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Marine diatom *Navicula jeffreyi*: from biochemical composition and physico-chemical surface properties to understanding the first step of benthic biofilm formation

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Abstract

To understand the first step of marine benthic microbial mat formation and biofouling phenomena, caused by diatoms in the marine environment, the surface properties of the epipelic diatom *Navicula jeffreyi* were studied and the composition of its bound Extracellular Polymeric Substances (EPS) was determined. These parameters are determining factors for the initial adhesion step of diatoms to other constituents that start marine fouling. Surface energy of a diatom cell layer was determined using the sessile drop technique and highlights that diatoms show a moderate hydrophobic character (contact angle with water > 68°), no Lewis acid character ($\gamma^+ < 1$ mJ/m^2) and a low Lewis basic character ($\gamma^- = 16{,}1$ mJ/m^2). An extraction procedure using a cationic resin subtracted only the bound EPS. Biochemical assays showed that there were 2.5 times more proteins than sugars. The propensity of *Navicula jeffreyi* diatom to adhere to five different solid surfaces, showing a gradient in their hydrophobic and hydrophilic character, was measured. The attachment densities were high on hydrophobic surfaces such as polytetrafluoroethylene and very low on substrata with surface free energy over 40-50 mJ/m^2. Using a thermodynamic approach, the free energy of adhesion of the diatom to the five substrata was determined, and led to a very strong correlation with attachment densities for polytetrafluoroethylene, polyamide, polyethylene and stainless steel.

Keywords: Bioadhesion, Contact angle measurements, Diatom, Extracellular Polymeric Substances, Microalgae, Physico-chemical surface properties.

Introduction

Diatoms are the most common early autotrophic colonizers of surfaces in seawater and are an important constituent of the biofouling community in the marine environment, together with bacteria and other algae [1,2]. Diatoms form an ubiquitous group of unicellular microalgae characterized by their highly ornate, siliceous cell walls, associated with organic extracellular polymers [3]. Marine biofouling phenomena cause serious and costly problems for surfaces immersed in seawater, such as energy loss for boats, increased risk of mechanical failure for static marine structures as well as safety problems [4-7]. During their assemblage diatoms produce copious amounts of Extracellular Polymeric Substances (EPS), rich in proteins and carbohydrates [8,9], which form an adhesive mucilage and allow them to build and, at a later stage, to hold the biofilm together [10]. EPS also permit the attachment of other fouling organisms, such as bacteria, to the sediment or to immersed solid surfaces [11]. These EPS are secreted from a slit in the cell wall called the raphe. A common feature of adhesion in raphid diatoms is that adhesive mucilages appear to be processed in several complex steps over time, and these depend on the stage of adhesion as well as the nature of both the organism and the substratum [12]. Most fouling diatoms have an initial adhesive mucilage that they use for traction and movement (initial mucilage) and then a more permanent adhesive mucilage when they eventually settle down to divide and form a biofilm (biofilm mucilage). There are also several distinct types of EPS; motility, outer capsule, and matrix EPS, that can all participate in the adhesion process [12]. The goal of the present paper is to study the transitory physico-chemical interactions that occur between diatoms with initial mucilage and different substrata.

In general, adhesion of microorganisms is influenced by environmental (temperature, pH and ionic strength), interfacial (surface charge and hydrophobic or hydrophilic character of the microbial cells, chemical composition) and physiological factors (type of microorganisms) [13-15]. The roughness, or the mechanical properties, of the substratum [16], as well as the roughness of the cell surface, can also influence the adhesion of cells to surfaces. Among the parameters outlined, interfacial properties can be most readily altered by using solid surfaces with coatings that prevent adhesion of microorganisms.

Interfacial properties are linked to the surface energy of both the microorganism cell surface and the solid substrate surfaces, that is a measure of the capacity of a surface to interact spontaneously with other materials by forming new bonds. While the effect of the surface energy of solid substrata on the adhesion strength of diatoms has often been studied by employing widely different materials, ranging from urethanes and epoxies (high surface energy), to silicones and fluorinated materials (low surface energy) [6,10,17-19], the measurement of the surface energy of diatom cells themselves has rarely been performed [20]. We feel that such measurements could help in understanding the first step of benthic biofilm formation, and thus help to prevent adhesion of microalgae to surfaces submerged by the sea.

In this report, the first part is devoted to a study of the surface properties of the epipelic diatom *Navicula jeffreyi*, a diatom largely involved in the formation of microphytobenthic biofilms. The cell length, width, and volume of *Navicula jeffreyi* are respectively equal to 11.18 ± 0.82 µm, 7.56 ± 0.60 µm and 366.71 ± 85.50 µm3/cell (mean± SE) (n = 6) [21]. Diatom cells were grown on an orbital shaker, so that they do not form a biofilm, and then freeze-dried, in order to have diatoms with their initial mucilage only. The surface energy of the diatom was determined by applying the sessile drop technique to a cohesive layer of diatom cells resuspended in water and was calculated using the Lifshitz van der Waals acid-base (LW-AB) method. The second part of the report evaluates the propensity of these diatom cells to adhere to five different solid surfaces showing a gradient in their hydrophobic and hydrophilic character. The role of the initial EPS in adhesion was also investigated, in particular, by extracting the bound polymers and characterizing their biochemical composition. Finally the experimentally observed adhesion of *N. jeffreyi* diatom to the five different substrata was compared with that predicted by a thermodynamic approach that uses the calculation of the free energy of adhesion.

