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Uptake and effects of microplastic particles in selected marine microalgae species; Oxyrrhis marina and Rhodomonas baltica

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Environmental Toxicology and Chemistry
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Dionis Joachim Lyakurwa

ABSTRACT

It is estimated that large numbers of microplastics are present in the marine environment and their concentrations are expected to rise in a foreseeable future. Interactions, uptake, and excretion rates as well as effects of microplastics on biota remain largely unknown. Here we used two algae species to investigate 1) the uptake of 10 µm virgin polystyrene (PS) microbeads and 1-5 µm green fluorescent (GF) microbeads in the dinoflagellate *Oxyrrhis marina* Dujardin (12-30 µm); and 2) interactions and effects of 10 µm virgin polystyrene (PS) microbeads in the cryptophyte *Rhodomonas baltica* Karsten (5-10 µm). *O. marina* (fed *R. baltica* 1:6 ratio of cell number) were exposed to 75 or 7500 microbeads/mL of GF (1-5 µm) or PS (10 µm) particles under continuous mixing for 60 min. Measurements of particle concentrations, volumes and size distributions were done at 0, 10, 25, 40 and 60 min using a coulter counter. *R. baltica* (2000 cells/mL) were exposed to 75, 750 and 7500 PS particles/mL and cultured in 40 mL vials on a plankton wheel at 24°C for 264 h. Particle concentrations, chlorophyll content, and pH were measured at 24 h intervals until the algae density levelled off at the maximum carrying capacity of the system. At the end of the experiment, samples of the microalgae-microplastics mix were examined visually using light/fluorescence microscopy to confirm ingestion of microbeads by *O. marina* or interactions of *R. baltica* with PS microbeads. Our results showed that *O. marina* ingests both PS and GF microbeads at the expense of microalgae uptake (*R. baltica*), and, at high PS concentration, serious adverse effects on *O. marina* were observed characterized by food replacement and loss of motility. Possibly *O. marina* preferred PS, due to particle size and/or polymer type. A non-monotonic dose-response relationship was observed between PS microsphere concentration and *R. baltica* production rate, which strongly correlated with pH in the exposure media as well as fluorescence and hence chlorophyll production. Shading and biofouling are possible mechanisms of action of microplastics impacts on autotrophic microalgae such as *R. baltica*.

Keywords:

microplastics; microalgae; *Rhodomonas baltica*; *Oxyrrhis marina*; uptake; toxicity

Abbreviations

GF	green fluorescent microbeads
HDPE	high density polyethylene
HOC	hydrophobic organic contaminant
LDPE	low density polyethylene
PAH	polycyclic aromatic hydrocarbon
PCB	polychlorinated biphenyl
PPE	polyethylene
PET	polyethylene terephthalate
POP	persistent organic pollutants
PP	polypropylene
PS	polystyrene
PVC	polyvinyl chloride
rpm	revolutions per minute

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1 INTRODUCTION

1.1 Plastics

1.1.1 Plastics: production, types, and additives

Plastics are polymers made from natural organic products such as cellulose, coal, salt, crude oil, and natural gas. The crude oil for example, is first processed and distilled into groups of lighter fractions made up of mixtures of hydrocarbons. The monomers that result from these fractions, such as ethylene and propylene, can be linked together through polymerization processes to larger plastics polymers (Bolgar et al., 2015).

Global plastic production has increased from 0.5 million tonnes in 1950 to 260 million tonnes in 2007 (Europe, Plastic, 2008; O'Brine et al., 2010; PlasticsEurope, 2015), likely due to the versatile properties of plastics. Plastics can be moulded into countless products, making them a preferable raw material for many manufacturing industries. Due to their low cost, light weight and durability, plastics are widely used in a range of applications such as fishing nets and rods, domestic utensils, packaging materials, bags and many other consumer products (Hopewell et al., 2009). The six most common types of plastics, polyethylene, polypropylene, polyvinyl chloride, polystyrene and polyethylene terephthalate are usually used for primary packaging and construction materials, thereby contributing 90% of the total global plastic production (Europe, Plastics, 2012) (Victoria, 2004).

During plastic manufacturing, chemical additives are added to yield the desired properties for the target use. These additives include antifogging, reinforcing and antistatic agents, blowing agents, colorants, fillers, lubricants, nucleating agents, optical brighteners, heat and light stabilizers, antacids, antimicrobials, antioxidants, chain-breaking, photo- and hydroperoxide deactivating antioxidants, dehydrating agents, light screening pigments and UV absorbers (Bolgar et al., 2015; Guart et al., 2011). The resulting polymer properties and structure bear functional combination of basic monomers and chemical additives used (Bolgar et al., 2015).

1.1.2 Marine plastic litter: sources, composition, and distribution

Marine plastic litter has recently been much debated at both public and scientific arenas. Plastic debris is widely distributed in the world oceans, and appears to accumulate in five major oceanic patches or gyres (Andrady, 2011). During the 1979 survey in the Mediterranean Sea it was found that plastics accounted for 60-70% of the debris observed (Morris, 1980). Another survey of debris in the North Pacific Ocean revealed that about 86% of all observed debris was plastic

(Dahlberg et al., 1984). Marine plastic litter originates from anthropogenic activities such as fishing and poor management of plastic wastes. It is estimated that about 80% is land based and 20% comes from marine activities (Lebreton et al., 2012). In densely populated areas, especially with high input of sewage effluents or nearby plastic processing factories, plastic pollution can be an extensive problem (Browne et al., 2011). Jambeck et al. (2015) estimated that 2.7 million tons of plastic entered the ocean in 2010 and that emissions are likely to increase with time.

A considerable amount of the plastic litter has been identified as plastic particles of the 1 μm – 5 mm size range, referred to as microplastics (Andrady, 2011; Andrady et al., 2009; Isobe et al., 2017). These small-sized plastic particles are occurring in the marine environment as primary or secondary microplastics. Primary microplastics refers to pristine microbeads or other microplastic particle types which over a long time have been produced for use in personal care products like facial scrubs, exfoliators, toothpastes, in medicine as vectors for drugs and in air-blasting technologies (Gregory, 1996; Patel et al., 2009; Zitko et al., 1991). Microplastics incorporated in such consumable products, may later find their way into the marine environment mainly through the sewage system. In contrast to primary ones, secondary microplastics result from fragmentation of larger plastic objects due to natural weathering, mechanical stress as well as UV radiation (Andrady, 2003).

A recent study by Van Sebille et al. (2015) found higher concentrations of microplastics in the centres of the North Pacific and North Atlantic Ocean gyres, where most of the samplings are performed. It was estimated that between 7000 and 35,540 tons of microplastics in the size range 0.2 – 100 mm are afloat in the world's oceans, compared to 233,400 tons of larger plastic items. Even in remote Arctic areas with no apparent input of plastic wastes, higher concentrations of microplastics have been measured (Lusher et al., 2015). The presence of microplastics in arctic areas has been linked to long term scavenging and concentration of these particles, whereby they remain trapped inside the ice until it melts (Obbard et al., 2014). Environmental factors such as wind in combination with Ekman transport contribute to the accumulation of plastic in the centre of the plastic garbage gyres (van Sebille, 2015). These mechanisms may account for long distance transport of plastics to the Arctic. Lack of data especially from remote areas makes estimation of the total amount of microplastics in the ocean demanding (Van Sebille et al., 2015). Differences in sampling and analytical methods further hamper comparison of results (Phuong et al., 2016). Examples of methodological deficiencies include the use of sampling nets of large mesh size (≥300 μm) allowing smaller particles to pass through.

1.1.3 Behaviour and implications of plastic particles in the marine environment

Most microplastics are buoyant, thus under normal situations will be found suspended at the surface and few deeper in the water column of the sea (Van Cauwenberghe et al., 2015). The vertical distribution of plastics in the water column is however influenced by plastic type and especially by properties like density and sorption. For example, PE and PP have a lower density than seawater and are therefore expected to and remain suspended at the surface and in the planktonic region (Millero et al., 2008; Morét-Ferguson et al., 2010). More dense polymers like PVC and PET are assumed to sink, while PS is neutrally buoyant and remains suspended in the water column, sinks or floats depending on weather conditions (Bach et al., 2012; Wang et al., 2016). Only plastics with higher density than that of sea water (>1.02 g cm^{-3}) will sink, while those slightly exceeding the surface seawater density are expected to remain suspended at a depth where its density is equal to that of the surrounding seawater (Barnes et al., 2009a). Different mechanisms contribute to the vertical distribution of microplastics in the water column. Such processes include ingestion by marine organisms which may transport plastics down to the sediments. Biofouling that enables attachment of microorganisms on the surfaces of microplastics, contributes to an increase in microplastic density as well as weight, hence affecting their sinking rate (Cózar et al., 2014; Lobelle et al., 2011). Therefore, microplastics are not only found suspended in the water column and on shorelines but also in sediments (Browne et al., 2011; Van Cauwenberghe et al., 2013; Wang et al., 2016; Woodall, L. C. et al., 2014).

Table 1. Densities (g cm^{-3}) of different type of plastics commonly found in the marine environment, relative to seawater (SW) (salinity 3.5%). Modified after Morét-Ferguson et al. (2010) and Millero et al. (2008).

Plastic type	**SW**	**LDPE**	**HDPE**	**PP**	**PS**	**PVC**	**PET**
Density (g cm^{-3})	1.025	0.89–0.93	0.94–0.97	0.85–0.92	1.04–1.08	1.16–1.41	1.38–1.41

Recent studies suggest that several marine species are ingesting plastic particles of various types and can therefore be an important sink of plastic particles (Barnes et al., 2009b). Degradation and fragmentation processes turn larger plastic particles into increasingly smaller fragments, making them easily ingestible by different species including copepods (Cole et al., 2013).

1.2 Phytoplankton: role and distribution in the marine environment

Phytoplankton are floating communities of autotrophic or heterotrophic organisms, unicellular and microscopic (Verma et al., 2016). Similar to terrestrial plants, autotrophic phytoplankton contains photosynthetic pigments (chlorophyll and/or xanthophyll) and requires sunlight and

inorganic nutrients such as nitrates, phosphates and sulphur to synthesise their own food (Siegel et al., 2010). Phytoplankton can be grouped into diverse groups based on their size or feeding strategy. Most microalgae species are autotrophs that produce their own food, for example *Rhodomonas baltica* Karsten. In contrast, heterotrophic microalgae graze on microorganisms and other small particles in their size range, for example *Oxyrrhis marina* Dujardin. Depending on the species, sizes of phytoplankton species can range from a few micrometres to a few hundreds of micrometres (Suganya et al., 2016). Often there is good correspondence between the size and type of phytoplankton communities. For example, picophytoplankton species are < 2 μm across, while most diatoms and dinoflagellates belong to the micro phytoplankton/microalgae class (> 20 μm) (Devred et al., 2011).

Phytoplankton populations are commonly represented by members of the Cyanobacteria, Dionophyta, Chlorophyta, Euglenophyta, Haptophyta and Chrysophyta, Chriptophyta and Bacillariophyta (Pal et al., 2014) although the most common phytoplankton species are members of the phyla of the diatoms, dinoflagellates and cyanobacteria. Dinoflagellates have 2 flagella which are often used to propel the algal cell through the water. Their cell wall is composed of cellulose which has a groove around its equator in most species. They are the most abundant group and can produce harmful algal blooms (red tides) with some species producing neurotoxin. Diatoms lack flagella and they are a group of microalgae that normally are single celled but sometimes can appear as a colony of cells (Pal & Choudhury, 2014; Reynolds, 1984). All species of diatoms have an external cell wall, or theca, composed of silicon dioxide. In contrast to the two groups mentioned above, cyanobacteria are bacteria (prokaryotes). They also obtain their energy through photosynthesis, but lack a nucleus or membrane bound organelles, such as chloroplasts. They have a unique set of pigments used in photosynthesis, called phycobiliproteins (phycobilins). Cyanobacteria are also responsible for Harmful Algal Blooms (HABs) through the production of cyanotoxins (Catherine et al., 2013; Pal & Choudhury, 2014).

Most phytoplankton species dwell near the surface of open waters of lakes, rivers and oceans (Pal & Choudhury, 2014). The distribution of phytoplankton in both marine and inland water environments is mostly determined by two major factors; light and nutrient availability. Most microalgae are found suspended near water surfaces and in shallow waters along shorelines for easy access to sunlight which enables them to synthesize their food. Light is supplied from above and nutrients are often supplied from below. Therefore, in poorly mixed water columns,

algae can be heterogeneously distributed, with thin layers of algal biomass found near the surface, at more depth, or on the sediment surface (Klausmeier et al., 2001).

In a balanced marine pelagic ecosystem phytoplankton species are primary producers that provide food for a wide range of organisms including snails, shrimp and jellyfish (Falkowski, 1994). Phytoplankton biomass accounts for 1-2% of the global oceans carbon source, yet they fix between 30 and 50 billion metric tons of carbon annually (Falkowski, 1994). In the biological carbon pump, phytoplankton is responsible for the transfer of atmospheric carbon dioxide to the ocean. Carbon from carbon dioxide used during photosynthesis, is incorporated in the phytoplankton as carbohydrates and stored in their cells. Most of the stored carbon returns to near-surface waters when phytoplankton are eaten or decomposed, but some descend in the water column and eventually sediment on the ocean floor.

Due to their chemical diversity in body composition, microalgae play major roles in various aspects of human life (Spolaore et al., 2006). These include but are not limited to; enhancement or supplementing of nutritional value of human and animal feeds, cosmetics constituents, and biofuel production. To date microalgae are counted as one of the preferred feedstocks for biofuel production due to the diversity and quality of biodiesel produced by them (Suganya et al., 2016). In biotechnology studies, microalgae have shown an important application in bioplastic production (Hempel et al., 2011) and they are intended to be used as photosynthetic gas exchangers for space travellers in near future (Spolaore et al., 2006).

Given these key roles of phytoplankton, especially regarding the structure and supply of energy in pelagic marine food webs, it is essential to understand how microplastics in the marine environment may affect phytoplankton populations.

