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Oil spill dispersants induce formation of marine snow by phytoplankton-associated bacteria

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ABSTRACT

Unusually large amounts of marine snow, including Extracellular Polymeric Substances (EPS), were formed during the 2010 Deepwater Horizon oil spill. The marine snow settled with oil and clay minerals as an oily sludge layer on the deep sea floor. This study tested the hypothesis that the unprecedented amount of chemical dispersants applied during high phytoplankton densities in the Gulf of Mexico induced high EPS formation. Two marine phytoplankton species (*Dunaliella tertiolecta* and *Phaeodactylum tricornutum*) produced EPS within days when exposed to the dispersant Corexit 9500. Phytoplankton-associated bacteria were shown to be responsible for the formation. The EPS consisted of proteins and to lesser extent polysaccharides. This study reveals an unexpected consequence of the presence of phytoplankton. This emphasizes the need to test the action of dispersants under realistic field conditions, which may seriously alter the fate of oil in the environment via increased marine snow formation.

1. Introduction

In April 2010, the drilling rig Deepwater Horizon exploded in the northeastern Gulf of Mexico, killing 11 people and causing crude oil to flow from the Macondo well at 1500 m depth (National Commission on the BP Deepwater Horizon Oil Spill and Offshore Drilling, 2011). When the well was finally capped in August, 780 million liters of crude oil had spilled into the Gulf of Mexico (National Commission on the BP Deepwater Horizon Oil Spill and Offshore Drilling, 2011). Spill response included skimming, in situ burning, and use of chemical dispersants. An unprecedented volume of dispersants, 6.8 million liters, was used, of which approximately 4 million liters at the sea surface and 2.8 million liters by subsurface injection at the wellhead (BP, 2014a, 2014b).

During the spill, an abnormally large amount of marine snow was observed at the sea surface (Dell'Amore, 2010; Passow et al., 2012). The marine snow event resulted in a depositional pulse of particulate matter to the sediment, during which the sediment mass accumulation rates were 10-fold higher than rates observed over the past ~100 years (Brooks et al., 2015). An estimated 1200 mile^2 of deep ocean sediment (NRDC, 2015) was covered with a thick layer of oily material including sea snow and particulates (Brooks et al., 2015), which smothered benthic macrofauna and meiofauna (Montagna et al., 2013) and corals (White et al., 2012). There was an 80–93% decline in density of benthic foraminifera (Schwing et al., 2015), which could be due to changes in sediment redox conditions and a reduction of pore-water oxygen concentration, or PAHs and other toxic compounds in oil and/or dispersants (Hastings et al., 2014). Vertical mixing in the top layers stopped, indicating a shutdown of bioturbation (Brooks et al., 2015). The flocculent material on the sediment after the depositional pulse contained crude oil and surface materials, including phototrophic organisms (Hollander et al., 2014) and particulate matter. The depositional pulse, mediated by marine snow, brought surface materials down through the water column to the sea floor. This process was named MOSSFA: marine oil-snow sedimentation and flocculent accumulation (MOSSFA Steering Committee, 2013). Through the MOSSFA process, marine snow influenced the fate of the dispersed oil by concentrating it on the sea floor (Kinner et al., 2014). In this way, marine snow provides an alternative route by which even the deep sea benthic organisms can be exposed to dispersed surface oil.

Marine snow generally consists of aggregates including fecal pellets, mineral particles, live and dead bacteria, phytoplankton, and zooplankton (Wotton, 2004a). The separate particles can be glued together into aggregates by Extracellular Polymeric Substances (EPS) from various sources because of the stickiness of EPS (Wotton, 2004a). EPS excretion is a naturally occurring process, which can be performed by a wide variety of microorganisms, ranging from (cyano)bacteria (Arnosti et al., 2015; Fu et al., 2014; Gutierrez et al., 2013; Han et al., 2014) and fungi (Metzger et al., 2009) to diatoms and microalgae (Corzo et al., 2000; Mishra and Jha, 2009; Raposo et al., 2013).

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The main constituents of EPS are carbohydrates (Myklestad, 1995; Wotton, 2004a) although other constituents can include proteins, uronic acid, lipids, and humic substances (Al-Halbouni et al., 2009; McSwain et al., 2005; Wingender et al., 1999). One specific type of EPS is the Transparent Exopolymer Particles (TEP). These are distinct particles that are formed from dissolved carbohydrate polymers excreted by bacteria and algae (Decho, 1990; Passow and Alldredge, 1994; Stoderegger and Herndl, 1999). TEP can also be formed when bacteria hydrolyze mucus on the surface of diatoms (Smith et al., 1995). TEP are very sticky (Passow, 2002), and thus greatly contribute to the formation of marine snow (Alldredge et al., 1993). Larger marine snow flocs can form when TEP stick together, aggregate with other material such as organic compounds and clay particles, and eventually become negatively buoyant and sink (Simon et al., 2002; Wotton, 2004a).

Wotton (2004a, 2004b) described several causes for EPS excretion, such as attachment of bacteria in biofilms and protection against stressors. Excreted EPS protects bacteria and phytoplankton against several stressors, including extremely high or low salinity (Liu and Buskey, 2000; Mishra and Jha, 2009), other changes in the physico-chemical environment such as desiccation and light availability (De Philippis and Vincenzini, 1998; Han et al., 2014; Hill et al., 1994; Wotton, 2004a), exposure to oil and other chemicals (Passow et al., 2012) and silver nanoparticles (Joshi et al., 2012). Excess photosynthesis products due to overflow metabolism or nutrient limitation can be released via EPS (Corzo et al., 2000; Staats et al., 2000). EPS and marine snow are food sources for many pelagic and deep sea organisms since they consist of proteins and easily degradable carbohydrates (Decho, 1990). In this way, EPS and marine snow form a link in mass and energy transfer towards the deep sea ecosystems (Beaulieu, 2002; Simon et al., 2002; Wotton, 2004a).

