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Microplastic dispersal behavior in a novel overhead stirring aqueous exposure system

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ABSTRACT

Using nominal dose metrics to describe exposure conditions in laboratory-based microplastic uptake and effects studies may not adequately represent the true exposure to the organisms in the test system, making data interpretation challenging. In the current study, a novel overhead stirring method using flocculators was assessed for maintaining polystyrene (PS) microbeads (Ø10.4 μm; 1.05 g cm^{-3}) in suspension in seawater during 24 h and then compared with static and rotational exposure setups. Under optimized conditions, the system was able to maintain 59% of the initial PS microbeads in suspension after 24 h, compared to 6% using a static system and 100% using a rotating plankton wheel. Our findings document for the first time that overhead stirring as well as other, commonly used exposure systems (static) are unable to maintain constant microplastic exposure conditions in laboratory setups whereas rotation is very effective. This suggests toxicological studies employing either static or overhead stirring systems may be greatly overestimating the true microplastic exposure conditions.

1. Introduction

While there is an increasing quantity of data reporting microplastic concentrations in different marine environmental compartments, a comparable amount of microplastic effects data for marine organisms is currently lacking (Barboza et al., 2019; Botterell et al., 2019; Prata et al., 2019). This is evidenced by the first study attempting to evaluate the risk of microplastic pollution being conducted only in 2018 (Everaert et al., 2018), and where the scarcity of effects data was highlighted as a critical limitation. To enable improved microplastic risk assessment, there is a need to generate uptake and effects data using controlled exposures under laboratory conditions (Everaert et al., 2018).

Most toxicological studies with microplastic employ nominal dose metrics with the assumption that exposure is 100% and constant over time. However, it is doubtful this reflects the true exposure conditions as microplastic particles are unlikely to remain homogenously dispersed in the aqueous phase due to aggregation, settling and sedimentation processes (Alimi et al., 2018; Choi et al., 2019; Gambardella et al., 2017; Karami, 2017; Kowalski et al., 2016). As most microplastic is either positively or negatively buoyant, it is prone to floating or sinking during aquatic exposures, especially in static systems, creating exposure concentration gradients within the system (Karami, 2017). Furthermore, many polymers are inherently hydrophobic and exhibit surface charges in water that can reduce microplastic bioavailability due to homo-aggregation and adhesion to exposure vessel surfaces and analytical equipment (Fotopoulou and Karapanagioti, 2012; Kim et al., 2015).

Ideally, exposure systems employed in laboratory uptake and effects studies with aquatic organisms should ensure full and constant exposure throughout the study duration, otherwise direct quantification of microplastic bioavailability throughout the exposure period is necessary (Potthoff et al., 2017; Rehse et al., 2016). Some studies have attempted to implement approaches that maintain negatively buoyant microplastic in suspension, including addition of dispersants (Paul-Pont et al., 2016), water flow or air bubbling (Karami, 2017; Sussarellu et al.,

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2016), ultrasound (Fernández and Albentosa, 2019; Zhang et al., 2017), stirring (Canniff and Hoang, 2018; Van Cauwenberghe et al., 2015), horizontal/orbital shaking (Beiras et al., 2018), and rotation (e.g. plankton wheel) (Beiras et al., 2018; Beiras and Tato, 2019). All of these approaches exhibit limitations and are often species- and task-specific.

Microalgae are primary producers at the bottom of the food chain and a major global source of oxygen. As they tend to concentrate in slicks together with microplastic fragments in coastal areas (Gove et al., 2019), it is important to study the potential impacts of microplastic on these organisms. However, standard toxicity tests with microalgae (ISO, 2016; OECD, 2011) are predominantly designed to study the impact of water-soluble chemicals and recommend shaking or stirring by orbital or reciprocal shaker tables to keep algae in suspension and to facilitate transfer of CO_2. The methods for preparation of exposure solutions suggested by ISO 14442:2006 are also not suitable for maintaining plastic particles in suspension (ISO, 2006). A recent review indicates that previous studies assessing microplastic effects on microalgae have primarily used shaking or static setups (Prata et al., 2019). These exposure methods are, however, not able to maintain microalgae and microplastic in a homogenous suspension as they will typically create concentration gradients in the exposure medium of both algae and plastic particles.