1.3 Interactions, adsorption, and uptake of microplastics by microalgae

Previously reported (micro)plastic concentrations in ocean surfaces waters are lower than expected (Cózar et al., 2014). Several mechanisms have been suggested through which microplastics may sink to the sediments. Such processes include biofouling; a process whereby microorganisms like bacteria and microalgae accumulate on the surfaces of microplastics (Woodall, Lucy C. et al., 2014). Such interaction may also facilitate phytoplankton-microplastics agglutination. Adsorption of microalgae on microplastics makes the latter slightly denser relative to seawater, thereby affecting the sinking rate of both microalgae and buoyant microplastics (Ballent et al., 2013; Law et al., 2010; Thornton et al., 2002). Biofouling of microplastics may also lead to their increased uptake by biota (Andrady, 2011), including by heterotrophic microalgae. Under stressful conditions such as the absence of light or nutrient

deficiency in their environment, microalgae secrete polysaccharides molecules known as exopolysaccharides which can later coagulate, enabling aggregation between algal cells (Long et al., 2015; Staats et al., 2000). During aggregation, microplastics and/or aquatic pollutants can potentially be incorporated into these aggregates. In addition, one of the distinguishing characteristics of most microplastics is their hydrophobicity. Due to this property, microplastics are readily adsorbed on the surfaces of microalgae. Additives added to plastic during manufacturing process, may leach from ingested or surface adsorbed microplastics into internal components of the microalgae. Heterotrophic phytoplankton species such as *O. marina* feed on prey of their own size (Roberts et al., 2011). Thus, unlike larger plastic fragments, small plastic particles (microplastics) can easily be ingested. *O. marina* may also accidentally engulf microplastics through phagocytosis including aquatic pollutants attached on their surfaces (Van Cauwenberghe, 2016).

1.4 Toxic effects of microplastics on microalgae

Whether pristine microplastics are harmful to microalgae is currently not well studied. In a study by Sjollema et al. (2016), microalgae growth was negatively affected by exposure to different sizes of polystyrene particles (0.05, 0.5 and 6 μm) for 72 h. The polystyrene particles used in their study were either negatively charged or uncharged. Microalgae photosynthesis was not affected in either treatment tested at lower concentrations. At high concentrations (250 mg/L) microalgae growth was negatively affected (up to 45%) by uncharged polystyrene particles. The observed adverse effects showed an increase with decreasing polystyrene particle size.

Microplastics are larger than nanospheres but may possess the same chemical properties. In a study by Bhattacharya et al. (2010) the physical impact of charged polystyrene nanospheres was assessed by using a carbon dioxide depletion assay, and showed that the adsorption of plastic beads hindered algal photosynthesis. It was further suggested that the observed effects were possibly due to physical blockage of light and air flow by the nanoparticles.

In most studies, the effects of microplastics on microalgae are associated with a change in chlorophyll production, algal biomass or cell number, making them suitable parameters for assessing the toxicity of microplastics. Therefore, we choose the same parameters to assess the effects of microplastics on *R. baltica* and *O. marina.*

1.5 Study species

1.5.1 Oxyrrhis marina

O. marina is a dinoflagellate, usually with a measurable cell size between 15 and 40 micrometres which sometimes can reach up to 60 µm. This microalga is abundantly and globally distributed except for the polar regions where it is rarely found. *O. marina* is a species that is relatively simple to isolate and maintain in the laboratory, making it a suitable algal model for laboratory studies (Lowe et al., 2011). It is heterotrophic, hence it obtains nutrients externally by consuming various types of small organisms from its environment like microalgae, bacteria and other particles (Hansen, F. C. et al., 1996). Some of these particles are larger than the *O. marina* cell itself but with some preference for certain taxa.

1.5.2 Rhodomonas baltica

R. baltica is a photosynthetic cryptophyte and flagellated microalgae (5 µm to 10 µm). Cryptophytes are widely distributed in both freshwater and in the marine environment, thus considered as an important species group in primary food production (Lafarga-De la Cruz et al., 2006).

1.6 Aim of the Study

The main objective of this study was to investigate the interaction, uptake, and effects of 10 µm virgin polystyrene (PS) microbeads in two species of microalgae. The chosen algae models were the autotrophic *R. baltica* (5-10 µm) that is not assumed to have the ability to ingest food particles like microplastics, and the heterotrophic *O. marina* (12-30 µm) that feeds on other microalgae and small particles up to its own size. The size of microplastics (10 µm PS and 1-5 µm green fluorescent (GF)) used in the present study make the particles small enough to be ingested by *O. marina*. *R. baltica* was also chosen as a natural prey for *O. marina* during exposure to microplastics. In addition to PS, GF particles were selected for the experiment with *O. marina* for easy visualisation and localisation of microplastics inside *O. marina.*

More specifically, the investigation aimed to

- observe the interactions between *R. baltica* cells and microplastics
- measure the changes in chlorophyll production (fluorescence units) and algal cell number and biomass of the *R. baltica* exposed to microplastics
- compare feeding behaviour and preference of *O. marina* for microplastics and *R. baltica*
- localise the ingested microplastics (both polystyrene and green fluorescent microparticles) inside *O. marina* cells using light/fluorescence microscopy.

2 MATERIALS AND METHODS

All experiments and laboratory work were performed at NTNU's facilities at Sealab in Trondheim, Norway during the period November 2016 - February 2017. Prior to the exposure of the microalgae to microplastics, several pre-tests were conducted to establish suitable methods.

2.1 Method development (pre-tests)

2.1.1 Monitoring particle engulfment by O. marina using a coulter counter with manual mixing

The aim of this test was to determine whether it is possible to adequately measure R. baltica engulfment by O. marina using a coulter counter when samples are manually mixed. mL culture of O. marina mixed with R. baltica as preference prey at nominal concentrations of 2000 and 21000 cells /ml respectively (ratio of 1:7 O. marina: R. baltica), was prepared in 20 mL polystyrene coulter cups, VWR International AS, Haavard Martinsens vei, OSLO, Norway. The culture was left standing on the coulter counter for all 30 minutes of experimentation. However, manual mixing was done before each coulter run performed at intervals of 0, 3, 6, 10, 15 and 30 minutes. While the coulter cup is tightly closed with polyethylene lid, mixing was done gently by turning the cup and its contents up and down.

2.1.2 Monitoring particle engulfment by O. marina using a coulter counter with mechanical mixing

Here, we aimed to determine whether it is possible to adequately measure *R. baltica* engulfment by *O. marina* using a coulter counter with mechanical mixing. 150 mL culture of *O. marina* mixed with *R. baltica* as preference prey at nominal concentrations of 2000 and 21000 cells /ml respectively (ratio of 1:7 *O. marina: R. baltica),* was prepared in 200 mL glass coulter beaker. The culture was left standing on the coulter counter for all 30 minutes of experimentation with continuous mechanical mixing using mounted coulter stirrer at (a) high speed of 8 rpm (b) low speed of 6 rpm. Measurements of particle concentration and volume were done at intervals of 0, 3, 6, 10, 15 and 30 minutes.

2.1.3 Determining the right excitation, and emission wavelengths for fluorescence measurements

We intended to determine the right excitation and emission wavelengths are applicable for measuring chlorophyll levels (fluorescence) in our samples of *R. baltica.* Samples of *R.baltica* were obtained direct from main cultures maintained at Sealab and divided into six aliquots. By using Cary Eclipse fluorescence spectrophotometer from Varian, Inc. PA, California, USA; (a) excitation wavelengths were scanned from 400nm with the maximum emission wavelength was set to 750nm. (b) emission wavelengths were scanned with the excitation wavelength set to 440nm (wavelength in the region where 'chlorophyll a' absorbs the maximum blue light).

2.1.4 Testing growth of R. baltica in 40 mL culture vials

The aim was to determine whether *R. baltica* cultures can survive and grow in small 40 mL vials and whether sub-sampling these cultures affects *R. baltica* growth. *R. baltica* cultures were divided into two groups each containing 6 culture vials. The nominal concentration of *R. baltica* in each bottle was 2000 cells/mL. Samples for analysis were taken either by sub-sampling (SS) or by terminating the whole vial (RT). Six SS culture bottles were sub-sampled every 24 h for 216 h. Simultaneously 2 RT vials were terminated from the second group.

2.2 Uptake of microplastics by *Oxyrrhis marina*

2.2.1 Oxyrrhis marina

The dinoflagellate *O. marina* was obtained from the Culture Collection of Algae and Protozoa, SAMS Ltd. (Oban, UK) and fed *R. baltica* supplied by Dag Altin (BioTrix, Trondheim, Norway). Before taking samples from the main *O. marina* culture, feeding with *R. baltica* cells was retained and *R. baltica* cells were left to settle for approximately 24 h to reduce background signal from *R. baltica* cells and debris during particle counting. The top layer was then decanted into a new flask and microalgae concentration and size were determined using a Multisizer™ 3 Coulter Counter (Beckman Coulter Inc., Miami, FL, USA). The equivalent spherical size of the *O. marina* cells in this culture was 16.0 ± 2.4 µm.

2.2.2 Microplastics

Spheric polystyrene (PS) particles of 10.1±0.71 µm diameter and a density of 1.05 g/cm^3 were purchased from Polysciences Inc, Warrington, PA, USA supplied in 2.5% solids (w/v) aqueous suspension, crosslinked with divinylbenzene, and a concentration of 4.55 x 10^7 particles/mL. Green fluorescent (GF) microspheres of 1-5 µm nominal diameter and a density of 1.3 g/cm^3 were purchased from Cospheric LLC, Santa Barbara, CA, USA in dry powder form. These GF particles of unknown composition were chosen for ease of localisation of these MPs both in exposure suspensions and inside *O. marina* after uptake. Stock suspensions of both plastic particles were made using filtrated seawater, and stock solution concentrations were subsequently validated using the coulter counter and used to prepare exposure suspensions of the required concentrations.

2.2.3 Exposure

Table 2. Nominal concentrations of Oxyrrhis marina and Rhodomonas baltica used

Treatment	Control	Low	High
Conc. of PS (MPs/mL)	0	75	7500
Conc. of R. baltica (cells/mL)	21000	21000	21000
Conc. of Oxyrrhis (cells/mL)	3000	3000	3000
Number of replicates	6	6	6

Exposure of *O. marina* to microbeads was performed in a 200 mL glass beaker intended for use in a coulter counter while samples were mixed continuously at minimum speed (6 rpm). This mixing speed was established in a pre-test to keep microalgae and microbeads in suspension yet allowing *O. marina* to prey on *R. baltica*. A culture of *O. marina* mixed with *R. baltica* as favourite food at nominal ratio of 1:7 *O. marina: R. baltica* was exposed to two concentrations of the polystyrene (PS) microbeads for 60 min. The nominal initial concentrations of PS particles were 75 and 7500 MPs/mL. In addition, the same *O. marina*: *R. baltica* mix was exposed to the same nominal concentrations of the GF microspheres to simultaneously investigate the influence of microbead size on interaction and effects in microalgae and to locate plastic particles inside microalgae and in exposure solutions. A control group fed only *R. baltica* (21,000 cells/mL) was included in the experiment to enable comparison of feeding rates with *O. marina* exposed to microbeads and *R. baltica*. Measurements of the concentrations, volumes and sizes of microalgae and microbeads were performed at five time points during the experiment (0, 10, 25, 40 and 60 minutes). At the end of the experiment ingestion of MPs was visually confirmed by microscopy.

2.3 Interaction and effects of microplastics in *Rhodomonas baltica*

2.3.1 Rhodomonas baltica

A batch culture of *R. baltica* was made in a 4000-mL culture flask and allowed to reach exponential growth before they were exposed to MPs (image

1 c). *R. baltica* samples from this culture with a start concentration of 2000 cells/mL were then cultured in each 40 mL glass vial in 0.22 µm filtrated sea water supplemented with 1.5% nutrients and with addition of the respective microbead types and concentrations. During the experiment, all the vials were placed on a custom-made plankton wheel which holds in total 48 vials of 40 mL. Light was supplied from one side of the wheel by three white fluorescence lamps (PHILIPS TL-D 18W/840), with a total light intensity of 150 µmol m^{-2} s^{-1}, 100 µmol m^{-2} $s^{-1}$and 60µmol m^{-2} s^{-1}at the near-, middle- and far end of the wheel respectively. Light intensities were measure by Biospherical Instruments' Laboratory Quantum Scalar Irradiance Meter (QSL- 100). This instrument measures light intensity in terms of number of photons in the radiant energy between 400 nm and 700 nm. The values were later expressed as photosynthetic photon Flux Density (PPFD) or photon irradiance in the units of moles per square metre per second (mol/m^2/s) (Figure 1a). Rotation speed was set to 0.80 rpm and all exposure vials experienced the same light intensity regardless of their position on the plankton wheel. Sampling was done every 24 h for a total of 264 h culture time. At each sampling point, 10 mL of exposure solution was sampled from each exposure vial for analysis, with 5 mL destined for particle counting using the coulter counter and 5 mL for measurements of both pH and fluorescence (chlorophyll content). After every sub-sampling, the amount withdrawn was replaced with 10 mL growth medium (filtrated seawater + growth medium), containing the same microbead concentration as the subsampled bottle.

2.3.2 Exposure

The same 10 µm polystyrene microspheres (Polysciences Inc, Warrington, PA, USA) as described in section 2.1.2 were used to study interaction and effects in *R. baltica*. A total of 48 culture vials were placed on the plankton wheel. Nominal microbead concentrations were 75, 750 and 7500 MPs/mL for respectively the low, median, and high concentration. All samples from control 2 were used to investigate behaviour of MPs, particularly sedimentation. Filtrated seawater was included as control 3 to assess the quality of the filtrated seawater used as part of the growth medium.

Table 3. Overview of experimental setup with Rhodomonas baltica exposed to PS microbeads

Treatment	Algae + MPs			Control1 (algae alone)	Control 2 (MPs alone)			Control 3 (Sea water)
	Low	Medium	High		Low	Medium	High	
Nominal concentrations (MPs/mL)	75	750	7500	0	75	750	7500	0
Number of samples	6	6	6	6	3	3	3	3
Termination frequency	N/A	N/A	N/A	N/A	2	2	2	2
Total	6	6	6	6	6	6	6	6

Image 1. Some of the instruments used during experimentation. The experimental system is made up of a), the plankton wheel, b) heating/cooling bath to maintain the temperature of the plankton wheel's water bath constant, c) batch culture, d) Beckman coulter counter. e) the 40mL culture vials, f) square spectrophotometer cuvette and h) the Cary Eclipse fluorescence spectrophotometer.

2.3.3 Microalgae and microbead quantification

Characterization of the microalgae and microbead samples were performed by coulter counter analysis (plate 1d). The instrument employs the coulter principle to measure particle volume as a direct measurement of a particle's physical properties. Particles in an electrical field (in an electrolyte) passing an aperture or pore between two electrodes, causes an increase in the electric resistance correlated to the volume of the particle(s) The analyser then translates the resistance into volume or sphere diameter size distributions in one measurement, within a ~400

nm to 1600 µm window, and the results may be displayed as particle size, volume, mass, or number. This is referred to as the principle of Electrical Sensing Zone Method and is a globally accepted standard method for particle sizing (van der Plaats et al., 1983).

Five mL of microalgae sample was drawn from the culture and made up to 100 mL (20x dilution) using filtrated seawater (0.22 µm). All readings were made in a 200 mL standard coulter beaker at minimum stirring speed (6 rpm) to ensure uniform and continuous distribution of particles in the solution. The sample volume drawn by the coulter per suction and aperture diameter, were set to 1000 µL and 100 µm respectively.