Marine snow formation is a natural process that occurs widely, but has never been reported as excessively as was seen during the Deepwater Horizon oil spill. The unexpected massive production during the spill raises questions about its cause. One suggested explanation is that oil-degrading bacteria excrete EPS as dispersant to better degrade weathered oil by emulsification (Passow et al., 2012). However, pilot experiments in our laboratory showed that the production of EPS seemed to be connected to the presence of phytoplankton, and also dispersant application without oil addition induced marine snow production by phytoplankton communities. This leads to the hypothesis that the unprecedented use of chemical dispersants in the presence of phytoplankton has induced the highly increased marine snow formation during the oil spill. The composition of the produced EPS will determine its nutritional value and physical characteristics, and may also influence the interaction with the oil, affecting its ultimate fate and degradation rate in the oily sludge layer that settled on the sediment. Also, nutrient flows and oxygen use in the marine system will be impacted by the massive production of EPS and marine snow.

In this study, we investigate the EPS formation by marine phytoplankton cultures upon exposure to dispersants. We tested the roles of phytoplankton and associated bacteria with two types of marine phytoplankton: the green algae *Dunaliella tertiolecta* and the diatom *Phaeodactylum tricornutum*. The phytoplankton cultures with and without sterilization by antibiotics were exposed to dispersant. In addition, the phytoplankton-associated bacteria alone were tested after filtering out the phytoplankton. This filtrate too was tested with and without subsequent filter-sterilization. The formation and appearance of dispersant-induced EPS was studied and the composition of the EPS was biochemically characterized.

2. Materials and methods

2.1. Phytoplankton culture and treatment

Non-sterile cultures of *D. tertiolecta* (green algae, *Chlorophyceae*; hereafter called '*Dunaliella*') and *P. tricornutum* (diatom, *Bacillariophyceae*; hereafter called '*Phaeodactylum*') were provided by IMARES, part of Wageningen UR, Den Helder, The Netherlands. The cultures were grown on f/2 medium (according to the recipe provided by Culture Collection of Algae and Protozoa, Argyll, Scotland, United Kingdom), which was made with autoclaved artificial seawater (32 g/L AquaHolland artificial sea salt in demi water). The cultures were kept at 20 °C on a shaker (New Brunswick Innova 44, Eppendorf AG, Germany) at 80 rpm under continuous light of nine 15 W F15T8 Plant & Aquarium Fluorescent tubes (GE, Cleveland, OH, United States). Once a week the phytoplankton cultures were refreshed by transferring 40 mL into 200 mL fresh f/2 medium.

In order to create axenic cultures, phytoplankton cultures were treated with antibiotics (20 mL/L PenStrep Glutamine, Gibco by Life Technologies, Paisley, United Kingdom) for at least 7 days. Cell density was measured with a CASY cell counter Model TT (Roche Innovatis AG, Reutlingen, Germany). After selecting optimal absorbance and validation (Supplementary Material S1), absorbance at 670 nm was used to determine initial cell densities, measured with a Tecan Infinite M200 PRO spectrophotometer (Tecan Trading AG, Männedorf, Switzerland).

2.2. EPS formation experiments

All experiments were performed in 20 mL glass tubes with phytoplankton in an exponential growth phase. Autoclaved fresh f/2 medium (5.5 mL) was combined with 4 mL of the phytoplankton cultures or filtrates in autoclaved glass tubes. A dispersant dilution of 10 mL/L Corexit (Corexit EC9500A, kindly provided by Nalco, Sugar Land, Texas, USA) was prepared in f/2 medium. This was then filtered-sterilized through a 0.2 μm filter to remove any bacteriological contamination that could be in the Corexit stock, and 0.5 mL of the dispersant filtrate was added to each tube to make an experimental dispersant concentration of 0.5 mL/L in each tube. In pilot experiments (data in Supplementary Material S2), it was determined that a concentration of 0.5 mL/L Corexit induced rapid and consistent EPS formation in phytoplankton cultures. Considering an application rate of 1870–9354 l per km^2 (2–10 US gallons per acre) (US EPA, 1995) and taking into account that application will not be homogeneous, this concentration could be reached in the upper centimeters of the water column after surface application of dispersants. Since we were primarily interested in the mechanism of EPS production and its composition, we have chosen a relatively high dispersant concentration of which we were confident that EPS was produced. The tubes were capped with an aluminum cap, mixed, and placed at 20 °C under continuous light of a 90 W red-orange–blue-purple LED lamp (LEDSPECTRUM, Drunen, The Netherlands). The tubes were manually shaken twice a day and every day the visible EPS formation was noted (starting time of EPS appearance).

The following experimental conditions were tested for both phytoplankton species (Fig. 1): non-sterile phytoplankton culture (containing phytoplankton and associated bacteria; hereafter called 'non-sterile phytoplankton'), antibiotic treated phytoplankton culture (containing only phytoplankton; hereafter called 'antibiotic treated phytoplankton'), filtrate from non-sterile cultures (containing only bacteria, made by filtering through 0.45 μm filter to separate water with bacteria from algal cells; hereafter called 'phytoplankton filtrate'), and phytoplankton filtrate which was then filter-sterilized (0.2 μm filtered; hereafter called 'filter-sterilized phytoplankton'). In addition to the EPS formation experiments with phytoplankton, a free-living n-alkane degrading bacteria (*Rhodococcus qingshengii TUHH-12*) was tested for Corexit-induced EPS formation. This was tested because oil-degrading bacteria have been suggested to produce EPS upon exposure to oil, but their response to Corexit exposure has not yet been tested. Bacterial suspension in the exponential growth phase (1 mL) was diluted to 10 mL total volume of f/2 medium with 0.5 mL/L Corexit.

