

ALGAL RESPONSES TO MICRO- AND NANOPLASTIC EXPOSURE

**UNDERSTANDING THE ROLE OF EXTRACELLULAR POLYMERIC
SUBSTANCES**

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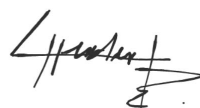
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The promoters,

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Laila Herder

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ABBREVIATIONS

PTC	Particle
NP	Nanoplastic
MP	Microplastic
MNP	Micro- and nanoplastic
PS	Polystyrene
EPS	Extracellular Polymeric Substances
WWTP	Waste Water Treatment Plant
NOM	Natural Organic Matter
BCP	Biological Carbon Pump
DOP	Dissolved Organic Phosphorus
DIP	Dissolved Inorganic Phosphorus
TSS	Total Suspended Solids
PAH	Polycyclic Aromatic Hydrocarbon
POP	Persistent Organic Pollutant
PCB	Polychlorinated Biphenyl
ROS	Reactive Oxygen Species
B-EPS	Bound EPS
LB-EPS	Loosely Bound EPS
TB-EPS	Tightly Bound EPS
SL-EPS	Soluble EPS
P/C	Protein-To-Carbohydrate
TBBPA	Tetrabromobisphenol A
GOE	Great Oxygenation Event
PET	Polyethylene Terephthalate
DPPC	Dipalmitoylphosphatidylcholine
DI	Deionized

JRC	Joint Research Centre
IRMM	Institute for Reference Materials and Measurements
CER	Cation Exchange Resin
ATR-FTIR	Attenuated Total Reflectance Fourier Transform Infrared
DLS	Dynamic Light Scattering
CCAP	Culture Collection of Algae & Protozoa
SD	Size Distribution
EN	Engineered Nanoparticles
HAB	Harmful Algal Bloom
AMR	Antimicrobial Resistance

ABSTRACT

While the potential threat of micro- and nanoplastics (MNPs) to marine ecosystems is gaining attention, research often utilizes unrealistically high exposure levels. This study bridges that gap by investigating the effects of environmentally relevant MNP concentrations on the saltwater microalgae *Rhodomonas salina*. It further explores the role of Extracellular Polymeric Substances (EPS) in mitigating these effects. Microalgae were exposed to a range of PET and aged-PET concentrations (10, 100, 1000, and 10000 particles/mL) to account for potential changes in polymer properties and additive release due to aging. Kaolin served as a control for non-plastic particle effects. Although the study's findings on algal growth didn't show a clear trend, they did reveal some intriguing patterns in EPS quantity and composition. Notably, at the low MNP concentrations used, there were no significant changes in total EPS amount. However, the Protein-to-Carbohydrate (P/C) ratio within the EPS displayed distinct responses depending on the exposure type. While the P/C-ratio remained stable for PET exposures, it significantly increased for aged-PET and kaolin at higher concentrations. Interestingly, kaolin exposure resulted in more and larger aggregates, whereas higher concentrations of PET and aged-PET led to fewer and smaller ones. Furthermore, at the highest exposure (10000 particles/mL), all treatments displayed a shift in aggregate composition, with a higher proportion of algal cells relative to EPS. These findings significantly improve risk assessment of MNPs under current marine pollution conditions. They also highlight the critical role of algal EPS in the aggregation of both microalgae and MNPs, ultimately influencing their environmental fate.

1. INTRODUCTION

1.1 Micro- and nanoplastics as emerging environmental contaminants

Over the past half-century, plastic production has skyrocketed, transforming industries and daily life. Its versatility and affordability have made it a go-to material for countless human needs (Oliveira and Almeida, 2019; Worm et al., 2017). This trend continues, with global annual plastic production exceeding 390 million tons in 2021, a 4% increase from the previous year (Plastics Europe, 2023). However, this surge in plastic use comes at a significant environmental cost. The ever-growing presence of plastics in our environment, both intentional and unintentional, poses a major threat to organisms (Oliveira and Almeida, 2019). Plastic pollution acts as a stressor, leading to adverse effects such as stunted growth, reproductive problems, and even death in various species (Chae and An, 2017).

While the issue of plastic debris in marine environments is not new, a recent area of growing concern is the breakdown of these plastics into much smaller particles. This fragmentation process can yield microplastics (MPs) (1 μm - 5 mm), and with further degradation, even nanoplastics (NPs) (<1 μm) (Zhu et al., 2021; da Costa et al., 2016). Collectively, these tiny plastic fragments are referred to as micro- and nanoplastics (MNPs). Adding to the problem is the increasing commercial production of NPs. Advances in nanotechnology allow scientists to manipulate plastic particles at the atomic level, equipping them with novel chemical and physical properties. These engineered NPs find applications in various fields, including pharmaceuticals, cosmetics, and medicine (Bundschuh et al., 2018).

The growing presence of NPs in our oceans is a significant cause for concern for multiple reasons. As plastic particles become smaller, their biological reactivity increases. This highlights the need to understand the extent of NP contamination and its potential consequences for marine life. Additionally, NPs behave very differently from larger plastics in the environment. Their small size allows them to remain suspended in the water column, making them more likely to spread, be mobile, and be readily taken up by marine organisms. This increased bioavailability raises concerns about NPs potential harm. Furthermore, the large surface area of NPs makes them ideal for adsorbing toxic chemicals from the surrounding environment. This adsorption process can further enhance the potential toxicity of NPs to marine biota (Gonçalves and Bebianno, 2021).

1.2 Importance of EPS production in marine microalgae

Microalgae are microscopic algae that can be found in both freshwater and marine systems. Representing the lowest trophic level, microalgae are the cornerstones of all aquatic ecosystems. As primary producers, they harness sunlight to synthesize organic biomass through photosynthesis. The biomass, produced by microalgae, serves as a crucial energy source for primary consumers like rotifers, copepods, and larval fish stages, which lack the ability to generate their own energy. Secondary consumers, in turn, rely on these primary consumers for their energy needs. This intricate web highlights the pivotal role of microalgae as the primary producers in sustaining healthy aquatic ecosystems (Gonçalves and Bebianno, 2021).

Marine microalgae can exist in two forms: either as unicellular organisms or organized into macroscopically visible colonies and filaments (Ferreira de Oliveira and Bragotto, 2022). Unicellular algae are enveloped in a protective layer of Extracellular Polymeric Substances (EPS), while those forming colonies or filaments are held together by the same EPS. EPS represent a diverse range of biopolymers, including polysaccharides, proteins, nucleic acids, and lipids. Collectively, these components form a hydrated matrix around algal cell walls, facilitating various functions. EPS contribute to adhesion, water retention, serve as an energy source, act as surfactants, and enhance sorption processes. Their substantial carbon content makes them a critical organic carbon source for marine organisms across the food chain. Furthermore, EPS display significant flocculating properties, with their composition and constituents determining the rate of flocculation (Xiao and Zheng, 2016).

In past research, the production of EPS by microorganisms has been suggested as a potential detoxifying mechanism. The study of Zhou et al. (2016) showed that in the presence of silver nanoparticles, microbes secreted increased amounts of EPS, correlating with greater tolerance to these particles. It has been proposed that the EPS create a protective barrier around the cells, preventing the internalization of nanoparticles. This research seeks to further unravel the protective role of EPS against NPs and the significance of EPS composition in relation to its detoxifying potential. Additionally, it also seeks to examine the impact of EPS on the aggregation dynamics of micro-algae.

In summary, understanding the role of EPS in marine microalgae is fundamental to comprehend their adaptability and responses to environmental changes, particularly the rising presence of NPs. Investigating the significance of EPS in detoxification processes and their potential role in mitigating the impact of NPs on these foundational organisms offers critical insights into the health and resilience of aquatic ecosystems. Moreover, examining how EPS influences the aggregation of microalgae can provide a deeper understanding of their ecological interactions and stability within aquatic environments.

2. LITERATURE REVIEW

2.1 Nanoplastics in aquatic environments

2.1.1 Sources and types of nanoplastics

Types of nanoplastics

NPs are typically categorized into two types: primary and secondary NPs. Primary NPs are intentionally manufactured in nanoscale dimensions for various industrial and consumer applications, such as cosmetics or composite materials. An example of primary NPs are polystyrene (PS) NPs. They consist of nanoscale particles with a PS core and various functional groups which enable the customization of plastics to possess specific properties tailored to particular applications. In the case of PS NPs, common functionalizations include anionic carboxylated (COOH) and cationic unsaturated amino (NH₂) functional groups, which determine the chemical reactivity and surface charge of the NPs (Mendoza et al., 2018).

The second type are secondary NPs, which result from the degradation of larger plastic polymers (Piccardo et al., 2020). This breakdown can occur both before entering the environment, such as the fragmentation of synthetic fibers during the washing of clothing, or afterward, due to the wear-and-tear of plastic items exposed to environmental factors, including sunlight and waves (da Costa et al., 2016).

This literature review primarily focuses on nanoplastics. However, relevant studies on nanoparticles, a broader category encompassing nanoplastics, will also be included. Though nanoparticles encompass various materials, the insights gained from these studies can be highly valuable for understanding the effects of nanoplastics on algae.

Sources of nanoplastics

An estimated 19 to 23 million metric tons, equating to 11% of the global plastic waste generated in 2016, found its way into aquatic ecosystems. Given that plastic is a human-made product, its primary source is the terrestrial environment. Roughly 75-90% of plastic pollution originates from land-based sources (Figure 2.1: 1a), while 10-15% can be attributed to marine activities like fishing, aquaculture, and oil and gas exploration (Figure 2.1: 1b) (Allen et al., 2022). Terrestrially sourced plastic eventually reaches the aquatic environment through natural waterways, sewage and drainage systems, wind-driven transport, or human neglect (Wayman and Niemann, 2021).

Some plastics are introduced into the environment in their micro-/nano-scale form, often through the operations of Waste Water Treatment Plants (WWTPs) (Allen et al., 2022). While several wastewater treatment processes have demonstrated their effectiveness in removing MPs, the same level of efficiency may not be extended to smaller nano-sized particles. Notably, major sources of micro- and nano-sized plastic polymers in aquatic ecosystems have been traced back to cosmetics and cleaning products that enter domestic wastewater (da Costa et al., 2016). These products may not be adequately removed by WWTPs, contributing to their presence in the environment. Moreover, industrial discharges from the plastic manufacturing process and the wear of vehicle tyres are additional contributors to the presence of these micro- and/or nano-sized plastic particles in aquatic ecosystems (Allen et al., 2022).

In addition to NPs introduced directly into the environment, a substantial contribution arises from the degradation of larger plastic items within the environment. The various mechanisms through which plastics degrade are explored in greater detail in Section 2.1.2.

2.1.2 Behaviour and fate of (nano)plastics

Degradation of plastic

Plastic polymers that enter the environment are susceptible to abiotic and/or biotic degradation. Abiotic degradation processes result in a loss of structural and mechanical properties, leading to the creation of smaller particles with increased surface area, making them more vulnerable to subsequent degradation. Key abiotic degradation processes include: i) hydrolysis, ii) mechanical degradation, iii) thermooxidative degradation and iv) photodegradation (da Costa et al., 2016).

- i) Hydrolysis refers to induced bond-breaking reactions, due to the presence of water (Mattsson et al., 2018).
- ii) Mechanical degradation (Figure 2.1: 2a) is the breakdown of plastic polymers when they are subjected to mechanical stress from waves, rocks, sand, and other forces or substances in the aquatic environment (Mattsson et al., 2018).
- iii) Thermooxidative degradation (Figure 2.1: 2c) is the slow oxidative breakdown that takes place at moderate temperatures (Mattsson et al., 2018).
- iv) Photodegradation (Figure 2.1: 2d) of plastic polymers involves photooxidation, where exposure to UV light from the sun initiates free radical reactions and chain scission, resulting in a loss of the polymer's mechanical properties. The increased brittleness makes the material more susceptible to fragmentation, thereby enhancing the available surface area for further photooxidation and other degradation pathways (Wayman and Niemann, 2021).

Biotic degradation of plastics, commonly referred to as biodegradation (Figure 2.1: 2b), is a process where living organisms, typically microbes, actively dismantle the plastic polymer structure (Mattsson et al., 2018). Ultimately, plastic polymers can be broken down

into oligomers and monomers, which are capable of undergoing mineralization. These breakdown products pass through the semi-permeable outer membrane of microorganisms, which assimilate them as a source of carbon or nitrogen. The rate and extent of biodegradation depend on several factors, including for example prior biotic degradation, environmental conditions, the type of plastic and the variety and quantity of microorganisms (da Costa et al., 2016).

Colloidal stability of nanoparticles

Upon introduction into the environment, nanoparticles interact with a diverse range of dissolved or particulate, inorganic, or organic compounds. This interaction significantly influences their aggregation and, consequently, their colloidal stability. (Bundschuh et al., 2018) Colloidal stability, refers to the stability of dispersed particles within a colloidal system (Encyclopedia Britannica, 2024). The colloidal stability of nanoparticles in seawater plays a pivotal role in determining their fate and subsequent effects in aquatic systems. Colloids themselves are defined as any particle having a linear dimension between 1 nm and 1 μ m. These colloids can be found in solution under isolated or aggregated forms, depending on various environmental conditions such as pH, ionic strength, and dissolved organic matter (Gigault et al., 2018). Understanding the colloidal behaviour of NPs is crucial to comprehend their behaviour in marine systems.

Dispersion of nanoplastics

In terms of dispersion, there is a significant distinction between macro/micro-sized particles and NPs. Larger macro/microplastics are mainly influenced by their buoyancy properties, which depend on factors like density and shape. Depending on the balance between upward and downward forces, these particles will either float, sink, or, in rarer cases, be neutrally buoyant (Wayman and Niemann, 2021). While buoyancy is a key factor, other variables can also significantly impact the dispersion of macro/microplastics. Biological factors such as biofilm growth, alongside particle characteristics such as shape and surface properties, and environmental factors like temperature and light intensity, all play pivotal roles in shaping the dispersion patterns of MPs (Vercauteren et al., 2024). In contrast, nanosized particles are primarily governed by collisions with water molecules and other present ionic species, which may prevent the particles from sedimenting. The resulting effect of this interaction is a random motion throughout the solution, called Brownian motion (Gigault et al., 2018). Due to this colloidal behavior, NPs can disperse widely throughout the entire ocean, including the water column and sediments. As a result, NPs have the potential to impact various oceanic ecosystems and niches (Wayman and Niemann, 2021).

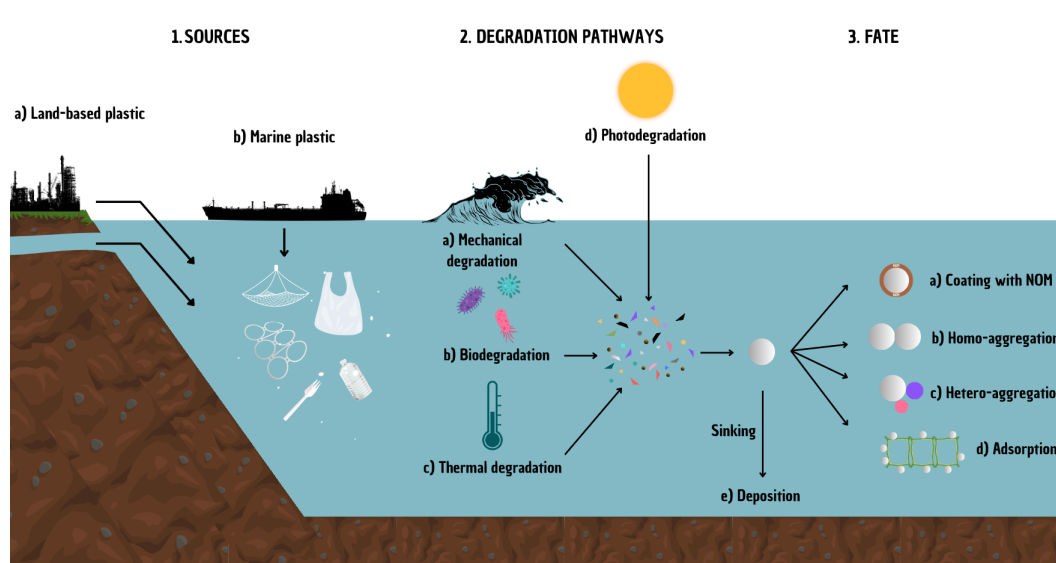


Figure 2.1: Sources, degradation and fate of NPs. (Based on Wayman and Niemann (2021); Bundschuh et al. (2018))

Detection and quantification

Optical and chemical methods commonly employed for MP analysis are not suitable at the nanoscale due to sensitivity issues as well as physical and optical limits (Wayman and Niemann, 2021). This difficulty in quantifying NPs in marine environments restricts research on the effects of environmentally relevant concentrations on marine organisms. Additionally, standard sampling tools like plankton nets, which have micrometer-sized mesh, are ineffective at capturing NPs, complicating sampling efforts (Wayman and Niemann, 2021).

While direct measurements of NP concentrations in oceans remain complicated, estimates have been derived. These estimates often rely on data regarding MP concentrations (Piccardi et al., 2020). According to mass conservation principles, the fragmentation of spherical MPs ranging from $0.1 \mu\text{m}$ to 5 mm into 100 nm NPs would theoretically yield particle concentrations exceeding those of currently observed MP concentrations by over 10^{14} times (Besseling et al., 2018). For instance, research by the Flemish Marine Institute identified a concentration of $1.5 \text{ MPs} / \text{m}^3$ in the North Sea (Everaert et al., 2022). Using the 10^{14} factor, this translates to an estimated $1.5 \times 10^{14} \text{ NPs} / \text{m}^3$. While rough, such estimates provide a means to approximate real NP concentrations, enabling experiments with more environmentally relevant concentrations.

Overall, further research and development of suitable analytical techniques are crucial not only to comprehensively address the NP problem but also to enable accurate measurements, which are essential for effective risk assessment in marine ecosystems.

2.2 The role of marine microalgae

As mentioned earlier, algae play a crucial role as primary producers, serving as the cornerstone of the aquatic food chain. In addition to their primary producer function, microalgae

also contribute significantly to carbon fixation, oxygen production, nutrient cycling, and the formation of marine snow.

2.2.1 Biological carbon pump

Macro- and microalgae serve as primary producers within aquatic ecosystems, both contributing to the pivotal role played by phytoplankton in the Biological Carbon Pump (BCP). This biological pump represents a set of processes by which inorganic carbon (e.g., carbon dioxide) is fixed into organic matter via photosynthesis and then sequestered away from the atmosphere, primarily through transport into the deep ocean (De La Rocha and Passow, 2014). The BCP's efficiency is determined by phytoplankton physiology and community structure, which are influenced by the physical and chemical conditions of aquatic ecosystems (Lobus and Kulikovskiy, 2023).

Although phytoplankton biomass constitutes only about 1-2% of the total global plant carbon, they annually fix between 30 and 50 billion metric tons of carbon, representing approximately 40% of the total carbon fixation (Falkowski, 1994). The implications of this BCP are profound for climate dynamics. The biological uptake of carbon dioxide (CO₂) by microalgae offers an effective mechanism for recycling both natural CO₂ sources (such as forest fires, volcanic eruptions, and organic matter decomposition) and anthropogenic CO₂ emissions (including those from power plants and automobiles) (Onyeaka et al., 2021). This underscores the critical importance of understanding how microalgae, a key component of the BCP, respond to changing environmental conditions. Such comprehension is essential for predicting future atmospheric CO₂ concentrations and climate dynamics (Lobus and Kulikovskiy, 2023).

2.2.2 Oxygen production

During the Great Oxygenation Event (GOE) around 2.4 billion years ago, atmospheric oxygen concentrations rapidly increased due to the activity of cyanobacteria, fundamentally transforming Earth's atmosphere (Dvořák et al., 2023). This pivotal event introduced substantial oxygen production, paving the way for the emergence and development of eukaryotic organisms. More specifically, it is the chloroplast, believed to have originated from ancient prokaryotic algae through endosymbiosis with early eukaryotic cells, that plays a crucial role in oxygen production across all plant species (Rai et al., 2000). When considering all types of algae present in rivers, lakes, and oceans combined, algae contribute approximately 50% of the Earth's total oxygen production (Chapman, 2010).

2.2.3 Nutrient cycling

Phytoplankton, including microalgae and cyanobacteria, play a crucial role in nutrient cycling, involving nitrogen, phosphorus and silicon among others. This involves the uptake and recycling of nutrients which significantly influences the overall nutrient balance in marine ecosystems.

Nitrogen (N)

Microalgae assimilate inorganic N compounds, transforming them into organic N within their cellular structures (Lobus and Kulikovskiy, 2023). These processes are essential for maintaining the N balance in the oceans, contributing significantly to the overall health and well-being of aquatic ecosystems. The N cycle controls the water productivity in marine environments. Unlike other major elements (e.g. Si), it is required by all groups of microalgae because it is essential for cell growth and development. Marine microalgae mediate key processes associated with the biogeochemical N cycle, primarily involving the assimilation of nitrate (NO_3^-), nitrite (NO_2^-), and ammonium (NH_4^+) and the biological fixation of N_2 .

Phosphorus (P)

Marine microalgae efficiently assimilate bioavailable P, encompassing dissolved inorganic phosphorus (DIP) and dissolved organic phosphorus (DOP). The efficiency of P assimilation by microalgae and cyanobacteria is so high that its concentration in water may decrease to zero during phytoplankton blooms. Furthermore, microalgae serve as significant contributors to intracellular P accumulation, primarily in the form of polyphosphates. The sedimentation of P-rich biogenic detritus further aids in the removal of P from the global biogeochemical cycle (Lobus and Kulikovskiy, 2023).

Silicon (Si)

Si plays a crucial role as both a nutritional and structural component for various aquatic organisms, including diatoms, sponges, and radiolarians (Cassarino et al., 2024). Primary minerals and river run-off constitute the primary sources of Si in the water column. Consequently, silicifiers absorb significant quantities of Si, along with N, P, and inorganic carbon. The Si cycle is intricately linked to the carbon cycle, as the presence of Si significantly impacts the growth and development of microalgae, especially diatoms. The optimal development of these microalgae is essential for effective carbon sequestration (Lobus and Kulikovskiy, 2023).

2.2.4 Marine snow

Organic matter, produced by primary producers in the euphotic zone, can either pass through the food web or be transported to deeper ocean layers as marine snow. Marine snow encompasses a variety of particles, including detritus (such as zooplankton fecal pellets), organic matter (residues and secretions from plankton), inorganic components, and amalgamated mixtures of these elements. Serving as a crucial component of the biological carbon pump, marine snow plays a pivotal role in the vertical transfer of carbon and nutrients in the ocean. Alterations in the community structure of both zooplankton and phytoplankton have the potential to impact the quantity and composition of marine snow, thereby influencing the efficiency of the marine carbon pump (Trudnowska et al., 2021).

2.3 The interaction between marine microalgae and nanoplastics

Examining the exposure of lower trophic levels to NPs is of significant importance for several reasons: (i) these levels serve as the entry point for pollutants into the food web, (ii) any adverse effects on primary producers can alter food resources, potentially leading to detrimental consequences for higher consumers and overall food web functions, and (iii) the interactions between NPs and microalgae may carry implications for biogeochemical cycling within marine ecosystems (Reichelt and Gorokhova, 2020).

2.3.1 The effects of nanoplastics on microalgae

Effect of nanoplastics on algal growth and photosynthetic activity

Previous research with microalgae has established that exposure to NPs can lead to inhibited growth, reduced photosynthetic activity, and oxidative stress (Yang et al., 2021). Yang et al. (2021) conducted a series of experiments to examine the inhibitory effects of NPs on the microalgae *Chlorella pyrenoidosa*. They observed inhibitory effects on photosynthetic pigments, which became more pronounced with increasing concentrations of PS NPs. In microalgae exposed to 50 mg/L PS NPs for 48 hours, the inhibition rates for chlorophyll a, chlorophyll b, and carotenoid contents were 29.64%, 62.86%, and 50.83%, respectively. However, after 96 hours of exposure, these inhibitory effects gradually diminished (Yang et al., 2021).

Existing research on growth inhibition due to NP exposure yields mixed results. While a general trend suggests a positive correlation between NP concentration and the degree of inhibition, conflicting findings have been reported. Studies by Zhu et al. (2021) observed increasing inhibition percentages (21.0%, 29.0%, and 38.5%) with increasing NP concentrations (0, 50, and 100 mg/L). However, Ripken et al. (2020) found the opposite effect, with the lowest NP concentration (0.01 mg/L) leading to the highest inhibition rate. These findings underscore the complex interplay between various factors influencing the effects of NP exposure.

It's important to consider that research often uses NP concentrations that are higher than what's likely found in the environment. For example, the study by Yang et al. (2021) used spherical polyethylene (PE) NPs of 80 nm at a concentration of 50 mg/L. In contrast, estimates of realistic NP concentration based on degradation of MPs, like previously mentioned estimate of 1.5×10^{14} NPs/ m^3 , translates to a much lower concentration of around 0.04 mg/L for PE NPs of the same size. This is significantly lower than the 50 mg/L used in the study of Yang et al. (2021). This difference in concentration is important because research using more environmentally relevant NP levels often shows minimal effects on algal growth. This suggests that the observed effects in some lab studies might not translate to real-world scenarios.

Combined effect of nanoplastics with other chemicals on microalgae

Another notable issue revolves around the combined effect of NPs with other chemicals on microalgae in marine environments. These ecosystems contain various contaminants such as polycyclic aromatic hydrocarbons (PAHs), persistent organic pollutants (POPs), polychlorinated biphenyls (PCBs), and various other chemical constituents, including metals. The extensive hydrophobic surface of NPs facilitates the adsorption of these toxic compounds, potentially altering the overall exposure effects (Gonçalves and Bebianno, 2021).

Moreover, the presence of chemicals on NPs may originate from the manufacturing process, involving diverse substances such as additives, catalysts, and solvents. These chemicals could also contribute to the observed effects of NP exposure on microalgae (Oliveira et al., 2019).

The diversity in the types of NPs, chemicals, and microalgae used in studies has resulted in a lack of consistent conclusions. Reports suggest both antagonistic and synergistic interactions between NPs and other chemicals (Zhu et al., 2021).

Factors influencing the nanoplastic exposure effect on microalgae

Nanoplastic concentration Previous research has reported conflicting results regarding the effect of NP concentration on algal growth. While some studies observed a positive correlation between concentration and inhibition, others reported the opposite trend. Ripken et al. (2020) attributed this unexpected finding to a higher probability of NPs aggregating with other NPs at higher concentrations. This aggregation could limit the number of individual NPs available to interact with algal cells, potentially explaining the observed lower inhibition at higher NP concentrations.

Nanoplastic size The size of plastic particles significantly impacts the level of inhibition experienced by algae. A study by Sjollem et al. (2016), shows that smaller particles lead to more pronounced inhibition. In their experiment, exposing *Dunaliella tertiolecta* to 250 mg/L PS beads resulted in a 45% reduction in algal cell density for 0.05 μm particles, while the inhibition was only 11% for 0.5 μm beads. This suggests that smaller particles have a greater ability to penetrate algal cells, potentially causing more damage.

Nanoplastic charge The charge of NPs can also play a decisive role in their effects. Given that the cell surface of microalgae commonly bears a negative charge, positively charged NPs tend to interact more with microalgae through electrostatic interactions (Zhu et al., 2021). Feng et al. (2020) investigated this phenomenon by exposing *Mycrocystis aeruginosa* to positively charged, amino-modified PS-NH₂ NPs at varying concentrations (3.4 and 6.8 mg/L) for 48 hours. They observed significant growth inhibition, with cell growth reduction reaching 23.57% and 46.10% respectively. Conversely, exposure to negatively charged PS-NH₂ NPs at a much higher concentration (100 mg/L) did not show any observable inhibition. This contrast underscores the role of NP charge in determining interaction and potential toxicity towards algae.

Exposure duration The inhibitory effect of NPs on algal growth seems to diminish gradually with prolonged exposure (Zhu et al., 2021). Yang et al. (2021) observed this phenomenon when exposing *Chlorella pyrenoidosa* microalgae to 50 mg/L of NPs. While an initial 24-hour exposure resulted in a 27.73% inhibition of maximum growth, extending the exposure to 96 hours led to a reduced inhibition rate of 10.53%. The authors suggest this reduced inhibition may be attributed to the adaptability of the algal strain. This potential detoxification ability could involve mechanisms like regulating internal pressure, accelerating the degradation of damaged cellular components, and promoting cell proliferation, ultimately allowing the algae to mitigate the negative effects of NP stress.

Despite the established acute toxicity of NPs in various short-term studies, the chronic effects of NPs remain under-explored and largely unknown (Zhu et al., 2021).

Exposure time Furthermore, the timing of exposure also plays a crucial role. For instance, Mao et al. (2018) observed initial growth inhibition in *Chlorella pyrenoidosa*, followed by a reversal with growth exceeding the control group during the stationary phase. This suggests the potential for algal populations to adapt to the presence of nanoparticles and potentially restore their productivity over time (Reichelt and Gorokhova, 2020).

Particle effect A crucial consideration is the impact of suspended particles in exposure systems. According to Gordon and Palmer (2015), suspended particles with specific geochemistry and size ranges trigger diverse responses in aquatic organisms. In the context of algae, the presence of sediment particles can lead to growth inhibition, emphasizing the need to implement controls for particle effects in toxicity testing (Reichelt and Gorokhova, 2020). This precaution is essential to avoid falsely attributing general particle effects to the presence of NPs.

The direct influence of Total Suspended Solids (TSS) on algae involves the promotion of flocculation, leading to the sinking of algae. An indirect effect may be associated with a reduction in light transmission, which can decrease photosynthesis and, consequently, algal productivity. Conversely, nutrients associated with TSS have the potential to enhance algal productivity (Chapman et al., 2017). Concerning concentration, proposed thresholds range from 20 to 100 mg/L of suspended solids, below which the impact on biota is considered negligible.

Nanoplastic type Finally, it is crucial to emphasize that distinct types of NPs may exhibit diverse inhibition effects. While the majority of current research has centered on PS NPs, extending the exploration to different NP types is crucial for comparing their individual impacts (Zhu et al., 2021).

2.3.2 Cellular mechanisms of nanoplastic exposure in microalgae

Having explored some of the observed effects of NPs on microalgae, the focus now shifts to the underlying mechanisms driving these effects at a cellular level. Figure 2.2 graphically represents these mechanisms. The adsorption of NPs onto the cell surface decreases the

fluidity of the algal cell membrane, hindering the exchange of energy and substances with the environment. Additionally, adsorbed NPs may induce shading of sunlight, thereby reducing light availability for photosynthesis (Zhu et al., 2021). Wang et al. (2020) reported increased aggregation and growth inhibition during the early stages of NPs adsorption to algal cells. With prolonged exposure, heightened permeability, compromised mitochondrial function, and cell surface rupture were observed.

Furthermore, adsorbed NPs could stimulate the generation of Reactive Oxygen Species (ROS). When ROS levels exceed the natural balance, oxidative stress can occur. This can result in peroxidation of cell membrane lipids, as well as structural alterations and damage to the cell membrane/wall (Zhu et al., 2021).

NPs that adsorb onto the microalgae surface may be internalized through endocytosis or physical damage. These internalized NPs can trigger ROS production or interact with subcellular structures, such as chloroplasts. This interaction can weaken the photosynthetic electron transport chain, decrease the electron transport rate and carbohydrate metabolism, and reduce cellular esterase activity and neutral lipid content (Zhu et al., 2021).

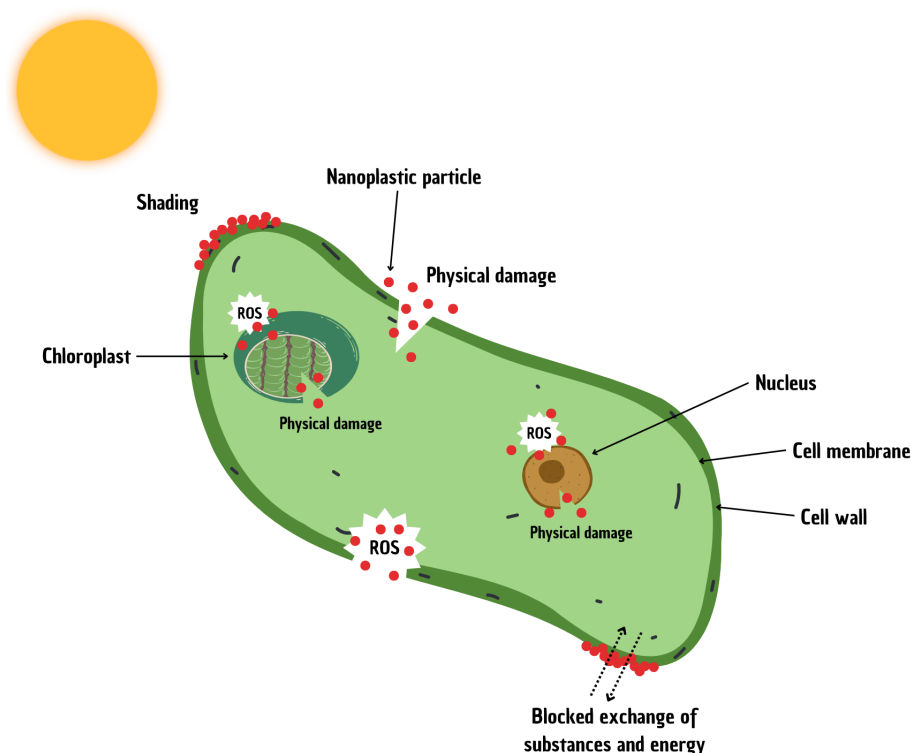


Figure 2.2: Mechanisms of NP toxicity to microalgae. (Based on Zhu et al. (2021))

2.4 Extracellular Polymeric Substances

2.4.1 Definition of EPS

EPS refer to large molecular compounds released by certain marine organisms outside their cells. Bacteria, archaea, and microeukaryotes are the primary groups of organisms known

to produce EPS. These polymers form a gel-like coating around the cells that acts as a defense against environmental stressors (Babiak and Krzemińska, 2021; Decho and Gutierrez, 2017). The microscope image in Figure 2.3 illustrates the encapsulation of algal cells within a layer of EPS.

EPS are categorized based on their binding force with cells. They can be divided into soluble EPS (SL-EPS) and bound EPS (B-EPS). SL-EPS dissolves into the solution, while bound EPS forms close associations with cells and is further classified into loosely bound EPS (LB-EPS) and tightly bound EPS (TB-EPS) (Ni et al., 2017). Most of the organic matter is distributed in TB-EPS, with a minor portion distributed in SL-EPS and LB-EPS.

