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Abstract

At low tide, intertidal mudflat biofilms cover large surfaces and are mainly responsible for the high productivity of these marine areas. In the European Atlantic coast, such biofilms are mainly composed of diatoms, especially *Navicula phyllepta*, bacteria, and microbial extracellular polymeric substances (EPS). To better understand interactions occurring between microorganisms, we first axenized a *N. phyllepta* culture with a new and simple protocol. Colloidal and bound EPS secreted by diatom cells during the exponential growth and the stationary phase were then harvested, and we tested their effects on the in vitro formation of biofilms by three marine bacteria. The latter had been isolated from a French Atlantic intertidal mudflat and were previously selected for their strong in vitro biofilm-forming ability. They belong to the *Flavobacterium*, *Roseobacter*, and *Shewanella* genera. *Navicula phyllepta*-bound EPS synthesized during the stationary phase specifically inhibited the biofilm formation by the *Flavobacterium* sp. strain, whereas they stimulated biofilm development by the two other strains. The EPS acted in all cases during the first stages of the biofilm establishment. Saccharidic molecules were found to be responsible for these activities. This is the first report on marine bacterial antibiofilm saccharides of microalgal origin. This work points out the complexity of the benthic natural biofilms with specific microalgae/bacteria interactions and underlines the possibility to use axenic diatoms as a source of bioactive compounds.

Keywords

Diatoms
Bacteria
Marine biofilms
Exopolysaccharides
Algae-bacteria interactions

Isabelle Lanneluc and Sophie Sablé contributed equally to this work.

Introduction

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Intertidal mudflats are among the most productive marine areas (Admiraal et al. 1984), and they may provide up to 50 % of the primary production of estuaries, in particular due to the formation of photosynthetic biofilms at their

surface (Underwood and Kronkamp 1999). By providing shelter and food, mudflats especially serve as pantry and nursery for many animal species of commercial interest, including fishes (Beck et al. 2001).

One of the peculiarities of intertidal mudflat biofilms lies in their transient character. During daily tidal emersion periods, autotrophic motile microalgae (microphytobenthos) move upward through the sediment to the surface to access the light they need for photosynthesis (Consalvey et al. 2004). When microalgae and heterotrophic prokaryotes associate at the surface, they secrete a matrix of mucilaginous extracellular polymeric substances (EPS) and form a biofilm surrounding the sediment particles (Decho 2000; Consalvey et al. 2004). During immersion and night, microalgae migrate downward into deeper sediment layers (Consalvey et al. 2004), part of the biofilm biomass is resuspended into the water column, and the temporary biofilm disappears (Colijn 1982; Serôdio et al. 1997).

This transient biofilm is thus mainly composed of microalgae (Admiraal et al. 1984; Underwood and Kronkamp 1999), prokaryotes (mainly bacteria) (van Duyl et al. 2000), and a variety of EPS (Underwood and Paterson 2003) constituting complex assemblages of polysaccharides, proteins, glycoproteins, uronic acids, lipids, and many other compounds (Underwood et al. 2004; Pierre et al. 2012). The EPS matrix is believed to limit sediment desiccation by maintaining high pore water content, to stabilize sediment layers, and to protect microorganisms against exogenous stresses (Flemming and Wingender 2010; Orvain et al. 2014). Motile epipelic diatoms secrete into the surrounding sediment 30 to 60 % of photoassimilated carbon as EPS (Underwood et al. 1995; Middelburg et al. 2000; Smith and Underwood 2000). In European intertidal mudflats, epipelic diatoms dominate the microphytobenthic community, representing up to 97 % of the biomass (de Jonge and Colijn 1994; Méléder et al. 2005). In the French Atlantic coast, the dominant species is *Navicula phyllepta* (Haubois et al. 2005). Whereas bacteria are also very abundant, with generally 10^9 cells per mL of sediment (Pascal et al. 2009), their diversity and activity remain largely uncharacterized (Gontang et al. 2007). The bacterial communities depend on the depth of sediment layers, and Gammaproteobacteria might be highly predominant (almost 90 %) in surface layers (Urakawa et al. 2000).

Within mudflat biofilms, multiple interactions such as synergy, competition, or defense occur between microalgae and prokaryotes. Although these interactions are recognized as essential to the biogeochemistry of marine

ecosystems (Amin et al. 2012), their study is limited. There is a strong correlation between diatom and bacterial abundances (Cammen 1991; van Duyl et al. 1999; Hamels et al. 2001). The presence of bacteria closely associated to diatom cells (referred to as "satellite" bacteria by Schäfer et al. 2002) seems essential for some diatom strains to their optimal growth in nature as well as in laboratory cultures (Bruckner et al. 2008, 2011; Amin et al. 2012). Diatom cell aggregation is also enhanced in the presence of bacteria (Bruckner et al. 2008; Lubarsky et al. 2010; Gärdes et al. 2011). Bruckner et al. (2008) and Windler et al. (2015) showed that bacteria can stimulate diatom biofilm formation, as biofilm formation was directly induced by the cell-free spent medium of bacteria, making modification of the diatom metabolism most likely. It has been shown that the carbon photosynthetically assimilated by diatoms is transferred into bacteria in few hours, indicating a rapid use of labile carbon sources, likely including EPS (Middelburg et al. 2000; Goto et al. 2001; Cook et al. 2007; Bellinger et al. 2009). To remain within the "phycosphere" where their growth is stimulated by algal EPS (Bell and Mitchell 1972), bacteria often need to attach to the surface of diatom cells. Mechanisms underlying attachment remain unclear, but they likely involve extracellular molecules such as polysaccharides and/or proteins that are also released by bacteria themselves (Amin et al. 2012). EPS are thus crucial intermediaries between bacteria and diatoms. However, interactions between diatoms and bacteria are not always beneficial: ~~M~~many diatoms have defense mechanisms against unwanted and/or algicidal bacteria (Amin et al. 2012). They secrete fatty acids, esters, and polyunsaturated aldehydes that can act as antibacterial compounds and influence the bacterial community structure (Lebeau and Robert 2003; Ribalet et al. 2008). However, some diatom-associated bacteria were shown to be resistant to bioactive polyunsaturated aldehydes, suggesting that satellite bacteria may have developed resistance to toxic molecules released by diatoms (Ribalet et al. 2008). To our knowledge, nothing is known about the effect of molecules excreted by diatoms in their immediate surroundings on the bacterial biofilm formation.

We previously selected three bacterial strains from intertidal mudflat biofilms of the French Atlantic coast that were able to attach to different surfaces and build biofilms with specific 3D structures (Doghri et al. 2015). These strains are appropriate models for deciphering the colonization of surfaces and the interactions between microorganisms in marine biofilms. Such knowledge is essential to the better understanding of biofouling which causes various and

costly damages to maritime human activities and industries. Here, we investigated the specific effects of diatom organic EPS on the formation of bacterial biofilms. A *N. phyllepta* isolate was selected since this species is one of the most abundant in temperate intertidal mudflats. To avoid the presence of EPS secreted by satellite bacteria, we axenized *N. phyllepta* prior to examine the effects of different EPS fractions on the bacterial models.

