Conditioning of self-assembled monolayers at two static immersion test sites along the east coast of Florida and its effect on early fouling development

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Among the first events after immersion of surfaces in the ocean is surface ‘conditioning’. Here, the accumulation and composition of the conditioning films formed after immersion in the ocean are analyzed. In order to account for different surface chemistries, five self-assembled monolayers that differ in resistance to microfouling and wettability were used. Water samples from two static immersion test sites along the east coast of Florida were collected at two different times of the year and used for experiments. Spectral ellipsometry revealed that conditioning films were formed within the first 24 h and contact angle goniometry showed that these films changed the wettability and rendered hydrophobic surfaces more hydrophilic and vice versa. Infrared reflection adsorption spectroscopy showed that the composition of the conditioning film depended on both the wettability and immersion site. Laboratory and field assays showed that the presence of a conditioning film did not markedly influence settlement of microorganisms.

Keywords: static immersion; conditioning film; self-assembled monolayers; field experiments; protein adsorption; Ulva linza

Introduction

When surfaces are immersed in the ocean, adsorption of proteins and other macromolecules (dissolved organic carbon, DOC) is among the first processes and a so-called conditioning film is formed. Between the different adsorbing molecules and species, cooperative and competing effects may occur and affect the formation of biofouling communities. Thome et al. (2012) showed that the presence of adsorbed DOC affected the settlement of zoospores of Ulva linza. The formation and composition of conditioning films have been investigated by a number of groups and their formation was observed as early as 1975 by Loeb and Neihof (1975) and later by Meyer et al. (1988); Bradshaw et al. (1997); van der Aa and Dufrene (2002) and Jain and Bhosle (2009). The major components of the conditioning layers were glycoproteins, humic acids (Loeb and Neihof 1975), proteins (Compère et al. 2001; Bakker et al. 2004), lipids, nucleic acids, polysaccharides, aromatic amino acids (Taylor et al. 1997), and several unspecified macromolecules (Zaidi et al. 1984). The concentration and composition of dissolved organic carbon (DOC) varied depending on seasonality and the salinity of the surrounding water, reaching a maximum in late winter to early spring (Cadée 1982). This in turn could have an impact on the conditioning films (Cadée 1982; Bakker et al. 2004). While the composition and seasonal variations in the conditioning layers have been studied in the past, there is still a lack of understanding of whether conditioning film formation is affected by the properties of immersed substrata and to what extent the presence of conditioning layers affects the initial accumulation of fouling organisms.

In the present study, natural seawater was collected from two static immersion test sites along the east coast of Florida, USA, at two different times of the year: summer 2012 (June) and winter 2010 (December). The two test sites were chosen since the population density and the seasonal variation of organisms have been studied extensively at both sites (Swain et al. 2006; Zargiel et al. 2011; Sweat and Johnson 2013; Zargiel and Swain 2013; Ralston and Swain 2014). The water was used to precondition self-assembled monolayers (SAMs) with different end group chemistries. SAMs are particularly useful well-defined model surfaces (Bain and Whitesides 1989; Ulman 1996) as they allow the fine-tuning of the chemistry of surfaces while maintaining their mechanical properties. These properties affect colonization by marine organisms (Callow et al. 2000; Schilp et al. 2007; Ekblad et al. 2008; Cao et al. 2010;

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Thome et al. 2012), thus SAMs were used in the present study to understand the influence of surface chemistry on conditioning and the initial attachment of microorganisms.

Spectral ellipsometry (SE) and water contact angle goniometry (CA) were used to investigate the formation of the conditioning layers, while infrared reflection absorption spectroscopy (IRRAS) was used to determine chemical differences in the adsorbed conditioning layers. To understand the relevance of a conditioning layer for early colonization by the settling stages of fouling organisms, SAMs were preconditioned in seawater for 24 h and along with pristine (ie unconditioned) samples, settlement assays with zoospores of Ulva linza were performed. In a different experiment, surfaces conditioned with natural water and pristine samples were immersed for 24 h during the summer at the static immersion test site to investigate the influence of a formed conditioning film on early settlement of fouling organisms in the field.

Materials and methods

Contact angle goniometry

Sessile drop (Millipore, Schwalbach, Germany) water contact angles were measured with a custom-built contact angle goniometer under ambient conditions. The reported values are the average of three measurements taken for different samples without the tip being in contact with the droplet. The error bars represent the standard error (SE) of the mean.