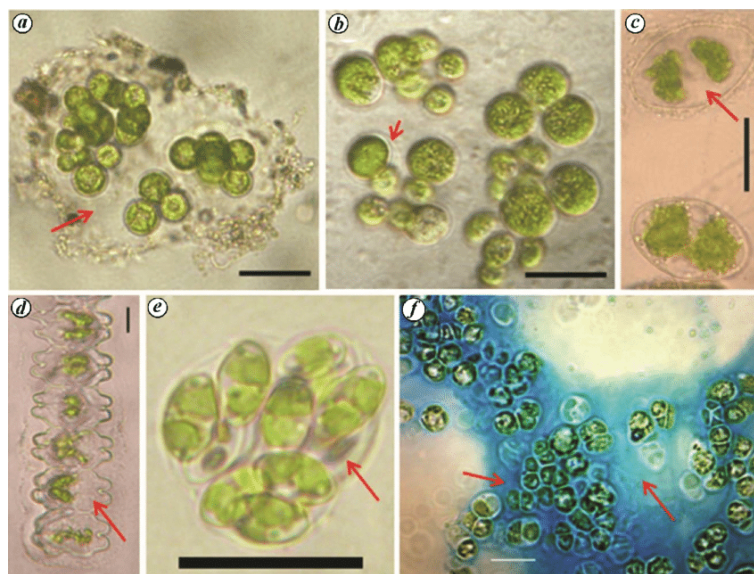


Figure 2.3: Green microalgae enclosed in EPS (red arrows). (a) *Bracteacoccus* sp., (b) *Chlorococcum humicola*, (c) *Cylindrocystis obesa*, (d) *Desmidium* sp., (e) *Oocystis lacustris* and (f) *Dictyosphaerium chlorelloides*. Scale bar = 10 μm . (Retrieved from Kumar et al. (2018))

2.4.2 Composition of EPS

The chemical composition and structure of EPS are diverse and depend on the species and growth conditions. Polysaccharides and proteins are the primary components, with fatty acids, nucleic acids, humic acids, amino acids, and other molecules present in smaller quantities. Key functional groups in EPS include carboxyl, hydroxyl, and amino groups, making EPS predominantly negatively charged (hydrophobic) due to the prevalence of hydrolyzed functional groups (Babiak and Krzemińska, 2021). Polysaccharides are mostly found in TB-EPS, while proteins are mostly found in SL-EPS and LB-EPS (Deng et al., 2022).

Polysaccharides, constituting 45-95w% of EPS, are a major component, with glucose and galactose being the most common sugar units observed in microalgal EPS (Babiak and Krzemińska, 2021). The primary roles of polysaccharides in EPS include adhesion, water retention, and sorption (particularly for charged polysaccharides), in addition to serving as a reservoir of nutrients and energy (Xiao and Zheng, 2016). Proteins represent 0.5-16.9 w% of microalgal EPS (Babiak and Krzemińska, 2021). Non-enzymatic proteins in EPS also play crucial roles in adhesion, water retention, sorption, and nutrient supply. Enzymatic proteins

are notably significant for their enzymatic activity, capable of breaking down exopolysaccharides and thereby serving as a carbon and energy source for the cells (Xiao and Zheng, 2016).

EPS composition can be variable depending on the source. Bacterial EPS exhibits a higher protein content. EPS of phytoplankton is generally richer in polysaccharides, resulting in a low Protein-to-Carbohydrate (P/C) -ratio. This P/C-ratio plays a crucial role in how EPS interacts with its surroundings. While proteins possess both hydrophilic and hydrophobic sides, their hydrophobic nature is generally dominant due to the abundance of specific amino acids with hydrophobic side chains. In contrast, polysaccharides are primarily hydrophilic due to their polar oxygen groups (Santschi et al., 2020).

Stressful conditions not only trigger an increase in the overall amount of EPS produced by algae, but also alter its composition. Studies show this shift in composition depends on the specific stressor. Deng et al. (2022) observed a rise in polysaccharide content, particularly in TB-EPS, when the cyanobacterium *Aphanizomenon flos-aquae* was exposed to gradually increasing temperatures. Conversely, Li et al. (2023b) found elevated protein levels in the EPS of the marine diatom *Thalassiosira pseudonana* after exposure to the pollutant tetrabromobisphenol A (TBBPA). This protein increase was more pronounced in B-EPS than in SL-EPS, and it intensified with higher TBBPA concentrations.

These findings suggest that similar changes in EPS composition, particularly the P/C-ratio, may occur in the presence of environmental stressors like NPs. Acknowledging the potential impact of such alterations is crucial, as it could significantly influence the interactions and functions of EPS in aquatic ecosystems.

2.4.3 Function of EPS in algae

EPS enable diverse functions that enhance the metabolism, adaptability and survival of algal cells (Decho and Gutierrez, 2017). They can either be produced as metabolic waste or intentionally excreted to serve a wide array of purposes (Natarajan et al., 2023). EPS acts as a protective shield against stress and toxins, as a source of nutrients and energy, promote algal aggregation, and facilitate attachment to substrates (Xiao and Zheng, 2016; Li et al., 2023a).

The diverse functionalities of EPS underscore its valuable role for marine microalgae. However, EPS is not essential for the life of the cells that release them. Cells can survive and grow without EPS. Nevertheless, the secretion of EPS significantly enhances the survival, metabolic efficiency, and adaptation of algal cells (Decho and Gutierrez, 2017).

Constructive EPS

EPS play a foundational role in structuring algal communities. EPS generates a matrix enriched with polymers released from cell surfaces into the extracellular space, delivering structural support, stability, and contributing to the organization of algal cells within biofilms or colonies. The pivotal structural elements within the EPS matrix are the two major constituents: polysaccharides and proteins (Neu and Lawrence, 2010).

Adsorptive EPS

EPS possesses charged functional groups that enhance its adsorptive capabilities. Through this mechanism, the EPS matrix can adsorb various dissolved substances from the environment, including nutrients, metals, and pollutants. Hydrophobic constituents such as carbohydrates with acetyl or methyl groups and proteins with carboxyl or amino groups play a role in the sorption, exchange, and release of dissolved compounds within the EPS matrix (Neu and Lawrence, 2010).

Protective EPS

Mishra and Jha (2009) found that as salt concentration increased, *Dunaliella salina* produced more EPS. Similarly, Li et al. (2023a) investigated how EPS helps microalgae resist copper (Cu) and zinc (Zn) toxicity. They observed an increase in EPS when exposing *Chlamydomonas reinhardtii* to Cu and Zn. They also compared the response of *C. reinhardtii* exposed to these metals with and without EPS. Results showed that after removing EPS, the growth of microalgae was further inhibited by Cu and Zn, with EC50% reductions of $28.6 \pm 3.4\%$ for Cu and $19.1 \pm 2.6\%$ for Zn, indicating the role of EPS in metal tolerance.

Besides an increase in EPS quantity under stress, also changes in EPS composition were observed. Li et al. (2023a) observed a 2.4 times increase in the EPS polysaccharide content, and 2.1 and 2.5 times increase in the EPS protein content compared to the control, when exposed to respectively 5 mg/L of Cu and Zn.

Nutritive EPS

Algae depend on essential nutrients, including carbon, nitrogen, and phosphorous, to sustain their metabolic processes. EPS offer a diverse range of constituents with variable biochemical composition, making them an ideal nutrient source. Polysaccharides can serve as a carbon source, proteins contribute nitrogen, and extracellular nucleic acids provide access to phosphorous. Previous research has demonstrated that bacteria utilize EPS as a carbon source (Wolfaardt et al., 1995). Numerous studies have also highlighted the significant role of EPS in accumulating nutrients and carbon through sorptive processes, with subsequent utilization of these accumulated materials (Neu and Lawrence, 2010).

2.4.4 Interaction between nanoplastics and EPS

Various marine organisms release EPS into the surrounding water column. These detached EPS, along with other Natural Organic Matter (NOM), can form an eco-corona layer around NPs. An eco-corona refers to the diverse biomolecular coating that forms on the surface of NPs when they interact with NOM, particularly EPS (Junaid and Wang, 2021). Several factors influence this eco-corona formation, including characteristics of the NPs (like age and surface charge), properties of the EPS (such as composition and surface charge), and the environmental conditions (such as salinity, temperature, and pH). This eco-corona is important because it can alter the NPs' physicochemical properties, fate, how they interact

with living organisms (bioreactivity), and their overall impact on ecosystems (Junaid and Wang, 2021).

Most research suggests that eco-corona formation lessens the toxic effects of NPs on aquatic organisms. For example, Natarajan et al. (2023) found that an eco-corona formed from EPS significantly improved cell viability, reduced oxidative stress, and decreased membrane permeability in the marine algae *Chlorella sp.* exposed to PS NPs. Most studies attribute the decrease in toxicity to a reduction in ROS generation, as the EPS molecules sequester free radicals. However, some studies have presented contradictory results, underscoring the need for additional research to conclusively determine the role of eco-corona formation in the toxicity of NPs in aquatic environments (Junaid and Wang, 2021).

2.5 Aggregation

Algae, widespread in aquatic realms, naturally form clusters and colonies which sculpt ecosystem dynamics. As plastic pollution spreads through water bodies, the likelihood of interactions between microalgae and NPs increases (Zhu et al., 2021). EPS play a central role in these interactions, determining not only the initiation of interactions between plastics and microalgae but also shaping the subsequent fate and behavior of the formed aggregates. Unraveling the pivotal role of EPS in aggregate formation is crucial for addressing the pressing challenges posed by plastic pollution.

2.5.1 Aggregates

In the marine environment smaller particles are suspended in the water column whereas larger particles settle, controlling the vertical transport of material. These two particle classes are interconnected through processes such as aggregation, flocculation, and coagulation. Aggregation is a general term describing the clumping together of suspended particles into larger settling particles, driven by factors like collision and electrostatic interactions (Jackson and Burd, 1998). Flocculation (Figure 2.4: a) specifically occurs when particles aggregate due to bridging by polymeric substances like EPS. Coagulation (Figure 2.4: b), on the other hand, refers to the aggregation of particles with opposite charges, resulting from a reduction in inter-particle electrostatic repulsion (Concha A., 2013).

Particles can aggregate with particles of the same type, a phenomenon known as homo-aggregation. Alternatively, they can aggregate with different types of particles present in the water column, for example natural colloids (such as minerals, microorganisms, or proteins), resulting in hetero-aggregation. Given the low predicted environmental concentrations of nanoparticles, homo-aggregation of nanoparticles (Figure 2.1: 3b) is less likely due to the low probability of collision. hetero-aggregation of nanoparticles (Figure 2.1: 3c) is considered more environmentally relevant due to the abundance of natural colloids relative to nanoparticles (Bundschuh et al., 2018).

Several factors influence the hetero-aggregation of nanoparticles, including i) electrostatic interactions, ii) the presence of NOM, and iii) bridging processes (Bundschuh et al., 2018).

- i) Electrostatic interactions play a role in the kinetics of nanoparticles and natural colloid aggregation. These aggregation kinetics are typically faster when the natural colloids and nanoparticles are of opposite charge and slower when they have the same charge.
- ii) NOM can diminish hetero-aggregation through electrostatic and steric stabilization. The presence of a NOM layer (Figure 2.1: 3a) around the NP alters its charge and creates a physical barrier, hindering aggregation.
- iii) Bridging processes (Figure 2.1: 3d), facilitated by polymers, hydrogen bonds, or chemical bonds, can promote aggregation between natural colloids and nanoparticles.

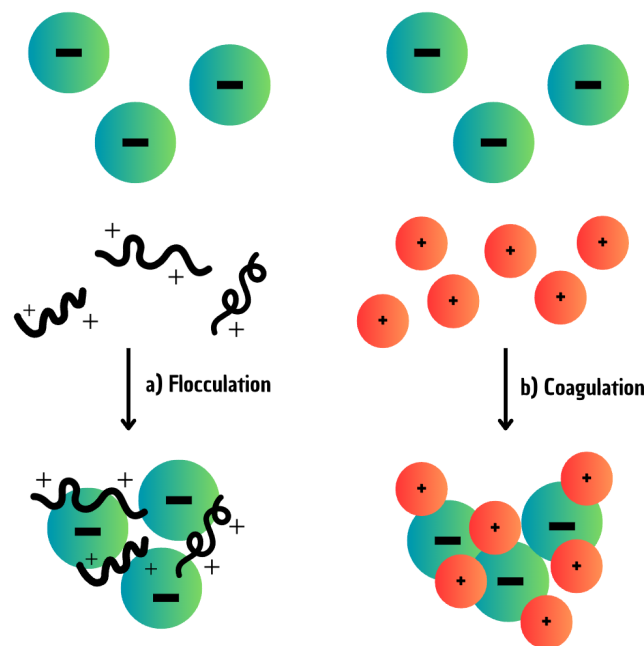


Figure 2.4: Mechanisms of flocculation and coagulation. (Based on Suopajärvi (2015))

2.5.2 Role of EPS in aggregation dynamics

This discussion explores how EPS influence aquatic aggregation by bridging microalgae and NPs, impacting charge dynamics and exhibiting adhesive properties. Moreover, it will discuss the impact of EPS composition, specifically focusing on the P/C-ratio.

Bridging

The charge of the particles in hetero-aggregates plays an important role. Algal cells typically bear a negative charge as a result of the prevalence of anionic functional groups (carboxyl, hydroxyl, and phosphate) on their surfaces. While EPS predominantly carry a negative charge due to the dominance of carboxyl and hydroxyl residues, they also harbor positive charges. This property enables them to act as bridges between algal cells and NPs (Figure 2.5). When an EPS layer surrounds algal cells, incorporating both negative and positive charges, it facilitates interaction with NPs irrespective of their charge (Babiak and Krzemińska, 2021).

Charge neutralization

In instances where algal cells or NPs possess similar charges, electrostatic forces cause repulsion. The presence of negative/positive charges in EPS can partially neutralize this charge (Figure 2.6), thereby mitigating the repulsion between positive/negative NPs or algal cells and promoting homo-aggregation (Babiak and Krzemińska, 2021).

Stickiness

The sticky nature of EPS can enhance the attachment of NPs on the EPS that has been secreted by certain marine organisms, resulting in aggregate formation. These aggregates exhibit an increased tendency to sink in the water column (Long et al., 2017).

Composition

Not only the presence of extracellular polymeric substances (EPS), but also its composition significantly influences aggregation dynamics. Higher protein content in EPS is linked with a higher relative hydrophobicity of the EPS. The protein and amino side chains of EPS induce interactions with hydrophobic substances in the water such as oil, organic pollutants, organic matter and plastics. Therefore EPS with higher a P/C-ratio typically leads to more aggregation (Santschi et al., 2020).

It has also been proven that EPS with a higher P/C-ratio tends to be stickier. This stickiness is another possible explanation for why EPS with a higher protein content can enhance aggregation (Shiu et al., 2020).

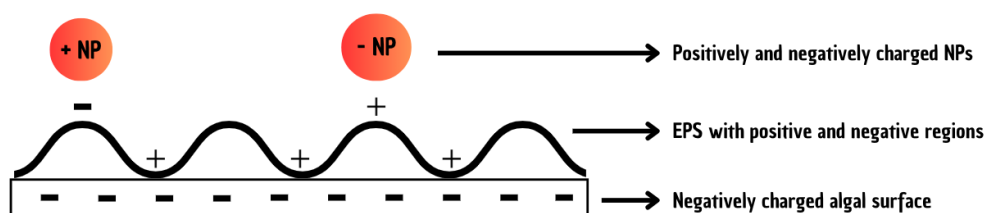


Figure 2.5: Mechanism of bridging. (Based on Babiak and Krzemińska (2021))

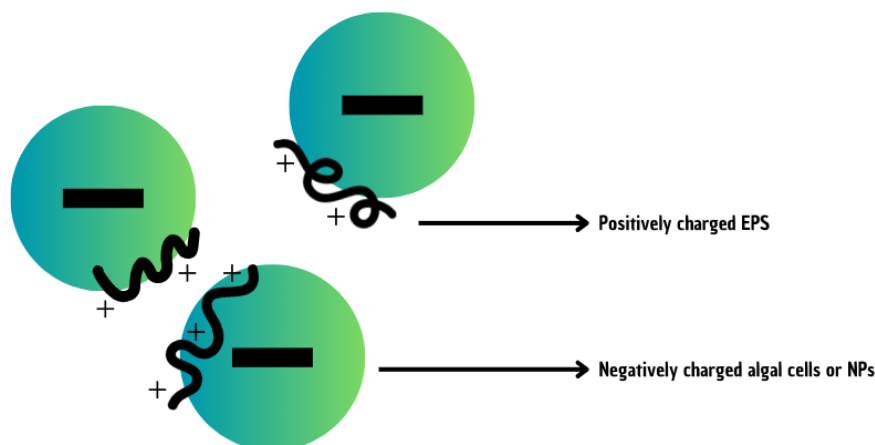


Figure 2.6: Mechanism of charge neutralization. (Based on Babiak and Krzemińska (2021))

2.5.3 The effects of aggregation

Homo-aggregation of nanoplastics

homo-aggregation of nanoparticles is strongly influenced by their concentration in the environment (Bundschuh et al., 2018). While the typically low environmental concentrations of NPs may limit the extent of homo-aggregation, it remains an important factor to consider. When NPs homo-aggregate, they form larger particles that tend to sink faster or become immobilized (Figure 2.1: 3e). This reduced mobility and bioavailability compared to dispersed NPs translates to less exposure for organisms in the water column, potentially mitigating some of the adverse effects (Gonçalves and Bebianno, 2021).

Homo-aggregation of algae

An experiment conducted by Mao et al. (2018) involved exposing the green single-celled microalgae *Chlorella pyrenoidosa* to PS MPs throughout an entire growth period. Their findings revealed an initial depression in phytoplankton activity during the initial exposure to MPs. However, in the later stages of the growth period, specifically from the end of the logarithmic phase to the stationary phase, a recovery of algal growth and activity was observed. The primary mechanism attributed to this recovery was the homo-aggregation of algal cells. The formation of algal communities through homo-aggregation served as a protective measure, reducing the algal cells' surface-to-volume ratio. This, in turn, minimized the adsorption of MPs on the surface of the cells (Mao et al., 2018).

Hetero-aggregation of nanoplastics and algae

Hetero-aggregates of algae and NPs have an increased tendency to settle to the bottom compared to the non-aggregated forms (Mao et al., 2018). The sinking behavior of these aggregates depends not only on their size but also on their shape and concentration (Gigault et al., 2018). This aggregation between NPs and algae can result in various adverse effects on algal cells, as previously described. Moreover, algae act as carriers within

these hetero-aggregates, transporting NPs downward in the water column, leading to an increased abundance of NPs in the sediment. NPs within these hetero-aggregates are also more likely to be consumed by zoobenthos (Yang et al., 2021).

3. OBJECTIVE

3.1 Problem statement

The well-being of life on our planet is intricately linked to the health of marine ecosystems (Fleming et al., 2019). Recognizing the critical role played by marine microalgae in sustaining these ecosystems, it is essential to grasp the repercussions of increasing nanoplastic (NP) exposure. Gaining insight into the impact of NPs on marine microalgae is pivotal for developing measures to protect their equilibrium and vitality, thereby safeguarding our oceans.

In recent years, the scientific community has raised concerns about the impact of NP pollution on marine ecosystems. Despite this growing awareness, studies on NP ecotoxicology show an uneven distribution across biological categories. In 2020, over half of the conducted studies exploring the ecotoxicity of NPs were focused on invertebrates (Piccardo et al., 2020). Fish accounted for the second-largest proportion at almost 17%, while algae comprised less than 13% of the studies (Piccardo et al., 2020). Consequently, there is still a lack of a comprehensive understanding of how marine microalgae respond to NP exposure.

Assessing the environmental impact of NPs is further complicated by limited research on realistic exposure scenarios. Most studies utilize spherical polystyrene (PS) NPs, neglecting the diversity of potential polymer types and shapes (Cunningham et al., 2023). Furthermore, current methods for detecting NPs in the environment face numerous limitations, resulting in a significant knowledge gap regarding actual environmental NP concentrations. As a result, many toxicity studies employ unrealistically high NP concentrations that are not representative of natural conditions (Zhu et al., 2021). Additionally, most research focuses on newly manufactured NPs, while real-world exposure often involves aged NPs that have undergone weathering processes. These aged NPs may have altered properties and different toxicity profiles compared to their newly manufactured counterparts (Cunningham et al., 2023).

The lack of knowledge on the effects of environmentally relevant NP exposure on microalgae makes it difficult to evaluate potential risks and create effective mitigation plans. To address these concerns, this study will investigate exposure conditions that better mimic the actual marine environment while also exploring understudied exposure conditions to fill current research gaps.

3.2 Research questions

This thesis seeks to enhance the understanding of marine microalgae's responses to environmentally relevant NP exposure, with a particular focus on the role of EPS. It explores how NP exposure affects the production and composition of EPS. Furthermore, the thesis examines the importance of EPS in the dynamics of algal aggregation, including the size and composition of the aggregates formed. Ultimately, it provides perspectives on how changes in EPS composition and aggregation might impact the broader marine environment. Various experimental setups and relevant literature are utilized to shed light on these topics.

To assess these problems two research questions are proposed:

- How does NP exposure affect the amount and composition of EPS produced?
- How do changes in the amount and composition of EPS, due to NP exposure, influence the amount and composition of aggregates formed?

4. MATERIALS AND METHODS

4.1 *Rhodomonas salina* culture

The brackish marine microalgae species *Rhodomonas salina* was used as test-organism. This culture was sourced from the Culture Collection of Algae & Protozoa (CCAP) in 2016 and has since been maintained in the Department of Animal Sciences and Aquatic Ecology at Ghent University.

R. salina was cultured in L1 medium as described by Guillard and Hargraves (1993). The L1 medium consisted of filtered seawater and 4 stock solutions (NaNO_3 , $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$, a trace element mix and a vitamin mix). The filtered seawater was made by combining 80% (v/v) natural seawater (from SEA LIFE, Bredene) with a salinity of 24 ppt, with 20% (v/v) deionized (DI) water, and then filtering the mixture through a $0.20\ \mu\text{m}$ filter. The stock solutions were prepared according to the L1 medium recipe from the CCAP. This media preparation recipe can be found in Appendix A.1.

4.2 Particle types and characteristics

The following sections will describe the different particle (PTC) types used in the exposure experiments, detailing key characteristics like size and concentration. For a thorough characterization of these particle types, comprising particle size distributions, SEM images, DLS assessments of zeta potential and hydrodynamic diameter, ATR-FTIR spectroscopic analyses, and details concerning PET additives, refer to Appendix B.

4.2.1 PET and aged-PET

Two types of NPs were used in the exposure experiments: virgin PET and aged PET. The aged PET was chemically weathered with potassium hydroxide. These plastics were sourced from the Institute for Reference Materials and Measurements (IRMM) at the Joint Research Centre (JRC) in Geel, Belgium. Concentration and size distribution (SD) of the plastic suspensions were assessed using the ClassizerONE (S1.4.34, EOS) by JRC in Milan, Italy. The virgin PET NP stock solution had a concentration of 1.6×10^6 PTCs/mL and a D_{10} , D_{50} and D_{90} of $0.57\ \mu\text{m}$, $0.72\ \mu\text{m}$ and $1.4\ \mu\text{m}$ respectively. The aged-PET NP stock solution had a concentration of 2.9×10^7 PTCs/mL and a D_{10} , D_{50} and D_{90} of $0.47\ \mu\text{m}$, $1.2\ \mu\text{m}$ and $3.6\ \mu\text{m}$ respectively.

When strictly defining NPs ($< 1\ \mu\text{m}$) and MPs ($1\ \mu\text{m} - 5\ \text{mm}$), the aged-PET and PET solutions cover not only NPs but also MPs. However, none of the particle diameters exceed $11\ \mu\text{m}$,

indicating that MP exposure falls within the smaller MP range. Henceforth, the term MNPs will be utilized to discuss the plastic exposures.

Dipalmitoylphosphatidylcholine (DPPC)

The PET MNPs used in this study arrived pre-dispersed in a solution containing 0.1% DPPC, a surfactant. While DPPC is essential for the initial dispersion of the PET particles, it is crucial to regulate its potential impact on the experiment by ensuring uniform DPPC concentrations across all samples. The 10000 PTCs/mL exposure concentration represented the highest concentration of PET in this study, and thus also the highest level of DPPC. Resulting in a concentration of 3.2×10^{-7} % after washing and diluting during preparation of the experimental set-up. Therefore, the DPPC levels in all other exposures were brought to this level to control for DPPC effects.

To achieve this, the PET solution was filtered over a 20 nm filter to retain PET MNPs but allow smaller DPPC molecules to pass through. The filtrate, containing the 0.1% DPPC solution, was diluted 1000-fold with L1 medium to achieve a final concentration of 0.0001% DPPC. Subsequently, this diluted 0.0001% DPPC solution was added in the required amount to all Erlenmeyer flasks, except those corresponding to the 10000 PTCs/mL PET exposure, to bring them to a uniform DPPC concentration.

4.2.2 Kaolin

Kaolin particles (Imerys, Polwhite E) were used as a control exposure to account for potential effects of non-plastic particles. To prepare the kaolin solution, a small amount of kaolin was mixed with deionized (DI) water to form a thick clay suspension. This suspension was subsequently filtered through a 5 μ m membrane to yield clay particles within a comparable size range to the aged-PET and PET MNPs.

The SD of the kaolin stock suspension was determined by placing a drop on a Bürker counter glass slide and capturing images of each grid. ImageJ software was utilized for image analysis, involving contrast adjustment and measurement of average particle size. The script used for this analysis is provided in Appendix C.1. The average particle size and particle count were then converted into a concentration of the kaolin suspension using the formula below. The ImageJ analysis yielded a concentration of 9.83×10^6 PTCs/mL.

$$\text{Cell density} \left(\frac{\# \text{ cells}}{\text{mL}} \right) = \frac{\text{Counted cells} * 10 \text{ (dilution factor)} * 10^3 \left(\frac{\mu\text{L}}{\text{mL}} \right)}{0.9 \mu\text{L (volume of the chamber)}}$$

The SD and concentration of the kaolin solution were also determined by the JRC. This yielded a concentration of 1.9×10^7 PTCs/mL and a D_{10} , D_{50} and D_{90} of 0.59 μ m, 1.2 μ m and 2.7 μ m respectively. These results confirmed that the kaolin particles exhibited a similar SD as the plastic solutions and that the concentration calculated with the Bürker counter was a suitable proxy for the real concentration.

4.2.3 Concentration series

Before being utilized in the experiments, the plastic solutions and kaolin solution were washed three times to remove any remaining suspension solution. This procedure involved the addition of L1 medium, followed by centrifugation, and subsequent removal of the supernatant. Following the washing steps, dilution series were prepared using L1 medium to attain exposure concentrations of 10, 100, 1000, and 10000 MNP PTCs/mL.

The exposure concentration values were based on the observed MP concentration in the North Sea of 1.5 MP/m^3 (Everaert et al., 2022), multiplied by a conversion factor assuming the complete degradation of a $250 \text{ }\mu\text{m}$ spherical PET particle into particles with a SD corresponding to the SD of the particles used in these experiments. This method is based on mass conservation principles as was proposed by Besseling et al. (2018). The complete calculations can be found in Appendix B.6.

Although this research uses concentrations measured in PTCs/mL, most studies report concentrations in g/L. Therefore, Table 4.1 below provides an estimate of the g/L concentration for each exposure group. This conversion uses the density of the exposure materials and the D_{50} diameter, assuming the particles are perfectly spherical.

Exposure	10 PTCs/mL in g/L	100 PTCs/mL in g/L	1000 PTCs/mL in g/L	10000 PTCs/mL in g/L
PET	$2.7 \cdot 10^{-9}$	$2.7 \cdot 10^{-8}$	$2.7 \cdot 10^{-7}$	$2.7 \cdot 10^{-6}$
aged-PET	$1.2 \cdot 10^{-8}$	$1.2 \cdot 10^{-7}$	$1.2 \cdot 10^{-6}$	$1.2 \cdot 10^{-5}$
kaolin	$2.4 \cdot 10^{-8}$	$2.4 \cdot 10^{-7}$	$2.4 \cdot 10^{-6}$	$2.4 \cdot 10^{-5}$

Table 4.1: Estimation of the exposure concentrations of PET, aged-PET and kaolin in g/L.

4.3 Experiment 1: Effect of MNP exposure on quantity and composition of algal EPS

This experiment investigated how exposure to MNPs affects the quantity and composition of EPS produced by *R. salina* cultures. To assess the quantity of EPS, the EPS will be extracted from the algal cells after MNP exposure and weighed after freeze-drying. To assess the composition of the EPS, ATR-FTIR will be utilized to obtain the absorbance spectra of the EPS and the ratio of the protein peak to carbohydrate peak will be determined. This analysis was conducted at different stages of algal growth: the beginning of exponential growth, the end of exponential growth, and the stationary phase. In Appendix D.2.1, a procedure employing salinity stress is elucidated as a means of method validation.

4.3.1 Set-up

Three particle types were used: PET, aged-PET, and kaolin. Each type was tested at four concentrations: 10, 100, 1000, and 10000 PTCs/mL. Erlenmeyer flasks were used, each containing a total volume of 50 mL consisting of: algal growth medium, algal culture, the

specific particle solution, and a DPPC solution. Each set of flasks included triplicates for each combination of exposure and concentration and 3 negative controls. A total of three identical sets of flasks were prepared, each corresponding to a specific growth stage. Figure 4.1 illustrates the set-up of one set of Erlenmeyer flasks.

4.3.2 Measurements

Set 1 corresponded to day 1-3 on which pH and cell count were measured daily. EPS was extracted on day 3. Set 2 corresponded to day 4-7 on which pH and cell count were measured daily and EPS was extracted on day 7. Set 3 corresponded to day 8-10 on which pH and cell count were measured daily and EPS was extracted on day 10. Figure 4.2 depicts the timeline of the measurements and extractions from day 1 to 10. Daily cell count and pH measurements followed the procedures outlined in Sections 4.5.1 and 4.5.2, respectively. The purpose of EPS extraction on days 3, 7, and 10 was to analyze the quantity and composition of EPS, as detailed in Section 4.5.3.

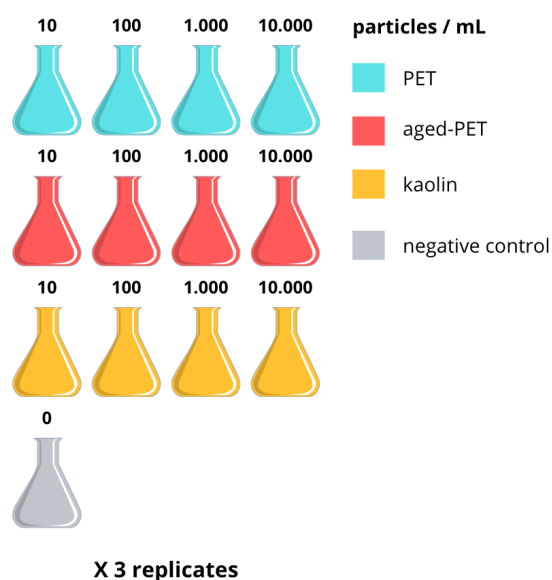


Figure 4.1: Visual representation of one experimental set.

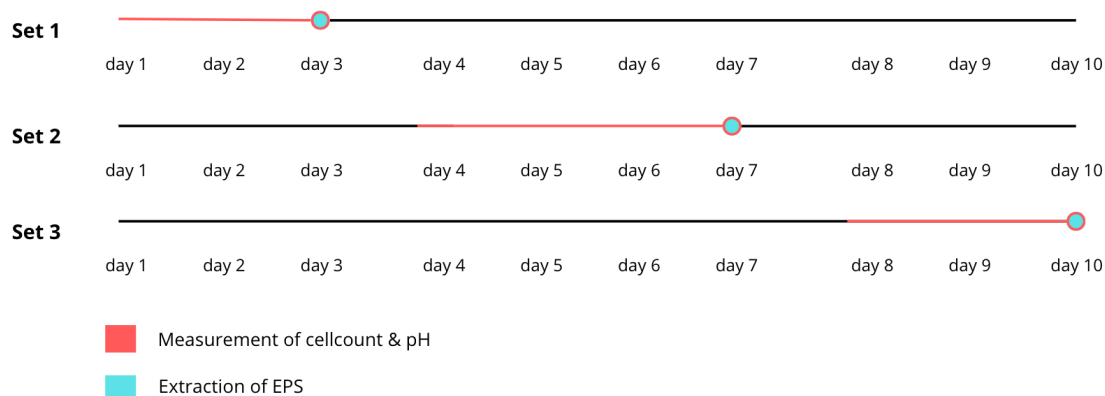


Figure 4.2: Timeline of the measurements and extractions from day 1 to 10.

4.3.3 Growth conditions

For the duration of the experiment the cultures were kept in a temperature controlled (20°C) room with a light:dark regime of 16:8 hours, at an intensity of 2500 lux. The position of the Erlenmeyers on the light bank was randomized and changed daily. To ensure good contact between the algae and the MNPs, the cultures were gently swirled once a day throughout the experiment.

4.4 Experiment 2: Effect of MNP exposure on aggregation

The second experiment investigated whether these potential changes in EPS quantity and composition due to MNP exposure affect the amount, size and composition of the aggregates that are formed. To assess the amount and size of the aggregates formed after exposure to MNPs, DLS will be used. To assess the composition of these aggregates, microscopy techniques are applied. In Appendix D.3.1, a procedure employing a chemical flocculant is elucidated as a means of method validation.

4.4.1 Set-up

For this second experiment the Erlenmeyer flasks were set up similar to Experiment 1, with three exposure types (PET, aged-PET, and kaolin) at four concentrations (10, 100, 1000, and 10000 PTCs/mL) but utilized a larger culture volume of 150 mL per flask (compared to 50 mL in Experiment 1). Each 150 mL flask consisted of: algal growth medium, algal culture, the specific particle solution, and a DPPC solution. Each exposure concentration was tested in triplicate flasks, and three negative controls were included. Unlike Experiment 1 with its focus on EPS at multiple growth stages, Experiment 2 focused on a single EPS extraction at the end of the exponential growth phase (day 10).

4.4.2 Measurements

Cell count and pH were monitored daily throughout the experiment (up to day 10) using the methods described in Sections 4.5.1 and 4.5.2. On day 10, 50 mL of the culture volume underwent EPS extraction to ascertain the quantity and composition of EPS, as detailed in Section 4.5.3. A second volume of 50 mL was allocated for Dynamic Light Scattering (DLS) measurements to evaluate aggregate amount and size distribution, as explained in Section 4.5.4. The final 50 mL was designated for microscopy analysis to assess aggregate composition, following the procedures outlined in Section 4.5.5.

4.4.3 Growth conditions

The growth conditions of Experiment 2 were identical to those in the first experiment.

4.5 Measurement techniques

4.5.1 Algal cell count

A Coulter counter (Z series Coulter Counter by Beckman Coulter) was used to monitor the growth of the algal cultures. The particle size was set at 4.000 μm . Since the algae were grown in a saltwater medium, a saltwater electrolyte solution was used for cell counting.

4.5.2 pH

A pH sensor (Consort, P407) was employed to monitor the pH levels of the algal cultures. This sensor was rinsed with DI water between each sample and was stored in a potassium bromide solution when not in use.