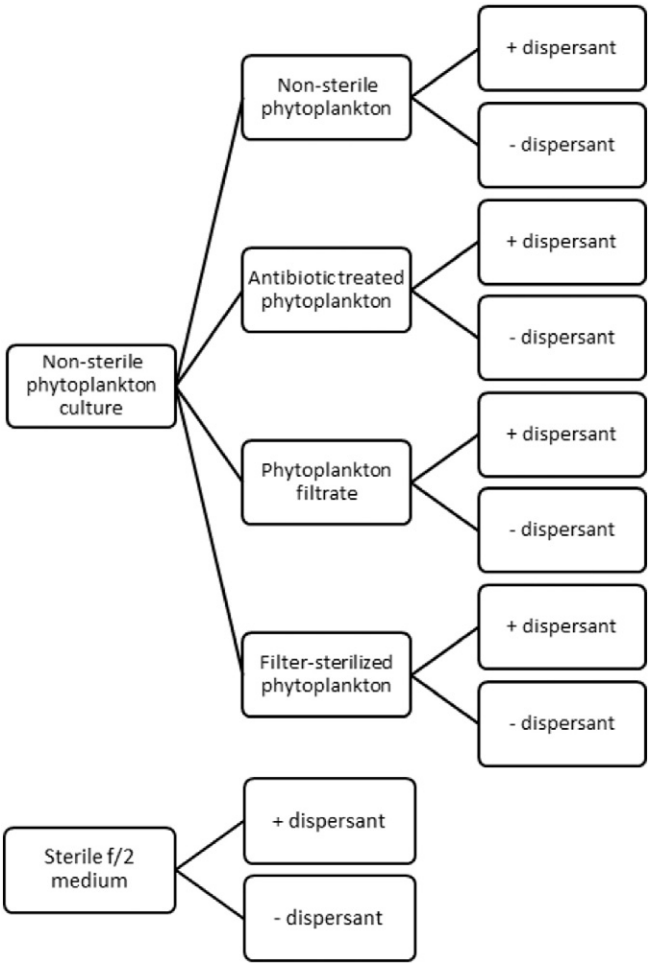

Fig. 1. Experimental setup of EPS formation experiment. All treatments were performed with two species of marine phytoplankton, *Phaeodactylum tricornutum* and *Dunaliella tertiolecta*, in triplicate.

2.3. EPS characterization

EPS was characterized visually, physically (dry weight) and biochemically (protein and polysaccharide content).

EPS for biochemical analyses was produced in 500 mL Erlenmeyer flasks with silicone stoppers with air exchange membrane. The flasks were autoclaved with 55 mL of f/2 medium. Then, 5 mL of a 10 mL/L dispersant dilution, which was 0.2 μm filter-sterilized, and 40 mL phytoplankton or 40 mL of phytoplankton filtrate was added. The final dispersant concentration was 0.5 mL/L. The flasks were incubated under the same conditions as the tubes during the EPS formation experiments. After 7 days, the EPS flocs were collected manually with a glass pipette. EPS from several flasks was combined and washed at least three times with demi-water to remove as much salt and phytoplankton cells as possible. The washed EPS was dried overnight in aluminum cups at 60 °C. Dry weight was measured by weighing aluminum cups containing EPS after drying and subtracting the weight of the empty cups. EPS production was expressed as ng dry weight per mL of exposure medium in the flasks.

Total carbohydrate content of dried EPS was measured with a phenol-sulfuric acid method based on Masuko's microplate adaptation of Dubois (Masuko et al., 2005). In short, the concentrated sulfuric acid hydrolyses all carbohydrates to monosaccharides, which then react with phenol. The absorbance of the reaction products is measured at 490 nm. We optimized the method by using a longer incubation time of 40 min to allow full hydrolysis of the dried EPS samples. The microplates were measured on a Tecan Infinite M200 PRO spectrophotometer (Tecan Trading AG, Männedorf, Switzerland), and D-Glucose was used as standard. All samples were tested in triplicate.

Monosaccharide composition of dried EPS was determined in a pilot test using orcinol-sulphuric acid hydrolysis and high performance anion exchange chromatography (HPAEC). Further analysis and quantification was done according to the method described by Englyst and Cummings (1984). Pre-hydrolysis in 72% (w/w) H_2SO_4 (30 °C, 1 h) was followed by hydrolysis in 1 M H_2SO_4 (100 °C, 3 h). The monosaccharides formed upon hydrolysis were derivatized to alditol acetates and analyzed by gas chromatography (GC) using inositol as an internal standard. The colorimetric *m*-hydroxydiphenyl assay (Ahmed and Labavitch, 1978) was used to determine the total uronic acid content. Samples were tested in duplicate, except the non-sterile *Dunaliella* sample where one duplicate was discarded for analysis because of problems with the internal standard and uronic acid measurement.

