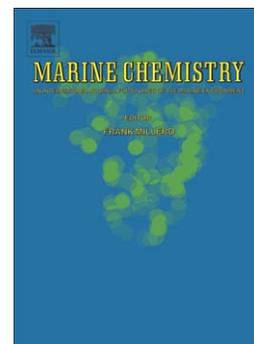# Accepted Manuscript

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Compositional Similarities and Differences between Transparent Exopolymer Particles (TEP) from two Marine Bacteria and two Marine Algae: Significance to Surface Biofouling

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Abstract

Transparent-exopolymer-particles (TEP) have been recently identified as a significant contributor to surface biofouling, such as on reverse osmosis (RO) membranes. TEP research has mainly focused on algal TEP/TEP precursors while limited investigations have been conducted on those released by bacteria. In this study, TEP/TEP precursors derived from both algae and bacteria were isolated and then characterized to investigate their similarities and/or differences using various advanced analytical techniques, thus providing a better understanding of their potential effect on biofouling. Bacterial TEP/TEP precursors were isolated from two species of marine bacteria (*Pseudidiomarina homiensis* and *Pseudoalteromonas atlantica*) while algal TEP/TEP precursors were isolated from two marine algae species (*Alexandrium tamarense* and *Chaetoceros affinis*).

Results indicated that both isolated bacterial and algal TEP/TEP precursors were associated with protein-like materials, and most TEP precursors were high-molecular-weight biopolymers. Furthermore all investigated algal and bacterial TEP/TEP precursors showed a lectin-like property, which can enable them to act as a chemical conditioning layer and to agglutinate bacteria. This property may enhance surface biofouling. However, both proton nuclear magnetic resonance (NMR) spectra and the nitrogen/carbon (N/C) ratios suggested that the algal TEP/TEP

precursors contained much less protein content than the bacterial TEP/TEP precursors. This difference may influence their initial deposition and further development of surface biofouling.

Keywords: transparent exopolymer particles, reverse osmosis, biofouling, marine, bacteria, algae

Introduction

Reverse osmosis (RO) membrane filtration has been widely applied in seawater desalination due to its low energy cost compared with thermal distillation. However, biofouling of membrane surfaces is still a challenge for seawater desalination application (Abd El Aleem et al. 1998). The biofouling on membranes is caused by substantial accumulation of biofilm formation. This biofilm can enhance the hydraulic resistance of filtration. In order to control the surface biofouling of membranes, a better understanding of the biofoulants is necessary.

The transparent exopolymer particles (TEP) have been reported to be one of the main components of membrane biofilms (Bar-Zeev et al. 2009, Berman 2010, Berman and Holenberg 2005). TEP are ubiquitous in all aquatic systems, and characterized as sticky substances mainly composed of acidic polysaccharides and stainable with alcian blue (Passow 2002b). TEP are operationally defined as larger than 0.4 μm, while the other substances chemically identical to TEP, but smaller than 0.4 μm, can be considered as TEP precursors. It has been reported that mucilage events in the Mediterranean Sea are related to the presence of TEP (Giani et al. 2012). If the seawater containing TEP is used as feed water for desalination plants, TEP can form a sticky biofilm on the membrane surfaces and thus enhance RO membrane fouling. In the Arabian Gulf, some desalination plants have been forced to reduce or shutdown operation during severe algal blooms (commonly called " red-tide") possibly due to issues associated with the production of high concentrations of TEP (Richlen et al. 2010, Villacorte et al. 2014).

TEP/TEP precursors and protobiofilms (suspended TEP with extensive microbial outgrowth and colonization) may have a major role in the initiation of aquatic biofilms (Bar-Zeev et al. 2012). These substances can form a chemical conditioning layer when they deposit on the membrane surface. This chemical conditioning layer can be thickened by the accumulation of deposited TEP/TEP precursors. A number of planktonic bacteria (first colonizers) can attach irreversibly on these TEP-conditioned surfaces and may agglutinate and grow on this conditioning layer. Consequently, a biofilm can be formed and enhanced with the growth of bacteria on the conditioning layer. When nutrients are not limited in the water, a continuous coverage of mature biofilm can develop within a short period of time (minutes to hours), eventually leading to biofouling.

Spontaneous self-assembly of dissolved precursors is a major process of TEP formation (Chin et al. 1998, Passow 2000). Algae have been generally considered to be a major source of TEP/TEP precursors in marine ecosystems (Passow 2002a, Wetz and Wheeler 2007), either releasing dissolved TEP precursors during exponential growth (Alldredge et al. 1993, Passow 2002a) or excreting TEP directly via sloughing and lysis of senescent cells (Beauvais et al. 2003). It has also been reported that algae-derived TEP could cause fouling of ultrafiltration (UF) and RO membranes (Villacorte et al. 2009). However, if effective pre-treatment processes (e.g., UF) are installed in front of the RO system, biofilms would be expected to be comprised mainly of TEP from bacteria growing in the system rather than TEP originally from seawater. Currently, most of the TEP related research regarding RO membrane biofouling is focused on algae derived TEP/TEP precursors. Although some studies showed the production of TEP by bacteria (Ortega-Retuerta et al. 2009, Passow 2002a), there is limited research regarding the composition of TEP/TEP precursors released from bacteria in either seawater or freshwater. The composition of bacterial TEP/TEP

precursors may also play a significant role in biofilm development. Furthermore, the compositional similarities and differences between algal and bacterial TEP/TEP precursors have thus far not been addressed, which may influence the fouling development on surfaces (such as membranes).

UF and RO membrane fouling caused by different organics have been reported (Li 2011, Rückel et al. 2011). Li showed that humics, sodium alginate and protein could cause UF membrane fouling; but the sodium alginate (model compound of polysaccharides) fouling layer was highly reversible by back flushing, while the bovine serum albumin (BSA, model compound of protein) fouling layer was much more irreversible and sticky on the membrane surface (Li 2011). The stickier fouling layer may promote the membrane biofouling afterwards. There is no research available regarding the impact of the composition difference between algal and bacterial TEPs on membrane fouling. The compositional difference between algal and bacterial TEP might influence the development of biofouling. Rückel and his colleagues investigated the formation of different fouling layers on polyamide thin film of common RO membranes for desalination (Rückel et al. 2011). It was shown that the adsorption of BSA on polyamide films is more than humics and sodium alginate, and the adsorption layer is much more difficult to rinse off as well. TEP/TEP precursors released from algae and bacteria in the ocean can be very different in composition, leading to different impacts on membrane fouling.

Depending on the compositional differences, one may identify which type of TEP/TEP precursor is the main foulant on the membrane of interest, and may be able to find a more appropriate pretreatment to reduce the membrane fouling problem or a better cleaning methodology to remove the biofilm. Therefore, this study was conducted to obtain a better understanding of similarities and differences of bacterial and algal TEP/TEP precursors, using advanced analytical tools, such as liquid chromatography-organic carbon detection (LC-OCD) analyses, fluorescence excitation-emission matrices (FEEM) and proton nuclear magnetic resonance (NMR) spectroscopy.

Materials and methods

Chemical reagents

Difco Marine agar 2216 (55.1 g/L) and broth 2216 (37.4 g/L), (Becton, Dickinson and Company) were used for the marine bacteria cultivation. A 0.1% (m/v) sodium azide (Sigma-Aldrich) solution was applied to limit the bioactivity of the isolated extracellular material during the desalting process via dialysis. To quantify the produced TEP/TEP precursors with a dye binding spectrophotometric assay, a 0.06% (m/v) alcian blue 8 GX (Sigma-Aldrich) staining solution was prepared by dissolving alcian blue in a 0.2 mol/L acetate buffer (pH4). The acetate buffer was prepared by mixing acetic acid and sodium acetate solution (Sinopharm Chemical Reagent Co. Ltd).