4.5.3 EPS

EPS extraction

To evaluate whether MNP exposure affects the amount and composition of EPS produced by *R. salina*, the EPS must be extracted from the algal cells. Various EPS extraction methods were tested to identify the most suitable technique for downstream experiments. The comparison of these methods is detailed in Appendix D.1. Based on the highest yield of EPS, the ultrasonic method was chosen as the most suitable method.

During this method, EPS is extracted by first centrifuging the algal suspension at 3800 rpm for 15 minutes using a centrifuge (Thermo Fisher Scientific Heraeus Magafuge 40R). The supernatant, containing LB-EPS, is discarded since the focus is on TB-EPS, which is more strongly associated with the algal cells and contains most of the organic matter. The resulting pellet is then resuspended in 10 mL of 0.05% NaCl per gram of wet biomass. This suspension is placed into an ultrasonic bath (VWR USC300T) at 80W for 10 minutes. Afterward, the suspension undergoes another centrifugation at 3800 rpm for 15 minutes. The resulting supernatant, considered the TB-EPS extract, is filtered through a 0.22 μm membrane and stored at -60°C until freeze-drying.

Refer to Figure D.1 in Appendix D.1 for a visual representation of these steps. This section of the Appendix also includes the additional experiments conducted to validate the integrity of the EPS extraction process.

EPS quantity

To determine the amount of EPS produced under different exposure conditions, a gravimetric approach was employed. Pre-weighed centrifuge tubes were used to collect the filtrate after the final centrifuging step in the EPS extraction process. Each tube was labeled and then freeze-dried along with the captured EPS. The weight of the empty tube was subtracted from the final weight to determine the dry weight of the EPS produced.

EPS composition

Attenuated Total Reflectance Fourier Transform Infrared Spectroscopy (ATR-FTIR) was used to investigate the chemical composition of the EPS. This method works by directing an infrared light beam through a crystal at an angle that causes it to reflect internally. The EPS sample, placed on the crystal surface, absorbs certain infrared wavelengths. These absorbed wavelengths correspond to the functional groups present in the EPS, revealing information about the types of chemical bonds within the sample.

To obtain the EPS absorbance spectrum, a small amount of the homogenized and freeze-dried EPS powder was placed on the ATR-FTIR crystal (Nicolet iN10 MX) for each exposure replicate. The number of replicates varied between experiments, with three replicates used for the first experiment and two for the second experiment, resulting in a total of two to three ATR-FTIR spectra per exposure replicate. The instrument settings were configured for 16 sample scans, 16 background scans and a resolution of 4.000 cm^{-1} . After the spectra had been obtained, a characteristic peak for carbohydrates ($\sim 1010\text{ cm}^{-1}$) and proteins ($\sim 1650\text{ cm}^{-1}$) in algal biomass was chosen (Ferro et al., 2019). Using Spectragryph (F. Menges, version 1.2.16, 2022), an optical spectroscopy software tool, the integrated area under each peak was determined after correcting for baseline variations. For carbohydrates, the integration region spanned wavelengths from 950 cm^{-1} to 1250 cm^{-1} . Similarly, the protein peak integration covered the 1500 cm^{-1} to 1750 cm^{-1} range. Finally, the P/C-ratio was calculated by dividing the integrated area of the protein peak by the integrated area of the carbohydrate peak. This ratio provides a relative measure of the protein content compared to the carbohydrate content in the EPS sample.

4.5.4 Aggregate size distribution

Dynamic Light Scattering (DLS) was utilized to determine the volume-based SD of particles in the samples. This method involves directing a laser beam onto the sample and detecting the scattered light resulting from the Brownian motion of suspended particles. Smaller particles exhibit more pronounced Brownian motion compared to larger ones, which influences the pattern of scattered light.

For the DLS measurements, a Mastersizer S instrument was employed. This apparatus includes a laser beam, a focusing lens (300 RF), and a flow cell. The algal culture and seawater mixture were contained in a round-bottom flask separate from the Mastersizer. A closed-loop circulation system was established using tubing and a peristaltic pump. The sample solution flowed from the bottom of the flask, through the flow cell within the Mastersizer S, and then returned to the top of the flask via another tube connected to the pump. The information about the scattered light collected by the detector was then sent to a computer. Following background subtraction and sample analysis, the DLS software provides data on the volume percentage of particles within a certain size range. Figure 4.3 is a schematic diagram of the experimental set-up used for DLS measurements.

To ensure a consistent concentration of algal culture the obscuration was considered. Obscuration, indicating the amount of light blocked by particles, should neither be too low, resulting in a weak signal and noise, nor too high, as excessive obscuration may cause

multiple scattering and signal distortion (De Cleyn et al., 2019). Algal culture was added incrementally to 120 mL of filtered seawater until an obscuration of 11% was achieved. The necessary volume of other samples to reach this 11% obscuration was calculated based on their cell count relative to the initial algal culture, then increased by 30% to ensure meeting the lower limit of 11% obscuration.

Refer to Appendix D.3.2 for some additional tests that were performed to validate this method.

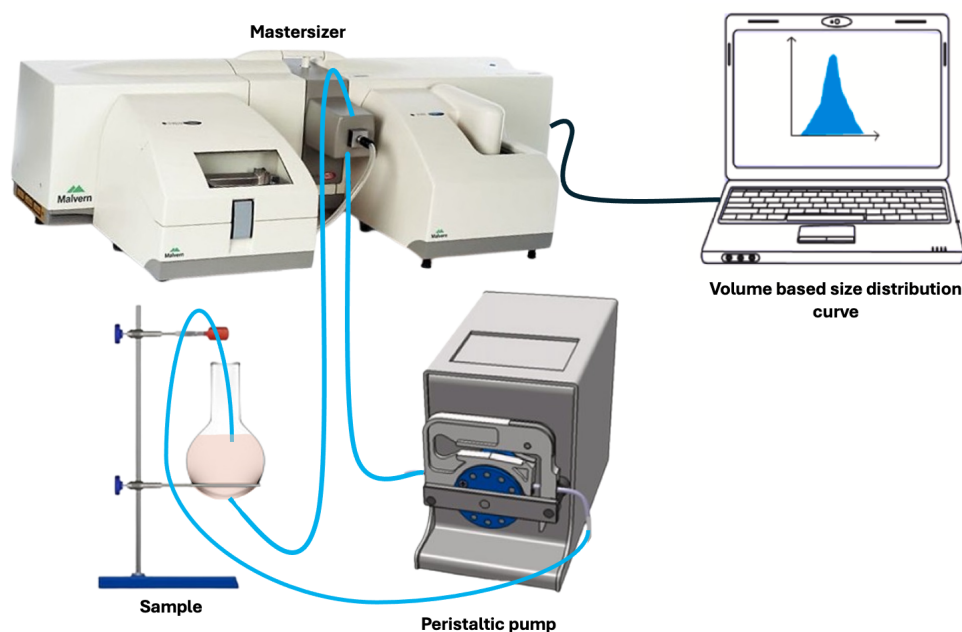


Figure 4.3: Schematic diagram illustrating the set-up for the DLS experiment. (Based on Ho et al. (2022))

4.5.5 Aggregate composition

Microscopy was used to quantify the percentage of algal cells present in aggregates as well as the percentage of EPS in association with aggregates. Two milliliter of each sample was diluted with 3 mL of L1 medium in a 6-well plate and allowed to settle for 1 hour to facilitate aggregate observation. The samples in the microplates were analyzed using 'Cytation 3 Cell Imaging Multi-Mode Reader' (BioTek Instruments Inc., Winooski, USA) to collect microscopic photographs of each well under a brightfield and autofluorescence (using Cy5.5 filter cube) setting. The high-resolution photographs were taken at 16 different points in a 4×4 matrix (Figure 4.4).

These images were then analyzed using a semi-automatic script developed in ImageJ which is provided in Appendix B.2. The script prompts the user to manually outline the aggregates in each image. For time efficiency, only 2 aggregates per photo were randomly selected for outlining, focusing solely on the control and the highest concentrations (10000 PTCs/mL) of each exposure. With 2 aggregates outlined per photo, 16 photos taken per well and 3 wells (representing 3 replicates) for each exposure, this resulted in 96 outlined aggregates per exposure condition.

4. Materials and methods

For the outlining process, some pre-defined rules were followed. Firstly, only aggregates containing both algal cells and visible EPS were outlined and counted. This ensured focus on aggregates where both components were potentially interacting. Secondly, all algal cells in direct contact with the EPS were included, regardless of their orientation. Finally, if an algal cell was connected to another algal cell that was itself directly in contact with the EPS, only cells connected with their long side to the neighboring cell were included in the aggregate. Cells with only short-side connections to the neighboring cell were excluded. These outlining criteria are visually represented in Figure 4.5.

After performing these manual outlining steps, the script automatically computed several parameters for each aggregate. These included the number of algal cells in the aggregate, the total area of the aggregate and the area of the aggregate occupied by algal cells.

Refer to Appendix D.3.3 for some additional tests that were performed to validate this method.

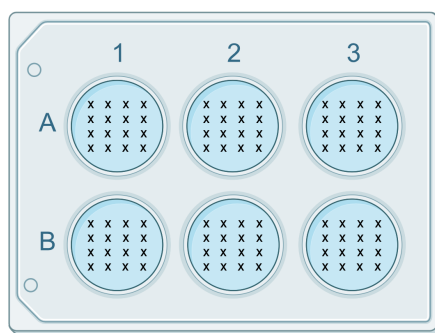


Figure 4.4: 4x4 pictures for each well of the six-well plate

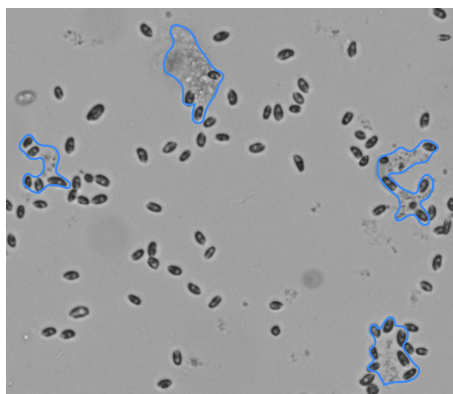


Figure 4.5: Example of the outlining of aggregates.

4.6 Data processing and statistical analysis

4.6.1 Data processing

The data obtained from the various experiments was implemented in Excel (version 16.82). Afterwards the data was read into RStudio (version 4.2.3) for further statistical analysis and visualization. All plots were made using the "ggplot2" package.

Algal growth data

The daily cell count measurements during the exposure experiments were implemented in Excel. Afterwards the data was read into RStudio for model fitting. The Baranyi growth model was selected based on its suitability for capturing the growth stages observed in the algal cell count data, resulting in a favorable fit (Baranyi and Roberts, 1994). The Baranyi model has following equation:

$$A = \text{time} + \frac{1}{\mu_{\max}} * \log(\exp(-\mu_{\max} * \text{time}) + \exp(-\mu_{\max}) - \exp(-\mu_{\max} * \text{time} - h_0))$$

$$\log(y) = \log(y_0) + \mu_{\max} - \log\left(1 + \frac{\exp(\mu_{\max} * A) - 1}{\exp(\log(K) - \log(y_0))}\right)$$

where: y_0 represents the initial abundance, $\mu_{\max}$ denotes the maximum growth rate, K signifies the carrying capacity and h_0 specifies the initial physiological state of organisms.

For model fitting, initial parameters and box constraints were selected to assist the optimization algorithm in locating a reasonable starting point in the parameter space. These values were determined based on the measured data.

	y_0	$\mu_{\max}$	K	h_0
Initial parameters	10000	1	3000000	1
Lower box constraints	90000	0.2	2000000	0
Upper box constraints	20000	1.2	4000000	10

Table 4.2: Initial parameter values and box constraints.

The model fitting process was iterated, with each subsequent run utilizing the coefficients from the previous run as initial parameters. Following three rounds of fitting, the R-squared value ($R^2 = 0.9968$) and sum of squares ($RSS = 0.0493$) reached stability. The high overall R^2 and low overall RSS indicate the goodness of the fit and the suitability of the Baranyi growth model. Individual growth curves were fitted to the cell count data for each combination of concentration, exposure, and replicate. The Baranyi model effectively captured the growth dynamics, as demonstrated in Appendix E.1. Subsequently, the maximum growth rate ($\mu_{\max}$) and carrying capacity (K) were derived from the fitted model. These growth parameters were utilized for visualization and statistical analysis.

Technical issues with the Coulter counter during Experiment 2 resulted in the inability to take measurements on days 3 and 9. To address this, the "zoo" package in RStudio was employed to manage the missing values. Linear interpolation was used to estimate cell counts for these missing days. Consequently, caution is taken in interpreting this data. Additionally, in Experiment 2, adjustments were made to accommodate the variations in growth data compared to Experiment 1. Specifically, the K value of the Lower box constraints was reduced from 2000000 to 1500000 cells/mL. Apart from this modification, the procedures remained identical and resulted in a high overall R-squared value ($R^2 = 0.9854$). However, the sum of squares (RSS) was slightly higher ($RSS = 0.1707$), potentially due to the more erratic nature of the cell count data in this experiment. This discrepancy is also

evident in the Baranyi fit plots in Appendix E.2, which capture the data points less well than those in Experiment 1.

EPS data

The weight of the extracted and freeze-dried EPS was implemented in Excel and read into RStudio. In RStudio the EPS weight was normalized by the cell count of the respective sample to obtain the amount of EPS per cell. This parameter was used for visualization and statistical analysis.

The with Spectragryph determined integrated peak areas for both the protein and carbohydrate peak were implemented in Excel and then read into RStudio. In RStudio, the ratio between the protein and carbohydrate peaks was calculated for visualization and statistical analysis.

DLS data

After conducting DLS measurements, the Mastersizer software generated volume percentage data at specific diameters for each sample in Excel. Figure 4.6 illustrates this, displaying the volume percentage plotted against the diameter for three samples. Additionally, the software provides the diameters for the main particle size classes. It's worth noting that the third peak around 200 μm was not consistently present in all samples and was excluded from further analysis. The first peak, approximately at 7 μm , is interpreted as representing single-celled algae. Meanwhile, the second peak, around 50 μm , is attributed to aggregated algae. The position and areas of these peaks serve as the parameters for subsequent analysis. Examining the position of the second peak provides insights into the average size of the aggregates. Furthermore, analyzing the ratio of the areas under the second and first peaks offers valuable information about the proportion of algal cells existing as aggregates versus individual cells.

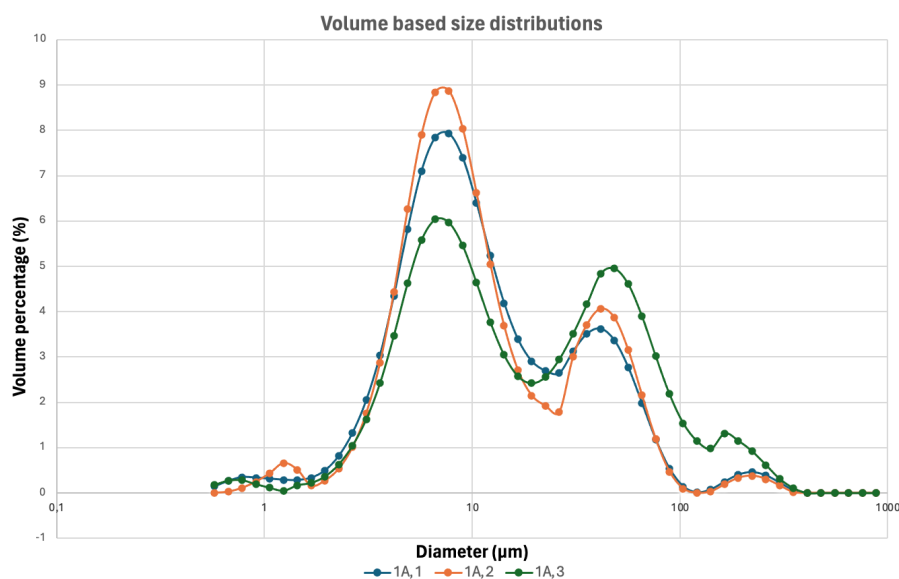


Figure 4.6: Example of the volume-based size distribution curve of three samples.

Microscopy data

Following the execution of the ImageJ script on all images, a CSV file was generated for each outlined aggregate, containing information on the number of algal cells in the aggregate, the total area of the aggregate, and the area of the aggregate occupied by algal cells. These CSV files were imported into RStudio, where the percentage of the aggregate area occupied by algal cells was calculated by dividing the area occupied by algal cells by the total area of the aggregate and then multiplying by 100. This parameter was subsequently utilized for visualization and statistical analysis.

4.6.2 Statistical tests

The same statistical protocol was applied consistently for growth data (K and μ_{max}), EPS weight, P/C-ratios of EPS, aggregate diameter, volume-based percentage ratio of aggregated algae to single celled algae, and the percentage of the aggregate area occupied by algal cells. Each dataset was divided into separate subsets for individual exposures and concentrations, facilitating the identification of significant differences between concentrations for a specific exposure and between exposures for a particular concentration. All datasets underwent statistical analysis using a significance level of 0.05, allowing for the attribution of significant differences within a 95% confidence level. Initially, a Shapiro-Wilk test evaluated the normality of each dataset, followed by a Levene's test to assess variance equality. If the data were normally distributed ($p > 0.05$) and no significant variance differences were detected ($p > 0.05$), a one-way ANOVA model was constructed for the dataset. Subsequently, if a significant difference was revealed between the group means ($p < 0.05$), a Tukey's HSD post-hoc test was conducted to pinpoint which means exhibited significant differences ($p < 0.05$). Alternatively, if either the Shapiro-Wilk test or the Levene's test indicated non-fulfillment ($p < 0.05$), a non-parametric Kruskal-Wallis test was employed to ascertain whether statistically significant differences existed among the group medians. Upon detecting significant differences with this test ($p < 0.05$), a post-hoc Dunn's test was utilized to identify which medians displayed significant differences ($p < 0.05$). In the Results, the p-values are reported along with the mean values when the Tukey test is used and the median values when the Dunn test is used. Additionally, the differences between means or medians are provided.

4.7 Generative AI-tools

ChatGPT (Version GPT-3.5) and Gemini (Google AI) were used throughout parts of this thesis for structuring, rephrasing and code refinement.

5. RESULTS

Due to the large number of experiments conducted, a vast amount of data was analyzed and tested for significant differences. All statistical tests were also performed across both exposures and concentrations. This resulted in many plots, which were not all included in these results for the sake of conciseness. The plots highlighting concentration variations within each exposure group were included. Although significant differences observed between exposures for a particular concentration are mentioned here, the figures for these comparisons can be found in Appendix F.

5.1 Experiment 1

5.1.1 Algal growth in response to micro- and nanoplastic exposure

Growth curves

The growth curves of *R. salina* depicted in Figure 5.1 offer insights into the cell growth over time, showcasing distinct growth phases. While the lag phase may not be prominently visible, there is evident exponential growth until day 6, followed by a plateau indicating the onset of the stationary phase at around 2.5×10^6 cells/mL. These growth curves, representing aged-PET, kaolin, and PET exposure across various concentrations, hint at potential effects of particle exposure on growth. Preliminary examination of the raw data suggests that exposure to kaolin particles stimulates growth compared to the control group, particularly during the exponential growth phase where the growth curves for the kaolin treatment surpass those of the negative control (0 PTCs/mL). In contrast, exposure to both PET and aged-PET MNPs appears to inhibit growth at certain concentrations, as evidenced by the growth curves falling below those of the control group at various concentrations. Detailed statistical analysis of these growth curves is presented in the subsequent section.

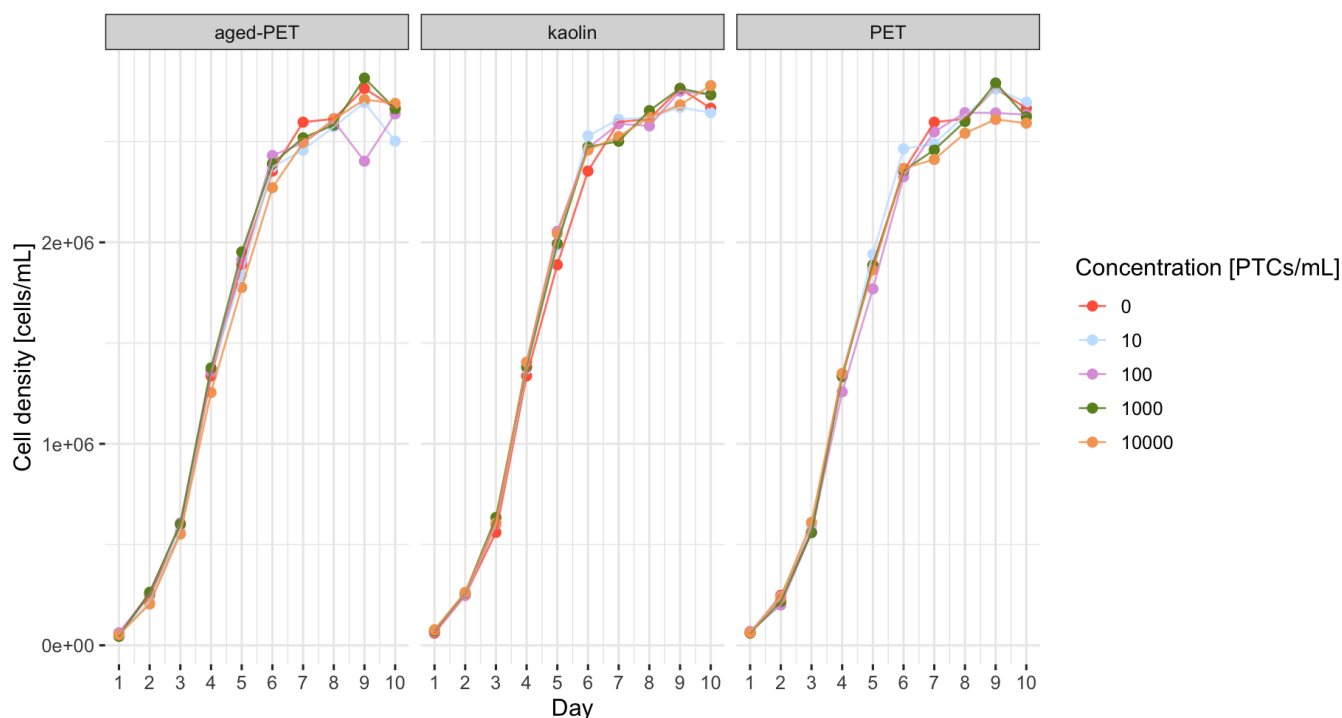


Figure 5.1: Growth curves of *R. salina* exposed to aged-PET, kaolin, and PET particles at concentrations of 10, 100, 1000, and 10000 PTCs/mL.

Growth models

To statistically compare algal growth across different exposures and concentrations over the entire growth period, the cell count data was subjected to fitting using the Baranyi growth model as described in Section 4.6.1. After fitting the maximum growth rate (μ_{max}) and carrying capacity (K) were derived from the fitted model.

Carrying capacities of aged-PET were consistently lower than those of PET and kaolin, except for the 10000 PTCs/mL PET exposure (Figure 5.2). Across all exposures and concentrations, the median carrying capacity consistently fell below the negative control's median carrying capacity of 2.642×10^6 cells/mL, except for the concentrations of 1000 PTCs/mL and 100 PTCs/mL of kaolin. Notably, a trend was observed where higher concentrations of aged-PET led to higher carrying capacities, while this trend appeared to be reversed for PET, and no clear trend was evident for kaolin. Specifically, for PET exposure, the mean carrying capacity at the concentration of 10000 PTCs/mL (2.523×10^6 cells/mL) was significantly lower than that at 0 PTCs/mL (2.629×10^6 cells/mL) and at 10 PTCs/mL (2.636×10^6 cells/mL) ($p = 0.02313$ and 0.01545 , $\text{diff} = -105775.99$ and -113123.15 , respectively).

5. Results

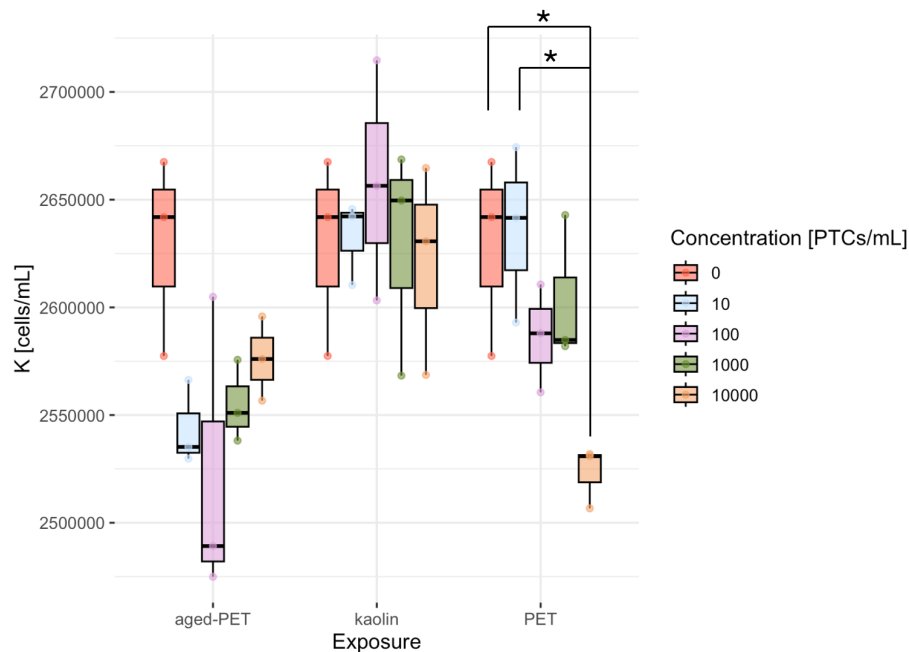


Figure 5.2: Carrying capacity (K) from from Baranyi growth model for aged-PET, kaolin and PET exposure. Individual data points are represented by dots (n=3 observations per boxplot). An asterisk (*) denotes a significant difference ($p < 0.05$) between concentrations within a specific exposure group.

The median maximum growth rates of aged-PET and kaolin consistently exceeded those of the negative control of 1.223 d^{-1} across all concentrations (Figure 5.3). However, the maximum growth rate of microalgae exposed to aged-PET at the concentration of 1000 PTCs/mL appears to be disproportionate compared to the rest of the data. This discrepancy appears to be attributed to the presence of two outliers, representing two replicates, for this exposure and concentration. Regarding PET exposure, the median maximum growth rate surpassed that of the negative control only at the 10000 PTCs/mL concentration, with a value of 1.242 d^{-1} . Notably, for kaolin exposure, there is a trend where increasing concentrations correspond to higher maximum growth rates, although no clear trends emerge for PET and aged-PET. Specifically, for kaolin exposure, the mean maximum growth rate at concentrations of 10000 PTCs/mL (1.271 d^{-1}) and at 1000 PTCs/mL (1.265 d^{-1}) is significantly higher than that of the negative control (1.223 d^{-1}) ($p=0.00528$ and $p=0.01187$, diff = 0.0480 and 0.0425, respectively).

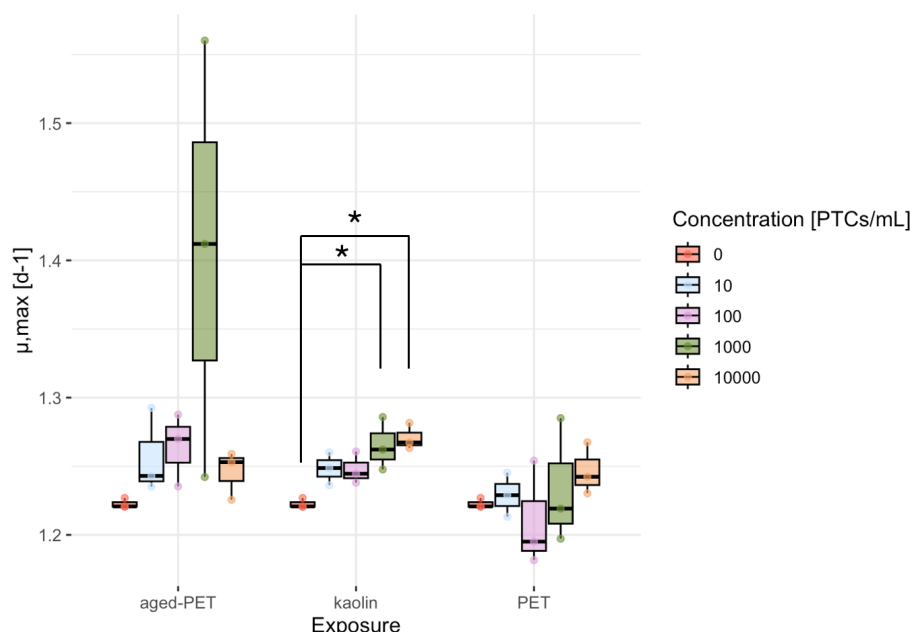


Figure 5.3: Maximum growth (μ_{max}) rate from the Baranyi growth model for aged-PET, kaolin and PET exposure. Individual data points are represented by dots ($n=3$ overervations per boxplot). An asterisk (*) denotes a significant difference ($p < 0.05$) between concentrations within a specific exposure group.

5.1.2 EPS production in response to micro- and nanoplastic exposure

Owing to technical challenges, data on EPS (weight and P/C-ratio) for the negative control was solely collected for the final set of Erlenmeyer flask (extraction 3). Consequently, all plots concerning EPS utilize the data obtained from this final extraction after 10 days of growth.

EPS quantity

To investigate whether exposure to MNPs affects the amount of EPS produced by *R. salina*, the EPS produced after 10 days extracted and weighed. This amount was then normalized by the number of algal cells. Except for aged-PET exposure at a concentration of 10 PTCs/mL, the median EPS production per cell for all exposures and concentrations is lower than that for the negative control of 0.182 ng/cell (Figure 5.4). Furthermore, except for the concentration of 10000 PTCs/mL, aged-PET exposure appears to result in a higher amount of EPS per cell compared to kaolin and PET exposure. EPS production appears to be stable across all concentrations for kaolin. In contrast, aged-PET shows a decrease in EPS production with increasing concentration, whereas PET exhibits an upward trend. However, no statistically significant differences were found in EPS production per cell across the concentrations for any of the exposures. It is worth noting that EPS production at the 1000 PTCs/mL PET exposure appears inconsistent with the rest of the data. This discrepancy is attributed to an unusually high EPS quantity, likely stemming from extraction process issues or weighing errors in this specific replicate.

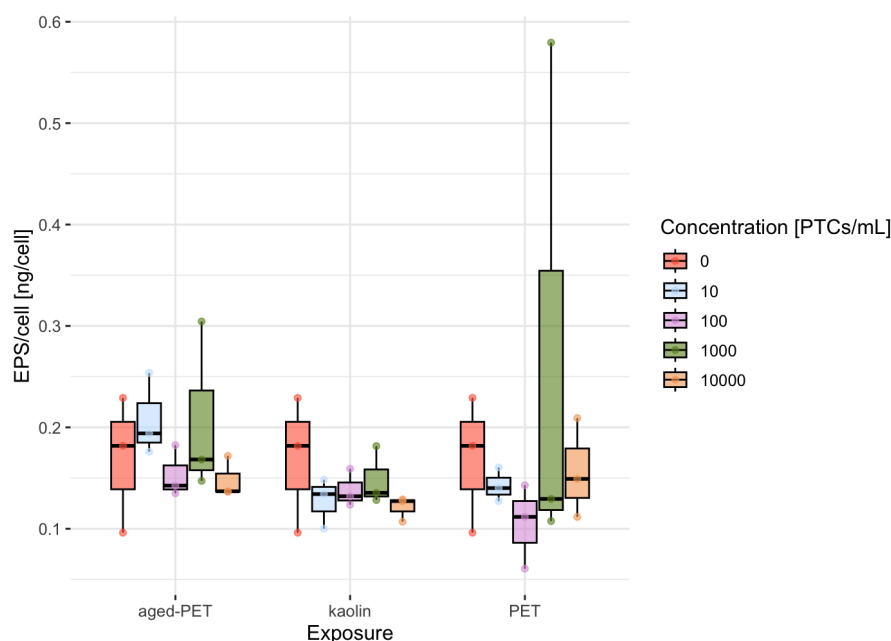


Figure 5.4: EPS production per cell after 10 days for aged-PET, kaolin and PET exposure. Individual data points are represented by dots (n=3 observations per boxplot).

EPS composition

To explore the impact of exposure to MNPs on the composition of EPS produced by *R. salina*, the P/C-ratio of the EPS was determined by calculating the ratio of the integrated peak area of proteins and carbohydrates from the ATR-FTIR spectrum. Across all concentrations, the mean P/C-ratios for kaolin and PET exposures consistently exceed those of the negative control of 0.58502 (Figure 5.5). This indicates a shift to a higher protein content compared to the negative control. Additionally, for kaolin exposure, the P/C-ratio appears to increase with concentration. The mean P/C-ratio of PET exposure at a concentration of 1000 PTCs/mL (0.704) is significantly higher than at 0 PTCs/mL (0.612) ($p = 0.00422$, $\text{diff} = 0.0920$). For microalgae exposed to kaolin, the median P/C-ratio at concentrations of 100, 1000 and 10000 PTCs/mL (0.69563, 0.70954 and 0.71774) are significantly higher than at 0 PTCs/mL ($p=0.00680$, $p=0.00584$ and $p=0.00124$, respectively).

Statistical test across concentrations showed that at a concentration of 10 PTCs/mL, the mean P/C-ratio of the aged-PET exposed microalgae (0.599) is significantly lower than that of kaolin (0.685) and PET exposed microalgae (0.669) ($p = 0.00056$ and $p = 0.00423$, $\text{diff} = 0.0864$ and 0.0706 , respectively). Similarly, at a concentration of 1000 PTCs/mL, the mean P/C-ratio of the aged-PET exposure (0.564) is significantly lower than that of the kaolin exposure (0.698) and that of the PET exposure (0.704) ($p = 0.00168$ and $p = 0.00053$, $\text{diff} = 0.134$ and 0.139 , respectively). Furthermore, at a concentration of 10000 PTCs/mL, the median P/C-ratio for the aged-PET exposure (0.644) is significantly lower than that for the kaolin exposure (0.718), while that of kaolin is significantly higher than that of PET (0.665) ($p = 0.00012$ and $p = 0.00876$, respectively). A visual representation of these findings can be found in Appendix F.1.3.

