Agglomeration of nano- and microplastic particles in seawater by autochthonous and de novo-produced sources of exopolymeric substances

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Abstract

Microplastics (<5 mm) have often been studied under *in-vitro* conditions where plastics have been investigated in isolation. However, in the natural environment microplastics readily form agglomerates conferring the particles with properties different to their pristine counterparts. Here, we examined the interaction of exopolymers with polystyrene nanoplastics and microplastics. Formation of plastic agglomerates was examined using simulated sea surface conditions. Flow cytometry coupled with microscopy revealed that nano- and microplastic particle spheres form agglomerates in seawater with a mucilagenous material and an associated microbial community. To characterise this material, differential staining methods revealed it to be glycoprotein in composition. Exposing increasing concentrations of a marine bacterial glycoprotein EPS to nano- or microplastics revealed that these types of polymers contribute to the formation of plastic agglomerates. This work highlights the importance of EPS on the fate of plastic and future research should take this into account when evaluating the impact of plastics.

Running title: Agglomeration of nano /microplastics by bacterial EPS

Keywords: Microplastic; Nanoplastic; Marine Snow; Exopolysaccharide; EPS; Glycoprotein; Marine Environment; Marine Pollution.

Introduction

Contamination of the natural environment by plastic debris is of increasing concern. With the global production of plastics increasing from 230 million tonnes in 2005 to 322 million tonnes in 2015 (PlasticsEurope, 2016), and considering the multitude of point sources for the entry of plastics into the global ocean and seas (Eriksen et al., 2014; Lechner et al., 2014), there has been a rise in studies attempting to better comprehend the risks this type of pollutant poses to marine ecosystems (see Andrady, 2011 for a review).

In the case of laboratory-based studies, the plastics used were often sourced from commercial suppliers, the reason for which often stems from the fact that they can be manufactured with a prescribed uniform size and of known chemical composition that allows for standardisation across studies. The composition of microplastics collected from the natural environment, however, can exhibit a different surface chemistry and form a different entity compared to their initial 'pristine' form (Fotopoulou and Karapanagioti, 2012; Li et al., 2018). This is largely attributed to the natural weathering process and interaction of the plastics with inert and living biogenic material upon their entry into marine waters. Hence, the use of pristine plastic particles in laboratory studies to ascertain their toxicological effects to marine organisms often discounts the fact that they are unlikely to exist in a pristine form after their entry into marine waters.

Following the entry of nano- and microplastics into marine waters, or their formation from disintegrating larger debris, the surface chemistry and/or physical state of the plastics will experience changes within hours and consequently these changes will have an influence upon their uptake/ingestion by marine organisms and subsequent toxicological effects. It is during these initial hours that a biofilm will start to develop on the plastics surface (Fletcher, 1977; Lobelle and Cunliffe, 2011), or if the particles are too small they would become enveloped as part of a new or existing biofilm (Ikuma et al., 2015; Martel et al., 2014). Zettler et al. (2013) reported that microorganisms are one of the first colonizers on the surface of plastic debris and form an environment that has been referred to as the 'plastisphere'. However, the diversity of the microbial communities associated with plastics is strongly influenced by the surrounding waters in which they are located, with plastic type and structure having a more minor influence (Amaral-Zettler et al., 2015; Oberbeckmann et al., 2018; Zettler et al., 2013).

Recent work by Canesi et al. (2015) and Hentschel (2015) observed microplastics in an agglomerated form in seawater. Whilst these studies did not explore the cause or mechanisms underlying this plastic agglomeration, the process may be analogous to the formation of marine snow in the ocean, which is a key component of the 'biological pump' that participates in the

redistribution of carbon in marine systems (Long and Azam, 2001; Shanks and Trent, 1980). In addition, the process of microplastics, specifically latex beads, forming into agglomerates has been referred to previously and used to monitor and record the 'stickiness' of various exopolymers in the ocean, though not in the context of marine plastic pollution (Mari and Robert, 2008). The encapsulation of plastic debris in marine snow was recently described for waters collected at Avery point, Conneticut, USA (Zhao et al., 2017) – particles of predominately polypropylene (PP), polystyrene (PS) and polyethylene terephthalate (PET) were found associated within the marine snow particles. If the formation of nano- and microplastic agglomerates proceeds in a similar way to marine snow, this would likely influence the buoyant density of the plastics within the water column, altering their sinking rate (Kooi et al., 2017; Lobelle and Cunliffe, 2011). For example, Long et al. (2015) reported that agglomeration of microplastics with diatoms significantly altered the sedimentation velocity of plastics from tens to hundreds of meters per day. The density of the plastic material, though, must be taken into consideration. For example, the common plastics identified within marine snow by Zhao et al. (2017) were PP, PS and PET, which have densities of 0.9, 1.04 and 1.38 g/cm^3, respectively. Since the density of seawater is 1.02 - 1.03 g/cm^3, each of these three plastic types would have a differential influence on marine snow buoyancy: PP would be expected to increase buoyant density of marine snow, whereas PS would have a marginal negative effect, and PET the greatest influence in lowering the buoyant density.