2.3.4 Fluorescence

Fluorescence was measured as a proxy for chlorophyll production using a Cary Eclipse fluorescence spectrophotometer from Varian, Inc. PA, California, USA (image 1 h), which includes corrected spectra, excitation and emission filters and extended range PMT detectors. Using fluorescence mode on advanced reads, the excitation and emission wavelengths were set at 462 nm and 685 nm, respectively with the excitation and emission slit sizes set at 5 nm and 10 nm, respectively, based on own pre-test results and results of previous studies. 3.5 mL of undiluted algae samples were analysed in triplicate by using standard cuvette, PS grade polystyrene (volume 4.5 mL, 10 mm path length and 45 mm high) supplied by VWR International AS, Haavard Martinsens vei, OSLO, Norway. Using culture medium as blank, the spectrophotometer was automatically zeroed each time before the measurement of a new sample.

2.3.5 pH

The pH value was determined for each sample at every sampling time point using a SensION™+ PH31 meter from Hach Willstätterstr, Dortmund, Germany. 5 ml subsample was obtained from each culturing glass vial and used for pH analysis before counting of the particles with the coulter counter. Before measurements were made, the pH meter was submitted to a three-point calibration (pH 4, pH 7 and pH 10) using the respective technical solutions.

2.4 Microscopy

All microscopic analysis and photoimaging of the microalgae and microplastics were done using a Nikon eclipse 90i. Bright field mode was chosen for PS particles while fluorescence mode (NIKON B2A filter, Leica EL6000 lamp, with OSRAM bulb; HXP R 120W/45C VIS) was employed to observe the GF and *R. baltica*, (due to their red fluorescence chlorophyll colour). Before sampling for microscopy, samples were left to settle for approximately 20 min

to allow particles to sediment to the bottom of container. A glass Pasteur pipette was used to draw a small amount of sample from the bottom of the vial. The drawn sample was put on a microscope slide, covered by glass cover, and then examined using the microscope.

2.5 Sedimentations studies and loss of microplastics

2.5.1 Sedimentations

Sedimentation studies were done with the microplastics control 2 samples to determine whether the quantities of microplastics are sticking on the walls of the 40 mL culture bottles. Three bottles were terminated from the plankton wheel at each time interval 0, 120 and 264 has previously described in (sub-section 2.2.2; table 3), and all content of each bottle was gently poured off. Bottles were then refilled with filtrated seawater up to 40 mL, followed by a vigorous shaking and finally the bottle content was analysed for microplastics using a coulter counter.

2.5.2 Loss of microplastics

This done to investigate whether samplings and other analytical tools (excluding coultercounter) are responsible for the controversial reduction in microplastics observed in the study with *Rhodomonas baltica*. The pH meter probe and sampling/refiling beaker were separately washed after every sampling and measurement of pH in the highest concentration (PS7500/mL exposed samples), using filtrated sea water. The volume of water was made up to 40 mL and analysed on the coulter counter.

2.6 Statistical analysis

All calculations and descriptive statistics including means and standard deviations were performed by Microsoft Excel 2016. Further data analysis, graphing and significance test were done using SigmaPlot, Systat Software Inc, version 13. One-way ANOVA was performed to test the significant differences among treatment groups compared to the control. In case of unequal variances among treatment groups, *one way ANOVA* on Ranks was performed. In addition, Bonferroni-test was performed for multiple comparisons versus the controls.

3 RESULTS

In the present section results are provided from both the method development tests (pre-tests) and the main experiments. Method development tests (sub-section 3.1) were required to determine an adequate method to monitor particle engulfment by *O. marina* using the coulter counter. In addition, background growth capacity of *R. baltica* in 40 ml glass vials needed to be tested before the effects of MPs could be assessed. Similarly, optimal fluorescence spectrophotometer slit sizes and excitation and emission wavelengths had to be determined to measure fluorescence in *R. baltica* samples as a proxy of photosynthetic activity. Results of the main experiments are presented in sub-sections 3.2 - 3.5.

3.1 Method development (pre-tests)

3.1.1 Monitoring particle engulfment by O. marina using a coulter counter with manual mixing

A reduction in *R. baltica* concentration was observed with a concurrent increase in the volume of *O. marina*, suggesting that (1) *R. baltica* were taken up by *O. marina*; (2) there was insignificant increase in volume of *O. marina* but significant increase might be observed at longer time of exposure (example 60 min) (3) a coulter counter can be used to assess particle engulfment by *O. marina*; (4) manual mixing of a combined suspension of *O. marina* and *R. baltica* appears to be adequate (Fig.1). However, large variation in concentration of *O. marina* was observed with manual mixing (Fig.2).

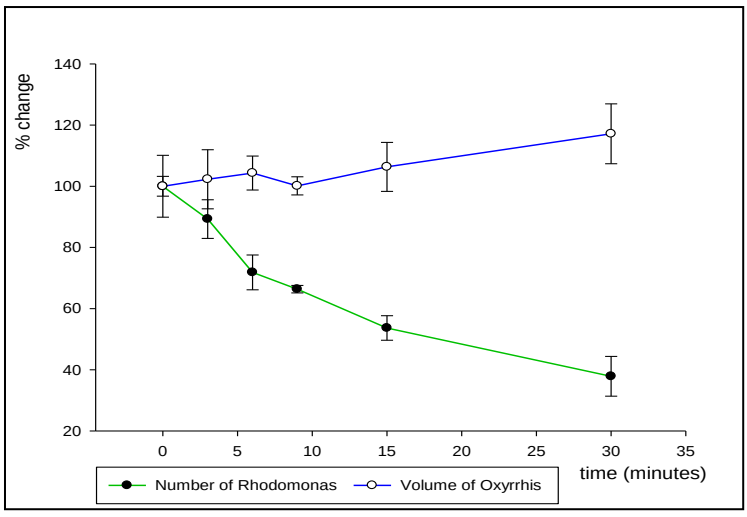

Figure 1. Percentage change in Rhodomonas baltica cell concentration compared to total Oxyrrhis marina volume with manual mixing (mean±SD; n = 4).

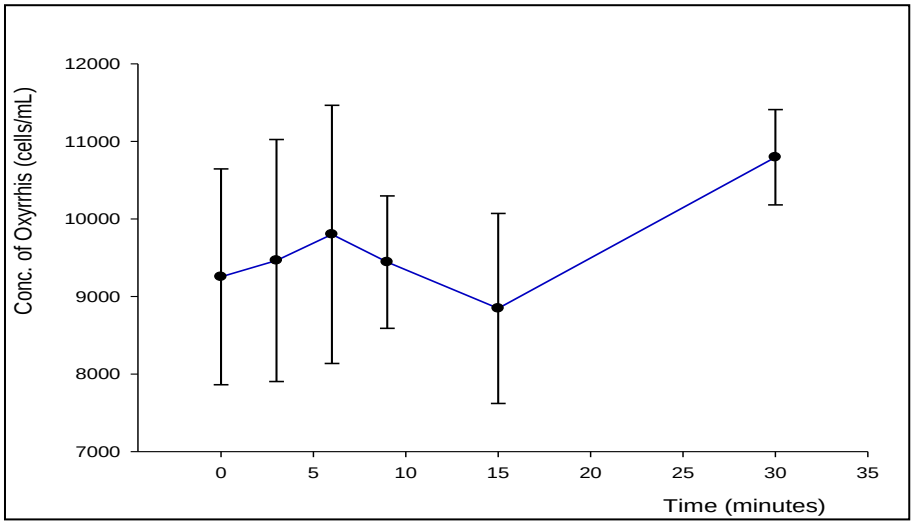

Figure 2. Changes of concentration of Oxyrrhis marina cells (cell/mL) over time after manual mixing (mean±SD; n=4).

3.1.2 Monitoring particle engulfment by O. marina using a coulter counter with mechanical mixing

The mechanical mixing at high speed of 8 rpm, apparently caused a total arrestment of *R. baltica* engulfment by *O. marina*, as the concentration *R. baltica* remained constant throughout the experiment (Fig.3). The sudden drop in *O. marina* cell volume after 10 min stirring at 8 rpm could be due to mechanical disruption of *O. marina* cells.

With mechanical mixing at low speed of 6 rpm, the results were closely related to that from manual mixing except (i) number of *R. baltica* cells seem to decrease slightly less than when we used manual mixing and (ii) Concentration of *O. marina* did not go up and down as compared to manual mixing (Fig. 4).

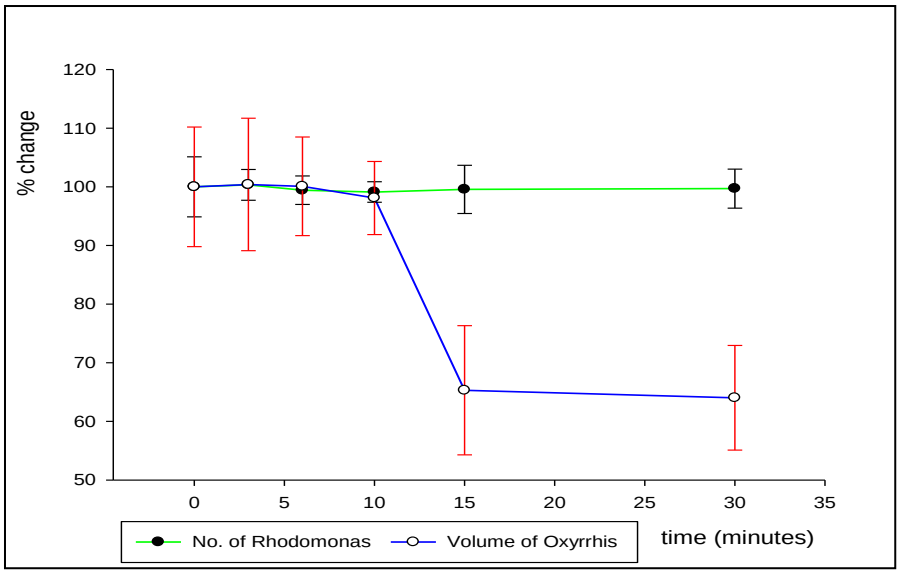

Figure 3. Percentage change of Rhodomonas baltica concentration (cells/mL) compared to total volume of Oxyrrhis marina (µm^3) with mechanical mixing speed of 8 rpm (mean±SD; n=4).

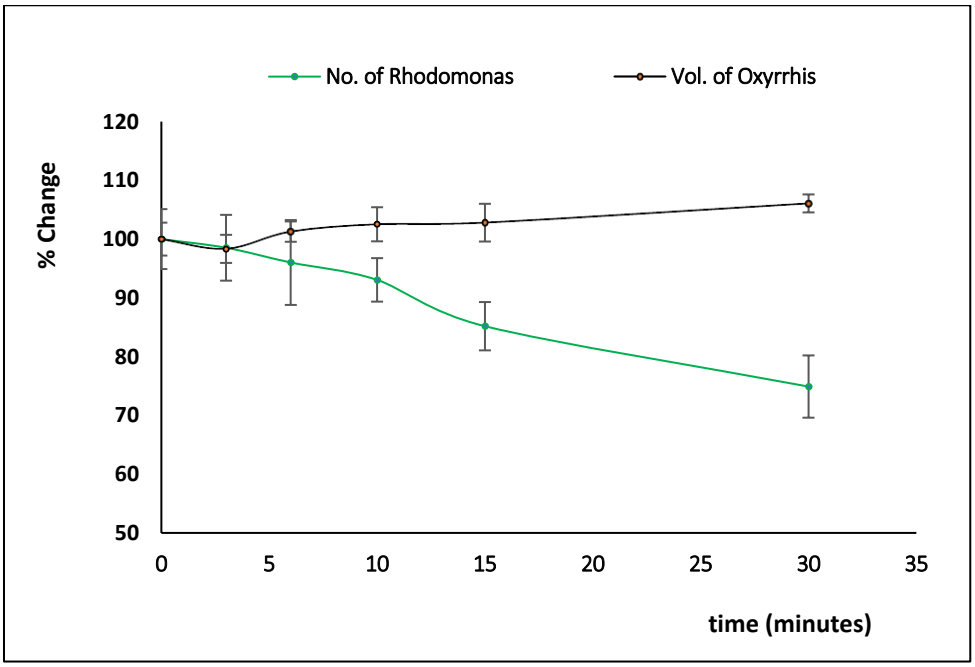

Figure 4. Percentage change in concentration of Rhodomonas baltica (cells/mL) compared to total volume of Oxyrrhis marina (µm^3) mechanical mixing speed of 6 rpm (mean±SD; n=4).

Due to high fluctuation in concentration of *O. marina* observed with manual mixing and the total arrest of *R. baltica* engulfment with the mechanical mixing at higher speed (8 rpm), we chose to work with mechanical mixing (low speed = 6 rpm) which has better and less variations than manual mixing. Mechanical mixing would also provide more uniform mixing of microplastics and reduce human error upon mixing. The 200 mL glass beaker used with mechanical mixing was also a better choice than the use of a 20 mL plastic beaker used in manual mixing. This is because the 20 mL coulter beakers are made up of plastics which may more easily allow microplastics and microalgae attachment to the walls as compared to glass material.

3.1.3 Determining the right excitation, and emission wavelengths for fluorescence measurements

Mean excitation and emission wavelength were found as 462 nm and 684nm respectively (fig5)

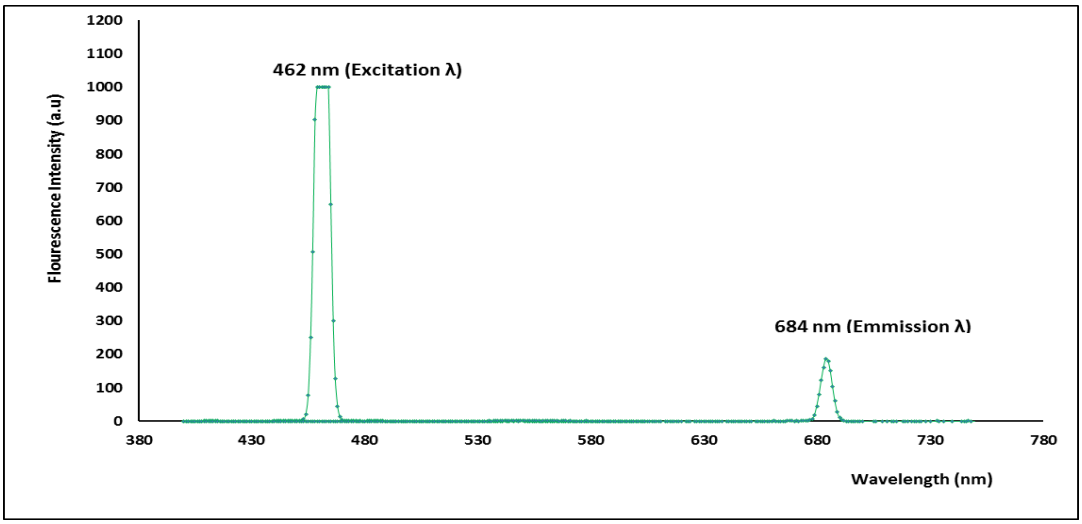

Figure 5. Mean excitation and emission wavelengths found after fluorescence scanning of different samples of R. baltica (n=6).