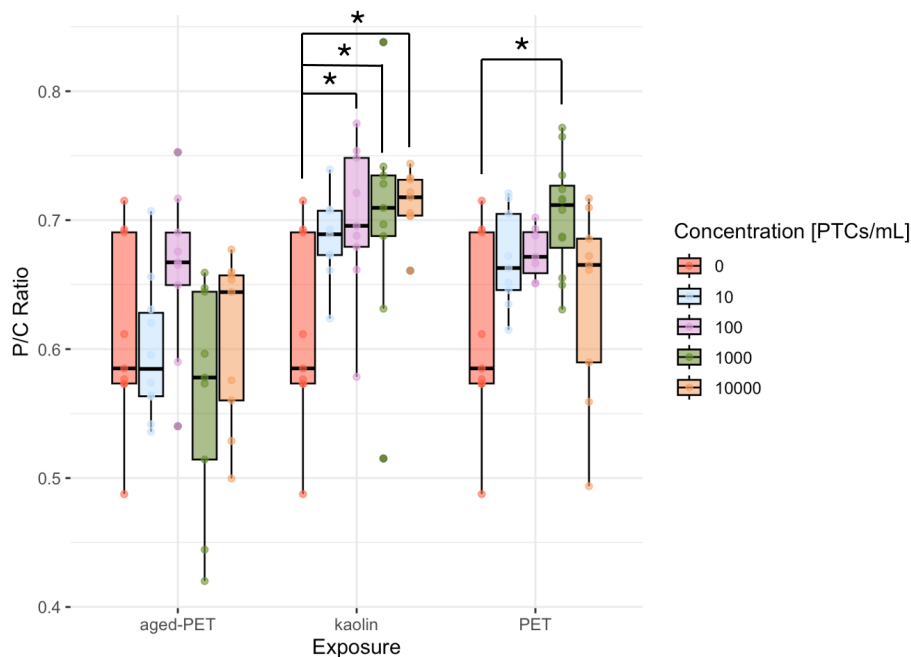


Figure 5.5: P/C-ratio of EPS extracted at day 10 for aged-PET, kaolin and PET exposure. Individual data points are represented by dots (n=9 observations per boxplot). An asterisk (*) denotes a significant difference ($p < 0.05$) between concentrations for a specific exposure group.

5.2 Experiment 2

After observing interesting changes in growth parameters and EPS quantity and composition, the aim was to investigate whether these alterations influence aggregation behavior. Hence, the second experiment replicated the procedures of the initial experiments to evaluate algal growth, EPS quantity, and EPS composition. This allowed for direct correlation with the newly conducted experiments, which determined the volume-based size distribution of the aggregates and their composition.

5.2.1 Algal growth in response to micro- and nanoplastic exposure

Growth curves

Like for the first experiment, the growth curves of *R. salina* were plotted using the raw data (Figure 5.6). Note that caution should be taken when interpreting the cell count data due to possible inaccuracies arising from measurement issues and interpolations of missing data as explained in Section 4.6.1. The growth curves appear more erratic compared to those of Experiment 1, as demonstrated by sudden fluctuations such as the drop in cell count on day 5. A notable difference is observed on day 10, where growth has not yet reached the stationary phase. While in the previous experiment, growth curves began to plateau at 2.5×10^6 cells/mL, in this experiment, they continue to increase at that same value.

5. Results

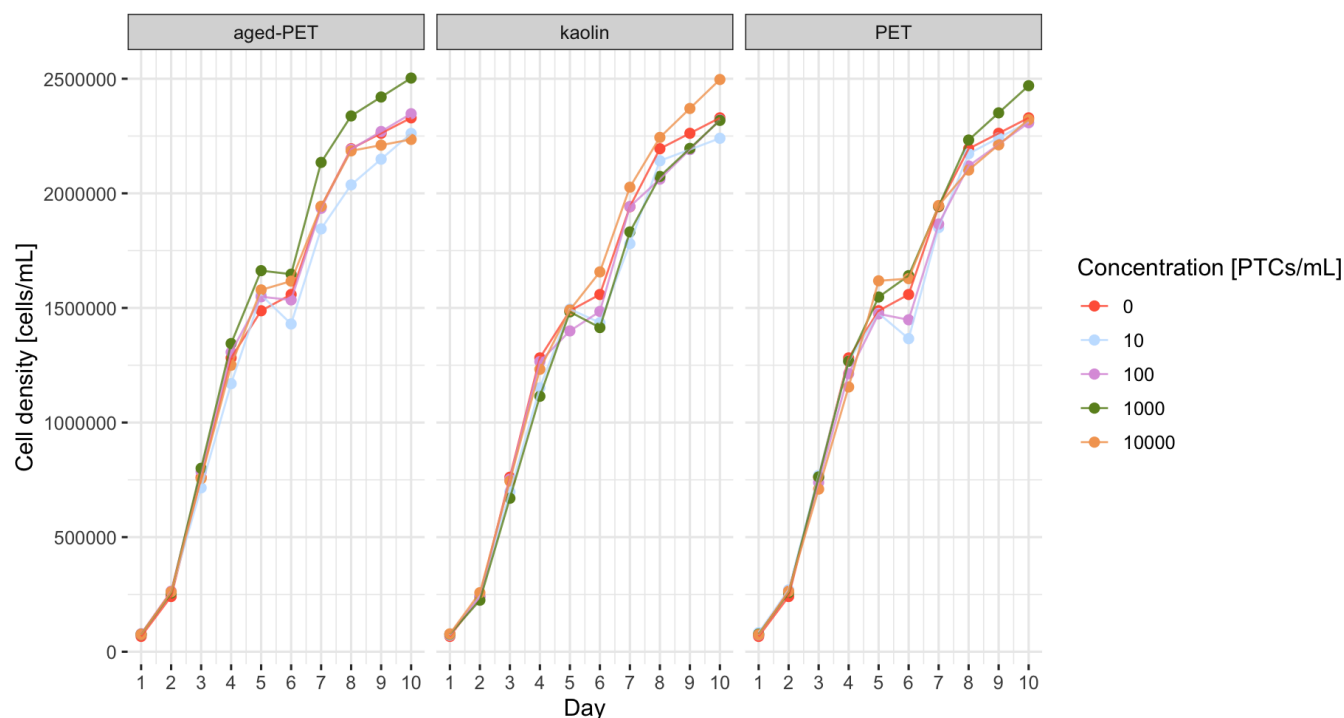


Figure 5.6: Growth curves of *R. salina* exposed to aged-PET, kaolin, and PET particles at concentrations of 10, 100, 1000, and 10000 PTCs/mL.

Growth models

Carrying capacities for PET exposure show an upward trend, suggesting that higher exposure concentrations lead to increasing carrying capacities (Figure 5.7). The median carrying capacity of most exposures is lower than that of the negative control of 2.018×10^6 cells/mL, except at the 1000 PTCs/mL and/or 10000 PTCs/mL concentration. For the aged-PET exposure, the median carrying capacity at the 1000 PTCs/mL concentration (2.128×10^6 cells/mL) is significantly higher than that of the 10 PTCs/mL concentration (1.898×10^6 cells/mL) and that of the 10000 PTCs/mL concentration (1.999×10^6 cells/mL) ($p = 0.00096$ and 0.02762 respectively).

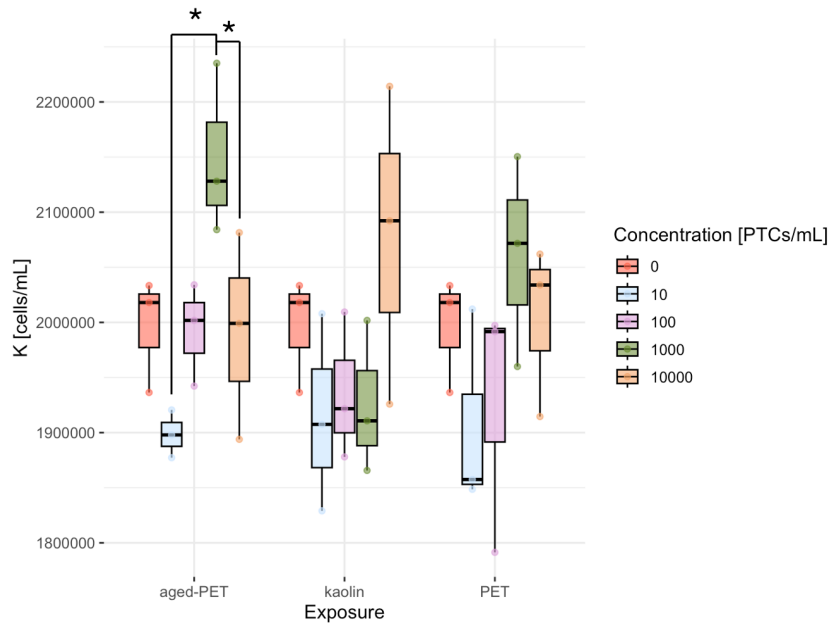


Figure 5.7: Carrying capacity (K) from the Baranyi growth model for aged-PET, kaolin and PET exposure. Individual data points are represented by dots (n=3 observations per box-plot). An asterisk (*) denotes a significant difference ($p < 0.05$) between concentrations for a specific exposure group.

The median maximum growth rate for the kaolin exposure falls below that of the negative control of 1.352 d^{-1} for all concentrations (Figure 5.8). For the PET exposure only the 10 PTCs/mL concentration has a higher median maximum growth rate than the negative control. For aged-PET exposure, both the 100 PTCs/mL and the 1000 PTCs/mL have a higher median maximum growth rate. No significant differences were found in the maximum growth rate across the different concentrations at any of the exposure groups.

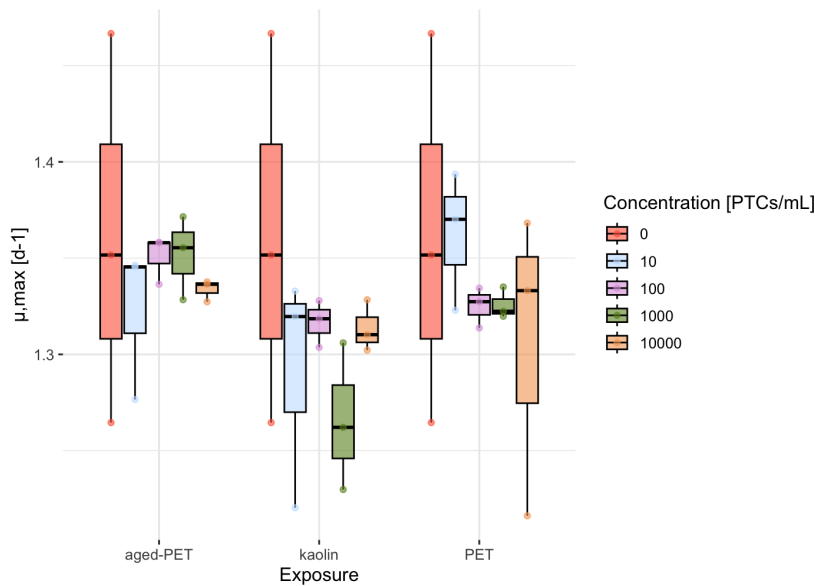


Figure 5.8: Maximum growth (μ_{max}) rate from the Baranyi growth model for aged-PET, kaolin and PET exposure. Individual data points are represented by dots (n=3 observations per boxplot). An asterisk (*) denotes a significant difference ($p < 0.05$) between concentrations for a specific exposure group.

5.2.2 EPS production in response to micro- and nanoplastic exposure

EPS quantity

Identical to Experiment 1, the EPS produced by *R. salina* after 10 days was extracted from the algal cells and weighed. This amount was then normalized by the number of algal cells on that day. Except for the 10 PTCs/mL PET exposure, all exposures at all concentrations exhibit increased EPS production per cell compared to the negative control of 0.099 ng/cell (Figure 5.9). Notably, for PET exposure, there is a trend of higher concentrations leading to higher EPS production. Conversely, for kaolin, no clear trend is observed, and for aged-PET, EPS production remains relatively stable across different concentrations. The EPS production at the 10000 PTCs/mL PET exposure appears to be inconsistent with the rest of the data. The raw data shows that this discrepancy is due to an unusually high EPS quantity, which might result from issues with the extraction process or errors in weighing this replicate. No significant differences were observed across concentrations for any of the exposure groups.

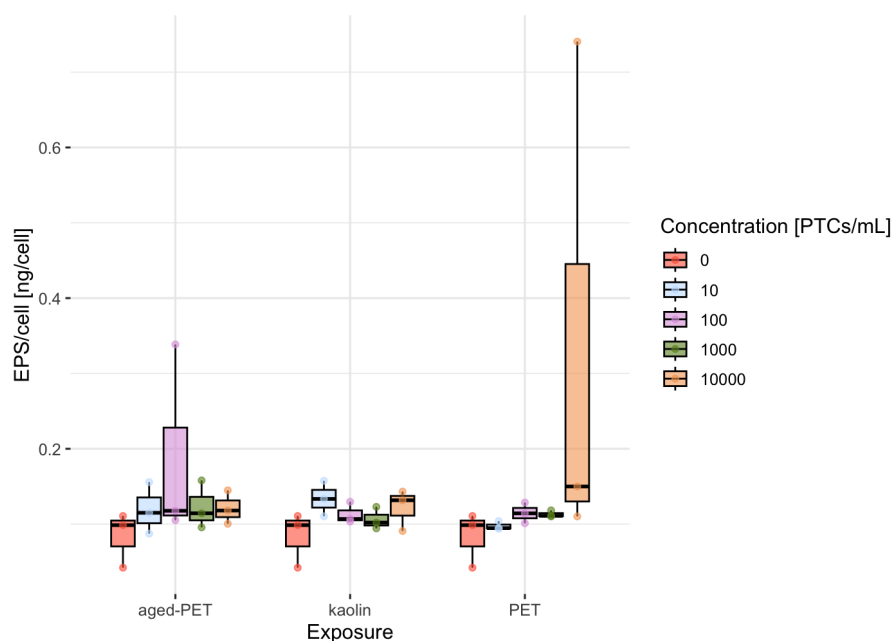


Figure 5.9: EPS production per cell after 10 days of exposure for aged-PET, kaolin and PET exposure. Individual data points are represented by dots (n=3 observations per boxplot).

EPS composition

Following the same procedure of Experiment 1, the P/C-ratio of the EPS was determined by calculating the ratio of integrated peak areas from the ATR-FTIR spectrum. For most exposure scenario's the median P/C-ratio is lower than that of the negative control of 0.620 (Figure 5.10). For PET exposures, the P/C-ratio remains fairly stable with no clear trend across different concentrations. In contrast, for the kaolin exposures, there is an upward trend in the median P/C-ratio with increasing concentration. The mean P/C-ratio of the

10 PTCs/mL concentration (0.347), is significantly lower than that of the 1000 and 10000 PTCs/mL concentration (0.535 and 0.629) ($p = 0.03893$ and 0.00091 , $\text{diff} = -0.18794$ and -0.28248 , respectively). Additionally, the mean P/C-ratio of the 0 PTCs/mL concentration is significantly higher than that of the 10 PTCs/mL concentration ($p = 0.00102$ and $\text{diff} = -0.26957$). For aged-PET exposure, excluding the 10000 PTCs/mL concentration, the P/C-ratio also appears to increase with increasing concentration. The median P/C-ratio of the 1000 PTCs/mL concentration (0.773) is significantly higher than that of the 10, 100 and 10000 PTCs/mL concentrations (0.477, 0.557 and 0.569) ($p = 0.00072$, 0.03931 and 0.01057 , respectively). Additionally, the median P/C-ratio of the 0 PTCs/mL concentration is also significantly higher than that of the 10 PTCs/mL concentration ($p = 0.01333$).

Statistical tests across the concentrations showed that for concentration of 10 PTCs/mL, the mean P/C-ratio of PET exposed microalgae (0.578) is significantly higher than that of kaolin (0.347) ($p = 0.00220$ and $\text{diff} = 0.23018$) (Figure F.8). At 1000 PTCs/mL, the median P/C-ratio of aged-PET exposures (0.773) is significantly higher than that of kaolin (0.561) and PET exposures (0.566) ($p = 0.00867$ and 0.02271 , respectively). A visual representation of these findings can be found in Appendix F.2.2.

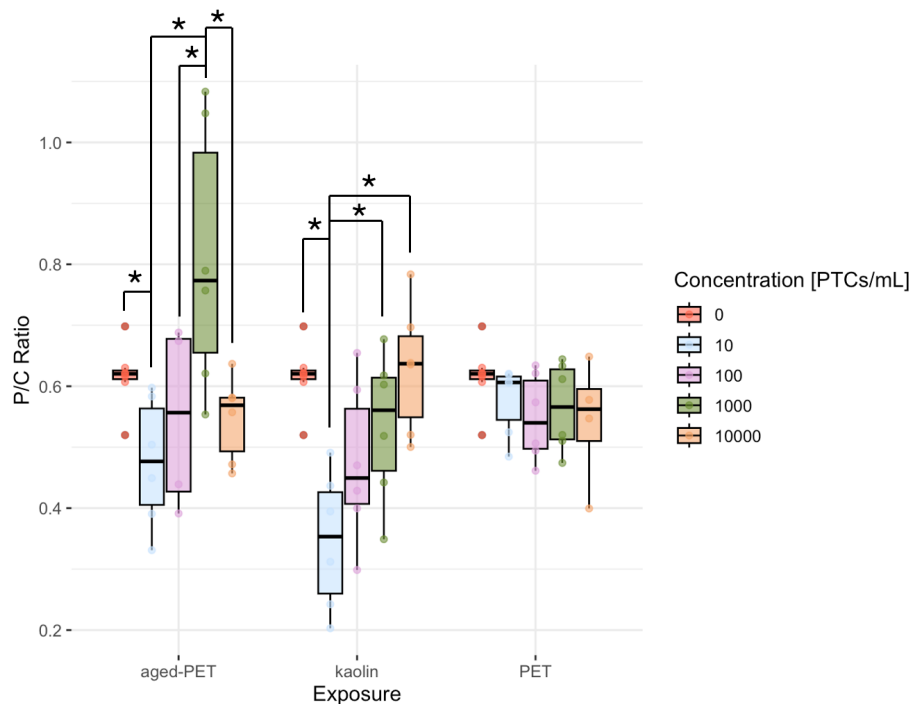


Figure 5.10: P/C-ratio of EPS extracted at day 10 for aged-PET, kaolin and PET exposure. Individual data points are represented by dots ($n=6$ observations per boxplot). An asterisk (*) denotes a significant difference ($p < 0.05$) between concentrations for a specific exposure group.

5.3 Aggregation dynamics in response to micro- and nanoplastic exposure

Aggregate size and quantity

To investigate whether changes in EPS quantity and composition affect the size and quantity of aggregates, volume-based size distribution curves were generated using DLS. In the case of aged-PET exposure, all concentrations result in smaller median aggregate diameters compared to the negative control of 39.780 μm (Figure 5.11). Conversely, for kaolin exposure, higher concentrations of 1000 PTCs/mL and 10000 PTCs/mL lead to larger aggregates than the control. However, for PET exposure, the median aggregate size of these concentrations is smaller than that of the negative control, while those of 10 PTCs/mL and 100 PTCs/mL are larger than the control. Specifically for kaolin exposure, the mean aggregate diameter at 10000 PTCs/mL (45.484 μm) is significantly higher than that for 0 PTCs/mL, 10 PTCs/mL, and 100 PTCs/mL (39.780 μm , 40.235 μm , and 39.506 μm , respectively) ($p = 0.01240$, 0.01507 , and 0.00647 , $\text{diff} = 5.415$, 5.249 , and 5.978 , respectively). For PET exposure, the mean aggregate diameter for the 10 PTCs/mL concentration (45.157 μm) is significantly higher than that of the 0 PTCs/mL, 1000 PTCs/mL and 10000 PTCs/mL concentrations (39.780 μm , 38.639 μm and 38.056 μm , respectively) ($p = 0.02585$, 0.00536 and 0.00292 , $\text{diff} = 5.088$, 6.518 and 7.101 , respectively). Additionally, for PET exposure, the mean aggregate diameter for the 100 PTCs/mL concentration (43.947 μm) is significantly higher than for 1000 PTCs/mL and 10000 PTCs/mL concentrations ($p = 0.02018$ and 0.01056 , $\text{diff} = 5.891$ and 5.308 , respectively).

Statistical test across concentrations showed that at the 10 PTCs/mL concentration, the mean aggregate size of PET exposures is significantly higher than that of aged-PET and kaolin ($p = 0.01120$ and 0.03259 , $\text{diff} = 6.28580$ and 4.92287 , respectively). At the 100 PTCs/mL concentration, the mean aggregate diameter of PET exposures is significantly higher than that of aged-PET ($p = 0.04775$, $\text{diff} = 5.65617$). Lastly, at 1000 PTCs/mL, the mean aggregate diameter of kaolin exposures is significantly higher than that of both aged-PET and PET exposures ($p = 0.01327$ and 0.02474 , $\text{diff} = 4.475$ and 3.885 , respectively). A visual representation of these findings can be found in Appendix F.2.3

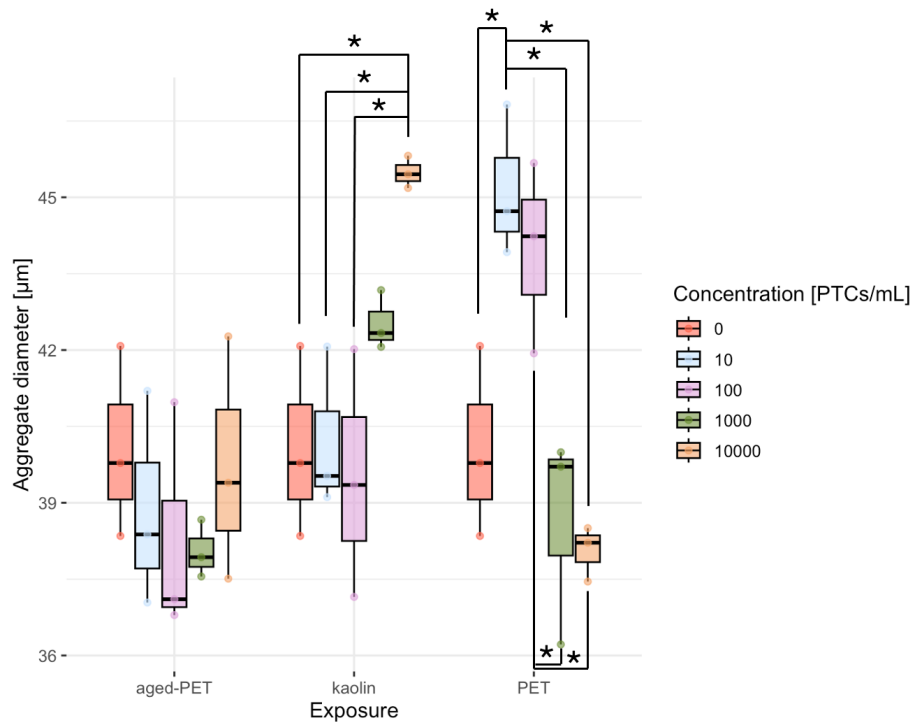


Figure 5.11: Diameter of the aggregated algae obtained from the volume-based size distribution curve for aged-PET, kaolin and PET exposure. Individual data points are represented by dots (n=3 observations per boxplot). An asterisk (*) denotes a significant difference ($p < 0.05$) between concentrations for a specific exposure group.

To gain additional insights into aggregation behavior, the abundance of the aggregates was also investigated. Specifically, the ratio of the area under the peak representing aggregated algae (at approximately $50 \mu\text{m}$) and that of single-celled algae (at approximately $7 \mu\text{m}$) was calculated. This ratio indicates the volume occupied by aggregates relative to single algae.

Compared to the negative control, exposure to aged-PET appears to reduce the proportion of algae in aggregates, with this reduction being concentration-dependent (Figure 5.12). Similarly, for PET exposure, there's a notable decline in algae aggregation with increasing concentrations. Conversely, with kaolin exposure, excluding the 10 PTCs/mL concentration, higher concentrations lead to a rise in the number of algae cells within aggregates. Specifically, in the PET exposure scenario, the mean volume ratio at the concentration of 10 PTCs/mL (5.166) significantly exceeds that at 10000 PTCs/mL (1.856) ($p = 0.04548$, diff = 3.310).

Statistical test across concentrations only showed a statistically significant difference at the concentration of 10 PTCs/mL. For this concentration the mean volume ratio of aggregates versus single-celled algae for PET exposures (5.166) is significantly higher than that for aged-PET exposures (2.306) ($p = 0.04545$, diff = 3.310). A visual representation of these findings can be found in Appendix F.2.4.

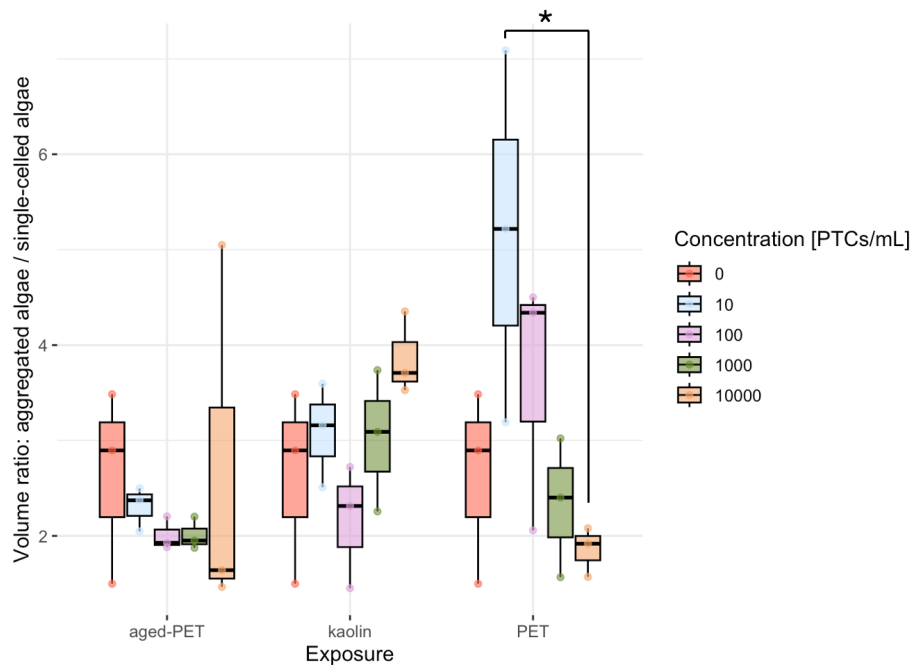


Figure 5.12: Ratio of the volume percentage taken up by aggregates versus by single celled algae for aged-PET, kaolin and PET exposure. Individual data points are represented by dots (n=3 observations per boxplot). An asterisk (*) denotes a significant difference ($p < 0.05$) between concentrations for a specific exposure group.

Aggregate composition

To explore if alterations in EPS quantity and composition affect the composition of aggregates, the percentage of aggregate area occupied by algal cells and EPS was determined through microscopy. This analysis focused solely on the 10000 PTCs/mL concentration to assess significant changes in aggregate composition at this highest, environmentally relevant level.

Figure 5.13 displays the percentage of the aggregate area occupied by algal cells on the left y-axis and the percentage occupied by EPS on the right y-axis, plotted against the exposure groups. All exposure groups have a higher median percentage of algal cells in the aggregates than the negative control at 58.04%. This means more algal cells were incorporated into the aggregates. The only significant difference is between the mean percentage of algal cells in the aggregates for the kaolin exposure (79.31%), which is notably higher than that of the negative control (56.37%) ($p = 0.02401$, $\text{diff} = 22.94$).

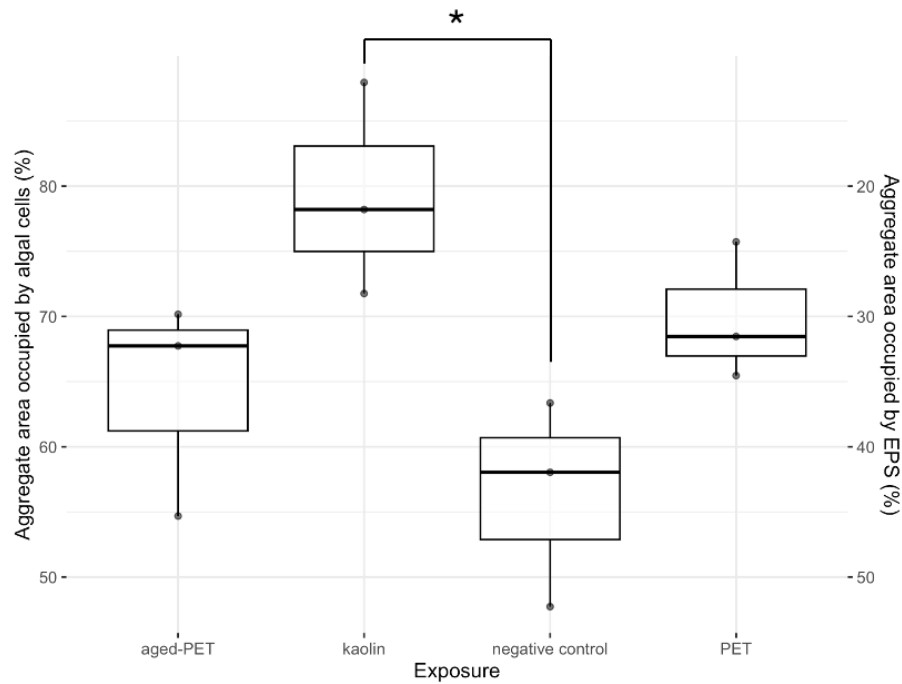


Figure 5.13: Percentage of the aggregate area occupied by algal cells and EPS for aged-PET, kaolin and PET exposure at 10000 PTCs/mL. Individual data points are represented by dots (n=3 observations per boxplot). An asterisk (*) denotes a significant difference ($p < 0.05$) between concentrations for a specific exposure group.

6. DISCUSSION

This study investigated the intricate relationship between plastic fragmentation and its repercussions on aquatic ecosystems. Specifically, it explored whether the production of EPS by microalgae serves as a protection mechanism against MNPs. Additionally, by examining how changes in EPS composition affect the aggregation behavior of algae and MNPs, this research investigates whether these alterations could potentially lead to reduced exposure of microalgae to MNPs.

6.1 Microalgal growth under micro- and nanoplastic exposure

The results indicate that exposure to PET, aged-PET, and kaolin generally reduces the carrying capacity of the algal population compared to the negative control, with a significant reduction observed only for PET. Due to challenges with cell count measurements and the delayed attainment of the stationary phase in Experiment 2, the discussion will focus on the growth data from Experiment 1. The minimal variation in carrying capacities across different concentrations for kaolin exposure suggests that the presence of natural particles does not significantly affect carrying capacity. Therefore, the decline in carrying capacity for aged-PET and PET is likely due to their specific properties rather than a general particle effect.

For PET, all exposure concentrations resulted in carrying capacities lower than the negative control. The carrying capacity decreases further with increasing concentration, suggesting a dose-response relationship where higher concentrations cause more adverse effects. Commonly described adverse effects include ROS generation, leading to oxidative stress when the natural balance is exceeded. Additionally, the adsorption of NPs onto the cell surface decreases the fluidity of the algal cell membrane, hindering the exchange of energy and substances with the environment (Zhu et al., 2021). However, a significant decrease in carrying capacity only occurs at the highest concentration, suggesting that a minimum concentration is needed to initiate these effects, likely between 1000 PTCs/mL and 10000 PTCs/mL.

Similar to PET, aged-PET consistently exhibited a lower carrying capacity compared to the negative control at all exposure concentrations. Notably, the carrying capacity for aged-PET was also generally lower than that of PET. This more pronounced decrease in carrying capacity for aged-PET might be attributed to the aged plastic polymer releasing more additives and chemicals (Luo et al., 2022). Meng et al. (2021) demonstrated that the natural and chemical aging of PVC can trigger the release of various toxic metal additives. When PVC was aged with 30% H₂O₂, up to 87% of the total concentration of metals could leach

out, depending on the type of metal. The study by Ni et al. (2023) demonstrated that the growth inhibition caused by PS-MNPs was significantly more pronounced for PS particles aged with natural sunlight compared to pristine PS particles. This supports the observation that aged plastics could have a bigger effect on algal growth. However, it is worth mentioning that Ni et al. (2023) also included the solution in which the plastic particles were stored, meaning that chemicals that leached out during storage were also introduced to the algal cultures. In contrast, in this study, the plastic particles were washed before being added to the algae, indicating that any chemical exposure must have resulted from chemicals leached during the 10-day exposure period.

In terms of the maximum growth rate (μ_{max}), it was found that μ_{max} increased under all exposure groups, though only significantly for kaolin. Under kaolin exposure, μ_{max} significantly increased compared to the negative control at the two highest concentrations. This suggests that the presence of particles can promote algal growth. Similarly, aged-PET exposure resulted in a higher μ_{max} than the negative control. For PET, μ_{max} exceeded that of the negative control at the lowest and the highest concentration.

These findings align with a study by Gorokhova et al. (2020), which exposed *Raphidocelis subcapitata* to kaolin, PET, and aged-PET. They observed an increased μ_{max} compared to the control for all these exposure groups at concentrations of 10 and 100 mg/L, with μ_{max} dropping below that of the negative control only at 1000 mg/L. This supports the conclusion that at the low concentrations used in this study, MNPs likely do not lead to a decreased μ_{max} . In fact, it appears that exposure to small concentrations of particles might even promote the algal growth rate. This notion finds support in the work of Wang et al. (2021), who deliberately induced stress responses in microalgae to enhance industrial microalgal production systems. They employed various stressors, such as sub-optimal levels of carbon and light, to augment the growth rates of algal strains. This approach reflects a form of natural evolution wherein algal genomes adapt to environmental changes (Wang et al., 2021). This is further supported by the study of Ni et al. (2023) which found that growth of microalgae was promoted by PS-MNPs (1 μm) at the beginning of exposure at low PS concentrations of 1-10 mg/L, resulting in a negative inhibition ratio. However, higher exposure concentrations of 10-50 mg/L immediately inhibited growth.

Overall, the literature on the effects of MNP exposure on algal growth presents mixed results due to the high variability in MNP sizes and concentrations. Some studies report inhibition of algal growth, but these typically involve high concentrations of MNPs. Conversely, studies utilizing more environmentally relevant concentrations (though still 100 times higher than those used in this thesis) generally observe no negative effects on algal growth. For instance, Long et al. (2017) employed polystyrene beads (2 μm) at 9×10^5 PTCs/mL, and Niu et al. (2021) used polyethylene microbeads (10–106 μm) at 1.25×10^6 . Both studies found no inhibitory effects. This supports the observation of ambiguous and mostly non-significant results on growth parameters in this study. However, what is intriguing is the overall elevated growth rate observed at these low concentrations. It is plausible that the minimal stress levels may have stimulated the algal cells, increasing their growth rate.

6.2 The role of EPS production in response to micro- and nanoplastic exposure

The findings regarding EPS production indicate that there are no significant differences or trends in EPS amount across exposures or concentrations. The results from Experiment 1 and Experiment 2 yielded varying results in terms of EPS production. Experiment 1 showed a slight decrease in EPS production compared to the negative control for PET, aged-PET and kaolin. This suggests that EPS production may decrease when algae are exposed to MNPs or natural particles, although this observation isn't supported by existing literature to the best of my knowledge. Conversely, Experiment 2 indicated a slight increase in EPS production, aligning with what most research suggests. Nevertheless, these changes were also not statistically significant.