1. Materials and Methods

1.1 Microalgal strain and growth conditions

The *N. jeffreyi* strain (CS-46/8) was from CSIRO Marine and Atmospheric Research (Australia). 20 mL of F/2 liquid medium (Sigma-Aldrich) were suspended in 1L of Artificial

Sea Water (ASW, Tropic Marin Sea Salts, Wartenberg, Germany, 30 Practical Salinity Units). 100 mg/L of sodium metasilicate were added to the medium then the pH was adjusted to 8.2. This solution was used as growth medium for *N. jeffreyi*. Cells were grown on an orbital shaker (50 rpm) and kept for 20 days at 18°C, in conditions of natural alternating day/night. After 20 days, corresponding to the end of the exponential growth phase, microalgal cells were harvested by centrifugation for 10 min at 6000 g and 4°C then washed twice with, and resuspended in NaCl 0,9 %. Cells were then collected and resuspended in 10 mL of ASW diluted 1: 1000. Finally, the suspensions were freeze-dried and stored at 4°C until they were used for the following experiments.

1.2 Extraction of the Extracellular Polymeric Substances (EPS) bound to *N. jeffreyi* cells

The extraction of EPS bound to *N. jeffreyi* cells was performed with Dowex resin, as this method was developed for culture pellets of *N. jeffreyi* cells and was shown to provoke the minimum release of internal compounds (protein, ATP) and the lowest proportions of glucose compared with the water-extracted EPS [22], from which the high glucose content must be inferred as contamination by the chrysolaminaran found in the vacuoles of the diatoms [23]. 20 mL of ASW were added to 30 mg freeze-dried cells. 5 g of activated Dowex (Marathon C, previously activated in Phosphate Buffer Saline for 1h in the dark) were gently mixed with the sample at 4°C for 1h in the dark. The solution was then centrifuged at 3500 g and 4°C for 10 min and the supernatant was collected, freeze-dried and stored at -80°C prior to biochemical analysis.

1.3 EPS composition

Total sugar and protein content of EPS and also sugar composition of polysaccharidic fraction of EPS were determined by previously used methods for EPS from the *Navicula* genus of diatoms [24-26]. Total sugar content was determined using the phenol-sulfuric acid assay, using glucose as standard [27]. Protein content was determined using the bicinchoninic acid assay, using bovine serum albumin as standard [28]. The sulfate content was measured by the Azure A assay [29], using dextran sulfate as standard.

The sugar composition of the bound EPS fraction was determined as follows. *N. jeffreyi* bound EPS fraction was dissolved in 2M HCl at 50 mg/mL and heated at 100°C for 20 h. Polysaccharides were completely hydrolyzed in monomers, then the preparation was freeze-dried and stored at -20°C. Analysis of the carbohydrate fraction was carried out by GC/MS using a Varian CP-3800 GC/Varian Saturn 2000. Operating conditions were based on the methodology of Pierre [26]: 200µL of pyridine and 200µL of BSTFA(N,O-bis(trimethylsilyl)trifluoroacetamide):TMCS(trimethylchlorosilane) (99:1) per mg of hydrolyzed EPS were added. The solution was mixed for 2 h at room temperature and injected into a DB-1701 J&W Scientific column (30 m, 0.32 mm, 1 µm). The helium pressure was 8.8 psi and the flow rate was 1 mL/min. The temperature of the injector was set at 250°C. The rise in temperature in the oven was programmed for a first step at 150°C, then an increment of 10 °C/min up to 200°C with a final step at 200°C for 35 min. The ionization was performed by Electronic Impact (70 eV), the trap temperature was set at 150°C, the transfer line temperature was defined at 180°C and the target ion was fixed at 40-650 m/z.

In order to check that the freeze-drying step for the diatom cells did not have any influence on this composition, the bound EPS extraction procedure was applied to diatoms cell with and without freeze-drying and the monosaccharide composition of polysaccharides was determined for both samples of bound EPS.

1.4 Determination of surface energy of substrata and diatom cells and the free energy of adhesion ΔG_{adh}^{Total} between substrata and diatoms

There are many thermodynamic approaches in the literature to evaluate the cells or solid substrata surface energy. All of them are based on contact angle measurements with different liquids with known surface tension, which were shown to give reproducible and accurate results for both inorganic material surfaces and microbial cell surfaces [30-32]. However the values of calculated surface energy from contact angles measurements depends on the followed approach: Fowkes, Equation of state, Geometric mean and Lifshitz van der Waals acid-base (LW-AB) approaches. In 2002, a remarkable study was published about the surface energy of 140 bacterial and 7 yeast cell surfaces, determined by the four different approaches mentioned above [30]. LW- AB was found to give the most consistent results. That is why we chose this last method to evaluate surface energy of the five different substrata and the epipelic diatom *N. jeffreyi*.

Five solid materials at monolithic and film state were used. The roughness and porosity of all these materials were considered as insignificant. Four materials were provided by Goodfellow: Stainless Steel AISI 316L (0.5 mm thick, noted SS316), Polytetrafluoroethylene (0.5 mm thick, noted PTFE), Polyamide-nylon 6 (0.5 mm thick, noted PA) and Polyethylene (0.5 thick, noted PE), and Glass (1 mm thick microscope slides) was provided by Thermo. The films were cut into 4 cm² pieces and washed in 2 % PCC-54 (v/v, phosphate-free surfactant, Thermo Scientific) for 10 min then rinsed 5 times with sterile milliQ water.

The surface energy of *N. jeffreyi* was measured by producing a uniform and cohesive layer of cells deposited on membrane filters [31]. The layer of cells was prepared by depositing 40 mL of microalga suspended in milliQ water (approximately 10^{10} cells/mL) on a cellulose triacetate membrane filter (with a pore diameter of 0.45 µm, Sartorius) by filtration of the suspension using low depression. The filters with the diatoms were then placed in a petri dish on the surface of a layer of 1 % agar (w/v) in water containing 10 % (v/v) glycerol to preserve constant moisture content [32]. The filters were stored for 30 min, at room temperature. Three separate filters from three different cultures were used and the results were averaged. The filters were then placed on empty Petri dish and allowed to air dry for 30 to 45 min [31,32]. After this drying time, same contact angles were measured for water droplets deposited on the diatom layer several minutes apart, indicating that water evaporation from the layer was achieved. The contact angle was consistent for 30 min up to 120 min of drying time.

