

Production of macroaggregates from dissolved exopolymeric substances (EPS) of bacterial and diatom origin

P V Bhaskar^{1*}, Hans-Peter Grossart^{2,3}, N B Bhosle¹ and Meinhard Simon².

¹National Institute of Oceanography, Dona Paula, GOA- 403 004, India.

²Institute of Chemistry and Biology of the Sea (ICBM), University of Oldenburg,
PO-Box 2503, D-26111 Oldenburg, Germany.

³ present address: Institute of Freshwater Ecology and Inland Fisheries, Alte Fischerhuette 2,
D-16775 Stechlin, Germany

* corresponding author

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Abstract

Exopolymeric substance (EPS) isolated from a pure culture of the marine bacterium *Marinobacter sp.* and the marine diatom *Skeletonema costatum* (axenic) were partially purified, chemically characterized and used as dissolved organic matter (DOM) for the production of macroaggregates. The role of organic particles such as transparent exopolymeric particles (TEP) and Coomassie stained particles (CSP) in the production of macroaggregates was experimentally assessed. Three experimental rolling tanks containing sterile medium with 1) EPS, 2) EPS + live diatom cells and 3) EPS + killed bacteria and 3 control tanks without any added EPS were used for macroaggregate production. Changes in abundance and average size of macroaggregates were monitored using image analysis, whereas TEP and CSP were enumerated microscopically. In the presence of microbial EPS, macroaggregates of a size of 23 to 35 μm^2 were produced. Aggregate size and abundance varied considerably with both time and source of EPS. No correlation was observed for macroaggregate size and abundance with either TEP or CSP. One-way ANOVA demonstrated significant differences in the variance of particle abundance and size in tanks having only EPS or in combination with living diatom cells. Our data suggest that production of macroaggregates was influenced by polymer chemistry and surface properties of colliding particles whereas TEP and CSP concentrations were influenced by molecular weight of EPS and the presence of growing cells. Interestingly, macroaggregates were formed in the near absence of TEP and CSP, highlighting the role of other unknown processes in the transformation of DOM to particulate organic matter (POM) in aquatic environments.

1. Introduction

Macroaggregates range from 500 μm to more than 10 cm in length [1] and are composed of live cells, cell debris, fecal pellets etc., that are often entrapped in a matrix of exopolymeric substances (EPS) [2, 3, 4]. Macroaggregates occur in various forms (amorphous aggregates, stringers, flocs, mats etc.) [5, 6] and are highly porous, hydrated and fractal in nature [7, 8]. Macroaggregates form an important fraction of both suspended and sinking particulate organic matter (POM) [9, 3, 10] and play an important role in biogeochemical transformation processes and trophic dynamics in aquatic environments. For example, macroaggregates produced during phytoplankton blooms sink to deeper waters, thereby transporting organic matter from the euphotic zone to the bottom sediments [11, 12, 13]. Such formation and sedimentation of macroaggregates is also vital in phytoplankton life-cycles [14]. Moreover, the composition and quality of these aggregates influence feeding and grazing rates of filter feeders [15], bacterial re-mineralization of organic matter, and POM-DOM conversion [16, 17, 18], thereby regulating the vertical flux of organic carbon [4].

Macroaggregate production is the result of both abiotic and biotic processes. Abiotic formation of macroaggregates from high molecular weight DOM may occur by 1) self-coagulation [19, 20], 2) bubble adsorption [21, 22] and 3) adsorption of DOM by charged non-living particles [23, 24]. In contrast, biotic processes include direct cell-cell binding [25] or aggregation, which is often mediated by exopolymeric substances (EPS) [26, 27, 28, 29, 30]. EPS serves as biological glue that alters the surface characteristics or the sticking coefficient (α) of colliding particles [27, 31]. EPS may also serve as a precursor for the production of TEP, which play a decisive role in aggregation processes [28, 32, 33]. Similarly, these surface-reactive polymers might also contribute to another class of protein rich particles called Coomassie stained particles (CSP) [34], whose role in aggregation processes is still largely unknown [35].

The role of microbial EPS and TEP in abiotic and biotic production of aggregates has been addressed in both field and laboratory experiments [33 and references therein]. However, most of these studies have discussed aggregation of growing microbial cells [3, 27, 28, 30] and there is little information on the production of macroaggregates from dissolved EPS in the absence of growing cells [32]. Earlier studies highlight that i) production of EPS does not necessarily warrant the production of macroaggregates [27], ii) *de novo* production of particles like TEP is influenced by EPS chemistry [22], and iii) TEP is an integral part of the aggregation process [28, 29, 33]. Based on these observations, we hypothesize that apart from the collision of particles, EPS chemistry and the presence of particles such as TEP and CSP is critical for *in situ* production and size distribution of macroaggregates.

In the present study, we carried out rolling tank experiments to produce macroaggregates using EPS isolated from a marine bacterium and diatom. The goals of the study were to evaluate 1) the effect of source and chemistry of EPS on production of macroaggregates and 2) the role of organic particles, e.g. TEP and CSP, in regulating aggregate abundance and size.

2. Material and methods

2.1. Isolation of EPS

A pure culture of *Marinobacter* was incubated for 14 days at 30 °C in 2 L flasks containing 1 L of basal salt supplement (BSS) medium (pH 7.6). The growth medium contained 3.5% NaCl, 0.075% KCl, 0.7% MgSO₄, 0.1% NH₄Cl and 0.02% CaCl₂ supplemented with 3% glucose, < 8 kDa fraction of yeast extract (0.05% final concentration) and peptone (0.1% final concentration). An axenic culture of the marine diatom *Skeletonema costatum* Greve (CCMP 1332) was grown in 6.5 L of sterile Guillard's *f/2* medium distributed equally in 2 L Schott Duran bottles. The culture was grown under a 12 h dark-light cycle at 15°C for 27 days.