Previous studies, however, have demonstrated increases in EPS production by algae under MNP exposure. For instance, the research findings of Gopalakrishnan and Kashian (2022) observed a dose-response relationship in *Chlamydomonas reinhardtii* exposed to high-density polyethylene microbeads (10 to 45 μm), where higher concentrations led to increased EPS production. However, their study employed a maximum concentration of 0.4 g/L, which is four orders of magnitude higher than the highest concentration used in this study. Additionally, the particle size they used is substantially larger than the MNPs used in this study, likely resulting in different interactions with algal cells. It is probable that the threshold concentration required to trigger a detoxification response has not been reached at the low concentrations used in this thesis, potentially explaining the absence of a trend for aged-PET and PET. The lack of a trend for kaolin in both experiments may be attributed to its natural composition, which may not stimulate a stress response and subsequent EPS production. No research was found indicating that natural particles also induce EPS production.

While EPS quantity appeared consistent across experiments, the EPS composition revealed significant differences between exposure groups and concentrations in both Experiment 1 and 2. However, the direction of these changes differed. Experiment 1 generally showed increased P/C-ratios for exposed groups compared to the control, while Experiment 2 exhibited lower P/C-ratios for plastic and kaolin exposure compared to the control. The observed differences in EPS production between the two experiments could stem from several factors. First of all, the algal cultures in the second experiment had not reached the stationary growth phase. The late exponential and stationary growth phases might be critical for stress-related EPS production and increases in P/C-ratios. However, Martinho de Brito et al. (2023) found lower protein content (indicated by ATR-FTIR) in EPS produced by cyanobacteria during the exponential and late-stationary phases. This suggests growth phase alone might not fully explain the observed differences. Another potential explanation might be the difference in nutrient availability. The delayed growth in the second experiment might have resulted in less nutrient depletion, leading to less stressed cultures and less pronounced P/C-ratio increases. However, Reignier et al. (2023) reported the opposite effect, nutrient limitation led to lower P/C-ratios in *Microcystis aeruginosa*. Consequently, the differences in P/C-ratios might have been related to slight inconsistencies in the EPS extraction procedure itself, unintentionally favoring the extraction of specific components. As shown

by Cho et al. (2012) small variations in sample dilution and shaking intensity could already impact the quantity and composition of the extracted EPS. Further investigation is necessary to reconcile these seemingly contradictory findings and understand the precise role of growth phase, nutrient availability, and EPS extraction procedures on the P/C-ratio of EPS produced by microalgae under MNP exposure.

While the experiments yielded contrasting findings regarding a P/C-ratio increase or decrease compared to the control, the way the P/C-ratios changed with increasing concentration stayed consistent across both experiments. For kaolin, higher exposure concentrations led to an increase in the P/C-ratio in both experiments. Similarly, aged-PET also showed an upward trend. This suggests that exposure to kaolin and aged-PET enhanced the protein content and/or reduced the carbohydrate content of the EPS. This increasing trend in the P/C-ratio aligns with the findings of Shiu et al. (2020). Their study demonstrated a significant increase in the P/C-ratio of EPS from *Thalassiosira pseudonana* in the presence of PS MNPs (1 μ m) even at low concentrations (10^{-3} mg/L), which are comparable to the highest concentration used in this study. Additionally, Shiu et al. (2020) showed a concentration-response relationship where the P/C-ratio of *Phaeodactylum tricornutum* increased with the concentration when exposed to PS NPs (55 nm and 1 μ m). This response began at levels higher than 0.1 mg/L. Shiu et al. (2020) attributed this observation to the significance of protein quantity in EPS as a measure of stickiness and relative hydrophobicity, which is crucial for chemically quenching or physically blocking hazardous substances.

The absence of a trend for PET in both experiments, unlike the upward trend observed for aged-PET and kaolin, may be attributed to the weaker negative charge of PET particles. With their stronger negative charge, aged-PET ($\zeta = -6.10 \pm 1.63$ mV) and kaolin ($\zeta = -12.8 \pm 1.12$ mV) exhibit a stronger affinity for EPS than PET ($\zeta = -3.54$ mV ± 3.02 mV), as noted by Junaid and Wang (2021). I propose that this enhanced affinity could facilitate their interaction with algal cells as they bind to the EPS surrounding these cells. Consequently, they might be more prone to inducing adverse effects. Sun et al. (2024) showed that various adverse effects including damage to chloroplasts, inhibited photosynthetic efficiency and electron transport, and suppressed activities of carbon fixation related enzymes can occur at environmentally relevant concentrations (0.1–10 mg/L) of PS-NPs. These adverse effects may trigger a stress response in the algae, leading to the secretion of EPS with a higher protein content, as described by Shiu et al. (2020). This sticky EPS could encapsulate MNPs to develop an eco-corona layer that changes the physicochemical properties, fate, bioactivity, and ecological impacts of these MNPs (Junaid and Wang, 2021). The impact of MNP charge on EPS affinity and consequently their ability to induce stress responses in microalgae should be further investigated in future research.

6.3 Influence of EPS changes on the aggregation behaviour of microalgae

Results from the aggregation experiments demonstrate that kaolin exposure prompts the formation of more and larger aggregates, while exposure to PET and aged-PET yields less and smaller aggregates. Initially, the aggregate diameter of kaolin-exposed aggregates remained relatively stable, but for the two highest concentrations, a notable increase oc-

curred. Additionally, kaolin exposure also increased the ratio of aggregated algae to single-celled algae. This suggests kaolin exposure led to not only larger, but also more aggregates. This observation is consistent with expectations, as clay minerals are often employed to counteract harmful algal blooms (HAB) by promoting the formation of large flocs, facilitating rapid settling (Mario R., 2001). Furthermore, this phenomenon may be linked to the elevated P/C-ratio of the generated EPS under kaolin exposure. Kaolin exhibits a clear and significant dose-response relationship, with higher concentrations correlating with higher P/C-ratios, which tends to be stickier and more hydrophobic (Shiu et al., 2020). This can promote hydrophobic interactions with other algae and with organic matter, thus favoring the formation of larger aggregates.

For PET, lower concentrations led to aggregates larger than the negative control, but for the two higher concentrations, the aggregate diameter significantly decreased. The changes for aged-PET were more subtle, although there appears to be a trend of smaller aggregates with increasing concentrations. Furthermore, the ratio of aggregated algae to single-celled algae decreased for both PET and aged-PET with increasing concentration. This trend was only significant for PET. The decrease in aggregate size for aged-PET and PET appears contradictory to findings from other studies. Demir-Yilmaz et al. (2022) exposed *Chlorella vulgaris* to high-density polyethylene MPs with a D_{50} of 6 μm and observed that in the control, cells produced only a small amount of EPS and small cell aggregates were present. However, after 7 days of growth in the presence of MPs, a large amount of EPS and large cell aggregates were observed.

A potential hypothesis for the observation that less and smaller aggregates are formed under PET and aged-PET exposure, is that the EPS was not primarily integrated into algal aggregates but rather into MNP homo-aggregates. This implies that the lower aggregate diameter for aged-PET and for the higher concentrations of PET result from the reduced presence of EPS associated with the algal aggregates, owing to the high concentration of EPS associated with the MNPs. This could be explained by the preferential binding of EPS to MNPs. To the best of my knowledge, no research directly compares the binding affinity of EPS with MNPs and algal cells. However, Junaid and Wang (2021) highlighted the high affinity between EPS and negatively charged PS NPs and noted the crucial role of EPS in the homo-aggregation of NPs. This interpretation finds further support in the findings of Natarajan et al. (2023), who observed that the aggregation of NPs was visibly intensified in the presence of EPS. They also noted that this strong aggregation of NPs with EPS might lead to a decreased bioavailability of NPs for sorption onto algal cells, as these large-sized homo-aggregated NPs tend to settle more readily. Additionally, they demonstrated that this phenomenon results in significantly reduced radical production, thereby diminishing the overall toxicity of the NPs. The larger size and greater abundance of aggregates observed under kaolin exposure may stem from the higher availability of EPS, which is not bound to MNPs. Additionally, the increased protein content in EPS under kaolin exposure could contribute to the enhanced aggregation.

At the highest concentration, the composition of aggregates for all exposures showed a larger percentage of the area occupied by algal cells rather than EPS, compared to the control. However, this increase was only statistically significant for kaolin. This suggests that across all exposures, the aggregates contain more algal cells relative to the negative control and less EPS. Since larger aggregates have smaller surface-to-volume ratios, it makes

sense that the large aggregates formed under kaolin exposure, likely due to the higher P/C-ratio of the EPS, would contain relatively more algal cells and less surrounding EPS. The relatively higher presence of algal cells versus EPS in aged-PET and PET aggregates supports the hypothesis that more EPS is in association with MNPs. However, it is important to interpret these findings cautiously since the microscopy images provide a 2D representation of a 3D structure, and EPS might be present underneath or on top of the algal cells without being counted as EPS in this assessment.

6.4 Linking microalgal growth, EPS production, and aggregation behavior

To gain a comprehensive understanding of the response of microalgae to MNP exposure, this study employed a combined approach, measuring growth, EPS production, and aggregation behavior simultaneously. This integrated approach allows us to directly link these factors and evaluate the environmental consequences of changes in algal aggregation and settling.

This study has shown that exposure to PET and aged-PET MNPs reduces the carrying capacity of algal cultures while increasing their maximum growth rates. This could be due to low-level stress inducing adaptive changes (Wang et al., 2021).

As microalgae grow, they excrete EPS into their surroundings to form a hydrated biofilm matrix (Xiao and Zheng, 2016). This biofilm potentially serves as a protective layer around the microalgae, shielding them from external stressors. Studies have shown that EPS can help microalgae resist toxic substances like copper and zinc (Li et al., 2023a) and that EPS production increases in response to high salt concentrations (Mishra and Jha, 2009). This study however, could not confirm that EPS production also increases under environmentally relevant MNP concentrations. However, significant increases in the P/C-ratio of the EPS were observed for all exposure groups. This suggests that both natural and plastic particles trigger protein production in EPS.

The increased aggregate diameter observed in the kaolin treatment group, accompanied by a significant rise in the P/C-ratio, strengthens the notion that EPS proteins play a crucial role in promoting aggregation. Studies by Shiu et al. (2020) and Wang et al. (2021) have demonstrated that proteins within EPS enhance stickiness and hydrophobic interactions, both essential factors for particle aggregation. It has been shown that this protein-mediated aggregation strategy serves as a protective mechanism, resulting in fewer MNPs directly embedded on the cell surface (Song et al., 2023). This enhanced formation of algal aggregates has the potential to increase settling rates and contribute to the biological pump by transporting more organic carbon to the deep ocean (Zhang et al., 2024).

Interestingly, exposure to PET and aged-PET resulted in fewer and smaller aggregates, even though the P/C-ratio increased. This might be explained by the way EPS interacts with MNPs. Junaid and Wang (2021) demonstrated that EPS has a high affinity for binding to MNPs, especially negatively charged ones. They propose that EPS can promote homo-aggregation of MNPs by forming a coating around them, called an eco-corona. Furthermore, they found that a higher protein content in EPS leads to aggregation of MNP at lower concentrations.

Additionally, Liu et al. (2019) has indicated that formation of an eco-corona on the MP surface reduces their affinity to microalgae. This suggests that EPS with a strong affinity for MNPs might form an eco-corona around the plastic particles, inducing their aggregation while leaving less EPS available for the aggregation of microalgae. This could explain the fewer and smaller aggregates for PET and aged-PET exposure. This finding also implies that MNPs may sink more rapidly, reducing their presence in the water column but increasing their abundance on the ocean floor.

6.5 Ecological consequences

This study demonstrates that exposing microalgae to environmentally realistic concentrations of MNPs can lead to substantial changes in EPS composition and aggregation patterns. These alterations, in turn, may impact the availability and transport of MNPs within aquatic ecosystems. Notably, it was suggested that there could be an increased sedimentation of algal and plastic aggregates. This would not only influence the exposure of microalgae to small plastics, but also that of other aquatic organisms.

This EPS-mediated aggregation, and sedimentation of MNPs can be beneficial for the pelagic ecosystem because this phenomenon not only serves as a trap for plastic particles but also adsorb other hazardous pollutants, participating in the self-cleansing capacity of aquatic bodies (Junaid and Wang, 2021). Conversely, it could lead to greater exposure to MNPs and its associated pollutants for benthic organisms when these aggregates accumulate on the seabed.

The formation of larger aggregates could facilitate the ingestion of MNPs by bottom-dwelling animals, effectively introducing them into the food chain (Ward and Kach, 2009). This is supported by a meta-analysis of Mason et al. (2022) which revealed that benthic organisms experience stronger negative impacts from MPs compared to pelagic biota. Their analysis highlighted significant negative impacts on development, reproduction, survival, growth, feeding and behavior across all life stages, with the strongest impact observed on seabed invertebrates. A reduction in the fitness or even mortality of these organisms could have cascading effects throughout the entire ecosystem.

Benthic invertebrates play a critical role in the remineralization of organic matter, a process that releases dissolved nutrients essential for the growth of benthic microbes, algae, fungi, and aquatic angiosperms (Covich et al., 1999). These organisms, in turn, provide the food source for herbivorous and omnivorous benthic invertebrates, creating a complex and interconnected food web (Covich et al., 1999). Therefore, increased exposure to MNPs, potentially leading to reduced populations of benthic invertebrates, could disrupt this delicate balance and have significant consequences for the entire benthic ecosystem.

However, there might also be some benefits related to the increased sinking of enlarged aggregates. EPS-rich aggregates could contribute to the biological pump, a vital process that transports organic carbon and nutrients to the deep ocean. This organic matter might fuel the benthos and contributes to the storage of carbon dioxide captured from the atmosphere (Cole et al., 2016). Additionally, the sticky nature of EPS can accelerate microbial

colonization of plastic aggregates (Shiu et al., 2020), potentially facilitating the degradation of some pollutants adsorbed onto the plastic (Oliveira et al., 2020).

These findings highlight the multifaceted consequences of MNP pollution on aquatic ecosystems. While some benefits might be associated to increased vertical transport of organic matter, the potential disruption of the benthic food web and the introduction of MPs into the food chain necessitate further investigation.

6.6 Limitations

This study identified changes in microalgae growth parameters like carrying capacity and maximum growth rate. However, these variations were inconsistent across experiments and statistically insignificant for most MNP exposure levels, making it difficult to conclusively link them to MNP exposure. While MNPs and kaolin particles might have influenced growth, it's important to acknowledge the substantial impact of the culture medium's physicochemical properties on microalgae growth, as highlighted by (Junaid and Wang, 2021). Although pH was monitored to minimize its confounding effects, ideally, additional parameters like temperature, ionic strength, and light intensity would be measured for each exposure group. Tracking these parameters alongside the microalgae response over time would provide valuable insight into the persistence of observed trends and their potential connection to MNP exposure. This comprehensive approach would strengthen the ability to differentiate genuine exposure effects from inherent fluctuations within the culture medium.

Another limitation of this study is the lack of MNP monitoring throughout the experiment. Previous research suggests that MPs can adhere to glassware, and that this tendency increases with culture age (Long et al., 2017). Without monitoring, we cannot determine the fate of the MNPs: were they adhering to the flasks, sinking, remaining on the surface, forming plastic aggregates (homo-aggregates), attaching to algal cells (hetero-aggregates), or staying suspended? This lack of information hinders our ability to interpret the observed effects on the microalgae. Future studies should prioritize quantifying the bioavailability and distribution of MNPs within the experimental systems, as highlighted by Long et al. (2017).

Finally, it's important to reiterate that this study focused on TB-EPS, the layer closest to the algal cell (Ni et al., 2017). While highly valuable in exploring EPS as a potential protective mechanism, TB-EPS represents only a small fraction of the total EPS. LB-EPS and SB-EPS detach more readily and, due to their abundance and higher protein content, likely play a more significant role in transporting MNPs and pollutants (Deng et al., 2022). Therefore, future research investigating the role of EPS in the vertical transport of MNPs and contaminants should encompass all EPS types.

6.7 Relevance of the experimental design

Drawing comparisons with other studies proved challenging due to methodological variations. By far the biggest portion of research on MNP toxicity towards algae utilizes PS as the

model plastic (Mao et al. (2018); Natarajan et al. (2023); Shiu et al. (2020)). While some studies explore the effects of PET, even fewer investigate the impacts of weathered PET. To the best of my knowledge, the study by Gorokhova et al. (2020) was the only one that used both PET and weather-PET as well as including kaolin particles. Furthermore, most existing research focuses on a single, uniform particle size (Shiu et al. (2020); Mao et al. (2018)), contrasting with our approach that employed a polydisperse plastic solution encompassing a range of sizes (Demir-Yilmaz et al. (2022)). Additionally, many studies assess acute toxicity over a short duration (Sjollem et al. (2016); Gorokhova et al. (2020); Niu et al. (2021)) rather than examining the effects on the entire growth period of the algal species (Mao et al. (2018); Deng et al. (2022)).

Moreover, most studies use elevated, non-environmentally relevant concentrations of MNPs, often several orders of magnitude higher (10^5 to 10^9 times) than those used in this thesis. While these studies provide valuable insights into potential effects at high MNP levels, their ecological relevance is limited. Only two of the studies referenced in this review utilized more environmentally relevant MNP concentrations (Long et al. (2017); Niu et al. (2021)). Both these studies reported no significant inhibitory effects on algal growth.

A comprehensive review of existing literature revealed a scarcity of studies investigating the chronic effects of polydisperse MNP solutions on microalgae at environmentally relevant concentrations, particularly for PET. Furthermore, the inclusion of weathered PET to simulate aging effects and kaolin as a control for non-plastic particles appears unique within the current research landscape. These considerations significantly enhance the ecological relevance of the present study. To build upon these findings and draw more robust conclusions regarding the potential toxicity of MNPs on microalgae, further research employing environmentally relevant exposure scenarios is highly encouraged.

6.8 Future perspectives

Microplastic research has seen a steep surge in recent years, with publications skyrocketing from a handful in 2010 to over 850 in 2019 (Zhu et al., 2021). While many studies report harm to aquatic life at high concentrations, these effects are unlikely in real-world scenarios since research using realistic concentrations shows minimal effects. This raises crucial questions about research prioritization. Are current research efforts on MNPs proportionate with their potential ecological threat or are there other environmental challenges that demand more immediate attention? Additionally, should some of the focus on plastic toxicity be redirected towards developing sustainable alternatives, or investigating the feasibility and effectiveness of Extended Producer Responsibility programs?

Despite these critical notes, this study did demonstrate significant changes in algal growth, EPS composition, and aggregation behavior even at low concentrations of a single type of MNP. However, in their natural environment, microalgae will encounter a complex mix of contaminants, including various plastic particles alongside chemicals and pharmaceuticals. These plastics can act as vectors, attracting and concentrating other pollutants on their surfaces (Amelia et al., 2021). This adsorbed chemical mixture can then be transferred to the algae, potentially leading to synergistic or antagonistic effects that are not captured

when studying single pollutants. Therefore, the combined effects of these contaminants are likely to be more pronounced than what was observed in this study.

Furthermore, while current MNP concentrations may not pose an immediate threat to microalgae, this doesn't guarantee long-term safety. With rising human populations, plastic production and environmental release are projected to increase. This study utilized realistic North Sea MP concentrations (1.5 MP/m^3), considering their fragmentation into NPs. However, concerning predictions suggest that by 2100, ocean MNP concentrations could reach $48.8 \text{ particles/m}^3$, a 33-fold increase compared to present day (Everaert et al., 2018). Such a steep increase necessitates close monitoring and reevaluation of potential risks to aquatic ecosystems, preferably incorporating multi-stressor exposures.

7. CONCLUSION

This study addressed critical gaps in micro- and nanoplastic (MNP) research by employing realistic exposure scenarios with weathered plastics and natural particle controls. It explored microalgal responses in terms of growth, EPS production, and aggregation to environmentally relevant concentrations of PET, aged-PET, and kaolin.

The findings highlighted the link between microalgal survival and the adoption of protective mechanisms under MNP exposure. Exposure to MNPs lowered the carrying capacity of algal cultures, while kaolin exposure resulted in an increase. Interestingly, maximum growth rate increased across all exposure groups.

Despite evidence suggesting stressful conditions can enhance EPS production, this study did not detect a significant increase in EPS production after 10 days of exposure for any treatment group. However, the composition of EPS did shift towards a higher protein-to-carbon (P/C)-ratio, particularly for kaolin exposure.

Aggregation data revealed a clear distinction between natural and synthetic particles. Exposure to kaolin particles led to the formation of larger and more numerous algal aggregates, likely due to the flocculating properties of clay and the increased production of sticky and hydrophobic EPS (indicated by the higher P/C-ratio). Notably, the area of these aggregates was dominated by algal cells, with a lower proportion of EPS. This can be explained by the larger size of the algal aggregates, leading to a lower surface-to-volume ratio and consequently less EPS bound to the surface.

In contrast, exposure to PET and aged-PET resulted in fewer and smaller algal aggregates. Additionally, the area of these aggregates contained a higher proportion of algal cells compared to EPS. This difference is likely due to the strong affinity of EPS for the negatively charged MNPs, leading to the formation of MNP homo-aggregates. This preferential binding leaves less EPS available to interact with algal cells, resulting in fewer and smaller algal aggregates.

Finally, this study considered the environmental implications of these findings. While the increased size and density of plastic aggregates might enhance their removal from the water column through sedimentation, this process could also lead to increased exposure and potential harm for benthic organisms, potentially disrupting the entire benthic food web.

Given the potential consequences for benthic communities, further research specifically investigating the effects of MNPs on these organisms is crucial. Additionally, future research should continue to employ environmentally relevant exposure scenarios, ideally incorporating multiple stressors to better mimic natural conditions.

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APPENDIX A

L1 MEDIUM

L1 Medium

Marine dinoflagellates

Stocks

- | | per litre |
|---|------------------|
| (1) NaNO ₃ | 75g |
| (2) NaH ₂ PO ₄ ·2H ₂ O | 5.65g |
| (3) Trace elements: There are 11 chemicals. Prepare Primary stock solutions first. Add first two chemicals separately in 500ml/dH₂O , allowing each to completely dissolve before adding the primary stocks in order: | |

	Chemical	Primary stock solutions 10ml dH ₂ O	Amount/Volume for <u>500ml</u> working stock solution
1	Na ₂ EDTA·2H ₂ O	-	2.18g
2	FeCl ₃ ·6H ₂ O	-	1.575g
3	CuSO ₄ ·5H ₂ O	0.0245g	0.125ml
4	Na ₂ MoO ₄ ·2H ₂ O	0.199g	1.5ml
5	ZnSO ₄ ·7H ₂ O	0.22g	0.5ml
6	CoCl ₂ ·6H ₂ O	0.1g	0.5ml
7	MnCl ₂ ·4H ₂ O	1.8g	0.5ml
8	H ₂ SeO ₃	0.026g	0.5ml
9	NiSO ₄ ·6H ₂ O	0.027g	0.5ml
10	Na ₃ VO ₄	0.0184g	0.5ml
11	K ₂ CrO ₄	0.0194g	0.5ml

(4) Vitamin mix:

	per litre
Cyanocobalamin (Vitamin B ₁₂)	0.0005 g
Biotin	0.0005 g
Thiamine HCl (Vitamin B ₁)	0.1g

Vitamin mix continued:

For ease of measuring first prepare primary stock solutions.
Into 950 ml of deionised water, dissolve the thiamine HCl and add 1 ml of the primary stocks. Bring volume to 1 litre with deionised water. Filter sterilise and store frozen.

Component	Primary Stock solution	Quantity	Molar Concentration in final medium
Thiamine HCl (B ₁)	-	100mg	2.96×10^{-7}
Biotin	0.5g L ⁻¹ dH ₂ O	1ml	2.05×10^{-9}
Cyanocobalamin (B ₁₂)	0.5g L ⁻¹ dH ₂ O	1ml	3.69×10^{-10}

Medium

per litre

Stock solution 1	1.0 ml
Stock solution 2	1.0 ml
Stock solution 3 (Trace elements)	1.0 ml
Stock solution 4 (Vitamin mix)	1.0 ml

Make up to 1 litre with filtered natural seawater. Adjust pH to **8.0** with 1M NaOH or 1 M HCl prior to autoclaving. For agar, add 15 g per litre Bacteriological Agar.
Autoclave at 15 psi for 15 minutes.

Reference

"*Stichochrysis immobilis* is a diatom, not a chrysophyte" Guillard and Hargraves (*Phycologia* 32.3(1993):234-236) – adapted for CCAP

Reviewed: 06th January 2023

APPENDIX B

PARTICLE CHARACTERISATION

B.1 SEM images

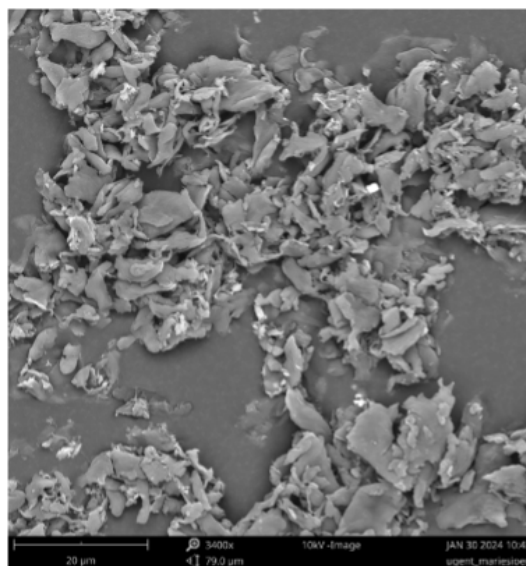


Figure B.1: SEM image of aged-PET particles.

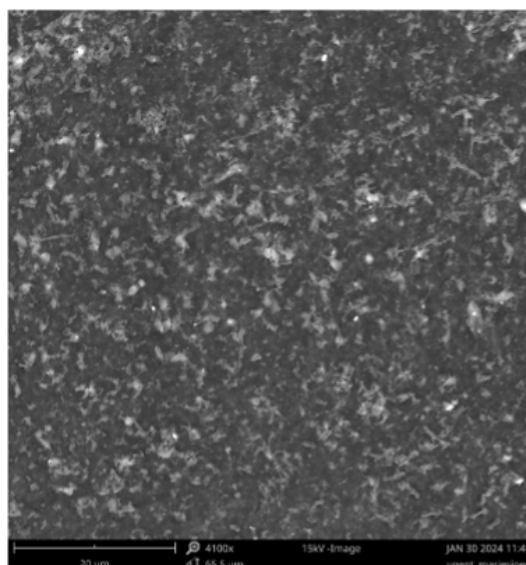


Figure B.2: SEM image of PET particles.

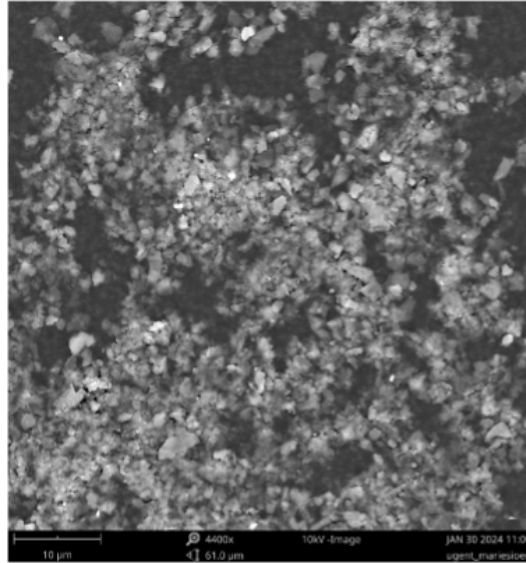


Figure B.3: SEM image of kaolin particles.

B.2 DLS data: hydrodynamic diameter and zeta potential

Particle type	Day	Parameter	Value
Kaolin	0	Hydrodynamic Diameter [nm]	2585 ± 303
Kaolin	11	Hydrodynamic Diameter [nm]	2178 ± 147
PET	0	Hydrodynamic Diameter [nm]	798 ± 240
PET	11	Hydrodynamic Diameter [nm]	$171 \pm 4,64$
weathered PET	0	Hydrodynamic Diameter [nm]	658 ± 167
weathered PET	11	Hydrodynamic Diameter [nm]	705 ± 78
Kaolin	0	Zeta potential [mV]	$-12,8 \pm 1,12$
Kaolin	11	Zeta potential [mV]	$-9,05 \pm 1,71$
PET	0	Zeta potential [mV]	$-3,54 \pm 3,02$
PET	11	Zeta potential [mV]	$-3,42 \pm 1,17$
weathered PET	0	Zeta potential [mV]	$-6,1 \pm 1,63$
weathered PET	11	Zeta potential [mV]	$-7,16 \pm 5,86$

Figure B.4: Summary of the DLS measurement data of the zeta potential and the hydrodynamic diameter, for each particle type, both measured in fresh medium, and 11 days old medium. (Measured at 10 V; with a RI of 1.33 for PET, and 1.57 for kaolin).

B. Model development coding

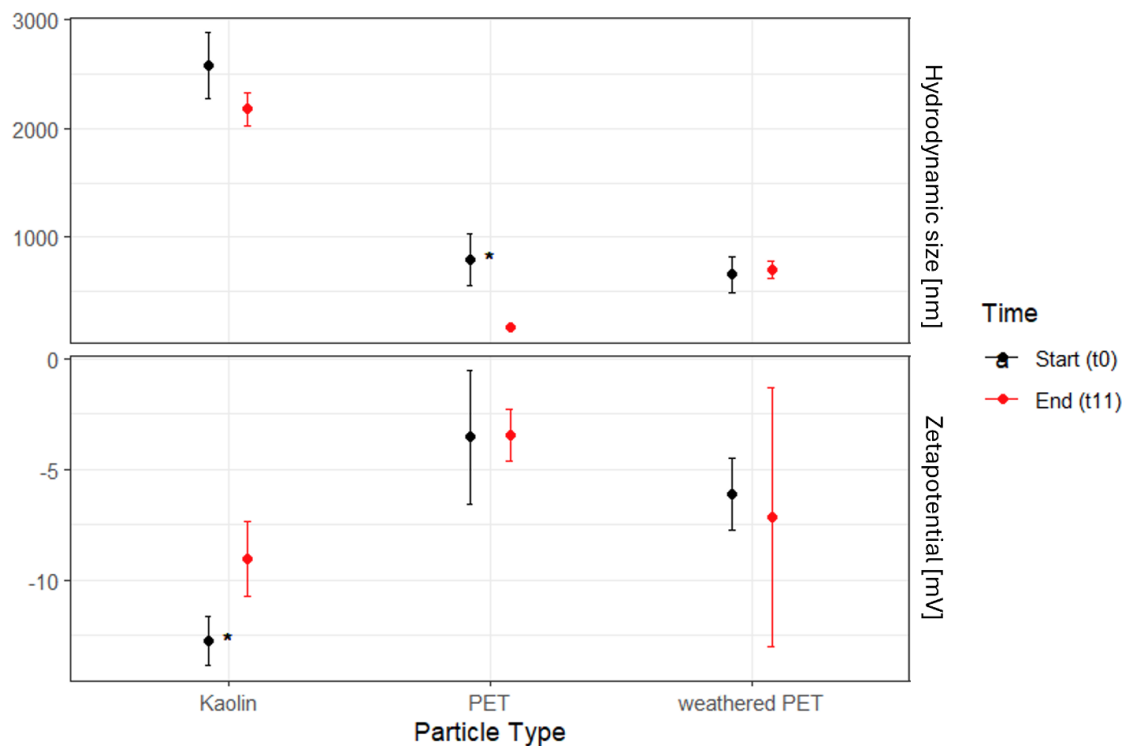


Figure B.5: Zetapotential and hydrodynamic size for each particle in medium on day 0, and medium on day 11. '*' indicates significant differences between day 0 and day 11.

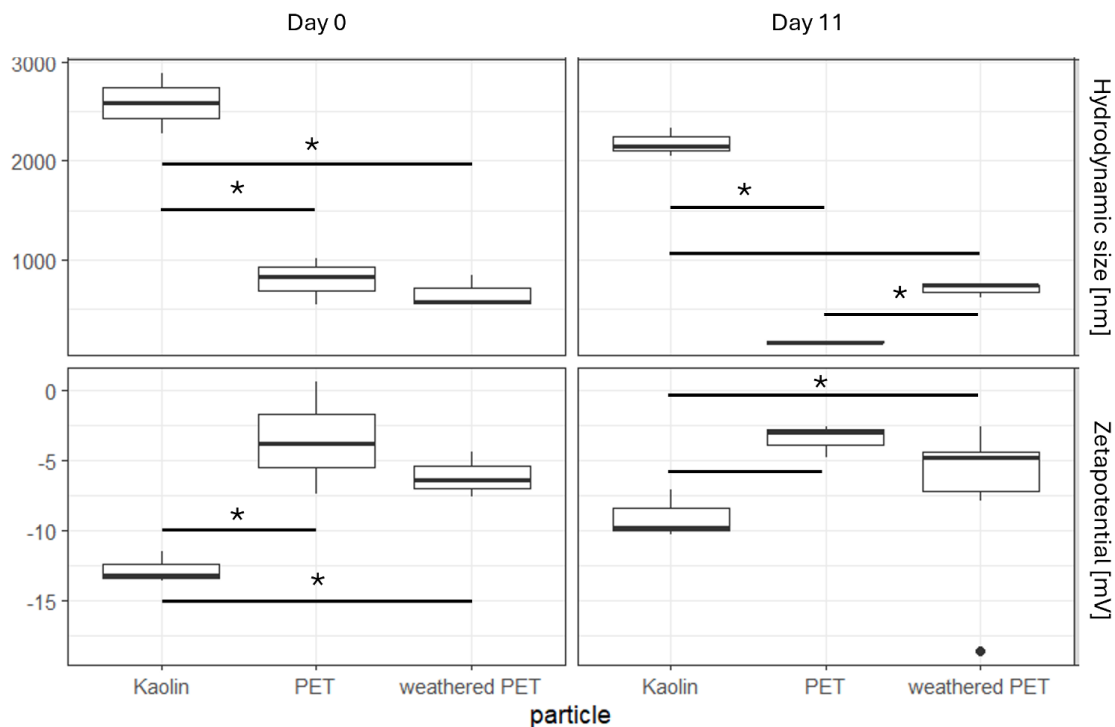


Figure B.6: Zetapotential and hydrodynamic size for each particle in medium on day 0, and medium on day 11. '*' indicates significant differences between the different particle types.

