Conditioning of surfaces by macromolecules and its implication for the settlement of zoospores of the green alga *Ulva linza*

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Conditioning, i.e. the adsorption of proteins and other macromolecules, is the first process that occurs in the natural environment once a surface is immersed in seawater, but no information is available either regarding the conditioning of surfaces by artificial seawater or whether conditioning affects data obtained from laboratory assays. A range of self-assembled monolayers (SAMs) with different chemical terminations was used to investigate the time-dependent formation of conditioning layers in commercial and self-prepared artificial seawaters. Subsequently, these results were compared with conditioning by solutions in which zoospores of the green alga *Ulva linza* had been swimming. Spectral ellipsometry and contact angle measurements as well as infrared reflection absorption spectroscopy (IRRAS) were used to reveal the thickness and chemical composition of the conditioning layers. The extent that surface preconditioning affected the settlement of zoospores of *U. linza* was also investigated. The results showed that in standard spore settlement bioassays (45–60 min), the influence of a molecular conditioning layer is likely to be small, although more substantial effects are possible at longer settlement times.

**Keywords:** self-assembled monolayers; protein adsorption; conditioning film; artificial seawater; zoospores, *Ulva linza*

**Introduction**

When a surface is exposed to natural water, macromolecules and ions rapidly adsorb from the solution, soon followed by the first fouling organisms. Biofouling on ships’ hulls leads to increased drag resulting in speed reduction (Schultz 2007; Schultz et al. 2011) and higher fuel consumption causing increased emission of greenhouse gases and particulates (Yebra et al. 2004). As the natural environment is a complex community of different molecules, microorganisms and spores/larvae of macrofoulers, both cooperative and competitive effects between the adsorbing/colonising species can occur. Soon after immersion in the ocean, conditioning layers are formed on the surface (Taylor et al. 1997; van der Aa and Dufrene 2002) consisting of organic and/or inorganic molecules which adsorb spontaneously within minutes to a few hours on the surface (Loeb and Neihof 1975; Jain and Bhosle 2009). Conditioning films formed from natural seawater are mainly composed of a mixture of glycoproteins, humic acids (Loeb and Neihof 1975), proteins (Compère et al. 2001; Bakker et al. 2004), lipids, nucleic acids, polysaccharide, aromatic amino acids (Taylor et al. 1997) and/or unspecified macromolecules (Zaidi et al. 1984). Baier (1973) was among the first to study the accumulation of molecules on germanium crystals exposed for 10 min in the seawater of Biscayne Bay (Florida, USA), using attenuated internal reflection infrared spectroscopy (ATIR), and he found that the conditioning film contained large amounts of proteins. Fluorescence measurements with water from different test sites indicated that humic acids may also be present (Loeb and Neihof 1975). Maki et al. (1990) investigated the growth of conditioning layers and found that the chemical composition and the quantity of adsorbed organic material depended on the chemistry of the surface during the first hours of immersion, but was completely independent of it after 4 h (Maki et al. 1990). However, the chemical nature of this adsorbed layer is still poorly understood (Compère et al. 2001). For a detailed understanding of cell/larval settlement, adhesion and colonization, it is desirable to know the kinetics of formation and the chemical composition of the conditioning layer. It is also important for interpreting the results of laboratory assays designed to compare the antifouling (AF) performance of experimental coatings.
In the present study the influence of surface conditioning on the settlement (attachment) of zospores of the green alga, *Ulva linza*, was examined over a wide range of settlement times. Spores of this species have been extensively used in comparative assays of the AF performance of experimental coatings (Callow and Callow 2011). However, a suspension of spores also contains molecules that may adsorb to the test surface and therefore may influence subsequent settlement of spores. These molecules originate from secretions by the spores themselves, but there will also be some residual molecules from the parent plants from which the spores are released. In the present work, ‘spore water’ (SP) composed of seawater in which spores released from plants had been swimming for 60 min, was used to precondition the substrata. Since a standard spore settlement assay is typically 45–60 min, SP contains the maximum amount of macromolecules available to condition a surface during the course of an assay. Conditioning by two types of commercial artificial seawater as well as self-prepared seawater was also evaluated. Spectral ellipsometry (SE), contact angle measurements (CA) and infrared reflection absorption spectroscopy (IRRAS) were used to reveal the formation and chemical nature of conditioning layers and the results were correlated with the effects on the subsequent settlement of zospores.

