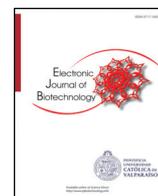




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Research article

Inhibition of *Nitzschia ovalis* biofilm settlement by a bacterial bioactive compound through alteration of EPS and epiphytic bacteria



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ABSTRACT

Background: Marine ecosystems contain benthic microalgae and bacterial species that are capable of secreting extracellular polymeric substances (EPS), suggesting that settlement of these microorganisms can occur on submerged surfaces, a key part of the first stage of biofouling. Currently, anti-fouling treatments that help control this phenomenon involve the use of biocides or antifouling paints that contain heavy metals, which over a long period of exposure can spread to the environment. The bacterium *Alteromonas* sp. Ni1-LEM has an inhibitory effect on the adhesion of *Nitzschia ovalis*, an abundant diatom found on submerged surfaces.

Results: We evaluated the effect of the bioactive compound secreted by this bacterium on the EPS of biofilms and associated epiphytic bacteria. Three methods of EPS extraction were evaluated to determine the most appropriate and efficient methodology based on the presence of soluble EPS and the total protein and carbohydrate concentrations. Microalgae were cultured with the bacterial compound to evaluate its effect on EPS secretion and variations in its protein and carbohydrate concentrations. An effect of the bacterial supernatant on EPS was observed by assessing biofilm formation and changes in the concentration of proteins and carbohydrates present in the biofilm.

Conclusions: These results indicate that a possible mechanism for regulating biofouling could be through alteration of biofilm EPS and alteration of the epiphytic bacterial community associated with the microalga.

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1. Introduction

Benthic diatoms are among the dominant forms reported in marine biofilms [1]. These microorganisms are unicellular algae that excrete extracellular polymeric substances (EPS) with a high carbohydrate content and are used in the formation of biofilms [2,3]. The formation of microbial biofilms on surfaces is one of the most important factors that affects the adhesion and growth of benthic diatoms [4,5,6]. The substrate upon which biofilms grow is determined by the topology [7,8] and chemical composition of the surface, light conditions, and water currents [9,10,11]. The production and composition of EPS differs in response to environmental conditions as well as the growth stage of the diatoms [12,13], suggesting that microalgae modulate their polysaccharide biosynthesis machinery to adapt to environmental conditions and allow them to adhere to surfaces [14].

In diatoms, EPS have different functions, with the most commonly reported ones including the stabilization of habitats, colony formation, adhesion and motility on the substrates [12,15]. The process of adhesion is different for diatoms than for bacteria, since they lack flagella and are therefore incapable of actively approaching a particular surface. However, biofilm-forming diatom species move by gliding via the excretion of extracellular polymeric substances from their raphe, an elongated slit in the cell wall [16,17]. It has been suggested that the observed rotational movements of diatoms can be produced by pulling off one pseudopod or stalk from the substratum with the help of extracellular polymeric substances. When a pseudopod or stalk is adhered to the substratum, the resulting torque causes a whole-cell rotational movement [18]. Although diatoms randomly settle, once in contact with the surface, they begin an active engagement where the initial reversible contact (primary adhesion) determines whether the diatom will continue its life cycle in that location (secondary adhesion) [19]. It has been suggested that the interaction between bacteria and diatoms has an important role in their ecological success [20]. Bacteria and diatoms interact in the

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phycosphere, where the bacteria can access algal exudates, such as polysaccharides, sugars, proteoglycans, small amino acids and glycoproteins [20]. These exudates can be species-specific and may determine the type of bacteria that associate with a diatom species [4]. The attachment of diatoms on surfaces makes them important fouling organisms due to their adherence to man-made substrates, which, in addition to bacteria, constitute a major problem for artificial structures immersed in the marine environment [4]. The recent ban on toxic antifouling biocides has underscored the need for the development of “environmentally friendly” strategies and has stimulated an active search for non-toxic natural marine antifouling compounds [21]. The bacterial strain *Alteromonas* sp. Ni1-LEM, first isolated by our lab from a natural substrate (red macroalgae; *Rhodomenia* sp. [22,23]) in northern Chile, produces an extracellular, thermostable compound that has a peptidic nature and a molecular weight lower than 3500 Da. This inhibitory compound showed antifouling activity against *Nitzschia ovalis*, a cosmopolitan benthic diatom often present in algal blooms [22,23]. The aim of this study was to characterize the antagonistic effect of the bioactive fraction secreted by *Alteromonas* sp. Ni1-LEM on microalgal biofilm development by altering the secretion of microalgal EPS. We also determined the phylogenetic affiliations of the diatom epiphytic communities with and without the antifouling compound by analyzing metagenomic 16S ribosomal DNA (rDNA) sequences.

2. Materials and methods

2.1. Bacterial culturing and isolation of the bioactive fraction

The strain *Alteromonas* sp. Ni1-LEM [23] was incubated in 1 L of M9 minimal medium at 20°C with constant stirring at 120 rpm for 4 d until the stationary phase was reached. The culture was centrifuged at 9632 g for 15 min at 4°C (Avanti J-25I, Rotor JA-14, Beckman Coulter, USA). Next, the supernatant was filtered through 0.2- μm nitrocellulose membranes (Sartorius, Germany) and concentrated 10-fold (10 \times) in a rotary evaporator (R200, Büchi, Switzerland) at 60°C. The concentrated supernatant was dialyzed at 4°C using a 1000-Dalton cut-off dialysis membrane (SpectraPor®, USA). The dialyze (SN10X) was filtered again through nitrocellulose membranes and stored under sterile conditions at 4°C until its later use. The protein concentration was determined using a BCA Protein Assay kit (ThermoScientific, USA) according to the manufacturer's instructions.

2.2. Diatom culture conditions

The benthic microalgae *Nitzschia ovalis* Arnott (14 μm \times 6 μm) was isolated from a commercial abalone (*Haliotis rufescens*) hatchery in Caldera, Chile (27°03'24"S-70°51'30"W) [23]. For culturing, 250- mL flasks containing 150 mL of Guillard f/2 medium [24] were inoculated with 1 \times 10⁵ cells mL⁻¹ of the non-axenic benthic microalga *N. ovalis* in pre-stationary phase. The monoalgal cultures were incubated for 5 d at 20°C under a light intensity of 100 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$, after which the cultures were sonicated for 15 s to separate the cells from the flask walls. The optical density of the cultures was determined every 24 h at 560 nm using a Halo20 Dynamica (Australia) spectrophotometer. The number of cells was determined by a direct count of ten fields at 20 \times magnification. For EPS extraction, 145-mL cultures of *N. ovalis* were grown and 5- mL samples were evaluated by microscopy. All experiments were run in triplicate using independent cultures.