The protein content of dried EPS was determined with the Dumas method (Schwenzfeier et al., 2011), which is based on combustion of the sample at high temperature, because of practical limitations when using common colorimetric protein assays for EPS. Protein assays such as Lowry and Bradford are incompatible with samples containing Corexit because of background fluorescence. The Pierce 660 nm protein kit in combination with Ionic Detergent Compatibility Reagent (ThermoScientific Pierce Biotechnology, Rockford, Illinois, USA) was compatible with samples containing Corexit. However, as the dried EPS could not be fully homogenized and dissolved, the Pierce protein assay could not be applied either. Therefore, the Dumas method was chosen. In this assay, the total nitrogen content was measured with a Flash EA 1112 N analyzer (Thermo Fisher Scientific Inc., Waltham, MA, USA) according to the manufacturer's protocol, with D-methionine as standard. The conventional ratio of nitrogen to protein of 6.25 was used (Schwenzfeier, 2013) since a specific ratio is not known for EPS. All samples were analyzed in duplicate.

Protein composition of EPS produced by non-sterile *Phaeodactylum* was visualized using SDS-PAGE under reducing conditions with ß-mercaptoethanol. Bio-Rad Mini-PROTEAN® TGX™ Any kD™ gels were run at 120 V on a Mini-PROTEAN® Tetra Cell system (Bio-Rad Laboratories Inc., Hercules, CA, USA). Protein standards of 10–250 kDa were used (Precision Plus Protein™ All Blue, Bio-Rad) and gels were stained with InstantBlue™ (Expedeon Protein Solutions, San Diego, CA, USA). Then, gels were scanned with a Bio-Rad GS-900™ Calibrated Densitometer.

2.4. Software and data analysis

SDS-PAGE results were analyzed with Image Lab™ 5.1. GraphPad Prism 5 was used to make graphs. Statistics were performed with IBM SPSS Statistics version 23. ANOVA tests and post-hoc Tukey tests were considered significant when $p < 0.05$.

3. Results

3.1. Phytoplankton culture

Both phytoplankton species grew well under the growth conditions provided (Supplementary Material S1). The biomass grew exponentially until the maximum density was reached after one week. The phytoplankton used for the EPS formation experiments was taken from the cultures 3 days after medium refreshment, so the phytoplankton was in the exponential growth phase.

3.2. EPS formation

Non-sterile cultures of both phytoplankton species exposed to 0.5 mL/L Corexit produced EPS flocs after ~3 days of incubation (Table 1). The flocs were fragile and fell apart upon shaking (Fig. 2).

Table 1
Characteristics of EPS produced by diatom *Phaeodactylum tricornutum* and green algae *Dunaliella tertiolecta*.

Sample	Formation of EPS flocs	Appearance of EPS flocs	Dry weight (ng/mL)	Total carbohydrates[a] (% of dw)	Total monosaccharides[b] (% of dw)	Nitrogen[c] (% of dw)	Proteins[d] (% of dw)	% of total mass explained (total carbohydrate + protein)
Non-sterile *Phaeodactylum*	Yes	Floc floating at surface; some flocs at bottom	18	2.6 (0.75)	2.6 (0.1)	4.3 (0.04)	26.6 (0.28)	29.2
Phaeodactylum filtrate	Yes	Floc floating at surface; some flocs at bottom	8	3.1 (0.56)	5.8 (0.1)	2.3 (0.004)	14.2 (0.03)	17.3
Antibiotic treated *Phaeodactylum*	No	-						
Filter-sterilized *Phaeodactylum*	No	-						
Non-sterile *Dunaliella*	Yes	Flocs floating at surface; some flocs at bottom	15	4.6 (0.38)	20.8*	5.0 (0.13)	31.2 (0.80)	35.8
Dunaliella filtrate	Yes	Flocs floating at surface; some flocs at bottom	13.5	2.5 (0.03)	3.8 (0.1)	3.4 (0.04)	21.0 (0.24)	23.5
Antibiotic treated *Dunaliella*	No	–						
Filter-sterilized *Dunaliella*	No	–						

All data are presented as average with standard deviation, except * where N = 1.
[a] Measured with phenol-sulphuric acid method, average with standard deviation.
[b] Measured with alditol-acetates method.
[c] Measured with Dumas method.
[d] Calculated from %N assuming a conversion factor of 6.25.

Phytoplankton filtrates of both species, without phytoplankton cells, produced EPS similar to the non-sterile cultures, with the phytoplankton cells still present, in terms of shape, size, and onset of floc formation. The amounts produced, however, were less in the filtrates than in the non-sterile cultures (Table 1, Fig. 3). In contrast, neither of the axenic cultures (antibiotic treated phytoplankton or filter-sterilized phytoplankton) produced EPS in any of the experiments. The free-living oil-degrading bacteria did not produce any EPS either.

Dry weight of the EPS formed per mL exposure medium ranged from 8 ng (*Phaeodactylum* filtrate) to 18 ng (non-sterile *Phaeodactylum*) (Table 1). For both species, dry weight of EPS produced by the non-sterile phytoplankton samples was consistently higher than those of the filtrates.

3.3. Biochemical composition of EPS

Table 1 shows the biochemical composition of dispersant-induced EPS produced by phytoplankton. Total carbohydrate content did not differ between EPS produced by non-sterile *Phaeodactylum* and *Phaeodactylum* filtrates (one-way ANOVA, $p = 0.433$, Supplementary Material S3-1). For *Dunaliella*, however, the phytoplankton samples had higher carbohydrate content than the filtrates (one-way ANOVA, $p = 0.001$, Supplementary Material S3-2). The total EPS monosaccharide content determined with the GC-based alditol acetates method was similar to the total carbohydrate content determined with the

Fig. 2. EPS flocs, formed by *Phaeodactylum tricornutum* filtrate. The flocs were floating at the surface (top picture) and at the bottom of the tube (bottom picture) while part of the EPS flocs had settled. The fragile flocs fell apart upon vortexing.