Spectral ellipsometry

To determine the thickness of the SAM and the adsorbed protein layer, spectral ellipsometry measurements were performed with a M44 (J.A. Woollam Co., Lincoln, NE, USA) operating in a wavelength range between 280 and 800 nm. As a light source, a Xenon lamp with a polychromatic spectrum was used. The organic film was modeled as a single layer using a Cauchy model with fixed refractive index of 1.45. On each sample three measurements were performed at different positions and the reported values are the average of these three measurements. The error bars represent the SE of the mean.

Infrared reflection absorption spectroscopy (IRRAS)

IRRAS data for the SAMs were recorded at atmospheric pressure with a Bruker VERTEX 80 FTIR-spectrometer equipped with a liquid-nitrogen cooled, narrow-band MCT detector and a purge gas generator (Bruker Optics, Ettlingen, Germany). All IRRAS data were recorded with a resolution of 2 cm\(^{-1}\) using the p-polarized part of radiation at an incidence angle of 80° relative to the surface normal. SAMs of perdeuterated 1-hexadecanethiol on Au were used as a reference in the IRRAS experiments.

Preparation and characterization of self-assembled monolayers

Self-assembled monolayers with different wettability were prepared from solutions of 1-dodecanethiol (DDT), 11-mercapto-1-undecanol (HUDT) and 11-amino-1-undecanethiol hydrochloride (AUDT), 11-(tridecafluorooctylxoxy) undecanethiol (FUDT) and hydroxy-PEG2000-thiol (PEG) (Table 1). The DDT, HUDT, AUDT and ethanol were purchased from Sigma Aldrich (Munich, Germany) and used without further purification. FUDT was purchased from ProChimia Surfaces Sp. z o.o. (Sopot, Poland). PEG was purchased from Rapp Polymere GmbH (Tübingen, Germany). Deionized water was purified with a MilliQ® plus system (Millipore). Thin films of polycrystalline gold were prepared by thermal evaporation of 5 nm of titanium as an adhesion promoter and subsequent deposition of 30 nm of gold (99.99 % purity) onto Schott float glass slides (Georg Albert PVD, Heidelberg, Germany). Evaporation was performed at a pressure of 2 x 10^{-7} Torr and a deposition rate of 1 nm s\(^{-1}\). The gold-coated glass slides used for the experiments were of extra-smooth quality with an rms roughness value of about 1 nm. The gold-coated slides were stored under argon until they were used. Prior to immersion, the samples were placed in a UV light-emitting reactor for a minimum of 1 h in order to remove adsorbed organics from the surface, then rinsed with absolute ethanol, ultrasonicated for 3 min and dried in a nitrogen stream. Then they were immersed in 1 mM ethanolic solutions of the SAMs of polycrystalline gold were prepared by thermal evaporation of 5 nm of titanium as an adhesion promoter and subsequent deposition of 30 nm of gold (99.99 % purity) onto Schott float glass slides (Georg Albert PVD, Heidelberg, Germany). Evaporation was performed at a pressure of 2 x 10^{-7} Torr and a deposition rate of 1 nm s\(^{-1}\). The gold-coated glass slides used for the experiments were of extra-smooth quality with an rms roughness value of about 1 nm. The gold-coated slides were stored under argon until they were used. Prior to immersion, the samples were placed in a UV light-emitting reactor for a minimum of 1 h in order to remove adsorbed organics from the surface, then rinsed with absolute ethanol, ultrasonicated for 3 min and dried in a nitrogen stream. Then they were immersed in 1 mM ethanolic solutions of the SAM in the dark at room temperature overnight. For the preparation of the AUDT samples 3 vol% NEt\(_3\) was added to the solution (Wang et al. 2005). After removal from the thiol solution, the SAMs were first rinsed with absolute ethanol, then ultrasonicated for 3 min and finally dried in a flow of nitrogen.