Our current understanding of the agglomeration of plastics, especially nanoplastics, in seawater is in a nascent phase and warrants considerable attention. In this study, we examined the formation of polystyrene nano- and microplastic agglomerates using methods for generating artificial marine snow (Shanks and Edmondson, 1989) with natural seawater collected from the northeast Atlantic. Since exoploymers (EPS), particularly transparent exoploymers (TEP), produced by marine microorganisms constitutes a major fraction of the total pool of dissolved organic matter (DOM) in the global ocean (Decho and Gutierrez, 2017; Hansell and Carlson, 1998; Jennings et al., 2017; Passow, 1994) and has been implicated in marine snow formation (Engel, 2004; Mari et al., 2017), it was evaluated for its potential to influence the formation of nano- and microplastic agglomerates.

Materials and Methods

Isolation of EPS produced by strain TGOS-10

The EPS produced by *Halomonas* sp. TGOS-10 was isolated by growing the strain in ZM/10 supplemented with glucose to a final concentration of 0.1% (w/v). For this,

exponentially-growing cells of the strain were inoculated into a 3 L Erlenmeyer flask containing 1 L of the growth medium. The flask was then incubated with shaking (75 rpm) at 21°C in the dark. Once growth had reached the stationary phase (2-3 days), the biomass was pelleted by centrifugation (4000 x *g*; 20 min) and the supernatant filtered (0.2 µm) to remove residual cells. Isolation of purified EPS was performed as previously described (Gutierrez et al., 2007). Briefly, KCl (to 7% w/v) was dissolved in the cell-free supernatant volume prior to the addition of two volumes of cold ethanol and the mixture left for 24 hours at 4°C. The precipitated material was then pelleted by centrifugation (4000 x *g*; 20 min), the supernatant disposed of, and then the pellet was extensively dialysed against milli-Q water (18 MΩ/cm quality) using a 1 kDa molecular-weight pore-size membrane (Spectra, Cole-Parmer, inc). The resultant purified EPS was then lyophilised and stored at room temperature in a sealed container until required.

Roller-bottle incubations

The potential for plastic particles to form agglomerates was investigated using a roller-bottle design similar to that by Shanks and Edmondson (1989). This method maintains the content of the bottles in constant gentle motion (~15-20 rpm) in order to simulate the natural water column, whilst also reducing the potential of agglomerates from settling to the container walls (Jackson, 1994, 2015).

Natural seawater experiments (NSE)

To determine if nano- and microplastic particles form into agglomerates in natural seawater, four experimental treatments were set up. The seawater for these experiments was collected from the Faroe-Shetland Channel (FSC) in the northeast Atlantic (60°38.12’ N, 4°54.03’ W) in September of 2015. Sea surface water samples were collected using Niskin bottles from a depth of 5 m and maintained at 4°C until used. For these NSE experiments, the first treatment (NSE-1) comprised filling 40 mL glass scintillation vials with 40 mL of the collected natural seawater (negative controls). The remaining treatments comprised filling scintillation vials with 40 mL of natural seawater and supplementing with polystyrene plastics of different sizes (See table S1 for details on plastics) to a concentration of 5 µg mL^{-1} in each treatment vial: 50 nm (for treatment NSE-2), 1 µm (for treatment NSE-3) and 10 µm (for treatments NSE-4).

In addition, the diameter and surface charge of the plastic used in these experiments was measured using a ZetaSizer (Nano-ZS, Malvern, UK). All manipulations were carried out aseptically and each treatment was performed using five replicates. The vials were sealed with Teflon coated septa, leaving a 2 mL headspace, and placed onto a low-profile roller table at a constant gentle rotation (~15-20 rpm) for 24 hours at 7°C, which is the *in-situ* temperature for the FSC at the time the seawater was collected.

At the end of the incubation period (24 hours), the contents of each vial were filtered through a 0.22 µm white Millipore filter, using a low-pressure vacuum to minimize the disruption of marine snow/plastic agglomerates. Each filter was then examined using a bright-field binocular dissection microscope. Three images (image area = 9 mm^2) were recorded for each of the five replicates, and the number of agglomerates formed and their size were measured using Fiji image analysis software (Schindelin et al., 2012).