2.3. Extraction of EPS

Two chemical methods (2.3.1 and 2.3.2) and one physical method (2.3.3) were used to extract EPS from *N. ovalis* biofilms.

2.3.1. Extraction with formaldehyde and NaOH (modified from Pan et al. [25])

The cell suspension was first extracted with formaldehyde (36.5%) for 1 h at 4°C followed by NaOH (1 M, 4°C, 3 h). The samples were centrifuged at 4873 g (4600 rpm, Rotanta 460R, Hettich, Germany), 4°C for 15 min. Next, the supernatants were filtered through a 0.45- μm membrane and concentrated in a rotary evaporator (R200, Büchi, Switzerland) at 60°C to a volume of 50 mL, which was subsequently dialyzed under sterile conditions for 24 h using 3500 Da MW cut-off dialysis bags (Snakeskin, Pierce, USA). During the dialysis, the water was replaced after 4, 8, 16 and 24 h. Once dialyzed, the EPS was lyophilized and stored at -20°C until further processed.

2.3.2. Ethanol precipitation (modified from de Brouwer et al. [26])

Soluble EPS was obtained by centrifuging 145 mL of culture at 3500 g for 15 min at room temperature. The supernatant was transferred to a bottle containing 435 mL of cold ethanol (96%), and the soluble EPS was allowed to precipitate overnight at -20°C. After centrifugation (15 min at 3500 g), the EPS pellet was lyophilized.

2.3.3. Extraction by ultrasonication (modified from Pan et al. [25])

The cell suspension was first ultrasonicated at room temperature at 37 kHz for 30 s using an ultrasound generator (Elmasonic S, Germany). The sonicated cell suspension was then centrifuged at 4873 g (4600 rpm, Rotanta 460R, Hettich, Germany) for 15 min at 4°C. Next, the supernatants were filtered through a 0.45- μm membrane and concentrated in a rotary evaporator (R200, Büchi, Switzerland) at 60°C to a volume of 50 mL, which was subsequently dialyzed under sterile conditions for 24 h using 3500 Da MW dialysis bags (Snakeskin, Pierce, USA). During the dialysis, the water was replaced after 4, 8, 16 and 24 h. Once dialyzed, the EPS was lyophilized and stored at -20°C until further processed.

2.4. Chemical analysis of EPS

The lyophilized EPS was analyzed for protein and carbohydrate content. The protein concentration was measured with a BCA Protein Assay Reagent kit (Pierce, USA) using bovine serum albumin as a standard. The total amount of carbohydrates in EPS samples was determined by the phenol-sulfuric acid method using glucose as a standard [27].

2.5. Adherence assays

To evaluate the adherence of *N. ovalis* to glass, and the effect of the bacterial supernatant on EPS, 125- mL flasks containing 50 mL of Guillard f/2 medium were inoculated with pre-stationary phase diatom cultures to a density of 1 \times 10⁵ cells mL⁻¹. Concentrated and dialyzed bacterial supernatant (SN10X) was added to a final concentration of 100 $\mu\text{g mL}^{-1}$ of total protein. The microalgae culture was incubated at 20°C under a constant illumination of 100 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$. As controls, flasks containing 50 mL of Guillard f/2 medium were inoculated with pre-stationary phase cultures of the microalga *N. ovalis* to a density of 1 \times 10⁵ cells mL⁻¹ without the addition of the concentrated and dialyzed bacterial supernatant. Coverslips were placed at the bottom of the flasks and were later recovered for microscopy analyses. All experiments were performed in triplicate using independent cultures. After diatoms were cultured for 24 and 48 h, EPS were extracted using the most efficient protocol. Each replicate was standardized to 2.5 mg mL⁻¹ for protein and carbohydrate analyses. The remaining EPS were stored for subsequent spectroscopy analyses.

2.6. FT-IR spectroscopy

Samples were prepared for by grinding the extracted EPS with potassium bromide and forming the mixture into a pellet. FTIR spectroscopy (Spectrum Two, Perkin Elmer) was used to read the absorbance from 650 to 4000 cm^{-1} (4 cm^{-1} resolution and a laser incidence of 20 repetitions) and detect the major structural groups of EPS present in the biofilm.

2.7. Nuclear magnetic resonance spectroscopy

Nuclear magnetic resonance (NMR) spectra were obtained using a Bruker Ascend TM (400 MHz for ^1H and 125 MHz for ^{13}C) at 55°C. Samples were exchanged three times with D_2O or DMSO-d_6 . Chemicals shifts were reported in ppm relative to sodium 2,2,3,3-tetramethylpropanoate for ^1H and CDCl_3 for ^{13}C NMR spectra.

2.8. Microscopy

Anionic carbohydrates were stained with Alcian blue for microscopic observations [28]. Glass slides from adherence bioassays were recovered and stained using 0.1% (w/v) Alcian blue in 0.5 M HCl (pH 0.5). Samples were incubated for 30 min then were carefully rinsed with water. The samples were observed with an epifluorescence microscope (BX51, Olympus). Scanning electron microscopy was performed using a HITACHI TM 3000 microscope fitted with a cryostage. Samples were coated with 10 nm of pure gold.

2.9. Epiphytic microbiota sampling for 16S rRNA iTag (Illumina amplicon tag) analysis

To evaluate the bacterial community associated with *N. ovalis* and determine the effect of the bacterial supernatant on its composition, 125- mL flasks containing 50 mL of Guillard f/2 medium were inoculated with pre-stationary phase cultures of the diatom to a density of 1×10^5 cells mL^{-1} , with concentrated and dialyzed (SN10X) bacterial supernatant added at a final concentration of 100 $\mu\text{g mL}^{-1}$ of total protein. The microalgae culture was incubated at 20°C under a constant illumination of 100 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$. Flasks containing 50 mL of Guillard f/2 medium were inoculated with pre-stationary phase cultures of the microalga *N. ovalis* to a density of 1×10^5 cells mL^{-1} were used as control, without the addition of the concentrated and dialyzed bacterial supernatant. Coverslips were placed at the bottom of the flasks and were later recovered for microscopic analyses. All experiments were run in triplicate using independent cultures. To recover the bacterial biomass associated with *N. ovalis* and free-living bacteria, 50 mL of each culture was sonicated for 10 min at room temperature after 0, 24, and 48 h of culturing and were subsequently filtered successively through 0.2- μm nitrocellulose membranes. The filters were stored at -20°C until the extraction of DNA.