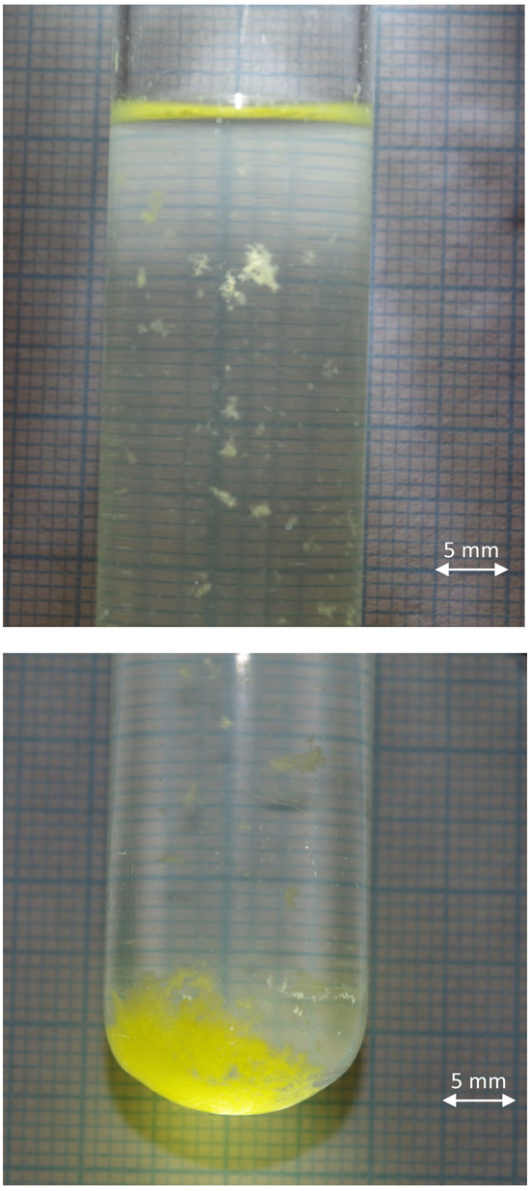

Fig. 3. EPS flocs, formed by non-sterile *Dunaliella tertiolecta*. The flocs were floating at the surface (top picture) and at the bottom of the tube (bottom picture). Tube was slightly shaken before taking the picture. The green color is an artifact produced by the camera lighting; in reality the flocs at the bottom were more brown in color.

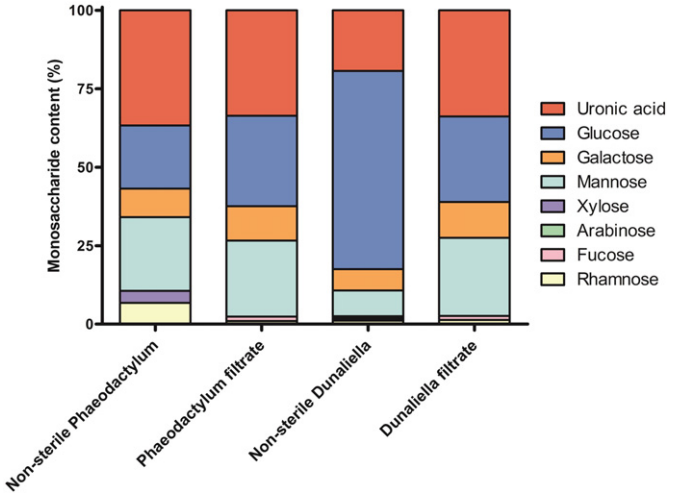

Fig. 4. Relative monosaccharide composition (% of total monosaccharide content) of EPS produced by filtrates and non-sterile *Phaeodactylum tricornutum* and *Dunaliella tertiolecta*.

plate-based phenol-sulfuric acid method. However, non-sterile *Dunaliella* EPS had carbohydrate content 5 times higher with the monosaccharide specific alditol acetates method than with the total carbohydrate phenol sulphuric acid method (20.8% of dry weight vs 4.6%, Table 1). It should be noted, however, that this higher percentage was based on a single measurement instead of duplicate measurements, due to a technical problem. The main difference in monosaccharide composition between non-sterile *Dunaliella* and the other three samples is the significantly higher relative and absolute glucose content (Fig. 4; Table S4; one-sample T-tests, $p < 0.001$, Supplementary Material S3-3). Non-sterile *Dunaliella* EPS also had significantly higher uronic acid and galactose content (one-sample T-tests, $p < 0.007$ and $p < 0.012$ respectively) than the other three samples. Mannose was significantly higher in non-sterile *Dunaliella* EPS compared to the non-sterile *Phaeodactylum* EPS (one-sample T-tests, $p < 0.011$). Characteristic of the non-sterile *Phaeodactylum* EPS was the presence of fucose and rhamnose (Fig. 4). Arabinose is only found in non-sterile *Dunaliella* EPS. In the filtrates, xylose was not found and rhamnose was present in minor amounts (Fig. 4, Table S4). The chromatogram from the pilot experiment using HPAEC (Fig. 5) shows, in a qualitative way, that EPS from both non-sterile species in general looked very similar. However, not all monosaccharides that were quantified using the alditol acetates method, were detected using HPAEC. A small fucose peak, however, was identified in the chromatogram for the non-sterile *Phaeodactylum* EPS while no fucose could be detected in the sample using alditol acetates method.