The contact angle (CA) measurements were performed with a goniometer G40 (Krüss, Germany) at room temperature (23°C) with an accuracy of ± 2°C, employing the sessile drop technique and using three pure solvents whose surface tension components were known (Table 1): distilled water (Infilco), diiodomethane 99% (Sigma-Aldrich) and formamide ≥ 99% (Sigma-Aldrich). During measurements, each probe was dropped on the cell layer or the solid surfaces, and CA were measured immediately for 10 s. Left and right contact angles in at least 3 locations were measured, with highest and lowest values discarded. The CA was calculated as the average of these values. According to the LW-AB approach, CA were converted into surface free energies using equation 1 (Eq.1) [33], which ignores spreading pressure and highlights Lifshitz-van der Waals and Lewis acid/base surface free energy components.

(1) $$\gamma_L^t\left(1+\cos\theta\right) = 2\left(\sqrt{\gamma_S^{LW}\gamma_L^{LW}} + \sqrt{\gamma_S^+\gamma_L^-} + \sqrt{\gamma_S^-\gamma_L^+}\right)$$

Here, γ^{LW}, γ^+ and γ^- are the Lifshitz-van der Waals, electron-acceptor (or Lewis-acid) and electron-donor (or Lewis-base) components of the surface free energy respectively; θ is the CA and the subscripts L and S denote the liquid and solid samples.

Equation 2 allows accessing to the Lewis acid-base components of the surface free energy:

(2) $$\gamma^{AB} = 2\sqrt{\gamma^+\gamma^-}$$

ΔG_{adh}^{Total} is obtained by first determining the surface properties of micro-organisms and substrata and the tension surface of the medium. Then, the total interfacial free energy of microbial adhesion is determined by the triple relationship between the organism (NJ), the substratum (S) and the liquid (L) [34,35]:

(3) $$\Delta G_{adh}^{Total} = \Delta G_{adh}^{LW} + \Delta G_{adh}^{AB}$$

$$\Delta G_{adh}^{LW} = \left(\sqrt{\gamma_{NJ}^{LW}} - \sqrt{\gamma_{S}^{LW}}\right)^2 - \left(\sqrt{\gamma_{NJ}^{LW}} - \sqrt{\gamma_{L}^{LW}}\right)^2 - \left(\sqrt{\gamma_{S}^{LW}} - \sqrt{\gamma_{L}^{LW}}\right)^2 \quad (4)$$

$$\Delta G_{adh}^{AB} = 2\left[\sqrt{\gamma_L^{+}}\left(\sqrt{\gamma_{NJ}^{-}} + \sqrt{\gamma_S^{-}} - \sqrt{\gamma_L^{-}}\right) + \sqrt{\gamma_L^{-}}\left(\sqrt{\gamma_{NJ}^{+}} + \sqrt{\gamma_S^{+}} - \sqrt{\gamma_L^{+}}\right) - \sqrt{\gamma_{NJ}^{-}\gamma_S^{+}} - \sqrt{\gamma_{NJ}^{+}\gamma_S^{-}}\right] \quad (5)$$

1.5 Microscopic analyses

Scanning Electron Microscopy (SEM) was used to examine the aspect of the microalgal cells after freeze drying. Samples were previously metallized by a layer of gold-palladium under vacuum. SEM observations were made with a JEOL 5410 JV SEM in high vacuum mode, using 2.0 KV as accelerating voltage.

1.6 *N. jeffreyi* attachment densities measurements

Lyophilized microalgae were re-suspended in ASW diluted 1:1000 to give a suspension of cells with a chlorophyll content of approximately 0,3 µg/mL measured by fluorimetry with acetone 90 %. Five milliliters of cell suspension were carefully pipetted into a quadriPerm microwell plate (Sarstedt) containing three pieces of material (4 cm^2) on the bottom. Two series were produced. After a set of different periods of attachment (2, 3, 5.5, 18, 24 and 48 hours, and 3 and 4 days) with slow orbital agitation (20 rpm, Rotamax 120, Heidolph) in the dark, the cell suspensions were carefully aspirated. Afterward, 5 ml of ASW diluted 1/1000 was pipetted into each well followed by slow shake back and forth five times to remove unattached cells. After washing, all the coupons were dried under a laminar flow hood for 1 h. Attachment densities were obtained by counting the adhered cells using a fluorescence microscope (Leica DMRB) combined with image software (Leica Application Suite v3.8).

The Y5 filter set was used (excitation 620-660 nm) and the λ used for emission was 700-775 nm. Counts were made for 20 fields (0.16 mm^2) randomly taken on the surface on each of the three replicate coupons. Cell settlement data are expressed as the mean number of cells adhered per mm^2. Each standard deviation is represented by error bars and calculated with raw data of three independent replicates.

2. Results and discussion

2.1 *N. jeffreyi* : from microscopic observations to biochemical and physico-chemical surface characterization

We used a freeze-drying treatment in this study because it allows the maintenance of microalga in a "fixed" state with initial mucilage. In the experiments presented here, this fixed state gives access to the initial step of attachment without having interference from polymeric metabolites (biofilm mucilage), which are synthesized when colonization occurs. The idea is to study the very early stage of attachment, that corresponds to a passive step, due to the net force of interaction between the diatom surface and the support that arises from the balance between van der Waals and Lewis acid-base forces. The diatoms are here considered as colloidal particles with bound initial adhesive polymers. It has to be kept in mind that these "living colloids", upon contact with a surface, will excrete supplementary adhesive polymers, allowing for strong adhesion to the surface and finally for irreversible biofilm formation. The ability of benthic diatoms to form biofilm is largely due to the secretion of these mucilaginous EPS from the raphe [12,36].

Scanning electromicroscopy (SEM) shows *N. jeffreyi* cells (Figure 1) with an intact structure, indicating that freeze-drying does not damage microbial cell surfaces. The SEM also shows the presence of EPS closely bound to the *N. jeffreyi* cells (Figure 1). The EPS detected by SEM in our samples correspond to the bound initial adhesive polymers that are very important mediators in the initial step of adhesion of diatoms to surfaces.