3.1.4 Testing growth of R. baltica in 40 mL culture vials

Due to limited sample size, termination (RT) vials series was discontinued after 96 h, while the sub-sampling group was sampled up to 216 h (fig. 6). Therefore, when the growth curves in the RT and SS groups were compared up to 9 h (Fig.6b), no differences were observed. Both fluorescence and pH values measured in the SS group were strongly correlated to the algae density (R^2 = 0.94 and 0.97 respectively) (fig.7).

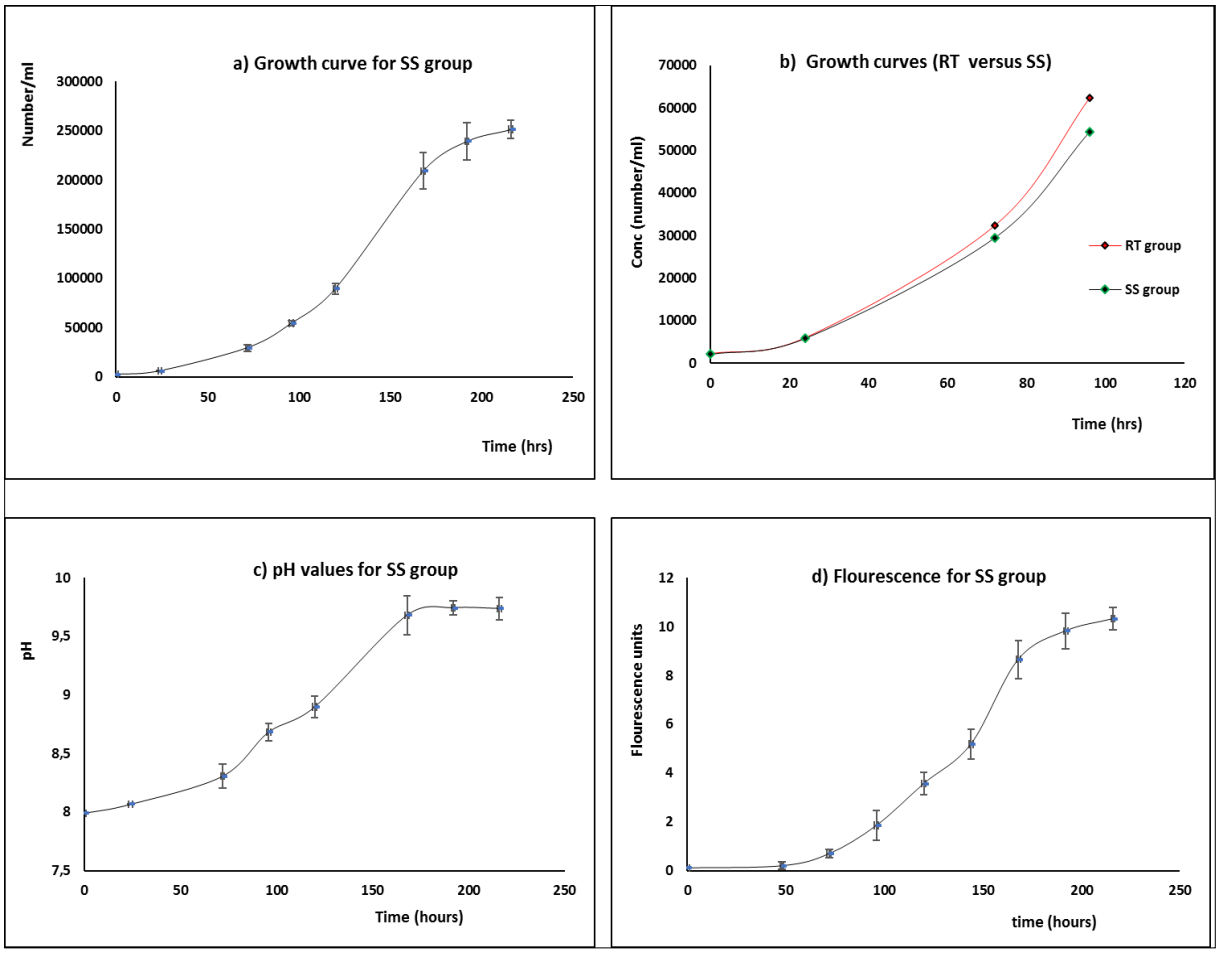

Figure 6. Growth curves of Rhodomonas baltica in 40 mL culture vials. a) Growth curve of R. baltica undergone sub-sampling (SS); b) Comparing growth curves from the subsampling group and the termination group (RT); C) pH curve for the sub-sampling group; and d) fluorescence curve from sub-sampling group. Symbols represent means, error bars standard deviation (n=6), except for RT group (n=3).

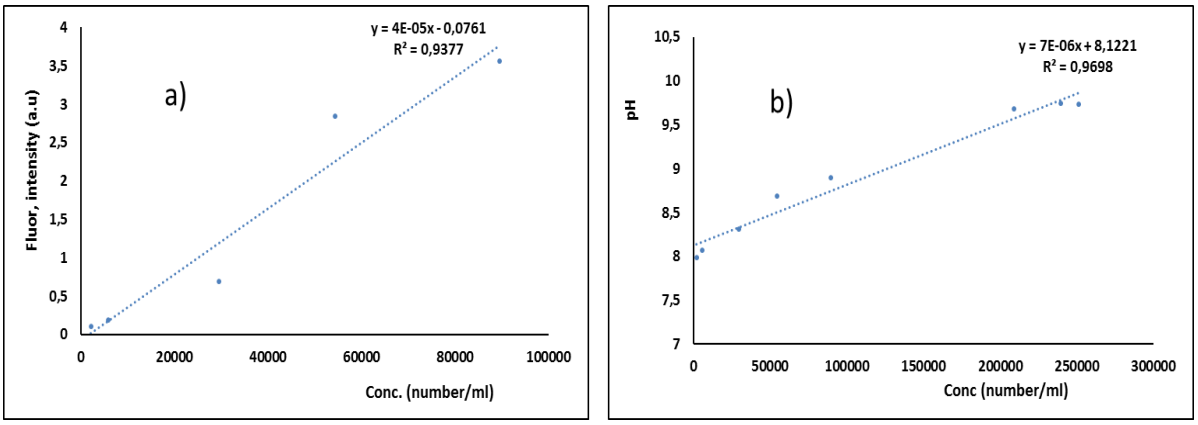

Figure 7. Regression analysis between a) the concentration of R. baltica (cells/mL) and fluorescence units b) concentration of R. baltica (cells/mL) and pH values, (n=6).

3.2 Uptake of microplastics by *Oxyrrhis marina*

3.2.1 Concentrations of R. baltica (cells/mL) and microplastics (PS/mL)

Compared to the control samples, there was a significantly less reduction in *R. baltica* number in the samples exposed to PS7500, followed by PS75, GF750 (ANOVA test, $p < 0.05$). The exception was the reduction in *R. baltica* exposed to GF75, which did not show a significant difference compared to the control at any time point (fig. 8a; table 4). A significant reduction in concentration of the microplastics was observed in all treatment groups after 40 minutes (ANOVA test, $p < 0.05$). However, high reduction was observed with exposure to PS 7500, followed by PS75, GF 7500 and finally GF75 (fig 8b; table 5).

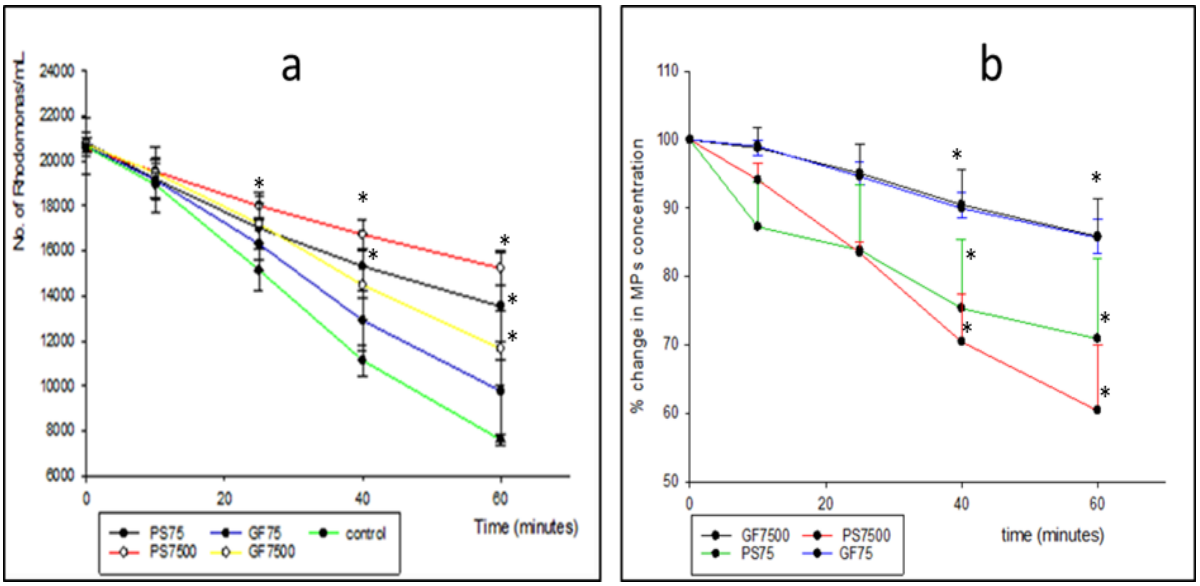

*Figure 8. Mean concentration ± SD of particles in the exposure system (a) R. baltica (cells/mL) and (b) microplastics (PS/mL) in different treatment groups (n=6). * indicates significant decrease in concentration of the particles compared to the initial values.*

Table 4. Significance tests (one-way ANOVA) between mean concentrations of R. baltica of the exposed and control. Values with significant difference from initial concentrations are asterisk marked.

Time	Comparison	PS7500	PS75	GF7500	GF75
25	Diff of Means	2853.833	2057.28	1864.333	1155.667
	t	4.794	3.456	3.132	1.941
	P-value	<0.001*	0.008*	0.018*	0.254
40	Diff of Means	5602	4195.833	3356.107	1778.5
	t	8.126	6.086	4.868	2.58
	P-value	<0.001*	<0.001*	<0.001*	0.065
60	Diff of Means	7629	5925.833	4057.357	2164.667
	t	7.876	6.118	4.189	2.235
	P-value	<0.001*	<0.001*	0.001*	0.138

Table 5. Concentration differences of microplastics at different time points with the exposure on *O. marina*

Treatment grp	Time (min.)	Diff of means	t	p-value	P<0.05
GF75	0 vs. 60	14,310	5,692	<0,001	Yes
	0 vs. 40	10,023	3,987	0,005	Yes
GF7500	0 vs. 60	14,145	14,993	<0,001	Yes
	0 vs. 40	9,512	10,083	<0,001	Yes
	0 vs. 25	4,903	5,197	<0,001	Yes
PS75	0 vs. 60	29,105	5,850	<0,001	Yes
	0 vs. 40	24,664	4,957	<0,001	Yes
	0 vs. 25	16,132	3,243	0,033	Yes
PS 7500	0 vs. 60	39,614	12,614	<0,001	Yes
	0 vs. 40	29,568	9,415	<0,001	Yes
	0 vs. 25	16,544	5,268	<0,001	Yes

3.2.2 Volume of O. marina (µm^3)

There was difference in initial volumes of *O. marina* among treatment groups (fig. 9), obviously due to initial culture status as each sample was separately drawn from the main culture and at different time. Significant increase in volume of single cells between initial (time 0) and final (time 60) volumes of *O. marina* was observed in all treatment groups (ANOVA test, $p < 0.001$), except for those exposed to 75 PS/mL concentration (ANOVA test, $p = 0.846$), table 6.

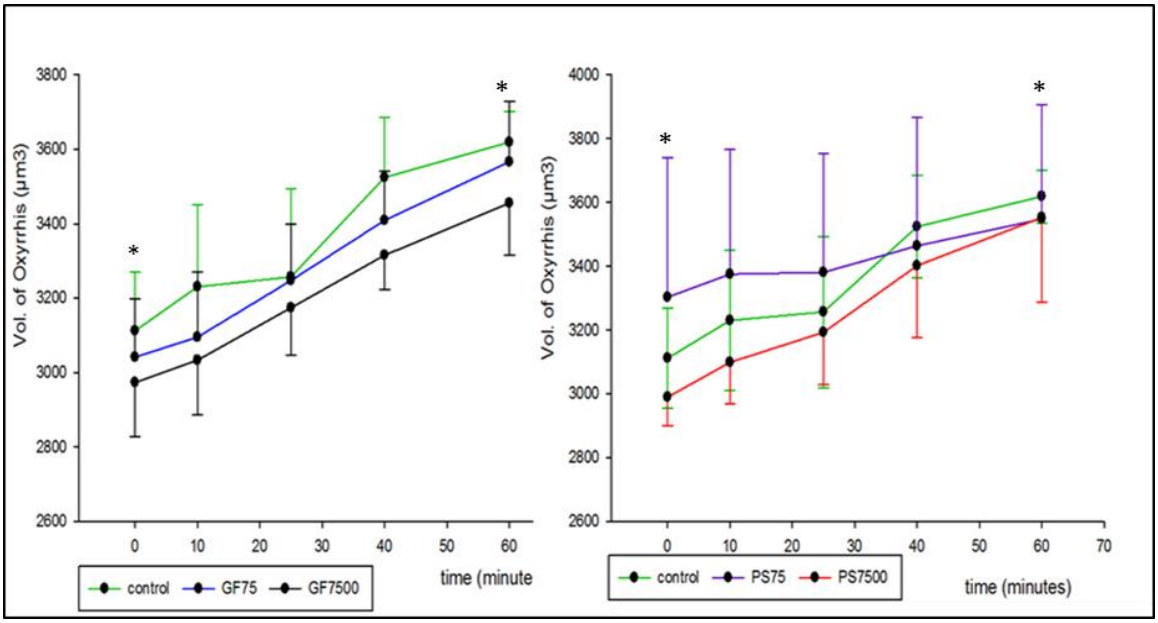

*Figure 9. Change in individual cell volume (µm^3) of Oxyrrhis marina in the four treatment groups compared to control over 60 minutes (mean±SD; n=6). * indicates significant difference in volume between time points (initial and final).*

Table 6. Differences between volumes (μm^3) of single cell of *Oxyrrhis* at different time points. Only comparisons showing significant differences are depicted except for PS75, which did not show any significance.