**Materials and methods**

**Preparation of self-assembled monolayers**

All chemicals, such as ethanol, 1-dodecanethiol (DDT), 11-mercapto-1-undecanol (HUDT) and 11-amino-1-undecanethiol hydrochloride (AUDT) were purchased from Sigma Aldrich (Munich, Germany). 11-(tridecafluorooctyloxy)undecanethiol (FUDT) was purchased from ProChimia Surfaces Sp. z o.o. (Sopot, Poland). Hydroxy-PEG2000-thiol (PEG) was purchased from Rapp Polymere GmbH (Tübingen, Germany). Deionized water was purified with a MilliQ® plus system (Millipore, Schwabach, Germany). 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). Deionized water was purified with a MilliQ® plus system (Millipore, Schwabach, Germany). 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 $2 \times 10^{-7}$ Torr and a deposition rate of 1 nm s$^{-1}$. The gold-coated glass slides used for the experiments were of extra-smooth quality. The rms roughness value was 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 organic adsorbents 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 self-assembled monolayer (SAM) molecules in the dark at room temperature overnight. 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 measurements**

Sessile drop (Millipore) 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).

**Spectral ellipsometry**

To determine the thickness of the SAM and the adsorbed protein layer, spectral ellipsometry measurements were performed with a M44 (JA Woollam Co., Inc.) operating in a wavelength range between 280 and 800 nm. The organic film was modeled as a single Cauchy layer with fixed refractive index. The ellipsometric measurements were performed on an instrument type M44 from the JA Woollam Co. As the light source, a xenon lamp with a polychromatic spectrum was used. On each sample three measurements were performed at different positions and the reported value is the average of these three measurements. The error bars represent the SE.

**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. All IRRAS data were recorded with a resolution of 2 cm$^{-1}$ using p-polarized radiation at an incidence angle of 80° relative to the surface normal. SAMs of perdeuterated 1-hexadecanethiol on Au were used as reference in the IRRAS experiments.

**Surface conditioning**

Conditioning film formation was evaluated with a given surface and the chosen type of water in a similar manner to established protein resistance assays (Prime and Whitesides 1991; Herrwerth et al. 2003). The SAM coated surfaces were cut into pieces of 25 x 25 mm$^2$. 
The samples were immersed in a 50 ml glass bottle in 5 ml of self-mixed salt water solution (see below) composed of salts at concentrations found in seawater (Kester et al. 1967) for 15 min to allow the surfaces to saturate with water which has the same salinity as seawater. Then 15 ml of the seawater to be tested were added. After different times (10 min, 20 min, 30 min, 60 min, 90 min, 2 h, 4 h, 6 h, 8 h, 12 h, 24 h, 48 h) the solution was diluted with 500 ml of MiliQ water (MQ), rinsed with MQ and dried in a stream of nitrogen. The prepared samples were directly used for spectral ellipsometry, contact angle measurements, IRRAS and XPS.

Zoospore settlement assay

Plants of *U. 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 3 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 2 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 × 10^6 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 quadruperm dishes (Greiner-One), each containing one test surface. The dishes were incubated in darkness at room temperature for 45 min (standard assay) or different periods as specified. 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 deionised water and allowed to air dry. Spores were counted by autofluorescence of chlorophyll using a Zeiss epifluorescence microscope (Callow et al. 2002). Fifty counts were made on each of three replicate slides (n = 90) except for the kinetic experiment (Figure 5), where the spore count at each point is the mean of 30 counts from one slide.

Preparation of liquids used for conditioning of surfaces

Tropic Marin® (TM) and Instant Ocean® (IO) were made up to the manufacturer’s instructions, then filtered through a 0.22 μm Millipore filter. Seawater made from individual salts (Salt Water; SW) was made up freshly using MiliQ water as described by Kester et al. (1967), Table 1. All chemicals were of analytical grade and the salt water was filtered through a 0.45 MiliPore filter after preparation.