B.3 ATR-FTIR spectra

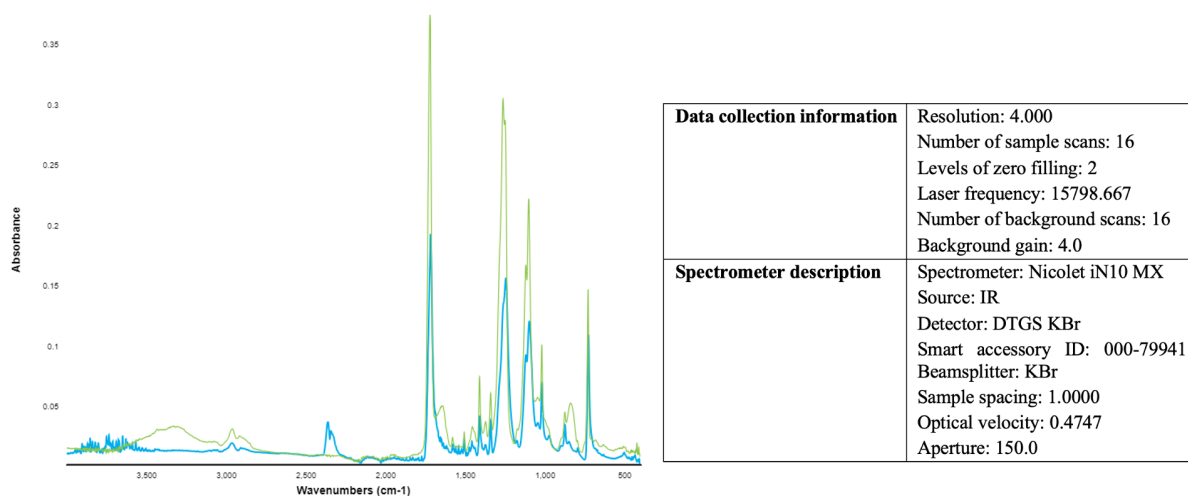


Figure B.7: IR spectrum of aged-PET (blue) and PET (green), including instrument specifications.

B.4 Additives



Polymer



Powder

Polyethylene terephthalate - Powder

Polyester, PET, PETP

Product Code: **ES30-PD-000132**

Maximum Particle Size: **300µm**

Additives: **maximum 1ppm Acetaldehyde**

Crystallinity: **>50%**

Inherent Viscosity: **0.80 dL/g**

Structure: **Semi Crystalline**

Colour: **Natural**

Weight: **100g - 20000g**

Polyethylene terephthalate

Polyethylene terephthalate, is the most common thermoplastic polymer resin of the polyester family and is used in fibres for clothing, containers for liquids and foods, and thermoforming for manufacturing, and in combination with glass fibre for engineering resins.

Material Properties for Polyethylene terephthalate

Chemical Resistance

Acids - concentrated: **Good-Poor**

Acids - dilute: **Good**

Alcohols: **Good**

Alkalies: **Poor**

Aromatic hydrocarbons: **Good-Fair**

Greases and Oils: **Good**

Halogenated Hydrocarbons: **Good-Poor**

Halogens: **Fair-Poor**

Ketones: **Good-Fair**

Electrical Properties

Dielectric constant @1MHz: **3**

Dielectric strength (kV mm⁻¹): **17.0**

Dissipation factor @ 1kHz: **0.0020**

Surface resistivity (Ohm/sq): **10¹³**

Volume resistivity (Ohmcm): **>10¹⁴**

Mechanical Properties

Coefficient of friction: **0.20 - 0.40**

Hardness - Rockwell: **M94-101**

Izod impact strength (J m⁻¹): **13.0 - - 35.0**

Poisson's ratio: **0.370 - 0.440**

Tensile modulus (GPa): **2.00 - 4.00**

Tensile strength (MPa): **80.00 , for biax film 190-260**

Physical Properties

Density (g cm⁻³): **1.300 - 1.400**

Flammability: **HB**

Limiting oxygen index (%): **21**

Radiation resistance: **Good**

Refractive index: **1.580-1.640**

Resistance to Ultra-violet: **Fair**

Water absorption - equilibrium (%): **< 0.70**

Water absorption - over 24 hours (%): **0.100**

Thermal Properties

Coefficient of thermal expansion ($\times 10^{-6} \text{ K}^{-1}$): **20.000 - 80.000**

Heat-deflection temperature - 0.45MPa (C): **115**

Heat-deflection temperature - 1.8MPa (C): **80.0**

Lower working temperature (C): **-40 - -60**

Specific heat (J K⁻¹ kg⁻¹): **1200.0 - 1350.0**

Thermal conductivity (W m⁻¹ K⁻¹): **0.15 - 0.40 @23°C**

Upper working temperature (C): **115 - 170**

Related Product Data

Form

Powder

Small particles with an approximately defined size range. Those materials described as alloy precursors are not true alloys - they are made by sintering a blend of powders of the component metals to achieve alloying by diffusion. The resultant cake is ground



and sieved to the required particle size range. Unless otherwise stated, the particle sizes shown are for guidance only. We do not guarantee either any particular size distribution between the quoted minimum and maximum sizes, or a specific particle shape.

Tolerances

Maximum Particle Size:

Mean Particle Size:

Mean Particle Size (FSSS):

Minimum Particle Size:

Material

PET - Polyethylene Terephthalate

Polyethylene terephthalate, is the most common thermoplastic polymer resin of the polyester family and is used in fibres for clothing, containers for liquids and foods, and thermoforming for manufacturing, and in combination with glass fibre for engineering resins.

Type

Polymer

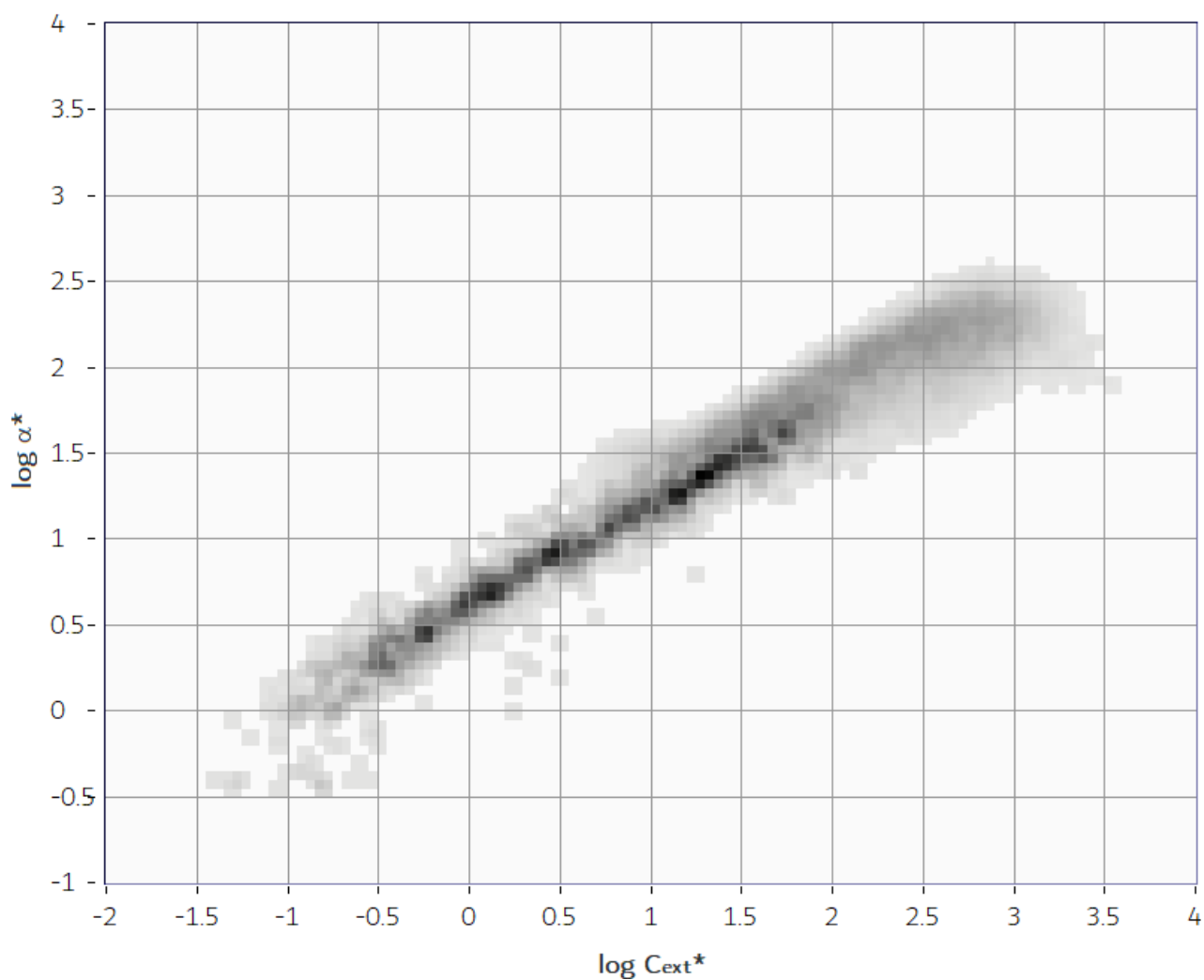
A carbon based material which is built up from a series of smaller units (monomers). The choice of the monomers and the final molecular weight (i.e. size) of the polymer govern the mechanical and physical properties of the resultant polymer.



B.5 Sample reports

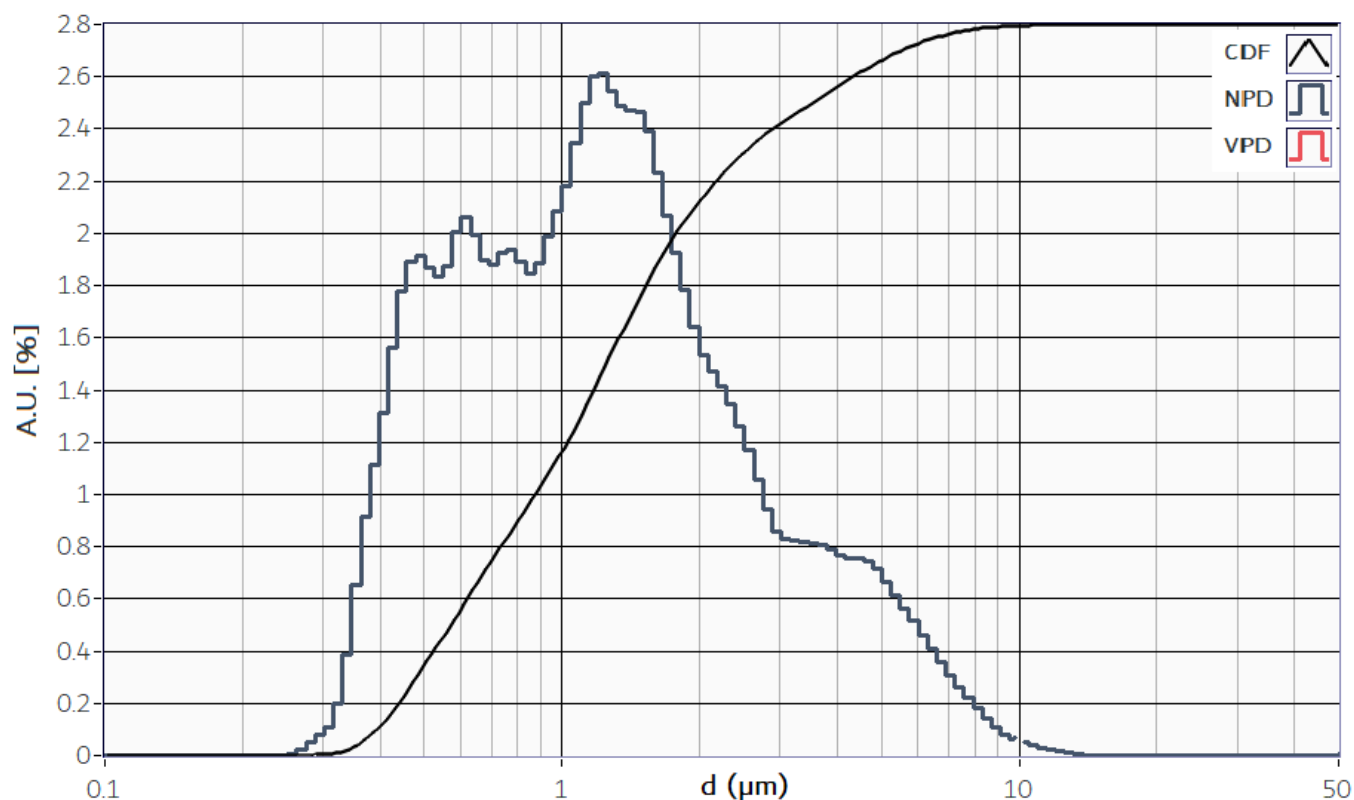
Aged-PET

Filename:	20240307_105956_aPET R2		
Operator:	User	Company:	Company
Acquisition Date:	07/03/2024 10:59:56	Analysis date:	07/03/2024 11:43
Solvent:	1.33 Water, Methanol	Particle Model:	Refractive Index
Device model:	CLASSIZER ONE	S/N:	CS08219AAB
SW Version:	ClassizerONE S1.4.42	Accessories:	



Flux (mL/min)	4.0	Ultrasounds power	0.00
Measure code	MC01617	Acquisition mode	Manual
Lotnumber		Analytical measure	1
SOP	Standard	Measure replicate	1
Stirrer speed	0.00	Analyzed vol. (mL)	17.33

Particle Size Distribution



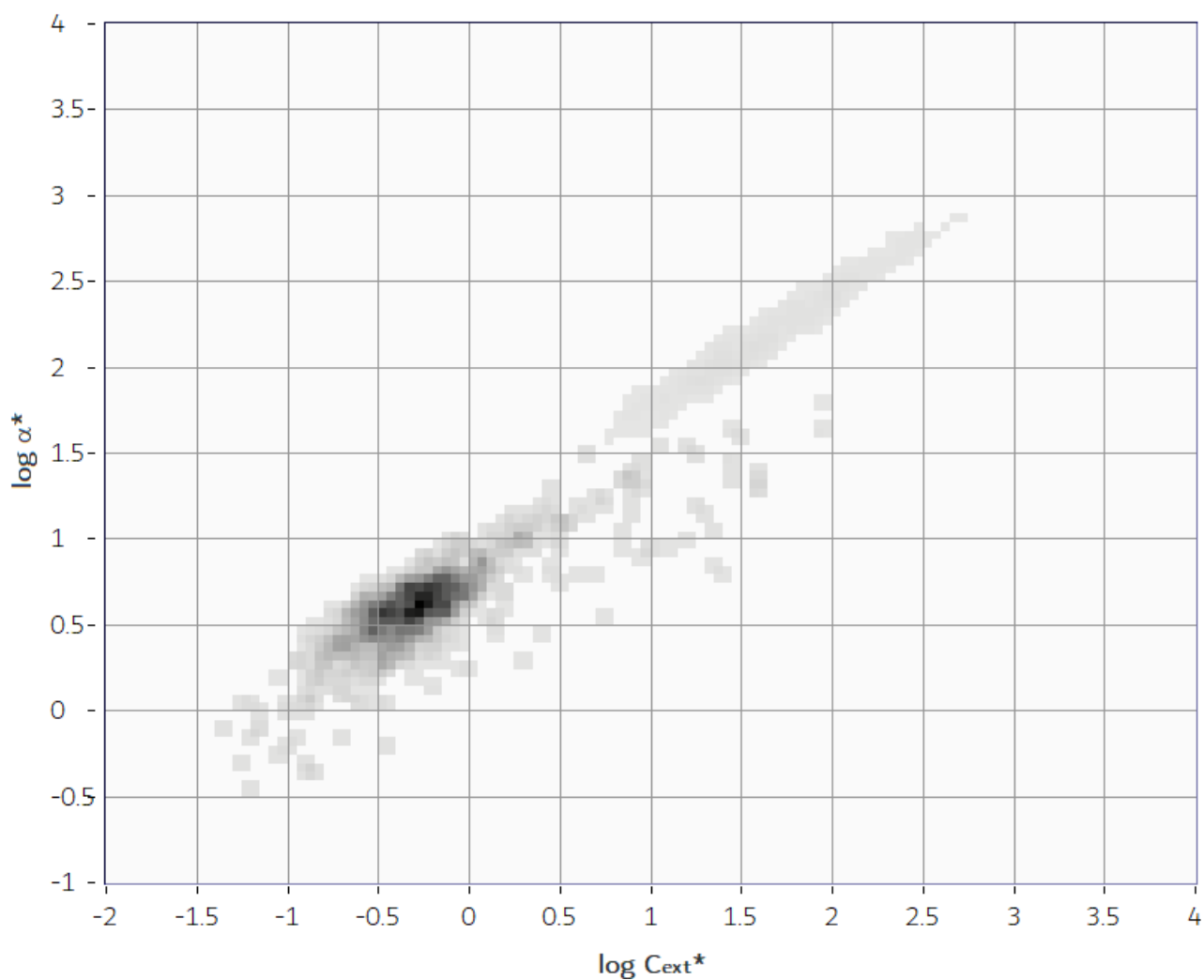
Conc. PTCs/mL	2.9E+7	RI	1.46 + i 0.00
Dilution	50	RI CV	0.04
Selection %	100.0	RI Std. Dev.	0.05
LUT superpos.	1.00	RI Skewness	0.18
PTCs validated	14583	Dim. Avg	1.7 μm
Avg Log[Re S]	1.18	Dim. Peak	1.2 μm
Avg Log[Im S]	1.31	Dim. CV	0.91
Sigma Log[Re S]	1.10	D10	0.47 μm
Sigma Log[Im S]	0.66	D50	1.2 μm
Mode Log[Re S]	1.25	D90	3.6 μm
Mode Log[Im S]	1.35	D[4,3]	6.3 μm
Comment	dilution 400uL in 20 mL		

Oversize Detection

Dimensional Class	Meas Num Conc [ptc/mL]	Scaled Num Conc [ptc/mL]	Relative Num Conc [%]	Expert Advice on Statistics
Overall	5.86E+5	2.93E+7	100%	Good
> 0.5 µm	5.17E+5	2.59E+7	88%	Good
> 0.7 µm	4.34E+5	2.17E+7	74%	Good
> 1.0 µm	3.47E+5	1.73E+7	59%	Average
> 2.0 µm	1.45E+5	7.26E+6	25%	Average
> 5.0 µm	3.04E+4	1.52E+6	5%	Fair
> 10.0 µm	1.03E+3	5.17E+4	0%	Poor

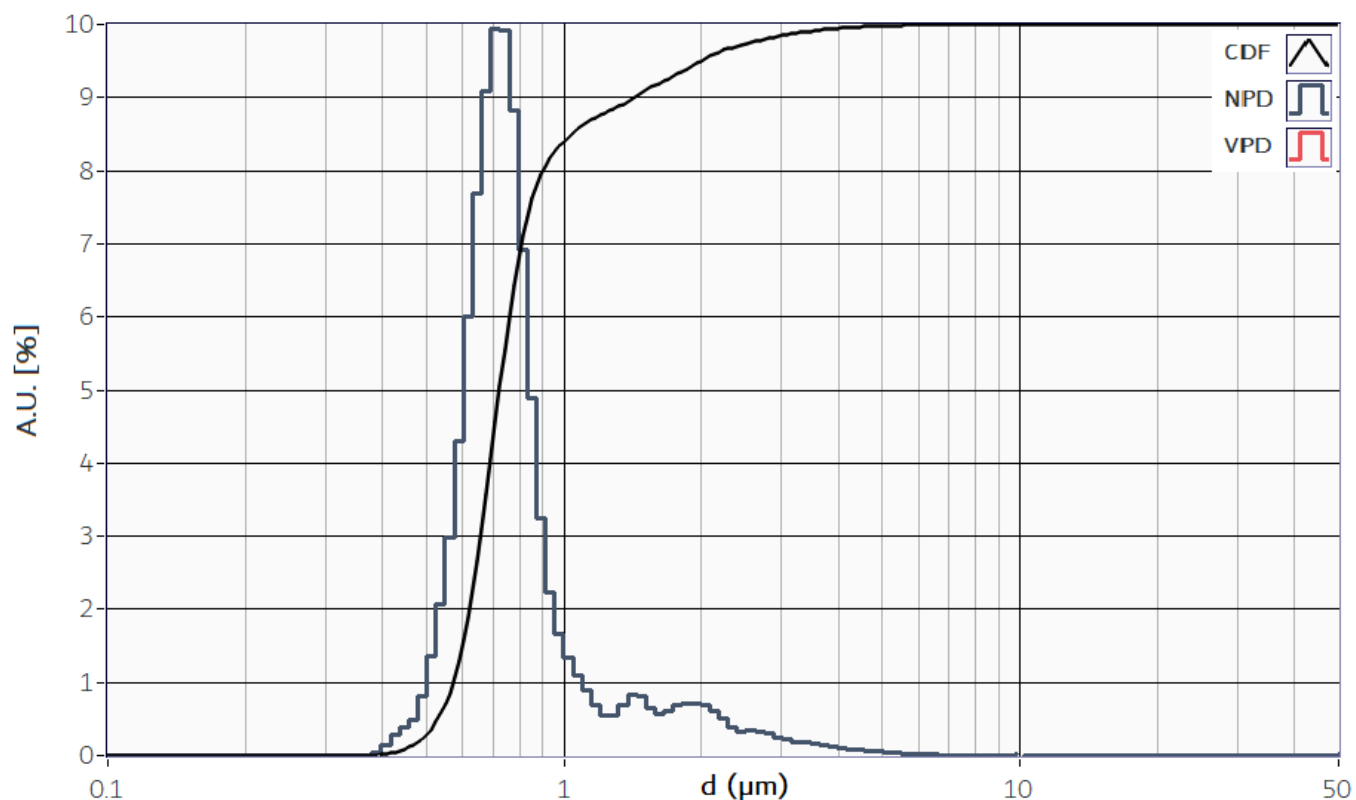
PET

Filename:	20240307_113335_PET R2		
Operator:	User	Company:	Company
Acquisition Date:	07/03/2024 11:33:35	Analysis date:	07/03/2024 11:44
Solvent:	1.33 Water, Methanol	Particle Model:	Refractive Index
Device model:	CLASSIZER ONE	S/N:	CS08219AAB
SW Version:	ClassizerONE S1.4.42	Accessories:	



Flux (mL/min)	4.0	Ultrasounds power	0.00
Measure code	MC01621	Acquisition mode	Manual
Lotnumber		Analytical measure	1
SOP	Standard	Measure replicate	1
Stirrer speed	0.00	Analyzed vol. (mL)	17.40

Particle Size Distribution



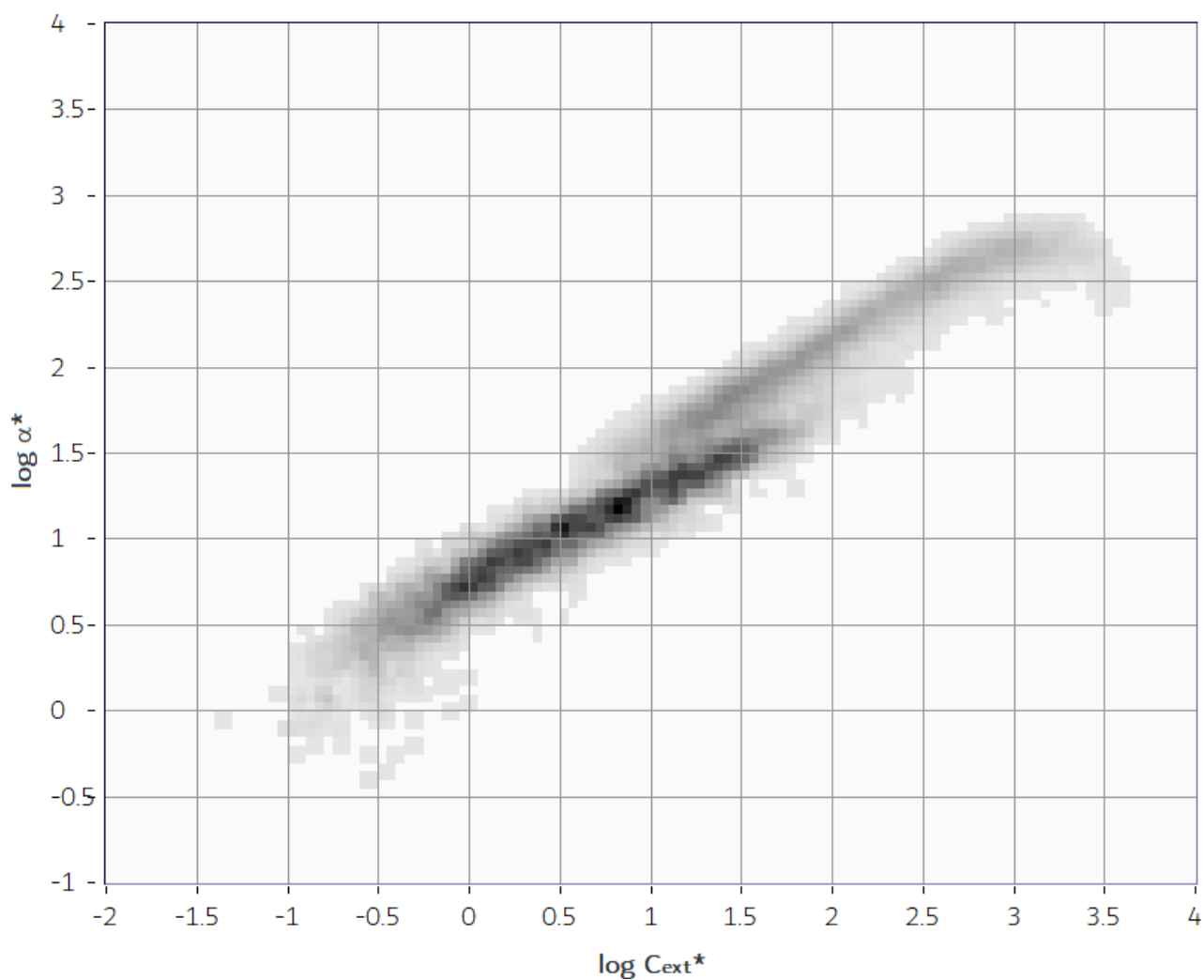
Conc. PTCs/mL	1.6E+6	RI	1.39 + i 0.00
Dilution	6	RI CV	0.03
Selection %	100.0	RI Std. Dev.	0.04
LUT superpos.	1.00	RI Skewness	0.65
PTCs validated	4859	Dim. Avg	0.91 μm
Avg Log[Re S]	-0.09	Dim. Peak	0.71 μm
Avg Log[Im S]	0.74	Dim. CV	0.64
Sigma Log[Re S]	0.68	D10	0.57 μm
Sigma Log[Im S]	0.51	D50	0.72 μm
Mode Log[Re S]	-0.30	D90	1.4 μm
Mode Log[Im S]	0.60	D[4,3]	3.7 μm
Comment	dilution 3.8mL in 22.8 mL		

Oversize Detection

Dimensional Class	Meas Num Conc [ptc/mL]	Scaled Num Conc [ptc/mL]	Relative Num Conc [%]	Expert Advice on Statistics
Overall	2.59E+5	1.55E+6	100%	Average
> 0.5 µm	2.53E+5	1.52E+6	98%	Average
> 0.7 µm	1.61E+5	9.65E+5	62%	Average
> 1.0 µm	4.34E+4	2.61E+5	17%	Fair
> 2.0 µm	1.38E+4	8.27E+4	5%	Fair
> 5.0 µm	5.03E+2	3.02E+3	0%	Poor
> 10.0 µm	2.04E+1	1.22E+2	0%	Poor

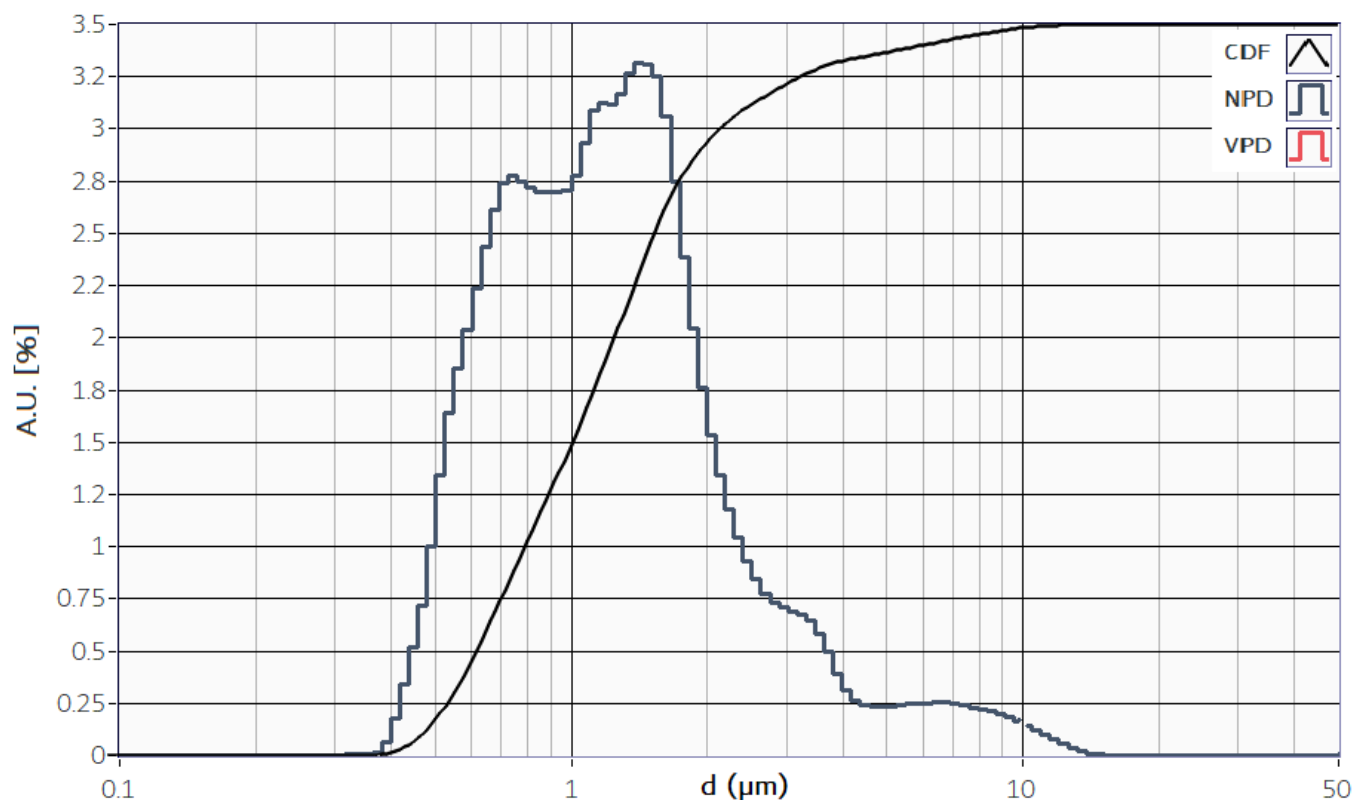
Kaolin

Filename:	20240307_111747_kadine R2		
Operator:	User	Company:	Company
Acquisition Date:	07/03/2024 11:17:47	Analysis date:	07/03/2024 11:44
Solvent:	1.33 Water, Methanol	Particle Model:	Refractive Index
Device model:	CLASSIZER ONE	S/N:	CS08219AAB
SW Version:	ClassizerONE S1.4.42	Accessories:	



Flux (mL/min)	4.0	Ultrasounds power	0.00
Measure code	MC01619	Acquisition mode	Manual
Lotnumber		Analytical measure	1
SOP	Standard	Measure replicate	1
Stirrer speed	0.00	Analyzed vol. (mL)	17.40

Particle Size Distribution



Conc. PTCs/mL	1.9E+7	RI	1.42 + i 0.00
Dilution	20	RI CV	0.03
Selection %	100.0	RI Std. Dev.	0.05
LUT superpos.	1.00	RI Skewness	0.52
PTCs validated	16718	Dim. Avg	1.6 μm
Avg Log[Re S]	0.96	Dim. Peak	1.4 μm
Avg Log[Im S]	1.34	Dim. CV	0.95
Sigma Log[Re S]	0.95	D10	0.59 μm
Sigma Log[Im S]	0.63	D50	1.2 μm
Mode Log[Re S]	0.80	D90	2.7 μm
Mode Log[Im S]	1.15	D[4,3]	8.1 μm
Comment	dilution 1mL in 20 mL		

Oversize Detection

Dimensional Class	Meas Num Conc [ptc/mL]	Scaled Num Conc [ptc/mL]	Relative Num Conc [%]	Expert Advice on Statistics
Overall	9.41E+5	1.88E+7	100%	Good
> 0.5 µm	9.15E+5	1.83E+7	97%	Good
> 0.7 µm	7.75E+5	1.55E+7	82%	Good
> 1.0 µm	5.77E+5	1.15E+7	61%	Good
> 2.0 µm	1.68E+5	3.36E+6	18%	Average
> 5.0 µm	3.80E+4	7.60E+5	4%	Fair
> 10.0 µm	5.51E+3	1.10E+5	1%	Poor

B.6 Particle concentration calculations

The calculations are based on the particle size distributions (as documented in Appendix B.5), and mass conservation principles. Calculations were performed to ascertain the quantity of MNPs resulting from the complete degradation of a single 250 μm plastic particle into MNPs with an equivalent size distribution to that of the plastic stocks used in these experiments. This involved converting the size distribution to a mass distribution and subsequently rescaling it to ensure that the total mass equaled that of one 250 μm plastic particle.