		Diff of means	t	p-value	P<0.05
control	60 vs. 0	507,000	4,878	<0,001	Yes
	60 vs. 10	507,000	4,878	<0,001	Yes
	60 vs. 25	389,000	3,743	0,010	Yes
	60 vs. 40	362,000	3,483	0,018	Yes
	40 vs. 0	412,000	3,964	0,005	Yes
GF 75	60 vs. 0	525,000	5,842	<0,001	Yes
	60 vs. 10	471,000	5,241	<0,001	Yes
	40 vs. 0	368,000	4,095	0,004	Yes
	40 vs. 10	314,000	3,494	0,018	Yes
GF7500	60 vs. 0	482,000	6,325	<0,001	Yes
	60 vs. 10	422,000	5,537	<0,001	Yes
	60 vs. 25	281,000	3,687	0,011	Yes
	40 vs. 0	342,000	4,488	0,001	Yes
	40 vs. 10	282,000	3,700	0,011	Yes
PS75	There is no any significance			0,846	NO
PS 7500	60 vs. 0	563,000	5,232	<0,001	Yes
	60 vs. 10	454,000	4,219	0,003	Yes
	60 vs. 25	360,000	3,345	0,026	Yes
	40 vs. 0	412,000	3,829	0,008	Yes

3.2.3 Concentrations (cells/mL) of O. marina

No significant differences were found between initial and final concentrations of *O. marina* in any treatment groups (ANOVA test, $p > 0.05$), table 7. Apparent differences among initial concentrations of *O. marina* in the three groups exposed to GF were observed. However, there was no statistical significance difference found between them (ANOVA test, $p > 0.05$).

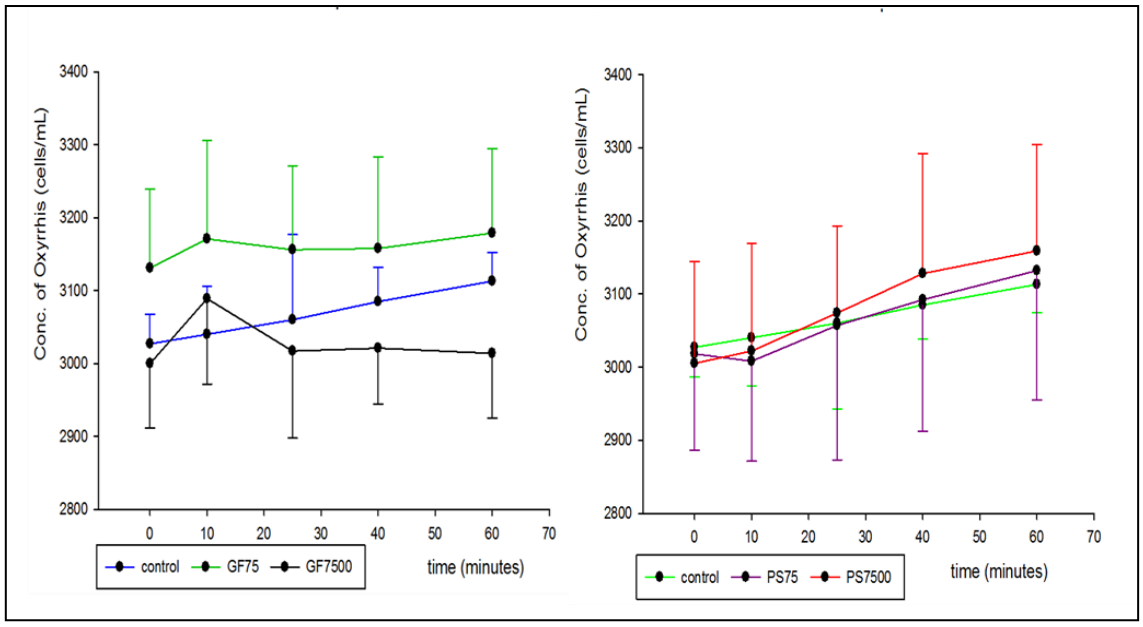

Figure 10. Concentration of O. marina (cells/mL) in the four treatment groups compared to control over 60 minutes (mean± SD; n=6).

Table 7. Significance levels attained to differences between initial (0 minute) and final (60 minutes) concentrations (cells/mL) of *O. marina*

	p-value	P<0.05
Control	0,221	no
GF 75	0,966	no
GF7500	0,574	no
PS75	0,667	no
PS 7500	0,305	no

3.2.4 Microscopy study on the ingestion of microplastics by O. marina

To confirm that both *R. baltica* and microbeads are engulfed by *O. marina* we examined all the exposed samples using both fluorescence and bright field microscope. Our observations depicted by image 2 and 3, showed that *O. marina* has engulfed microplastics at different capacities (1 to 4 PS particles, or several GF particles). However, *O. marina* slightly increased as the number of engulfed microplastics increased, although their sizes did not differ much.

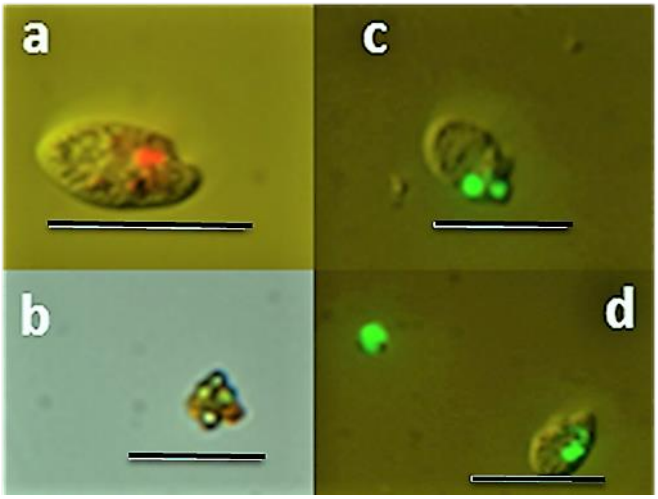

Image 2. Localisation of green fluorescent microplastics (GF particles) inside and outside O. marina cells using fluorescence microscopy. (a) *O. marina cells with only food (Rhodomonas baltica); (b) GF particles complexing with brown material assumed to be a bolus of excretes from O. marina; (c) O. marina cell with several GF particles inside; and (d) some free GF particles suspended in the culture medium. All scale bars are 20µm long.*

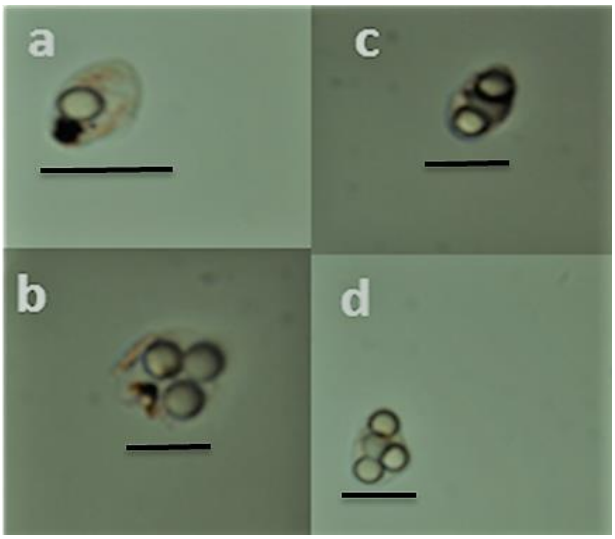

Image 3. Localisation of polystyrene (PS) microplastics inside O. marina cells using light microscopy. (a) O. marina cells with only one PS particle; (b) O. marina cells with three PS particles; it is not sure whether the cell has ruptured but it is likely; (c) O. marina cell with two PS particles (d) O. marina cells with four PS particles inside and still intact; All scale bars are 20µm long.

3.3 Interactions and effects of microplastics in *Rhodomonas baltica*

3.3.1 Algae Concentration (number of cells/mL)

All algae cultures from the four treatment groups (control, low, median and high) exhibited normal growth curves (figure 11). The median concentration (750PS/mL) showed highest growth rate with significances to the control at 216, 240 and 264-time point. Samples exposed to high concentration (PS7500/mL) exhibited the lowest growth rate with significantly different from to the control group from 120hours to 264hours (ANOVA test, $p < 0.05$). However, the growth of samples from the lowest concentration of 75PS/mL (black curve), did not show any significant difference to the control (ANOVA test, $p = 0.086$).

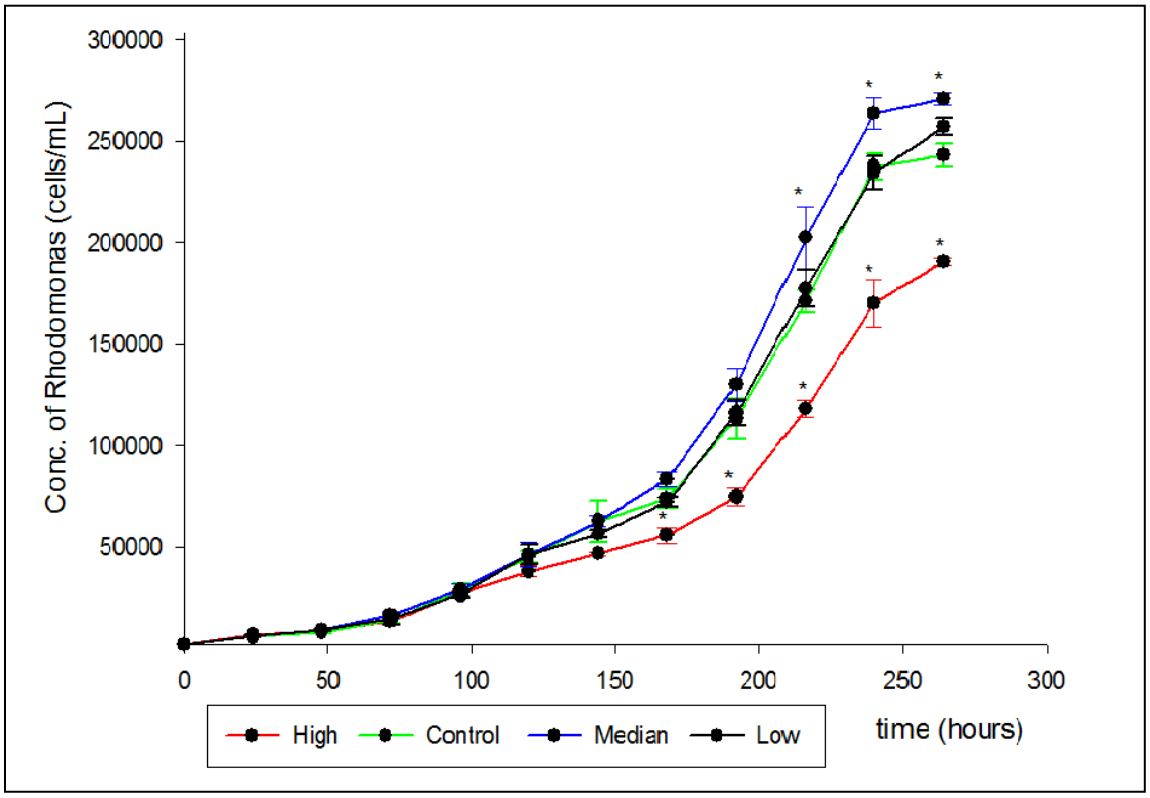

Figure 11. Concentration of Rhodomonas (cells per mL) in the four treatment groups compared to control (Mean± SD; n=6). Asterisk (*) indicate significant difference from the control.

3.3.2 Concentration of microplastics – (microplastics/mL)

Concentrations of PS microsphere were monitored each time of sampling and an unexpected decrease from the samples exposed to both the highest and medium concentration was observed between 0 to 72-hour time intervals (fig. 12). Recovery of the microplastic concentration is

gradually restored to its initial concentration after 264 hours. This phenomenon was apparently attributed by loss and sedimentation of microplastics as studied in section 3.4 and 3.5.

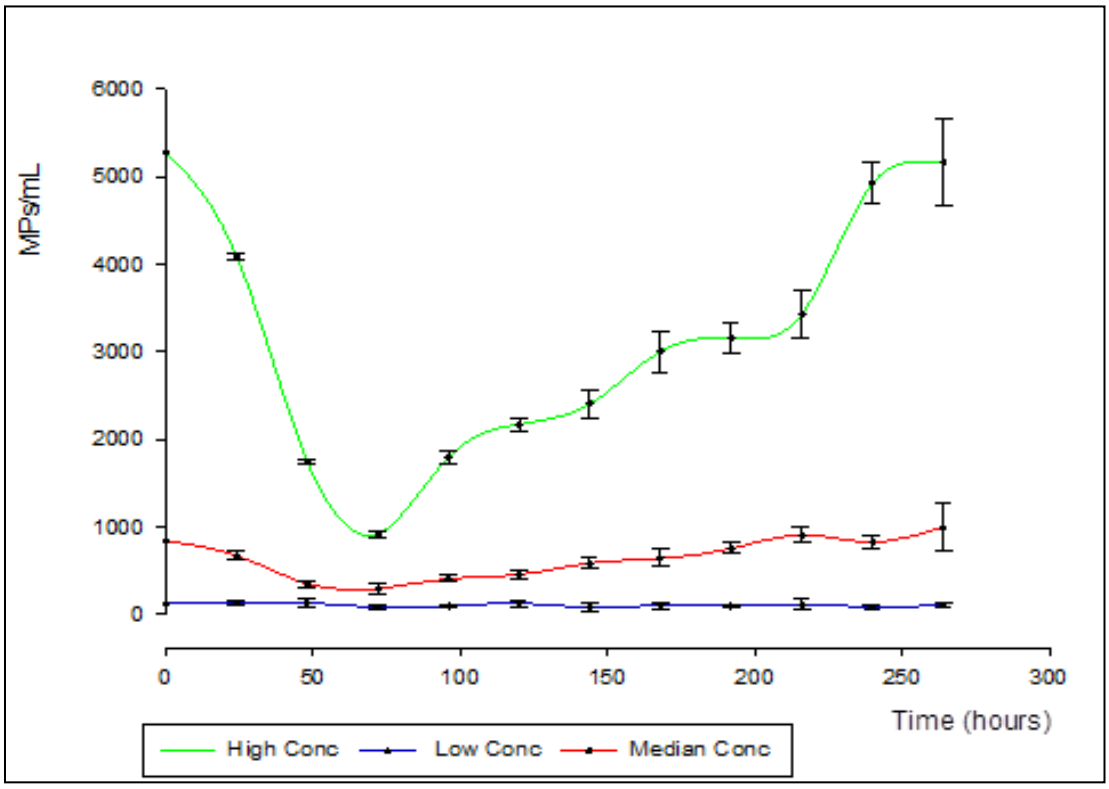

Figure 12. Concentration PS microsphere (microplastics per mL) in the three treatment groups (Mean± SD; n=6).