2.10. Extraction of DNA from the epiphytic microbiota

Extraction of genomic DNA was performed with the PowerBiofilm DNA Extraction Kit (MoBio, USA) from the filtration membranes, which were cut into pieces and transferred to vials provided by the kit, following the manufacturer's instructions. The DNA was dissolved in TE buffer and stored at -20°C for later analysis. The integrity of the DNA was assessed by electrophoresis in 1% agarose gels and viewed with a UV transilluminator. The concentration and purity of the DNA were determined by measuring its absorbance at 260 and 280 nm to obtain the 260/280 ratio using a BioSpec-nano UV-VIS spectrophotometer (Shimadzu, USA). The DNA was later used as template for PCR.

2.11. Amplification of the 16S rRNA gene

An approximately 1500-bp fragment of the 16S rRNA gene was amplified using the 8F (5'-AGAGTTTGATCTGGCTCAG-3') and 1392R (5'-ACGGGCGGTGTGTAC-3') universal primers. A reaction mixture was prepared in a final volume of 25 μL that contained: 10 μL of 5X GoTaq PCR buffer (Promega, USA); 3 μL of 25 mM MgCl_2 ; 1 μL of 10 mM dNTPs; 0.3 μL of 5 U μL^{-1} Go-Taq DNA polymerase (Promega, USA); 5 pmol μL^{-1} of each primer; and 10 ng of DNA template obtained from the 0.2 μm and 3 μm filters. The PCR was performed in an Applied Biosystems thermal cycler using the following protocol: initial denaturation: 95°C for 5 min; 30 cycles at 94°C for 45 s, 56°C for 1 min, and 72°C for 2 min; and a final extension step at 72°C for 7 min. The PCR product obtained was used as template for the amplification of the hypervariable V3–V4 region using the following primers: 537F 5'-CAGCMGCCGCGTAATWC-3' and 785R 5'-TACNVGGGTATCTAATCC-3' [29].

2.12. 16S rDNA iTag sequencing and analysis

Metagenomics iTag 16S sequencing was conducted by the Research and Testing Laboratory (RTL; Lubbock, Texas, USA) using the MiSeq instrument with Illumina protocols and reagents (Illumina, San Diego, USA). All PCR products used sequencing templates adjusted to 20 ng μL^{-1} . The sequence libraries were generated by RTL using the primers described above, with barcoded sequences in each library recovered by sequencing from the V3–V4 region. Raw fastq files were provided by RTL for analysis and were curated and analyzed (Table S1 and Table S2).

2.13. Metagenomics rapid annotation using subsystem technology MG-RAST analysis pipeline

Fastq files were uploaded to the Metagenomics Rapid Annotation using Subsystem Technology (MG-RAST) server for annotation [30]. In MG-RAST, sequences were subject to quality control, which included dereplication, dynamic trimming, length filtering (>2) and ambiguous base filtering (>5). Sequence reads were clustered into **operational taxonomic units** (OTUs) with a distance cutoff of 0.03 (OTU 0.03), and the clusters with a bootstrap support of at least 80% (after 1000 iterations) were assigned into taxa using the Ribosomal Database Project (RDP). The data were compared using a maximum e-value of $1e^{-20}$, a minimum identity of 97%, and a minimum alignment length of 50 bp. To determine the rate of diversity, rarefaction curves were generated with the Vegan package [31] in R [32]. The Shannon index, rarefaction index and diversity index alpha were calculated as previously described by Park et al. [33].

3. Results

3.1. Microalgae culturing and extraction of biofilm EPS

The growth curve of *N. ovalis* and the comparison of different extraction procedures to isolate soluble carbohydrates from the diatom is shown in Fig. 1. Cell-normalized amounts of soluble EPS yields varied significantly with the extraction method used. The daily EPS yield for each extraction procedure is shown in Table 1. The highest concentrations of bound EPS were obtained by ultrasonication at 37 Hz. The precipitation with ethanol (EtOH) method after centrifugation yielded approximately half of these yields, while the formaldehyde and sodium hydroxide (NaOH) extraction method resulted in even lower yields. Depending on the extraction method used, it was not possible to establish a dependent relationship between the amount of EPS and cell number. The amount of EPS obtained by ultrasonication decreased with the culture time, unlike what was quantified by the other two methods. The highest

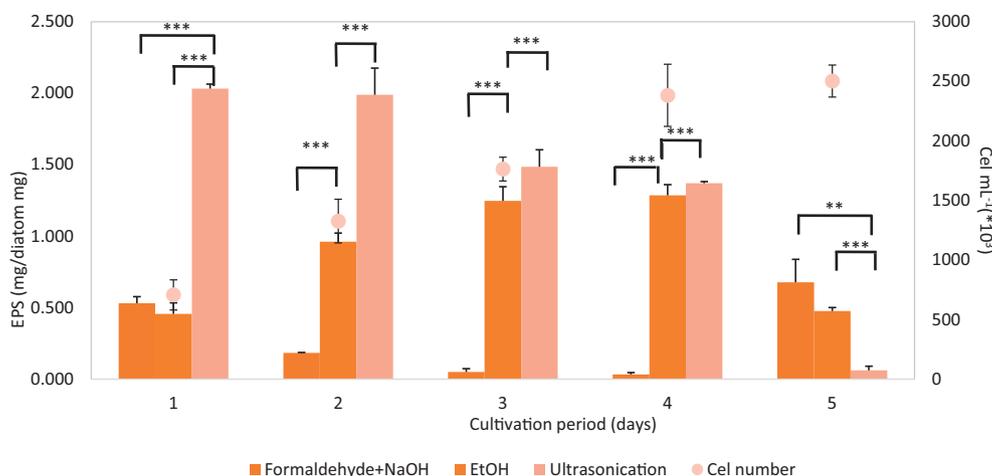


Fig. 1. Soluble EPS in *N. ovalis* cultures sampled for 5 days. Bars: soluble EPS. Circles: cell density. Dark orange: formaldehyde and NaOH. Orange: EtOH precipitation. Pink bars: ultrasonication. Data points indicate the mean values and error bars represent standard deviations for culture replicates for each treatment (n = 3) (Bonferroni test; $P > 0.05$; $*P < 0.05$; $**P < 0.01$).

