Phytoplankton Exopolymers Enhance Adhesion of Microplastic Particles to Submersed Surfaces

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Abstract
Intense pollution of marine environments with plastic waste, including micro- and nanoplastics, is a new and poorly studied threat measured in tens of million tonnes annually. Despite a huge scale of the problem, almost nothing is known about pathways and mechanisms of involvement of micro- and nanoplastics in marine food webs, trophic processes, global biogeochemical cycles. In this study, a hypothesis is considered and experimentally verified about the role exopolymers from marine phytoplankton play in flocculating micro- and nanoplastics and forming their aggregates in marine environments to transfer and deposit them further in bottom sediments. In experiments with non-axenic cultures of the cryptophyte Rhodomonas salina (RHO) and the green alga Tetraselmis suecica (TET) exposed to micro-polystyrene particles (MP, 4.3 μm diam., about 0.4 × 10^6 particles/ml, 16 mg/L), microalgal exudates were shown to promote MP flocculation and immobilization on vertical glass surfaces. The highest levels of MP were “cleared” from the medium by the TET culture which released more extracellular polysacharides. Hetero-aggregation of MP and algal cells was not observed, probably owing to turbulent mixing and cell motility. Abundant bacterial consortia released in the cultures (up to 9 × 10^6 cells ml^-1) could be an additional source of exopolymers and serve an agent of MP flocculation and adhesion. Thus, the results obtained highlight the potential for phytoplankton exudates to interact with micro- and nanoplastics, and potentially affect their bioavailability and vertical transport in marine environments.

Key words: microplastics, nanoplastics, flocculation, adhesion, phytoplankton, exopolymers, Tetraselmis, Rhodomonas, flow cytometry.

Introduction
The pollution of the marine environment by microplastic (MP) debris with a size below 5 mm (Arthur et al., 2008) poses a serious, ever-increasing threat to marine ecosystems (Barboza et al., 2019). The scale of the global MP contamination is huge, from 5 to 13 million metric tons annually (Jambeck et al., 2015). Moreover, it is steadily and rapidly growing, with MP accumulating in the oceans for several decades due to chemical inertness and durability of polymers (Woodall et al., 2014). According to modern estimates, about 10^{14} MP particles are drifting in marine waters (Van Sebille et al., 2015). Their direct consumption by marine organisms have been reliably confirmed in experiments (Cole et al., 2011; Farrell and Nelson, 2013; Sussarellu et al., 2016). Field studies have revealed presence of MP in fishes (Boerger et al., 2010; Lusher et
Microalgal cultures. The experiments were conducted with cultures of cryptophytic (Rhodomonas salina - RHO) and green (Tetraselmis suecica - TET) microalgae (obtained from the Microalgal Culture Collection at IBSS RAS, Sevastopol). The choice of these species was due to an abundant production of transparent exopolymer particles (TEP) by their cultures, especially in the stationary phase. Besides, TET is a well-known flocculant agent of bio-flocculation method which can fasten the harvesting of non-flocculant microalgae like Chlorella sp. and Nanochloropsis sp. (Kawaroe et al., 2016).

Non-axenic stock cultures were grown in Walne medium. They were incubated on an orbital shaker (S-3.02M.20, Latvia) at 75 rpm, under natural light conditions, without aeration, at 21°C. Cell density was monitored by flow cytometry. At the beginning of the experiments, the microalgal concentrations were about 10⁶ cells/mL in all experimental vessels.

Microplastics. Polychromatic fluorescent microspheres (MS) made of polystyrene (4.3 μm diam., 1.05 g/cm³, 491 nm excit., 554 nm emiss. – Polysciences, Inc.) were used as MP stock in the experiments. The initial suspension of MS was diluted with sterile culture medium to a concentration of 0.4 × 10⁹ MS/mL equivalent to about 17.5 g/L. Before use, the working solution was thoroughly mixed with a vortex to evenly distribute MS in the solution.

Experimental design. To prepare experimental suspensions, the microalgal cultures (20 ml) in the stationary phase and the MS working solution (30 μl) were poured (in three replicates) into experimental beakers with 10 ml of sterile growth medium. In the reference vessels, the medium was used instead of the cultures. In all the incubations, the start MS abundance and mass were about 0.36 × 10⁶ MS/mL and 15.9 mg/L, respectively.