Materials and methods

Diatom and bacterial strain isolation and culture

Benthic diatom *Navicula phyllepta* Kützing (Culture Collection Yerseke, CCY9804) cells were grown in batch cultures at 20 °C in sterile artificial F/2 seawater medium (Tropic Marin salt, Guillard and Ryther 1962) with a light intensity of 75 μmol photons m^{-2} s^{-1} and a 16-h light/8-h dark photoperiod (white fluorescent tubes, L58W/840, Osram, Germany). Diatom cultures were started with a concentration of 0.3 μg chlorophyll *a* (Chl *a*) mL^{-1} using a Chl *a* spectrophotometric quantification (Jeffrey and Humphrey 1975). Specific growth rates, μ (day^{-1}), were calculated from regression of the natural logarithm of the Chl *a* (mg mL^{-1}) during their exponential growth phase. During its growth, *N. phyllepta* forms a biofilm in the flask bottom that can be visually observed.

Benthic bacteria We used strains *Flavobacterium* sp. II2003, *Roseobacter* sp. IV3009, and *Shewanella* sp. IV3014. They were isolated from the intertidal temperate mudflat biofilm of the Marennes-Oléron Bay (French Atlantic coast) and selected for their ability to form biofilms on different surfaces, under static and dynamic conditions (Doghri et al. 2015). Strains were conserved as frozen stocks with 25 % glycerol at −80 °C. For all tests, the bacteria were grown at 22 °C in Zobell broth (Bio-Rad pastone 4 g L^{-1}; Bio-Rad yeast extract 1 g L^{-1}; sea salts Sigma 30 g L^{-1}) supplemented with agar (12 g L^{-1} Biokar) for solid medium.

Axenization of *N. phyllepta*

Different axenization strategies were investigated, combining mechanical cell separation and antibiotic treatments (Fig. 1 and Table 1). Based on Shishlyannikov et al. (2011), two pretreatments of the diatom suspensions were tested and compared with a non-pretreated control (Fig. 1, “Physico-chemical pretreatments”). The suspensions, pretreated with or without Triton X-100, were filtered through a 5-μm polycarbonate membrane (Millipore,

USA) put on a Whatman membrane by using a manual filtration unit (Sartorius, Germany). Diatom cells were then washed with artificial seawater and transferred to F/2 medium. Different antibiotics, alone or in combination (Table 1), were added to the pretreated or the control suspensions, and diatom cells were grown as described above, with soft shaking at regular time intervals (Fig. 1, "Antibiotic treatments"). The final concentrations of the tested antibiotics are described in Table 1. Diatom cells were then harvested by filtration, rapidly washed with culture medium, and resuspended in F/2 medium. The degree of axenization of diatom cell suspensions was then immediately checked by (i) bacterial counting with epifluorescence microscopy after 4′6-diamidino-2-phenylindole (DAPI) staining and (ii) counting the number of colony forming units (CFU—culturable bacteria) after plating diatom cell suspensions on Zobell agar (Fig. 1, "Checking"). For the microscope counting, diatom cell samples were fixed in formaldehyde (final concentration 2 %) and diluted in di-sodium pyrophosphate 10 mM with 0.1 % of Tween 80 in order to enhance detachment of bacteria from the diatoms (Lavergne et al. 2014). After incubation for 30 min at 4 °C, samples were stained with DAPI (1 mg L^{-1}) for 15 min and filtered through a 0.2-µm polycarbonate membrane (Nuclepore Track-Etch Membrane, Whatman Schleicher & Schuell, USA). The membranes were mounted on a glass slide in a non-fluorescent oil drop, and bacterial cells were counted with an epifluorescence microscope (Leica DMRB, mercury steam light, magnification ×1000, filter UV A Leica) on an average of five fields of 0.0415 mm^2 each.

Fig. 1

Key steps of the axenization protocol of the *N. phyllepta* diatom culture. Protocol finally selected (*bold arrows*)

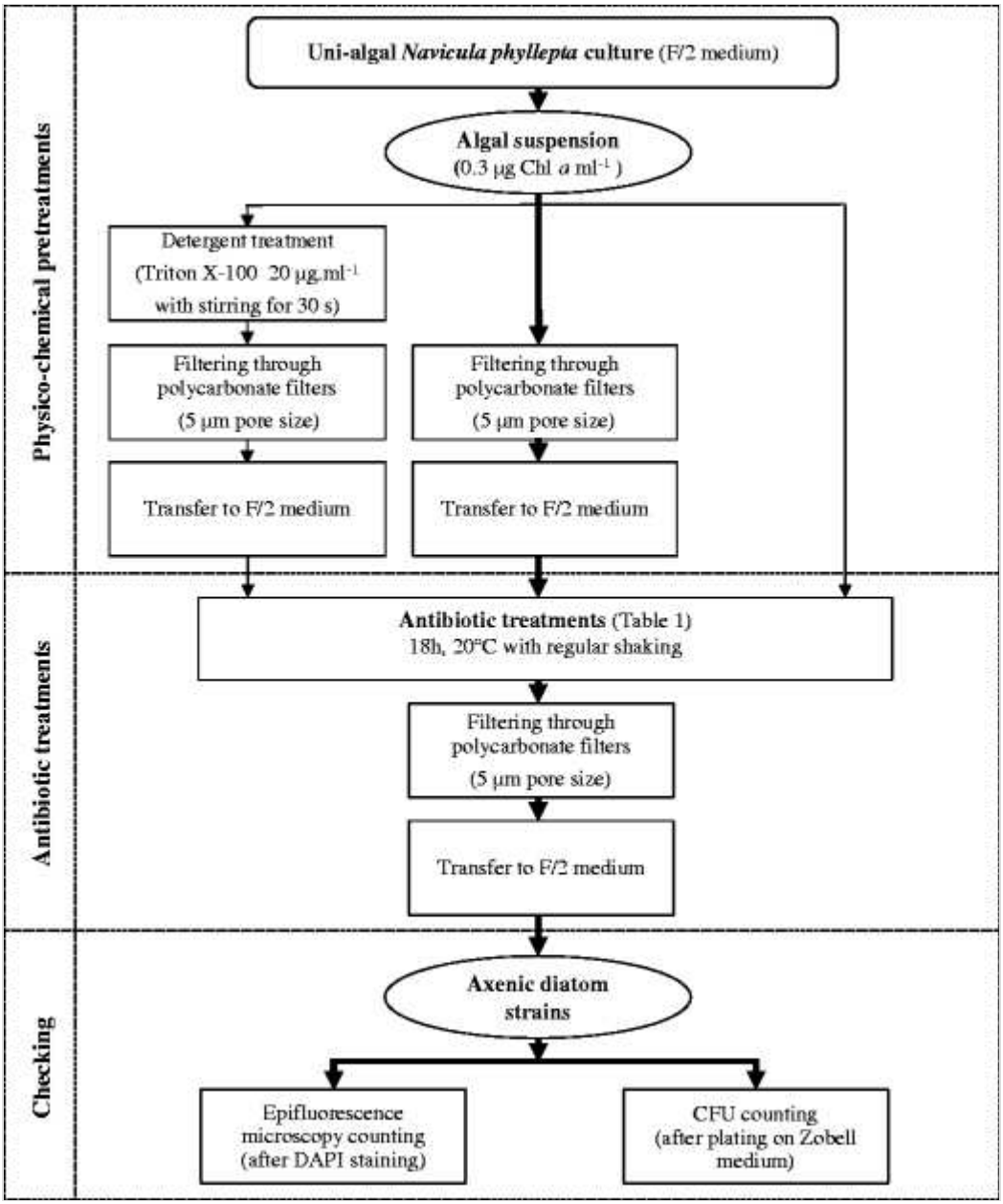


Table 1

Antibiotics tested alone or in combination for the axenization of the *N. phyllepta* cultures