The protein content of the non-sterile phytoplankton EPS was significantly higher than the protein content of the EPS from filtrates for both species (Table 1; one-way ANOVA with post-hoc Tukey test, $p < 0.002$, Supplementary Material S3-4). *Dunaliella* EPS had significantly higher protein content than *Phaeodactylum* EPS (Supplementary Material S3-4). SDS-PAGE indicated that most of the EPS-proteins produced by *Phaeodactylum* (32–48%, Table 2) were polypeptide chains with apparent molecular mass of 110 kDa (Fig. 6). Other bands with high intensity appeared at molecular masses of about 64, 43 and 17 kDa. These molecular masses are in concordance with Czaczyk and Myszka (2007), who state that EPS proteins have molecular masses between 10 kDa and 200 kDa. The band at 250 kDa reflects non-protein particles that did not penetrate the SDS-PAGE gel. At 10 kDa, the analysis program

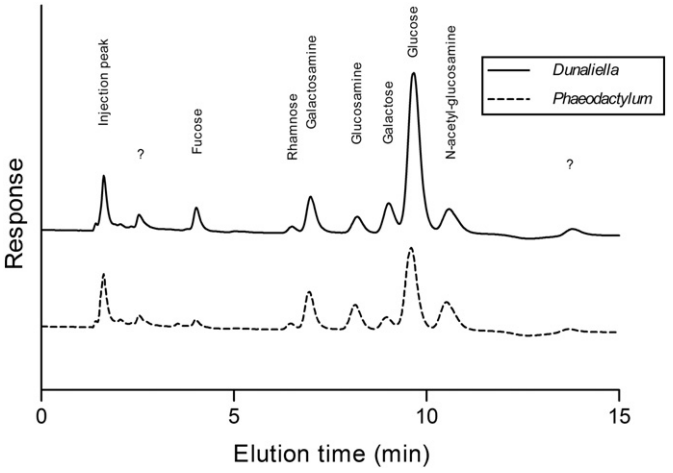

Fig. 5. HPAEC chromatograms of non-sterile *Dunaliella* (solid black line) and *Phaeodactylum* (dotted black line) EPS. A question mark indicates that this peak was not identified.

Table 2
Relative protein content of *Phaeodactylum tricornutum* EPS sample, based on band intensity on SDS-PAGE gel. Table shows how much of the total intensity corresponded to the molecular masses of the bands. R1 and R2 are replicates of the same EPS sample.

R1		R2	
Molecular mass (kDa)	%	Molecular mass (kDa)	%
250.0	10.6	250.0	11.0
110.2	31.9	108.4	48.4
63.9	2.1	63.4	4.1
42.6	6.9	41.1	1.8
37.5	0.6	31.0	3.3
31.2	0.9	17.1	4.1
17.2	3.4	10.0	27.2
10.6	43.6		

calculated a relative mass of 43.6% and 27.2% for the replicates. However, no clear bands can be seen in Fig. 6, so we consider these values as not representative of a specific protein mass.

4. Discussion

This study shows that EPS is produced by marine phytoplankton cultures upon exposure to the dispersant Corexit EC9500A. This EPS production does not occur in axenic cultures, while bacteria separated from the phytoplankton also produced EPS, although to a lesser extent. They especially produced less protein, and had a different monosaccharide profile.

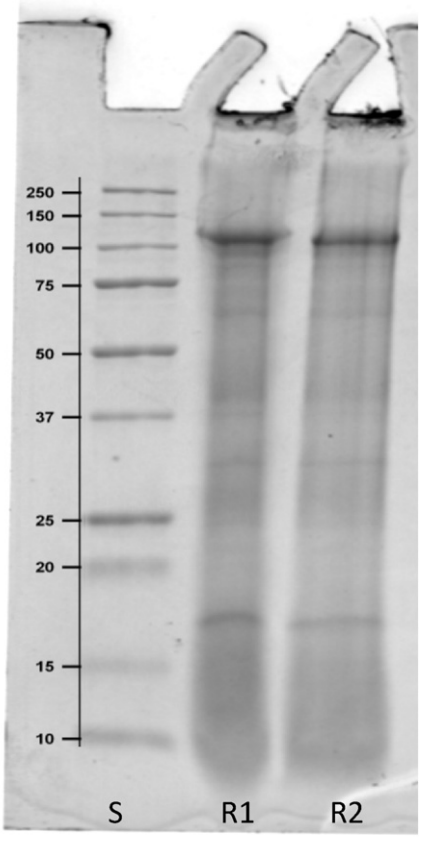

Fig. 6. SDS-PAGE gel of EPS produced by non-sterile *Phaeodactylum tricornutum*. S: Protein standards with molecular weight marker on the left; R1 and R2: replicates of the same EPS sample.

4.1. Role of bacteria in EPS production

Our experiments reveal that bacteria associated with the phytoplankton are the organisms responsible for the actual formation of the EPS, and the amount of EPS formed is greater in the combined presence with phytoplankton. In situ, phytoplankton always occurs in combination with bacteria, either in symbiosis or more loosely connected. Fu et al. (2014) found marine snow production when seawater, containing both bacteria and phytoplankton, was exposed to oil and dispersant separately and in combination. As they did not include a bacteria-only or phytoplankton-only treatment, they could not yet distinguish between roles of phytoplankton and bacteria. Our experiments demonstrate that the bacteria living in association with the phytoplankton cultures are responsible for the actual formation of the EPS, while phytoplankton seems to provide (additional) energy for the EPS production by the bacteria. The free-living oil-degrading bacteria we tested (*R. qingshengii TUHH-12*) were not capable of producing EPS. It has been shown that other free-living bacteria can produce EPS, but they always need a source of energy. From freshwater bacteria it is known that they can produce EPS such as alginate from waste water (Lin et al., 2010; Lin et al., 2013). Possibly, free-living oil-degrading bacteria use oil as energy source to produce EPS, as suggested by Passow and colleagues (Passow et al., 2012).