At the end of their respective incubation periods, bacteria and diatoms were harvested by centrifuging the cultures at 10,000 rpm and 3,000 rpm at 4°C, respectively. The supernatant was filtered through 0.22 µm Millipore filters, concentrated using a 3 kDa MW spiral membrane filter attached to a tangential flow filtration unit (Amicon, USA) and the retentate was further reduced in volume (< 50 ml) at 35°C using a rotary evaporator. EPS was precipitated overnight in absolute ethanol at 4°C. The precipitate was then re-dissolved in small quantities of deionised water and dialyzed under sterile conditions for 48 h through 8 kDa MW dialysis bags (Spectrochem, USA). During the dialysis, the water was replaced every 8 h. Once dialyzed, the EPS was lyophilized and stored at -20°C until further processing.

2.2. Chemical characterization

The lyophilized EPS was analyzed for organic carbon and nitrogen, carbohydrates (TCHO), proteins, uronic acids, and sulphates. The carbon and nitrogen content of the EPS were analyzed using a ThermoFinnigen CNH/S analyzer. TCHO of bacterial EPS was analyzed by the phenol-sulfuric acid estimation [36] whereas the monosaccharide (aldose) composition of both EPS was analyzed using an HPLC-PAD system equipped with a CarboPac PA 10 column (Dionex, USA), using 20 mM NaOH as an eluent [37]. Proteins were estimated by the bicinchoninic acid method [38]. The uronic acid content of the EPS was measured by a modified meta-hydroxy biphenyl method [39]. The sulfate content was estimated following the BaCl₂-gelatin method described by Dodgson and Price [40]. The carbohydrate, protein and uronic acid concentration of EPS derived from the two sources are expressed in terms of organic carbon.

2.3. Production of macroaggregates

In order to produce macroaggregates, two separate rolling tank experiments were carried out, one with bacteria-derived EPS (Bacteria-experiment), and one with diatom-derived EPS (Diatom-experiment). In each experiment, six sterile rolling tanks (1.5 L each), three

controls and three experimental tanks, were filled with sterile f/2 medium. Care was taken to ensure that no bubbles were retained in these tanks.

In the Bacteria experiment, one of the control tanks contained only sterile f/2 medium and served as negative control. In the second and third control tanks, killed *Marinobacter* cells (killed-bacterial control) and live diatom (*Skeletonema*) cells (diatom control) were added to the sterile medium, respectively. The three experimental tanks contained sterile medium in combination with a) only *Marinobacter* EPS, b) *Marinobacter* EPS + killed *Marinobacter* cells and c) *Marinobacter* EPS + live diatom cells (Fig. 1). In the Diatom experiment, the experimental conditions were the same as in the Bacteria experiment, except that EPS derived from *S. costatum* was used as DOM source. The rolling tanks were incubated in the dark at a speed of 2 rpm on a rolling table [41]. The final EPS concentration in all the experimental tanks was 300 $\mu\text{mol-C L}^{-1}$. Although it was necessary to keep the experimental conditions similar and yet as close to natural conditions as possible, it was not possible to simulate abiotic aggregation with live bacterial cells. The experiments were carried out in the dark, which restricted diatom growth and yet allowed the aggregation of live diatoms under abiotic conditions. However, killed bacterial cells were used chiefly to avoid artifacts from biotic aggregation [32] and breakdown of the bacterial EPS (DOM) by the live bacteria [42], thereby altering the abiotic aggregation process.

2.4. Image analysis

Prior to every sampling, rolling tanks were illuminated with side-arm focus lamps and the images of the samples were captured for 30 s using a Sony 900CCD handy cam (680K gross pixels). In order to analyze the images, a 250x250-pixel window (1 pixel= 60 μm) was chosen and the images were gray-scale transformed to highlight all aggregates within each window. The transformed images were then used to analyze the aggregate abundance and size using SIS image analyzing software (SIS analysis, Germany). The equivalent spherical diameter (ESD)

was calculated by using the image analyzing software, which was then used to estimate the equivalent spherical area (mm^2). All detected aggregates were grouped into different size-classes ranging from 0.2 mm^2 to 100 mm^2 . The aggregate abundance in each size class of the experimental and control tanks was summed up to yield the total aggregate abundance. The total aggregate abundance in the experimental tanks was then corrected from the aggregate abundance in the corresponding control tanks. The particle area was calculated as the mean area of all measured aggregates in all size-classes.

2.5. TEP and CSP sampling

A known volume (8 ml) of the sample was removed at regular intervals and replaced with an equal volume of sterile medium to maintain the volume of the medium and to avoid air bubbles inside the rolling tanks. Duplicate sub-samples (2 ml each) were filtered onto polycarbonate membrane filters ($0.2 \mu\text{m}$ pore size) [41] under vacuum ($< 10 \text{ mbar}$ pressure) to enumerate TEP and CSP. The TEP samples were stained with $0.22 \mu\text{m}$ pre-filtered 0.02% Alcian blue prepared in 0.06% glacial acetic acid (pH 2.5) [41]. Similarly, CSP samples were stained with 1 ml 0.04% Coomassie brilliant blue (G-250) according to Long and Azam [34]. The samples were then filtered dry, placed over a drop of oil on a frosted slide (Cytoclear TM, Poretics Corp., USA), and enumerated by using a Zeiss Axioplan microscope under bright field illumination and a magnification of 100x to 200x. The abundance of TEP and CSP in the experimental tanks was corrected from that in the control tanks.

2.6. Statistical analyses

A simple regression analysis was carried out to assess the relationships between the various parameters. One-way ANOVA for unequal data sets was used to assess the differences in the abundance of aggregates, TEP, and CSP and size of aggregates between the two experiments. All statistical analyses were carried out using Statistica 5.0 software.