In order to extract cell bound *N. jeffreyi* EPS a cationic resin was used. Extraction protocols can distinguish a range of EPS types, depending on their degree of interaction with diatom cells: (1) colloidal fractions, corresponding to the EPS excreted into the medium, (2) bound fractions, corresponding to the EPS surrounding the cells and (3) residual fractions, corresponding to the internal EPS [26]. Total sugar and protein content assays showed that the bound EPS had 2.5 times more proteins than sugars. Sulfated sugars were not detected. This composition is consistent with the works of various authors who have shown that adhesive EPS are composed of cross-linking proteins (probably glycoproteins), polysaccharides and phenols with covalent o-linkages [37,38]. These surface polymers are directly involved in the physico-chemical surface properties of microorganisms.

The monosaccharide composition of the polysaccharidic fraction of bound EPS from *N. jeffreyi* was also determined. In order to check that the freeze-drying step used to fix the diatom cells did not have any influence on this composition, the bound EPS extraction procedure was applied to diatom cells with and without freeze-drying and the monosaccharide composition of polysaccharides was determined for both samples. The results were very similar whether or not the diatoms had undergone freeze-drying (Table 2). The monosaccharidic composition (expressed in % w/w) was 17 % of galacturonic acid, 0 % of sulfated sugars and 83% of neutral carbohydrate. The neutral monosaccharidic composition was dominated by glucose (36.1 % of the total fraction) but contained other sugars such as rhamnose (8.9 %), mannose (18.4 %) and galactose (19.5 %). This composition is similar to the one described for two other benthic diatoms, *Cylindrotheca closterium* and *Navicula salinarum* [38].

We then characterized the surface energy of *N. jeffreyi* cells using contact angle (CA) measurements.

Diatom layers were prepared by filtering their suspensions (c.f. Materials and Methods), in a way that the cells covered the filter surface homogeneously and formed a cohesive layer.

The measured CA from *N. jeffreyi* layers are detailed in Table 3. Based on these CA measurements, equations 1 and 2 allowed the determination of the surface energy components of filter alone and of N. jeffreyi cells (Table 4). Results show that there are significant differences between the data obtained with the membrane filter alone (controls) and those measured with the *N. jeffreyi* layers deposited on the same membrane, for the different individual surface energy components: γ^t, γ^- and γ^+. The CA of water with the *N. jeffreyi* layers (68.6°) shows that the microalgae surface, in general, is moderately hydrophobic.

The layer of diatoms presented a γ^t value equal to 36.7 mJ/m², a γ^{LW} value equal to 31.8 mJ/m² and a γ^{AB} value equal to 4.8 mJ/m². It showed no Lewis acid character ($\gamma^+ < 1$ mJ/m²) and an average Lewis basic character ($\gamma^- = 16.1$ mJ/m²). The predominance of the electron-donor character is an indication of the nature of the chemical groups exposed at the surface of diatoms. Indeed, Lewis basic character is often attributed to neutral or slightly charged basic chemical groups such as carboxylate (COO^-), amine (NH_2), phosphate (PO_4^-) groups of phospholipids or lipoproteins [39,40].

In the case of *N. jeffreyi*, the Lewis basic character of the diatom surface may derive, in part, from galacturonic acid and proteins in bound EPS.

In conclusion, the results of this first part demonstrate that the diatom cells of *N. jeffreyi* are moderately hydrophobic and also show an average Lewis basic character. In the second part, we demonstrate that these surface properties are linked to the propensity of the diatom to adhere to different solid surfaces, presenting a gradient in hydrophobic and hydrophilic character.

2.2 Initial bioadhesion of *N. jeffreyi* is strongly affected by the surface properties of the substrata

Prior to carrying out initial bioadhesion measurements, an optimization of the method for diatom attachment measurement was carried out. The highest densities of attached cells were reached between 24-48h periods and then remained almost constant, as seen in Figure 2. Furthermore, data showed no significant difference between a 24 h- and a 48 h-adhesion time and the spatial distribution of cells on the different surfaces was homogeneous until 48 hours of contact between cells and surfaces with very rare visible aggregates (no more than 10 cells stuck together).Thus a period of 24 h adhesion time in the dark was chosen. Cells were then counted after their adhesion to different surfaces (c.f. Materials and Methods and Table 4). The initial affinity was calculated by means of the initial slope of each curve before 18h attachment. *N. jeffreyi* adhered with a speed of 10 and 12 cell.mm^{-2}.h^{-1}on glass and polyamide-nylon (PA), respectively, and 77 and 78 cell.mm^{-2}.h^{-1} on stainless steel (SS316) and polyethylene (PE), respectively. Finally, *N. jeffreyi* adhered most quickly on polytetrafluoroethylene (PTFE), with a speed of 121 cell.mm^{-2}.h^{-1}.

To assess the effect of solid surfaces properties on the diatom-substratum interaction, physical properties of the surfaces were measured. The surface energies, calculated from measured CA (Table 3), of the five substrata used in this study are listed in Table 4. The obtained γ^{tot} values were in the range of 14.7-56.4 mJ/m², γ^{LW} in the range of 15.9-40.4 mJ/m², γ^{+} in the range of 0.1-1.3 mJ/m², and γ^{-} in the range of 1.9-54 mJ/m². As expected, the results for the five different surfaces showed reasonably good agreement with the literature data, within experimental error [41]. The present data indicate that the five substrata form a gradient with decreasing surface hydrophobicity and increasing hydrophilicity. PTFE has the lowest γ^{tot} and γ^{LW} values, equal to 17.1 ± 0.9 mJ/m² and 15.9 ± 0.6 mJ/m² respectively, and negligible γ^{+} and γ^{-}. Next, PE has a γ^{tot} equal to 32.5 ± 2.7 mJ/m², then, SS316 with γ^{tot} of 40.6 ± 0.4 mJ/m², PA with γ^{tot} of 42.4 ± 0.9 mJ/m² and glass with γ^{tot} of 56.2 ± 0.3 mJ/m².