Flow Cytometry

A random subset of NSE samples were examined using flow cytometry to monitor the agglomeration of the plastics after 24 hours. Only plastics smaller than 1000 nm were used to prevent blockages within the injection port of the instrument. Therefore, an addition of a 500 nm plastics treatment was added to increase scope of the investigation. This was achieved using a BD LSR Fortessa multi-colour cell analyser (Biosciences, UK). The nano- and micro plastics were assessed on forward scatter (FC), side scatter (SC) and fluorescence intensity (Alexa Fluor 488, Cyan-green colour; excitation: 495 nm; emission: 519 nm). Plastic spheres were recorded based on their FC and fluorescent intensity using 10000 events or a 2 minute duration if this number of events could not be achieved. For the smaller 50 nm nanoplastics, FC was not an appropriate method of detection due to the small size of the particles, therefore only fluorescence intensity was used. Solutions of the nano- and microplastics were analysed to examine the mean size and fluorescent intensity of the plastics in a singlet form. These singlet stocks were used to set the gating to ensure that the majority of the singlet nano-plastics were measured in gate Q4. This was repeated after 24 hours incubation within the natural seawater to determine if agglomeration had occurred. Agglomeration was measured by changes in event numbers occurring in gate Q2 and Q3, which measured increases in size and fluorecscnt intensity, respectively (more plastics per aggregate). Gate Q1 measured for an increase in the size of agglomerates with no increase in fluorescence, therefore a mostly marine snow aggregate with little to no increase in plastic occurance. All samples were filtered using a 100 µm gauze to prevent system blockages, though this limited the size of the agglomerates (to

<100 μm) that could be investigated by this method. All data was analysed and reported using the FlowJo software (v10.3.0.) The relative proportion of events within each gate was reported for both singlet and agglomerated samples.

Synthetic seawater experiments (SSE)

To determine the influence of bacterial EPS on the formation of plastic agglomerates, the partially purified EPS from *Halomonas* sp. TGOS-10 was dissolved in sterile ONR7a synthetic seawater (Dyksterhouse et al., 1995) to 1500 μg mL^{-1}. This EPS working volume was aliquoted into a series of 22 mL glass Hungate tubes to achieve the following final concentrations of the EPS in ONR7a to 20 mL total volume per tube: 0, 0.1, 1, 10, 100 and 1000 μg mL^{-1}. To each tube, 10 μL of the 1.0 μm microplastics stock solution (5 μg mL^{-1} final concentration) was added. Each EPS treatment was prepared in triplicate, and all the tubes were incubated on the roller table for 24 hours at 7°C. After 24 hours, each tube was filtered through 5.0 μm Nucleopore filters under a low-pressure vacuum to recover microplastic spheres incorporated into any agglomerates (>5 μm) that formed, whilst 'pristine' microplastics – i.e. those not associated with agglomerates, would pass through the filters with the filtrate.

Sedimentation Velocity Experiments (SVE)

The influence of plastics on the buoyant density of marine snow particles was measured and compared to marine snow particles containing no plastic. Seawater for these experiments was collected at a depth of 1 m from the Leven docks on the Firth of Forth estuary (56°11.11' N, 03°00.09' W) on October 17, 2016, using sterile Duran bottles. For these sedimentation velocity experiments (SVE), a series of amber 40 mL scintillation glass vials were filled with 40 mL of the seawater and supplemented with fluorescent plastic particles of diameter 50 nm (SVE-1 treatment), 1 μm (SVE-2 treatment), or 10 μm (SVE-3 treatment). The final concentration of plastic spheres in each treatment vial was 5 μg mL^{-1}. The control treatment comprised the same seawater without the addition of plastics (SVE-4). All experimental manipulations were carried out aseptically and each treatment was performed in triplicate. The tubes were sealed using Teflon septa, with a 2 mL headspace provided and placed onto a roller table at a constant gentle rotation (~15-20 rpm) for 7 days at 10°C.

After 7 days, the experiment was terminated and the whole volume from each of the treatments was filtered through 5.0 μm Nucleopore filters and then immediately examined

using a binocular dissection microscope. Each visible agglomerate that was identifiable under the microscope was carefully manipulated using a blunt needle to separate it from nearby agglomerates and to allow size and area measurements to be taken. Each agglomerate was individually transferred into a 2 L sedimentation tube containing sand-filtered natural seawater for sedimentation velocity measurements. Measurements for sinking rate were of individual agglomerates after allowing them to equilibrate for up to 30 seconds and then letting them sink vertically within the tube. The time taken for each agglomerate to sink 40 mm was visually observed and recorded. Agglomerates that came within 1 cm of the sedimentation tube walls, or that were observed to disintegrate, were discounted. The sedimentation velocities of at least ten individual agglomerates were examined for each SVE treatment.

Microscopy

To directly visualise the agglomerates under the microscope, a separate approach to the NSE experiments was conducted. For this, a series of clear glass Hungate tubes containing 22 mL of FSC water were prepared and further amended with 10 µL of either 50 nm, 1 µm, or 20 µm plastic spheres to give a final plastic concentration of 5 µg mL^{-1} for each plastic size per tube. Each Hungate tube was wrapped in aluminium foil to eliminate potential light-induced influences, sealed with Teflon-coated septa and placed onto a low-profile roller table at a constant gentle rotation (~15-20 rpm) for 7 days at 7°C.