Contact angle goniometry revealed that a series of surfaces with sessile water contact angles between 24° and 112° were obtained (see Table 1). This is the key to the study, as formation of conditioning was studied as a function of wettability. Thicknesses as determined by spectral ellipsometry were in the range of 1–2 nm. The values measured for the hydrophobic DDT and FUDT surfaces were in good agreement with literature values (Bain et al. 1989; Zhu et al. 2001). Also the IR spectra obtained showed the typical methylene vibrations at 2,920 cm\(^{-1}\) and 2,851 cm\(^{-1}\) (FUDT) and 2,919 cm\(^{-1}\) and 2,850 cm\(^{-1}\) (DDT), indicating an excellent crystalline order of the films (Porter et al. 1987; Nuzzo et al. 1990; Harder et al. 1998; Arnold et al. 2001; Tielens et al. 2008). The thickness of the AUDT SAM of 1.5 nm was
also in the expected range (Wang et al. 2005; Schilp et al. 2008). The methylene vibrations of the AUDT coatings were observed at 2,927 cm\(^{-1}\) and 2,856 cm\(^{-1}\), respectively, indicating in agreement with literature spectra on similar SAMs a less well-ordered monolayer (Nakano et al. 2003; Chen et al. 2006). The measured thickness for HUDT samples is smaller than those found in the literature (Frutos et al. 2000) and also the IR spectra suggest disordered layers since the position of the stretching mode appears at 2,927 cm\(^{-1}\). This is unusual, as HUDT SAMs are known to assemble well and form crystalline surfaces (Nuzzo et al. 1990; Bertilsson and Liedberg 1993; Tielens et al. 2008). A post-experimental analysis of the components revealed unusually high amounts of dithiols in the base material, which might have altered the kinetics of SAM formation and a too low packing density was achieved. Nonetheless, the film thicknesses of the HUDT SAMs are only slightly (18%) below those for the DDT surfaces, so that complete coverage of the surface with a slightly lower packing density and order can be expected. The PEG surfaces had a higher thickness of ~3 nm due to the much longer thiol molecules. Also here, very disordered films are formed due to the short aliphatic spacer, which was used to avoid coil-to-brush transition (Tokumitsu et al. 2002; Schilp et al. 2007; Thome et al. 2012). In summary, the hydrophobic SAMs were rather crystalline and a shift to more disordered SAMs occurred towards the hydrophilic surfaces.

Table 1. Thiol molecules used to form self-assembled monolayers, static water contact angle and thickness as determined by contact angle goniometry and spectral ellipsometry.

| Chemistry | Name | Label | Water contact angle [°] | Ellipsometric thickness [nm] |
|-----------|------|-------|-------------------------|-----------------------------|
| SH-(CH\(_2\)_11-(O-(CH\(_2\)_2))\(_{44}\)-OH | Hydroxy-PEG 2000-thiol | PEG | 31 ± 4 | 3.1 ± 0.6 |
| SH-(CH\(_2\)_11)-OH | 11-Hydroxy-1-undecylthiol | HUDT | 24 ± 3 | 0.9 ± 0.1 |
| SH-(CH\(_2\)_11)-NH\(_2\) | 11-Amino-1-undecylthiol | AUDT | 57 ± 4 | 1.5 ± 0.2 |
| SH-(CH\(_2\)_11)-CH\(_3\) | Dodecanethiol | DDT | 101 ± 2 | 1.1 ± 0.2 |
| SH-(CH\(_2\)_11-O-(CH\(_2\)_2)-(CF\(_2\)_5)- | (Tridecafluorooctyloxy) | FUDT | 112 ± 3 | 1.6 ± 0.2 |

The values are the means of 12 measurements; errors are the SD.

Preparation of the seawater samples used in experiments

The different types of seawater used in the experiments were either prepared from pure salts (salt water; SW) or collected from natural sources (‘Sebastian water’ (SeW) and ‘Canaveral water’ (CW)). The artificial seawater (SW) was made up freshly using MilliQ water as described by Kester et al. (1967); Table 2. All chemicals were of analytical grade and the SW was filtered through a 0.45 µm Millipore filter after preparation.

Table 2. Salt mixture used to prepare 35.00% self-mixed salt water (SW) (artificial seawater according to Kester et al. (1967)).

| Salt | g l\(^{-1}\) |
|------|-------------|
| NaCl | 23.926 |
| Na\(_2\)SO\(_4\) | 4.008 |
| KCl | 0.677 |
| NaHCO\(_3\) | 0.196 |
| KBr | 0.098 |
| H\(_3\)BO\(_3\) | 0.026 |
| NaF | 0.003 |