3. Results

3.1. Chemical composition of EPS

The bulk chemical composition of EPS derived from *Marinobacter* sp and *Skeletonema costatum* is given in Table 1. Both bacterial and diatom EPS were more enriched in proteins than carbohydrates but also contained substantial proportions of uronic acids and sulfates. The diatom EPS had a relatively higher amount of sulfates and a higher C:N ratio as compared to the bacterial EPS (Table 1). The bacterial EPS had a lower amount of aldoses (TCHO) than the diatom EPS with glucose as the most abundant neutral monosaccharide, followed by galactose (Table 1). Mannose/xylose and fructose constituted < 1 nmol/mg EPS and pentoses (ribose and arabinose) and deoxy sugars (rhamnose and fucose) were not detectable. However in diatom EPS, other aldoses including rhamnose, mannose/xylose and fucose were also present in substantial fraction along with glucose and galactose (Table 1).

3.2. Macroaggregate abundance

All tanks showed small particles at the beginning of the experiment. These particles mainly originated from precipitated salts formed while sterilizing the medium. In the control tanks, these particles did not form larger aggregates (Table 2) and therefore aggregate abundance in experimental tanks were corrected for the particle abundance in the control tanks. Temporal variations of control-corrected aggregate abundance in both experiments did not follow any specific trend and varied with the treatments (Fig. 1a & b). In the Bacteria experiment, the aggregate abundance in the tanks containing only bacterial EPS and bacterial EPS + live diatom cells decreased in the first 20 to 42 h (Fig. 1a). Thereafter, the aggregate abundance in the tank containing only bacterial EPS increased to 444 aggregates per l⁻¹ at 99 h. The abundance of aggregates in the experimental tank declined subsequently and was lower than that in the negative control. On the other hand, aggregate abundance in tanks having bacterial EPS + live diatom cells increased steadily after 42 h to reach a maximum of 400

aggregates l^{-1} at 140 h. In contrast, no large-scale temporal variations in aggregate abundance occurred in tanks having bacterial EPS + killed bacterial cells (Fig. 1a) and the abundance generally varied from 0 to 56 aggregates l^{-1} (Table 2).

In the Diatom experiment, the abundance of aggregates in the tank containing only diatom EPS showed peaks after 20 h (506 aggregates l^{-1}) and 102 h (181 aggregates l^{-1}) (Fig. 2b). The aggregate abundance in tanks having diatom EPS + live diatom cells increased to 168 aggregates l^{-1} within 8 h of incubation and decreased thereafter to numbers lower than those of the diatom control at 64 h. Thereafter, there was a sharp increase in aggregate abundance after 90 h (344 ml^{-1}) and remained so until the end of the experiment. In contrast, aggregate abundance in tanks containing diatom EPS + killed bacterial cells showed small-scale variations and was mostly lower than that recorded in the killed bacterial control tank.

3.3. Macroaggregate size

In the Bacteria experiment, the aggregate size generally increased with time in tanks having bacterial EPS with or without killed bacteria. The macroaggregate size was generally larger (1.2 mm^2 at 0 h to a high of 22.7 mm^2 by the end of the experiment) in tanks containing only bacterial EPS (Fig. 2a). Similarly, the aggregate size in tanks containing bacterial EPS + killed bacterial cells increased from 0.7 mm^2 to 13.2 mm^2 after 164 h (Fig. 2a, Table 2). In contrast, aggregate size in tanks with live diatoms + bacterial EPS did not vary much and remained relatively small (0.05 to 1.7 mm^2) throughout the course of the experiment (Table 2).

In the Diatom experiment, tanks containing diatom EPS only and diatom EPS + live diatoms produced large macroaggregates after 102 h (34.5 mm^2) and 90 h (26.7 mm^2), respectively. In both approaches, the mean aggregate size was ~ 20 mm^2 at the end of the experiment (Fig. 2b). No such increase in aggregate size was observed in tanks with diatom EPS + killed bacteria.

In contrast, the aggregate size in the control tanks of both experiments showed only little variation during the course of the experiment. Moreover, the aggregates formed were generally smaller than those in the experimental tanks (Table 2).

3.4. TEP and CSP abundance

Dynamics in control-corrected TEP and CSP abundance differed greatly in both experiments. In the Bacteria experiment, abundance of TEP and CSP declined with time in all treatments. In tanks containing only bacterial EPS, TEP abundance increased sharply from 0 to 4.6×10^3 TEP ml^{-1} within 20 h followed by a decrease to 0.3×10^3 ml^{-1} after 42 h. Thereafter, TEP abundance showed small-scale variations and decreased slightly towards the end of the experiment (Fig. 3a). TEP abundance in tanks containing bacterial EPS + killed bacteria increased from 0 to 0.7×10^3 ml^{-1} within 42 h and thereafter decreased constantly until the end of the experiment. In tank having bacterial EPS + live diatom cells, TEP abundance showed small-scale variations for the first 70 h, followed by a continuous increase to 0.6×10^3 ml^{-1} (Fig. 3a) (Table 2) at 140 h. In comparison to TEP, variations in the abundance of CSP in the Bacteria experiment were more consistent and generally decreased with time. CSP concentrations often exceeded those of TEP in most of the tanks (Fig. 4a). The CSP concentrations in tanks containing bacterial EPS, bacterial EPS + killed bacterial cells and bacterial EPS + live diatom cells ranged from 0 to 2.6×10^3 ml^{-1} , 0 to 1.9×10^3 ml^{-1} and 0 to 0.9×10^3 ml^{-1} , respectively (Table 2).

In the Diatom experiment, TEP abundance after blank correction was nearly zero in all tanks during most of the sampling period. TEP in tanks with only diatom EPS were noticeable only at 28 h and at the end of the experiment (Fig. 3b) and TEP abundance varied from 0 to 5.1×10^3 ml^{-1} (Table 2). In contrast, abundance of CSP in the same tank was generally greater than that of TEP and peaked after 20 h (3.2×10^3 CSP ml^{-1}) and at the end of the incubation (13.9×10^3 CSP ml^{-1} , Fig. 4b). On the other hand, TEP abundance in tanks containing diatom EPS and killed bacterial cells or live diatoms, and CSP abundance in diatom EPS + killed bacterial cells

showed very little variation over time (Fig. 4b; Table 2). In contrast, CSP abundance in tanks containing diatom EPS + live diatoms peaked after 8h and 64 h.