carbohydrate and protein content in the EPS was observed at 24 and 48 h (Table 2). The localization of the carbohydrates in the biofilm was visualized microscopically using Alcian blue to stain anionic sugars (Fig. 2). The soluble carbohydrates were typically present as mucilage that filled the space between diatom cells (Fig. 2H). The growth of the diatom was quantified for the first five days, during which time the biofilm was established. During this time, it was possible to observe the increasing adherence of the diatoms to the bottom of the flask (Fig. 2A–E).

3.2. Colorimetric assays of exopolysaccharides

The composition of the EPS was also significantly affected by the extraction method (Table 2). Protein could not be undetected in EPS samples prepared by the ultrasonication and ethanol precipitation methods, indicating that these methods extracted little protein. Although a low yield of EPS was obtained by formaldehyde and NaOH extraction, the protein and carbohydrate content was highest in the samples prepared by this method.

3.3. Adherence assay

The effect of bacterial supernatant on *N. ovalis* adherence was evaluated. The biofilm of the diatom control culture was tightly attached to the surface of the coverslip that was placed at the bottom of the flask during the first 24 h. Microscopic examinations of biofilm samples using SEM showed that when the diatom was cultured with the bacterial supernatant, the biofilm was loosely attached and the number of cells present was low. (Fig. 3). Additionally, EPS nanowire-like filamentous structures were visible in the SEM images for the control culture but were not visible in diatom cultures in which the bacterial supernatant was added (Fig. 3C–D). There was not significant difference observed between the amount of EPS obtained by ultrasonication with the diatom control culture and the diatom cultured with the bacterial supernatant at 24 h. At 48 h, the amount of

Table 1
EPS yield from different extraction methods. Two-way ANOVA, Bonferroni test $P > 0.05$.

Day	Ethanol precipitation (mg of EPS/g of diatom)	Formaldehyde and NaOH (mg of EPS/g diatom)	Ultrasonication (mg of EPS/g diatom)
1	0.455 ± 0.045	0.531 ± 0.045	2.032 ± 0.032
2	0.961 ± 0.035	0.183 ± 0.004	1.989 ± 0.19
3	1.246 ± 0.058	0.05 ± 0.023	1.485 ± 0.12
4	1.285 ± 0.043	0.033 ± 0.012	1.37 ± 0.011
5	0.476 ± 0.014	0.678 ± 0.016	0.061 ± 0.003

EPS obtained from the *N. ovalis* culture with the bacterial supernatant decreased (Table 3). A significant increase was observed compared to the control by quantifying the carbohydrate (Fig. 4A) and protein (Fig. 4B) concentrations in samples treated with the bacterial supernatant. (Bonferroni test Ns $P > 0.05$; $*P < 0.05$; $**P < 0.01$; $***P < 0.001$).

3.4. Chemical characterization of EPS

The FTIR spectra of soluble EPS from the *N. ovalis* control and with the bacterial supernatant showed similar characteristics: strong peaks at approximately 3389–3284, 2921, 1102–1099 cm^{-1} for O–H stretching, N–H bend, C–H bend and C–O stretching, respectively (Fig. 5). The band in the 1100 cm^{-1} region corresponds to C–O–C and C–O present in carbohydrates. The peaks at approximately 800–900 cm^{-1} correspond to a sensitive anomeric configuration of glucose, suggesting that the peaks observed at 870 and 711 cm^{-1} may be assigned to different glycosidic linkages. For EPS obtained from the microalga cultured with the bacterial supernatant, the FT-IR spectrum was similar to the EPS control, but the glycosidic bond area was altered and the signal at 871 and 934 cm^{-1} disappeared. It appears that are different compounds of different chemical nature. The FT-IR results revealed that the primary structural components of the diatom EPS (both for the control and with the bacterial supernatant) were carbohydrates. However, the N–H bend indicated the presence of proteins.

3.5. Metagenomic analysis of bacterial community characterization

Metagenomic analysis provided evidence for significant genetic diversity of the bacterial community associated with the diatom *N. ovalis*. For population analysis, rarefaction curves were plotted

Table 2
Carbohydrate and protein yield in soluble EPS extracted by three different methods. Two-way ANOVA, Bonferroni test $P > 0.05$.

Day	Carbohydrates ($\mu\text{g}/\text{mg}^{-1}$ of EPS)			Protein ($\mu\text{g}/\text{mg}^{-1}$ of EPS)		
	EtOH precipitation	NaOH	Ultrasonication	EtOH precipitation	NaOH	Ultrasonication
1	0.318	0.048	0.007	ND	0.052	ND
2	ND	0.084	0.011	0.003	0.091	0.001
3	0.011	0.222	0.059	ND	0.028	ND
4	0.002	2.588	0.033	ND	0.201	ND
5	0.023	0.016	0.024	ND	0.010	0.008