The two sources of natural seawater used were from two static immersion test sites along the east coast of Florida. The ‘Sebastian’ site is located in the Indian River Lagoon about 5 km north of the Sebastian inlet, Florida (27° 53’ 59.04”N, 80° 28’28.31”W). The conditions at this site are estuarine, being influenced by both freshwater tributaries and small tidal fluctuations. The salinity at this site ranges from 18 to 37 ppt, with an average of 30 ppt. The temperature ranges from 8 to 34°C, with an average of 26°C. The ‘Canaveral’ site is located at Port Canaveral (28° 24’28.30”N, 80° 37’ 39.78” W). The water conditions here are influenced by the ocean, which has open access to the Port, and is an area of high fouling activity (Swain and Zargiel, unpublished data). The salinity at the Canaveral site ranges from 31 to 37 ppt, with an average of 35 ppt. The temperature ranges from 14 to 29°C, with an average of 25°C. The water was collected at a depth of ~0.3 m below the air–water interface and first filtered through a 63 µm filter to remove larger particles before filtering through a 0.45 µm syringe filter to remove the remaining organisms and particulates from the water. Macromolecules including proteins would pass through the filter and remain in the solution. The ‘Sebastian water’ had a pH of 8.25, the ‘Canaveral water’ a pH of 8.04. The water samples were frozen and stored at –20°C. For each conditioning experiment the water was defrosted freshly and used within a few days.
Surface conditioning

Conditioning film formation from filtered (0.45 μm) seawater was analyzed on all five surfaces following established protocols (Thome et al. 2012) derived from those for protein adsorption assays (Prime and Whitesides 1991; Herrwerth et al. 2003). For surface analysis, the SAM-coated glass surfaces were cut into square pieces of 25 x 25 mm². Slides for biological testing were used as whole slides (25 x 75 mm²). First, the samples were immersed in a 50 ml glass bottle with 5 ml of artificial salt water (SW) solution (see Table 2), for 15 min. This volume was sufficient to cover the slides completely and allowed the surfaces to saturate with water of the same salinity as seawater. Then 15 ml of the seawater to be tested were added. After different times (1 h, 3 h, 6 h, 24 h, 48 h), the seawater was diluted with 500 ml of MilliQ® water (MQ) to avoid Langmuir–Blodgett film formation during transfer through the air–water interface. The slides were rinsed with MQ water to remove residual salts and dried in a stream of nitrogen. The dried samples were used for spectral ellipsometry, contact-angle measurements and IRRAS. The results showed that this protocol produced conditioning films of significant thickness and served as a sensible approach, considering the lacking in situ spectroscopic capabilities in the field. This procedure faces a small risk that loosely bound components might have been removed during preparation, which is an issue that could be solved by future in situ ATR-IR spectroscopy studies.

Zooplankton settlement assay

Plants of Ulva linza were collected from the beach at Llantwit Major, South Wales. Zoospores were released from fertile tips into filtered (0.22 μm) artificial seawater (Tropic Marin; TM). The spore suspension was filtered through three layers of nylon mesh (100, 50 and 20 μm) to remove debris. The beaker containing the spore suspension was plunged into ice to concentrate the spores (zoospores swim towards the bottom of the beaker), which were removed with a pipette. This procedure was repeated and then the spore suspension was filtered through two layers of nylon mesh (20 μm pore size). The spore suspension was kept on a magnetic stirrer and the absorbance adjusted to 0.15 at 660 nm (1.0 x 10⁶ spores ml⁻¹) with filtered (0.22 μm) TM. The spore suspension was used in bioassays within 10 min.

Ten ml of spore suspension were pipetted into individual compartments of quadriperm dishes (Greiner-One), each containing a SAM-coated glass microscope slide (25 x 75 mm²). The dishes were incubated in darkness at room temperature for 30 min. At the end of the incubation period, the slides were washed to remove unattached (motile) spores by passing the slide 10 times through a beaker of TM. The slides were fixed in 2.5 % glutaraldehyde in TM (10 min), then washed sequentially in TM, 50% TM and deionized water and allowed to air dry. Settled spores were visualized and counted by auto-fluorescence of chlorophyll using an AxiosVision 4 image analysis system attached to a Zeiss epifluorescence microscope (Carl Zeiss, Oberkochen, Germany) (20x objective, excitation and emission of 546 and 590 nm, respectively). The reported data are the average of 90 counts, 30 counts from each of three replicates of each sample type; error bars show 2 x SEs.