Step 1										
Microplastic pollution										
Concentration	1.5	MP/m³	PET density	1.38E-12	ng/nm³					
Size	250	µm								
Volume	8.18E+15	nm³								
	1.13E+04	ng								
Step 2										
1 microplastic particle completely degrades to particles with size distributions like the size distribution of the plastic material used in the experiment.										
Size distribution is derived from ClassizerONE data as reported in SI.1.										
Virgin PET										
Concentration	1600000	MNPs/ml								
Size class [nm]	cum [%]	Size class [nm]	cum [%]	Size class [nm]	% per class	# Ps	D [nm]	V [nm³]	Vt [nm³]	Mt [ng]
>500	98%	<500	2%	0-500	2%	32000	250	8.18E+06	2.62E+11	3.61E-01
>700	62%	<700	38%	500-700	36%	576000	600	1.13E+08	6.51E+13	8.99E+01
>1000	17%	<1000	83%	700-1000	45%	720000	850	3.22E+08	2.32E+14	3.19E+02
>2000	5%	<2000	95%	1000-2000	12%	192000	1500	1.77E+09	3.39E+14	4.68E+02
>5000	0%	<5000	100%	2000-5000	5%	80000	3500	2.24E+10	1.80E+15	2.48E+03
>10000	0%	<10000	100%	5000-10000	0%	0	7500	2.21E+11	0.00E+00	0.00E+00
										3.36E+03
Weathered PET										
Concentration	29000000	MNPs/ml								
size class [nm]	cum [%]	size class [nm]	cum [%]	size class [nm]	% per class	# Ps	D [nm]	V [nm³]	Vt [nm³]	Mt [ng]
>500	88%	<500	12%	0-500	12%	3480000	250	8.18E+06	2.85E+13	3.93E+01
>700	74%	<700	26%	500-700	14%	4060000	600	1.13E+08	4.59E+14	6.34E+02
>1000	59%	<1000	41%	700-1000	15%	4350000	850	3.22E+08	1.40E+15	1.93E+03
>2000	25%	<2000	75%	1000-2000	34%	9860000	1500	1.77E+09	1.74E+16	2.40E+04
>5000	5%	<5000	95%	2000-5000	20%	5800000	3500	2.24E+10	1.30E+17	1.80E+05
>10000	0%	<10000	100%	5000-10000	5%	1450000	7500	2.21E+11	3.20E+17	4.42E+05
										6.48E+05
Step 3										
Based on the mass distribution, we can calculate the weight of the particles in 1 ml of the stock solution as the sum of the total weight of each size class.										
Virgin PET: 1600000 MNPs have a total weight of:				3.36E+03	ng					
Weathered PET: 29000000 MNPs have a total weight of:				6.48E+05	ng					
Step 4										
Based on the mass of a 250µm MP [1.13E+04 ng], and the mass of 1 ml particles with known concentration and size distribution, we can calculate the number of MNPs after full degradation of the MP, based on mass conservation principles.										
MP [250 µm]	1.13E+04	ng								
Virgin PET	1.60E+06	MNPs/ml								
	3.36E+03	ng/ml								
	5.38E+06	MNPs								
1 MP [250 µm] would break down in 5.38E+06 MNPs with above-described size distribution										
Weathered PET	2.90E+07	MNPs/ml								
	6.48E+05	ng/ml								
	5.05E+05	MNPs								
1 MP [250 µm] would break down in 5.05E+05 MNPs with above-described size distribution										
Step 5										
Based on the measured MP concentration of 1.5 MP/m³, we assume following MNP concentrations:										
Virgin PET	8.07E+06	MNPs/m³								
	8.07	MNPs/ml								
Weathered PET	7.58E+05	MNPs/m³								
	0.76	MNPs/ml								

APPENDIX C

SCRIPTS

C.1 ImageJ script for kaolin particle analysis

```
open("C:/Gebruikers/lailaherder/Bureaublad/Thesis/Lab Protocols/Algen test/kaoline
    concentratie/4.tif");
run("8-bit");
run("Subtract Background...", "rolling=100 light");
//run("Brightness/Contrast...");
run("Apply LUT");
setAutoThreshold("Default no-reset");
//run("Threshold...");
//setThreshold(0, 166);
setOption("BlackBackground", true);
run("Convert to Mask");
run("Fill Holes");
run("Convert to Mask");
run("Analyze Particles...", " show=Overlay display clear");
saveAs("Results", "C:/Users/mrsioen/OneDrive - UGent/Documenten/Doctoraat nanoplastics/FW0
    /EX1 algae/experiment/POGING 3/partikels info/Kaoline2023/Results10.csv");
```

C.2 ImageJ script for experiment 2

```
dir=getDirectory("Choose Folder");
names=getFileList(dir);
run("Set Measurements...", "area area_fraction redirect=None decimal=3");

for (i=0; i<names.length; i++) {
    open(dir+names[i]);

    a = getTitle();
    if (matches(a, ".*Bright.*")) {
        b=replace(a, "Bright Field", "CY5");
        b=replace(b, "_1_", "_2_");

        open(dir+b);
        setAutoThreshold("Default dark");
        run("Analyze Particles...", "size=20-Infinity show=[Overlay Masks] clear include summarize
            ");
        wait(2000);
        selectImage(a);

        Dialog.create("Selection");
        Dialog.setLocation(2000, 1000); //set location of popupbox
        Dialog.addNumber("number of clusters=", 0);
```

```

Dialog.show();
d = Dialog.getNumber();

for (j=0; j<d; j++) {

    setTool("polygon");
    waitForUser("confirm selection");
    selectImage(b);
    setAutoThreshold("Default dark");
    run("Restore Selection");
    run("Analyze Particles...", "size=20-Infinity show=[Overlay Masks] clear display include")
    ;
    run("Measure");

    selectWindow("Results");

    totalfluo=0;
    cell=118; //set size of cell
    scale=2.40662724507814; //set scale

    for(k=0; k<nResults-1; k++) {
        totalfluo += getResult("Area", k);
    }
    setResult("Total_Area_fluo", nResults, totalfluo);
    setResult("Cell_num (#)", nResults, totalfluo/cell);
    setResult("Total_eps", nResults, getResult("Area", k)/scale);

    updateResults();

    saveAs("Text", "/Users/lailaherder/Desktop/Thesis/Experiment 2/Microscopie/Foto's/Results/
        "+a+"_"+j+1+".csv");

    wait(2000);
    selectImage(a);
}

close();
close();

}
else{
    close();
}
}

```

APPENDIX D

ADDITIONAL EXPERIMENTS

D.1 EPS extraction methods

To identify the most suitable EPS extraction method for downstream experiments, several techniques were compared. The exact steps followed are represented in Figure D.1.

Five EPS extraction methods were evaluated: heat, ultrasonic waves, a combination of both, chemical extraction with formaldehyde and sodium hydroxide, and Cation Exchange Resin (CER) based extraction. The extraction protocols were obtained from Pan et al. (2010) and Strieth et al. (2020). A 2L bag of algal culture was set-up to perform all methods with a consistent starting material.

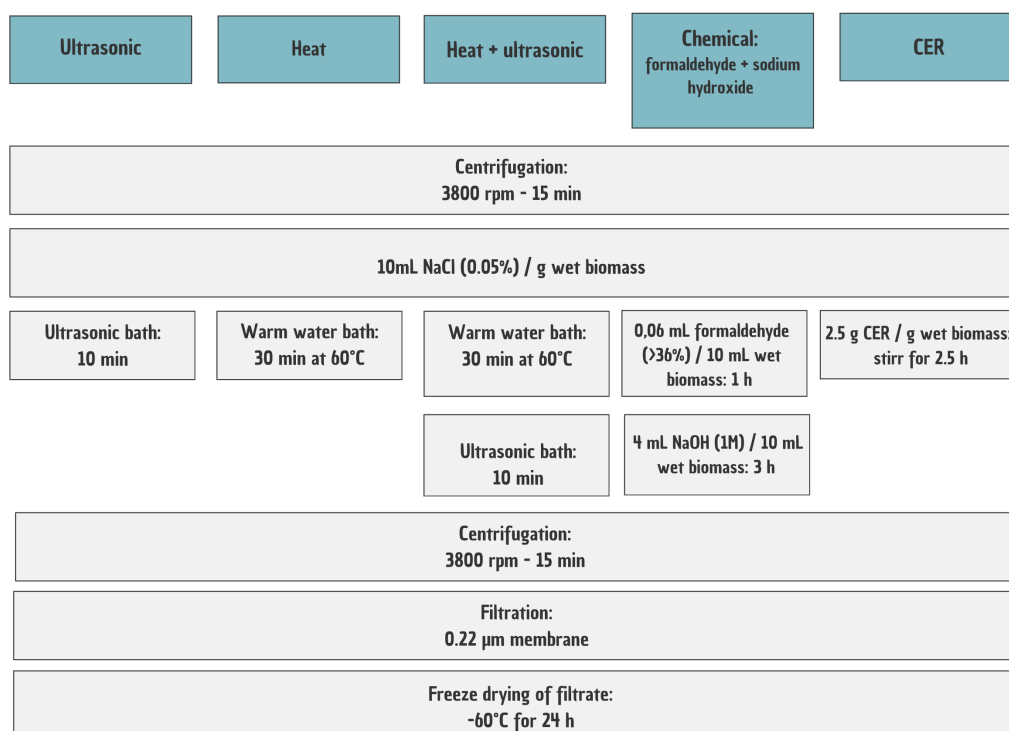


Figure D.1: EPS extraction methods. (Based on Strieth et al. (2020); Pan et al. (2010))

For all methods 50 mL algal suspension was first centrifuged (3800 rpm for 15 min) in a centrifuge (Thermo Fisher Scientific Heraeus Magafuge 40R). The supernatant was removed since this contains the LB-EPS but it is the TB-EPS that is still attached to the algal cells that is of interest. The pellet at the bottom was resuspended in NaCl (0.05%). For the ultrasonic

EPS extraction method	Average EPS yield
Ultrasonic	63 mg
Heat	18 mg
Heat + ultrasonic	20 mg
Chemical	23 mg
CER	0 mg

Table D.1: Average EPS yield (mg) from different extraction methods (2 replicates, 50 mL starting volume per replicate).

method the suspension was then put into an ultrasonic bath (VWR USC300T) at 80W for 10 min. For the heat based extraction method the suspension was put into a warm water bath (60°C for 30 min). For the ultrasonic and heat combination method the warm water bath was followed up by the ultrasonic bath. For the chemical extraction method formaldehyde (36.5%) was added to the suspension and put aside for 1 hour after which NaOH (1M) was added and put aside for another 3 hours. For the cation exchange method 2.5 g of [CER specifications] CER was added per g of wet algal biomass and stirred for 2.5 hours.

After these steps, all suspensions were centrifuged again (3800 rpm for 15 min). The supernatant, considered the TB-EPS extract, was filtered (0.22 μ m membrane) and stored at -60°C until freeze-drying. Following freeze-drying, the EPS yield from each method was compared using the weight that was obtained. The cation exchange method yielded no EPS and was excluded from further analysis. The EPS yield, averaged for 2 replicates and for a starting volume of 50 mL algal suspension, of the remaining methods is presented in Table D.1. The ultrasonic extraction yielded the highest EPS amount and was therefore chosen for subsequent experiments.

D.1.1 Additional experiments for EPS extraction

Control of cell lysis

To ensure the chosen EPS extraction method isolated only the extracellular polymeric substances (EPS) surrounding the algal cells, and not internal cellular material, several control experiments were conducted. Cell lysis, or bursting of the cells, could release intracellular components into the extracted EPS fraction, compromising the analysis of true EPS composition.

Three key parameters of the extraction protocol were evaluated for their potential to induce cell lysis: centrifugation speed/duration, NaCl solution temperature, and ultrasonication duration.

- Centrifugation: Algal suspensions were centrifuged at increasing speeds (2000, 2500, 3000, and 3500 rpm) and durations (10 and 15 minutes) followed by microscopic observation to assess cell integrity. All cells remained intact under these conditions. Therefore, the protocol using 3800 rpm for 15 minutes is considered safe for EPS extraction without cell lysis.
- NaCl temperature and volume: The effect of NaCl solution temperature on cell integrity was investigated. When using warm (60°C) 0.05% NaCl solution, microscopic obser-

uations revealed ruptured algal cells. In contrast, room temperature NaCl solution did not cause cell lysis. Based on this observation, room temperature NaCl solution will be used for resuspending algal biomass in future experiments. The volume of NaCl solution used was also tested. 4 different volumes (100, 200, 300, and 400 μL) of NaCl were added to the algal biomass after removing the supernatant. No cell rupture was observed with any volume tested, indicating NaCl treatment itself did not cause lysis.

- Ultrasonication duration: The effect of ultrasonication duration on cell lysis was evaluated using 5 and 10 minutes. Microscopic observations confirmed that neither duration caused cell rupture. Therefore, the protocol using 10 minutes of ultrasonication is considered safe.

Minimum culture volume for EPS extraction

To optimize resource usage, an experiment was conducted to determine the minimum volume of algal culture required for successful EPS extraction. Different culture volumes (50, 30, 20, and 10 mL) were extracted and freeze-dried. Only the 50 mL volume yielded a sufficient amount of EPS for further analysis. Lower volumes either produced no detectable EPS or yielded too little for consequent analysis. Therefore, 50 mL is considered the minimum culture volume required for successful EPS extraction using this protocol.

D.2 Experiment 1: Effect of MNP exposure on quantity and composition of algal EPS - additional experiments

D.2.1 Method validation: EPS production under salinity stress

An experiment employing salinity stress was conducted to validate whether stressful conditions can influence EPS production and alter its composition. Salinity was chosen as the stress factor because previous research, such as the work by Farghl et al. (2015), has shown that salt stress can impact various aspects of marine microalgae, including growth, photosynthetic pigment production, protein content, and carbohydrate content.

For this experiment, the salinity of pure seawater was measured at 31 ppt. A series of salinity levels was prepared by mixing seawater with DI water to create three lower salinities (23, 26, and 29 ppt) and three higher salinities (34, 37, and 40 ppt). Six Erlenmeyer flasks were filled with 200 mL each of the prepared dilution series. To each flask, 200 μL of stock solutions 1-4 were added, followed by spiking with algal culture. The cultures were then distributed into three replicates of 50 mL each for each salinity level.

Cell counts were determined using a Coulter counter every three days. On day 3, EPS was extracted using ultrasonication, and the samples were freeze-dried. After freeze-drying, the EPS was weighed and analyzed using ATR-FTIR spectroscopy. The limited sample size of this test made it difficult to assess whether there were definitive trends in the amount

and composition of EPS. However, the test did reveal variations in EPS production and composition, suggesting that they could yield interesting data for the full experiment.

D.3 Experiment 2: Effect of MNP exposure on aggregation - additional experiments

D.3.1 Method validation: flocculation with ferric chloride FeCl_3

To evaluate the effectiveness of light microscopy and DLS in detecting aggregation, a control experiment was conducted using ferric chloride (FeCl_3). FeCl_3 is a chemical flocculant that induces aggregation of algal cells through charge neutralization. Three Erlenmeyer flasks containing 100 mL of algal culture each were treated with three different concentrations of FeCl_3 (200, 300, and 400 mg/L).

These samples were examined under a fluorescence microscope using a DAPI filter (excitation 340/80nm, emission 425nm) to observe algal cells and EPS. While algal cells showed distinct auto-fluorescence, EPS was not directly observable with fluorescence microscopy. Attempts to stain EPS with Alician Blue dye did not result in a fluorescent signal. Nevertheless, samples treated with flocculant clearly demonstrated the formation of more and larger aggregates compared to the controls. This suggested that light microscopy effectively visualizes the quantity, size, and algal cell content of aggregates.

To validate the DLS method, the same samples were analyzed using a DLS instrument, providing volume-based size distribution for each sample. A notable difference in size distribution was observed between samples with and without flocculant. Flocculated samples displayed a notably greater average diameter, affirming the suitability of DLS for estimating aggregate size distributions.

D.3.2 Dynamic light scattering

Volume of algal culture

To determine the amount of algal culture needed for 120 mL of seawater, the obscuration level was considered. Obscuration indicates the amount of light blocked by particles. A low obscuration level may result in a weak signal and noise, while a high obscuration level may cause multiple scattering, distorting the signal (De Cleyn et al., 2019). To ensure consistent and optimal measurements across all samples (targeted obscuration: 11-30%), the initial algal culture was gradually added to 120 mL seawater until an obscuration of 11% was attained. The volume of subsequent cultures required to achieve an 11% obscuration in 120 mL seawater was calculated based on their cell count relative to the first algal culture, then increased by 30% to ensure reaching the lower limit of the target range. This approach ensured consistent sample concentrations for DLS measurements.

Circulation time

To assess the impact of sample circulation on size distribution, measurements were taken immediately after adding the sample and after extended circulation. These measurements revealed a shift in the peak of the size distribution for the longer circulated sample. The peak diameter decreased, suggesting that prolonged circulation might disrupt the aggregates. Consequently, during the actual experiment, the sample was added to seawater before activating the pump and starting the measurement.

Reproducibility

To evaluate the reproducibility of the DLS method, 27.5 mL of algal culture was added to 120 mL of seawater under identical conditions three times. The resulting volume-based size distributions were compared for their similarity, ensuring consistency in the measurement process. The high degree of similarity observed among the three distribution curves affirmed the reproducibility of this method.

D.3.3 Microscopy

Dilution optimisation

Prior to the experiment, different sample dilutions were tested: 2 mL algal culture + 2 mL medium, 2 mL algal culture + 1 mL medium, and 2 mL algal culture + 3 mL medium. The obtained images revealed that a dilution of 2 mL algal culture + 3 mL medium produced the most suitable results. Higher culture concentrations resulted in slightly overcrowded images.

Fixation selection

Both lugol solution and glutaraldehyde were evaluated for fixation, but both methods blocked the autofluorescence of the algal cells. As a consequence, subsequent experiments were conducted without fixation to preserve the inherent fluorescence of the algal cells.

EPS staining

Alician blue was initially tested as a stain for EPS. However, no fluorescence signal was detected. Therefore, EPS quantification was not based on fluorescence but determined by subtracting the fluorescence image from the brightfield image for each aggregate. This approach allowed for differentiation between algal cells and EPS based on their light scattering properties.

APPENDIX E

ADDITIONAL PLOTS

E.1 Baranyi growth model fitted to Experiment 1 data

Aged-PET data

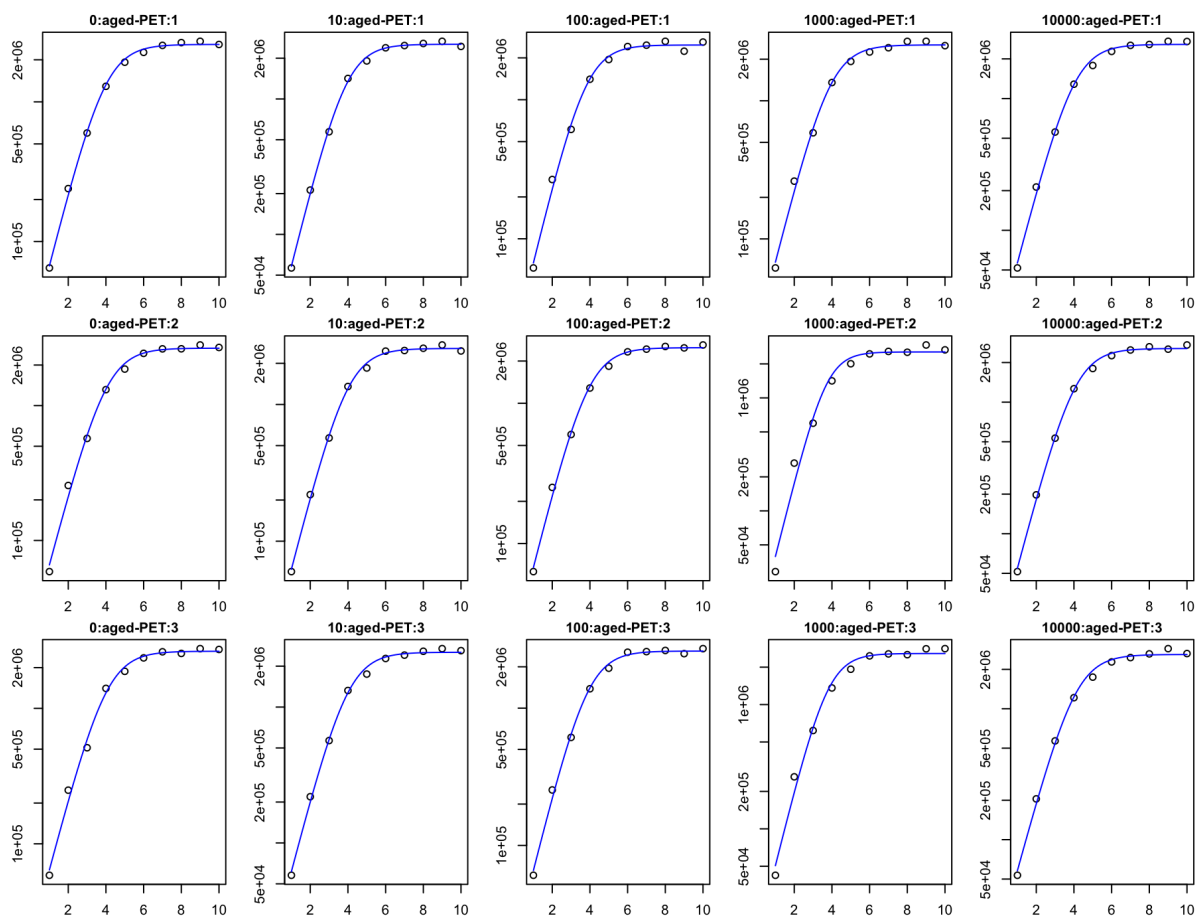


Figure E.1: Cellcount of *R. salina* exposed to aged-PET, fitted to the Baranyi growth model. The columns represent the various exposure concentrations. The rows represent the replicates.

PET data

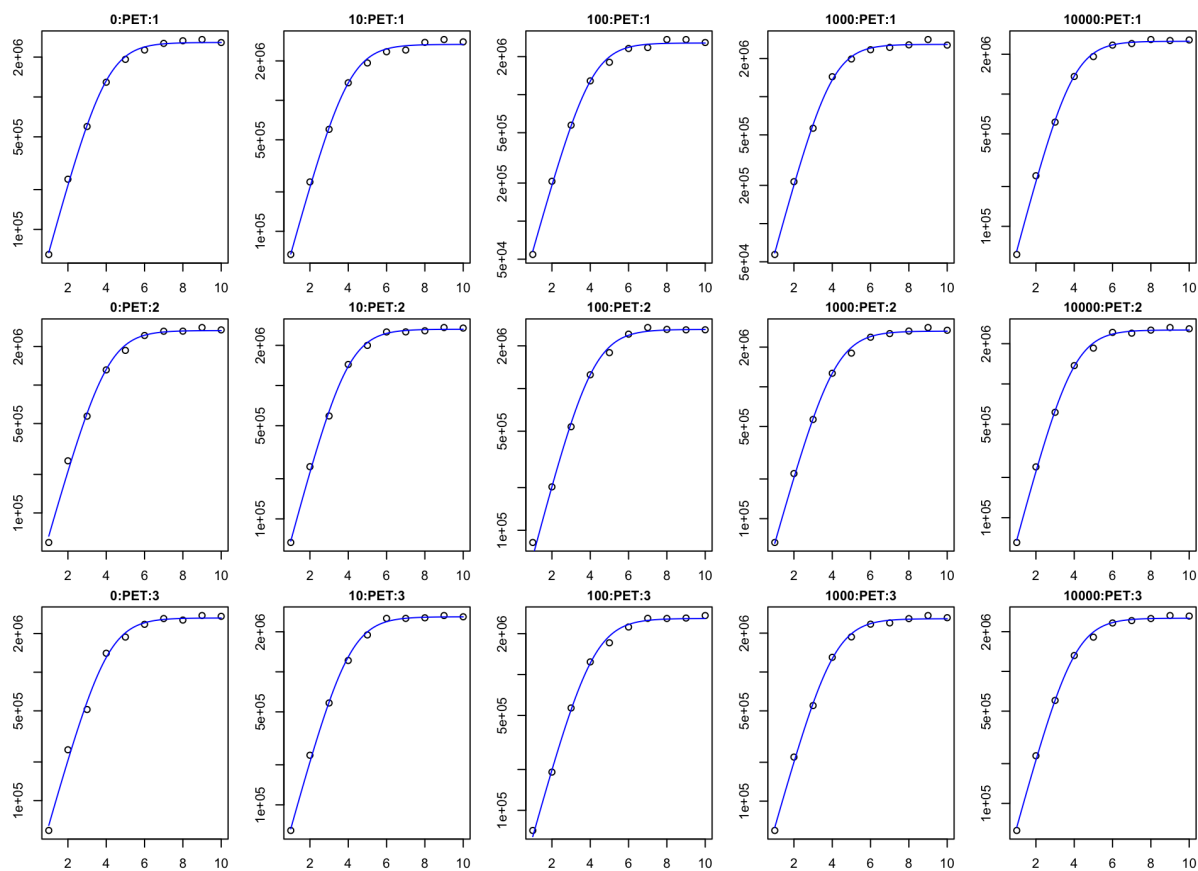


Figure E.2: Cellcount of *R. salina* exposed to PET, fitted to the Baranyi growth model. The columns represent the various exposure concentrations. The rows represent the replicates.

Kaolin data

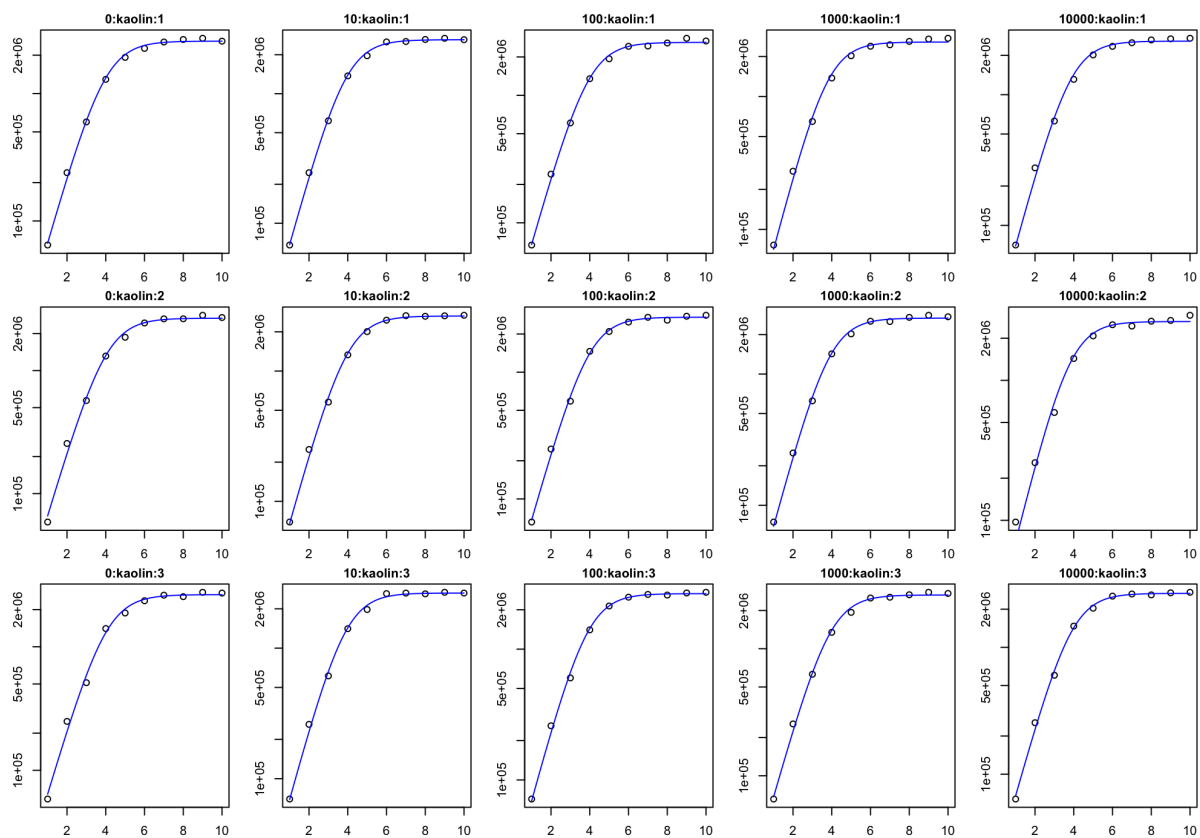


Figure E.3: Cellcount of *R. salina* exposed to kaolin, fitted to the Baranyi growth model. The columns represent the various exposure concentrations. The rows represent the replicates.

E.2 Baranyi growth model fitted to Experiment 2 data

Aged-PET data

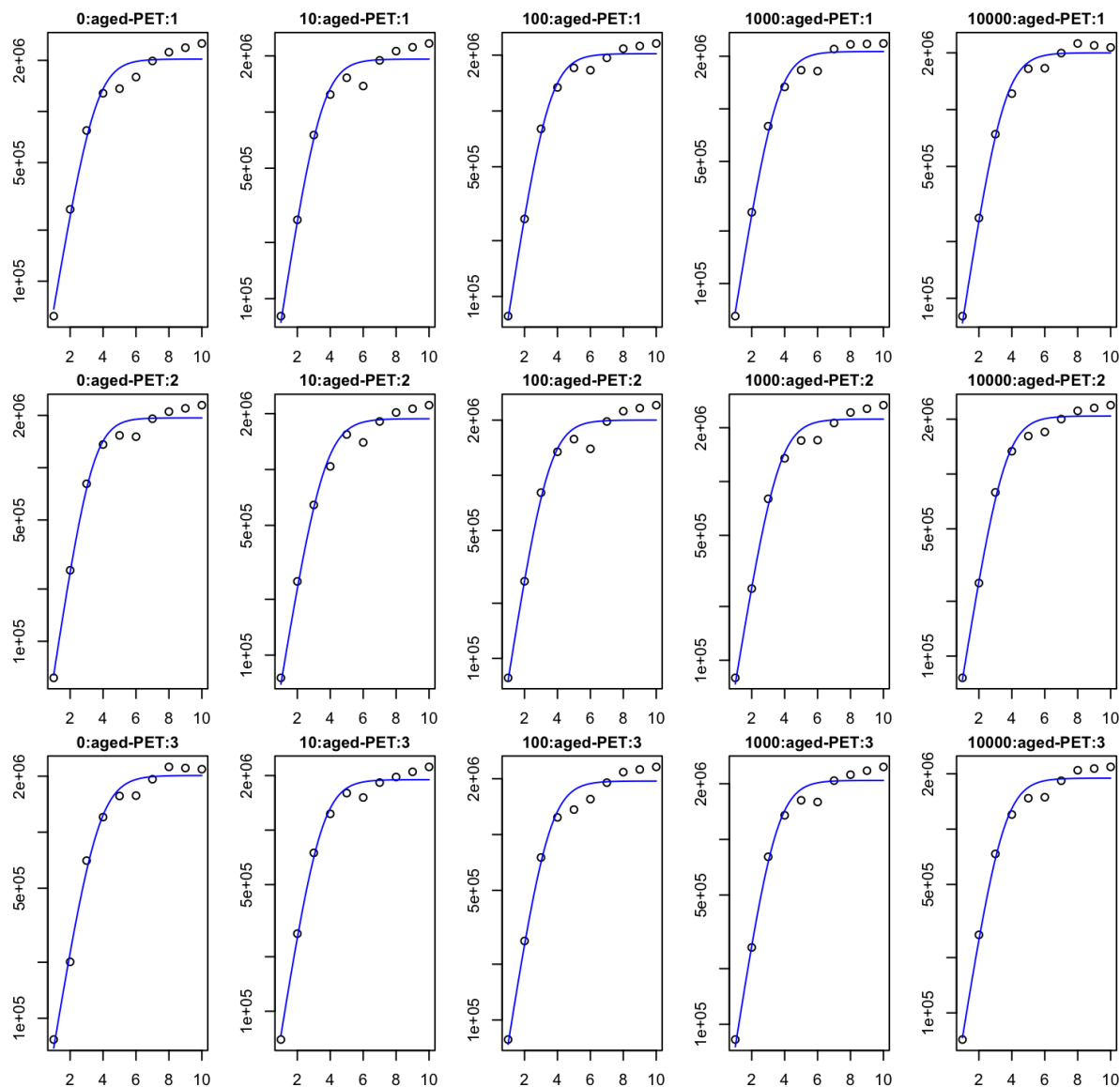


Figure E.4: Cellcount of *R. salina* exposed to aged-PET, fitted to the Baranyi growth model. The columns represent the various exposure concentrations. The rows represent the replicates.

PET data

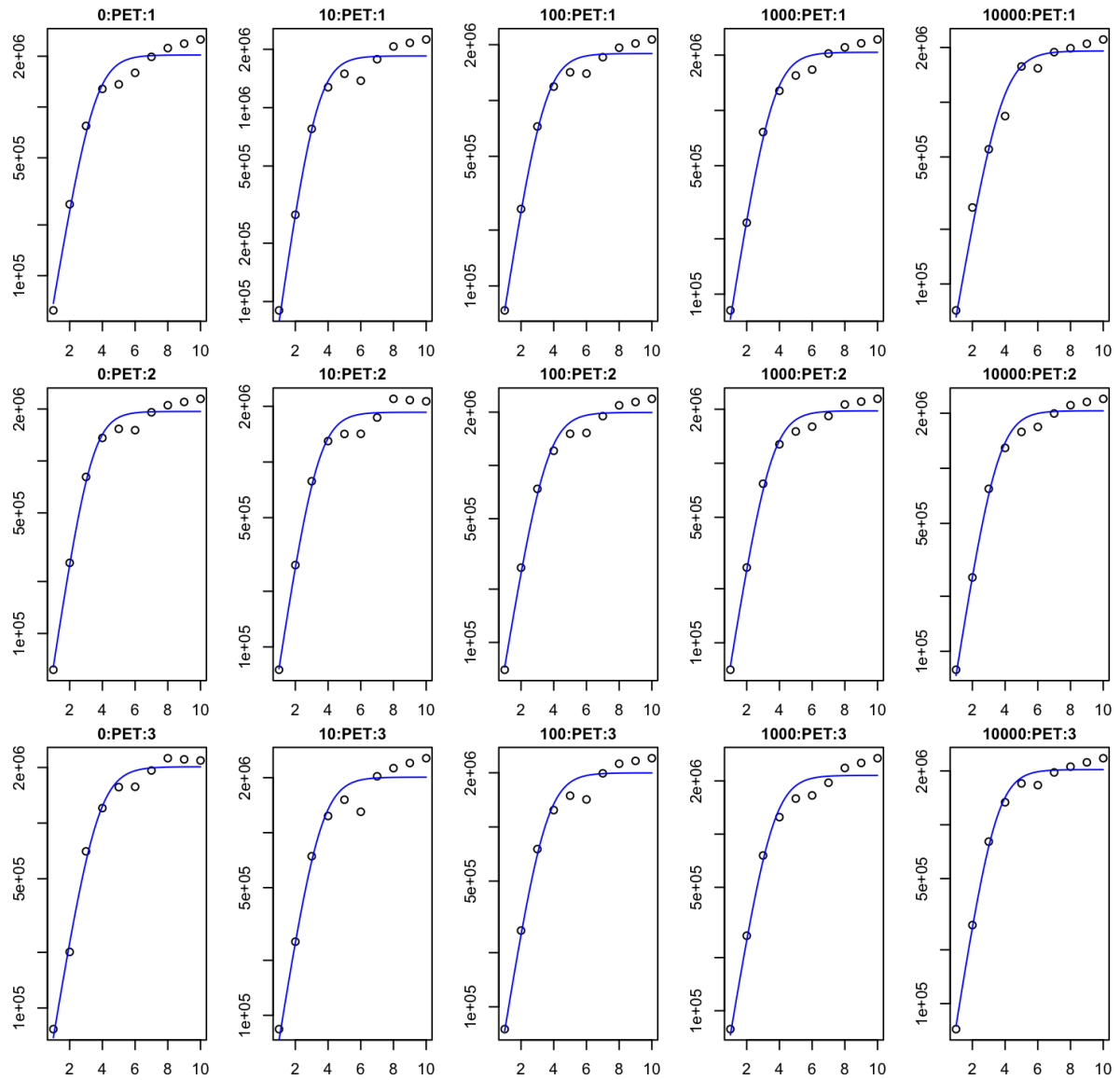


Figure E.5: Cellcount of *R. salina* exposed to PET, fitted to the Baranyi growth model. The columns represent the various exposure concentrations. The rows represent the replicates.