To assess the influence of solid surface properties on the adhesion of diatom *N. jeffreyi*, the mean cell densities of attached cells on the five different solids were quantified. The attachment densities of diatoms decreased with an increase in the total surface energy γ^{tot} of the substratum (Table 4). When γ^{tot} is over 40 mJ/m², diatom adhesion is minimized. There were significant differences between attachment densities on the tested surfaces. The values of attachment densities ranged from 2160 ± 110 cells/mm^2 at 17.1 mJ/m^2 to 225 ± 29 cells/mm^2 at 56.2 mJ/m^2 after 24 h attachment. The effect of the total surface energy on the adhesion of the diatom *Navicula closterium* MMDL533, using a series of more or less silanized glass slides as model surfaces, has been measured by other authors [34]. Our data profiles are similar to those of Li and co. [34] who examined initial attachment after 5.5h. We confirm here the preference of *N. jeffreyi* for hydrophobic surfaces.

In another earlier study, marine fouling diatoms *Navicula perminuta* were found to adhere more strongly to hydrophobic surfaces than to hydrophilic surfaces. This behavior was ascribed to the physicochemical properties of their extracellular adhesives [14]. *Navicula perminuta* cells were also shown to adhere more strongly to hydrophobic materials thanks the hydrophobic segments of their EPS [42]. A similar conclusion was obtained for the diatom *Amphora* [17], whose cells were found to attach more strongly to hydrophobic surfaces. However, in another study, it was shown that *Navicula* diatom cells adhered with comparable strength to a hydrophobic elastomer and a hydrophilic mineral [36]. This result was explained by the presence of either different EPS macromolecules, different segments on these macromolecules, or even different regions on the same macromolecule being likely to mediate adhesion of *Navicula sp.* [36]. More generally, hydrophobic regions of adhesive exopolymers correspond to hydrophobic polypeptides and lipids, whereas hydrophilic regions correspond to hydrophilic saccharides on glycoproteins or polysaccharides [10]. In the case of *N. jeffreyi*, bound EPS were found to include 2.5 times more proteins than sugars, which is in accordance with the hydrophobic character of the diatom.

In general, diatom adhesion is weaker on hydrophilic surfaces when compared to hydrophobic surfaces [6,17,42], in good agreement with the results obtained here.

The current study also addresses the question of whether the composition of EPS is similar between initial and biofilm EPS for the same type of the diatom species and under the same growth conditions. Numerous studies have been carried out to evaluate the differences in biochemical metabolites in planktonic and biofilm cells of bacteria [10, 43]: differences in carbohydrate profiles for EPS of planktonic and biofilm cells of marine diatom *Amphora rostrata*, grown in batch culture, were highlighted. It has also been reported that, for some diatoms, the adhesive properties of their EPS are unrelated to the amount of exopolymer produced [10], suggesting that the chemical composition of EPS does not vary over time for a particular type of diatom species grown under particular conditions. The results of the present study confirm that diatom adhesion is strongest to hydrophobic surfaces. It should be noted that the data obtained in this study used freeze-dried diatoms with initial mucilage after passive attachment in the dark, while all other studies used diatoms with biofilm mucilage after active settlement in the light. The data appears to suggest that the adhesive properties of bound EPS remain constant between initial and biofilm EPS.

When considering the relationship between attached diatom cell density and the different surface energy components of the five substrata (Table 4), it appears that the best correlations are observed between cell density and van der Waals component, on the one hand, and electron acceptor component on the other hand. In the case of the electron donor component, a correlation was obtained between cell density and the four substrata PTFE, PA, PE and SS316 (the point corresponding to the glass substratum was not aligned with the others).

Finally, we tested whether it is possible to predict how a diatom can adhere to a substratum by calculating the free energy of adhesion between the microalgae and the solid surfaces.

We used a Lifshitz van der Waals acid-base (LW-AB; [30]) thermodynamic approach to determine the free energy of adhesion ΔG_{adh}^{Total} of *N. jeffreyi* to the five different support materials (c.f. Materials and Methods). This parameter is of crucial importance and may allow the prediction of the initial adhesion of microorganisms, as the adhesion process will be favored if the process itself causes the thermodynamic function to decrease ($\Delta G_{adh}^{Total} < 0$).

Using equations (3), (4) and (5), the values of the total interfacial free energy of adhesion of *N. jeffreyi* to the five studied substrata and its components (ΔG_{adh}^{LW} and ΔG_{adh}^{AB}) were calculated and are presented in Figure 3. When considering the relationship between attached diatom cell density and the different contributions of the free energies of adhesion to the five substrata, it appears that the best correlations are observed between cell densities and ΔG_{adh}^{Total} on the one hand (Fig. 3) and ΔG_{adh}^{AB} on the other hand (Fig. 3), for all the substrata except for glass which is not aligned with the others. In the case of ΔG_{adh}^{LW}, its contribution to ΔG_{adh}^{Total} is insignificant (Fig. 3). The negative values of ΔG_{adh}^{Total} and ΔG_{adh}^{AB} actually lead to a strong adhesion of *N. jeffreyi* to PA, PE, PTFE and SS316 surfaces. The adhesion test reveals a close correlation between the surface hydrophobicity and ΔG_{adh}^{Total} and the attachment of *N. jeffreyi*: the more hydrophobic the substratum is, the more strongly *N. jeffreyi* adheres. For glass, the positive values of ΔG_{adh}^{Total} and ΔG_{adh}^{AB} unexpectedly correspond to a weak but significantly positive adhesion of *N. jeffreyi* to the hydrophilic surface, at a similar level to PA, for which a ΔG_{adh}^{Total} value equal to -28 mJ/m^2 was calculated (Fig.3). This adhesion to glass may be due to possible local attractive electrostatic interactions, which are not explicitly included in the thermodynamic approach used in the present study.