To quantify the rate of MS adhesion, glass slides were placed in all the experimental and reference vessels. They were immersed in the suspension and fixed in the upright position. Thus, the following series of incubations were set up: RHO + MS, TET + MS and MS alone (the control), each in triplicate (9 vessels and 27 slides in total).
The experimental suspensions were incubated for 8 days under the same conditions as the cultures. The shaking speed was chosen in such a way as to prevent the hetero-aggregates from settling too quickly and, at the same time, to avoid their destruction and washing the MS away from the glass slides. For the flow cytometric analysis, suspension samples (1 ml) were collected in all the vessels after 1, 4 and 8 days of the experiment. Additionally, a slide was taken from each vessel to enumerate the attached MS. Before the analysis, the slides were immersed twice in distilled water in order to wash off the non-attached MS, and dried at 40°C.

**Microscopy and visualization of exopolymers.** Suspended/settled MS aggregates (including their biopolymer matrix) and slide-attached MS were studied using the microscope Nikon Eclipse TS100-F equipped with the Ikegami ICD-848P camera and a Nikon D5100 SLR digital camera, in light and fluorescence (blue-light excitation) modes. At least, 20 fields of view were photographed on both sides of each slide for further MS enumeration and their density calculations. Image analysis (MS counts) was performed using ImageJ Software (Version 1.48).

Microalgal exopolymers were stained using Alcian Blue, a polysaccharide marker (Alldredge et al. 1993, Passow, Alldredge 1995). Experimental suspensions were dried on glass microscope slides and stained using Alcian Blue (3% in acetic acid, pH 2.5) for 20 min, then rinsed in water and dried (Staats et al., 1999). The same protocol was applied to visualize exopolymer films on the slides.

**Flow cytometry.** MS, microalgae and bacteria were enumerated using a Cytomics™ FC 500 flow cytometer (Beckman Coulter Inc., USA) equipped with a 488-nm argon laser, and the CXP software package. MS and microalgae were enumerated in unstained samples by gating the cell/MS populations on 2-parameter cytograms of forward light scattering (FS) and red autofluorescence (FL4, 675 nm), both on 4-decade logarithmic scales (Fig. 1, B and C, left plots). Additionally, formation of hetero-aggregates of MS and microalgae was monitored on dot plots of FL4 versus green fluorescence (FL1, 525 nm) but not revealed (Fig. 1, B and C, right plots).

Bacterial abundance was determined in the subsamples stained with SYBR Green I (Molecular Probes, USA) according to a standard protocol (Marie et al., 1997, Gasol, Del Giorgio, 2000). Working solution of the stain was prepared in a dilution of 10^{-2} and stored frozen at −20 °C. The final SYBR Green I dilution in the subsample was 10^{-4}. Staining was performed in the dark for 30 minutes immediately before cytometric measurements. Bacteria were gated on 2-parameter cytograms of FS versus green fluorescence (FL1 channel, 525 nm) on 4-decade logarithmic scales.

Bacteria with a high content of nucleic acids (HNA bacteria) were considered physiologically active and identified by their high green fluorescence signal (channel FL1, 525 nm) after their staining with SYBR Green I (Lebaron et al., 2002; Servais et al., 2003).

The concentrations of MS and cells were calculated from the flow rate (15 to 60 μL min⁻¹), counting time (60 to 480 s), and the number of events in the target gate (at least 3000). The quality of measurements was controlled using the Flow-Count™ fluorospheres (Beckman Coulter Inc., USA) at a known concentration in the sample.

**Results**

**Dynamics of microalgae and MS in the suspension.** For the first day of the incubation, both the cultures exhibited a decrease in their abundances down to about 0.2 × 10^5 cells/ml (Fig. 2, left plot). Then, the growth rate of TET exceeded sufficiently those of RHO, with their abundance achieved the value of 3.4 ± 1.0 × 10^5 cells/ml (± SD here and below) by the end of the experiment. The number of suspended microspheres (MS) rapidly decreased in all the experimental vessels, including the control (with no microalgae), due to: (i) adherence to walls and glass slides, (ii) sedimentation to the bottom as a part of aggregates. The highest rate of removing the MS from the suspension was observed in the vessels with TET (Fig. 2), the lowest one – in the reference vessel without the microalgae.