3.5. Statistical analyses

With the exception of tanks containing EPS with live diatoms, no significant correlation and specific trend could be established between TEP and macroaggregate abundance and size (Table 3). Macroaggregate abundance was significantly correlated with macroaggregate size only in tanks having diatom EPS + live diatoms. The CSP abundance had no correlation with either macroaggregate size or abundance in any of the experiments.

Significant differences, however, were observed between the abundance of macroaggregates produced from different EPS sources ($F=15.9$; $p<0.001$) and for tanks containing EPS + live diatoms ($F=12.3$; $p<0.01$). On the other hand, the size of macroaggregates produced by EPS derived from different sources varied from moderate ($p<0.05$) to significant ($p<0.01$) in all three experiments. The abundance of TEP and CSP varied significantly with EPS source and the presence of killed bacterial cells.

4. Discussion

EPS derived from various sources behave differently due to the dissimilarity in their chemistry [43, 44]. Since the presence of EPS alters the particle stickiness, thereby influencing the production of macroaggregates [1, 27], it is apparent that the chemistry of EPS might play a decisive role in regulating aggregate production. In the present study, EPS derived from bacterial and diatom cells showed considerable differences in their chemistry. Both bacterial and diatom EPS had a lower amount of TCHO than proteins (Table 1). Such high concentrations of proteins in EPS may play an important role in flocculation process [45]. Moreover, the presence of xylose + mannose, rhamnose along with higher concentrations of sulfate in the diatom EPS may substantially impart the gelling properties of EPS [22, 44]. On

the other hand, the higher concentrations of uronic acids in bacterial EPS impart an overall negative charge to the EPS, thereby enhancing their binding properties [46, 47]. Mechanisms of aggregation are also influenced by the presence of ionogenic entities like uronic acid and sulfate which allow EPS to behave like a polyelectrolyte [48]. Thus, it is apparent that the differences in the EPS derived from these two sources may explain the differences in macroaggregate formation.

Temporal variations in aggregate abundance indicate both aggregation and disaggregation processes occurred. For example, an increase in aggregate size in tanks having only EPS (of bacterial or diatom origin) from 20 to 46 h (Figs. 2a & b) with a concomitant decrease in particle abundance (Figs. 1a & b) suggests that smaller aggregates were coagulating to form fewer and larger aggregates. At some instances, however, aggregate counts increased with negligible changes in the mean size of aggregates, suggesting production of newer particles either *de novo* or by disaggregation of larger particles. The temporal variation in abundance of aggregates produced by the bacterial EPS was less than that of the diatom EPS (Fig. 1a & b), suggesting that aggregates derived from EPS of the former were more stable than those derived from EPS of the latter. The stability of aggregates is influenced by the gelling capacity of the EPS-bonding between the functional groups of the various constituents, the properties of the organic or inorganic surfaces and the structural conformation of the EPS. Hence, observed differences in the aggregate abundance and size can be attributed to variations in EPS chemistry and tertiary structure.

The variations in aggregate abundance were in contrast to the continuous increase in abundance of visible aggregates observed in earlier studies. For example, Elzone particles in seawater cultures increased from $3 \times 10^3 \text{ ml}^{-1}$ to $\sim 12 \times 10^3 \text{ ml}^{-1}$ over the incubation period [32]. Similarly, visible flocs formed during aggregation of *Chaetoceros* cells also increased with incubation time [28]. However, these studies were carried out using either growing cultures of

microorganisms in the laboratory or during phytoplankton blooms in the natural environments. In our study, except for tanks containing live diatoms whose growth was restricted to reduce artifacts, living organisms were excluded by working under sterile conditions. Therefore, increase in particle abundance was not due to changes in biomass but solely due to physical and chemical aggregation processes.

Control tank-corrected aggregate abundance and size in tanks containing only EPS (bacterial or diatom) changed dramatically over time (Figs. 2a & 2b). The control tanks contained particles $< 1 \text{ mm}^2$ in size at the start of the experiments, which did not change much over time. On the other hand, the addition of EPS to the experimental tanks increased the amount of particles at zero time in both experiments (Figs. 1a & b), which might be due to precipitation of EPS in the presence of salts in the medium [49]. Interestingly, a similar increase in the macroaggregate abundance at 0 h was not observed when EPS was mixed with killed bacteria (Figs. 2a) and live diatoms (Fig. 2b). Both, killed bacterial cells and live diatom cells were added as nuclei to accelerate aggregate formation. These cells serve as surfaces that might have adsorbed the EPS upon addition, thereby reducing its co-precipitation along with the salts. Although both bacterial and diatom EPS contained moieties that impart binding and gelling properties in varying amounts, their aggregation pattern was also different. For example, diatom EPS took a longer time (46 h) to form larger sized aggregates whereas bacterial EPS gave rise to relatively smaller aggregates at a faster rate (20 h). Moreover, one-way ANOVA showed statistically significant differences in aggregate production and size between tanks having only EPS ($F= 15.9; p<0.001, F= 9.2; p<0.01$) and EPS + live diatoms ($F= 12.3; p<0.01, F= 9.1; p<0.01$). Thus, the source and chemistry of EPS appears to have influenced the aggregation pattern under experimental conditions.

Another important factor in aggregation process is the surface property of the colliding particles [1]. Since the rate of macroaggregate production is related directly to the stickiness of

the colliding particles [50] and its collision rate (which is a function of shear and particle density) [51, 52], certain changes in the size of macroaggregate could be expected following coagulation theory. Bacterial EPS led to production of larger aggregates only with killed bacteria whereas diatom EPS produced larger aggregates only with live diatoms as nuclei. Such cell specific aggregation behavior might arise from interactions between the cell wall and the ionic components of EPS. The gram-negative bacterial cells have a surface membrane over the cell wall, which is rich in proteins, lipopolysaccharides, phospholipids, and divalent cations [53]. On the other hand, diatoms have a cell wall rich in proteins, carbohydrate components, and uronic acid apart from siliceous matter [28]. The distribution of these compounds on the cell wall and their ability to bind with EPS or other particles may have influenced the aggregation process.