The current study therefore investigated for the first time the viability of a novel overhead stirring method as a potential cost-effective approach for maintaining microplastic particles in homogeneous suspension during future toxicological studies with microalgae. To investigate the role of exposure vessel composition on loss of microplastic from the aqueous phase, the overhead stirring studies were conducted using glass, polypropylene (PP) and stainless steel beakers as exposure vessels. Parameters including surface to volume ratio, initial PS suspension concentration, stirring speed and the use of dispersants were also investigated. The concentration of negatively buoyant polystyrene (PS) microbeads (average diameter 10.4 μm; density 1.05 g cm^{-3}) remaining in suspension over a 24 h exposure period was determined in all experiments using a Coulter counter. Finally, the overhead stirring method was evaluated against two other exposure systems commonly used for assessing microalgae toxicity; a static exposure and rotation of sealed exposure vessels.

2. Materials and methods

2.1. Materials and chemicals

PS microbeads (average diameter 10.4 μm, density 1.05 g cm^{-3}) were purchased from Polysciences, Inc. (Warrington, PA, USA) and were provided as 2.5% solids (w/v) in aqueous suspension with minimal residual surfactant (Tween or SDS; information supplied by Polysciences, Inc.). These microbeads were selected as they have been used extensively in microplastic research as a proxy for environmental microplastic particles. A stock suspension was prepared in a glass bottle at a concentration of ~800,000 PS mL^{-1} in sterivex filtered (0.22 μm) natural seawater (FSW), taken from 90 m depth from Trondheimsfjord (Norway). The stock solution was kept in a cold room at ~5 °C. Prior to use, the bottle was shaken vigorously. Every sample was pipetted individually and diluted in FSW to reach the target concentration. Dispersants polyvinylpyrrolidone (PVP40), Tween20 and gum arabic were purchased from Merck Sigma-Aldrich (Darmstadt, Germany).

2.2. Overhead stirring exposure parameter assessment

Overhead stirring is used in Coulter counters to keep particles and cells, including microalgae and protozoans, in suspension during the analysis of samples. The current study evaluated the capacity of overhead stirring to maintain PS microbeads in suspension and as a potential method for future toxicity testing of microplastic using phytoplankton, with relevance to other unicellular organisms and potentially some zooplankton species. To allow for simultaneous overhead stirring of several samples at the same rotational speed, two flocculators (JLT 6 flocculator, VELP Scientifica, Italy) were used in the current study. The system comprised 6 rotating rods with fixed, non-interchangeable solid mixing plates in stainless steel.

A range of extrinsic parameters (exposure vessel material, initial microplastic concentration, surface to volume ratio, stirring speed and addition of dispersant) were then systematically varied to investigate their influence on the ability of the overhead stirring exposure system to maintain full and constant microplastic suspension concentrations. The impact of exposure vessel material on microplastic adsorption/loss was investigated by conducting the tests in borosilicate glass beakers (600 mL), PP beakers (600 mL) and stainless steel beakers (1000 mL), all purchased from VWR International. The influence on the free fraction of PS microbeads in suspension due to each of the other parameters tested was assessed using overhead stirring in glass beakers (600 mL). Initial microplastic concentration was investigated using nominal PS microplastic concentrations of 2200, 8000, 15,000, 22,000 microbeads mL^{-1} (2238 ± 318, 8265 ± 77, 15,299 ± 1296, 22,133 ± 511 microbeads mL^{-1} measured). Surface to volume ratio of the exposure media was investigated using initial volumes of 300, 350 and 400 mL in identical glass beakers (representing ratios of 0.87, 0.81 and 0.76, respectively), and stirring speed was investigated using speeds of 12, 60, 125 rpm. Finally, the influence of three different dispersants, PVP40, Tween20 and gum arabic, was investigated over the following concentration range: 0.025, 0.25, 2.5, 25 μM. This corresponded to 1, 10, 100 and 1000 mg mL^{-1} for PVP, 0.0279, 0.279, 2.79 and 27.9 μL L^{-1} for Tween20, and 0.00625, 0.0625, 0.625 and 6.25 g L^{-1} for gum arabic.