Possibly, Corexit acted as an energy and carbon source for the filtrate with bacteria in our experiments. Corexit contains hydrocarbons in amounts up to 30% w/w (Nalco, 2005) (Campo et al., 2013; Fu et al., 2014; García et al., 2009). *Halomonas* species are both oil-degrading and EPS-producing (Gutierrez et al., 2013), and were present in oil-aggregates produced during the Deepwater Horizon oil spill, together with other Gammaproteobacteria (Arnosti et al., 2015). Both groups of bacteria produce EPS, possibly as dispersant to be able to enhance degradation of weathered oil.

4.2. Composition of the EPS

With the methods applied, around 35% of the EPS mass could be accounted for. This incomplete mass balance could be due to either the suitability of methods for quantification of the polysaccharides and proteins present in EPS, or additional compounds that are present besides polysaccharides and proteins. These issues are discussed below.

4.3. Quantification of polysaccharide and protein content

In the spectrophotometric analysis of the phenol-sulfuric acid method, different sugar monomers have different absorption characteristics. This means that this method provides the most accurate results when applied to samples containing only the carbohydrate that is used as standard (Brummer and Cui, 2005). D-Glucose was used as a standard in our assay. As a consequence, polysaccharides are expressed as glucose-equivalents. The choice for glucose as standard can greatly influence the results if most carbohydrates present are not glucose. As can be seen in Fig. 4 and Table S4, glucose accounts for 20% (*Phaeodactylum*)–63% (*Dunaliella*) of all monosaccharides. Relating all carbohydrates to glucose underestimates the actual amount of sugars in the EPS. Ideally, every monosaccharide should be known and would have its own standard, but for practical reasons that is unfeasible. The alditol acetates method provides more information about the polysaccharide content and monosaccharide composition, but only for seven monosaccharides and uronic acid, so it does not provide a full monosaccharide characterization. So this too will underestimate the monosaccharide content.

Proteins were determined using the Dumas method based on the total nitrogen content. Because a protein-specific conversion factor was not available, a general nitrogen-to-protein conversion factor of 6.25 was applied to determine the total protein content. This factor of 6.25 is the widely accepted general value, assuming that protein contains 16% nitrogen and an insignificant amount of non-protein nitrogen

(NPN) (Conklin-Brittain et al., 1999). Many studies and food guidelines continue to use this default factor (Mariotti et al., 2008), even though there are indications that this is not appropriate for many materials (Diniz et al., 2001; Lourenço et al., 2004; Mariotti et al., 2008), in particular for plant material. Plants contain a significant amount of NPN, increasing the nitrogen content to up to 23% instead of the assumed 16% (Conklin-Brittain et al., 1999). The general conversion factor of 6.25 (100/16) will then overestimate the amount of protein. Assuming a nitrogen content of 23%, a conversion factor of 4.35 (100/23) would give a better estimate. Using 4.35 lowers the protein content in our samples from 14.2–31.2% (Table 1) to 9.9–21.7%. However, without data about NPN and actual nitrogen content of our EPS samples there is no justification to use any other conversion factor than the general factor. Also, we do not expect a lot of plant material in the EPS produced, especially not in the filtrates without phytoplankton. An underestimation of the amount of protein is made when the percentage nitrogen in proteins is less than 16%. However, this seems unlikely because almost all of the conversion factors (for foodstuffs) published are below 6.25 (Mariotti et al., 2008). The FAO considers the error in using the general factor compared to specific factors to range from −2% to +9% (FAO, 2003). For our samples, the range of protein content would change from 14.2–31.2% to 14.0–30.5% (lower estimate, −2%) and 15.5–34.0% (higher estimate, +9%).

Considering all the uncertainties about using the general conversion factor, and without information about specific N-to-protein factors for proteins in EPS, the colorimetric methods are most suitable since the assumed conversion factor can be avoided. This method, however, only is applicable to dissolved EPS but not to dried EPS flocs that do not dissolve. Also, the SDS-PAGE results serve as an indication that peptides are present in the sample, but it is not possible to identify specific proteins in the sample based on this method. To calculate specific conversion factors, information about the amino acid composition of the EPS is necessary. Amino acid composition can be determined by more advanced proteomics techniques (reviewed by Miller et al. (2014)), and, for example, Ultra High Performance Liquid Chromatography (UHPLC) separation and mass spectrometry detection (Nemkov et al., 2015).

4.4. Possible other EPS contents

Polysaccharides often are considered to be the main components of EPS produced by microorganisms in general (Myklestad, 1995; Wotton, 2004a). However, using the phenol-sulfuric acid method, we found that only a minor part of the dry weight of EPS was polysaccharides (<5% of dry weight) and a larger part was proteins (up to 31.2% of dw). Microbial EPS can also consist of proteins, glycoproteins, lipoproteins, nucleic acids and lipids (Gutierrez et al., 2007; Wingender et al., 1999). For practical reasons, we could only analyze polysaccharides and proteins in this study. It is recommended for future studies to also analyze other possible components.