Production of bacterial extracellular material

Raw seawater from the Red Sea was used for the marine bacteria isolation. Seawater was spread on a marine agar plate for one-day cultivation at 30℃. Afterwards, two single colonies with different appearances on this marine plate

(two types of bacteria) were separately selected and transferred to two sterilized marine agar plates for further cultivation/purification. The shape of colonies on each agar plate was checked again for uniformity after cultivation. The purified marine bacteria with uniform colonies were then sampled for identification via Sanger sequencing.

In terms of extracellular material production (including TEP/TEP precursors), the two identified marine bacteria were spiked into two separate autoclaved flasks containing 50 mL marine broth solution, respectively. After 4 days incubation at room temperature, 1 mL solution was transferred via a sterilized pipet to an autoclaved flask containing 500 mL marine broth solution. The solution in the one-liter flask was incubated for 7 days on a shaker rotating at 160 rpm, at room temperature.

Centrifugation is a common method to obtain a good bacteria and medium separation in biotechnology. The broth solution was centrifuged at 9000 rpm for 5 minutes to separate bacteria and media after incubation, and the settled bacteria were discarded. The supernatant was recovered and checked active bacterium-free by observing agar plates with supernatant incubated for 2 days.

Afterwards, sodium azide was added in the supernatant to reach a concentration of 0.1% (w/v). The solution was transferred to a 3500 Da MWCO membrane bag (Spectrum Laboratories, Inc.) for a 4-day dialysis against Milli-Q water. The Milli-Q water for dialysis was renewed daily to maintain the driving force. After dialysis, the isolated bacterial extracellular material was lyophilized for storage.

Production of Algal extracellular material

Algal extracellular material was obtained from two marine algae species, namely: *Alexandrium tamaren*se (CCAP 1119/32) and *Chaetoceros affinis* (CCAP 1010/27). The two marine algal species were selected to represent the two common types of bloom-forming algae in the ocean (i.e., dinoflagellates and diatoms). *Alexandrium tamarense* is a marine dinoflagellate identified as one of the major causative species of "red tide" or harmful algal blooms (Anderson et al. 2012). *Chaetoceros affinis* is a marine diatom known to be one of the major producers of extracellular polysaccharides including TEP/TEP precursors in the ocean and has been reported in various occasions to cause marine mucilage due to massive production of TEP (Giani et al. 2012, Myklestad 1995, Passow 2002a). There are more bloom forming species of algae (100-300 species) in the ocean but we selected this two species because they have been studied extensively in the field of oceanography and algal research. The isolation protocol of algal extracellular material from the two algal cultures was described by (Villacorte et al. 2013). In brief, algae were inoculated in sterilized synthetic seawater containing nutrients and trace elements. The synthetic seawater (SSW) was prepared according to the typical ion composition in seawater (TDS 34 g/L and pH 8 ± 0.2). Both algal cultures were incubated at 20°C under an artificial light source (fluorescent lamp) with 12/12 h light/dark regime, and continuous slow mixing on a shaker. After incubation, the algae cells were removed by 5μm filtration with low vacuum (-0.2 bar) to separate algal organic matter from algae cells with minimum breakage of cells, and then the

extracellular material in supernatant was dialyzed in membrane bags of 3500 Da MWCO to remove salts. The dialyzed material was lyophilized for storage.

Sanger sequencing

DNA of the cultured bacteria was extracted with a "DNeasy Blood & Tissue Kit " (QIAGEN Company), and then the polymerase chain reaction (PCR) for extracted DNA was conducted with 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') primers. Afterwards, the produced PCR product of each sample was purified with a "PCR Purification KIT" (QIAGEN Company) for Sanger sequencing. The sequences of cultured bacteria were registered in Genbank, National Center for Biotechnology Information (NCBI), United States, with accession numbers of KF019205 and KF019206 for SW1 and SW2 marine bacteria, respectively.

Alcian blue assay

A modified alcian blue assay was used for TEP measurement (Arruda Fatibello et al. 2004). A volume of 5 mL of isolated extracellular material after dialysis was added to a 10 mL volumetric flask. Afterwards, 0.5 mL of 0.06% (m/v) alcian blue solution was added and the volume was made up to 10 mL with Milli-Q water. The volumetric flask was stirred for 1 min. The resulting suspension was then centrifuged at 3000 rpm (2160×g) for 30 min and the absorbance of the supernatant (representing alcian blue solution in excess) was measured in a UV spectrometer (UV-2550, SHIMADZU) at a wavelength of 610 nm. There is a direct relationship between alcian blue removed and the TEP concentration. Two sets of solutions with different xanthan gum concentrations (0, 2.5, 5, 8, 10 µg/mL) were prepared for the establishment of a calibration curve. The TEP concentration was expressed as the equivalent of xanthan gum concentration.

The xanthan gum calibration curve (Figure 1) displayed a linear relationship, with an R-squared value of 0.977, within the concentration range of 0-10 mg xanthan gum eq./L, which is comparable with results of other research (Arruda Fatibello et al. 2004).

Figure 1: TEP calibration curve with xanthan gum.

Total organic carbon

Total organic carbon (TOC) of the isolated TEP substances was measured by using a Shimadzu TOC-VCPN TOC analyzer. Since the algae and bacteria cells have been removed either by filtration or by centrifugation during the isolation of TEP substances, no further pre-filtration was conducted before TOC analyses.

Fluorescence Excitation-Emission Matrices (FEEM)

A fluorescence spectrometer (FluoroMAX-4 from Horiba) was used in this study to obtain measurements. The FEEM measurements were performed at an excitation wavelength ranging from 240 to 500 nm with 5 nm increments and an emission wavelength ranging from 290 to 600 nm with 5 nm increments. The slit widths were set to 5 nm for excitation and emission. The spectrum data series from FEEM were processed using MatLab program. Background signals were eliminated by subtracting the signals of the Milli-Q water from those of the samples.

To detect which fractions of isolated extracellular material are TEP/TEP precursors, each isolated extracellular material was analyzed in FEEM two times: 1) isolated extracellular material after dialysis and 2) after intensively mixing the isolated extracellular material with alcian blue dye for 1 min, centrifuging the mixture 3000 rpm (2160×g) for 30 min, and removing formed precipitates by 0.45 μm filters. Then the remaining supernatant was determined again in FEEM. If the peaks shown in the first measurement are eliminated in the second run, it means those peaks represent TEP/TEP precursors.

Liquid Chromatography-Organic Carbon Detection-Organic Nitrogen Detection (LC-OCD-OND)

A LC-OCD-OND analysis instrument (DOC-LABOR Dr. Huber, Karlsruhe, Germany) was used in this study to characterize the isolated TEP precursors as described by other researchers (Huber et al. 2011). The principle of LC-OCD-OND is size exclusion chromatography with combination of organic carbon and nitrogen detection. Mobile Phase (a phosphate buffer, 12.5 g KH_2PO_4 +7.5 g $Na_2HPO_4×2H_2O$ to 5 L, Fluka, #30407 + #30412) was pre-cleaned by UV-Oxidation in an annular UV-reactor and delivered to an autosampler and then to the chromatographic column (250mm_20 mm, TSK HW 50S, 3000 theoretical plates, Toso, Japan). The chromatographic column is a weak cation exchange column based on the polymethacrylate basis. Before chromatographic separation, the samples were pre-filtered with 0.45 micron filter. The first detector after chromatographic separation was a fixed wavelength (254nm) UV-detector (UVD), and then the second detector organic carbon detector (OCD). Before entering the OCD, the sample was acidified with phosphoric acid at the flow rate of 0.2 mL/min to convert carbonates to carbonic acid. A side stream after UVD was diverted to the organic nitrogen detector (OND) for nitrogen analyses. Before analysis, the samples were stored in a refrigerator at 4℃ until the measurements were conducted. The samples were diluted 10 times to ensure the concentrations were within the detection range of 1-5 mg/l DOC, and dilution correction was made.