The baseline conditions in all experiments were a PS microplastic concentration of approximately 2200 mL^{-1} (2286 ± 246 microbeads mL^{-1}), a medium volume of 300 mL and a rotation speed of 12 rpm. In all experiments, the quantity of microplastic remaining in suspension was measured after 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 and 24 h using a Coulter counter (Fig. 1, red curves). In addition, PS suspension vessels were shaken vigorously after the last measurement at 24 h and analysis by the Coulter counter confirmed all PS microbeads introduced to the exposure vessels were still present.

2.3. Exposure method assessment

The efficiency of the overhead stirring method to maintain spherical polystyrene (PS) microbeads in suspension for 24 h using the optimal set of extrinsic parameters was compared to a static exposure system and a rotating plankton wheel. The static system comprised the addition of PS microbeads to 300 mL of FSW to obtain a final concentration of 2238 ± 318 PS/mL in a glass beaker, gently mixed and put in the Coulter counter for 24 h without overhead mixing. In the rotating plankton wheel study, a total of 33 0.5 L borosilicate glass bottles (VWR International, Oslo, Norway), representing 11 sets of triplicates (one for each sampling point), were fixed onto the rotating axel. For each triplicate of 0.5 L bottles, a 2 L stock suspension of nominally 2200 PS mL^{-1} (2109 ± 36 PS mL^{-1} measured) was prepared in a 2 L borosilicate glass bottle. The three 0.5 L bottles were filled almost to the brim, leaving space for a small air bubble to increase turbulent mixing during rotation and enhance the suspension of the PS microbeads. In addition, 100 mL of the remainder of the 2 L of PS suspension was transferred to a Coulter beaker to measure the concentration of PS microbeads used in the study. Samples were then set to rotate at a speed of 0.8 rpm for a 24 h exposure period. The quantity of MP remaining in suspension was measured in all exposure systems (static, overhead stirring and rotating) at the same frequency as during the overhead stirring exposure parameter assessment. All quantification by Coulter counter was performed in triplicate.

