 micro-PS mL^{-1}			900 000 micro-PS mL^{-1}		
Days	**Free micro-PS**	**Hetero-aggregates**	**Flask walls**	**Free micro-PS**	**Hetero-aggregates**	**Flask walls**
3	79.1 ± 4.3	0.3 ± 0.1	20.6 ± 4.3	81.9 ± 0.4	0.1 ± 0.0	18.0 ± 0.3
5	79.2 ± 3.0	0.7 ± 0.3	20.1 ± 3.2	80.9 ± 1.2	0.4 ± 0.0	18.7 ± 1.2
7	64.9 ± 6.7	1.3 ± 0.5	33.8 ± 7.1	79.4 ± 3.9	0.6 ± 0.1	20.0 ± 3.8
10	45.5 ± 5.6	1.9 ± 0.6	52.6 ± 6.1	58.7 ± 1.6	0.9 ± 0.1	40.4 ± 1.5
12	34.9 ± 9.2	2.9 ± 0.9	62.3 ± 10.0	46.5 ± 2.3	1.5 ± 0.1	52.0 ± 2.4
14	22.1 ± 3.6	3.0 ± 0.8	74.9 ± 4.4	28.9 ± 3.3	1.2 ± 0.6	69.9 ± 3.3
19	7.7 ± 1.2	1.0 ± 0.2	91.3 ± 1.4	8.5 ± 2.0	0.7 ± 0.0	89.9 ± 2.0

Supplementary table 4:

Species	Condition	Exponential growth phase duration	FSC Initial	FSC mid-exponential	FSC stationary
C. neogracile	Control	22	75.3 ± 2.9	73.6 ± 0.8	80.5 ± 1.1
	Micro-PS		68.9 ± 1.4	71.3 ± 0.7	82.1 ± 0.6
T. lutea	Control	15	62.4 ± 1.9	43.9 ± 0.3	30.8 ± 0 .5
	Micro-PS		56.5 ± 1.85	42.2 ± 0.1	30.4 ± 0.4
H. triquetra	Control	23	114.1 ± 3.8	114.3 ± 1.2	110.4 ± 1.1
	Micro-PS		112 ± 2.4	113.8 ± 3.6	107.6 ± 0.9

Supplementary table 5:

Species	Condition	Exponential growth phase duration	SSC Initial	SSC mid-exponential	SSC stationary
C. neogracile	Control	22	13.0 ± 0.9	7.5 ± 0.4	6.4 ± 0.5
	Micro-PS		11.8 ± 0.9	7.5 ± 0.3	7.1 ± 0.3
T. lutea	Control	15	14.1 ± 0.9	7.6 ± 0.2	4.8 ± 0.1
	Micro-PS		23.6 ± 9.1	10.1 ± 0.6	5.1 ± 0.1
H. triquetra	Control	23	185.9 ± 2.2	139.5 ± 7.8	145.6 ± 1.8
	Micro-PS		176 ± 2.2	139.4 ± 11.0	145.2 ± 4.6

Supplementary table 6:

Species	Condition	Exponential growth phase duration	FL3 Initial	FL3 mid-exponential	FL3 stationary
C. neogracile	Control	22	17.5 ± 0.3	14.6 ± 0.1	14.1 ± 2.3
	Micro-PS		18.4 ± 0.1	14.5 ± 0.1	9.3 ± 3
T. lutea	Control	15	7.5 ± 0.2	10.4 ± 0.2	4.5 ± 0.1
	Micro-PS		7.4 ± 0.2	9.7 ± 0.1	4.6 ± 0.1
H. triquetra	Control	23	12.5 ± 0.4	17.6 ± 0.4	13.9 ± 0.4
	Micro-PS		12.4 ± 0.1	18.4 ± 0.4	15.2 ± 1.4

Supplementary table 7:

Species	Condition	Exponential growth phase duration	90 000 micro-PS mL^{-1}	900 000 micro-PS mL^{-1}
C. neogracile	Control	22	0.227 ± 0.004	0.227 ± 0.004
	Micro-PS		0.227 ± 0.011	0.235 ± 0.003
T. lutea	Control	15	0.450 ± 0.004	0.450 ± 0.004
	Micro-PS		0.440 ± 0.005	0.440 ± 0.005
H. triquetra	Control	23	0.206 ± 0.006	0.206 ± 0.006
	Micro-PS		0.198 ± 0.005	0.195 ± 0.005