Organic particles such as TEP are considered important for aggregate production [27, 32, 1]. In our experiments, the abundance of TEP was low compared to earlier reports in which TEP was produced from DOC [32, 22, 54]. TEP were virtually absent in the Diatom experiment (Table 2) whereas in the Bacteria experiment their abundance decreased over time, suggesting that TEP were either effectively scavenged by colliding particles or increased in size without *de novo* synthesis of new TEP.

The low amounts of TEP in the present study- especially in the Diatom experiment, were intriguing (Table 2) because both bacterial and diatom EPS have fair amounts of sulfates and uronic acid, which are potential precursors for the production of TEP [22, 33]. Moreover, the diatom EPS was rich in monosaccharides like rhamnose and fucose that have been reported to be common in TEP [33]. One reason for the observed low numbers of TEP could be the high molecular weight of the EPS used. Leppard et al [55] reported that precursors of organic colloids are a few nanometers in size, which can be lost during dialysis [33]. In the present study, the EPS used as a precursor for TEP production were dialyzed through 8 kDa MW cut-

off dialysis bags prior to aggregation experiments. Therefore, it is possible that a large fraction of TEP precursors was lost during dialysis of diatom EPS. On the other hand, bacterial EPS are rich in high molecular weight colloids that get retained in the dialysis bags, therefore capable of producing TEP. The low numbers of TEP in tanks having EPS with killed bacteria and live diatoms as compared to those with only EPS might be due to adsorption of organic matter on the cell surface. Due to their surface properties (see above), both dead and live cells can scavenge TEP from the solution [19], thereby reducing the abundance of TEP in the medium.

The abundance of TEP, CSP and aggregates was relatively high in tanks with EPS only but neither TEP nor CSP showed any correlation with aggregate abundance and size (Table 3). Interestingly, in the absence of live diatom cells, TEP did not show any significant correlation with any other parameter suggesting that the aggregation of live diatoms was controlled by the production of new TEP. Similar observations have been reported during phytoplankton blooms in natural waters [56] and mesocosm experiments [57]. Despite its greater abundance than TEP in almost all experiments, CSP abundance had no correlation with aggregate abundance and size in any of the experiments. Field observations of aggregation during phytoplankton blooms also suggest that CSP have negligible role in macroaggregate production [58]. In contrast, variations in chemical properties of EPS as well as changes in surface properties of source particles appear to be critical for aggregate formation and may explain some of the variability in aggregation patterns observed in the field.

Conclusions

The rolling tank experiments showed that EPS of bacterial and diatom origin lead to production of aggregates under exclusively abiotic conditions. The rate of aggregation, size, and stability of aggregates varied with the source of EPS and were influenced by differences in polymer chemistry. The addition of particles such as killed bacterial cells or live diatoms did not increase aggregate abundance and/or size, highlighting the importance of appropriate

binding between the EPS and surface of colliding particles. TEP seem to play only a significant role in aggregation processes in the presence of live diatoms indicating that specific surface properties of diatoms and *in situ* production of TEP by live diatoms facilitate the association of TEP and diatom cells, influencing aggregate production. In the absence of live diatoms, organic particles such as TEP and CSP seem to play an insignificant role in aggregate production. This observation implies the importance of other so far unexplained aggregation mechanisms in aquatic environments. These abiotic processes may also increase aggregation in the absence of living primary producers and hence alter DOM-POM transformation processes and the vertical flux of POM.

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Legends to Figures:

Fig. 1: Variations in macroaggregate abundance in bacterial experiment (a) and diatom experiment (b). Bacterial experiment used bacterial EPS and diatom experiment used diatom EPS as DOM.

Fig. 2: Variations in macroaggregate size in bacterial experiment (a) and diatom experiment (b). Details of experimental conditions are as described in Fig. 1.

Fig. 3: Variations in Transparent exopolymer particles (TEP) abundance in bacterial experiment (a) and diatom experiment -II (b). Details of the experiment are as in Fig. 1.

Fig. 4: Variations in Coomassie stained particles (CSP) abundance in bacterial experiment (a) and diatom experiment (b). Details of the experimental conditions are as in Fig. 1.

Table 1: Chemical characteristics of EPS derived from *Marinobacter* sp. and *S. costatum*.

Parameters	<i>Marinobacter</i> sp.	<i>S. costatum</i>
TCHO*	168.9	28.3
Proteins*	438.9	35.8
Uronic acid*	175.8	46.2
Sulfates*	26.9	215.4
C:N (w:w)	2.8	8.1
Glucose [@]	24.8	104.5
Galactose [@]	5.0	32.3
Xylose/Mannose [@]	0.9	32.9
Fucose [@]	----	5.3
Ribose [@]	----	----
Rhamnose [@]	----	12.1
Arabinose [@]	----	----
Fructose [@]	0.4	8.9

* : $\mu\text{g}/\text{mg}$ EPS; @ : nmol/mg EPS

Table 2: Range, mean, standard deviation (SD) and median of macroaggregate size, abundance, TEP and CSP produced during the aggregation experiments using EPS derived from a *Marinobacter* sp. (Bacteria experiment) and *S. costatum* (Diatom experiment).