Tubes were visually inspected daily using a blue light transilluminator to monitor for signs of agglomerate formation. Agglomerates that were visible to the eye after 7 days were collected using a glass Pasteur pipette, mounted on a glass slide and then carefully washed with phosphate-buffered saline (10 mM Phosphate, 137 mM NaCl, and 2.7 mM KCl) for subsequent staining of agglomerates. This was performed with the amino-acid specific dye Coomassie Brilliant Blue G (CBBG) at pH 7.4 (Long and Azam, 1996) or with the cationic copper phthalocyanine dye Alcian Blue (AB) at pH 2.5 (Alldredge et al., 1993). AB is commonly used for staining acidic sugars of EPS or TEP in seawater, whereas CBBG is used for staining the proteinaceous component of these polymeric substances. Following staining, each agglomerate was counterstained with the nucleic acid stain acridine orange at 4 µg mL^{-1} to examine for the presence and abundance of prokaryotic cells.

Statistics

To determine if plastic size influenced the abundance of formed agglomerates, the numbers of agglomerates formed in the NSE experiments were examined using a Kruskal-Wallis test, as

these data did not meet the assumption required for parametric analysis. An additional Wilcoxon post-hoc test was carried out to determine the difference between each of the means. The equivalent spherical diameter (ESD) of the agglomerates were compared by an analysis of variance test (ANOVA) for each plastic sphere size used; the data was log transformed to meet the assumptions for parametric analysis. A Tukey post-hoc test was carried out to identify which groups were significantly different.

For the SSE experiments, the data was analysed using a polynomial regression analysis to infer any relationship between EPS concentration and total number of agglomerates that formed. A pairwise t-test was used to test for differences between mean ESD and EPS concentration. A polynomial regression analysis was used to infer any relationship between surface tension and EPS concentration. The difference in sedimentation velocity for agglomerates, containing different sizes of plastic spheres, was analysed using a pairwise t-test of the ratios between size and sedimentation velocity. All statistical analyses were performed using R (R Core Team, 2014).

Results and Discussion

Plastic characterisation

The individual characteristics of the plastics used were recorded using dynamic light scattering (DLS) and zeta potential (Table 1). The smaller plastics, purchased as 50 nm and 500 nm were found to have a diameter that was more than double that expected on the pack. This may be due to the size and density of these plastics, as at this size the PS plastics were starting to exhibit a sedimentation effect. As the movement of the larger plastics was more than could be attributed to just Brownian motion, the DLS method was not suitable for accurately measuring this dimension in this case. However, for the smaller plastics (50 and 500 nm), these were collodially stable, permitting a more accurate measure of their size. This colloidal stability matched the zeta potential measurement obtained, with the smallest plastic size measured corresponding to having the largest zeta potential measured. As the size of the plastics increased, the charge reduced until the value of ±30 mV was achieved, at which point the colloidal stability was affected enough to permit agglomeration.

Formation of nano- and microplastic agglomerates in seawater

The formation of agglomerates occurred within 24 hours in all the NSE treatments, as confirmed by flow cytometry (Figure 1). It was clear that the stock 50 nm particles were tightly grouped together in Q4 (Figure 1A; 99.6%). However, after 24 hours of exposure to natural

seawater, agglomeration could potentially be measured (Figure 1B) with the proportion of events measured in Q4 dropping to 94.4%. While this was only a marginal difference in number, the plot indicates that the distribution of the size and fluorescent intensity was beginning to shift from that observed from the 50 nm stock solution. This sized plastic is below the detectable limits of this cell analyser (FC), therefore these data are only an indication that agglomeration may be occurring.

The 500 nm plastics appeared to have formed density spots of both singlets and agglomerates (Figure 1C and 1D) in both Q4 and Q3 respectively, during both pre- and post-incubation. This indicates that some agglomeration had taken place prior to the incubation of the plastics in seawater. In this case, the proportions of the events measured in each gate are more indicative of agglomerate formation as 60.0% of events are in Q4, showing most the 500 nm plastics are unbound in singlet form; with the remaining 39.9% of events showing some agglomeration already. However, following 24 hours incubation in the natural seawater the proportion of plastics in Q3, indicating agglomeration, had risen from 39.9% to 93.9%. For the largest plastic measured (1000 nm; Figure 1E and 1F) the difference between pristine plastic and incubated plastics was not clear. The proportions in Q4 and Q2 were similar, before and after incubation, with 87.1% and 82.1% of events in Q4 (singlet) and 12.8% and 15.4% present in the gate believed to display the agglomerates. In addition, there appears to be an increase in FC of the events in Q4 as the contour plot started to become evident in Q1. This indicates that larger agglomerates were forming, yet their fluorescent signature was either not detected by the cell analyser or aggregates were being formed with non-fluorescent particles.