The LC-OCD-OND analysis can roughly provide information regarding the molecular weight distribution of organic material in the samples. Similar to the FEEM analyses, by analyzing the isolated extracellular material with LC-

OCD-OND before and after reacting with alcian blue dye, the parts of isolated extracellular material that are TEP precursors and its corresponding molecular weight can be determined.

Haemagglutination assay

A Haemagglutination assay is usually utilized to investigate the lectin activity of substances. Lectins are carbohydrate-binding proteins (Damme et al. 1998) which can bind the carbohydrates within cell membranes. To understand whether one substance has lectin-like properties (potential of agglutination), red blood cells are normally added to the target substance for agglutination and compared with the negative and positive controls. Since cell membranes of both bacteria and red blood cells contain carbohydrates, a Haemagglutination assay can be used to determine the bacteria-agglutination ability of TEP substances in this study.

A 2% (m/v) suspension of sheep red blood cells (Sigma-Aldrich) dissolved in 1x concentrated phosphate buffer saline (Sigma-Aldrich) and 1% (m/v) concanavalin-A from canavalia ensiformis (Sigma-Aldrich) was used for the Haemagglutination assay.

The assay was carried out in 20-mL glass tubes by adding 0.5 mL of a 2% suspension of sheep red blood cells to 0.5 mL samples or control solution. The control solution contained a negative control (Milli-Q water) and a positive control (Concanavalin A). After adding red blood cells, the agglutination was allowed to develop at room temperature for 12 hours. The cells in the negative control solution do not agglutinate and slide down to form a compact bottom or ring at the center of the curved glass tube, while the cells in positive control solution agglutinate and form a complete carpet of cells covering the bottom of tubes. Samples showing a complete carpet similar to the positive control indicate that the samples contain lectin-like substances. Substances with lectin-like properties have the potential to agglutinate bacteria, and then promote surface biofouling.

Solution state ^1H nuclear magnetic resonance (NMR) spectrometry

^1H NMR analyses were conducted to understand the composition of TEP/TEP precursors isolated from different bacteria and algae species. Each NMR sample was prepared by dissolving 5-10 mg of lyophilized compounds in 650 μl of deuterated water D_2O and then 0.6 mL of the solution was transferred to 5 mm NMR tubes. NMR spectra were acquired at 298 K using a Bruker 700 AVANAC III spectrometer equipped with a Bruker CPTCI triple resonance cryo-probe with an ATM module (5 mm CPTCI 1H-13 C/15N/D Z-GRD) probe. In order to generate comparable data all NMR experiments were carried out under the same experimental conditions where temperature for all experiments was kept at 298 K. The ^1H NMR spectra were recorded by collecting 1k scans with a recycle delay time of 3s. To suppress the water peak, each spectrum was induced using an excitation sculpting pulse sequence using a standard (zgesgp) program from the Bruker pulse library. Exponential line broadening of 1 Hz was applied before Fourier Transformation. Bruker Topspin 2.1 software was used in all experiments to collect and analyze the data.

Results and discussion

Identification of marine bacteria

Isolated DNA of different bacterial samples (DNA lengths of 1350 base pairs) was sequenced and then identified via BLAST (on-line DNA identification software provided by NCBI, United States). The isolated SW1 and SW2 bacteria showed a 99% similarity to *Pseudidiomarina homiensis* and *Pseudoalteromonas atlantica*, respectively. Both bacteria are gram-negative. *Pseudidiomarina homiensis* and *Pseudoalteromonas atlantica* are known marine bacteria (Corpe 1972, Kwon et al. 2006), and *Pseudoalteromonas atlantica* has been reported as a primary producer of biofilms.

In biofouling studies performed in the San Diego Bay and in the open ocean off the coasts of California, New Jersey, and Florida (Corpe 1972), *Pseudoalteromonas atlantica* was shown to be a primary biofilm-forming bacteria, attaching to sterile slides and producing voluminous amounts of EPS. Following biofilm formation by *Pseudoalteromonas atlantica*, stalked and/or filamentous bacteria with more limited nutrient utilization capabilities attached to the surface, and these organisms were followed by microalgae and sessile protozoa (Corpe 1972). *Pseudidiomarina homiensis* has been reported to be a dominating species on a biofouled RO membranes extracted from a desalination treatment plant in the local region (Jeddah, Saudi Arabia) (Khan et al. 2013).

Normalized TEP concentration of isolated extracellular material

Normalized TEP concentrations were calculated by dividing the TEP concentrations of samples by corresponding TOC concentrations. The TEP and TOC of the different organisms were harvested and determined at the same growth time point (stationary phase). Figure 2 shows the normalized TEP concentrations of marine algal and bacterial extracellular material. It is clear that all the tested algal and bacteria species produced TEP.

A higher normalized TEP concentration indicates the isolated extracellular material from the corresponding algae/bacteria have more functional groups for alcian blue dye staining, which means more TEP within the per unit released organic carbon. Although all investigated bacteria and algae produced TEP, the amount and structure of TEP released is highly species-specific. TEPs are generally considered to be acidic polysaccharides, since alcian blue dye stains the carboxyl and sulfate half-ester groups of the acidic polymers (Mopper et al. 1995, Myklestad 1995, Passow and Alldredge 1995). It has been reported that the types and amount of excreted polysaccharides by phytoplankton are species-specific (Myklestad 1995). Even though some diatom species produce polysaccharides containing similar residues of rhamnose, fucose and galactose, they exhibit major differences in terms of structure. The differences in type, amount and structure of polysaccharides excreted may determine the number of carboxyl and sulfate half-ester groups (binding sites) within each diatom species for staining with alcian blue dye, and consequently affect its normalized TEP concentration. The highly species-specificity of TEP production observed in this study may be due to the differences in type, amount and the structure of polysaccharides excreted as well.

A higher normalized TEP concentration does not necessarily mean a higher TEP production capability. The production capability depends on the TOC production per unit increase in bacterial and algal cells, so the TEP production capacity of different bacteria and algae can only be addressed by monitoring the TEP production trends during species' growth under the same culturing conditions.

Figure 2: Normalized TEP concentration of isolated algal and bacterial extracellular material, expressed as mg-xanthan gum equivalent/mg-carbon.

FEEM of isolated extracellular material

The left and right hand side of Figure 3 show the FEEM spectra of isolated extracellular material of different species before and after the removal of TEP/TEP precursors, respectively. TEP/TEP precursors are generally considered to be composed of acidic polysaccharides, and polysaccharide substances do not fluoresce; but humic-like and protein-like substances do fluoresce. Hence, only proteins and humic substances contained within the isolated extracellular material will give a signature signal in the FEEM spectra. As shown in Figure 3, all the isolated extracellular material displayed one main peak within the 300-350nm emission and 250-300 nm excitation wavelength ranges in FEEM spectra before removing TEP/TEP precursors. Besides this main peak, some of them also exhibited minor peaks within the 250-260nm emission and 380-480 nm excitation, 320-340 nm emission and 390-420 nm excitation ranges. The main peak was in the range which is generally considered as protein-like substances, while the other two minor peaks were in the range of humic-like substances reported by (Villacorte et al. 2012). According to the FEEM spectra, both the isolated marine bacterial and algal extracellular material contain protein-like substances and a minor amount of humic-like substances which may have been partially removed due to dialysis during extracellular material isolation.