Antibiotics	Solvent(s) (%/%)	Final concentration (µg mL^{-1})
Ap	Ethanol/water (50/50)	100
Tc	Ethanol/water (50/50)	50
Sm	Water	100
Km	Water	50
Km + Sm	Water	100/100

Ap ampicillin, *Tc* tetracycline, *Sm* streptomycin, *Km* kanamycin, *Cip* ciprofloxacin, *Ipm* imipenem

Antibiotics	Solvent(s) (%/%)	Final concentration (µg mL^{-1})
Ap + Sm	Ethanol/water (25/75)	100/100
Ap + Tc + Km	Ethanol/water (25/75)	100/50/30
Ap + Tc + Sm + Km	Ethanol/water (25/75)	100/50/100/30
Cip	Water	5
Ipm	Water	98

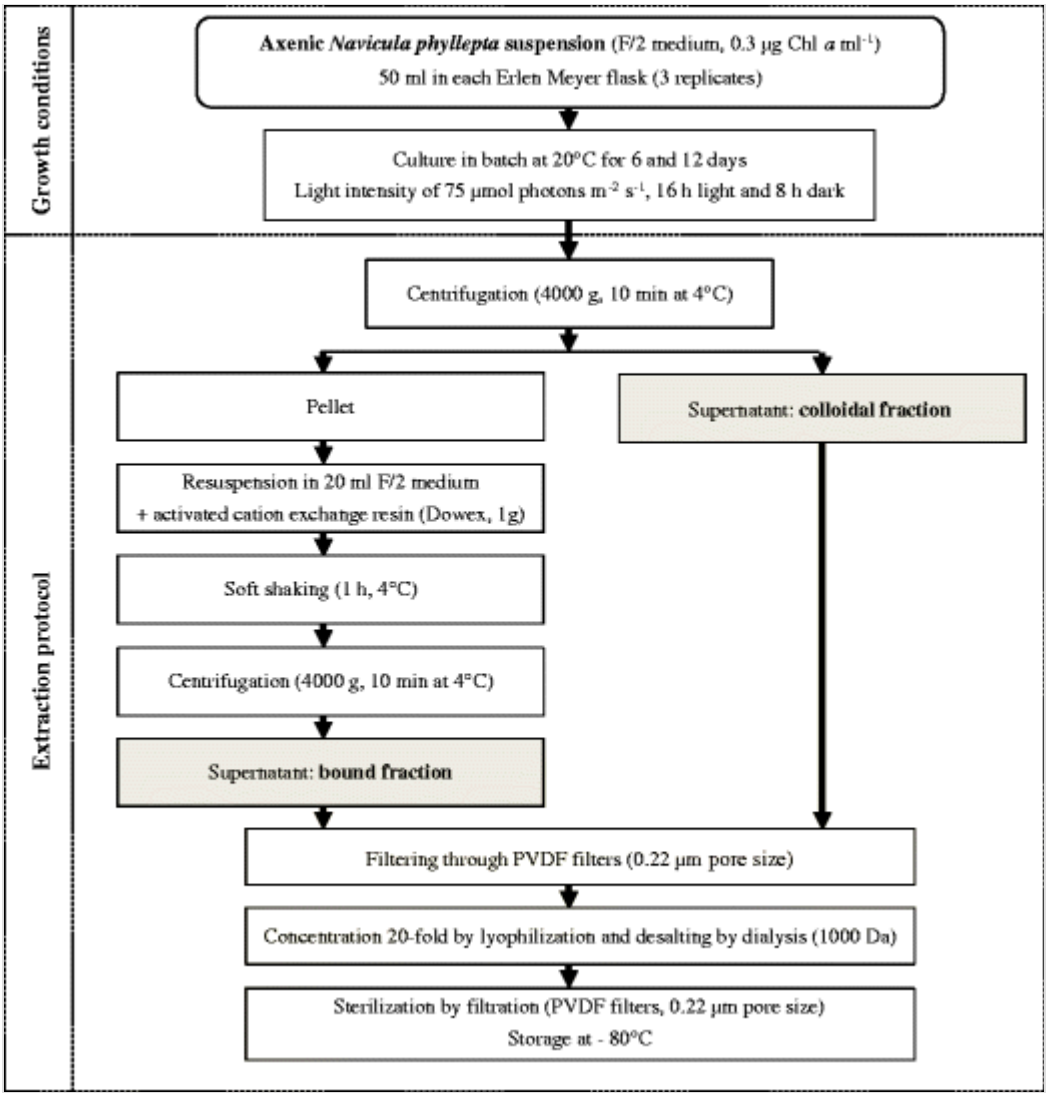
Ap ampicillin, *Tc* tetracycline, *Sm* streptomycin, *Km* kanamycin, *Cip* ciprofloxacin, *Ipm* imipenem

Extraction of EPS from axenic cultures of *N. phyllepta*

The detailed protocol was adapted from Takahashi et al. (2009) and is summarized in Fig. 2. The axenic *N. phyllepta* suspension was cultured during 6 days (D_6, middle of the exponential phase, 1.16 µg Chl *a* mL^{-1}) and 12 days (D_{12}, stationary phase, 1.24 µg Chl *a* mL^{-1}) in order to collect the colloidal and bound EPS. D_6 and D_{12} samples were centrifuged to harvest the supernatants containing the colloidal EPS fraction. To extract EPS bound to the diatom cells, the pellets were resuspended in F/2 medium containing activated cation exchange resin (Dowex Marathon C Na^+, Sigma-Aldrich, USA) and left under gentle shaking. After centrifugation, both colloidal and bound EPS fractions were filtered (Millipore PVDF filters, USA) and lyophilized. The dried fractions were resuspended in 1 mL ultra-pure water. The salt excess was removed by dialysis (molecular cutoff of 1000 Da, Spectrum Labs, USA) against ultra-pure water, and EPS samples were then stored at −80 °C until further use.

Fig. 2

Protocol for the extraction of EPS from axenic *N. phyllepta* cultures



Antibacterial assay

Antibacterial (i.e., bactericidal or bacteriostatic) activity of EPS was tested by using the agar well diffusion assay previously described by Sablé et al. (2000). Sterile glass rings (4-mm inside diameter) were placed on solid nutrient plates (15 mL of Zobell agar inoculated with 10^7 of the target bacteria) and filled with 30 µL of the EPS 20× samples to be tested. The plates were incubated at 22 °C for 48 h to allow bacterial growth and EPS diffusion. The presence of a halo around the glass cylinder indicates an inhibition of bacterial growth if the halo is clear (without cell growth) or eventually a stimulation if the halo is denser than the remaining plate (with cell growth).