Spore water (SP) was made from a suspension of swimming spores (3 l) prepared as described above. The suspension (1 × 10^6 spores ml^-1) was kept stirred for 1 h (to prevent spores settling) before filtering 250 ml aliquots through 10 cm GFA filters (Whatman) using vacuum suction. Microscopic examination of the filtrate showed that no spores had passed through the filter. The SP was not passed through a 0.22 micron filter to avoid removing macromolecular aggregates/colloids that might serve as a source of conditioning molecules. Although the spore water was not sterile, bacteria were not detected after streaking aliquots onto marine agar and were not detected by epifluorescence microscopy (after staining with Syto 13) on slides that had been immersed in spore water for 24 h. The filtrate was aliquoted into 50 ml Falcon tubes and flask frozen by plunging into liquid nitrogen. The tubes were stored at −20°C.

Results and discussion

Self-assembled monolayers were prepared on 30 nm thin gold films deposited on ultrasmooth Nexterion glass slides (Schott). Five different self-assembled monolayers were prepared, which varied in terms of protein resistance and wettability (Table 2). Sessile drop contact angles varied between 27° and 112° and were in good agreement with literature values, where film thicknesses of 1–2 nm were obtained by spectral ellipsometry (Bain et al. 1989; Zhu et al. 2001; Schilp et al. 2009). Only the PEG surfaces had a higher thickness of ~7 nm because of the much longer thiol molecules.

Deposition from different types of artificial seawater and seawater conditioned by spores (SP) was investigated on dodecanethiol (DDT) surfaces. Two

Table 1. Salt mixture used to prepare 35.00% self mixed salt water (artificial seawater according to Kester et al. (1967)).

| Salt       | g l^-1 |
|------------|--------|
| NaCl       | 23.926 |
| Na₂SO₄     | 4.008  |
| KCl        | 0.677  |
| NaHCO₃     | 0.196  |
| KBr        | 0.098  |
| H₂BO₃      | 0.026  |
| NaF        | 0.003  |
commercial sources of artificial seawater were used, viz. Tropic Marin (TM) and Instant Ocean (IO), together with a non-commercial seawater equivalent (‘salt water’ (SW)), prepared by mixing the major separate mineral salts found in natural seawater (Kester et al. 1967). In order to compare the different seawaters, DDT SAMs were used as a non-protein resistant model surface with hydrophobic properties. The conditioning film was characterized after various incubation times by spectral ellipsometry and contact angle measurements. The formation of a conditioning layer was most prominent for SP (Figure 1). The thickness of the formed protein layer was determined to be \( \sim 25 \, \text{Å} \). This measurement is consistent with the values found in literature when proteins are deposited from different buffered protein solutions, e.g. ribonuclease A (RNase A), pyruvate kinase, fibrinogen and lysozyme (Prime and Whitesides 1991, 1993; Schilp et al. 2007). Spectral ellipsometry showed that the formation of the conditioning film was insignificant (in terms of thickness) before 8 h. However, at longer timescales (48 h) a weak, but noticeable conditioning of the surfaces could be recognized for IO. As expected, SW, consisting only of mineral salts, showed no accumulation.

While hydrophobic surfaces, such as the DDT SAM, are known to readily adsorb a conditioning film (Prime and Whitesides 1991, 1993; Schilp et al. 2007), the question arose, to what extent spore conditioned water was able to condition the other four surface chemistries. The data collected in a second experiment are plotted in Figure 2 and show that a conditioning film is formed on all surfaces, except PEG which featured a decrease in film thickness. With the exception of PEG, the rate of film growth only changed slightly with the surface chemistry. Irrespective of whether surfaces were hydrophilic or hydrophobic, all seemed to be conditioned rapidly by biomacromolecules. For PEG, the thickness values were negative, which could point to a degradation of the surface over time. The time scale of increase in thickness broadly matched the changes in the contact angle after conditioning (Figure 2b). The contact angle changed for all surfaces on a similar time scale of \( \sim 5–10 \, \text{h} \). Interestingly, the hydrophobic surfaces became more hydrophilic, while the hydrophilic HUDT surface became more hydrophobic. These data are in agreement with results reported by Pratt-Terpstra et al. (1987). They showed that the contact angle of surfaces with different hydrophobicities changed after being conditioned by bovine serum albumin (BSA). For a hydrophilic surface the contact angle increased from \( 26^\circ \) to \( 59^\circ \) and for a fluorinated hydrophobic surface, it decreased from \( 107^\circ \) to \( 97^\circ \) after conditioning with BSA.