All the protein-like peaks were mostly eliminated in the FEEM spectra of extracellular material after removing TEP/TEP precursors, indicating that most isolated extracellular materials which were associated with protein-like substances have reacted with alcian blue dye and were removed by the 0.45µm filtration. Therefore, except for some minor humic substances, most of the isolated extracellular material associated with protein-like substances was TEP and/or TEP precursors. It suggested that TEP/TEP precursors may be acidic polysaccharides associated with protein-like substances (e.g. glycoprotein).

Figure 3: FEEM spectra of isolated extracellular material before (left) and after removing TEP/TEP precorsors (right) for: *Pseudidiomarina homensis* (SW1) and *Pseudoalteromonas atlantica* (SW2); algal TEP: *Alexandrium tamarense* (AT) and *Chaetoceros affinis* (CA).

LC-OCD-OND of isolated dissolved extracellular material

The LC-OCD results of all extracellular material are shown in Figure 4. The chromatograms on the upper part of the Figure 4 are the dissolved extracellular material from different algal and bacterial species before reacting with alcian blue and forming precipitates. All these chromatograms showed a peak at about 30-min retention time. According to the manufacturer of the LC-OCD, the compounds detected with a rapid elution have high molecular weights. The molecular weight of the detected compounds decreased with the increase of retention time (Huber et al. 2011). Peaks at 30 minutes can be assigned to the biopolymer fraction. The biopolymer fraction could be proteins and/or polysaccharides, which is considered to be the main foulant of membranes (Huber 1998, Laabs et al. 2006).

The chromatograms of the dissolved extracellular material after reacting with alcian blue are shown at the lower part of Figure 4. The biopolymer fraction detected before the reaction with alcian blue was eliminated from the chromatograms, indicating the dissolved biopolymers that are TEP precursors. One peak at around 55-min retention time was detected in all chromatograms after reacting with alcian blue. However, this was the signal of alcian blue dye residues, which corresponded to the chromatogram of pure alcian blue dye.

Since the LC-OCD-OND analyzer consists of three detectors; organic carbon, UV and organic nitrogen; the carbon and nitrogen content of samples can be measured together (Huber et al. 2011). It is suggested by data in the Figure 4 that the detected biopolymers are TEP precursors. The carbon and nitrogen content of TEP precursors of different species, obtained from LC-OCD-OND analyses, is shown in Table 1. Moreover, the N/C ratios of the TEP precursors in different species are compared in Figure 5. In general, bacterial TEP precursors exhibit a higher N/C ratio than algal TEP precursors. TEP precursors of both bacteria have N/C ratios around 0.35 (*Pseudidiomarina homiensis* showed an N/C ratio of 0.44), while the N/C ratio of the algal samples ranges are between 0.05-0.1. This indicates that the algal TEP precursors contain less protein content than those of the bacteria. In one study regarding the C:N ratio of TEP in the northwestern Mediterranean Sea (Mari et al. 2001), an average C:N ratio of 20 within a year has been reported, but the average value was elevated because of the extremely high C:N ratio (up to 70) observed in summer. Most of the the year, the C:N ratios of TEP in the northwestern Mediterranean Sea ranged between 12-14, corresponding to the 0.83-0.7 N:C ratios are close to the values of algal TEP precursors detected in this study. Therefore, the data suggest that TEP in natural water may be dominated by algal extracellular material.

Figure 4: LC-OCD chromatogram of bacterial and algal dissolved extracellular material before (upper) and after (lower) reacting with alcian blue and forming precipitates.

Figure 5: N/C ratio of biopolymer fraction in bacterial and algal samples, measured by the LC-OCD-OND.

Table 1: Carbon and nitrogen content of isolated algal and bacterial TEP measured by LC-OCD-OND.

	Carbon (ppb)	Nitrogen (ppb)
Alexandrium tamarense	560	43
Chaetoceros affinis	1893	69
P. homensis	781	336
P. atlantica	2394	830

Lectin activity of isolated TEP/TEP precurosrs

Figure 6 displays the results of the agglutination test. Isolated extracellular material from both algae and bacteria formed a similar even carpet within the positive control (Concanavalin A), indicating all of them have lectin-like properties. The results of the FEEM and LC-OCD measurements indicate that although there were some minor humic-like substances existing in the isolated extracellular material, most of the isolated extracellular material was TEP/TEP precursors. Therefore, the lectin-like activity of the extracellular material very likely indicated a similar activity within TEP/TEP precursors of investigated algae and bacteria species.

Figure 6: Agglutination test of bacterial and algal TEP substances.

^{1}H NMR of isolated TEP/TEP precursors

^{1}H solution-state NMR spectra at 700 MHz for bacterial and algal TEP/TEP precursors are shown in Figure 7. Chemical shift assignment and interpretation of different peaks are shown in Figure 7. This analysis was carried out based on the data published by Simpson et.al (Simpson et al. 2011). In general, peaks observed between 0.7-2.3 ppm, 2.5-5.2 ppm and 6.4-8.5 ppm chemical shift can be viewed as aliphatic, carbohydrates, and amide/aromatic substances groups, respectively. Within the aliphatic group, the peak at 0.8-1.0 ppm could be assigned to methyl groups mainly from peptides. The presence of peptides provides substantial proof for the existence of proteins in samples. On the other hand, peaks at 1.5 ppm and 2.2 ppm could be assigned to $CH_2\gamma$ to COOH and β to COOH, respectively. Peaks at 3.7 ppm, 4.3 ppm, 5.0 ppm and 5.3 ppm could be assigned to methoxyl (lignin), α-protons peptides, anomeric (carbohydrate) and double bonds, respectively. In terms of the amide/aromatic group, the peaks at 6.6 ppm, 7.1 ppm and 8.2 ppm were assigned to lignin aromatics, aromatic amino acid side chains and amide in peptides, respectively. As shown in highlighted area of Figure 7, all bacterial TEP/TEP precursors exhibited clear signals for methyl groups mainly from peptides and aromatic amino acid side chains, indicating the presence of protein substances in those samples. On the other hand, there were no or a very limited number of methyl groups mainly from peptides and aromatic amino acid side chains, observed for all algal TEP/TEP precursors. Results of the LC-OCD-OND data only provides a general understanding of the N/C ratio of TEP precursors, but there is no

substantial proof to clarify that the nitrogen is from protein. By conducting the solution state ^{1}H NMR spectrometry, the presence of proteins is thereby confirmed.

In order to quantitatively investigate the compositional difference of samples, each NMR chemical shift assignment in the spectra (standing for different compounds mentioned above) was integrated and compared with one specific chemical shift assignment as a reference. Methyl groups mainly from peptides within the 0.8-1.0 ppm chemical shift were used as the reference in the integration. The integrated value of methyl mainly from peptides itself was 1.0 because it is equal to the reference. The relative integration values of other chemical shift assignments were determined by comparing their integration area with the reference. Afterwards, the total area of all assignments can be summed, and the percentage of each assignment can be calculated. The calculated percentage composition is shown in Figure 8. TEP/TEP precursors from both marine bacteria contained more methyl groups mainly from peptides, lignin aromatics, aromatic amino acid side chains and amide in peptides than the algal TEP/TEP precursors. Algal TEP/TEP precursors contain more methoxyl (lignin) and anomeric (carbohydrate) material compared to the bacterial TEP/TEP precursors. These materials belong to the carbohydrates group. Since proteins are composed of one or more chains of amino acid, more lignin aromatics, aromatic amino acid side chains and amide in peptides detected in the bacterial TEP/TEP precursors, probably indicates more protein substances in the bacterial TEP/TEP precursors as well.