Effect of the diatom EPS on bacterial biofilm formation

Microtiter plate assay: static conditions According to Pitts et al. (2003), the EPS fractions were mixed to bacterial suspensions during the adhesion step. After overnight growth (Zobell broth, shaking at 150 rpm,

22 °C) and harvesting by centrifugation (7000×*g*, 10 min), bacteria were resuspended in artificial seawater (for the control) or in EPS 20× fractions to a final optical density at 600 nm (OD_{600}) of 0.25. Of the resulting suspensions (10^8 CFU mL^{-1}), 150 µL was then loaded per well of a 96-well microtiter plate (MICROTEST 96, Falcon, USA). After a bacterial attachment step of 2 h at 22 °C, the wells were gently washed three times with artificial seawater, and 150 µL Zobell medium were added to each well. After incubation at 22 °C for 24 h, the bacterial biofilms were washed three times with artificial seawater, stained with a 0.8 % crystal violet solution for 20 min, and rinsed with ultra-pure water until the wash liquid was clear (10 times on average). Crystal violet was then eluted from attached bacteria with 96 % ethanol (150 µL $well^{-1}$), and the absorbance was measured with a plate reader at 595 nm (FLUOstar Omega, BMG Labtech, Germany) to quantify the biofilms.

Flow cell assay: dynamic conditions Bacterial biofilms were grown on glass slides in continuous culture three-channel flow cells (channel dimensions 1 by 4 by 40 mm, Technical University of Denmark Systems Biology, Denmark) as described by Pamp et al. (2009). Flow cells were inoculated with 24-h old bacterial cultures diluted in artificial seawater (for the control) or in EPS 20× fractions to a final OD_{600} of 0.1. Bacteria were allowed to attach to the glass slide during 2 h at 22 °C without medium flow. Channels were washed to remove non-attached bacteria by applying a flow of artificial seawater for 15 min at a rate of 2 mL h^{-1}, and biofilm growth was allowed for 24 h at 22 °C under a constant flow (2 mL h^{-1}) of Zobell medium. The biofilms were then stained with 5 µM Syto 61 Red and observed by confocal laser scanning microscopy (CLSM) using a TCS-SP2 system (Leica Microsystems, Germany). The biofilm stacks were analyzed with the COMSTAT software (developed in MATLAB, Heydorn et al. 2000) to estimate the maximal and the average thicknesses (µm) as well as the biovolume (μm^3 μm^{-2}) of each biofilm.

Biochemical assays and characterization of the diatom active EPS fraction

Proteins assays were carried out with a bicinchoninic acid protein assay kit (Sigma-Aldrich, USA), using bovine serum albumin as a standard (Smith et al. 1985). The sugar content was determined using the phenol-sulfuric acid assay, using glucose as standard (Dubois et al. 1956). To elucidate the biochemical nature of the active component(s) of diatom EPS 20× fractions,

different treatments were performed to digest proteins, lipids, nucleic acids, and saccharides. Proteinase K or Pronase E was added to a final concentration of 1 mg mL^{-1}, and the reaction was incubated for 1 h at 37 °C. Lipase acrylic resin form was used at a final concentration of 2 mg mL^{-1}, and the reaction was incubated for 48 h under shaking at 37 °C. DNaseI (100 μg mL^{-1}) or RNaseA (25 μg mL^{-1}) was added for 12 h at 37 °C. $NaIO_4$ was used to hydrolyze saccharides by cleaving the C–C bonds and by oxidizing the carbon of vicinal hydroxyl groups (Mack et al. 1996; Bendaoud et al. 2011). We improved the classical treatment (20 mM $NaIO_4$, incubation for 12 h at 37 °C) by adding a neutralization step of $NaIO_4$ with ethylene glycol (1:100) (Babor et al. 1973) for 2 h at 37 °C followed by a final dialysis step (1000 Da). Each treated EPS fraction was mixed to bacterial suspension during the adhesion step, and a microtiter plate assay was performed as previously described.

Statistical analyses

All values presented in the "Results" part are the average of three independent experiments. In order to analyze differences between a sample and the corresponding control, Student's *t* tests were performed. ANOVA tests gave the same results.

Results

Axenization of the *N. phyllepta* diatom from associated bacteria

The uni-algal *N. phyllepta* cultures were non-axenic, and the initial suspension (0.3 μg Chl *a* mL^{-1}) contained in average about 10^6 bacteria mL^{-1} (Table 2). In order to test the effect of EPS specifically secreted by *N. phyllepta* and to avoid interference with EPS synthesized by the bacteria associated with diatom cells, it was crucial to remove bacteria present in these cultures. Different antibiotic treatments as well as pretreatments potentially increasing the effectiveness of antibiotics were then investigated (Fig. 1). While the detergent treatment associated with the filtration reduced the bacterial load by 40 % (Table 2), it considerably affected the viability of the diatom cells (Chl *a* in cultures decreased by more than 50 %) and the ability of *N. phyllepta* to form a biofilm (visual observation of a brown layer of diatom cells in the flask bottom). The filtration without detergent treatment reduced the bacterial contamination by up to 26 % (Table 2) without affecting viability and culture aspect of diatoms. This pretreatment was therefore chosen. After validation of this step, different antibiotics (ampicillin [Ap],

ciprofloxacin [Cip], imipenem [Ipm], kanamycin [Km], streptomycin [Sm], and tetracycline [Tc]) were tested, alone or in combination. The treatments including Ap, Tc, or Ipm were quickly discarded owing to the modification of the visual aspect (i.e., pigmentation), the ability to form a biofilm, and the growth rate of the diatom cultures. The results obtained with the most promising antibiotics are shown in Table 2. Cip and Sm treatments reduced the bacterial contamination by 94 % according to microscope counting and by 60 and 73 % according to agar medium plating, respectively. Km treatment also reduced significantly the bacterial contamination, in particular the bacteria growing on agar medium. Nevertheless, the strongest effect was exerted by the Km + Sm mix which completely removed bacterial contamination.

Table 2

Enumeration of bacteria associated with *N. phyllepta* before and after physico-chemical and antibiotic treatments

	Microscope counting (bacteria mL^{-1})	**Counting by plating on agar medium (CFU mL^{-1})**
Initial algal suspension without treatment	$1.1 \times 10^6 \pm 1.4 \times 10^4$	$1.9 \times 10^5 \pm 3.1 \times 10^4$
	~~$1.9 \times 10^5 \pm 3.1 \times 10^4$~~	
Physico-chemical pretreatment		
Filtering	$8.5 \times 10^5 \pm 10^4$	$1.4 \times 10^5 \pm 2.7 \times 10^4$
Detergent + filtering	$6.8 \times 10^5 \pm 1.3 \times 10^4$	$1.1 \times 10^5 \pm 2.4 \times 10^4$
Antibiotic treatment after filtering[a]		
Cip	$6.5 \times 10^4 \pm 1.1 \times 10^4$	$7.5 \times 10^4 \pm 7.8 \times 10^3$
Sm	$6.2 \times 10^4 \pm 6.1 \times 10^3$	$5.1 \times 10^4 \pm 10^2$
Km	$5.1 \times 10^4 \pm 1.1 \times 10^4$	$4.0 \times 10^2 \pm 7$
Km + Sm	No bacterium detected	No bacterium detected
Cip ciprofloxacin, *Sm* streptomycin, *Km* kanamycin		
[a]Antibiotics not toxic for *N. phyllepta*~~;~~		

To summarize the selected protocol of axenization, the algal suspension was first filtered, treated with Km and Sm for 18 h, again filtered, and transferred

in new culture medium. The growth rate of the obtained axenic diatom cultures was ~1.2 day^{-1}, similar to untreated diatom cultures (data not shown).