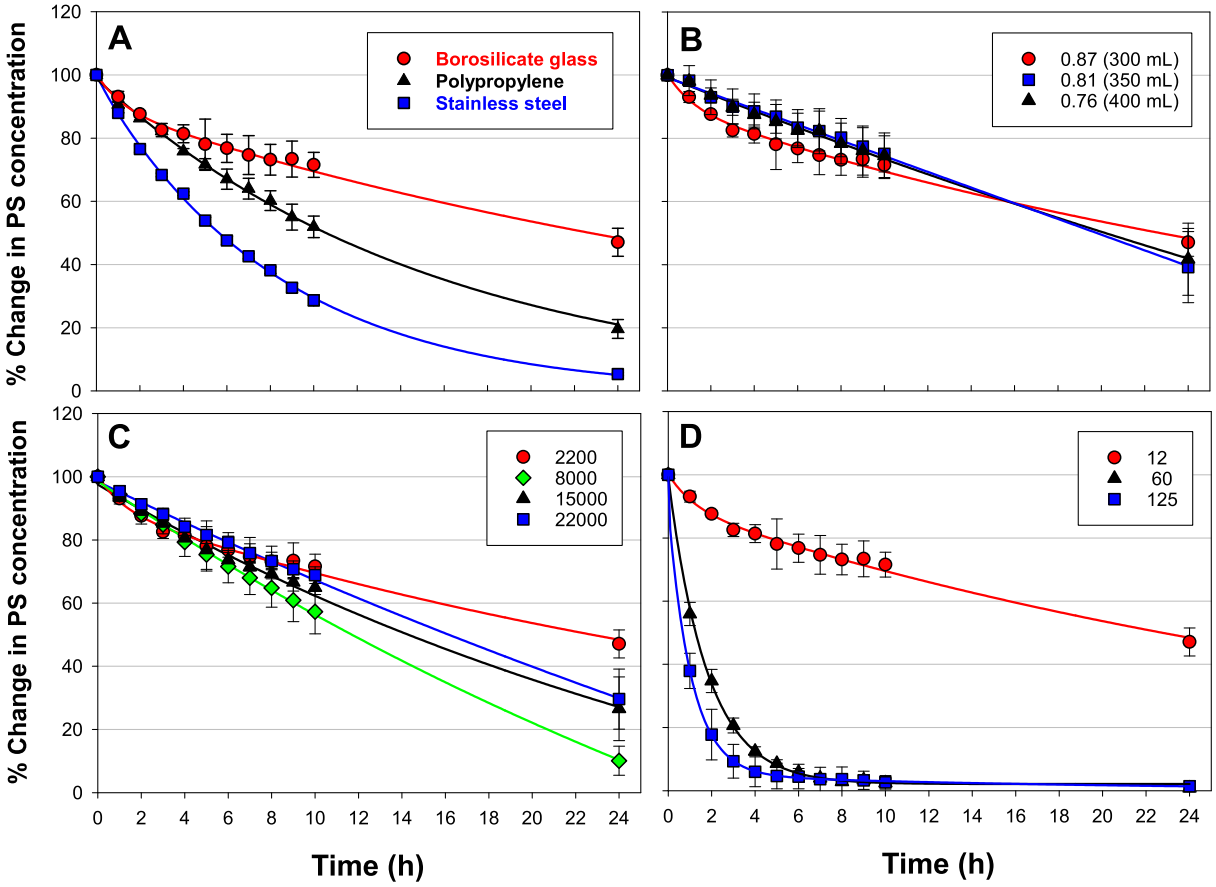

Fig. 1. Exposure parameter assessment for overhead stirring. Effects of exposure vessel material (A), surface area to volume ratio (and volume (mL)) (B), initial PS microbead concentration (beads mL^{-1}) (C), and stirring speed (rpm) (D) on PS microbead concentration (% of initial concentration) during 24 h. Symbols depict means, error bars SD (n = 3).

2.4. Microplastic analysis

PS microplastic concentrations were determined in all experiments with a Multisizer™ 3 Coulter Counter (Beckman Coulter Inc., Miami, USA) equipped with a 100 µm aperture. For the static setup study, a glass beaker containing PS suspension was inserted and maintained in the Coulter counter for the whole 24 h sampling period. For the overhead stirring study individual vessels were transferred directly from the flocculators to the Coulter counter at each sampling time point and microplastic suspensions stirred at comparable speed during analysis, after which they were returned to the flocculator. Overhead stirring during analysis with the Coulter counter was used to maintain the same conditions as closely as possible with those of the flocculators. For the rotation study using plankton wheels, 100 mL of exposure medium from each 0.5 L bottle was carefully poured into a Coulter beaker immediately after removal from the plankton wheel and analyzed while applying overhead stirring during analysis. Stirring speed during quantification with the Coulter counter was identical to that used for the samples generated using the flocculators. For each sample, the average concentration of particles and the corresponding average particle diameter (10.4 µm) and volume was determined from triplicate measurements. Quantification of particles seemingly larger than the 10.4 µm PS microbeads used in the present study was also performed as they may indicate the formation of microbead homo-aggregates. The presence of PS aggregates in the medium was also checked visually.

3. Results and discussion

With the aim of counteracting the reduction in the PS concentration over time when using the overhead stirring method, a number of extrinsic exposure conditions were investigated. In the first of these studies, the influence of exposure vessel material (glass, metal and plastic) was assessed. Clear differences in the concentration of PS microbeads remaining in suspension were observed over the 24 h exposure period (Fig. 1A). The glass vessel performed the best, with a PS microbead concentration of 47% remaining after 24 h, while a greater reduction in PS microbead concentration was observed for the plastic (20 ± 3% remaining) and the stainless steel (5 ± 0.5% remaining) vessels. It is interesting to note that while the metal beaker resulted in the largest decrease in suspended PS, it also exhibited the lowest variation among replicates (Fig. 1A). Furthermore, the metal beakers had a brushed surface interior, which might result in both higher friction and a larger surface area for the microplastic to adhere to than in the glass and PP vessels. A stainless steel beaker was tested to include a material with a different microstructure than glass, but should not be used in a microalgae toxicity test as it is impermeable for light required for algae growth. The PP beaker exhibited also lower light permeability than glass. Despite efforts to use vessels of similar volume, the volume of the metal beaker was larger (1000 mL) than that of the other 2 material types (600 mL). However, a separate study established that the vessel surface to PS suspension volume ratio has no significant effect on the suspended PS concentration over 24 h (Fig. 1B). It was not within the scope of the present study to investigate the physicochemical properties that could explain the differences in PS bead distribution observed between the different material exposure vessels. However, sorption in general is affected by microplastic surface properties such as point of zero charge, surface area and pore volume, surface topography, functional groups and acid-base behavior (Fotopoulou and Karapanagioti,