In summary, the ^{1}H NMR analyses showed that bacterial TEP/TEP precursors contain more protein substances, while the algal TEP/TEP precursors contain more carbohydrates, consistent with the results of LC-COD-OND analyses in terms of N/C ratio.

Figure 7: ^{1}H solution-state NMR spectrum at 700 MHz of isolated TEP substances from different bacteria and algae.

Figure 8: Fraction assignments and integration of bacterial and algal samples (based on the chromatography of proton NMR).

Similarity between algal and bacterial TEP/TEP precursors

The isolated marine bacteria and algae species produced TEP/TEP precursors, although the production capacity is different depending on species. It is logical that different species have a different TEP production capacity, because it is related to the living style of each specific species. However, the important thing is that both bacteria and algae can produce TEP/TEP precursors, which may be responsible for the biofouling of surfaces within the marine environment (and SWRO membranes).

TEP/TEP precursors are generally considered to be acidic polysaccharides (Passow 2002b) due to their stainability with cationic alcian blue dye. Because it is suggested by the FEEM results that most of protein-like material is associated with TEP/TEP precursors for all algae and bacteria species, TEP/TEP precursors are probably a mixture of polysaccharides and proteins (glycoprotein, for example). However, the polysaccharides and protein content within each type of bacterial and algal TEP/TEP precursors cannot be elucidated by only FEEM analyses. LC-OCD results showed that the isolated marine bacterial and algal TEP precursors contained a significant amount of dissolved macromolecular organics which is defined as "biopolymer" by the manufacturer of the LC-OCD (Huber et al. 2011). The particulate TEP larger than 0.4 micron was probably removed.

Furthermore, it is very likely that all investigated bacterial and algal TEP/TEP precursors possess lectin-like property. This means that both algal and bacterial TEP/TEP precursors have the potential to agglutinate bacteria. The lectin-like property of TEP/TEP precursors is an important factor in causing the biofouling of surfaces and membranes. Since these TEP/TEP precursors are sticky, they can form a chemical conditioning layer on the surface of RO membranes when they reach the membrane. This TEP layer does not only promote the settlement of bacteria, but also provides an ideal environment with sufficient nutrients for the growth of settled bacteria, since the TEP substances contain polysaccharides and protein (necessary carbon and nitrogen source for bacterial growth). Because of the sticky properties of TEP substances, other necessary nutrients, such as phosphates and trace medals may also be collected on the TEP layer, encouraging the growth of bacteria. During the growth, the settled bacteria can produce TEP substances as well.

Therefore, bacterial TEP substances have the same potential as algal TEP substances to initialize surface biofouling, which has long been neglected.

Difference between algal and bacterial TEP/TEP precursors

As previously discussed, the FEEM results revealed that algal and bacterial TEP/TEP precursors are probably a mixture of polysaccharides and proteins, possibly as glycoprotein. However, it is not clear which type of material is dominant in the different TEP/TEP precursors found in the FEEM analysis alone.

A higher protein concentration in bacterial TEP/TEP precursors compared to algal ones was detected by the LC-OCD-OND analyses (reflected as N/C ratios in biopolymers fraction) along with proton NMR analyses. The LC-OCD-OND analyses revealed the ratio between carbon and nitrogen of samples, indicating a high Nitrogen content in the bacterial TEP precursors. This high nitrogen content is probably contributed by the higher protein contents in the bacterial samples. ^{1}H NMR spectrum analysis provided further substantial proof for the presence of more protein contents in the bacterial than algal TEP samples. It has been claimed that TEP/TEP precursors are mainly acidic polysaccharides. However, the analyses show that it is associated with protein-like material. Depending on the original source of TEP/TEP precursors, the association of protein-like material varied significantly. Investigated bacterial TEP/TEP precursors possess more protein than those of investigated algae. Considering the differences in

terms of thickness and reversibility of a membrane fouling layer caused by sodium alginate (carbohydrates) and BSA (protein), the compositional differences between algal and bacterial TEP/TEP precursors should be given greater consideration. Regarding the biofouling potential, the initial deposition of TEP/TEP precursors on the submerged surfaces in the ocean may be different for algae and bacteria, due to the different properties between polysaccharides and proteins (such as hydrophobicity and surface charge density).

FEEM or LC-OCD-OND analyses alone have limitations on providing a complete picture of the composition of TEP substances. The FEEM only reveals the protein-like and humic-like substances of the extracellular material; while LC-OCD-OND can just provide a rough molecular weight distribution and a hint of the presence of protein-like material. The combination of FEEM and LC-OCD-OND, in addition to NMR data, provides a comprehensive understanding of the characteristics of different TEP substances, in terms of aromatic property, molecular weight distribution, and functional group composition.

Conclusions

In this study, the composition of TEP/TEP precursors produced by two marine bacteria and two marine algae species were investigated. Although these two bacteria and two algae species do not represent all bacteria and algae, the investigated bacteria and algae species have been reported as dominant species on biofouled RO membranes, or species producing significant amount of TEP in the ocean. In summary, both of the two marine bacteria and two marine algae produce TEP/TEP precursors, consistent with some previous studies. All isolated bacterial and algal TEP precursors from the four bacterial and algal species contain high molecular weight biopolymers. Furthermore, all isolated bacterial and algal TEP/TEP precursors exhibit lectin-like properties, which give them the potential to agglutinate bacteria and may enhance biofouling of membrane surfaces. However, compared with the two isolated algal TEP/TEP precursors, the two isolated bacterial TEP/TEP precursors possess higher nitrogen content, indicating that there is more protein in the isolated bacterial compared to the algal TEP/TEP precursors. This compositional difference between TEP isolated from these four common TEP producing marine bacteria and algae has not been previously reported, and may help in the development of appropriate pretreatment designs to control surface biofouling caused by different TEP/TEP precursors.