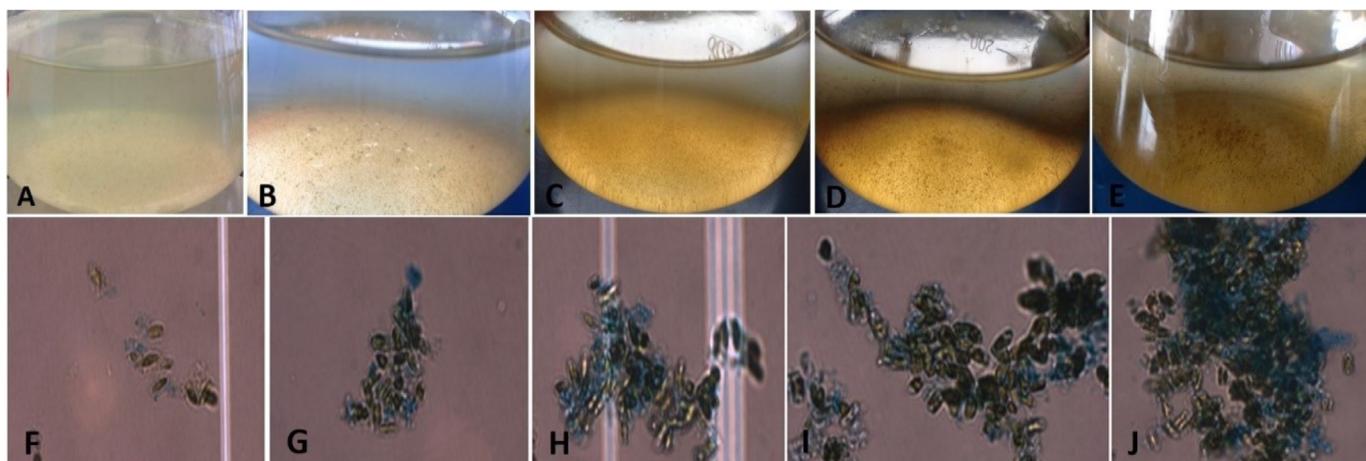


Fig. 2. Micrographs of *N. ovalis* stained with Alcian blue to visualize soluble EPS. A and F: d 1; B and G: d 2; C and H: d 3; D and I: d 4; E and J: d 5.

by means of read sampling (with 10,000 iterations, at an interval of 1000 reads) and by the calculation of OTUs observed at distance of 0.03. The results were used to characterize the genetic diversity of the community by calculating species richness parameters. Shannon and alpha indices (Table S3) are presented.

As can be observed in Fig. 6, the bacterial community in the control culture was dominated by members of the phylum Bacteroidetes (55.1% at day 0 and decreasing to 5.7% after 48 h) and Proteobacteria (43.3% at day 0 and increasing to 94.2% at 48 h). In comparison, in cultures with the bacterial supernatant, the epiphytic community decreased from 26.1% of Bacteroidetes at day 0 to 0.3% at 48 h. In contrast, the phylum Proteobacteria increased from 71.6% at day 0, to 99.5% at 48 h (Table 4). In both diatom cultures, the class

γ -Proteobacteria was represented primarily by bacteria of the genus *Marinobacter* (36.2%), and the class Bacteroidetes was represented by *Salinibacter* (22.9%). For the diatom cultured with the bacterial supernatant, we identified bacteria of the genera *Marinobacter* (40%), *Psychrobacter* (37.4%), *Salinibacter* (6.6%) and *Acetobacter* (3.9%).

4. Discussion

4.1. Protein and polysaccharide in EPS

EPS are key components required for biofilm formation by microalgae. While EPS are primarily used for adhesion to surfaces and between cells, they can also serve as a source of nutrients for

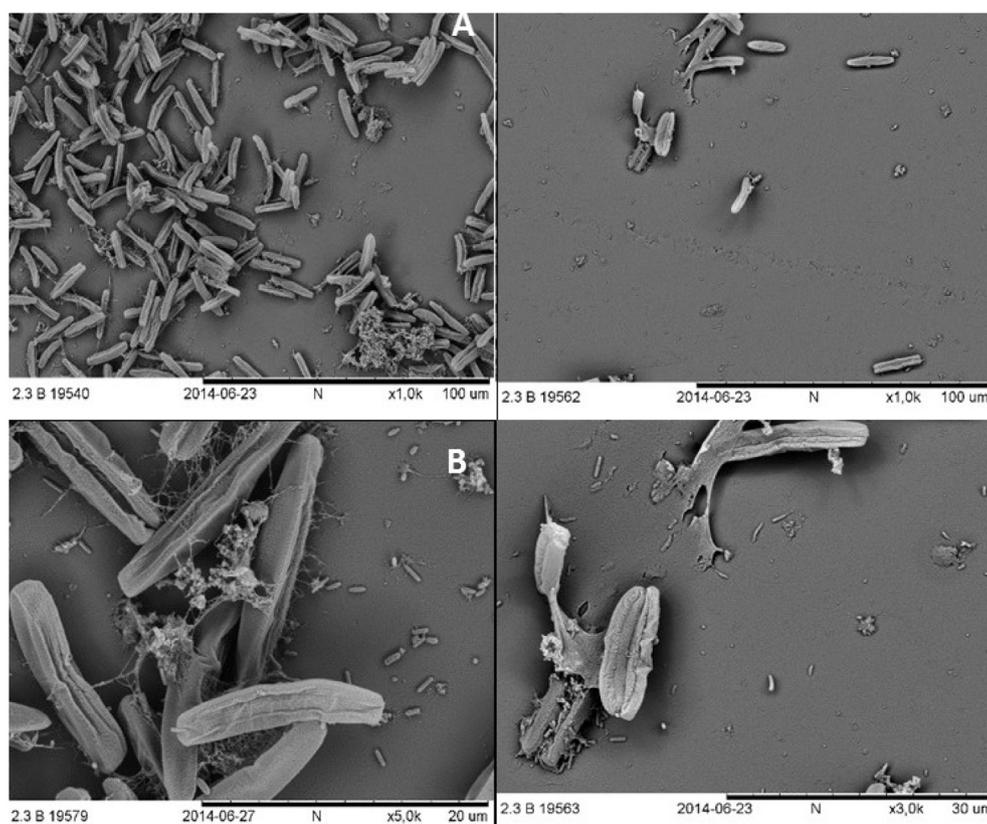


Fig. 3. Scanning electronic microscopy of the adherence assay for *N. ovalis*. Diatoms were cultured for 24 h with the bacterial compound. A–B: *N. ovalis* control; C–D: *N. ovalis* cultured with the bacterial supernatant ($100 \mu\text{g mL}^{-1}$ total protein). Scale bar: A and C: 100 μm ; B and D: 20 μm .

Table 3

EPS yield extracted from a *Nitzschia ovalis* Arnott control and with added bacterial supernatant. Two-way ANOVA. Bonferroni test $P > 0.05$.