3.3.3 pH measurements in the culture medium

The pH values from each treatment group seem to increase as time increases (fig 13). Samples exposed to high concentration (7500 PS/mL) of microplastics (red line), is showing significantly lower pH values compared to other treatments and control (ANOVA test, $p < 0.005$). While the samples exposed to median concentration is showing significanly higher pH values only 216 to 264 hours (ANOVA test, $p < 0.005$), the lowest concentration did not have any significant difference in pH values compared to the control (ANOVA test $p = 0.74$)

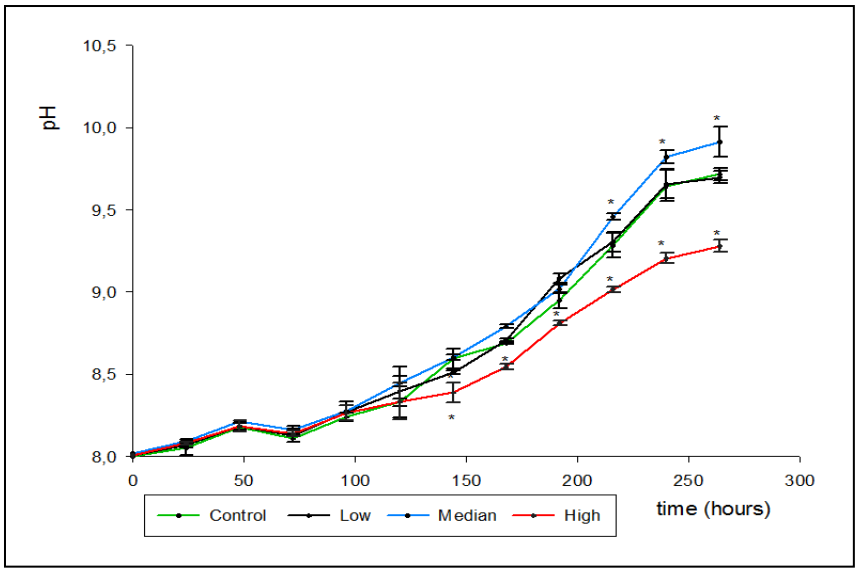

Figure 13. pH values measured in the treatment groups compared to control (Mean ± SD; n=6).

3.3.4 Measurement of chlorophyll production (fluorescence)

Fluorescence units were observed to increase relative to time, algae growth, and pH (fig.14). Samples exposed to higher concentration of microplastic (7500PS/mL) were showing significance lower values compared to the control group at 144, 216- 264 hours' time interval (ANOVA test, $p < 0.05$). However, after the maximum pH and stationery phase was attained (240 hrs), all samples from the three treatment groups were exhibiting significantly lower fluorescent values compared to the control.

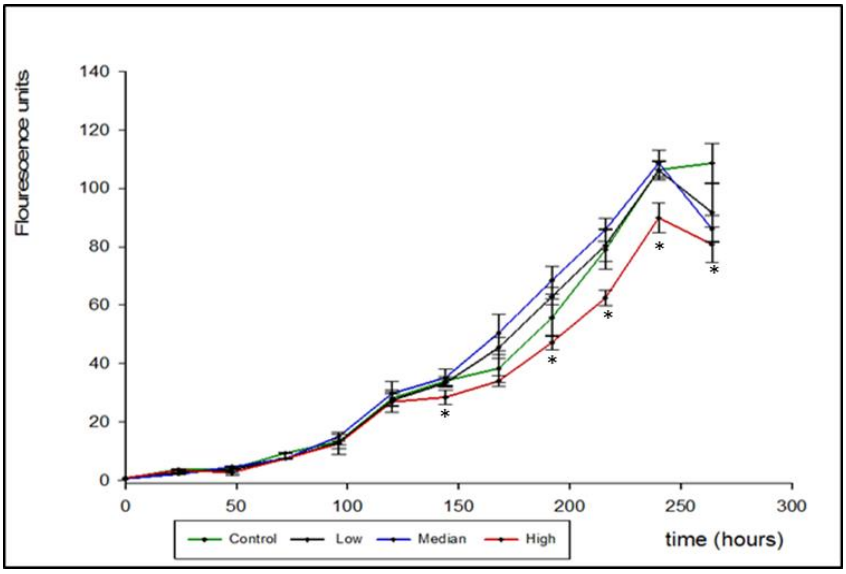

Figure 14. Fluorescence values (a.u) measured in the R. baltica cultures exposed to the three concentration of PS microspheres (low, median, high) and control (Mean ± SD; n=6).

Microscopic observations on effects of microplastics on R. baltica

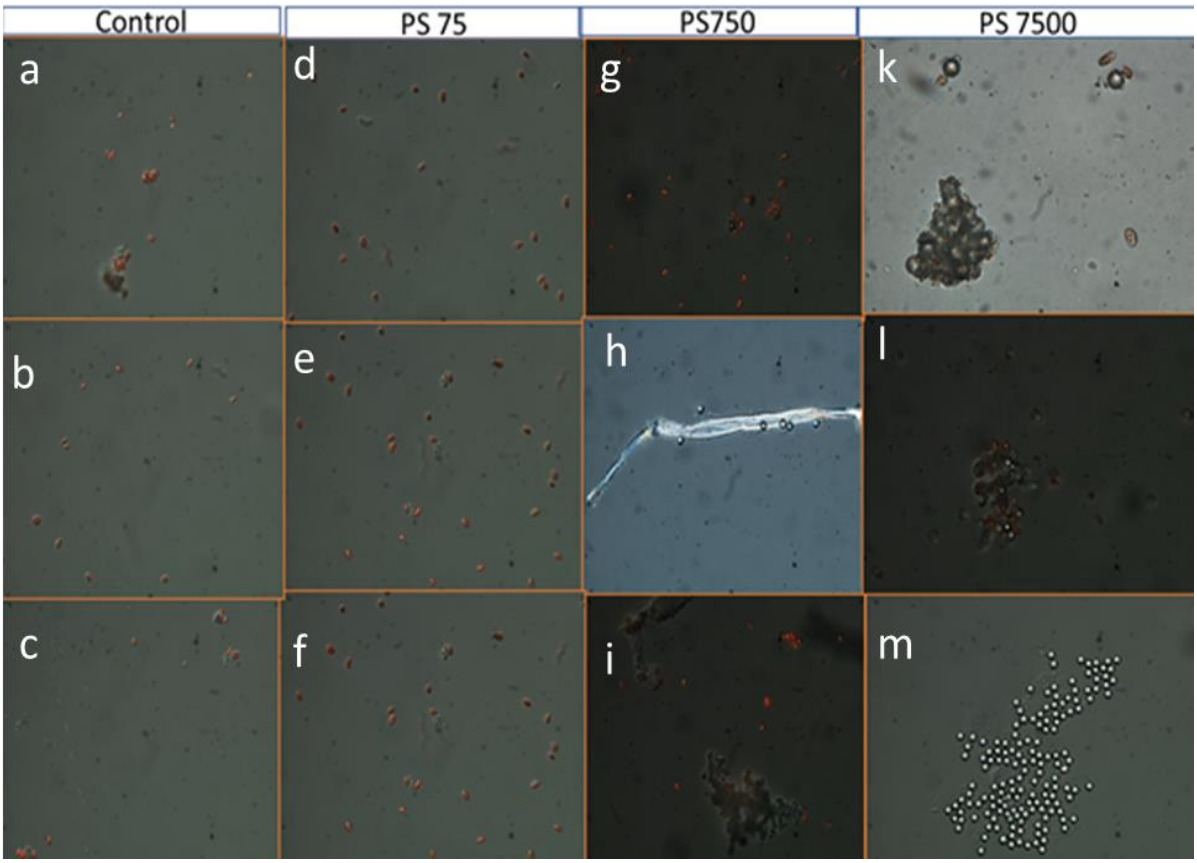

Image 4. Microscopic observations on interaction between R. baltica polystyrene microspheres after 264 hours. (abc) Control samples; Algae cells are normally distributed with very few artefacts like paired/agglutinated algae cells. (def) Samples exposed to 75PS/mL do not differ significantly from the control. No any algae-to-PS-agglutination can be observed in these samples. (ghi) Algae samples exposed to 750 PS/mL algae; agglutinations of up to 3 or 4 cells are observed. Some PS particles attached to fibres are also seen (h), with larger artefacts assumed to be biofilms (i). (klm) Algae samples exposed to 7500PS/mL are showing large mass of clumped materials. Microplastics are seen agglutinated with some remains from the R. baltica but not live or whole algae cell (k), some algae cells are observed trapped inside this complex material (l). Mass of PS particles can be seen attached together by a very thin film (m).

3.4 *Sedimentations studies and loss of microplastics*

Due to limit of quantification of the microplastic by coulter counter and the high dilution factor, the number of microplastics quantified from the 75PS/mL sample apparently remain constant at 5 PS/mL each time. Mean concentration of microplastics sediment on walls of vials seems to increase with time (fig 15).

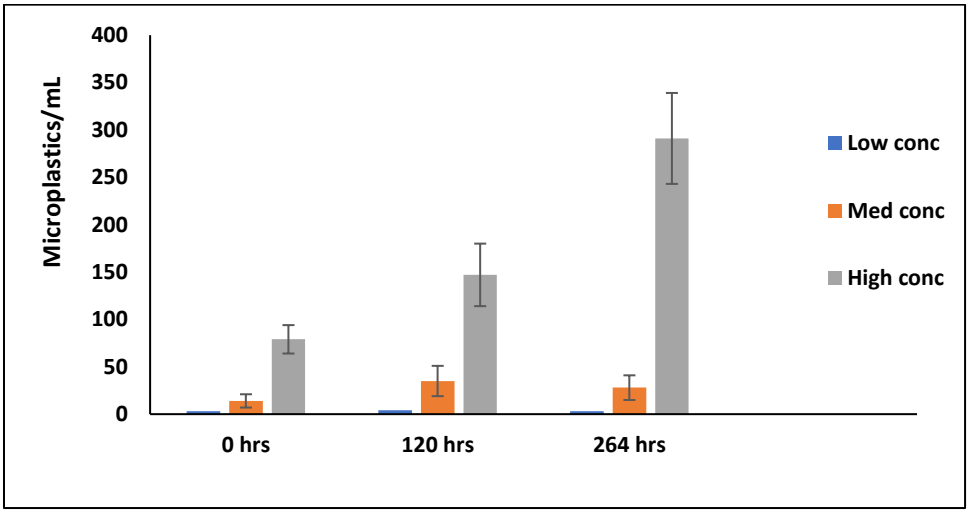

Figure 15. Mean concentration ± SD of microplastics sediment inside 40 mL culture bottles. (n=3).

The loss through refilling was 267PS/mL, while that from dipping the pH probe was 40 PS/mL respectively (fig 16), making total of 40. 9% microplastic lost per millimetre from each bottle of the PS7500 exposed samples.

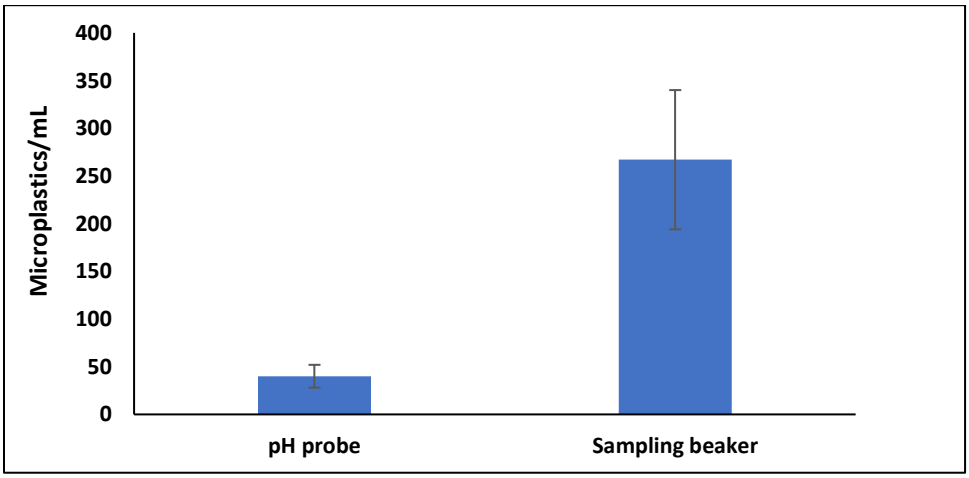

Figure 16. Concentration of microplastic lost through sampling beaker and pH probe (Mean ± SD; n=3).

4 DISCUSSION

4.1 Uptake of microplastics by *Oxyrrhis marina*

4.1.1 Concentrations and volume of microalgae and microplastics

Among the measured parameters, concentration of *R. baltica* and microplastics, and the cell size/volume of *O. marina* were identified in the initial pilot test to be the best markers to monitor the uptake of microplastics by *O. marina*. The strong correlation ($R^2 = 0.96$) between change in volume of *O. marina* and concentration of particles is because *O. marina* increases in size/volume immediately after having engulfed particles. Therefore, the decrease in particles' concentrations in the medium is a strong indication of ingestion by *O. marina*. This was characterised by the observed significant increase in volume ($p < 0.001$) of *O. marina* after 60 minutes, for all treatments except the samples exposed to 75 PS/mL concentration (table 6).

In the 60-minute experiment, concentrations of both *R. baltica* and microplastics were observed to be decreasing with increase in time (fig. 8), assumed to be the result of grazing by *O. marina*. However, the percentage change in particle concentration varied between treatments. The concentration of *R. baltica* varied seemingly in a concentration-dependent and bead-type-dependent manner, where the least reduction was associated with PS high concentration exposure, followed by the PS low concentration, the GF high concentration, the GF low concentration, and then control (Figure 8 a). Higher decrease in concentration of *R. baltica* was thus observed in samples exposed to GF microbeads, which showed less decrease in concentration compared to the PS concentration over the period (figure 8 b, appendix 4). The measured concentrations of the green fluorescence GF microplastics were much higher than the nominal concentrations, probably largely an artefact caused by additional similar-sized particles originating from both *R.baltica* and *O.marina* stock solutions. Even though the initial concentrations of green fluorescence particles were measured to be higher than expected (appendix 2), there was a statistically significant percentage reduction over the exposure time both for the high and low concentration media ($p < 0.001$). Therefore, a considerable proportion of these particles were anyway lost from the system (culture solution).