2012). The results show that glass, the most commonly used exposure vessel material in laboratory studies, performed best and that less conventional exposure vessels appear to offer no advantage in terms of maintaining a constant microplastic exposure concentration. However, the 53% decrease in suspended PS microbead concentration after 24 h highlights the challenges of maintaining constant exposure conditions even when using relatively conventional exposure vessels.

In the study investigating the influence of initial PS microbead concentration on the suspension stability, there was no significant variation between the tested concentrations (nominally 2200, 8000, 15,000 and 22,000 microbeads mL^{-1}) over the first 5 h (Fig. 1C). In this period, suspended PS microbead concentrations all decreased by a similar percentage irrespective of the initial concentration. However, after 5 h the curves for the different samples began increasingly to diverge from each other (Fig. 1C). The lowest start concentration, 2200 PS mL^{-1}, showed the highest detectable PS microbead percentage after 24 h: 47%, compared to 27–30% for the higher start concentrations. This trend towards greater suspension stability at lower concentrations is consistent with previous studies, especially those from the field of nanomaterial fate and effects (Keller et al., 2010). Although relatively little is known about the aggregation of plastic particles in liquid media, higher concentrations favor increased frequency of physical interactions between the particles and facilitates the formation of MP aggregates (Karami, 2017). It should be noted that the nominal baseline PS microbead concentration of 2200 PS mL^{-1} is extremely high compared to average microplastic concentrations estimated for the water column in the marine environment (< 1 particle m^{-3}) (Everaert et al., 2018). However, the concentration of 2200 PS mircobeads mL^{-1} was selected to ensure PS concentrations would not drop below 200 particles mL^{-1} during 24 h.

In an experiment investigating the influence of stirring speed on PS microbead suspension stability, the lowest speed tested (12 rpm) was found to maintain the highest suspension concentration over time (Fig. 1D). However, this stirring speed still resulted in a loss of 53% of the PS microbeads from suspension after 24 h. At increased stirring speeds of 60 and 125 rpm, the loss of the PS microbeads from aqueous suspension occurred much faster than at 12 rpm and resulted in 99% removal after 24 h. It is suggested that higher stirring speeds actually drive the settling of the PS microbeads due to the formation of a slight vortex in the center of the exposure vessel. At low speeds the overhead stirring method offers a clear advantage over static exposure systems but can perform more poorly if high stirring speeds are employed.

In the final experiment, the combination of dispersant and overhead stirring was assessed with three different dispersion agents (PVP40, Tween20 and gum arabic) at a range of concentrations. Although the highest concentration of Tween20 (27.9 μL L^{-1}) exhibited the highest PS microbead suspension concentration, PVP40 showed the greatest overall positive effect on maintaining PS in suspension, especially during the first 10 h of exposure (Fig. 2A). However, this was not necessarily concentration-dependent over the concentration range employed in the current study, and variation in PS suspension concentration between replicates was higher for PVP40 than either Tween20 or gum arabic. Furthermore, no beneficial effect of PVP40 was observed at the end of the 24 h exposure period relative to exposures without dispersant. In contrast, Tween20 exhibited negligible effects on the suspended PS concentration in the period 0–8 h, but increased suspension stability from 8 to 24 h in a concentration-dependent manner for dispersant concentrations ≥ 2.79 μL Tween L^{-1} (Fig. 2B). Gum arabic elicited no positive effects on the suspended PS concentration throughout the entire 24 h exposure period (Fig. 2C).