Hour	Control		Treatment with Bacterial supernatant	
	EPS (mg/mg of diatom)	SD	EPS (mg/mg of diatom)	SD
24	0.191	0.067	0.259	0.052
48	0.227	0.015	0.122	0.023

heterotrophic bacteria. Marine benthic diatoms secrete large amounts of EPS, both for mobility and in response to environmental conditions [34,35,36]. The EPS of diatoms are primarily composed of polymers rich in carbohydrates and are important for cells found in marine sediments, increasing the stability of these environments [37,38]. Because of these aspects of EPS, their production is an essential means by which diatoms enter the marine food chain and affect the physical environment [39].

The optical microscopy, SEM and quantification of carbohydrates and protein results indicated that the bacterial supernatant secreted by *Alteromonas* sp. Ni1-LEM altered the structure of the EPS of *N. ovalis*. These compounds are fundamental to algae adhesion to a substrate and the formation of biofilms. According to the chemical characterization of EPS, the control samples had a different structure than those obtained from microalgae co-cultivated with the bacterial supernatant. Given the complexity of the signals, it is not possible to define the exact composition of the EPS analyzed, but the main components were carbohydrates and proteins.

The Dubois technique is based on the degradation of monosaccharides to 3-carbon compounds (hydroxymethyl) such that a furfural complex is formed with phenol. Although the protein denaturation depends on the temperature, at close to 41°C the disruption of interactions may occur. It been reported that the presence of 4 amino acids (cysteine, cystine, tryptophan and tyrosine) are responsible for the formation of a colorimetric complex with BCA. Therefore, the bacterial supernatant produced by *Alteromonas* sp. Ni1-LEM may disrupt EPS by acting upon the exopolysaccharide matrix, thus inhibiting the adherence of *N. ovalis*. For the EPS extraction methodology with NaOH, the use of formalin allows for the cells to be fixed when it reacts with amino, hydroxyl, carboxyl and sulfhydryl groups in the proteins present in the cell membrane, thus preventing cell lysis and avoiding intracellular contamination. The NaOH increases the pH, resulting in the dissociation of acidic groups in the EPS and the repulsion between negatively charged groups, increasing their solubility in water [40]. Thus, it is possible that the

obtained supernatant possesses EPS with a higher concentration of these polymers, unlike the other two methods. For this reason, although the protocol had a lower performance than the other two methods, obtained samples had a higher concentration of total protein and carbohydrate, which are the assayed parameters when assessing EPS extraction protocols.

The composition of EPS may vary depending on the extraction method [3,40,41]. According to Comte et al. [42], an increase in pH due to the presence of NaOH resulted in separation between acidic groups in EPS, and thus more EPS were extracted. Liu and Fang [40] showed that treatment of EPS with formaldehyde and NaOH was the most efficient method. However, the EPS obtained from diatoms have two general compositional characteristics: they are formed by heteropolysaccharides that may be sulfated; and they contain rhamnose, fucose, galactose, glucose, mannose, xylose and/or uronic acids and arabinose at very low amounts. Furthermore, diatomaceous EPS have large proportions of methylpentose compared to intracellular soluble polysaccharides and cell walls [14,43]. To date, the structure of diatomaceous EPS has not been determined, and only a few studies have presented information about the links present in these polysaccharides. However, available information shows a wide variety of links found in the EPS of diatoms, with many characteristic glycosyl residues of branched structures [41].

The results showed that the bacterial supernatant acted by disrupting the EPS matrix to inhibit the adhesion of microalgae and that the disruption of the formation of biofilm with other microalgal cells altered the composition of the epiphytic bacteria community.

4.2. Bacterial community analysis

Interactions between diatoms and bacteria is regarded as a key factor in biofilm formation, in addition to other biotic and abiotic factors that influence diatom EPS secretion, e.g., the stage of their cell cycle, the surface material [4] or available nutrients [39]. Bruckner et al. [44] demonstrated that bacteria have the potential to control diatom growth, EPS secretion and biofilm formation, whereas diatoms provide organic matter necessary for bacterial proliferation.

Studies examining the bacterial communities of diatom cultures have demonstrated that Proteobacteria and Bacteroidetes are the primary heterotrophic bacterial phyla associated with diatoms. Within these phyla, specific genera (e.g., *Alteromonas*, *Flavobacterium*, *Roseobacter* and *Sulfitobacter*) appear to be strongly associated with diatoms based on their repeated occurrence in different studies [45,46,47,48]. For example, over the course of 7 months, observations of bacterial community dynamics and their correlations to phytoplankton showed

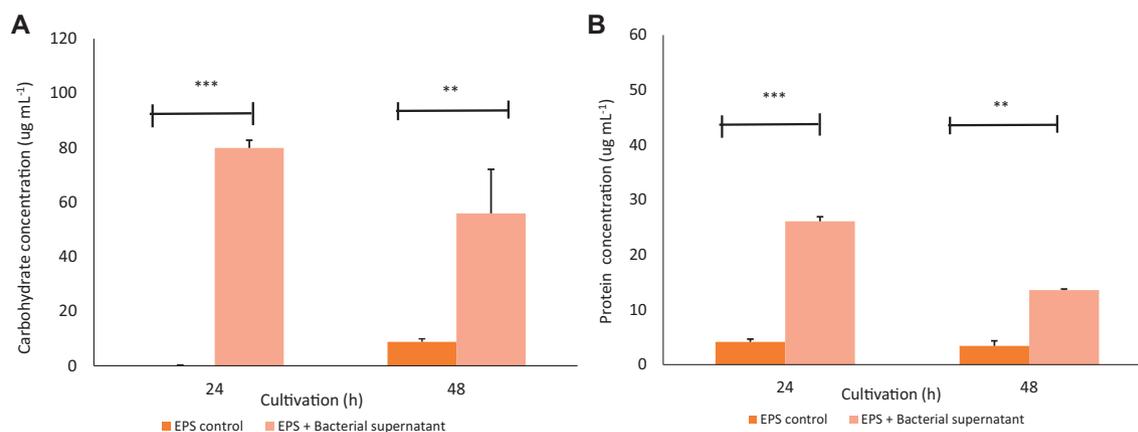


Fig. 4. (A) Quantification of total carbohydrates in soluble EPS extracted from *N. ovalis* cultures. Orange bar: control. Pink: EPS extracted from *N. ovalis* cultured with the bacterial supernatant. The bars on the columns represent standard error. Significant differences were determined using the Bonferroni test (Ns $P > 0.05$; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$). (B) Quantification of total protein in soluble EPS extracted from *N. ovalis* cultures. Diagonal lines: control. Dots: EPS extracted from *N. ovalis* cultured with the bacterial supernatant. The bars on the columns represent standard error. Significant differences were determined using the Bonferroni test (Ns $P > 0.05$; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