Agglomerates formed from the NSE experiments were examined using a bright field microscope. The size and quantity of the agglomerates were recorded. There was a significant difference in the size of the agglomerates when different sized plastics were used (Figure 2A; ANOVA, $F_{(1\text{-}753)} = 12.746$, $p = <0.001$). However, as shown in Figure 2B, there was overall no effect conferred by the size of the plastics to the number of agglomerates measured. Furthermore, the larger agglomerates that formed in vials containing 50 nm plastic spheres were the only treatment that reported differing agglomerate ESDs from the control treatment (pairwise t-test, $p = < 0.001$; mean ESD (SD): NSE-1 = 121.84 μm (83.70); NSE-2 = 197.64 μm (132.16); NSE-3 = 122.9 μm (92.07); NSE-4 = 122.25 μm (106.67)). These results demonstrate that agglomerates containing larger plastic spheres were not significantly different from those formed in control incubations (NSE-1).

Whilst we are unable at present to definitively explain why the 50 nm plastic spheres promoted the formation of larger agglomerates, we offer possible hypotheses. The plastic

spheres that were added to the treatments had been standardised for mass per volume, which would result in a greater number of plastic spheres present in the 50 nm treatment (10^{10} mL^{-1}) compared to the 1 μm (10^{6} mL^{-1}) and 10 μm (10^{3} mL^{-1}) treatments. This higher plastic particle number in the former treatment will have altered the collision probability between the spheres and particulate organic matter (e.g. EPS, TEP) in forming agglomerates (Alldredge and McGillivary, 1991; Alldredge et al., 1993). The initial stages of agglomerate formation would have favoured the higher particle density within the NSE-2 treatment. Over time, it may be expected that the rate of agglomerate formation in these incubations would sharply drop and allow the agglomerates that formed in the other treatments to possibly reach an equal size to that observed in the NSE-2 treatment. Furthermore, the shear stress experienced by agglomerates would be expected to decline over time, and in turn increase the probability of larger agglomerates to form (Jackson, 2015). Additionally, the dilution effect in the ocean water column will likely reduce the collision rate of plastic particles to forming agglomerates compared to that occurring in a confined volume as in a laboratory setting (Alldredge and McGillivary, 1991). Therefore, examination of particulate density as well as mass density is an important variable to be investigated in future studies.

The total number of agglomerates that formed in these NSE incubations did not significantly differ as a function of plastic particle size (Figure 2B; Kruskal-Wallis, $H = 4.9$, d.f $= 3$, $p > 0.05$). In addition, all four treatments also presented a high level of variability in these data (mean ± SD): NSE-1 = 27.62 ± 26.44; NSE-2 = 33.50 ± 14.14; NSE-3 = 46.15 ± 26.73; NSE-4 = 32.78 ± 17.94.

Overall, the total numbers of agglomerates detected across all treatments ranged between 33-47 per mL. The controls had a mean of 27 agglomerates per mL; though smaller than the treatments this was not significant. Given the relatively small size of these agglomerates (100-200 μm in diameter), the concentrations of agglomerates that formed were not unexpected; numerous small particle clusters will form initially and then over time will collide and stick together to form fewer, yet larger, agglomerates(Logan and Wilkinson, 1990). As the agglomerates grow larger and fewer in number, the fact that they reached a maximal abundance and size may be explained by the finite concentration of EPS in these experiments. In addition, the larger agglomerates may experience a greater shear stress, resulting in an equilibrium state of more abundant yet smaller agglomerates present within these microcosms (Barton et al., 2014). As there was no significant difference in the number of agglomerates formed between the plastic size and control treatments, this is evidence that the plastic is not influencing the formation of agglomerates differently to that of marine snow in the controls. While nanoplastics

have yet to be recorded in the natural marine environment, concentrations of microplastics from highly polluted areas have been recorded at ~10^5 particles m^{-3} (Lozano and Mouat, 2009). While this concentration is lower than the particle densities used in the microcosms in this study containing the 10 μm plastic spheres (10^9 particles m^{-3}), the microplastics used in the microcosms are 1/10th of the diameter of those reported by Barton et al. (2014). Therefore the final volume of the smaller plastics was was one order of magnitude greater than that currently observed for larger plastics.