Dispersants have frequently been employed in particulate fate and effects studies to help maintain particle dispersions or suspensions (Paul-Pont et al., 2016; Renzi et al., 2019). However, their use requires a careful evaluation of the impact of the dispersant aid on the test organisms, while unknown effects due to modified bioavailability of particles may occur (Potthoff et al., 2017). Furthermore, mechanical forces are the main driver of microplastic dispersion in the natural environment and there are concerns over the environmental relevance of fate and effect studies that use such chemical dispersants (Karami, 2017). The three dispersants used in the present study were selected because they do not elicit any significant toxicity on plankton species (Gao et al., 2012; Paul-Pont et al., 2016). The use of equimolar concentrations of the three dispersants resulted in the lowest mass or volume per volume concentration for Tween20, yet only this dispersant appeared to elicit a concentration-dependent effect on the suspended PS microbead concentration.

As the suspended PS concentration decreased over time, increasing numbers of 13.1- and 15.0 μm-sized particles appeared during all of the 24 h studies (except for the experiments with the plastic and metal vessels, where aggregate levels remained constant over 24 h at approximately 3% of the total PS microbead load), suggesting aggregation of the PS microbeads was occurring. This was supported by the volumes of these larger particles corresponding to the expected volumes of aggregates comprising 2 and 3 (1178 and 1767 μm^3, respectively) of the individual 10.4 μm PS microbeads (589 μm^3) used in the study. There were no measurable aggregates in the range 15.0–60 μm diameter, while any aggregates larger than 60 μm would not be detected due to the use of the 100 μm aperture in the coulter counter. Furthermore, all treatments using the overhead stirrer exhibited visible PS aggregates, both at the surface of the exposure medium and on the vessel floor under the stirring rod. The number of visible aggregates was considerably higher in samples with higher PS microbead concentrations and faster stirring speeds, which promote an increased frequency in particle-particle interactions in the system. The visible PS microbead aggregates disappeared during the analysis of these samples with the Coulter counter, during which particles were kept in suspension using an overhead stirrer with a helix-like rod end, suggesting the aggregates were very loosely bound together. All samples were shaken vigorously after the last measurement at 24 h to resuspend any settled PS microbeads. Analysis by Coulter counter confirmed all PS microbeads introduced to the exposure vessels were still present and that any small or large aggregates formed could be easily broken up into individual microbeads.

Results from the comparative study of static, overhead stirring and rotating exposure systems are presented in Fig. 3. The rotating exposure system was clearly the best at maintaining the PS microbeads in suspension over a 24 h period, with suspended concentrations remaining at approximately 100%. In contrast, the overhead stirring without the use of dispersant and static exposure systems exhibited average PS microbead suspensions of 47% and 6%, respectively after 24 h. As reported above, all treatments using overhead stirring exhibited visible PS aggregates, both at the surface of the exposure medium and on the vessel floor under the stirring rod. This suggests mixing by overhead stirrer using a stirring rod fitted with a solid, flat metal plate creates currents in the exposure medium that can lead to local microplastic gradients. In contrast, no such PS aggregates were observed in bottles rotated by plankton wheel, indicating PS bead suspensions are mixed more efficiently by rotation than by stirring. It should be noted that different mixing speeds were employed during overhead stirring (12 rpm) and rotation (0.8 rpm), which may in part explain the differences in microplastic mixing efficiency observed between these two methods. The plankton wheel rotation speed of 0.8 rpm was selected as a speed fast enough to maintain microplastic fragments in suspension and slow enough to cause minimum stress to aquatic biota. The overhead stirring speed of 12 rpm was dictated by the flocculators employed herein as it was the lowest setting at which the mixing rods would rotate at constant speed. In addition, transfer of the exposure medium (100 mL) from each rotating exposure bottle to the Coulter counter beaker may have resulted in additional mixing that may have produced higher PS microbead suspension concentrations. Even so, rotation is clearly much more efficient at keeping microplastic particles in suspension than the other methods tested. Despite the negative buoyancy