Thus, the thermodynamic analysis for hydrophobic substrata such as PTFE, PA, PE and SS316 gives a good prediction of initial diatom cell attachment. This thermodynamic model is a potentially very interesting tool for predicting the initial adhesion of diatoms on all types of hydrophobic or moderately hydrophobic surfaces.

Conclusion

In the present paper, the initial interaction between diatom cells and different substrata, with very different hydrophobic and hydrophilic surface properties was studied. Diatom cells were grown on a shaker so that they did not form a biofilm, and then freeze-dried, in order to have diatoms with their initial mucilage only. A chemical attraction occurred between these diatom cells and the substrata, which was predicted by the free energy of adhesion between the two components. The free energy was calculated from the surface energy of both diatom cells and surface substrata, using a thermodynamic approach. In general, the more hydrophobic the surface, the more strongly *N. jeffreyi* adheres to it. We observed very weak attachment to surfaces with a total surface energy superior to 42 mJ/m^2. This paper constitutes an original study of the transitory physico-chemical attraction between diatom cells containing bound initial EPS and the substratum. This leads to an initial contact between the two components, which was called ‘‘the first kiss’’ by Wetherbee and represents “an active commitment by raphid diatoms to attach and activates adhesion mechanisms specifically designed for subsequent binding to the substratum” [12]. One previous study about physico-chemical surface properties of microalgae has been performed in 2013 [20] and showed interesting correlations between surface properties and the cell-cell interactions, estimated by their propensity to form colonies. The present study provides information for a better knowledge of cell-surface interaction for a particular species of diatom. Both cell-cell and cell-surface interactions are very important parameters for diverse biotechnological applications including algal biomass production and marine biofouling prevention.

Competing interests

The authors declare that they have no competing interests.

Author’s contributions

GLK conducted supports and diatom surface energy measurements, diatom adhesion measurements, SEM observations and IR measurements, she drafted the manuscript, GP carried out EPS extraction, sugar and protein assays and drafted the manuscript, MNBF supervised supports and diatom surface energy measurements and revised the manuscript, JMZ carried out determination of sugar composition of bound EPS by GC/MS, MB is in charge of diatom culture, TM supervised the bound EPS extraction and their subsequent analysis, the study was coordinated by MG, who also contributed to the data analysis and revised the manuscript.

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Figure Legends

Figure 1: Scanning electron micrographs of *Navicula jeffreyi*. White arrow highlights the bound EPS.

Figure 2: Kinetic study of the adhesion of Navicula jeffreyi on five different substrata (Glass, PTFE, PE, PA and SS316; see Materials and Methods).

Figure 3: The values of the total interfacial free energy of adhesion of *N. jeffreyi* to the five studied substrata and its components are given. Total (ΔG_{adh}^{Total}, black dots), acid/base (ΔG_{adh}^{AB}, grey dots) and Lifshitz van der Waals (ΔG_{adh}^{LW}, empty dots) interfacial free energy of adhesion of *Navicula jeffreyi* on five different substrata.