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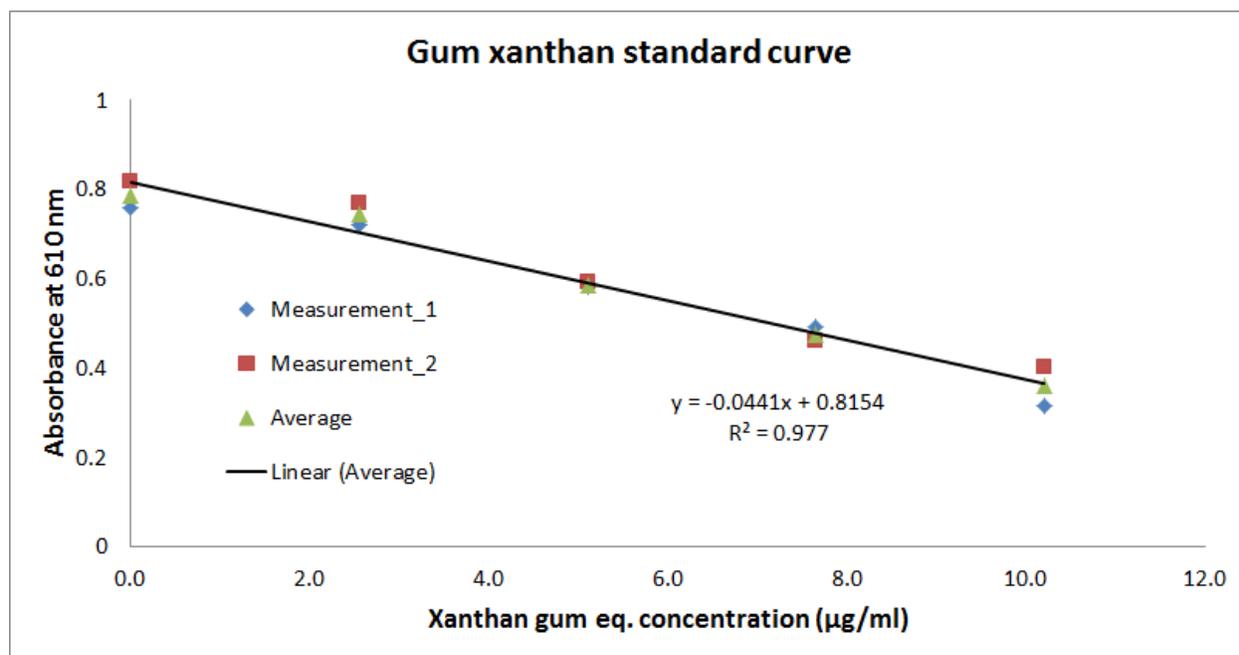
Figure 1