Kaolin data

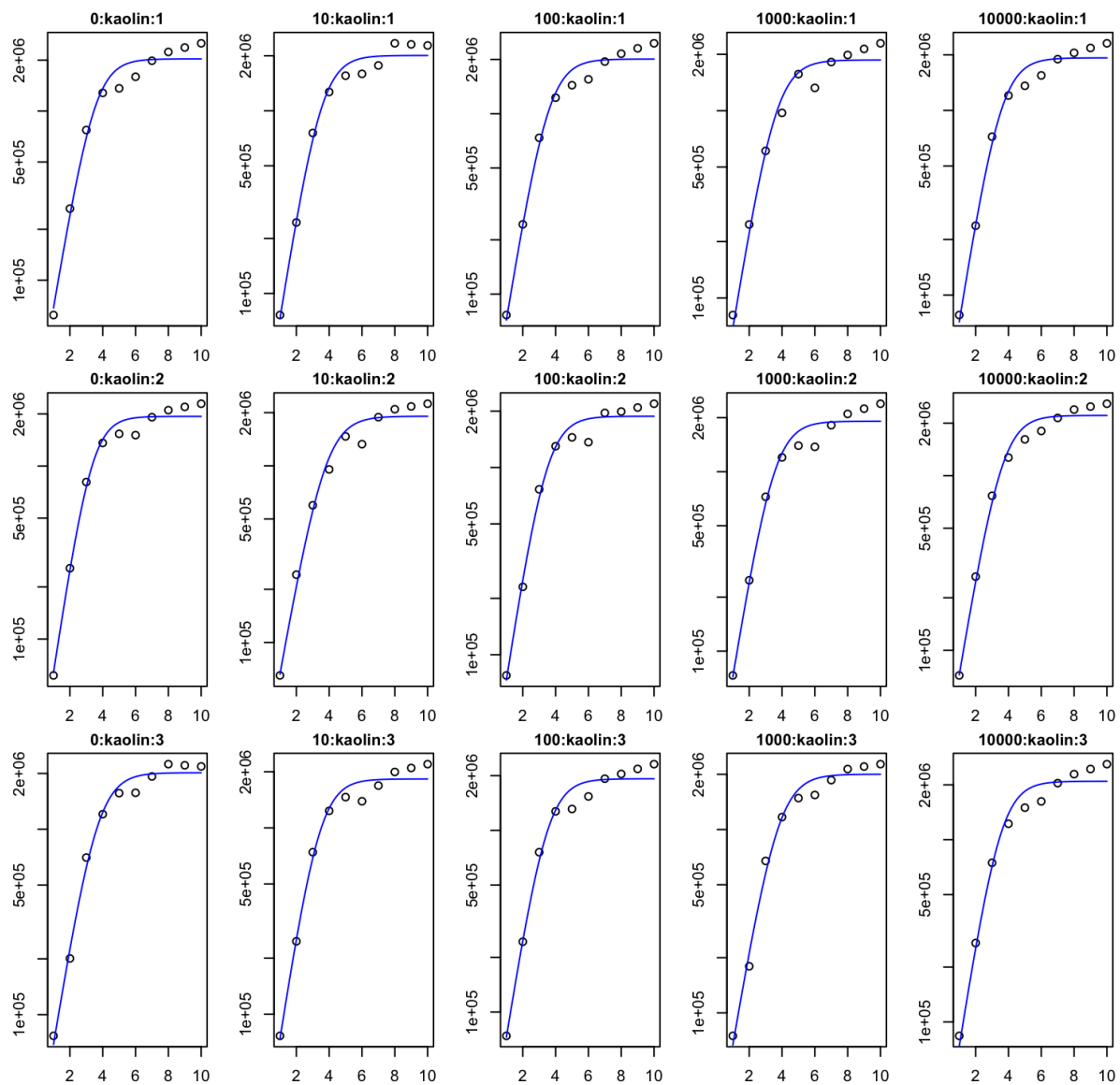


Figure E.6: Cellcount of *R. salina* exposed to kaolin, fitted to the Baranyi growth model. The columns represent the various exposure concentrations. The rows represent the replicates.

APPENDIX F

ADDITIONAL RESULTS

F.1 Additional results from Experiment 1

F.1.1 Growth models

Across all concentrations except for 10000 PTCs/mL, aged-PET exposed microalgae exhibit the lowest carrying capacity, followed by PET, with kaolin exposures displaying the highest carrying capacity (Figure F.1). The median carrying capacity of PET and aged-PET exposure appears substantially lower than that of the negative control for concentrations above 10 PTCs/mL. Notably, at concentrations of 10000 PTCs/mL and 10 PTCs/mL, there are significant differences in carrying capacity across exposure groups. At 10000 PTCs/mL, the mean carrying capacity for kaolin exposure (2.621×10^6 cells/mL) is significantly higher than that of PET (2.523×10^6 cells/mL) ($p = 0.02031$, $\text{diff} = 98167.98$). At 10 PTCs/mL, both kaolin and PET exposures exhibit significantly higher carrying capacities (2.633×10^6 cells/mL and 2.636×10^6 cells/mL) compared to aged-PET (2.543×10^6 cells/mL) ($p = 0.02063$ and 0.01737 , $\text{diff} = 88961.00$ and 92511.45 , respectively).

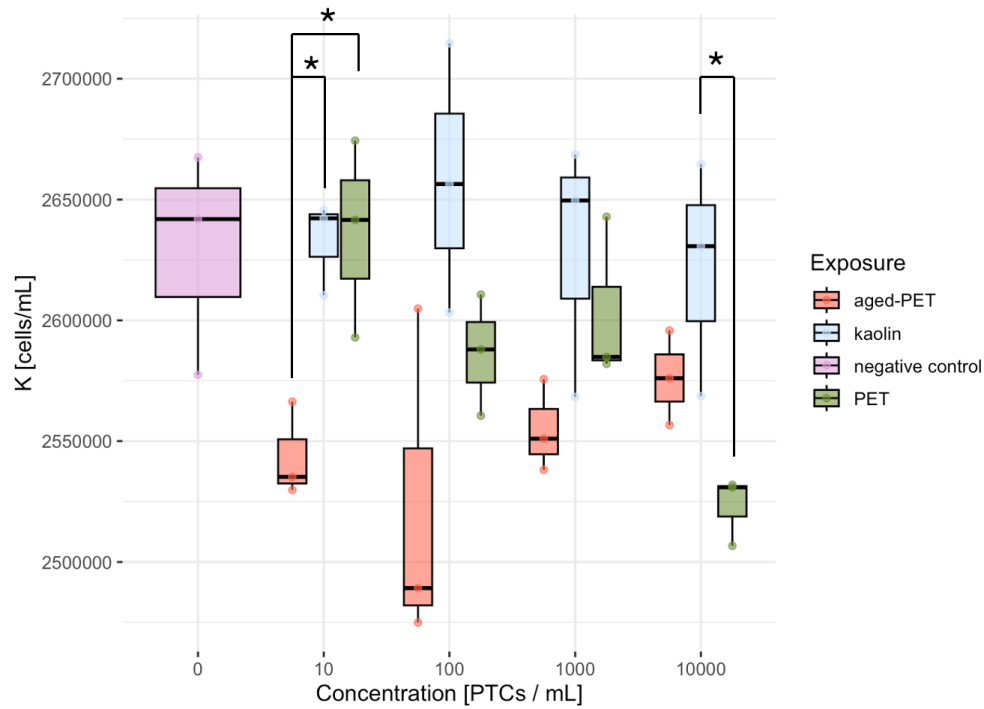


Figure F.1: Carrying capacity (K) from the Baranyi growth model for the exposure concentrations 10, 100, 1000, and 10000 PTCs/mL. Individual data points are represented by dots (n=3 observations per boxplot). An asterisk (*) denotes a significant difference ($p < 0.05$) between exposure groups for a specific concentration.

Across all concentrations, the median maximum growth rate for PET exposure is lower than that for kaolin and aged-PET exposure (Figure F.2). Similar to Figure 5.3, the growth rate of aged-PET exposed microalgae at the concentration of 1000 PTCs/mL appears to be disproportionate compared to the rest of the data. Furthermore, the median maximum growth rate for aged-PET and kaolin exposure is consistently higher than that of the negative control. Interestingly, there are no significant differences in the maximum growth rate across the different exposures at any of the concentrations.

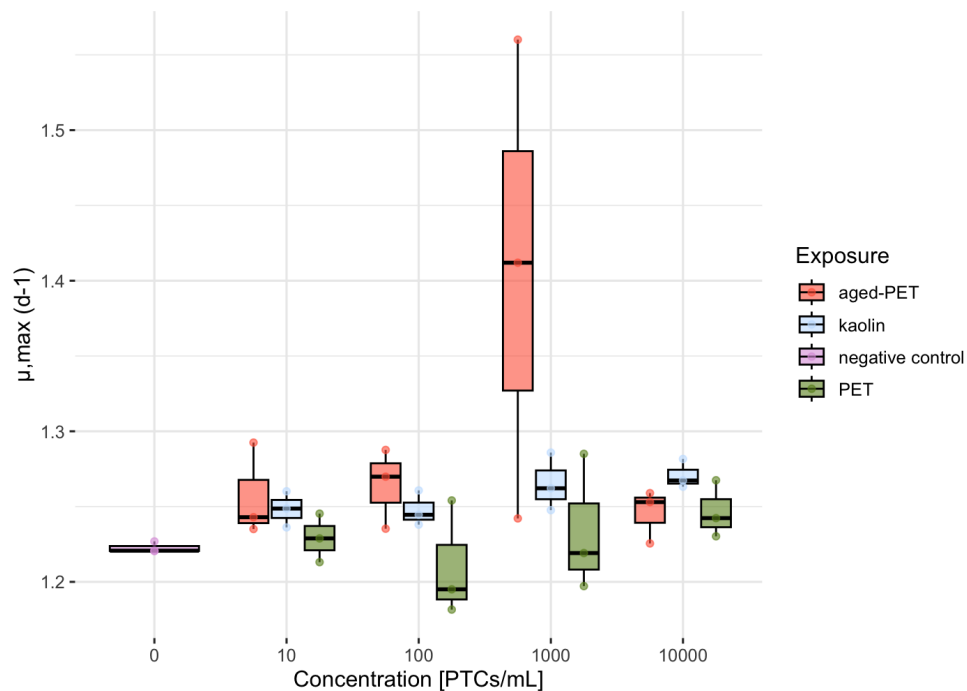


Figure F.2: Maximum growth rate (μ_{max}) from the Baranyi growth model for the exposure concentrations 10, 100, 1000, and 10000 PTCs/mL. Individual data points are represented by dots (n= 3 observations per boxplot).

F.1.2 EPS quantity

With the exception of aged-PET exposure at a concentration of 10 PTCs/mL, the median EPS production per cell for all exposures and concentrations is lower than that for the negative control of 0.182 ng/cell (Figure F.3). Furthermore, except for the concentration of 10000 PTCs/mL, aged-PET exposure appears to result in a higher amount of EPS per cell compared to kaolin and PET exposure. At a concentration of 10 PTCs/mL, the mean EPS per cell for aged-PET exposure (0.208 ng/cell) is significantly higher than that for kaolin exposure (0.128 ng/cell) ($p = 0.03460$, $\text{diff} = 8.0331\text{e-}11$).

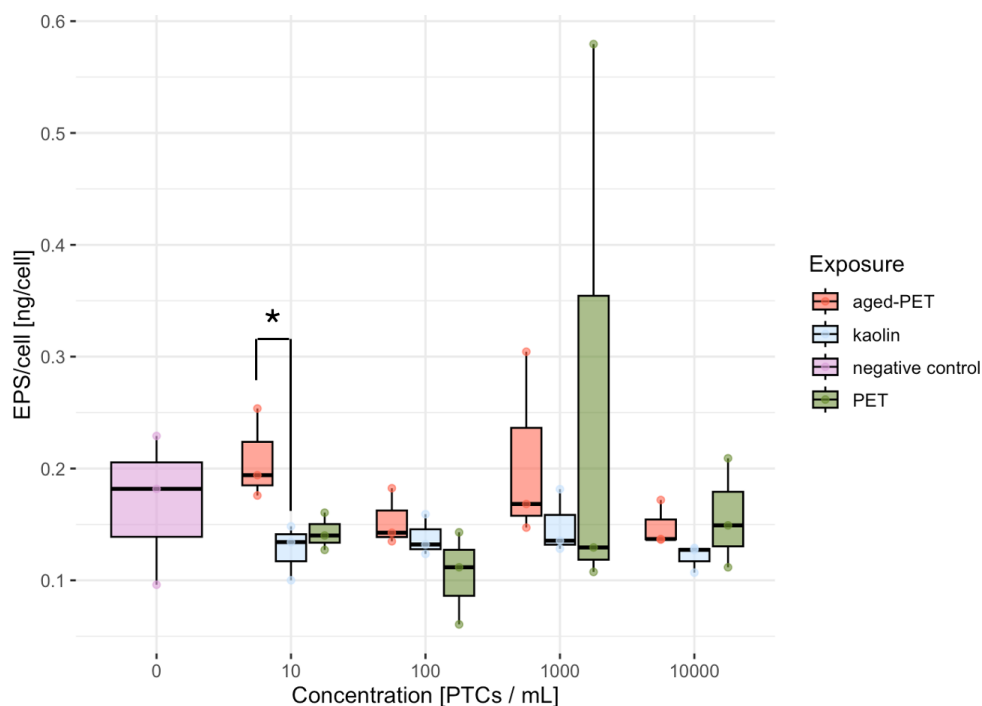


Figure F.3: EPS production per cell after 10 days of exposure across the exposure concentrations 10, 100, 1000, and 10000 PTCs/mL. Individual data points are represented by dots (n=3 observations per boxplot). An asterisk (*) denotes a significant difference ($p < 0.05$) between exposure groups for a specific concentration..

F.1.3 EPS composition

At a concentration of 10 PTCs/mL, the mean P/C-ratio of the aged-PET exposed microalgae (0.599) is significantly lower than that of kaolin (0.685) and PET exposed microalgae (0.669) ($p = 0.00056$ and $p = 0.00423$, diff = 0.0864 and 0.0706, respectively) (Figure F.4). Similarly, at a concentration of 1000 PTCs/mL, the mean P/C-ratio of the aged-PET exposure (0.564) is significantly lower than that of the kaolin exposure (0.698) and that of the PET exposure (0.704) ($p = 0.00168$ and $p = 0.00053$, diff = 0.134 and 0.139, respectively). Furthermore, at a concentration of 10000 PTCs/mL, the median P/C-ratio for the aged-PET exposure (0.644) is significantly lower than that for the kaolin exposure (0.718), while that of kaolin is significantly higher than that of PET (0.665) ($p = 0.00012$ and $p = 0.00876$, respectively). Generally, exposure to any particle type seems to result in an increase in the protein content and/or a decrease in the carbohydrate content of the EPS compared to the negative control.

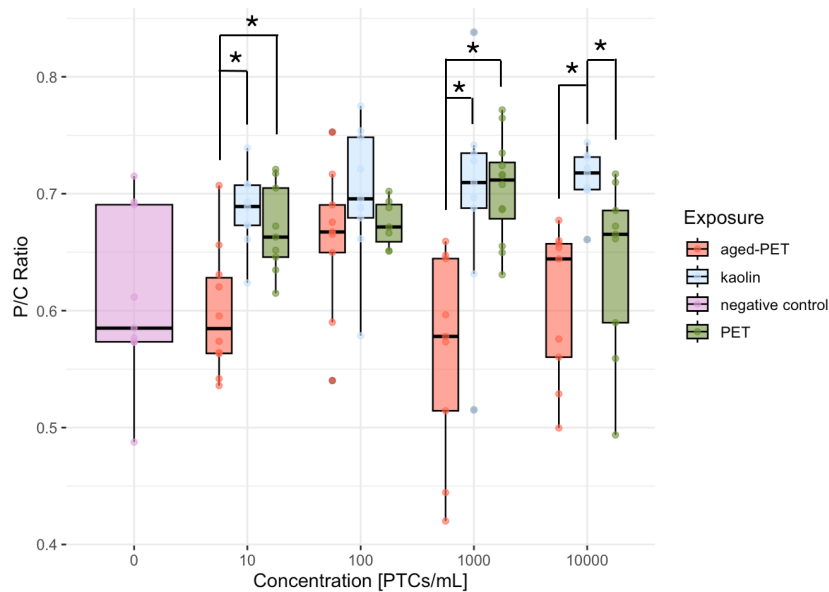


Figure F.4: P/C-ratio of EPS extracted at day 10 for the exposure concentrations 10, 100, 1000, and 10000 PTCs/mL. Individual data points are represented by dots (n=9 observations per boxplot). An asterisk (*) denotes a significant difference ($p < 0.05$) between exposure groups for a specific concentration.

F.2 Additional results from Experiment 2

Growth models

There appears to be a trend where higher concentrations correspond to higher carrying capacities (Figure F.5). However, at the concentration of 10000 PTCs/mL, the carrying capacity for aged-PET and PET exposure drops below that of the 1000 PTCs/mL concentration. At the 1000 PTCs/mL concentration, the mean carrying capacity of aged-PET exposed microalgae of 2.149×10^6 cells/mL is significantly higher than that of kaolin exposed ones of 1.926×10^6 cells/mL ($p = 0.03568$, $\text{diff} = 223062.92$).

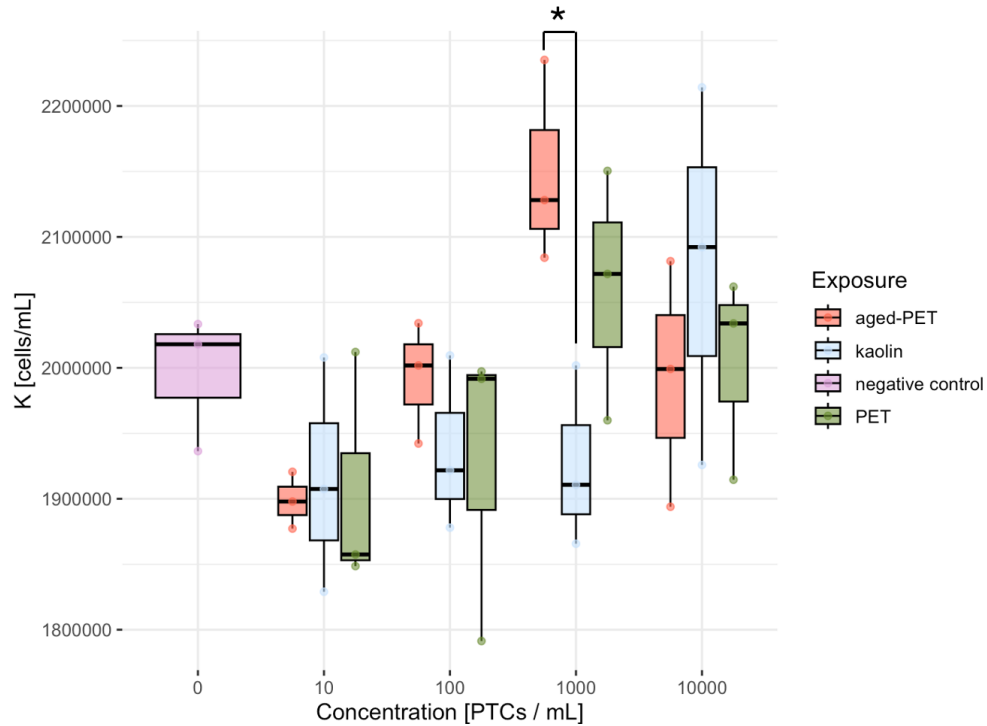


Figure F.5: Carrying capacity (K) from the Baranyi growth model across the exposure concentrations 10, 100, 1000, and 10000 PTCs/mL. Individual data points are represented by dots (n=3 observations per boxplot). An asterisk (*) denotes a significant difference ($p < 0.05$) between exposure groups for a specific concentration.

Across all concentrations, the median maximum growth rate of kaolin exposures is smaller than that of PET and aged-PET exposures (Figure F.6). Furthermore, the median maximum growth rate of aged-PET exposures consistently surpasses that of PET exposures, except at the 10 PTCs/mL concentration. Particularly at the 100 PTCs/mL and 1000 PTCs/mL concentrations, the mean maximum growth rate of aged-PET exposures (1.351 and 1.352, respectively) is significantly higher than that of kaolin exposures (1.317 and 1.266, respectively) ($p = 0.02827$ and 0.01557 , $\text{diff} = 0.0342$ and 0.0858 , respectively).

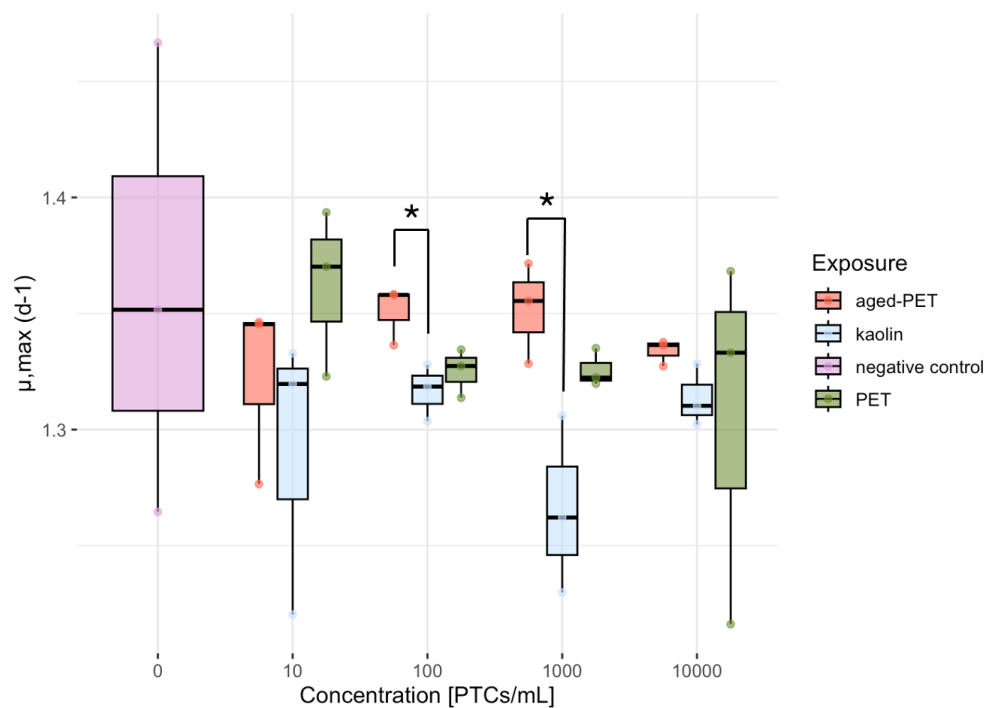


Figure F.6: Maximum growth rate (μ_{max}) from the Baranyi growth model across the exposure concentrations 10, 100, 1000, and 10000 PTCs/mL. Individual data points are represented by dots (n=3 observations per boxplot). An asterisk (*) denotes a significant difference (p < 0.05) between exposure groups for a specific concentration.

F.2.1 EPS quantity

No statistically significant differences were found in the EPS production per cell across the different exposures at any of the concentrations (Figure F.7).

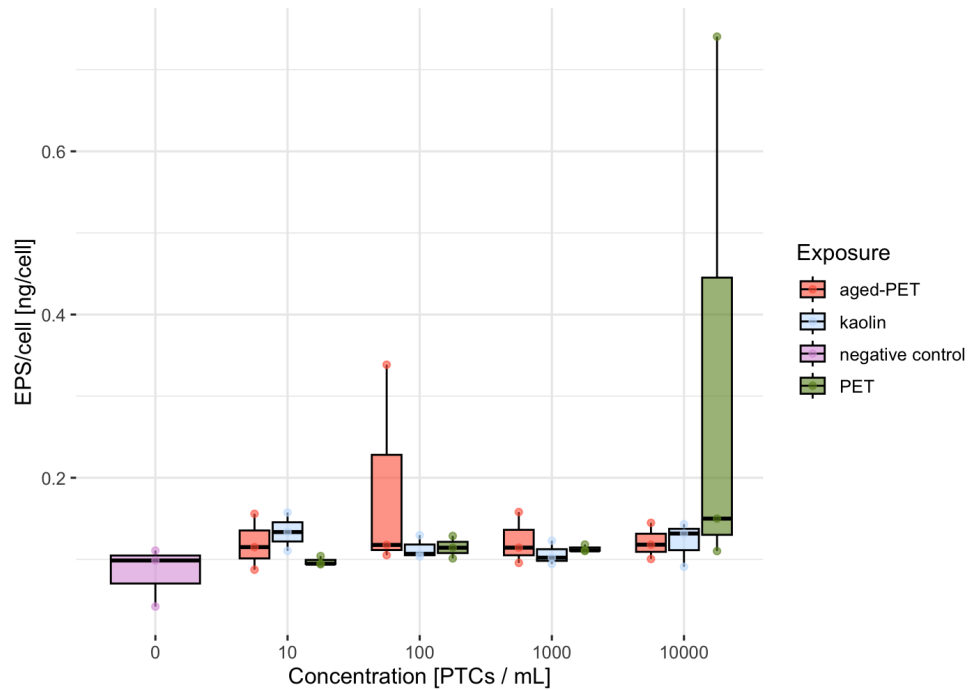


Figure F.7: EPS production per cell after 10 days of exposure across the exposure concentrations 10, 100, 1000, and 10000 PTCs/mL. Individual data points are represented by dots (n=3 observations per boxplot).

F.2.2 EPS composition

At a concentration of 10 PTCs/mL, the mean P/C-ratio of PET exposed microalgae (0.578) is significantly higher than that of kaolin (0.347) ($p = 0.00220$ and $\text{diff} = 0.23018$) (Figure F.8). At 1000 PTCs/mL, the median P/C-ratio of aged-PET exposures (0.773) is significantly higher than that of kaolin (0.561) and PET exposures (0.566) ($p = 0.00867$ and 0.02271 , respectively).

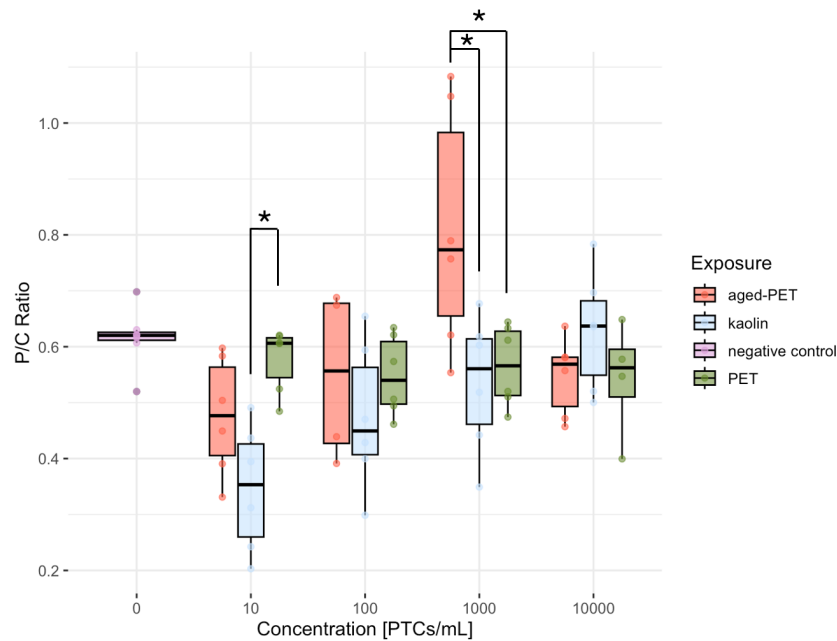


Figure F.8: P/C-ratio of EPS extracted at day 10 for the exposure concentrations 10, 100, 1000, and 10000 PTCs/mL. Individual data points are represented by dots (n=6 observations per boxplot). An asterisk (*) denotes a significant difference ($p < 0.05$) between exposure groups for a specific concentration.

F.2.3 Aggregate size

At all concentrations except for the 10000 PTCs/mL, aged-PET exposures have the lowest median aggregate diameter (Figure F.9). At concentrations 10 PTCs/mL and 100 PTCs/mL PET exposures have a notably higher median aggregate diameter. Conversely, at concentrations 1000 PTCs/mL and 10000 PTCs/mL it is the median aggregate diameter of kaolin exposures which is notably higher than the other exposures. At the 10 PTCs/mL concentration, the mean aggregate size of PET exposures is significantly higher than that of aged-PET and kaolin ($p = 0.01120$ and 0.03259 , $\text{diff} = 6.28580$ and 4.92287 , respectively). At the 100 PTCs/mL concentration, the mean aggregate diameter of PET exposures is significantly higher than that of aged-PET ($p = 0.04775$, $\text{diff} = 5.65617$). Lastly, at 1000 PTCs/mL, the mean aggregate diameter of kaolin exposures is significantly higher than that of both aged-PET and PET exposures ($p = 0.01327$ and 0.02474 , $\text{diff} = 4.475$ and 3.885 , respectively).

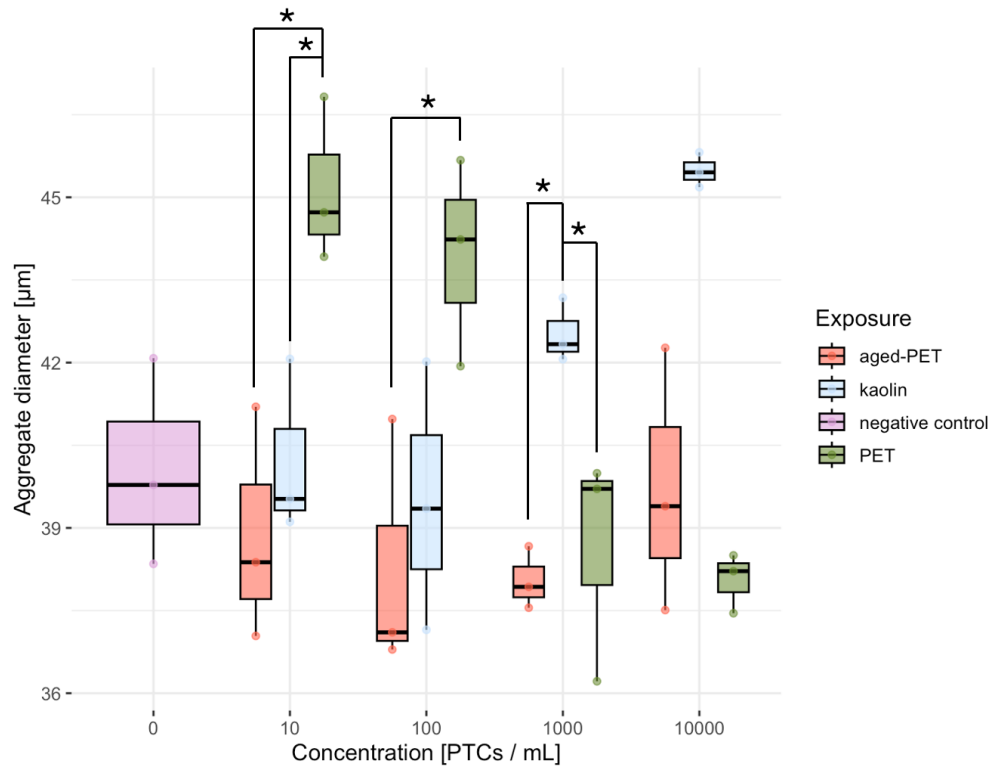


Figure F.9: Diameter of the aggregated algae obtained from the volume based size distribution curve for the exposure concentrations 10, 100, 1000, and 10000 PTCs/mL. Individual data points are represented by dots (n=3 observations per boxplot). An asterisk (*) denotes a significant difference ($p < 0.05$) between exposure groups for a specific concentration.

F.2.4 Aggregate quantity

For concentrations of 10 PTCs/mL and 100 PTCs/mL, there is a trend where the median volume ratio of PET exposures is the highest, followed by kaolin exposures, and lastly aged-PET exposures (Figure 5.12). However, at concentrations of 1000 PTCs/mL and 10000 PTCs/mL, this trend shifts, with the highest median volume ratio for kaolin exposures, followed by PET exposures, and lastly aged-PET exposures. At the concentration of 10 PTCs/mL, the mean volume ratio of aggregates versus single-celled algae for PET exposures (5.166) is significantly higher than that for aged-PET exposures (2.306) ($p = 0.04545$, $\text{diff} = 3.310$).

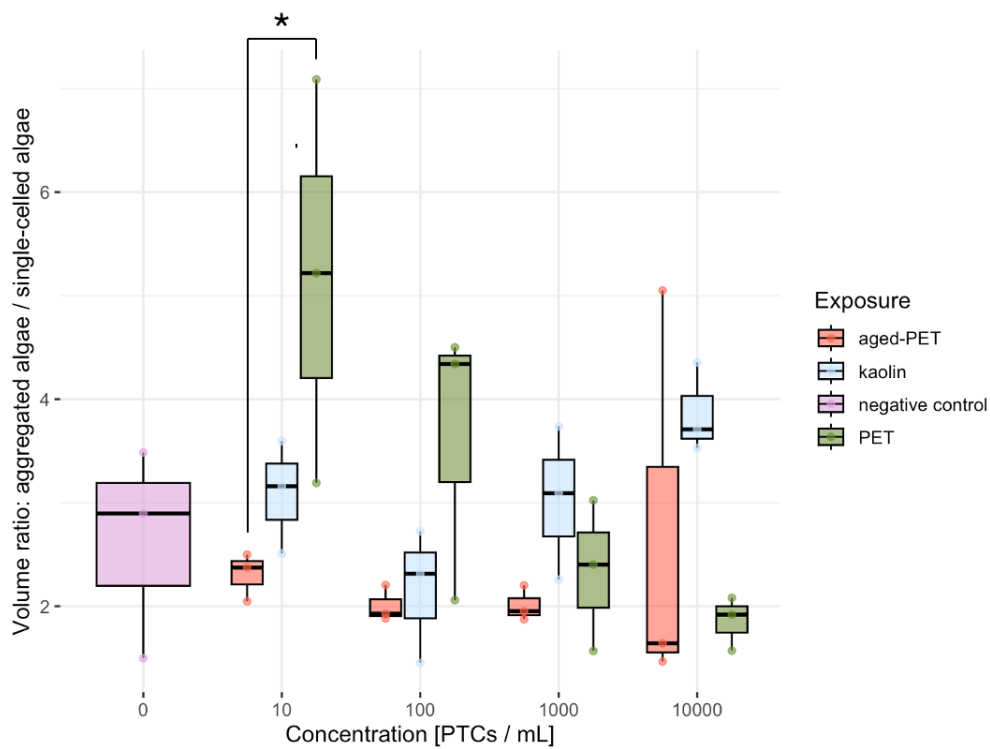


Figure F.10: Ratio of the volume percentage taken up by aggregates versus by single celled algae for the exposure concentrations 10, 100, 1000, and 10000 PTCs/mL. Individual data points are represented by dots (n=3 observations per boxplot). An asterisk (*) denotes a significant difference ($p < 0.05$) between exposure groups for a specific concentration.