Figure 1

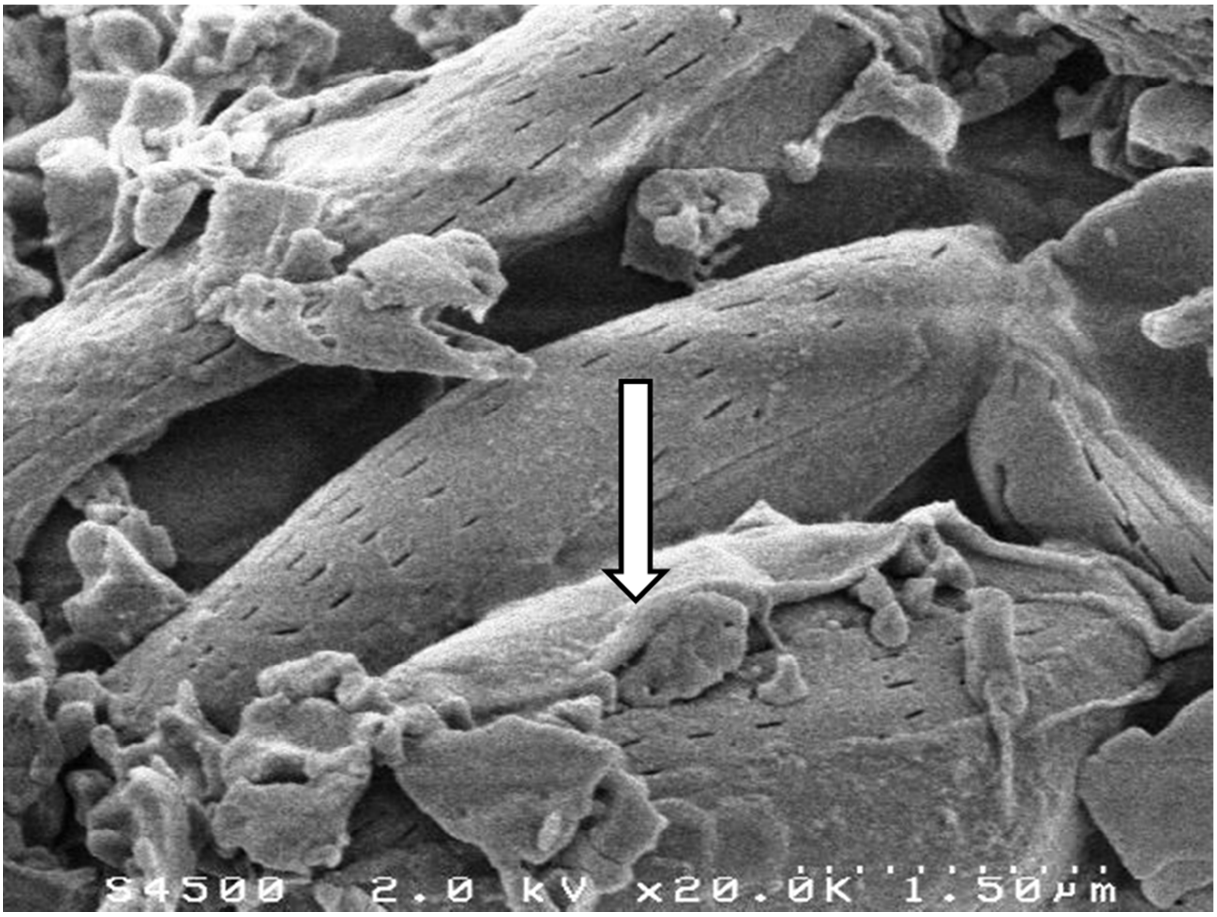
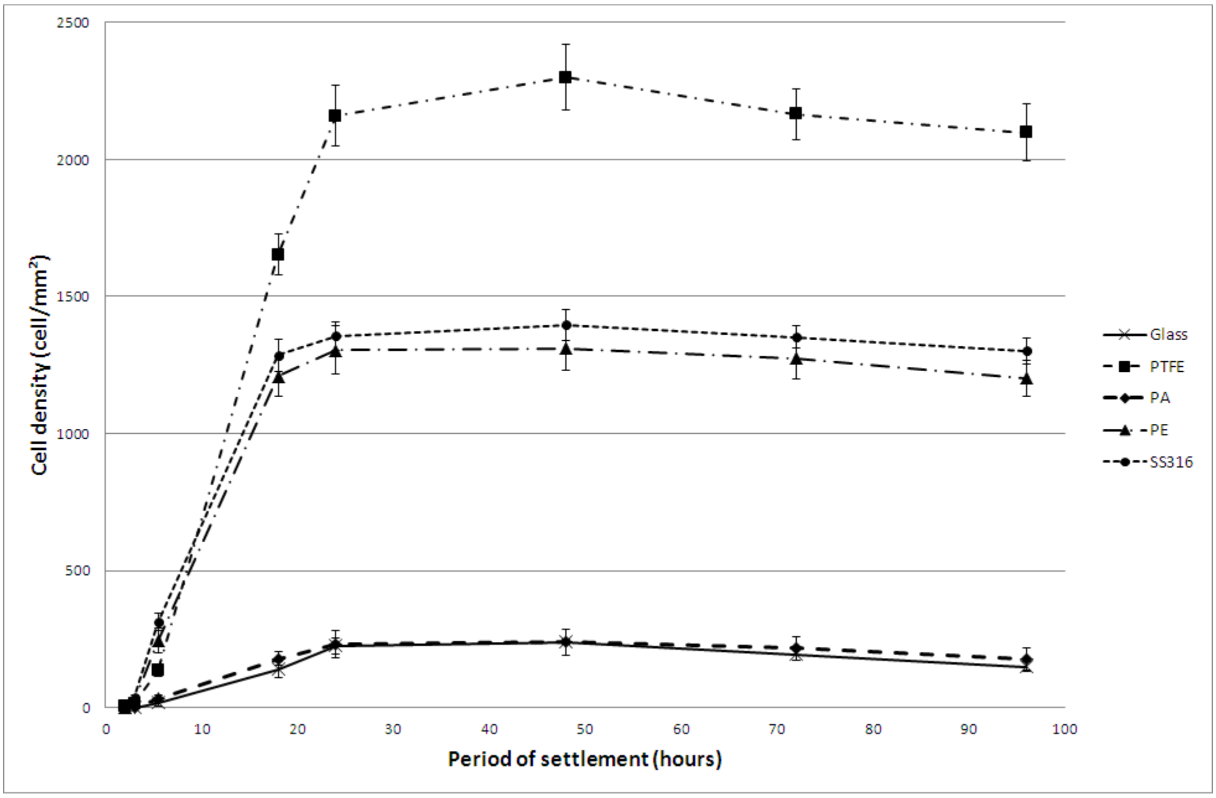


Figure 2



2500
2000
1500
1000
500
0
0
10
20
30
40
50
60
70
80
90
100
Cell density (cell/mm²)
Period of settlement (hours)
Glass
PTFE
PA
PE
SS316