In addition, components might be captured in the EPS matrix such as salt and phytoplankton cells. A high salt content will reduce the polysaccharide and protein content relative to the dry weight of EPS. However, our conductivity measurements of the water collected from the washing steps showed that three washing steps removed >97% of salt, and four washing steps removed >99% of salt. Since at least three, but mostly four or more, washing steps were used, it is not expected that the presence of salt contributed significantly to the total EPS mass. No phytoplankton cells were seen microscopically after four washing steps, even though the washed *Dunaliella* EPS had a distinct green color (Figure S5-1 in Supplementary Material). Therefore, it cannot be excluded that chlorophyll was still present in the EPS and contributed to the total EPS mass.

4.5. A specific category of EPS: alginate

Alginates are a family of sugar polymers produced by seaweeds and bacteria of several genera, such as *Pseudomonas* and *Azotobacter* (Hay et al., 2010; Remminghorst and Rehm, 2006). Alginates contain mannuronic acid (M) and guluronic acid (G) units arranged in irregular patterns of MM, MG, and GG blocks (Lin et al., 2013). Although not chemically analyzed, our EPS samples closely resemble the alginate-like exopolysaccharides (ALE) found in flocculent aerobic sludge from a wastewater treatment plant with an estimated M/G ratio and an ALE yield of 20% ± 2 of dw (Lin, personal communication).

Lin et al. (2010) found that when D-glucose was used as standard in the phenol-sulfuric acid assay, the carbohydrate content of alginate would be underestimated by 62.6% compared to using sodium alginate as standard. Taking this underestimation into account increases the total polysaccharides found in our sample from 2.5–4.6% of dw to 4.1–7.5% of dw.

4.6. Dispersant concentrations after application

Our experimental dispersant concentration was 0.5 mL/L which, as explained before, is a high but feasible concentration in the field. After aerial application of dispersant, a maximum concentration of about 0.01 mL/L (13 ppm) Corexit was measured at 0.6 m depth (George-Ares and Clark, 2000). Dioctyl sodium sulfosuccinate (DOSS) is the major surfactant component in Corexit (Kujawinski et al., 2011). DOSS was found during the Deepwater Horizon oil spill at a maximum of 229 μg/L at the surface, 0 m depth (Gray et al., 2014). Given that DOSS represents 10% of the total Corexit formulation applied, this equals 2.42 μL/L Corexit at the surface. As no information was provided about the time between application and sample taking, the concentration of 0.01 mL/L at 0.6 m depth indicates our experimental concentration is within a realistic range after dispersant application. It would be interesting to study both the gradient of dispersant concentration over the water column, hours and days after application. The lowest effect concentration of dispersant to induce EPS formation still needs to be studied, and will also depend on factors like species and density of algae and symbiotic bacteria, light intensity (turbidity of the water), nutrient availability, temperature, salinity, etc. This is very important for oil spill responders to know, but was beyond the scope of this study.

4.7. Consequences of increased EPS formation in the marine environment

Increased formation of EPS and marine snow can have serious consequences in the field situation, both for the fate and transport of oil, as well as for the ecological impacts after an oil spill. As was seen during the Deepwater Horizon oil spill, marine snow can aggregate with dispersed oil droplets and suspended particles (Passow et al., 2012). These aggregates are negatively buoyant and sink to the sea floor. One of the response measures taken during the Deepwater Horizon spill was to flush the Mississippi river to limit influx of oil into estuaries (Bianchi et al., 2011). This increased both the amount of suspended particles and the nutrients in the GoM, increasing the likelihood for a MOSSFA event (Vonk et al., 2015). This can explain the effects at the local and regional benthic community level (Montagna et al., 2013; Schwing et al., 2015).

Since phytoplankton and bacteria are ubiquitous in the marine environment, marine snow formation can potentially occur whenever large amounts of dispersant are used during algal bloom periods. Vonk et al. (2015) reviewed 52 large oil spills, and found indications that also during two other large oil spills the MOSSFA process has occurred, namely the IXTOC I blowout in the Gulf of Mexico in 1979–80, and possibly the Santa Barbara blowout near California in 1969. Sediment core sampling at these locations can reveal depositional pulses which can be related to a specific event by isotope dating, as was demonstrated for the Deepwater Horizon blowout (Brooks et al., 2015). In the Summer of 2015, a cruise of the C-IMAGE consortium went to the site of the IXTOC I blowout and sampled both sediments and biota (fish) to look for evidence of MOSSFA processes, and indeed found evidence for such an event during this spill (Vonk et al., 2015). This suggests that MOSSFA processes were

not unique to the Deepwater Horizon blowout but occurred more widespread in situations with massive dispersant application than previously thought. This also highlights the need to include sampling of the benthic community during and after oil spills, and preferably before oil drilling takes place as well to get baseline data. Even deep sea benthic communities can be damaged following oil spills, and their ecological relevance still is hardly known. In addition, analysis of protein and polysaccharide concentrations in sediment cores which show depositional pulses can give further insight in the role of EPS and marine snow in this deposition.

Our findings demonstrate that phytoplankton and their associated bacteria can rapidly produce large amounts of EPS when exposed to chemical dispersant. This mechanism is highly relevant to take into account for the spill response decision makers. Basing field applications of dispersants solely on efficacy tests performed under standard laboratory conditions in pure seawater without phytoplankton and suspended particles can have unexpected and unwanted consequences. Dispersants should not only be tested for their oil-dispersing effectiveness and for their direct toxicity, but also indirect effects on, for example, benthic ecosystems should be considered, as well as their performance under less standard (e.g., Arctic, including ice) conditions. Potential side-effects of applying oil spill dispersants, like increased marine snow formation and subsequent transport of oiled marine snow with particles to the benthic system, should therefore be considered in the decision making process.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.marpolbul.2016.01.005.

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