In order to obtain more detailed information about the chemistry of the conditioning layers, infrared reflection absorption spectroscopy (IRRAS) was applied (Figure S1 in Supplementary Information). [Supplementary material is available via a multimedia link on the online article webpage.] In the area of 3000 cm\(^{-1}\) the CH\(_2\) vibrations of the methylene backbone of the aliphatic SAMs can be seen in all spectra. While the intensities of the methylene vibrations remained for the different incubation times, the amide I and amide II peaks in the area of 1650 cm\(^{-1}\) respectively 1740 cm\(^{-1}\) increased during formation of

| Chemistry                        | Name                           | Label  | Water contact angle [°] | Ellipsometric thickness [nm] |
|----------------------------------|--------------------------------|--------|------------------------|-------------------------------|
| \( \text{SH-(CH}_2\text{)}_2\text{(O-(CH}_2\text{)}_4\text{)-OH} \) | Hydroxy-PEG 2000-thiol         | PEG    | 27                     | 7.38                          |
| \( \text{SH-(CH}_2\text{)}_{11}\text{)-OH} \)                  | 11-Hydroxy-1-undecanethiol     | HUDT   | 33                     | 1.09                          |
| \( \text{SH-(CH}_2\text{)}_{11}\text{-NH}_2 \)                  | 11-Amino-1-undecanethiol       | AUDT   | 60                     | 1.93                          |
| \( \text{SH-(CH}_2\text{)}_{11}\text{-CH}_3 \)                  | Dodecanethiol                  | DDT    | 104                    | 1.06                          |
| \( \text{SH-(CH}_2\text{)}_{11}\text{-O-(CH}_2\text{)}_2\text{(CF}_2\text{)}_5\text{-CF}_3 \) | 11-(tridecafluoroctyloxy)undecanethiol | FUDT   | 112                    | 1.44                          |
the conditioning layer. This suggests that the conditioning layer contained macromolecules with amide groups, most likely proteins/glycoproteins. Figure 3 shows the amide bands in greater detail after incubation for 48 h in SP for all four surfaces. It can be seen that the peak shapes and relative intensities of amide I and II peaks showed slight differences. On AUDT and HUDT especially, the amide bands were very pronounced, indicating large amounts of protein or proteinaceous components on the hydrophilic surfaces. The two hydrophobic surfaces, DDT and FUDT, showed completely different spectra, and in both cases the amide bands were comparably weak. As ellipsometric thicknesses were in a similar range for all four surfaces (DDT slightly higher), it may be concluded that the conditioning layers on hydrophobic surfaces have lower amounts of proteinaceous components compared to the hydrophilic surfaces. These absolute thicknesses are very similar to literature values of surfaces conditioned with natural seawater (Dexter et al. 1975; Marszalek et al. 1979). If the development of the intensities of the amide bands is plotted against time it can be seen that the kinetics of adsorption of molecules with amide groups were different for the different chemistries (Figure 4). Interestingly, the AUDT surface especially attracted amide-containing molecules quickly at the beginning of the conditioning phase. However, the low protein contents on the hydrophobic surfaces did not allow a more detailed kinetic analysis.

In agreement with the ellipsometric data, it can be noted that PEG surfaces deteriorated over time. The peaks at 1122–1343 cm$^{-1}$, typical of ethylene glycol units (Pasquali et al. 2008; Wu et al. 2010), decreased with increasing incubation time in SP. The CH$_2$ peaks at 2888 cm$^{-1}$ were also reduced (Figure S1b in Supplementary information). [Supplementary information is available via a multimedia link on the online article webpage.]. As this did not happen for all other
SAMs and was in agreement with spectral ellipsometry, it indicated that only the ethylene glycol coating deteriorated over time. As no amide peaks grew, the film did not appear to be completely degraded and up to 48 h a certain resistance to conditioning was retained.