**MS immobilization on the glass surface and aggregate formation.** MS surface density increased over the experiment in all the vessels (Fig. 2, right plot). The maximum (about 800 MS/mm²) was achieved in the TET suspension that provided evidence of the highest efficiency of MS immobilization by this species. Correspondingly, the lowest rate of MS attachment to the glass slides was registered in the reference vessels (Fig. 2).
Figure 1. Cytograms of the cultures of Chaetoceros neogracile (A; data from Long et al., 2017), Rhodomonas salina (B; our data) and Tetraselmis suecica (C; our data) exposed to fluorescent polystyrene microspheres. Gating: MS – microspheres, CHA – Ch. neogracile, H-A – hetero-aggregates of Ch. neogracile and microspheres (according to Long et al., 2017), RHO – Rh. salina, TET – T. suecica.
Figure 2. General scheme of processes in the experimental and reference (CNL) vessels: Dynamics of *Rhodomonas salina* (RHO), *Tetraselmis suecica* (TET) and fluorescent microspheres (MS) in the medium (left plot); Immobilization of MS on slide surface (right plot and photos). Error bars are standard deviations.

Figure 3. Aggregates of 4.3-µm fluorescent microsphere settled at the bottom of the experimental vessels with *Rhodomonas salina* (RHO), *Tetraselmis suecica* (TET) and the reference vessel (CNL) on the fourth day of experiment. Micrographs were obtained in light (top) and fluorescent (bottom) modes. Arrows show organic matrix stained by Alcian Blue. The microspheres fluoresce in green.
Figure 4. Gating of the total (Bac) and high nucleic acid (HNA) bacteria in the space of forward scatter (FS) and green fluorescence (FL1). Results obtained for the experimental vessels with *Rhodomonas salina* (RHO), *Tetraselmis suecica* (TET) and the reference vessel (CNL).
Besides MS accumulation on the vertical surfaces (vessel walls and glass slides), single MS and their aggregates (including detritus and exopolymer matrix) settled at the vessel bottom (Fig. 3). Coagulation of MS and cells, and hetero-aggregate formation were not observed owing to turbulent mixing and cell motility. The MS aggregate matrix was well stained by Alcian Blue (Fig. 3) that demonstrated the key role of microalgal exopolymers in the particle flocculation. In the reference vessels, MS flocculated at much lower rates, and their aggregates did not contain a noticeable amount of organic matter despite relatively high abundances of bacteria, another potential source of exopolymers.

**Bacterial abundance and activity.** The bacterial consortia revealed in the experimental vessels by flow cytometry differed sufficiently in their green fluorescence profiles, with the highest heterogeneity being in RHO culture (Fig. 4). At the final stage of the experiment, the bacterial abundances reached $8.8 \pm 0.3 \times 10^6$ cells/ml (TET) and $8.8 \pm 0.3 \times 10^6$ cells/ml (RHO) in the experimental vessels, and $1.4 \pm 0.7 \times 10^6$ cells/ml in the reference ones (Fig. 5). The HNA-bacteria representing physiologically active part of the bacterial consortium, contributed roughly about 60% (control), 80% (TET) and 90% (RHO) to the total bacterial abundances in the corresponding vessels (Fig. 4).

![Fig. 5. Bacterial abundance (N) and a portion of HNA-bacteria in the bacterial consortium (HNA%) in the experimental vessels with *Rhodomonas salina* (RHO), *Tetraselmis suecica* (TET) and the reference vessel (CNL) at the final stage of the experiment. Error bars are standard deviations.](image)

**Discussion**

According to a conceptual framework suggested in this study for understanding the fate of microplastics in marine environments, the pollutant is not only involved in the food chain and trophic processes but, in an alternative pathway, escapes the food chain through sedimentation (Woodall et al., 2014; Lagarde et al., 2016; Long et al., 2015, 2017). Our results show that in the presence of phytoplankton able to produce extracellular polysaccharides, the rate of adhesion of microplastic particles to submersed surfaces and suspended aggregates can sufficiently grow thus, promoting microplastics vertical transfer to and deposition in bottom sediments. This strengthens the hypothesis about the role exopolymers from marine phytoplankton play in removing microplastics from the pelagic food web.