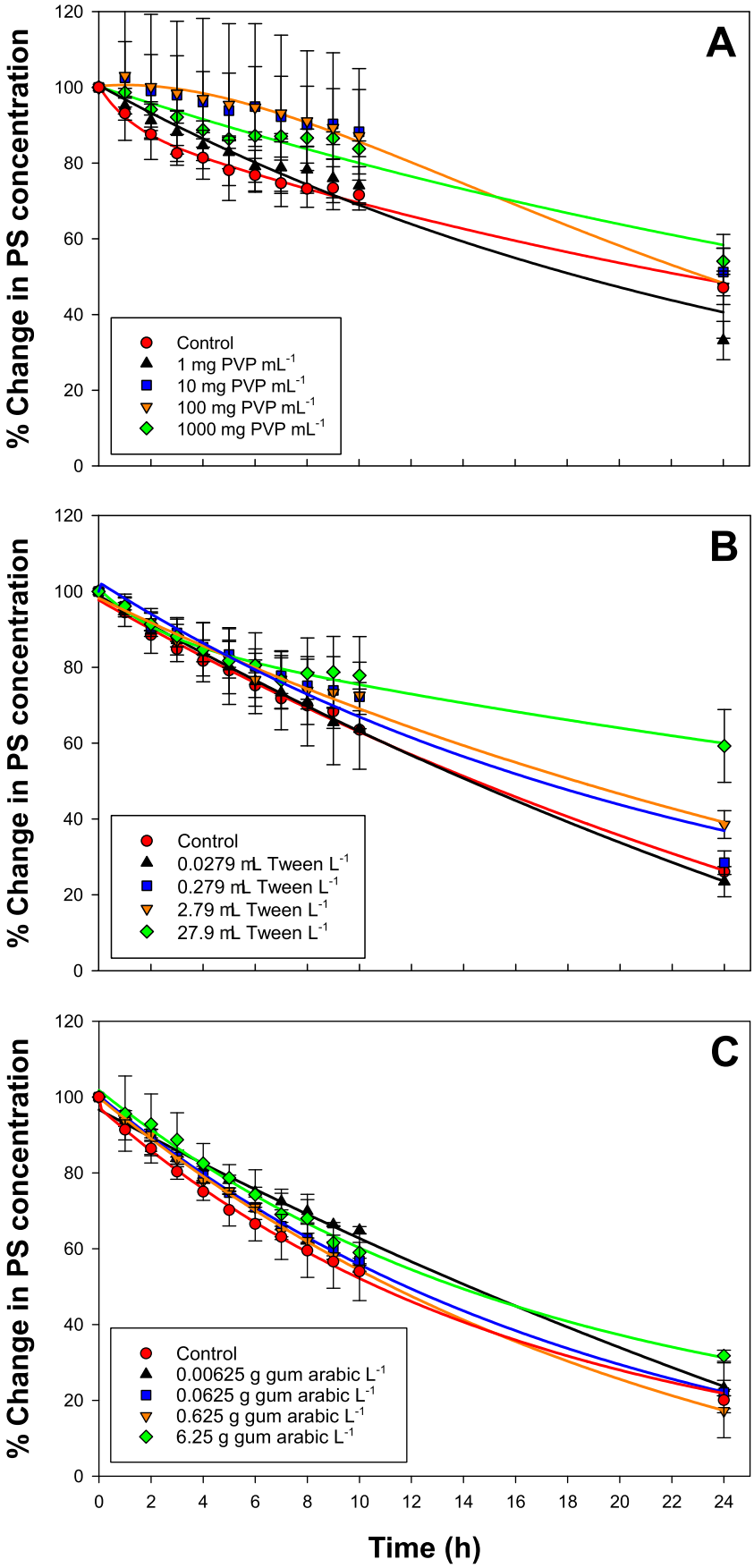

Fig. 2. Dispersant assessment for overhead stirring. Effects of 0.025, 0.25, 2.5, 25 μM polyvinylpyrrolidone (PVP40) (A), Tween20 (B), and gum arabic (C) on PS microbead concentration (% of initial concentration) during 24 h. Symbols depict means, error bars SD (n = 3).