Figure 2
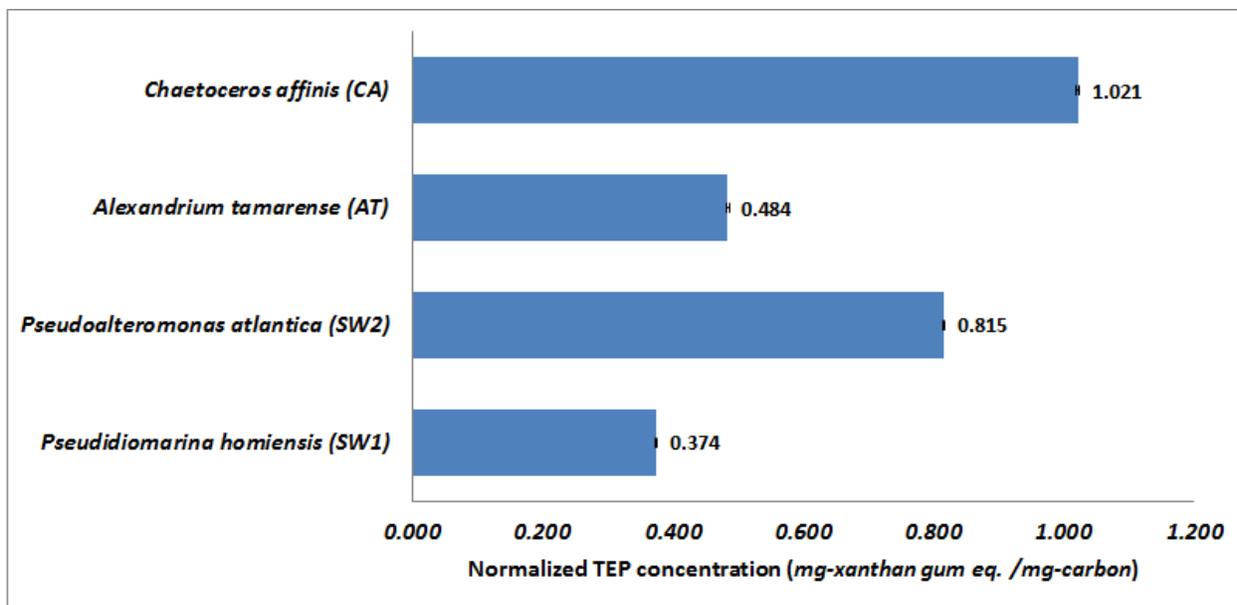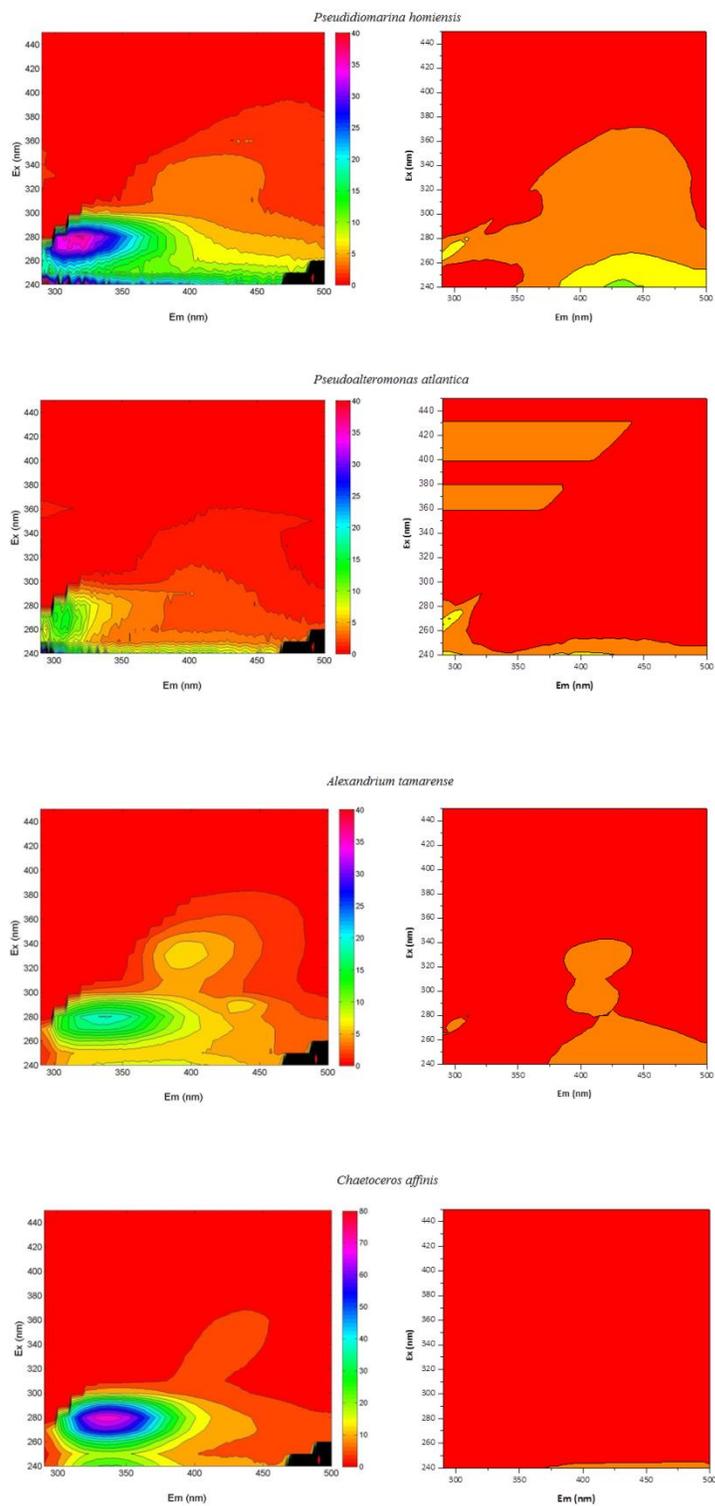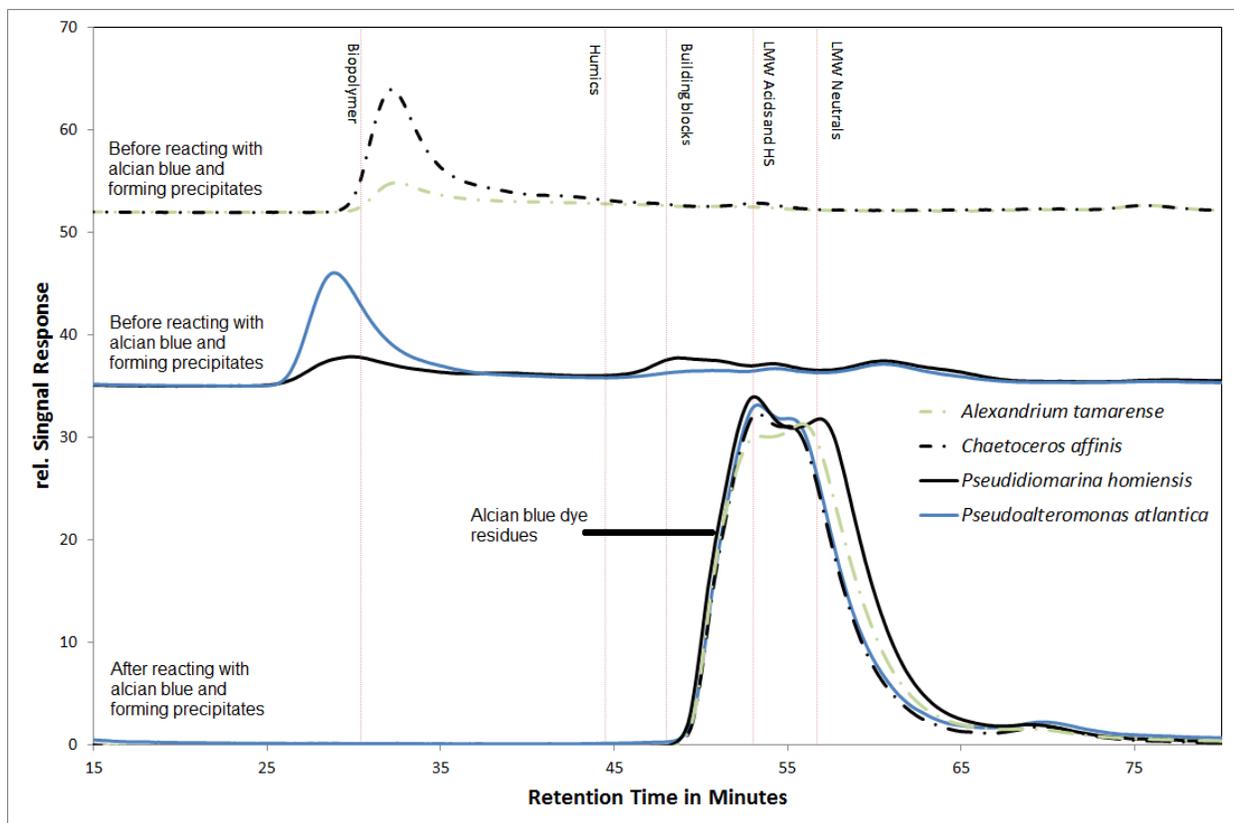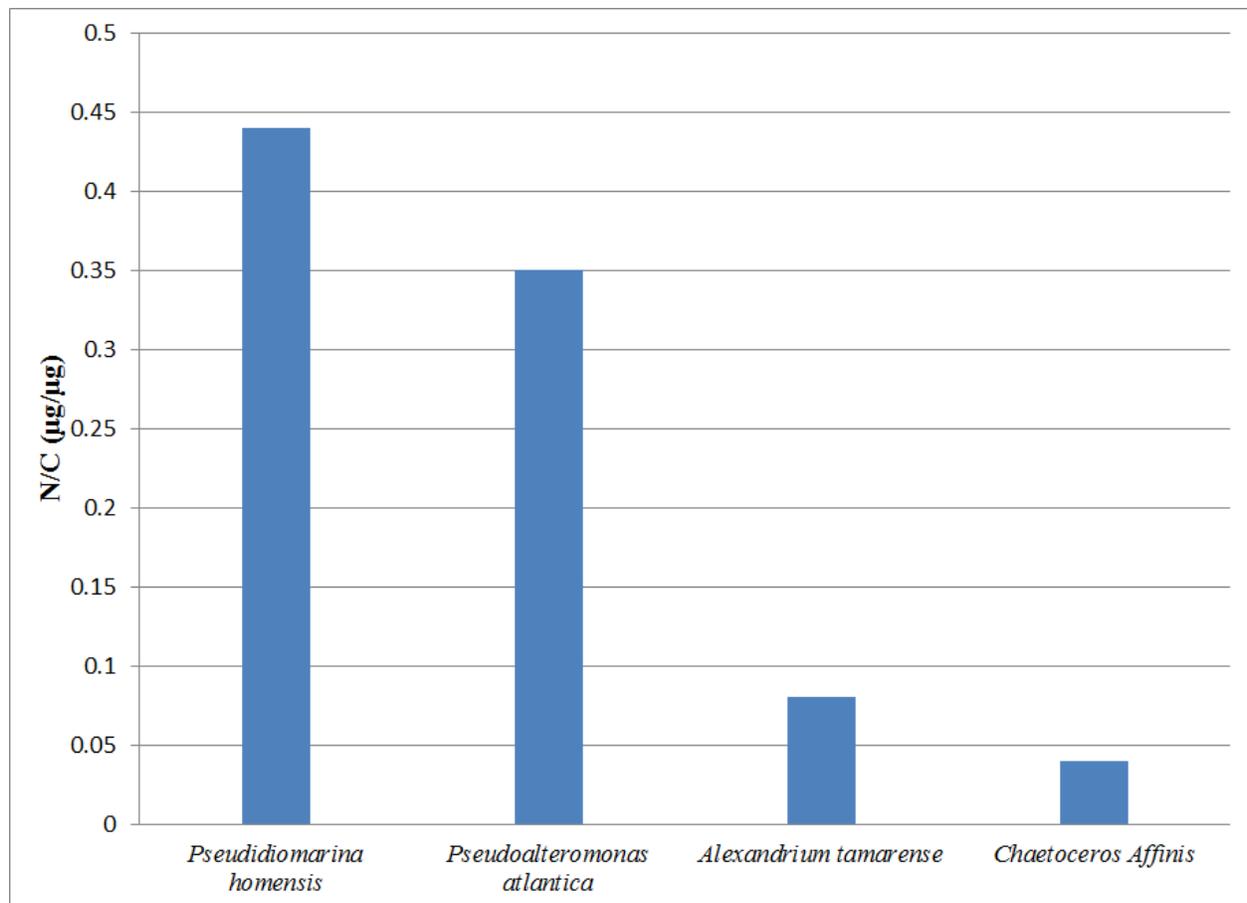
Figure 3

Figure 4

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Figure 5

Negative control, Positive control, *Pseudidiomarina homensis*, *Pseudoalteromonas atlantica*, *Alexandrium tamarense*, *Chaetoceros affinis*