Effects of the *N. phyllepta* EPS fractions on bacterial biofilm formation

In order to observe biological effects, *N. phyllepta* EPS were concentrated. Based on the EPS protein and saccharide contents, a 20× concentration was chosen, to reach concentrations slightly higher than what was observed in the intertidal mudflats where *N. phyllepta* is the major diatom (Pierre et al. 2012).

Antibacterial assays, performed with the agar well diffusion techniques, did not exhibit any clear halo characteristic of inhibition of bacterial growth. These experiments then showed that no EPS fractions from diatom cultures had a bactericidal or bacteriostatic activity against the three tested marine bacterial strains: *Flavobacterium* sp. II2003, *Roseobacter* sp. IV3009, and *Shewanella* sp. IV3014. Moreover, no stimulation of the bacterial growth (halo denser than the remaining plate) was observed around the deposit of EPS fractions.

We then examined whether EPS fractions affected biofilm formation. In static conditions (on polystyrene surface, microtiter plate assay, quantification of biofilms by spectrophotometry), the D_6 colloidal and bound EPS fractions (Fig. 3a), as well as the D_{12} colloidal EPS fraction (Fig. 3b), did not show any significant activity on bacterial biofilm formation. On the contrary, the D_{12} bound EPS fraction showed significant effects: ~~C~~compared with the not treated control biofilms, the biofilms treated with this EPS fraction were reduced by more than 40 % for *Flavobacterium* sp. II2003 ($t = -13.63$; $p < 0.01$) and were increased by more than 30 % for *Roseobacter* sp. IV3009 ($t = 6.97$; $p < 0.05$) and *Shewanella* sp. IV3014 ($t = 11.99$, $p < 0.01$) (Fig. 3b). In a second set of experiments, the activity of the D_{12} bound EPS fraction on bacterial biofilm formation was further tested by growing the biofilms under dynamic conditions (on glass surface, flow cell assay, observation of biofilms by confocal laser scanning microscopy). The main aim of this set of experiments was to confirm EPS effects obtained under static conditions. The antibiofilm activity against *Flavobacterium* sp. II2003 was also observed in the second conditions. Compared with the untreated sample, the treated biofilm was very thin: −76 % of the biovolume ($t = -6.82$,

$p < 0.01$), −47 % of the average thickness ($t = -5.89$, $p < 0.01$), and −60 % of the maximal thickness of the biofilm ($t = -13.20$, $p < 0.001$), and it lost its mushroom-like 3D structures (Fig. 4a). Under dynamic conditions, the *Roseobacter* sp. IV3009 biofilm seemed to be still stimulated compared with the untreated sample, but this positive effect of D_{12} bound EPS was not significant anymore ($t = 1.41$, $p = 0.24$ for the biovolume, $t = 1.78$, $p = 0.25$ for the maximum thickness, and $t = 2.28$, $p = 0.09$ for the average thickness) (Fig. 4b). However, the stimulating effect of D_{12} bound EPS was maintained for *Shewanella* sp. IV3014 (Fig. 4c): ~~A~~an increased biovolume (+40 %, with $t = 3.59$, $p < 0.05$) and increased average thickness (+43 %, with $t = 4.64$, $p < 0.01$) and maximal thickness (+37 %, with $t = 3.62$, $p < 0.05$) were observed, without modification of the hairy architecture compared with the control biofilm. The results obtained under static and dynamic conditions were thus consistent for *Flavobacterium* sp. II2003 and *Shewanella* sp. IV3014.

Fig. 3

Effect of EPS fractions from axenic *N. phyllepta* cultures on bacterial biofilm formation in static conditions. *Flavobacterium* sp. II2003, *Roseobacter* sp. IV3009, and *Shewanella* sp. IV3014 were mixed during the adhesion step in microplates with the EPS fractions collected after 6 (D_6) or 12 (D_{12}) days of the *N. phyllepta* growth. Biofilms were stained with crystal violet and quantified by measuring absorbance at 595 nm. **a** D_6 EPS fractions and **b** D_{12} EPS fractions. Untreated control: bacterial biofilm without any diatom EPS fraction. The data represent mean values ± SD of three replicates. Each treated biofilm was compared with the corresponding untreated control biofilm. $*p < 0.05$ or $**p < 0.01$, significant differences, only observed with D_{12} bound EPS fractions (**b**)