		Bacteria experiment						Diatom experiment					
Parameters		Control	EPS	KB-Control	EPS + KB	Diatom Control	EPS + Diatom	Control	EPS	KB-Control	EPS + KB	Diatom Control	EPS + Diatom
Abundance (l ⁻¹)	Range	181-1668	0-444	37-162	0-56	62-362	0-400	131-381	0-506	75-312	0-38	131-937	0-344
	Mean	711	173.6	95	12.5	318	128.5	234	163	139	3.75	422	95
	SD	527	188	44	24.8	116	140.5	83	160	101	11	219.8	128
	Median	519	144	106	0	65.6	193	212	153	150	0	381	6.3
Size (mm ²)	Range	0.5-9.5	1.2-22.7	0.6-2.8	0.7-13.2	0.09-5.9	0.05-1.7	0.14-1.3	0.6-34.5	0.07-1.03	0.3-1.2	0.08-1.27	0.8-26.7
	Mean	2.17	10.67	1.67	3.8	0.97	0.7	0.45	13.7	0.56	0.68	0.79	7.2
	SD	2.8	6.69	0.8	4.2	1.9	0.6	0.39	15.4	0.3	0.39	0.43	9.9
	Median	1.2	9.4	1.3	1.5	0.34	0.38	0.35	1.6	0.63	0.57	0.88	0.97
TEP abundance (x 10 ³ l ⁻¹)	Range	0.14-4.6	0-4.6	0.14-1.8	0-0.7	0.4-0.8	0-0.6	0-1.8	0-5.1	<0.5	< 0.5	0-5.6	0-0.3
	Mean	1.48	1.6	0.55	0.78	0.33	0.52	0.23	0.6	---	---	1.21	0.03
	SD	1.48	1.6	0.58	0.52	0.22	0.18	0.26	1.6	---	---	2.15	0.09
	Median	1.09	1.04	0.27	0.74	0.27	0.5	0	0	---	---	0.13	0
CSP abundance (x 10 ³ l ⁻¹)	Range	0.3-2.5	0-2.6	0.14-1.08	0-1.9	0.4-1.12	0-0.9	0-3.6	0-13.9	<0.5	< 0.5	1.2-9.5	0-8.7
	Mean	1.15	1.6	0.63	1.21	0.8	0.69	1.55	2.6	---	---	4.1	0.97
	SD	0.77	1	0.34	0.94	0.23	0.53	1.29	4.42	---	---	2.4	2.74
	Median	1	1.3	0.67	1.14	0.86	0.64	1.5	1.6	---	---	3.4	0

Table 3: Correlation coefficient (r-values) matrix of particle abundance, size, TEP and CSP abundance produced using EPS in the experimental tanks using EPS derived from a *Marinobacter* sp. (Bacteria experiment) and *S. costatum* (Diatom experiment)..

Parameters		Bacteria Experiment			Diatom Experiment		
Tanks		Size	TEP	CSP	Size	TEP	CSP
EPS	Abundance	-0.32	0.17	0.61	-0.36	-0.36	-0.32
	Size		-0.01	-0.21		0.22	0.59
EPS + Killed Bacteria	Abundance	-0.05	-0.05	-0.17	---	--	--
	Size		-0.57	-0.45		--	--
EPS + live diatoms	Abundance	-0.47	0.71*	-0.11	0.91***	0.78**	0.45
	Size		-0.16	-0.46		0.89***	0.15

*: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$.

--: Since the macroaggregate abundance, TEP and CSP counts were negligible; correlations were not carried out in this experiment.

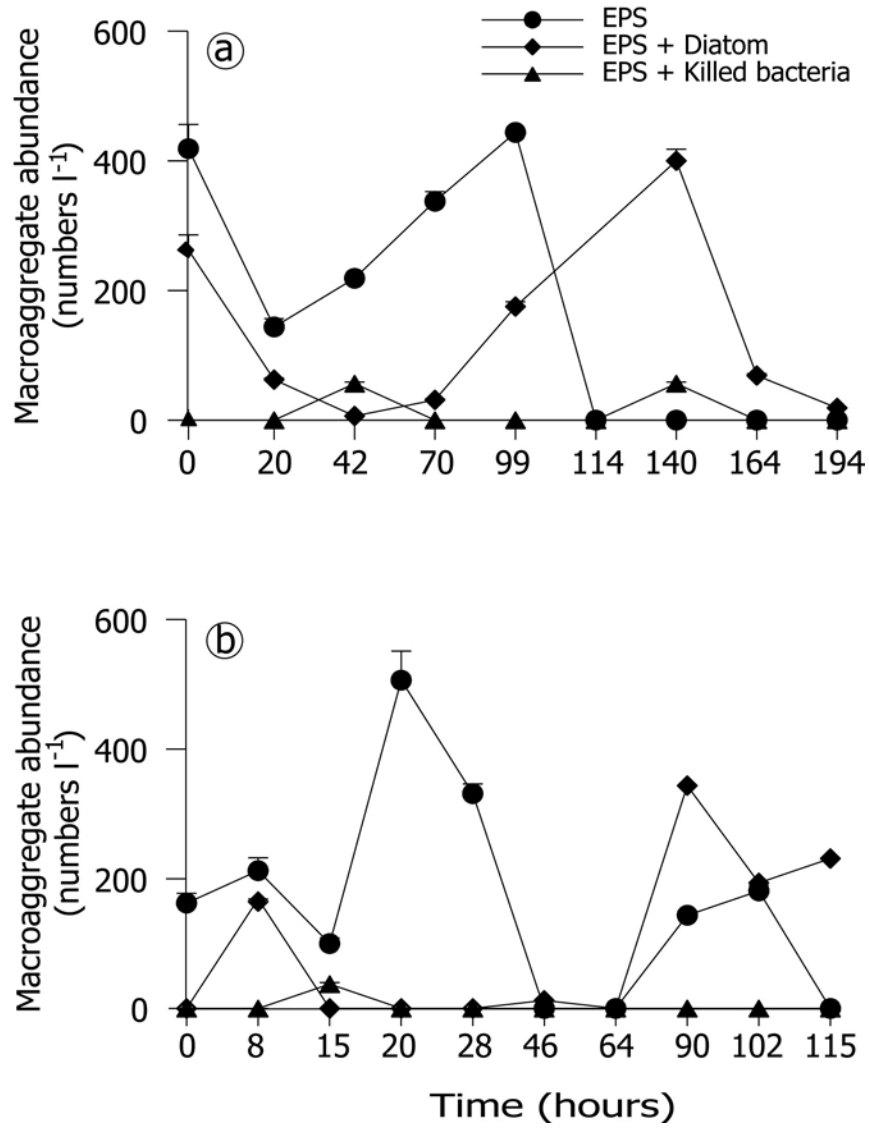


Fig.1 Variations in macroaggregate abundance in bacterial experiment (a) and diatom experiment (b). Bacterial experiment used bacterial EPS and diatom experiment used diatom EPS as DOM.