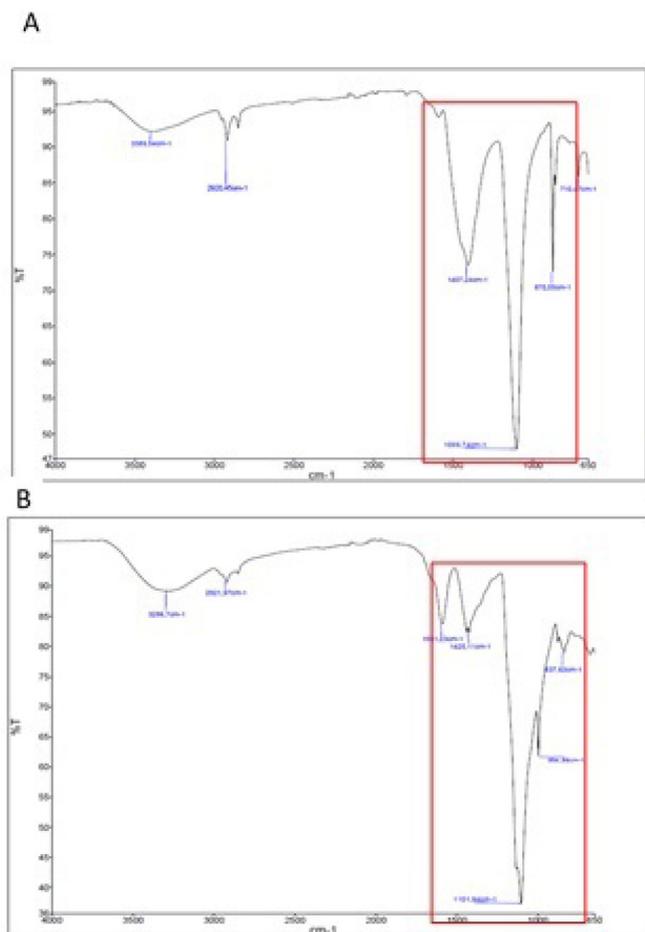


Fig. 5. Chemical analysis EPS extracted on day 1. FT-IR Spectra of EPS from day 1 of the *N. ovalis* culture. The red boxes indicate the differences in the signals. A: control culture; B: treatment with the bacterial supernatant (SN10X).

that *Roseobacter*, *Sulfitobacter*, and members of *Cytophaga* were among the most abundant bacterial genera present, in both the attached (>5- μm) and free-living (5- to 0.22- μm) fractions. In addition, bacterial numbers correlated more strongly with the abundance and diversity of diatoms than with other groups of phytoplankton, such as dinoflagellates [49].

Roseobacter, *Flavobacterium* species and members of the Gammaproteobacteria are the most dominant bacteria in phytoplankton blooms, and the abundance of these groups (determined by 16S rRNA iTag) often correlates with patterns of succession of the phytoplankton population [14,50]. The results of taxonomic classification showed that the bacterial community was mainly composed of members of the phyla Proteobacteria and Bacteroidetes. Both microalga cultures, the control and cultured with antifouling compound, had an abundance of α and γ Proteobacteria. These organisms are aerobic or facultative anaerobes and may use organic compounds, such as proteins and polysaccharides, as an energy source.

Marine Bacteroidetes have great potential in the metabolic degradation of proteins, bacterial cell wall components and polymer degradation [51]. Bacteroidetes has also been found to contribute to 15–30% of DMSP assimilation in the North Atlantic Ocean and in the Gulf of Mexico [52,53,54], which could represent a synergistic relationship between the members of the epiphytic community, characterized by the presence of producing microorganisms and other metabolizers of DMSP. These results relate to the epiphytic community of *N. ovalis* that use DMSP produced by the diatom.

Furthermore, it has been reported that this osmolyte, produced by phytoplankton and algae, provides protection to bacteria from salt stress [52,55], reflecting a symbiotic relationship between these microorganisms. These findings may account for the changes observed in the microbiota associated with the diatom during growth, where some species were displaced are replaced by others with appropriate adaptive capacities for the environmental conditions. For example, *Marinobacter* sp. could degrade organic matter available in cultures from microalgae that were not attached to the surface due to the action of the bacterial supernatant.

Flavobacteriaceae was another family present in the analysis. Although much less abundant than microalgae in the culture, studies suggest that Flavobacteria are more abundant in areas of upwelling, in oceans of temperate to polar and coastal regions and that bacterial communities in the open ocean usually contain 10–20% of Bacteroidetes, most of which are Flavobacteria [50,56]. In the context of phytoplankton blooms, the abundance of Flavobacteria is typically highest during their decline, and it is proposed that a central role for members of this group is the conversion of compounds of high molecular weight to compounds of low molecular weight [57,58]. In fact, the content analysis of genes from sequenced isolates, as well as the genomes of uncultivated environmental representatives, predicts that at least some *Flavobacterium* members are better suited to the use of complex substrates rather than simple monomeric compounds [59].

The ability of *Flavobacterium* to utilize a wide range of biopolymers, including polysaccharides and proteins [60], as carbon sources and primary energy may explain the various interactions that these bacteria appear to have, not only with eukaryotic phytoplankton but also marine vertebrates and mammals [61]. Although only a few metatranscriptomic and metaproteomic studies have been carried out so far, which have differed in terms of dominant phytoplankton species, geochemical conditions and geographical location, the common functional patterns are evident, especially for *Flavobacteria* [58,62,63].