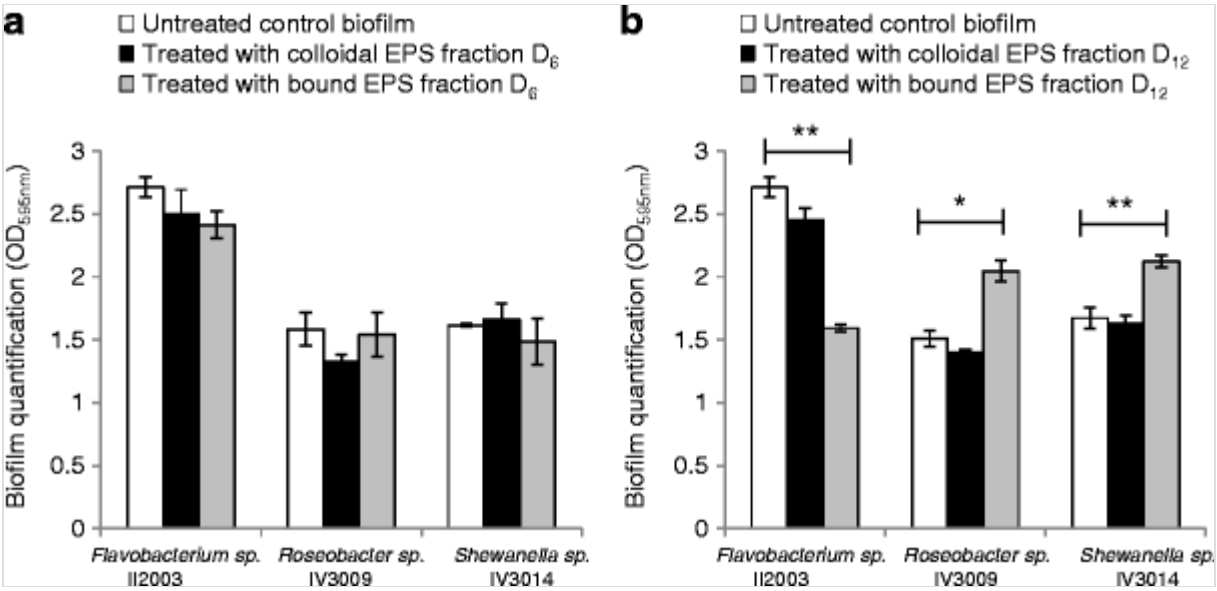
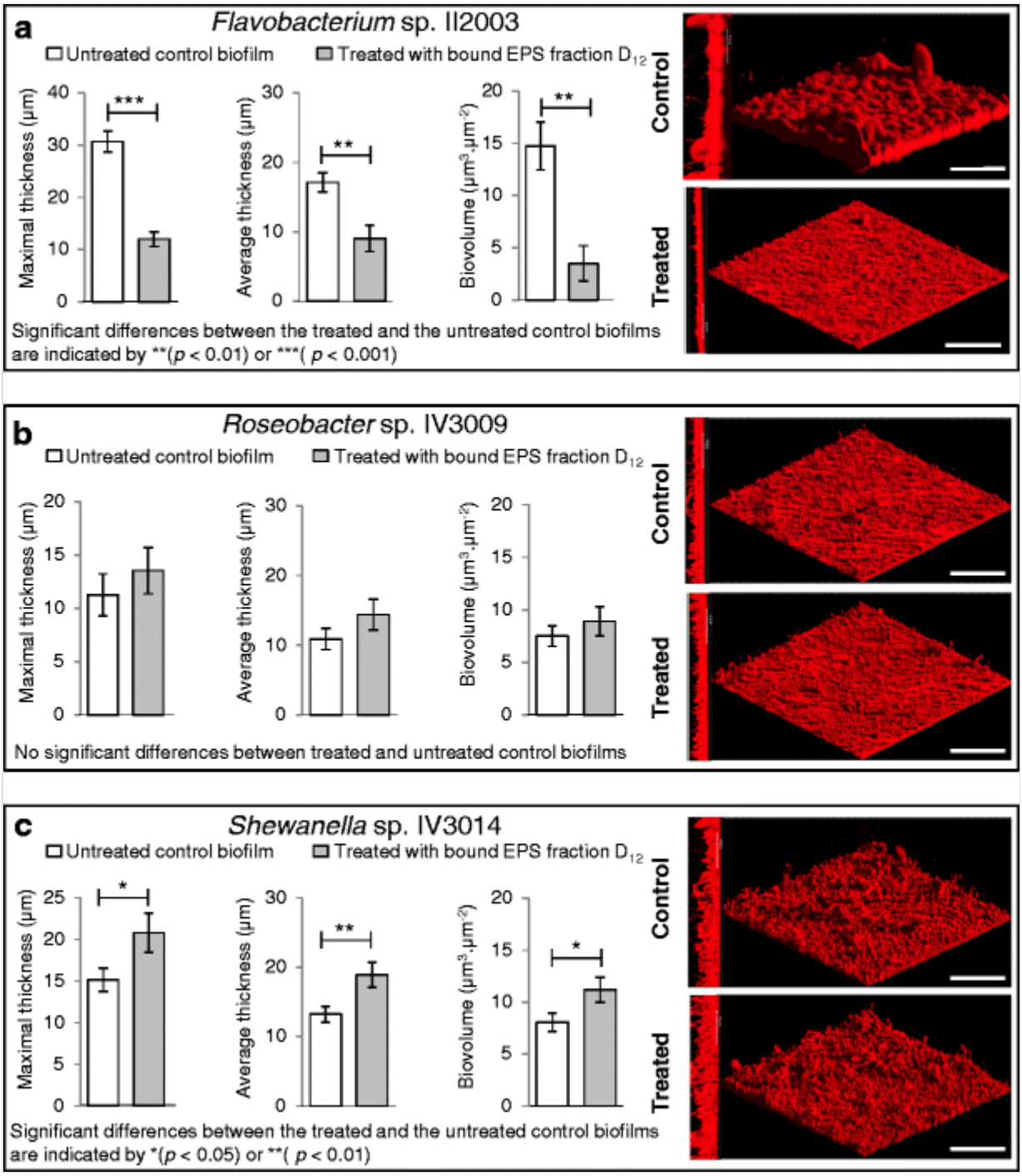


Fig. 4

Effect of EPS bound fraction D_{12} from axenic *N. phyllepta* cultures on bacterial biofilm formation in dynamic conditions. Biofilms of *Flavobacterium* sp. II2003 (**a**), *Roseobacter* sp. IV3009, (**b**) and *Shewanella* sp. IV3014 (**c**). 3D images of the biofilms (*top* and *side views*) are shown on the *right* (*bars* = 67.3 μm) and the corresponding COMSTAT analysis output on the *left*. Untreated control biofilm: bacterial biofilm without diatom EPS fraction. The data represent mean values ± SD of three replicates



Biochemical characterization of the *N. phyllepta* active EPS fraction

The carbohydrate and protein contents of all EPS fractions were quantified (Table 3), in order to compare the active and inactive fractions. It is noticeable that carbohydrate and protein quantities were higher in D_{12} than in D_6 fractions. The measure of the chlorophyll content of the cultures allowed us to evaluate the number of diatoms and then to show that the quantities of carbohydrates and proteins per cell were higher in the D_{12} fractions than in the D_6 fraction. Moreover, the carbohydrate content was higher than the protein content in all fractions, and the active fraction (D_{12} bound EPS) was

the most concentrated in sugars. *Navicula phyllepta* D_{12} bound EPS fraction was further analyzed. It was submitted to different treatments to gain information on the chemical nature of the putative active compounds (Fig. 5). Neither proteases nor lipase nor nuclease treatments significantly affected the activity of the D_{12} bound EPS fraction. Only the $NaIO_4$ treatment significantly reduced the antibiofilm activity of the D_{12} bound EPS fraction, by 90 % ($t = 18.53$, $p < 0.001$), against *Flavobacterium* sp. II2003, and totally prevented the stimulating activity in *Roseobacter* sp. IV3009 ($t = -13.8$, $p < 0.001$) and *Shewanella* sp. IV3014 ($t = -39.5$, $p < 0.001$). $NaIO_4$ very efficiently hydrolyzes saccharides by oxidizing the carbons bearing vicinal hydroxyl groups and cleaving the C–C bonds (Mack et al. 1996; Jiang et al. 2011). These results indicated that the active compounds of the D_{12} bound EPS fraction were saccharidic molecules.