Field experiments were performed in June 2012 at the ‘Sebastian’ test site. Samples were pre-incubated in filtered seawater from the ‘Sebastian’ test site (‘Sebastian water’ (SeW)) for 24 h. Then conditioned surfaces and pristine surfaces (without conditioning) were immersed in the lagoon for 24 h. The immersion time chosen was a compromise to allow sufficient microorganisms to populate the surface whilst minimizing conditioning of the pristine surfaces. For each experiment two replicates of each surface were immersed. The experiment was carried out twice on two consecutive days (day 1 (d1), day 2 (d2)). The test samples were arranged as shown in Figure 1 using commercially available plastic microscope slide holders (Carl Roth, Karlsruhe, Germany) and held in place by rubber bands. All samples had the same orientation and distance from each other and the terminating slide, number 20, faced glass. Each slide holder contained four surfaces of each chemistry; two pristine and two conditioned. The layout ensured that environmental factors such as shear, solar irradiation and salinity had the same influence on all samples. The samples were immersed ~0.5 m below the air–water interface. After 24 h, the samples were removed from the water without passing through the air–water interface using a bucket full of water. Within this bucket the samples were transferred underwater into the containers for fixation. For the analysis of the thicknesses and the contact angle of the adsorbed conditioning films, three measurements were performed and plotted against the incubation time (Figure 2). For the analysis of the settlement density of the organisms in the field test (see Figure 6), 2 pristine and 2 conditioned slides were immersed each day. The mean counts are based on 60 fields of view (FOV) per sample. Error bars are the SE of the mean of both samples (SEM, n = 120).

Sample fixation and analysis

The natural seawater in the holders containing the samples was exchanged with freshly filtered (0.45 μm) seawater and adjusted to 200 ml. For fixation, 30 ml of a 37% (v/v) formaldehyde solution were added to give a final concentration of 4.8%. After 1 h, the containers were flooded with filtered seawater before gradually
decreasing the salt concentration by dilution with distilled water. After this treatment the samples were air-dried. The samples were viewed by phase contrast microscopy (TE-2000, Nikon) using a 15x objective. To evaluate settlement density, a FOV of 1,340 x 1,004 μm was used and for each sample 60 FOVs, each stitched together from four single FOVs (670 x 502 μm), distributed across the whole surface were obtained. For the identification of the different species on the samples, a FOV of 670 x 502 μm was used and for each sample 20 FOVs were evaluated. The most common organisms found on the samples were identified to genus and the common genera were counted, namely, *Navicula*, *Amphora*, *Bacillaria*, *Cylindrotheca*, *Mastogloia*, *Coscinodiscus*, *Plagiotropis*, *Licmophora*, *Brachysira*, *Triceratium* and *Nitzschia*. The genera *Mastogloia*,

Figure 1. Experimental layout of the static immersion test at the ‘Sebastian’ test site. (a) Image of samples in the microscope slide holder fixed with rubber band. (b) All samples have the same orientation, the same distance and the terminating slide (number 20) faced an uncoated glass slide. (c) Arrangement of samples in the holder.

Figure 2. Conditioning of self-assembled monolayers with different chemical termination by natural seawater from two test sides (‘Sebastian’, ‘Canaveral’) after different immersion times showing the thickness of the conditioning layer (a, b) and contact angle (c, d) of the conditioned surfaces at two different times of the year (summer 2012 (June) and winter 2010 (December)). Each point is the mean of three ellipsometric or contact angle measurements. Error bars are the SEM.
*Coscinodiscus*, *Plagiotropsis*, *Licmophora*, *Brachysira*, *Triceratium* and *Progonia* were found only occasionally and thus were grouped as ‘others’. The subclass of protozoa was counted as peritrichous ciliates.

**Results and discussion**

Filtered seawater, collected from two different sites (‘Sebastian’ and ‘Canaveral’) at two different times of the year (summer 2012 (June) and winter 2010 (December)), was used to investigate the formation of conditioning films on the five different surfaces. The conditioning films were characterized after various incubation times by spectral ellipsometry and contact angle goniometry. As shown in Figure 2, a conditioning film was built up over time on all surfaces except PEG. In contrast, PEG showed a decrease in film thickness. Statistical analysis of three measurements with the Student’s *t*-test revealed that no statistical difference between film thicknesses on chemically identical substrata could be obtained for ‘Sebastian water’ and ‘Canaveral water’ after 48 h immersion (*p* > 0.05). Also, no difference in the film thicknesses on the chemically different surfaces between June 2012 (water temperature: 29°C) and December 2010 (water temperature: 22°C) could be detected (*p* > 0.05). Only HUVT surfaces immersed in ‘Sebastian water’ showed a significant difference between June 2012 (8 ± 1 Å) and December 2010 (13 ± 1 Å) (*p* < 0.05) (see Figure 2a and b). No major changes in the rate of film growth on the different surface chemistries were found, and irrespective of whether surfaces were hydrophilic or hydrophobic, all were rapidly conditioned by biomacromolecules.