Decho [64] showed that bacterial polysaccharides secretions enhance the survival and competitive success of microbial cells under natural conditions. A study by Shen et al. [65] suggests that natural heterotrophic bacterial communities have a role in the development of *Microcystis* blooms in natural waters. This is due to the production of EPS in non-axenic *M. aeruginosa*-created microenvironments that probably served to link both cyanobacterial cells and their associated bacterial cells into mutually beneficial colonies. *Microcystis* colony formation facilitates the maintenance of high biomass for a long time, and the growth of heterotrophic bacteria was enhanced by EPS secretion from *M. aeruginosa*. Windler et al. [28] observed that capsule and biofilm induction can be achieved by the addition of bacterial spent medium, indicating that soluble hydrophobic molecules produced by the bacterium may mediate the interaction between diatoms and bacteria. These studies suggest that the presence of heterotrophic bacteria can stimulate the EPS release by *N. ovalis*, since increasing EPS production may be a defensive response of diatoms to environmental stresses, which is a key aspect of biofilm formation. The extracellular molecules released by diatoms may be involved in the attachment of bacteria to the surface of the microalgae [66]. The bacteria would recognize the presence of the diatom and initiate attachment to EPS, which may also act as a nutrient source for bacteria. Several strains of bacteria have been found to influence EPS production when added to bacterial-free cultures of the diatom *Thalassiosira weissflogii*. These bacteria attached to the diatom and subsequently induced diatom cell aggregation. The mechanism appears to be highly dependent on the identity of the bacteria [67]. Bacteria also release exopolysaccharides in response to the presence of phytoplankton, likely to initiate attachment. Rinta-Kanto et al. [61] found evidence for this mechanism using next generation sequencing (NGS) technology to examine the transcriptional response of

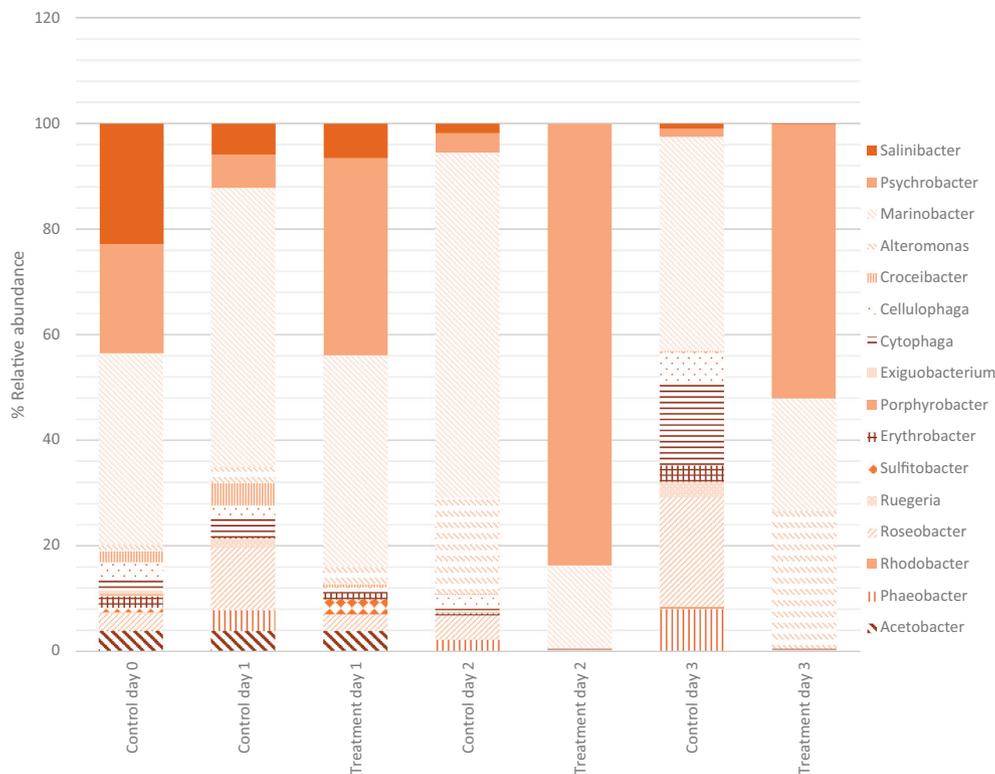


Fig. 6. Relative abundance of bacteria in the epiphytic community associated with the microalga *N. ovalis*. Nc: Control culture; SN: diatom cultured with the bacterial supernatant. The incubation time is also indicated.

heterotrophic bacteria to an induced diatom-dominated bloom in a microcosm compared to that of a non-bloom control. The authors observed significantly more abundant bacterial transcripts in the bloom than in the control, which corresponded to genes involved in dissolved organic matter (DOM) and organic acid uptake, as well as cell surface adhesion, such as EPS production.

5. Conclusions

This work is the first to investigate the relationship between the EPS produced by *Nitzschia ovalis*, the epiphytic bacterial community and diatom adhesion to a substrate. The yield of EPS from biofilms varied greatly with the extraction method. A gentle ultrasonic pre-treatment doubled the extraction yield without significant modification of the composition of EPS. However, the chemical method using paraformaldehyde and sodium hydroxide was a better method for carbohydrate and protein quantification. Metagenomic analysis revealed taxonomic diversity of bacteria associated with *N. ovalis*, and this diversity changed in response to the presence of the bacterial supernatant. Our results suggest that the culture supernatant

produced by *Alteromonas* sp. Ni1-LEM disrupts the structure of the EPS matrix, which may alter the composition and diversity of the epiphytic community and inhibit the adhesion of diatoms. This change in the bacterial community associated with *N. ovalis* has implications for the relationship between the bacteria and diatom. The results from our study can partially explain the role of EPS in biofilm formation and the composition of bacterial communities associated with *N. ovalis*, although the mechanisms behind the changes in the EPS composition and bacterial community resulting from the application of the antifouling compound requires further investigation.

Conflict of Interests

The authors declare no conflict of interest.

Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ejbt.2018.03.002>.

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Table 4
Bacterial dominance grouped by Phylum sampled from *Nitzschia ovalis* Arnot cultured alone (control) and with the bacterial treatment.

Phylum	Control		Treatment with bacterial supernatant	
	0 h	48 h	0 h	48 h
Bacteroidetes	55.1%	5.7%	26.1%	0.3%
Proteobacteria	43.3%	94.2%	71.6%	99.5%
Cyanobacteria	1.6%	0.1%	ND	ND
Actinobacter	ND	ND	0.9%	ND
Firmicutes	ND	ND	0.9%	0.4%
Other	0.01%	0.02%	<0.01	<0.01

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