Table 3

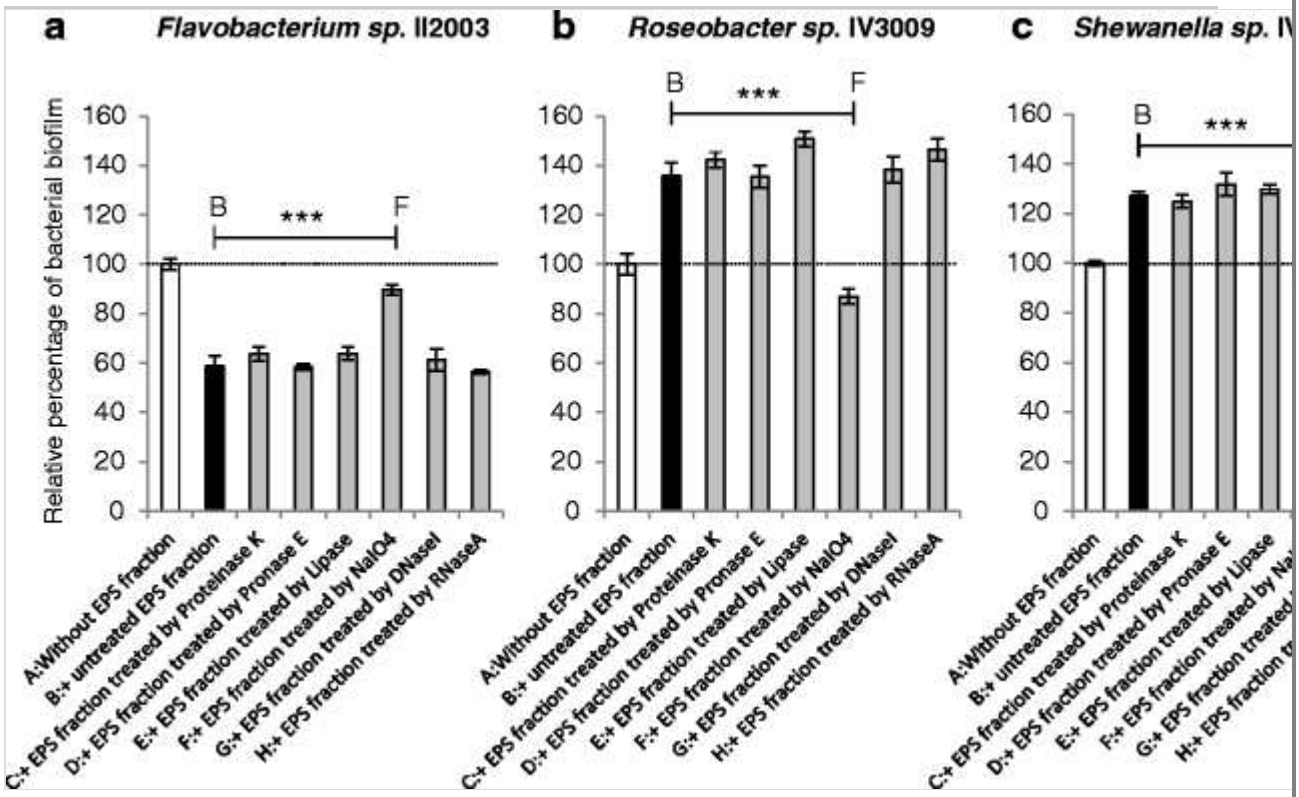
Determination of protein and carbohydrate contents of the *N. phyllepta* EPS 20× fractions and estimation of these contents per diatom cell

	D_6 EPS fractions		D_{12} EPS fractions	
	Colloidal	Bound	Colloidal	Bound
Proteins (mg L^{-1})	36.3 ± 2.9	45.9 ± 2.3	114.0 ± 8.7	151.0 ± 5.4
Proteins (mg $cell^{-1}$)	6.2×10^{-8}	7.9×10^{-8}	1.8×10^{-7}	2.4×10^{-7}
Carbohydrates (mg L^{-1})	45.0 ± 3.5	198.0 ± 9.1	178.0 ± 6.9	690.0 ± 2.4
Carbohydrates (mg $cell^{-1}$)	7.7×10^{-8}	3.4×10^{-7}	2.9×10^{-7}	1.1×10^{-6}

Fig. 5

Effect of various treatments on the activity of the *N. phyllepta* D_{12} bound EPS. The inhibiting or stimulating effects of the *N. phyllepta* D_{12} bound EPS were determined using the microtiter plate assay. Biofilms were stained with crystal violet and quantified by measuring absorbance at 595 nm. The "100 %" values on the *Y*-axis are given to the biofilms grown without EPS fraction (**aA**). Samples + untreated EPS fraction (**bB**) correspond to reference values of the inhibiting or stimulating effects of the *N. phyllepta* EPS on the bacterial biofilm formation. The data represent mean values ± SD of three replicates. The effect of each treated EPS fraction (**c**–C to **h**H) was compared with the effect of the untreated EPS fraction (**b**B). Highly significant differences were observed, only between not treated *N. phyllepta* D_{12} bound EPS and EPS treated with $NaIO_4$. These differences are indicated by *** ($p < 0.001$) on the *upper part* of the

figure. *T* values are −18.53, 13.8, and 39.5 for *Flavobacterium* sp. II2003 (**a**), *Roseobacter* sp. IV3009 (**b**), and *Shewanella* sp. IV3014 (**c**), respectively



Discussion

Successful strategy to remove the satellite bacteria from the N. phyllepta cultures Axenization of diatom cultures by the removal of bacteria was essential to ascertain that the studied active compounds were indeed produced by *N. phyllepta* and not by its associated bacteria. Depending on the diatom strain and its growth form, the axenization protocol can be very different (Bruckner and Kroth 2009). In particular, benthic diatoms are often difficult to axenize because of the mucilage that surrounds the cells (Bruckner and Kroth 2009). Moreover, many diatoms in co-culture with bacteria grow denser and faster than their axenic counterparts (Bruckner et al. 2008). We achieved to axenize the intertidal benthic diatom *N. phyllepta*, without changing its main biological properties (aspect, growth, and biofilm formation), thanks to a combination of antibiotics (kanamycin + streptomycin) used after a filtering step. This is in accordance with Bruckner and Kroth (2009) who noticed that for most freshwater benthic diatoms, physical separations and antibiotic treatments have to be combined to fully remove associated bacteria. They succeeded in removing satellite bacteria from some diatom strains with a combination of penicillin G, streptomycin, and chloramphenicol without affecting their biological properties in cultures.

However, this combination could not be used in our case since chloramphenicol inhibits the growth of *Navicula* (Khandeparker et al. 2014). Recently, Windler et al. (2012) described a simple protocol for freshwater benthic diatom axenization including treatment of the cultures with the antibiotic imipenem. Here, the imipenem treatment generated a disturbance of *N. phyllepta* biofilm formation. To optimize our axenization method, diatom cells were treated during the latency phase of the growth curve, during which the bacterial load is low. This approach allowed us to overcome problems due to the excretion of protective substances (such as EPS) by bacteria and diatoms during the growth phase (Flemming and Wingender 2010; Orvain et al. 2014). Additionally, resuspending the cells by shaking at regular intervals enhanced the efficiency of the method by disturbing the diatom-bacterium biofilm formation and thus by decreasing the protection toward antibiotics.

EPS produced by N. phyllepta exhibit activity on bacterial biofilm formation The main objective of our work was to investigate the effects of the EPS produced by axenic diatom *N. phyllepta* on the ability of three benthic bacterial models to form a biofilm. We worked intentionally with a concentration of EPS slightly higher than in the environment such as intertidal mudflats in order to better highlight biological activities. The examined bacterial strains reacted to one or several molecules secreted by *N. phyllepta*, suggesting that a direct diatom-bacterium contact was not required, as reported before (Windler et al. 2015). *Navicula phyllepta* produced the active molecule(s) when cultivated separately from bacteria, which means that the synthesis and the secretion of these molecules were not induced by bacterial presence and/or activity. In our experimental conditions, the secretome part of *N. phyllepta* active on the early stages of bacterial biofilm formation was the bound EPS fraction harvested during the stationary phase of diatom growth. Its main begotten effects were (i) a strong inhibition (more than 40 %) of the biofilm formation of *Flavobacterium* sp. II2003 under both static and dynamic conditions and (ii) a stimulation of the biofilm formation, again under both conditions for *Shewanella* sp. IV3014 (up to 40 %) and only under static conditions for *Roseobacter* sp. IV3009. The results first depend on the bacterial strains and, to a lesser extent, on the experimental approach. In our case, it is likely that the difference in the surface properties between static (polystyrene) and dynamic (glass) conditions may influence early stages of surface colonization and subsequent biofilm formation of the bacterial strains (Dang and Lovell 2000; Jones et al. 2006; Lee et al. 2008). It was therefore essential to investigate different conditions of growth and/or biofilm formation to confirm (or not) the significance of the positive or the negative

effect of the bound EPS fraction on the bacterial biofilm.