Some general trends were in line with earlier studies (Thome et al. 2012). Conditioning of surfaces by spore water (artificial seawater (TM) in which swimming zoospores of *U. linza* had been incubated) also revealed that the rate of accumulation did not depend on the surface chemistry. In addition, the film thicknesses obtained were in the same range as those observed for the natural seawater used in the present experiments. Furthermore, the decrease in film thickness for PEG surfaces was also observed in both studies.

The increase in film thickness as measured by spectral ellipsometry seems to be connected with the changes in the CA after conditioning (see Figure 2c and d) and occurred during the first 10 h after immersion. Interestingly, the magnitude of the CA decrease for the hydrophobic surfaces was larger in June 2012 than in December 2010, while the change in wettability of the hydrophilic surfaces was in the same range, independently of the time of the year.

To obtain more detailed information about the chemistry of the conditioning layers formed on the different surfaces, infrared reflection absorption spectroscopy (IRRAS) was applied to samples conditioned with ‘Canaveral water’ and ‘Sebastian water’ from the June 2012 experiment (see Figure S1 in Supplementary Information) [Supplementary information is available via a multimedia link on the online article webpage]. As expected, the symmetric and asymmetric CH2 stretching vibrations of the methylene backbone of the aliphatic SAMs were observed in all spectra. While the intensities of the methylene vibrations remained almost constant for the different incubation times, the carbonyl (1,745 cm−1, C=O stretching vibrations), amide I (1,650 cm−1, most likely C=O stretching vibrations of the peptide bond), and amide II (1,540 cm−1, N–H bending and C–N stretching vibrations) increased during the formation of the conditioning layer (peak assignment according to Myrskog et al. (2010) and Winter et al. (2011)). This suggests that the films were composed of macromolecules containing carbonyl and amide groups, ie most likely proteins, glycoproteins, lipids and polysaccharides (Loeb and Neihof 1975; Taylor et al. 1997; Compère et al. 2001). Figure 3 shows the carbonyl and amide bands after 48 h incubation in ‘Sebastian water’ and ‘Canaveral water’ for all four alkanethiol surfaces. PEG is not shown in this Figure because no carbonyl and amide bands were present on this surface. PEG will be discussed separately. It can be seen that the shapes and relative intensities of the carbonyl, amide I and amide II peaks showed slight differences. Especially on the hydrophilic AUDT and HUVT samples, the carbonyl and amide I bands (1,745 cm−1 to 1,650 cm−1) were very pronounced, indicating the presence of proteins or proteinaceous components. An interesting point to note is that the shape of the carbonyl bands at 1,745 cm−1 differed between AUDT samples immersed at the ‘Sebastian’ and ‘Canaveral’ test sites. The peaks seemed to be a summation of at least two components, which reflects differences in the degrees of hydration and hydrogen bonding of the carbonyl groups (Blume et al. 1988; Lewis et al. 1994; Lewis and McElhaney 1998) and indicates a different composition of the conditioning layer. Interestingly, the similar overall carbonyl and amide peak intensities of both films on AUDT are in line with the spectral ellipsometry data, revealing a similar conditioning layer thickness. In contrast, the two hydrophobic surfaces, DDT and FUDT, showed very weak carbonyl and amide bands after immersion in ‘Canaveral water’. While the spectra for FUDT after immersion in ‘Sebastian water’ showed comparably weak peaks, the conditioning film formed on DDT showed pronounced amide modes.

Plotting the intensities of the alkyl, carbonyl and amide bands against immersion time reveals that the molecules adsorbed on the different surface chemistries at different rates (Figure 4). While the intensity of the alkyl bands increased only weakly and was in general weak for all surfaces (Figure 4a), the intensity of the
carbonyl band (Figure 4b) and also the amide I and amide II bands (Figure 4c) depended on the surface chemistry. Both carbonyl and amide-containing components were highest on the hydrophilic AUDT sample and slightly weaker on the hydrophilic HUDT. Interestingly, the intensity of the carbonyl (panel b) and amide peaks (panel c) on the hydrophobic samples (DDT and FUDT) remained weak. This was surprising as all surfaces showed the formation of conditioning layers of similar thickness. Furthermore, in previous studies where artificial seawater (TM) conditioned by spores of *U. linza* was used to form a conditioning film, a strong increase in these bands was observed (Thome et al. 2012).