Quantitatively, in the control group reduction in *R. baltica* density amounted to 67%, while for the other treatment groups the reduction was 52% (GF75), 44% (GF7500), 35% (PS75) and 25.8% (PS7500). However, the volume of *O. marina* did not increase as much in the control, compared to the PS7500 (Appendix 3). An explanation may be that following ingestion by *O.*

marina, the *R. baltica* cells are easily digested and the remains excreted as waste. On the contrary PS particles engulfed by *O. marina* remain unchanged and may accumulate inside the dinoflagellate cells. This may be associated with the observed higher increase in volume in *O. marina* exposed to the high-concentrated PS particle solution. Accordingly, the highest reduction in microplastic concentration was observed in the highest concentration PS7500 (7500 particles/min), followed by PS75, GF7500 and finally GF75. Although the percentage consumption in GF7500 and GF75 are somehow similar, numerically more microplastic has been consumed in GF7500 than in GF75. Hence, reduction in *R. baltica* density was generally inversely proportional to the concentration of the MP concentration (35% PS75 versus 25% PS7500, and 52% GF75 versus 43.5% GF7500). The number of microplastics engulfed by *O. marina* decreased with decrease in concentration for both the GF and PS particles. Numerically, after 60 minutes of exposure 2818, 58, 982 and 10 MPs/mL were removed from the PS7500, PS75, GF7500, and GF75 solutions, respectively.

Examination by microscopy of the exposed dinoflagellate cells supports the considerations done above. *O. marina* exposed to both the GF75 and GF7500 solutions, as well as the PS7500 solution, had visible microplastics inside. (images 1 and 2). On the contrary, no *O. marina* cells from the 75PS/mL exposed group were observed having microplastic inside. Possibly there was low or no ingestion of microplastics from this low concentration (75PS/mL). Or, due to the transparent colour of the PS, the chance for recognizing single *O. marina* cells with microplastics from this low concentration might have been very low. The uptake of PS microplastics from the PS7500 exposed samples, ranged from one to four PS particles per *O. marina* cell (image 2). Because of the space required by the PS particles, cells with 3 or 4 PS particles were visibly enlarged and completely immobile. The GF particles engulfed by *O. marina* were less visible and therefore more difficult to count, but an approximate number could be estimated facilitated by the fluorescence light emitted by these microplastics. However, most *O. marina* cells from the GF exposed samples were actively swimming around and did not show any sign of reduced mobility, possibly due to the difference in size between the PS and GF microbeads.

The non-significant increase in number (growth) of *O. marina* observed throughout the exposure period, is related to limited time (60 minutes) as compared to the time required for growth (division cycle) of this heterotrophic dinoflagellate. The fastest growth rate for *O.*

marina reported in literature was 1.0/day when feeding the dinoflagellates *Amphidinium carterae* and lower than 1.0/day when fed two other algae species (Montagnes et al., 2010). So, after 60 minutes we do not expect a measurable increase in cell number of *O. marina* fed *R. baltica*.

4.1.2 *Implications of the uptake of microplastics by O. marina*

The observed effects of ingestion of plastic particles by *O. marina* can be discussed under three major categories;

a. Effects due to selective feeding by *O. marina* (food displacement)

The low effect by GF on *O. marina* motility compared to PS particles shows the difference in feeding preference among microplastic particles (selective feeding by *Oxyrrhis*). In their studies, both Hammer et al. (1999) and Wootton et al. (2007) showed that *O. marina* discriminates food based on biochemical composition, size and physical properties. Hammer et al. (1999) found that *O. marina* feeds more on relative large particles (4µm) than smaller ones (1µm). Therefore, the efficiency of micro-particle ingestion by *O. marina* observed in the present study can be related to the size, density and polymer type. The size of polystyrene microplastics (10.1±0.71 µm) used in this experiment was twice as big as the green florescent (GF) particles (1-5µm). Therefore, we are predicting that due to food selection and preferences, *O. marina* may select microbeads rather than *R. baltica* resulting in food displacement effects. Nevertheless, factors such as biofouling may influence the uptake of microplastics, resulting in reduced energy and potentially death (Andrady, A. L., 2011).

b. Loss of diversity and balanced ecosystem

O. marina dwell in estuaries and bays. In highly populated areas, these places receive excessive amounts of domestic wastes, that incorporate elevated levels of microplastics from consumer products (Browne et al., 2011). The observed effect of the presence of microplastics, including reduced energy intake and decreased motility of *O. marina* cells may lead to a quantitative reduction in their marine populations. If numbers of microalgae predators (including *O. marina*) decline, a relative increase in diatom and other dinoflagellate preys may occur, resulting in seasonal algal bloom (Hansen, P. J., 1991; Strom et al., 1997). In addition, pelagic grazers other than *O. marina* are considered as an important source of dissolved organic carbon in the marine environment (Strom et al., 1997). Although *O. marina* is not a common species in the open

ocean, as a model organism it reflects what could happen if other species are exposed to similar type and concentration of microplastics. Moreover, microplastics which has been engulfed and accumulated may easily be transferred to *O. marina* predators.

c. Modification of the microplastics

The term modification, as used here, refers to the possibility that *O. marina* may add biomaterials on the surfaces of microbeads after ingesting them. Such biomaterial may be sticky and ultimately induce formation of complexes of microplastics and cellular remains from *O. marina*. After ingestion of several microplastics the cell may rupture and release such complex to the near environment. The bolus of microplastics captured in image 1 b may be an example of this kind of complexes.

This complex mixture of cellular remains on microplastics from *O. marina* may have implications as far as biofouling of microplastics is concerned. Biofouling has for example been associated with the fate of microplastics in marine environment (Andrady, A. L., 2011; Fazey et al., 2016). However, the impact resulting from this modification may solely depend on the stability of the resulting microalgae-microplastic complex. Particle-to-particle attachment may reach a size that could be mistaken for food by higher marine organisms such as fish and shrimps. The presence of digestive and biological materials from *O. marina*, would also carry some chemotactic agents attracting predators analogous to *O. marina*.

4.2 Interactions and effects of microplastics in *Rhodomonas baltica*

4.2.1 Concentration of particles in the system

The actual concentrations of polystyrene microplastics varied slightly from the initial nominal values. For instance, the initial nominal concentration of the highest concentration was 7500 PS/mL, but it was measured as 5300PS/mL. The primary error may have resulted from wrong estimation of the concentration of the microplastics from the stock solution or poor mixing during such measurements. This error may have been caused by challenging sampling and handling techniques particularly considering the size of the samples, dilution procedures, sampling equipment and sedimentation of microplastics. Microplastic properties and behaviour like sedimentation on the vial walls would also be a probable cause of imprecise estimation of

the concentration. A controversial decrease in microplastic concentration from the samples exposed to highest concentration was observed between 0 to 72-h time intervals (Fig. 20). After studying probable causes of such variation, it was found that most of the microplastics from the sampled portion sediment on the walls of the sampling beaker, glass pipette and pH meter probe. The total loss of microplastics was quantified and it was established that approximately 40.9% of the nominal concentration was lost from the system (*Figure 16*). The sampling beaker was then removed from the procedure and coulter counting was done before pH measurements to improve accuracy of measurements. Also, the large glass pipette was replaced by a regular 5000 µl microtip pipette.

4.2.2 pH and temperature

While no significant variation in temperature was found, pH has shown to have a key role in algae growth rate and ageing. In the results generated during the method development tests, pH values >9.7 were associated with the initiation of stationary phase, and subsequently death. In the method development test, this pH of 9.6 was attained after 144 h compared to 240 h in the main study. A significant and strong correlation ($R^2 = 0.99$, $p<0.0001$) was found between pH and growth, measured as an increase in cell number, as well as between pH and chlorophyll production, measured as fluorescence (Appendix 11). Regardless of slight temperature variations in the system, no significant correlation was found between temperature and any parameter measured (Appendix 11). This means temperature did not influence algal growth, chlorophyll production and change in pH in the exposure system.

4.2.3 Interactions between microalgae and microplastics

Microscopy study of *R. baltica* samples exposed to different concentrations of PS microplastics showed a normal distribution of microalgae cells in the control samples, with very few artefacts such as paired/agglutinated algae cells. Samples exposed to 75PS/mL did not differ significantly from the control. Not any algae-to-microplastic agglutination was observed in these samples. After exposure to 750 PS/mL, algae-to-algae agglutinations of up to 3 or 4 cells were observed. Some PS particles attached to fibres were also observed, and larger artefacts assumed to be biofilms. A large mass of clumped materials was observed in algae samples exposed to 7500PS/mL. These materials were characterised as microplastics agglutinated with background debris from *R. baltica* and to some extent algae cells were observed trapped inside these complex matrices, which likely have a shading effect.

Generally, the extent of algae aggregation increased with the increase in PS concentration. Microplastics agglutination was more pronounced at higher microplastic exposure concentrations, with some algae cells trapped inside the formed plastic-plastic complexes. A similar study was performed using polypropylene (PP) and high-density polyethylene (HDPE) and a freshwater microalga, *Chlamydomas reinhardtii*. After 20 days of contact, only in the case of PP hetero-aggregates constituted of microalgae, microplastics and exopolysaccharides were formed. This complex (hetero-aggregate) was suggested to be a potential pathway through which MPs are brought to the sediments (Lagarde et al., 2016). Although differences in exposure time, experimental species, and polymer type may contribute to variations, we demonstrated that PS microbeads may cause similar effects as those reported by Lagarde et al. (2016) in another species of microalgae (*R. baltica*). The trapping of microalgae inside microplastic complex, accounts for shading of algae cells from light which can lead to reduced chlorophyll production and growth rate.

4.2.4 Effects on chlorophyll production (fluorescence)

Figure 14 shows only samples exposed to high microplastic concentration (7500PS/mL) exhibited significantly reduced fluorescence values compared to the control group ($p<0.001$). The differences were specifically observed during the exponential growth phase (between 120 and 240 h time interval). However, after the maximum pH and stationery phase was attained (240 h), samples from all three treatment groups exhibited significant decrease in fluorescent compared to the control. The observed effects on chlorophyll production are closely related to the shading effect thought to occur in PS7500 exposed samples. Microalgae cells require optimal light conditions for chlorophyll synthesis and cellular growth. Shading will allow less light to reach the algae cells, hence the reduced fluorescence values observed for the R. baltica exposed to PS7500/mL concentration.

4.2.5 Effects on algae growth

R. baltica from all treatment groups showed normal sigmoid algal growth curves, including the first three distinct phases (lag, exponential and stationery). While the medium concentration (750PS/mL) showed a slightly higher growth rate than the control, the lowest concentration did not exhibit any significant difference compared to the control. Growth of *R. baltica* exposed to the medium concentration differed significantly from the control only at 216, 240 and 264 h time points (Figure 11). However, *R. baltica* exposed to high concentration (PS7500/mL),

exhibited significant lowered growth compared to the control group from 120 h to 264 h ($p < 0.001$). We found a strong positive association ($R^2 = 0.979$) between fluorescence units (chlorophyll production) and increase in cell number. Using *Dunaliella tertiolecta*, Sjollema et al. (2016) observed a slight reduction (11%) in growth caused by uncharged 0.5 µm PS microbeads, and inhibition of the algal growth rate by 13%. A similar study by (Besseling et al., 2014) using PS nanoplastics (0.07 µm) reported growth inhibition of the microalgae *Scenedesmus obliquus* by 2.5% at higher concentrations (1 g/L) of particles. Despite the smaller size of plastic particles used in the latter two studies, the trend of effect on algae growth can be compared to our study as both studies, like ours, reported reduction of growth only at the higher MP concentrations. In the present study, the reduction in growth observed in the PS 7500 exposed group, is generally associated with the shading effect, and the low chlorophyll values as stated in section 4.2.2 above.

4.2.6 Sedimentation and biofouling effect on microplastics

The sedimentation study with the samples containing microplastics alone showed that about 260 PS/mL, 25 PS/mL and < 5 PS/mL from PS7500, PS750 and PS75 stuck on the walls of the 40 mL culture vials (fig.16) after 120 h. This may be a main reason to uneven distribution of the microplastics in the system. Assuming this also happened in the exposure groups containing microalgae, the bioavailability of PS was significantly reduced. This may explain the lack of effects on microalgal growth observed for the medium PS concentration.

As stated above, biofouling contributes to plastic distribution of microplastics in water column. Microscopy study (Image 4) showed that in samples exposed to high concentration revealed clustering of microplastics by a thin film assumed to be bacterial or background from *R. baltica* stock solution. The attachment of microplastics to different surfaces including fibres present in the solution (Image 4) indicates uneven distribution of these particles in the system. This might have decreased availability of these particles to interact with microalgae in the water column.

4.3 Scientific and environmental relevance of the study

The nominal concentrations of both microalgae used in this study may be within the range of the normal environmental relevant concentrations. Depending on nutrients, season and availability of predators, algae concentration in nature may vary from zero to millions of cells per millilitre. During algae blooms the concentrations of microalgae increases exponentially with some algal blooms recorded to contain more than 1,000,000 cells per millilitre of water

(Cosper et al., 1987). However, such high concentrations are yet to be reported on the algae species used in this study. The highest concentrations of microplastics (> 80 µm) reported in the environmental was 100 particles per millilitre (Magnusson and Norén, 2014, Isobe et al., 2017). However, sampling nets predominantly of large mesh size (<300 µm) have been used, thus concentrations of microplastics <300 µm present in the environment may be much higher (Magnusson and Norén, 2014, Phuong et al., 2016). Several publications have used elevated concentrations compared to what is reported to be in the environment (Cole et al., 2015, Bhattacharya et al., 2010, Lee et al., 2013).

5 CONCLUDING REMARKS

O. marina ingests both PS and green fluorescent microbeads at the expense of microalgae uptake (*R. baltica*). At high PS concentration, serious adverse effects on *O. marina* were observed characterized by food replacement and loss of motility.

A non-monotonic dose-response relationship was observed between PS microsphere concentration and *R. baltica* production rate, which strongly correlated with pH in the exposure media as well as fluorescence and hence chlorophyll production. Shading and biofouling are possible mechanisms of action of microplastics in autotrophic microalgae such as *R. baltica*.

Recommendations

Improved methodological procedures may help reducing errors that were observed in this study. Increased sample size and space on the plankton wheel will reduce statistical errors due to low sample size (increase the statistical power). Improved sampling techniques will help to keep a constant number of microplastics in the system, thus reaching a clear conclusion on the effects. Replacement of the small 40 mL vials with bigger ones, will allow enough subsamples that requires no or less dilution, thus reducing errors that may arise from dilution procedures.

It is important to extend this study and look at the photosynthetic efficiency of the microalgae, which would give a better interpretation of the intrinsic effects resulting from exposure to microplastics.