The active N. phyllepta compounds are bound EPS carbohydrates

The biochemical characterization of the active bound EPS fraction demonstrated that the inhibiting/stimulating activity can be assigned to saccharidic compounds, which were very abundant in the active fraction. In fact, the carbohydrate concentration in both bound and colloidal algal EPS fractions was approximately 4-fold higher during the stationary phase (D_{12}) than during the exponential growth phase (D_6). This is in accordance with Underwood and Paterson (2003) who described that the concentrations of extracellular carbohydrates in the medium significantly increased during the transition from exponential growth to stationary phase for benthic diatoms, including *Navicula* species, when grown in batch culture. Additionally, the amount of proteins was lower (up to 4.6-fold less for bound EPS) compared with the amount of carbohydrates, which is in agreement with previous reports on *Navicula* strains (Staats et al. 1999; Underwood and Paterson 2003; Scholz 2014). EPS (colloidal and bound fractions) extracted directly from mudflat microphytobenthic biofilms also predominantly consisted of polysaccharides, and only small quantities of proteins were present (Underwood and Paterson 2003; Pierre et al. 2012).

Potential mode of action of the N. phyllepta EPS on the bacterial biofilm formation

In the phycosphere model of Bell and Mitchell (1972), bacterial growth and metabolic activity were proposed to be stimulated by microalgal extracellular substances. Algal organic substances are generally considered as energy and carbon sources for bacteria (Bellinger et al. 2009), and EPS might favor bacterial metabolism by supplying nutrients (Middelburg et al. 2000; Goto et al. 2001; Cook et al. 2007; Bellinger et al. 2009). Although no stimulation of bacterial growth has been observed in solid culture medium, the biofilm formation of *Shewanella* sp. IV3014 and *Roseobacter* sp. IV3009 was enhanced by *N. phyllepta* EPS. Indeed, failing to directly act on cell multiplication, microalgal polysaccharide could act as signaling molecules that modulate the expression of genes involved in biofilm lifestyle of recipient bacteria (Rendueles et al. 2013). Diatom EPS are also assumed to facilitate cell attachment to a surface and to protect cells against desiccation and different environmental stress (Lubarsky et al. 2010; Orvain et al. 2014), thus enhancing the development of associated bacteria biofilms.

In our work, *N. phyllepta* polysaccharides showed also a powerful biofilm inhibition activity on *Flavobacterium* sp. II2003, without any antibacterial activity, which leads to ascribe the *N. phyllepta* polysaccharide activity as

specifically antibiofilm. Several studies identified antibiofilm molecules from bacteria, and exopolysaccharides were specifically shown (i) to interfere with bacterial adherence to surfaces and biofilm formation (Valle et al. 2006; Jiang et al. 2011), (ii) to modulate expression of genes (such as curli genes) related to biofilm formation in bacteria (Kim et al. 2009; Rendueles et al. 2013), or (iii) to disrupt established biofilms (Qin et al. 2009; Jiang et al. 2011). However, very few polysaccharides from microalgae were described as negatively impacting bacteria or other microorganism activities, and none of them were studied in the marine context. They are (i) polysaccharides isolated from the *Chlorella* (green microalga) and *Spirulina* (cyanobacterium) inhibiting the binding of *Helicobacter pylori* to gastric mucin in vitro (Loke et al. 2007) and (ii) a sulfated polysaccharide named naviculan and isolated from *Navicula directa* displaying antiviral activities against herpes simplex and influenza A viruses (Lee et al. 2006). Recently, it has been shown that changes in the composition of carbohydrates produced by the diatom *Asterionellopsis glacialis* reduced bacterial abundance (da Silva et al. 2016).

As regards to the physico-chemical aspect of biofilm formation, the stimulating or inhibiting effect of microalgal EPS can also be due to modifications of the substratum (abiotic surface) and/or of the bacterial cell surface (Grossart et al. 2006; Rendueles et al. 2013). Studies utilizing culture supernatants or purified polysaccharides as surface coating provide further evidence that microbial polysaccharides modify the wettability and the charge of abiotic surfaces and hence affect the interaction of bacteria with the substratum (Valle et al. 2006; Rendueles et al. 2011). Additionally, polysaccharides are suspected to change the physical properties of gram-negative and gram-positive bacteria cell surfaces such as the cell surface hydrophobicity (Sayem et al. 2011). These properties are essential during the adhesion phase of biofilm formation and the further development of microcolonies (O'Toole et al. 2000).

Interest for the diatoms to modulate the formation of bacterial biofilms and potential biotechnological applications Our experiments with axenic diatom secretomes and different benthic bacterial biofilm models clearly demonstrate that specific interactions between algae and bacteria are instrumental for bacterial biofilm formation. Specific algae-bacteria interactions have largely been neglected so far but are presumably as important as, for instance, nutrient supply and grazing in controlling the development of microphytobenthic biofilms (Grossart et al. 2006). In our work, it is striking that the active diatom bound EPS fraction showed two

opposite effects on bacterial biofilm formation, depending on the strains. One can hypothesize that *N. phyllepta* would favor some satellite bacteria for its own (or reciprocal) benefit and, conversely, would inhibit attachment of others, useless or detrimental to diatom cells (Amin et al. 2012). Bound EPS might be closely involved into the formation of the extracellular matrix of the microphytobenthic biofilm, facilitate attachment of the cell to a surface, and also improve cell-cell interactions between bacteria and diatoms (Lubarsky et al. 2010; Pierre et al. 2012). It strongly suggests the existence of complex physico-chemical interactions between microorganisms in natural biofilms, especially in our model, i.e., the transitory biofilm forming at the surface of sediment of intertidal mudflats during daily emersion. These interactions could allow a process of selection/adaptation to changing environmental conditions, and populations able to co-exist or to exploit algal/bacterial species would be promoted, as it has been shown for estuaries (Haynes et al. 2007).

The study of marine biofilms raises a growing interest, especially for human activities, and marine diatoms are considered as attractive sources of new active compounds for biotechnological applications (Fu et al. 2015). *Flavobacterium* species are important pathogens in aquaculture setting (Duchaud et al. 2007) and have been detected in industrial, domestic, and medical environment biofilms (Basson et al. 2008). In that framework, it is of particular interest to identify a diatom secretome that strongly inhibits the biofilm formation by a *Flavobacterium* strain. On the contrary, *Shewanella* sp. IV3014 and *Roseobacter* sp. IV3009, whose biofilm formation is positively impacted by *N. phyllepta* secretome, belong to genera known to be dominant among the pioneers in marine environments (Salta et al. 2013). It is crucial to understand the first steps of marine biofilm formation in order to efficiently prevent biofouling or conversely to stimulate biofilm formation, which improves for instance larval settlement and metamorphosis of farmed oysters (Yu et al. 2010). Therefore, the molecules of the *N. phyllepta* EPS bound fraction responsible for *Shewanella* sp. IV3014 or *Roseobacter* sp. IV3009 and *Flavobacterium* sp. II2003 biofilm stimulation and inhibition, respectively, need to be identified.

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