High bacterial abundances revealed in the experimental vessels pointed to another potential source of the aggregate matrix. Bacteria do release exopolysaccharides with sticky properties (Bhaskar et al., 2005; Passow, 2002), and algae-bacteria consortia can be highly efficient in producing EPS, TEPs, and promoting
aggregation (Alldredge et al., 1993; Passow, 2002). Hence, it seemed reasonable for us to suppose that bacterial exudates or debris could also serve an agent of MS adhesion to glassware in the experiment. Besides, lysis of algal cells may have released some organic substances possibly acted as glue as it was shown earlier in experiments with *Heterocapsa triquetra* (Long et al., 2017).

In a recent study of mechanisms of microplastics aggregation (Long et al., 2017), the so-called hetero-aggregation between 2-µm polystyrene particles and the diatom *Chaetoceros neogracile* (>8 µm in size) was identified by flow cytometry as an addition cluster on cytograms (Fig. 1, A). These findings were in well agreement with field observations of plastic particles associated with diatoms, including the pelagic genus *Chaetoceros* (Reisser et al., 2014; Zettler et al., 2013). However, in our experiments with TET and RHO, adhesion of MS to microalgae were not documented (Fig. 1, B and C), probably due to: (i) high motility of the flagellated cells; (ii) lower ratio between the cell and MS sizes (they were of comparable dimensions). The latter appears to be a factor controlling phytoplankton-microplastics interactions. Smaller plastic particles seem to stick to a phytoplankton cell easier and, at the same time, to be a threat for the cell. Microplastics do not usually affect microalgal physiology (growth rate, autofluorescence of pigments) (Davarpanah and Guilhermino, 2015; Lagarde et al., 2016; Sjollema et al., 2016; Long et al., 2017), while nanoplastics can have a dramatically negative (including cytotoxic) effect on physiological functions of the cell, apparently due to contact with the cell wall/membrane, blocking of pores and channels (Bhattacharya et al., 2010) and even penetration through lipid membranes (Rossi, Monticelli, 2014).

Despite the cell surface did not contribute to the MS immobilization in our experiments, the rate of MS removal from the suspension was high due to their adhesion to any available solid surfaces, either MS clumps and TEP or glassware. The outcome of the competition between these substrates seems to depend on a number of factors like turbulent mixing and fragility/ephemerality of the aggregates, that in its turn complicates the experiment. If the lack of mixing in the experimental vessels results in a rapid sedimentation of suspended matter and, in fact, makes it impossible to study formation and growth of aggregates in the water column, then the use of a shaker can cause destruction of the aggregates and a complete halt in the process of their formation. Nevertheless, even highly turbulent conditions cannot prevent exopolymer-mediated adhesion of cells and particles onto immersed solids. In this regard, the use of artificial substrates with a controllable surface area seems to be a promising methodological approach which allows one to manipulate the process of immobilization of microplastic particles under experimental conditions.

**Conclusions**

1. The presence of phytoplankton exopolymers in the medium led to a significant increase in the rate of adhesion of microplastic particles on submerged surfaces, while the magnitude of the effect was species-specific. The green alga *Tetraselmis suecica* ensured maximum adhesion rate.

2. Given comparable sizes of microplastic particles and microalgal cells, their coagulation and formation of the so-called hetero-aggregates did not occur probably due to: (i) turbulent mixing of the medium; (ii) cell motility.

3. In the non-axenic cultures of *T. suecica* and *Rhodomonas salina*, high abundances of free bacteria (8.8 × 10⁶ and 5.6 × 10⁶ cells/ml, respectively) were revealed, which could also be a source of exopolymers and serve as flocculation and adhesion agents for plastic particles.

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