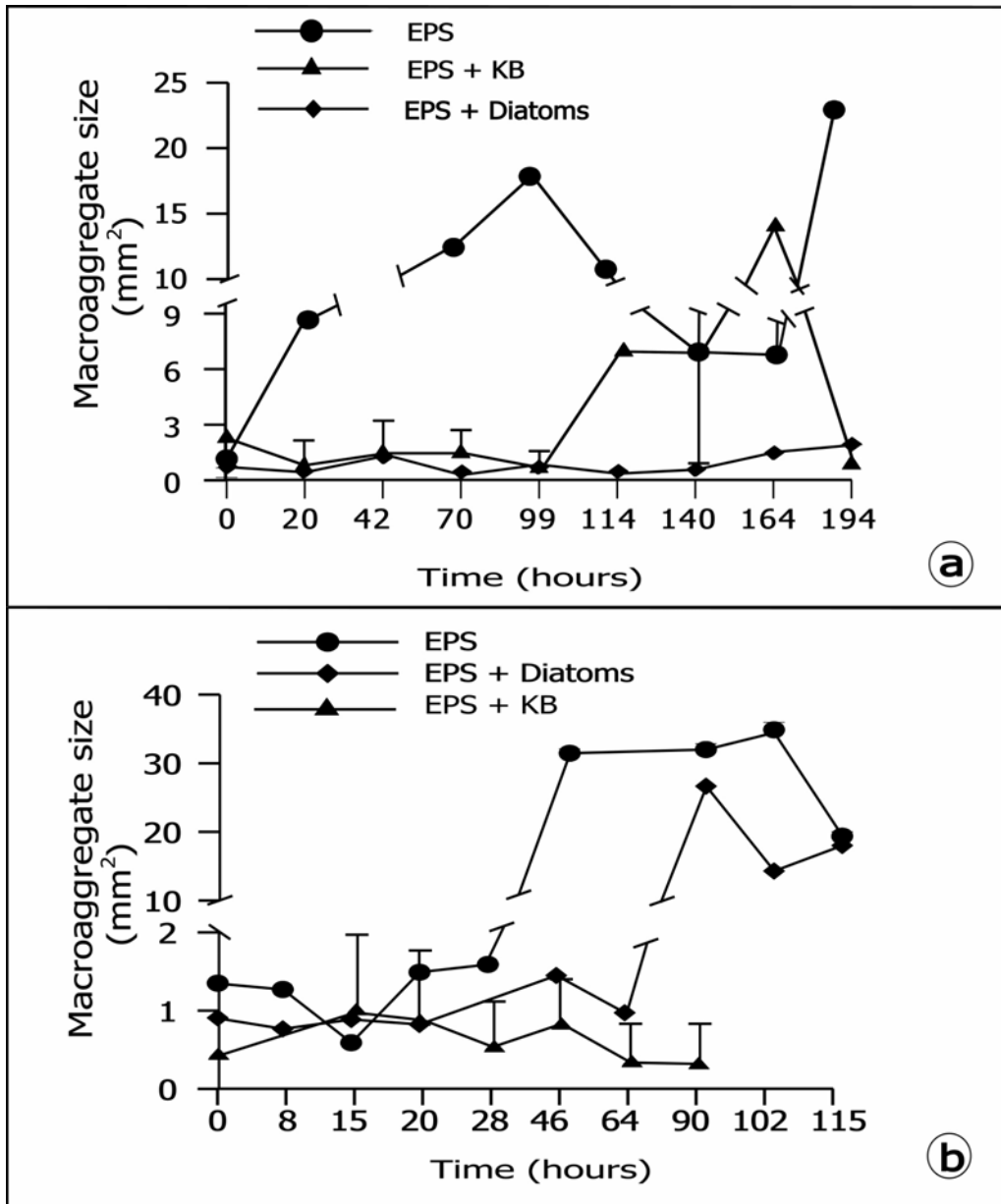


Fig.2 Variations in macroaggregate size in bacterial experiment (a) and diatom experiment (b). Bacterial experiment used bacterial EPS and diatom experiment used diatom EPS as DOM.