In agreement with the ellipsometry data, it can be noted that PEG surfaces deteriorated over time (see Figure S2). The peaks in the range of 1,122 cm\(^{-1}\) to 1,343 cm\(^{-1}\), typical bands of the C-O-C stretching, CH\(_2\) twisting and CH\(_2\) wagging modes of the ethylene glycol units (Valiokas et al. 1999; Pasquali et al. 2008; Wu et al. 2010), decreased with increasing incubation time. Also, the symmetric and asymmetric CH\(_2\) stretching bands at 2,888 cm\(^{-1}\) were reduced. Despite the degradation of the PEG, no amide peaks were observed during the first 48 h of immersion. Thus, a certain resistance against accumulation of molecules was retained even though the integrity of the coating was affected. After an extended incubation time of seven days, the PEG film completely disappeared and a conditioning film was detected on the surface (Figure S2).

While spectral ellipsometry revealed that the conditioning layer had a similar thickness on all tested surfaces (except PEG), IRRAS measurements revealed that the conditioning layers on the different surface chemistries showed variations in their chemical nature. As shown in a previous study (Thome et al. 2012), a conditioning film formed after incubation in spore water (ie filtered (0.22 μm) TM seawater in which spores of *U. linza* were allowed to swim for 1 h) influenced the subsequent settlement of spores. Thus, the question arose as to whether settlement of spores was also influenced by the presence of a conditioning layer formed from ‘Sebastian water’. Therefore surfaces were pre-immersed in ‘Sebastian water’ (SeW) and salt water (SW) (control) for 24 h. A standard settlement assay was conducted on

Figure 3. Carbonyl (1,745 cm\(^{-1}\)), amide I (1,650 cm\(^{-1}\)) and amide II (1,540 cm\(^{-1}\)) intensity on different surfaces after conditioning for 48 h with (a) ‘Sebastian water’ (SeW), (b) ‘Canaveral water’ (CW) from June 2012.

Figure 4. Intensity of the alkyl (a), carbonyl (b), amide I and amide II bands (c) on chemically different alkanethiol surfaces plotted against incubation time in ‘Sebastian water’ and ‘Canaveral water’ from June 2012. Error bars are the SEM of three spectra.
both surfaces conditioned by ‘Sebastian water’ (SeW) and salt water (SW) as well as pristine (non-immersed) coatings.

Figure 5 shows the settlement density of spores of *U. linza* after a 30 min assay. The density of spores on pristine SAMs with different wettabilities corresponds to data observed in previous studies, which showed that hydrophobic surfaces were more attractive for settlement than hydrophilic surfaces (Schilp et al. 2007; Mieszkin et al. 2012; Thome et al. 2012). PEG as a resistant standard showed extremely low settlement with < 20 spores mm⁻². The data show that pre-incubation for 24 h in salt water (SW) had no influence on the settlement density of zoospores compared with the pristine samples. Thus, incubation in SW for 24 h, a medium with high osmotic strength, did not change the ‘attractiveness’ of the SAMs for spore settlement.

Incubation in ‘Sebastian water’ led to some changes in spore settlement density for DDT and AUDT, but for HUDT and FUDT the density of settled spores appeared to be dominated by the underlying SAM. Statistical analysis with the Student’s t-test revealed that there was no statistical difference between the pristine samples and those incubated in salt water (SW) for DDT, HUDT and AUDT (*p* > 0.05). For FUDT there was a small difference between the samples (*p* < 0.05). A significant influence of ‘Sebastian water’ (SeW) could be detected for DDT and AUDT (*p* < 0.05). For HUDT and FUDT there was no significant difference (*p* > 0.05). It is interesting to note that the thickness of the conditioning layers was larger on DDT (17 Å), FUDT (15 Å), and AUDT (15 Å) surfaces compared with HUDT (8 Å) (*p* < 0.05) (see Figure 2). The IRRAS data for the conditioning films deposited on DDT and AUDT showed a much stronger amide I peak (see Figure 3a) compared with HUDT and FUDT. A comparison with literature reports on molecules that are involved in surface conditioning in the ocean shows that these carbonyl-containing substances are most likely composed of glycoproteins, humic acids (Loeb and Neihof 1975), proteins (Compère et al. 2001; Bakker et al. 2004), nucleic acids, and polysaccharides. The present data clearly show that the surface chemistry determines the composition of the molecules that absorb on the surface.