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Appendices

Appendix 1. Mean ± SD concentrations of *R. baltica* cells/mL in the five treatment groups of the experiment with *O. marina*

Time (min.)	Control	GF75	GF7500	PS75	PS7500
0	20634±404	20666±1269	20725±342	20820±470	20547±346
10	18948±573	19149±1463	19467±393	19189±885	19527±577
25	15148±916	16304±1173	17205±877	17013±1394	18002±624
40	11126±666	12905±1353	14482±1530	15322±1440	16728±663
60	7603±250	9768±2221	11661±1644	13529±2411	15232±751

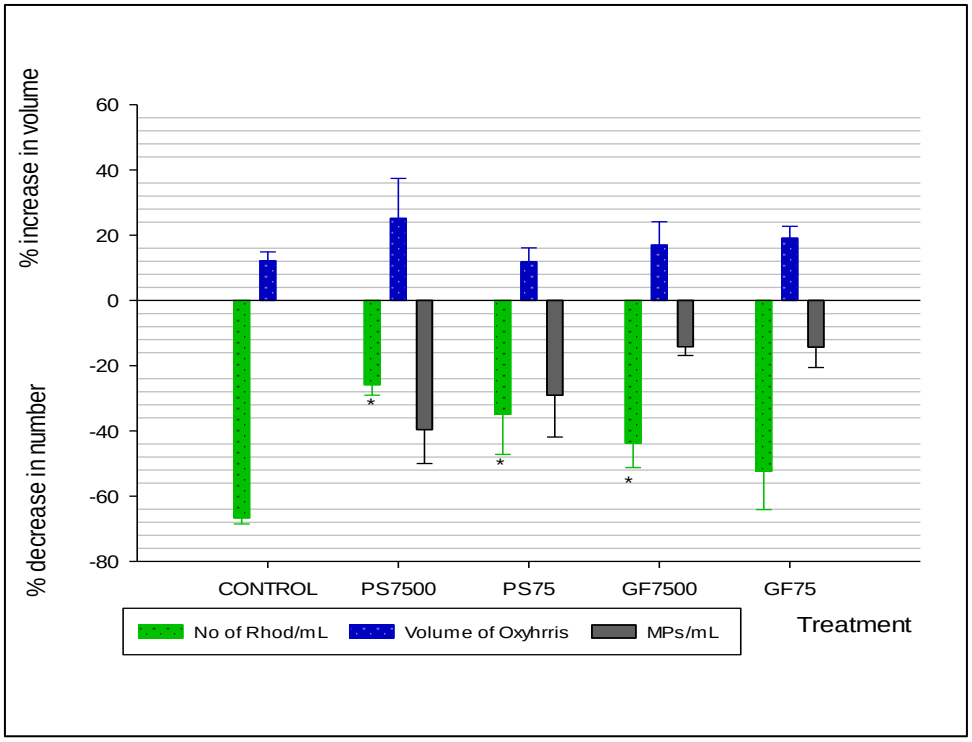
Appendix 2. Concentration of Microplastics measured from the four treatment groups on the effects of Microplastics on Oxyrrhis marina

Time	GF75	GF7500	PS75	PS7500
0	9245±6047	15406±7512	201±30	7142±601
10	9225±6168	15250±7481	175±30	6720±571
25	8910±6149	14656±7178	168±31	5960±517
40	8585±6140	13967±6918	150±25	5028±656
60	8216±5944	13267±6647	142±30	4315±803

Appendix 3. Percentage change in concentration and volume of particles among treatment groups after 60 minutes

Treatment	Rhodomonas/mL	Volume of *O. marina*	Microplastics/Ml
Control	-67±2	12±3	-
PS7500	-26±3	25±12	-40±10
PS75	-35±12	12±4	-29±13
GF7500	-44±7	17±7	-14±3
GF75	-52±12	19±4	-14±6

Appendix 4. Change in volume of O. marina compared to change in concentrations of both R. baltica and Microplastics in culture medium after 60 minutes. Error bars are presented as standard deviation



Appendix 5. Mean ± standard deviation concentrations (cell number/mL) of R. baltica culture at different time points

Time (hours)	Control	PS7500	PS750	PS75
0	2040±0	2040±0	240±0	2020±0
24	6211±280	6539±443	5932±427	6465±631
48	8249±786	8331±919	9013±610	9047±619
72	13787±1561	13539±902	16528±1673	14408±2757
96	28633±3356	27402±1179	29031±2864	26282±1753
120	45223±3100	38149±2490	46458±5997	46306±4730*
144	62620±10287	46728±1038	62780±2565	56662±1663*
168	73975±4645	55686±3819	83583±3662*	71841±2356*
192	113092±9933	74705±4220	129874±8403*	116015±6287*
216	171284±5493	118189±4127	202375±15103*	177538±8942*
240	237516±6700	170279±11688	260263±10810*	234426±8194*
264	246493±6628	185850±11674	262406±11338	248743±6129*

Comparing to the control, the median concentration has highest mean as from 48hrs. However, this difference was not statistically significant differences before 168 and after 240 hrs. The highest concentration has the lowest mean all over the culture time but with no significant differences before 96 h.

Appendix 6. Mean ± standard deviation of pH values measured in the four treatment groups

Time (hours)	Control conc.	Low conc.	Median conc.	High conc.
0	8±0	8.01±0	8.02±0	8.01±0
24	8.11±0.04	8.17±0.01	8.19±0.01	8.18±0.01
48	8.18±0.02	8.18±0.03	8.21±0.03	8.19±0.03
72	8.11±0.02	8.13±0.04	8.16±0.04	8.14±0.04
96	8.24±0.02	8.27±0.06	8.28±0.06	8.26±0.06
120	8.33±0.09	8.4±0.09	8.45±0.09	8.33±0.09
144	8.6±0.06	8.51±0.01	8.6±0.01	8.39±0.01
168	8.69±0.01	8.71±0.01	8.79±0.01	8.54±0.01
192	8.95±0.05	9.09±0.03	9.02±0.03	8.81±0.03
216	9.28±0.08	9.31±0.06	9.46±0.06	9.02±0.06
240	9.65±0.09	9.66±0.09	9.82±0.09	9.21±0.09
264	9.72±0.04	9.7±0.04	9.91±0.04	9.28±0.04

Appendix 7. Mean ± standard deviation total volume (μm^3) of O. marina in the five treatment groups

Time (min.)	Control	GF75	GF7500	PS75	PS7500
0	9034088±1	9514667±452	8920070±528	9982576±1476	8984076±4675
10	9145955±1	9810217±604	9357033±246	10171317±144	9360969±5290
25	9360979±1	10244583±51	9571640±469	10365865±155	9807818±3957
40	9883904±2	10758233±41	10014644±39	10736437±161	10659085±116
60	10130236±	11328350±54	10410445±47	11133551±145	11251365±129

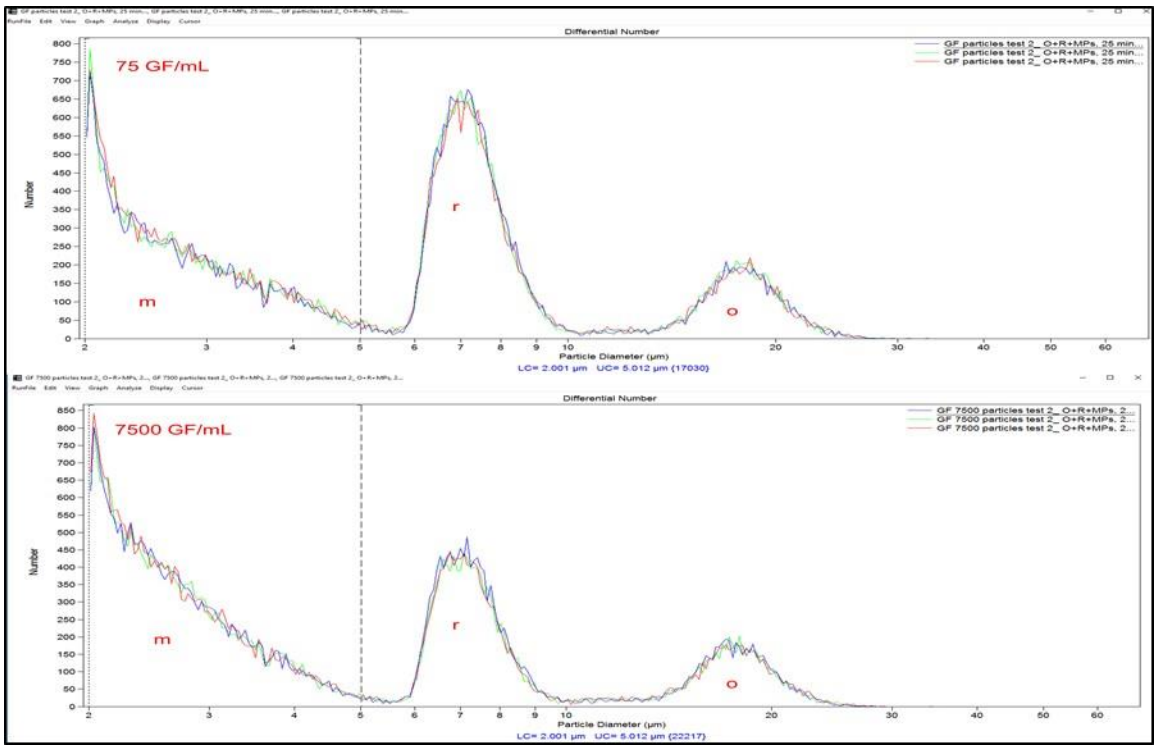
The volume of the *O. marina* seems to increase from as time increases in all treatment groups. The differences between initial (0 minutes) and final (60 minutes) volumes of *O. marina* are statistically significant for each treatment group (t-test, p = 0.05).

Appendix 8. Mean ± standard deviation fluorescence values measured in the four treatment groups

Time (hours)	Control	Low (PS75)	Median (PS750)	High (PS7500)
0	0.69±0	0.66±0	0.51±0	077±0
24	3.74±0.15	2.57±0.04	2.11±0.32	3.43±029
48	3.88±1.13	3.56±1.18	4.6±0.33	2.67±197
72	9.3±0.19	7.43±0.17	7.41±0.06	7.41±006
96	13.29±2.5	13.05±0.92	15.03±1.47	12.55±274
120	28.08±2.72	27.37±2.23	29.72±4.22	26.85±24

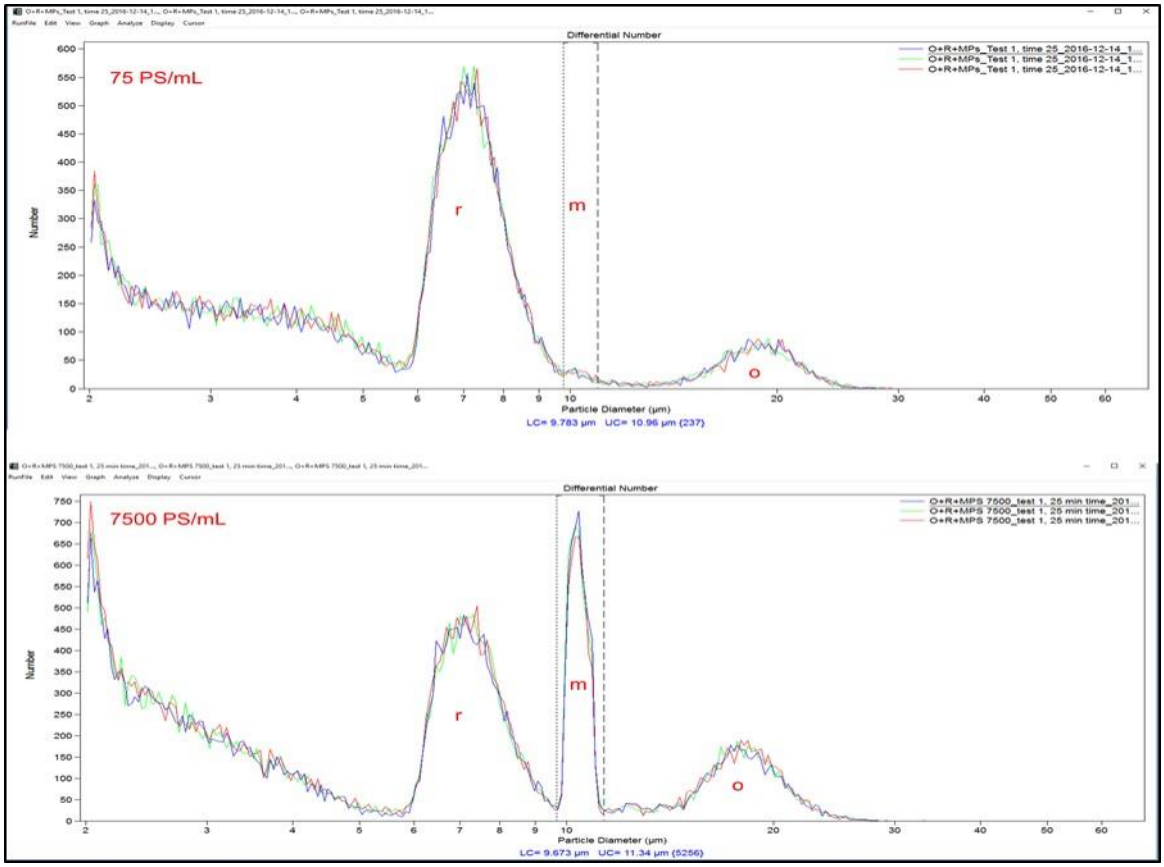
144	34.03±1.54	33.33±1.45	35.11±3.01	28.44±144
168	38.32±4.79	45.38±3.73	50.52±6.3	33.99±486
192	55.69±6.52	62.97±2.92	68.49±4.74	47.25±647
216	78.96±6.69	80.47±5.54	85.84±4	62.42±655
240	106.35±2.69	106.17±3.23	108.62±4.59	89.89±294
264	108.64±6.72	91.71±9.78	86.09±4.69	80.81±605

Appendix 9. Coulter counter (Multisizer^{TM} 3) outputs showing number of particles in different picks with the green fluorescence particles (m, r and o represent microplastics, Rhodomonas and Oxyrrhis respectively).



Number of microplastics in the overlaid M region are extremely high as compared to the nominal concentration of microplastics (17030 versus 75 and 22217 versus 7500). This higher increase of GF concentrations is particularly contributed by the background from both *Rhodomona*s and *Oxyrrhis* stock solutions. To eliminate this, a microplastics data were expressed as percentage change from the initial concentration.

Appendix 10. Coulter counter (Multisizer^{TM} 3) outputs showing number of particles in different picks with the polystyrene microplastics (m, r and o represent microplastics, Rhodomonas and Oxyrrhis respectively)



With polystyrene (PS) particles, the background from stock solutions did not seriously affect the number of microplastics measured in our exposed sample. However, the pick that represents PS75 particles was over shaded by the relative higher number of both *R. baltica* and *O. marina.* The highest dilution factor (20 times) dilution would have contributed to the observed low pick with the PS75. Therefore, we used diameter of (9.5 to 10.5µm) as our overlay region for this concentration.

Appendix 11. Coefficients of correlation between different parameters measured in the experiments with the effects on microplastics on *Rhodomonas baltica*

	temp	concentration	fluorescence	pH	
time	0.528	0.928	0.958	0.952	R^2
	0.0775	<0.0001	<0.0001	<0.0001	p-value
temperature		0.236	0.324	0.297	R^2

		0.46	0.304	0.348	p-value
concentration			0.979	0.99	R^2
			2.76E-08	7E-10	p-value
fluorescence				0.991	R^2
				5.78E-10	p-value