Microscopic examination of microplastic agglomerates

Microscopic examination of the 20 μm plastic agglomerates that formed in our experiments with natural seawater from the FSC were found to be free from natural particulate matter, such as plankton casts and faecal matter, that is normally observed associated with marine snow (Smetacek, 1985). The agglomerates were barely visible to the naked eye during the initial 24 hours of the incubations, but clearly visible when illuminated under blue light transillumination, which was indicative that the agglomerates were largely composed of microplastic particles. At day 7 (the termination of the experiment), the agglomerates visually appeared larger (~0.5–1 mm) – a size that is consistent with the size definition for marine snow (>0.5 mm) in the ocean. When observed under the light microscope, these 7-day agglomerates were almost entirely composed of the plastic particles, held together by what appeared to be a biopolymeric substance. When stained with the polysaccharide-specific dye AB (Figure 3A), or with the amino acid-specific dye CBBG (Figure 3B), this confirmed the biopolymer enveloping the particles was of glycoprotein composition. Since EPS produced by marine bacteria is commonly of glycoprotein composition (Gutierrez et al., 2007; Hassler et al., 2011; Mancuso Nichols et al., 2004), we suspect that the biopolymer forming these microplastic agglomerates may predominantly be of bacterial origin. Whether it was present as part of the DOM pool in the water from the FSC at the time of collection, or it was produced via *de novo* synthesis by endogenous EPS-producing bacteria during these incubations, is unknown to us as we did not measure changes in EPS or TEP concentrations. Nonetheless, this glycoprotein polymer appeared to act as a 'glue' by way of interconnecting, encapsulating or trapping (like a net) the microplastic particles into an amorphous matrix. The presence and persistence of EPS has been shown to enhance the agglomeration of particulate matter and micro-algal cells (Grossart et al., 2006), and we show here that autochthonous EPS in seawater, in particular that of glycoprotein composition and possibly of bacterial origin, is a major component of nano- and microplastic agglomerates.

Staining the agglomerates with acridine orange revealed they contained a community of associated microorganisms which, based on their size (average 0.5-2.0 μm), were prokaryotes (Figure 4). Due to the background fluorescence contributed by the plastic particles, it was logistically impossible to provide an accurate microbial cell count associated with any one agglomerate. This was also confounded by the spatial localisation of cells likely hidden within and behind the agglomerates and their encapsulated plastic spheres. The EPS, and potentially also the associated microbial community assuming they are not plastic-degraders, may offer the microplastic particles a degree of protection from the weathering forces at sea. Coombes et al. (2011), for example, observed that the colonization of engineered materials by marine microorganisms (i.e. biofilms) can offer bioprotection from the weathering of such materials when left exposed at intertidal zones. Other studies have also reported bioprotective qualities of biofilms for other materials, such as silicates (Di Bonaventura et al., 1999; Gowell et al., 2015). Although we did not carry out a sequencing survey to analyse the phylogenetic identity and abundance of the microbial community associated with these microplastic agglomerates, this is a key area for future investigation as it could reveal insight into a functional bioprotective role, as well as whether the agglomerates might act as carriers for the transportation of pathogens (Lyons et al., 2005).

Role of bacterial EPS in microplastic agglomerate formation

From our observations of plastic agglomerate formation in the NSE incubations and analysis of their composition, which revealed they are composed largely of glycoprotein biopolymer, we hypothesised that marine bacterial EPS may be a key agent in the agglomeration of plastic particles within the marine environment. To evaluate this, we used a glycoprotein polymer produced by a marine EPS-producer, *Halomonas* sp. TGOS-10. Using increasing concentrations (0.01 to 1000 μg mL^{-1}) of this *Halomonas* EPS when dissolved in ONR7a synthetic seawater, we found a significant relationship between the numbers of microplastic agglomerates that formed and the concentration of EPS (Regression analysis, R^2_{adj} = 0.054, p = 0.022). Higher numbers of agglomerates formed as a function of increasing EPS concentration, from the lowest EPS concentrations used (0.01 μg mL^{-1}) to 1000 μg mL^{-1} (Figure 5A). EPS concentrations higher than 1 μg mL^{-1} did not yield significantly higher numbers of agglomerates relative to the untreated controls. This may be due to the finite number of unbound plastic particles becoming a limiting factor at these higher EPS concentrations – i.e. there was likely no available unbound microplastic particles to enhance the size of existing agglomerates in these incubations that contained EPS at >1 μg mL^{-1}. Hence, whilst the size of plastic

agglomerates had been visually observed to increase in size over time in our microscopic examination of incubations with just natural seawater, our results with a purified form of bacterial EPS suggests that its concentration in seawater may contribute a limiting factor in microplastic agglomerate formation.

Noteworthy was the number of agglomerates recorded for the negative control, which was the highest across all the treatments. While the EPS clearly appears to act as a binding agent, specifically in holding the plastic particles together into agglomerates, it is feasible that EPS may impart a bio-dispersant function, potentially limiting the hydrophobic properties of the plastic surfaces, which at low EPS concentrations may outweigh the 'sticky' properties of the polymer. Therefore, in the absence of the EPS in the control treatment, the hydrophobic chemistry of the plastic spheres may be enough to mediate efficient agglomerate formation. This resulted in 136.07 (±34.33) agglomerates that formed per mL in the EPS negative control. We emphasize, however, that these negative controls are not a realistic representation of the natural seawater environment because they were devoid of biopolymers that are a key feature to any natural aquatic environment. With surface seawater from the North Sea having a reported DOC concentration of ~65–75 μM (Thomas et al., 2005) and EPS contributing to ~65% of this carbon budget (Underwood et al., 2010), the calculated EPS concentrations of 350–750 μg mL^{-1} are comparable to those used in this study. Therefore, whilst pristine plastics can agglomerate in EPS-free synthetic seawater, our results highlight the importance of marine EPS in the formation of plastic agglomerates in the ocean, and we have shown here, for the first time, that this occurs with nanoplastics. Hence, nano- and microplastics encapsulated within a matrix of natural seawater biopolymer should be considered in any study deemed to understand the fate of these pollutants in marine environments.