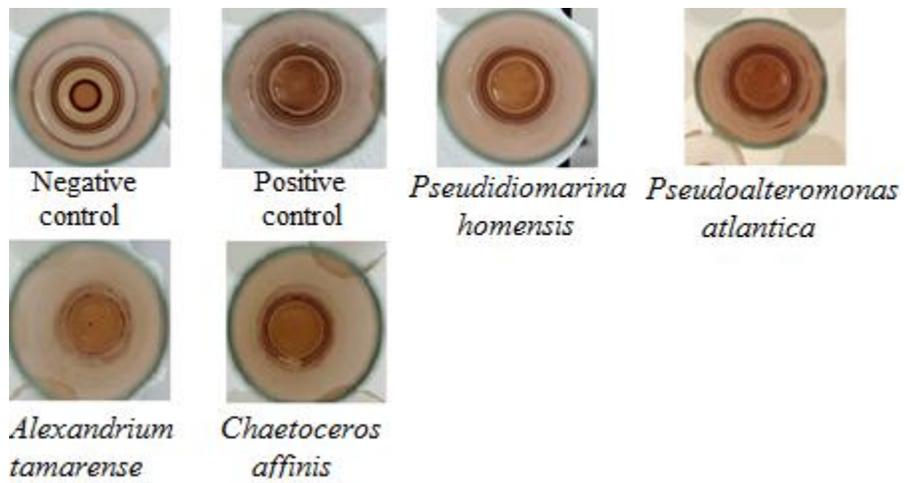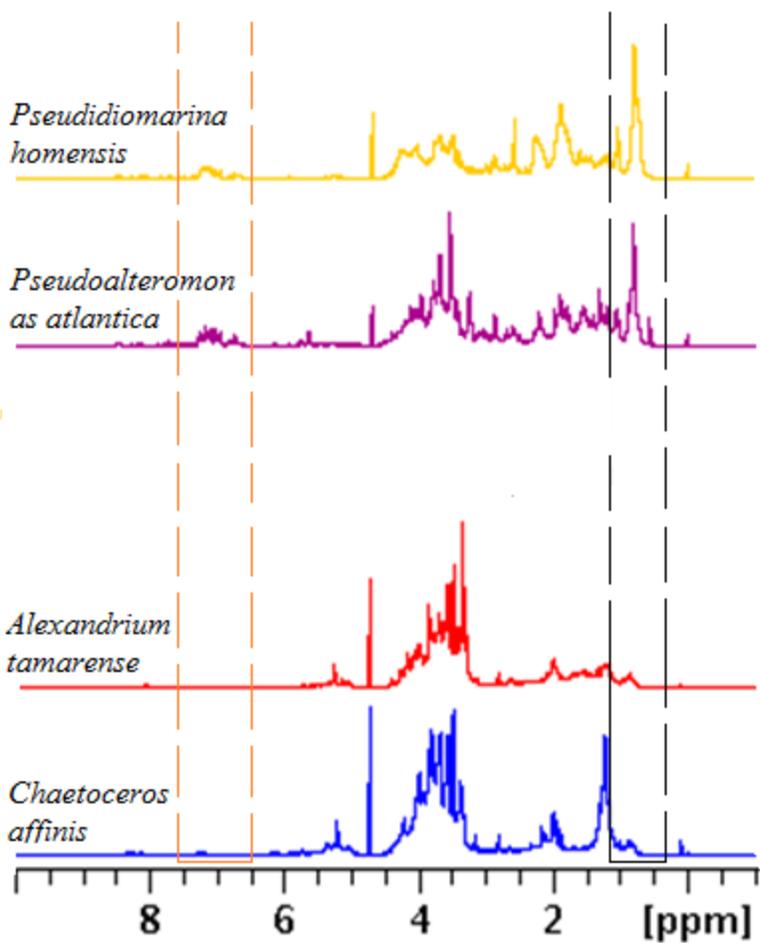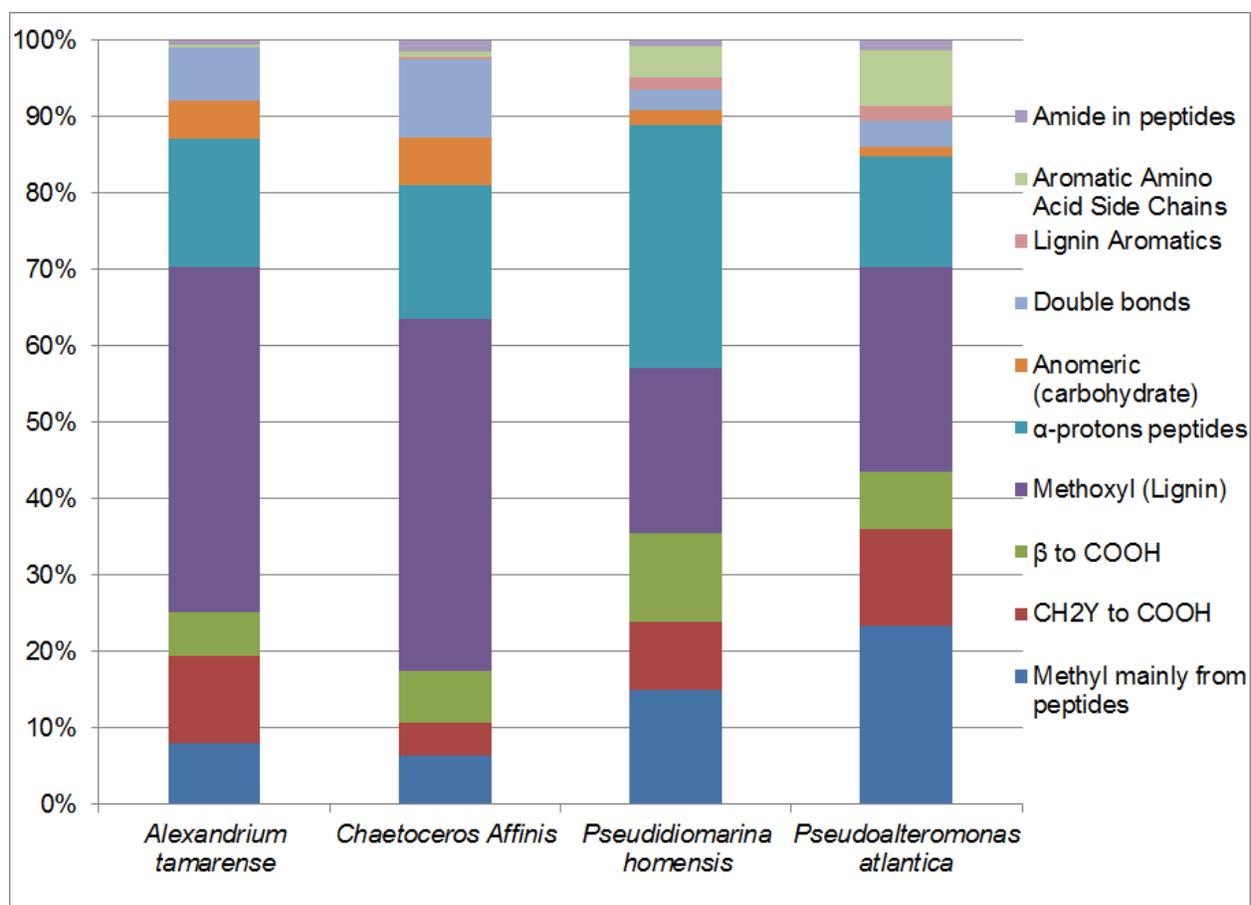Figure 6

Figure 7

Figure 8

Highlights

- Bacterial & algal TEPs contain high amount of high-molecular-weight biopolymers

- Bacterial & algal TEPs have lectin-like property enhancing bacteria agglutination

- Algal TEPs are compositional different from bacterial TEPs, may influencing surface biofouling