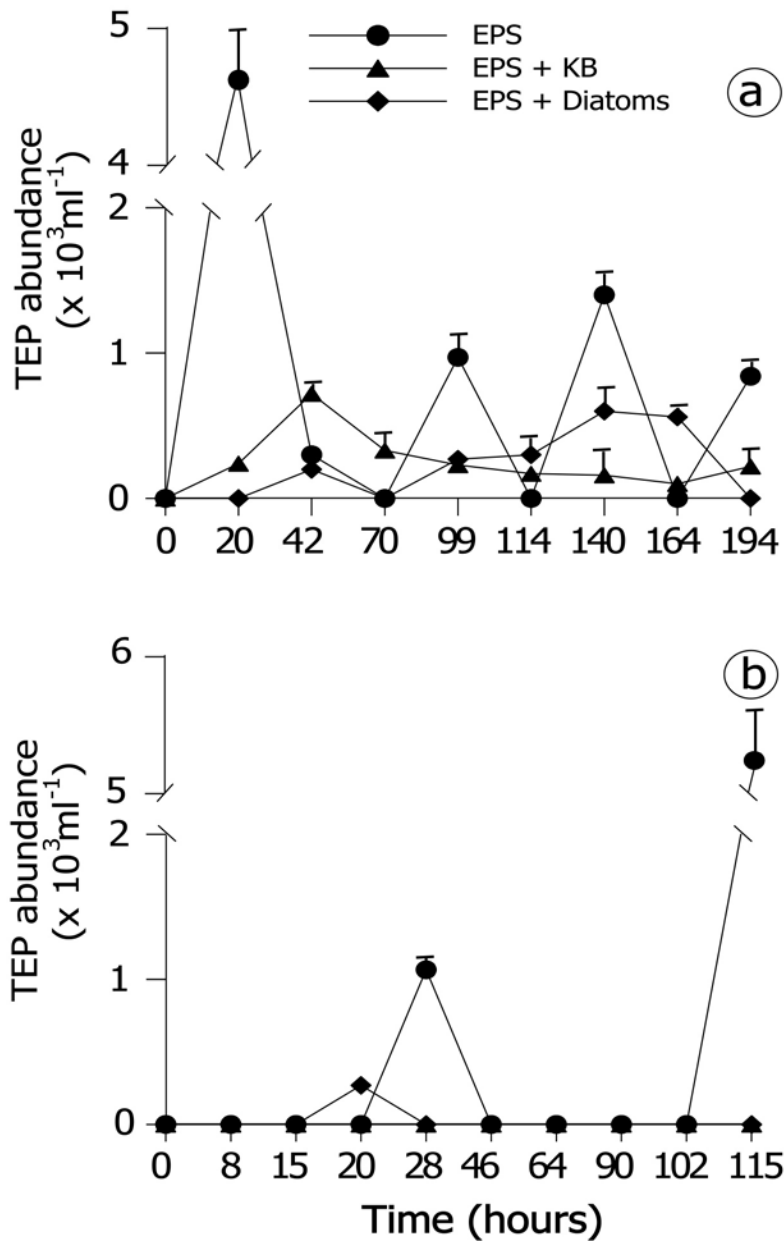


Fig.3 Variations in transparent exopolymeric particle (TEP) abundance in bacterial experiment (a) and diatom experiment (b). Bacterial experiment used bacterial EPS and diatom experiment used diatom EPS as DOM.

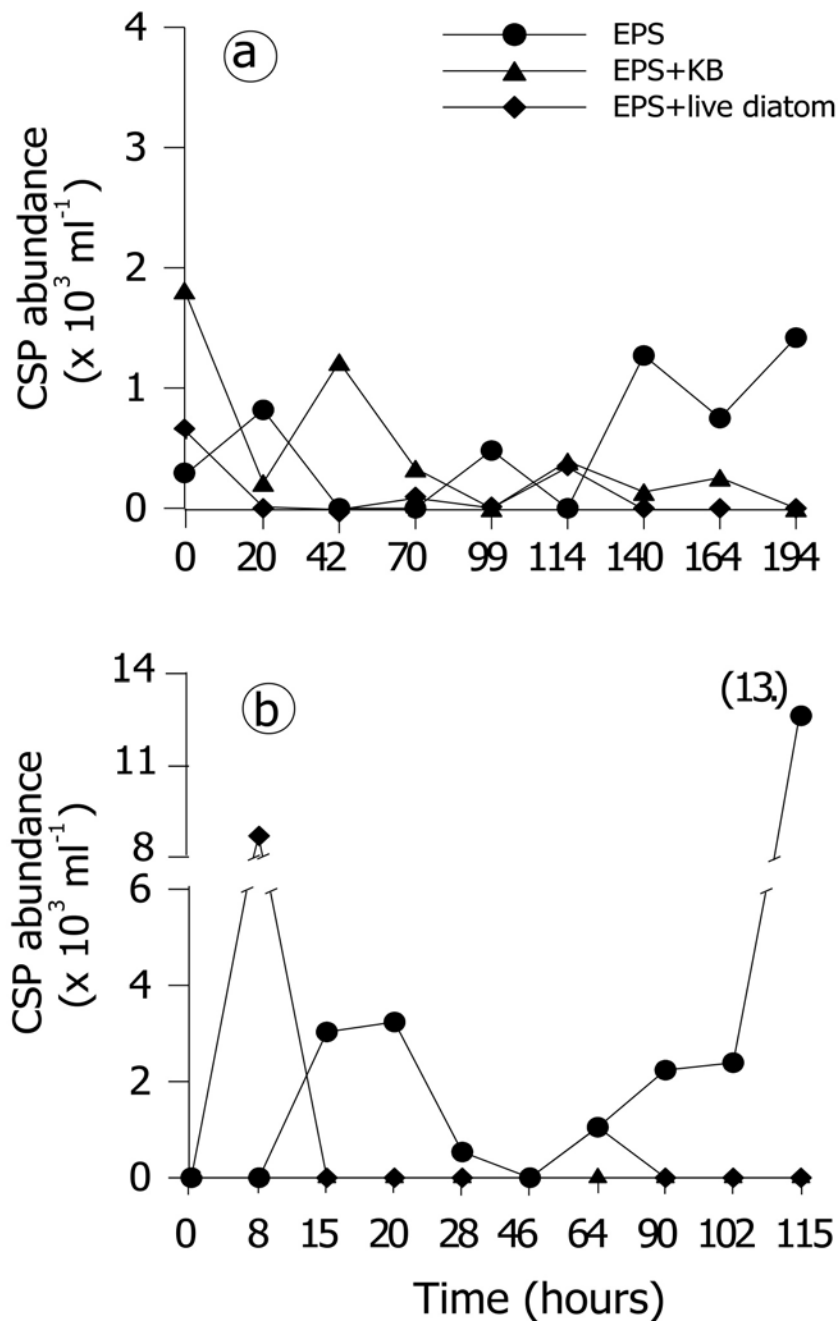


Fig.4 Variations in Coomassie stained particle (CSP) abundance in bacterial experiment (a) and diatom experiment (b). Bacterial experiment used bacterial EPS and diatom experiment used diatom EPS as DOM.