618 Figure 3

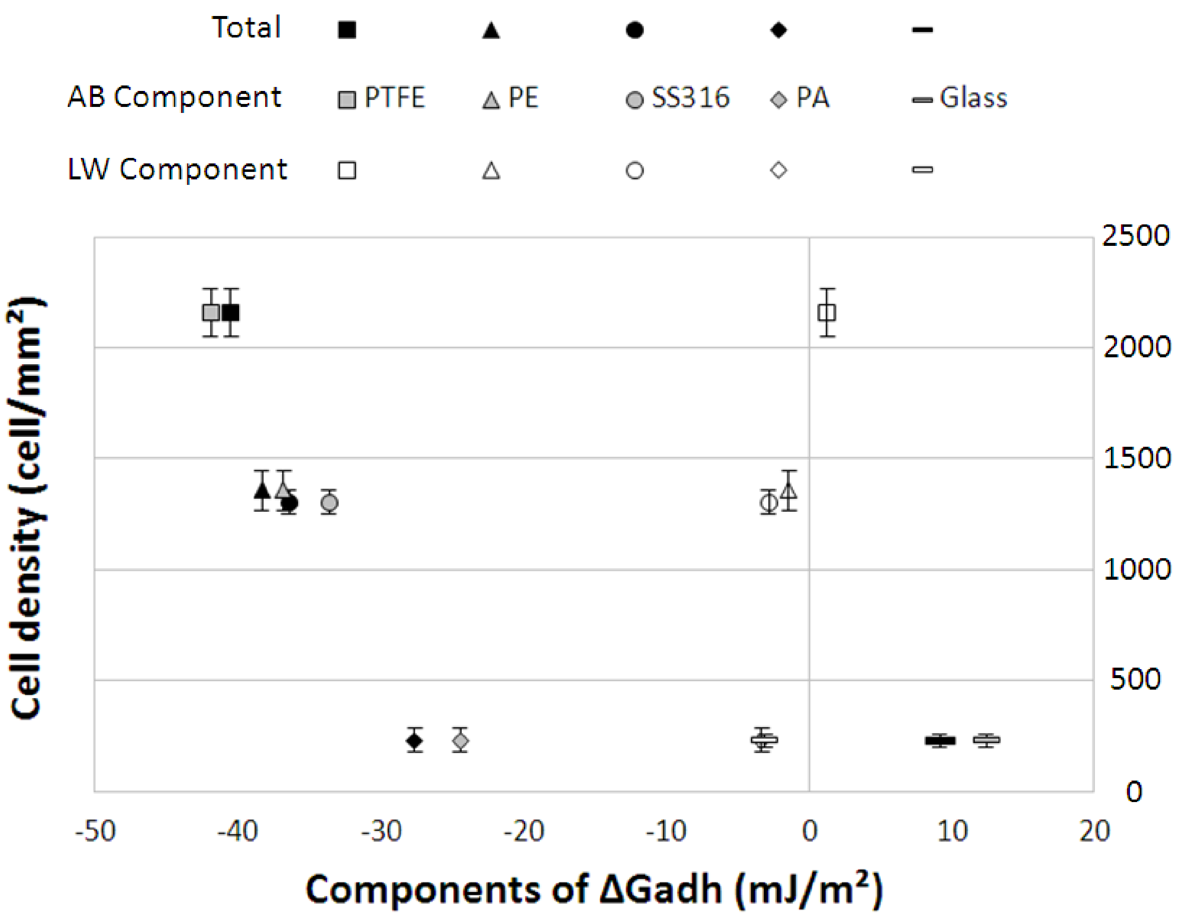


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Table 1: Surface tension components of the different test solvents used in the contact angle measurements: total (γ^t), Lifshitz-van der Walls (γ^{LW}), electron-acceptor (γ^+) and electron donor (γ^-) components.

Test liquids	**Purity**	**Surface energy (mJ/m²)**			
		γ_L^t	γ_L^{LW}	γ_L^+	γ_L^-
Water	MilliQ	72.8	21.8	25.0	25.0
Diiodomethane	> 98 %	50.8	50.8	0.0	0.0
Formamide	> 99 %	58.0	35.6	2.3	39.6

Table 2: Monosaccharide composition (% w/w) of polysaccharidic fraction of bound EPS from cultures of *N. jeffreyi* (end of the exponential growth phase), after extraction through Dowex-resin, with and without a freeze-drying step before extraction. Values are mean ±SD of three samples from a culture of *N. jeffreyi*, the variability within true sample replicates of the biochemical analysis was less than 5%.

Monosaccharide	**with freeze-drying**	**without freeze-drying**
Galacturonic acid	17±3	15±4
Sulfated sugars	0±0.2	0±0.3
Neutral sugars	83±8.9	85±10.7
Glucose	36.1±4	34.5±3
Rhamnose	8.9±0.9	11.2±3
Mannose	18.4±2.1	21.3±4.1
Galactose	19.5±1.7	17.9±0.8

Table 3: Contact angle measurements of Stainless Steel AISI 316L (SS316), Polytetrafluoroethylene (PTFE), Polyamide-nylon 6 (PA) and Polyethylene (PE), Glass, membrane filters alone and *Navicula jeffreyi* layers previously deposited on cellulose triacetate membrane filters. The results presented are the average of at least 8 measurements done with each probe liquid for each surface, cell layer or solid substrata.

Sample	**Contact angle (°)**		
	Water	Diiodomethane	Formamide
SS316	78.7 ± 0.7	44.5 ± 1	53.6 ± 0.6
PTFE	110.9 ± 2.0	83.2 ± 1.2	99.4 ± 1
PA	70.9 ± 2.5	38.5 ± 1.5	52.4 ± 1.9
PE	99.7 ± 4	85.7 ± 1.5	58.7 ± 2.7
Glass	10 ± 1.6	40.5 ± 3	10 ± 0.1
Membrane Filter	56.2 ± 1.8	44.0 ± 0.5	49.9 ± 1.7
Navicula jeffreyi	68.6 ± 2.9	54.3 ± 2.1	56.2 ± 6.1

Table 4: Surface energy of membrane filters alone, *Navicula jeffreyi* layers previously deposited on cellulose triacetate membrane filters, the five selected substrata calculated from equation (1) and cell density mean values measured on ten different fields for the five substrata.

Sample	**Surface energy (mJ/m²)**					**Cell density (cell.mm²)**
	γ^{t}	γ^{LW}	γ^{AB}	γ^{+}	γ^{-}	
Membrane Filter	39.8 ± 1.0	37.5 ± 0.3	2.2 ± 3.4	0.1 ± 0.1	28.6 ± 1.2	
Navicula jeffreyi	36.7 ± 4.1	31.8 ± 1.1	4.8 ± 2.9	0.4 ± 0.1	16.1 ± 2.1	
PTFE	17.1 ± 0.6	15.9 ± 0.6	1.2 ± 0.3	0.2 ± 0.1	1.9 ± 0.5	2160 ± 110
PE	32.5 ± 2.7	29.3 ± 1.5	3.2 ± 1.2	0.8 ± 0.3	3.4 ± 1.6	1356 ± 88
SS316	40.6 ± 0.4	37.3 ± 0.8	3.3 ± 0.3	0.6 ± 0.1	5.0 ± 0.6	1305 ± 52
PA	42.4 ± 0.9	40.4 ± 0.8	2.0 ± 0.2	0.1 ± 0.0	11.5 ± 1.5	231 ± 51
Glass	56.2 ± 0.3	39.3 ± 1.6	16.8 ± 1.9	1.3 ± 0.3	54.1 ± 0.2	225 ± 29