We had expected that at lower concentrations of EPS, smaller sized agglomerates would form, and the converse occur at higher EPS concentrations. Our results, however, did not show this. The mean ESD of microplastic agglomerates showed no difference across the range of EPS concentrations evaluated (ANOVA, $F_{(6\text{-}10906)} = 1.227$, $p = 0.289$; Figure 5B). As the number of agglomerates increased with increasing concentrations of EPS, it was assumed that the EPS was acting as a cohesive agent. This, however, did not appear to significantly influence the size of the agglomerates, which may be due to sheer forces that the agglomerates would have experienced within the bottles during these incubations (Jackson, 2015; Long et al., 2015). Furthermore, as this experiment employed a single size and concentration of plastic spheres, the experimental set up was standardised for both mass concentration as well as particle numbers. Therefore, this lack of any significant difference in agglomerate ESD supports the

hypothesis that the larger agglomerates reported from the NSE experiments with the 50 nm spheres could be due to particle numbers being higher, and the resulting increase in surface area as a binding site, compared to the other treatments; as particle densities in synthetic seawater were uniform, it would explain the apparent uniformity in agglomerate ESD.

Although we did not perform viscosity measurements, we noted by visual inspection that the viscosity of the ONR7a medium increased, especially at the higher EPS concentrations. An increase in viscosity could impede particle mobility and collision in the liquid medium. Additionally, an increase in the viscosity of the solution could exert a sheer stress on the agglomerates, sufficient to affect the maximal size the agglomerates could otherwise potentially reach – smaller agglomerates would remain stable under the prevailing conditions (Alldredge et al., 1990; Tiselius and Kuylenstierna, 1996).

To ascertain if increasing concentrations of the *Halomonas* EPS affects the surface tension of the ONR7a liquid medium, and potentially also influence microplastic agglomerate formation, we measured the surface tension of each of the EPS solutions. Figure 6 shows the surface tension for the various EPS concentrations in ONR7a. At the lower EPS concentrations used (0.0 – 1 μg mL^{-1}), the surface tension remained relatively constant, averaging around 72.6 to 73.6 mN m^{-1}. However, at EPS concentrations of 10 μg mL^{-1}, the surface tension increased to 78.5 mN m^{-1}, and further increased to 84.4 mN m^{-1} when an EPS concentration of 100 μg mL^{-1} was used. At the highest EPS concentration measured (1000 μg mL^{-1}), however, the surface tension of the liquid was 77.9 mN m^{-1}, which was 6.5 mN m^{-1} lower compared to that for the EPS concentration at 100 μg mL^{-1}. Overall, there was a significant relationship between surface tension and EPS concentrations employed in the SSE treatments (R^2_{adj} = 0.153, p <0.001). The effects of surface tension on particle agglomeration have been studied within the fields of particle analysis and fluid dynamics, which show that increasing surface tension is related to the increased agglomeration of oil associated minerals in solution (Duzyol and Ozkan, 2010; Ozkan et al., 2005).

Influence of plastic encapsulation on marine snow sinking velocities

Seawater from the Firth of Forth, and the particulates and plastics therein, bound with the authchthonous EPS material to form agglomerates. Agglomerates comprising mainly of natural particulate matter exhibited reduced cohesive stability. As observed for several of the control agglomerates and those with 50 nm plastics, they broke apart easily into smaller sizes. Therefore, only three agglomerates from each of these treatment types were assessed in these

SVE experiments. The larger plastic particles (1 and 10 μm spheres) were incorporated into agglomerates and found to maintain a structural integrity throughout the experiment. During the experiment, two of the agglomerates, both containing 10 μm plastic spheres, adhered to or came within 1 cm of the walls of the sedimentation tubes. Due to the increase in drag that this can exert on a particle (Winet, 1973), they were excluded from further analyses.

The nano-scale (50 nm) plastic spheres formed agglomerates that were smaller than those observed in incubations containing the micron-scale (1 and 10 μm) plastic spheres and in the control incubations with no plastic added. The small size of these agglomerates appeared to have influenced their sinking velocities, which were 39.4 m day $^{-1}$, 113.4 m day $^{-1}$, 77.4 m day $^{-1}$ and 56.3 m day $^{-1}$, respectively for agglomerates formed in the 50 nm, 1000 nm, 10000 nm and control treatments (Figure 7). For comparison, we show our data alongside two well cited articles that report sedimentation velocities for artificial marine snow – 67.1 m day $^{-1}$ (Shanks and Trent, 1980) and 74.9 m day $^{-1}$ (Alldredge et al., 1990).