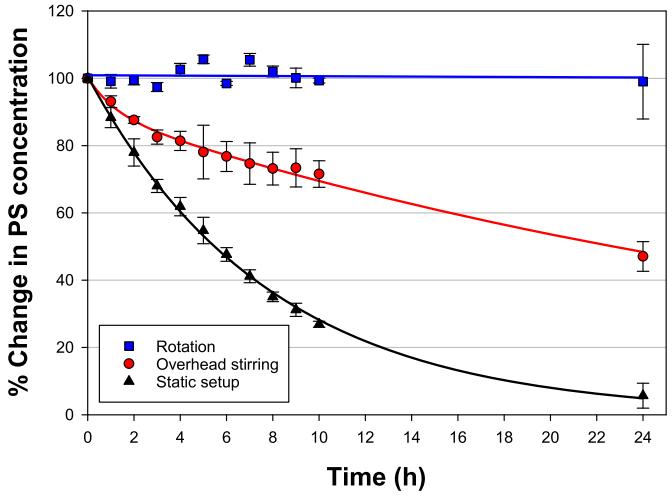

Fig. 3. Comparative PS microbead concentration trends (% of initial concentration) over a 24 h period in seawater using a static setup, the overhead stirring method and rotation by plankton wheel (mean ± SD; n = 3).

of many polymers, static and semi-static setups have frequently been used previously in microplastic exposure studies (Khan et al., 2015; Kokalj et al., 2018; Magni et al., 2018; Wang et al., 2019). Results from the current study show that the detectable fraction of PS microbeads shows an exponential decrease in seawater (Fig. 3), suggesting that exposure concentrations rapidly decrease compared to nominal concentrations. Despite providing energy into the exposure system, overhead stirring was also unable to maintain a constant PS microbead concentration in the exposure media. Although overhead stirring is not conventionally used in microplastic research, Coulter counters are usually equipped with a stirrer to prevent particles and cells from settling during analysis. However, our data suggest that it is ineffective over longer time periods. These results suggest studies employing either static or overhead stirring systems may be greatly overestimating the true microplastic exposure conditions.

Previous approaches to maintain negatively buoyant microplastic in suspension in toxicological studies include water flow or air bubbling, vigorous shaking, ultrasound and rotation. To our knowledge, this is the first assessment of overhead stirring for maintaining homogeneous microplastic suspensions for fate and effects assessment. In addition, no study has previously characterized the fate and distribution of microplastics in different exposure systems. Although overhead stirring maintains higher microplastic suspension concentrations relative to static systems, the results indicate the approach is not suitable for maintaining the initial exposure concentrations and form constant conditions with regards to exposure concentrations. However, the ability of overhead stirring to maintain microplastic particles in suspension may be improved by testing stirring rod ends that are of different forms compared to the solid, flat metal plate rod end that was used in the current study. For example, rod ends shaped like a helix, hook, or perforated flat plates may be more effective at maintaining non-aggregated suspensions. In addition, reducing overhead stirring speed < 12 rpm may further improve microplastic mixing efficiency. Nevertheless, if overhead stirring is used in toxicological studies it is essential to study the potential impact of this mixing method on performance and survival of the organisms employed in these studies. Future work includes exposure studies investigating the impact of microplastics on microalgal growth comparing overhead stirring with rotation. In addition, the dispersal behavior of more environmentally realistic microplastic fragments of different size and with different functional groups and surface characteristics should be studied, including weathered and biofouled microplastics.

4. Conclusions

For the first time it has been documented that the rotation of glass exposure vessels by plankton wheel currently remains the most effective method for maintaining a constant bioavailability of microplastic during the course of a laboratory experiment. Overhead stirring with glass vessels, slow stirring speeds and low initial microplastic concentrations (and possibly the use of Tween20) exhibited the best results for maintaining PS suspensions, but still resulted in a significant reduction in the nominal exposure concentration and the formation of fragile aggregates. If overhead stirring or other exposure systems that are unable to maintain full and constant exposure conditions are employed in aquatic organisms uptake and effects studies, it is essential that microplastic concentrations and the degree of particle aggregation are monitored throughout the exposure period.

CRediT authorship contribution statement

Iurgi Salaberria:Conceptualization, Methodology, Writing - original draft, Supervision, Funding acquisition.**Colette Nadvornik-Vincent**:Methodology, Investigation, Writing - original draft.**Giovanna Monticelli**:Methodology, Investigation, Writing - original draft.**Dag Altin**:Conceptualization, Methodology, Writing - original draft.**Andy M. Booth**:Writing - original draft, Project administration, Funding acquisition.

Declaration of competing interest

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

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