In addition to the laboratory assay with spores of *U. linza*, the question was asked whether colonization of the surfaces in the natural mixed-species environment was influenced by the presence of a conditioning layer. Therefore, surfaces were pre-immersed in ‘Sebastian water’ for 24 h prior to immersion in the ocean. Both conditioned surfaces and pristine coatings were simultaneously immersed at the ‘Sebastian’ static immersion test site on two sequential days, which had similar weather conditions.

The density of organisms that settled on the different surface chemistries without preincubation (pristine) and after 24 h preincubation in ‘Sebastian water’ (conditioned) is shown in Figure 6. Pristine and conditioned slides were immersed on consecutive days indicated by day 1 (d1) and day 2 (d2) (2 replicates in each set per day). It can be noted that the density of organisms on the pristine samples on d1 was approximately the same for all surface chemistries. On d2, the pristine hydrophobic
DDT and FUDT samples showed a higher attractiveness for settlement. The trend observed, ie that hydrophobic surfaces are more attractive for settlement than hydrophilic surfaces, supports the general trends reported for laboratory assays with single organisms. For example, spores of the green alga *U. linza* prefer to settle on hydrophobic compared with hydrophilic surfaces (Callow et al. 2000; Finlay et al. 2002; Schilp et al. 2007). On the hydrophilic HUDT and AUDT samples, the settlement density was independent of preconditioning, while on FUDT SAMs conditioning led to a reduction in spore settlement. Interestingly, the weakest carboxylic and amide modes in IRRAS were recorded for FUDT samples immersed in ‘Sebastian water’. Based on the present observations, it is concluded that neither surface chemistry nor conditioning affected the attached organisms, with the exception of FUDT on day 2. Furthermore, the settlement density of the cells/organisms was not altered in a consistent manner by the formation of a conditioning layer (Figure 6).

The distribution of the organisms that settled over a period of 24 h on pristine (0 h) and preconditioned (24 h) surfaces with different end group chemistries is shown in Figure 7. It can be noted that the most frequently observed species on all surfaces (30% to 40% of all observed organisms) was the diatom *Navicula* sp. The frequency of the other diatom genera (*Bacillaria*, *Amphora*, and *Cylindrotheca*) and peritrichous ciliates varied between 6% and 17% of all organisms, but the percentage occurrence of the different species was similar on all samples except HUDT, where the quantity of peritrichous ciliates was slightly reduced. No differences were observed in the occurrence of the different organisms between unconditioned (pristine, 0 h) and preconditioned (24 h) samples. The insensitivity of the initial attaching organisms to surface chemistry and conditioning is in agreement with findings of Hugget et al. (2009) who observed that the bacterial community composition of biofilms in Pearl Harbor, Hawaii, was similar on all samples, regardless of the initial surface wettability.

To summarize, the results have shown differences in the composition of the conditioning layers formed on SAMs of different hydrophobicity. However, the effect of the conditioning films on the subsequent settlement of organisms in both a laboratory bioassay and a short-term (24 h) field immersion appeared to be minimal. In an earlier study (Zargiel et al. 2011), non-biocidal fouling-release (FR) coatings including International Intersleek 700, International Intersleek 900 and Hempel Hempasil X3, immersed for 60 days at several static immersion test sites along the east coast of Florida, were fouled with similar diatom genera (eg *Amphora*, *Navicula*, *Bacillaria*, and *Cylindrotheca*) to those reported in the present study. A significant difference, however, was detected among the varying test sites concerning the diatom community composition, diversity and species richness (Zargiel et al. 2011). Additionally, FR coatings exposed at the ‘Canaveral’ site had significantly different diatom adhesion, abundance and community composition among the coating treatments. However, the differences were dependent on the date of sampling (Zargiel and Swain 2014) and may also have been influenced by bacteria and other microorganisms incorporated into the biofilm (Briand et al. 2012; Dobretsov et al. 2013;
Mieszkin et al. 2013; Ling et al. 2014). The question as to whether conditioning films formed over a longer period of time on non-biocidal FR coatings immersed in the ocean influence the settlement and adhesion strength of organisms still needs to be answered. It is likely that the effect of conditioning films on diatom settlement and adhesion strength is influenced by temporal variation in both the time (season) when conditioning films develop as well as the season in which subsequent biofilm communities develop.

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