Unlike the NSE and SSE experiments, in which agglomerates formed within 24 hours, we performed these SVE experiments for 7 days to allow for larger agglomerates to form within a size range reminiscent of marine snow (>500 μm). This, combined with the increased presence of particulate matter from the water used here from the Firth of Forth estuary, resulted in agglomerates that were markedly different in appearance and size.

As the sedimentation velocity of a sinking particle is directly proportional to its size, we could not investigate sedimentation velocity in isolation. Therefore, the ratio between the ESD and the velocity was assessed using a pairwise t-test to determine if any agglomerate was significantly different to those from treatments containing different sized plastics. Our results showed no significant difference as the sedimentation velocities were due to their size and not the plastic contained therein. Moreover, the size difference of the agglomerates was not significantly different between the different treatments and negative controls. The lack of any significant difference in sedimentation velocity means that, at least with these plastic spheres, the rate of DOM transport to the benthos is not affected. However, the density of different plastic types may contribute to influencing the sedimentation velocities of DOM in the ocean, as changes in ballast or buoyancy properties resulting from the abundance and densities of the incorporated plastics will influence sedimentation (Kooi et al., 2017; Lagarde et al., 2016). Moreover, the buoyant density of these agglomerates may be influenced by the composition or chemistry of the EPS or TEP – the glue holding them together – since this gel-like substance can significantly alter the sedimentation velocity of agglomerates (Azetsu-Scott and Passow,

2004; Kooi et al., 2017). However, the behaviour of these particles may not be consistent, as the time taken for the agglomerates to reach significant depths can facilitate changes in the biofilms, such as metabolisation of labile carbon or alterations to the microbial community capable of sustaining metabolic processes at bathymetric pressures. This can result in unpredictable migration velocity of the particles, which in turn would influence their sedimentation. Kooi et al. (2017) reports that plastic agglomerates can alter from positive to negative buoyancy, with the opposite being true also, in a cyclic manner resulting in an unpredictable trajectory. Therefore, any alteration to the buoyancy of the agglomerate may be mitigated or enhanced by the EPS, thus opening a new area of investigation that explores biofilm formation with various plastic types.

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Supplementary data

Details of some of the methods used in this study have been included in the supplementary documents.

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687 **Figures and Tables**

688

689 **Figure 1** Countour quadrant plots showing singlet and agglomerated plastics monitored
690 using flow cytometry. A, C and E are the initial stock solutions. B, D and F are after 24 hour
691 exposure to sea water in a roller bottle. The plastic sizes used were 50 nm (A, B), 500 nm (C,
692 D), and 1000 nm (E, F).

693

694 **Figure 2.** Microplastic agglomerates formed in natural seawater after 24 hours incubation.
695 The microplastic agglomerates were measured for size (A) and abundance (B) for four
696 treatments comprising of a control and three plastic sphere sizes (50 nm, 1 µm, and 10 µm).
697 Values shown are the replicate means (n = 5), and error bars represent standard error.
698 Significant differences between treatment and control are indicated by p value.

699

700 **Figure 3.** Microplastic agglomerates formed in natural seawater after a 7-day incubation
701 were visualised widefield light microscopy. The microplastic particles stained with Alcian Blue
702 (A) and Coomassie Brilliant Blue G (B), indicating the biopolymer is of glycoprotein
703 composition. Scale bars, 100 µm.

704

705 **Figure 4.** Nano- and microplastic agglomerates (green spheres) stained with the nucleic
706 acid-specific stain acridine orange (orange cells) and observed under a fluorescence
707 microscope. Plastic particles are: A = 20 µm, B = 1 µm and C = 50 nm plastic spheres; Scale
708 bars, 5 µm. Images are composites of images from filters FITC and Rhodamin (ex 440nm, em
709 510nm and ex 546 nm, em 580 nm, respectively).

710

711 **Figure 5.** Total number of microplastic agglomerates (A), and the mean equivalent
712 spherical diameter (ESD) of the microplastic agglomerates (B) that formed in treatments with
713 increasing concentrations of bacterial EPS. Values are the mean of triplicate measurements,
714 and error bars signify standard error. Significant differences between treatment and control are
715 indicated by p value.

716

717 **Figure 6.** Surface tension of ONR7a synthetic seawater containing increasing
718 concentrations of the *Halomonas* EPS and with/without added microplastics. Values are the
719 mean of triplicate measurements, and error bars signify 95% confidence interval.

720

Figure 7. Mean equivalent spherical diameter of agglomerates formed over a 7-day incubations (A), and sedimentation velocity of these agglomerates (B). Two well-cited data sets were added for comparison.

Table 1. The characteristic data of the nan- and micro-plastic particles used throughout the different experiments of this study.