After the detailed investigation of the formation and chemistry of the conditioning films, the question arose as to how settlement of spores of *U. linza* was influenced by the conditioning layers. To compare the results of conditioning from SP on the test surfaces with the settlement of zoospores, spore settlement on the pristine surfaces up to ~48 h was first investigated (Figure 5). Figure 5a gives an expanded view of the first 2 h of settlement. The general trend for settlement was consistent with previous findings by Callow et al. (1997) and Cooper et al. (2011), but the rates of zoospore settlement were different for the five different surfaces. The grey area in Figure 5a represents the time used for standard settlement assays with spores (Callow and Callow 2006). Figure 5b shows settlement over 48 h. While the increase in settled spore numbers continued for ~5–10 h, the settlement on the different surfaces (except for PEG) saturated at approximately the same value after ~20 h, independent of the surface chemistry. However, at these longer incubation times the values for spore settlement must be considered with caution since by 22 h many spores had started to germinate and some spores had settled on top of previously settled spores. These effects reduce the accuracy of the image analysis macro in discriminating individual spores and this probably explains the apparent decline in spore density at 36 h.

For PEG, the settlement of spores started after about 8 h, i.e. when the surface deterioration had advanced to the point where the surfaces were no longer inhibitory to settlement. With these exceptions, the kinetics of spore settlement occurred on a similar timescale as surface conditioning. These data support the standard practice of using only 45–60 min in the settlement bioassay for spores of *U. linza* when the intention is to compare the settlement rates on different types of AF coatings.

The observed saturation of spore settlement leads to the question whether this saturation is due to a full coverage of the surface or due to a depletion of the spore suspension. Table 3 shows the quantification of spores on the different surfaces after a long settlement time (36 h). For the calculation of coverage, the

| Surface | Number of adhered spores on whole surface area after 36 h | Percentage of spores out of the whole population which settled | Coverage |
|---------|------------------------------------------------------------|---------------------------------------------------------------|----------|
| PEG     | 2.98 ± 0.29 × 10⁶ spores                                   | 29.8 ± 2.9%                                                  | 3.1 ± 0.3% |
| HUDT    | 5.79 ± 0.29 × 10⁶ spores                                   | 57.9 ± 2.9%                                                  | 6.1 ± 0.3% |
| AUDT    | 4.14 ± 0.52 × 10⁶ spores                                   | 41.4 ± 5.2%                                                  | 4.3 ± 0.5% |
| DDT     | 5.30 ± 0.41 × 10⁶ spores                                   | 53.0 ± 4.1%                                                  | 5.5 ± 0.4% |
| FUDT    | 4.51 ± 0.48 × 10⁶ spores                                   | 45.1 ± 4.8%                                                  | 4.7 ± 0.5% |
| Theoretical maximum coverage if all spores settled | 1.00 × 10⁷ spores                                           | 100%                                                         | 10.4%     |
The projected area of a single spore was calculated to be \( \approx 1.96 \times 10^{-5} \text{ mm}^2 \) taking the diameter of a hydrated spore to be 5 \( \mu \text{m} \). However, it should be noted that this figure does not include the halo of adhesive that is secreted on settlement. The secreted adhesive spreads more on hydrophilic surfaces giving a mean diameter of the cell plus adhesive of around 8 \( \mu \text{m} \) for a CH\(_3\)-terminated SAM and 15 \( \mu \text{m} \) for a hydrophilic OH-terminated SAM (Callow et al. 2005). For a settlement assay, 10 ml spore solution are used with \( 1.00 \times 10^6 \) spores ml\(^{-1} \). Assuming that all spores settled on the test surfaces, they could cover an area of 196 mm\(^2\) (based on a diameter of 5 \( \mu \text{m} \)). Comparing this number to the area of the surface (1875 mm\(^2\)), which is available for settlement, (dimensions of the slides are 75 \( \times \) 25 mm) shows that the supply of spores could cover at most 10.4% of the surface. Saturation in the kinetic curves is observed at \( \approx 5 \times 10^6 \) spores, which means that only about 30–50% of the total spore population settled and the coverage of the surface was between 3.1 and 6.1%. Thus, in this case, the full coverage of the surface as observed in typical Langmuir kinetics (Langmuir 1917) is not observed, but rather, a depletion in the number of spores with the capability to settle occurs.

A kinetic analysis of spore settlement on glass surfaces by Callow et al. (1997) led the authors to suggest a reduced competence of the zoospore population to settle with time, ie within a population of ‘wild’ spores there is a range of propensity for settlement so that over time the population of spores in the assay becomes biased towards those with a reduced propensity to settle. Indeed, it was observed that even after 48 h a few spores were still swimming in the assay dishes. The analytical data shown earlier suggest that surface conditioning might influence settlement over long settlement times. Another effect, which is an unavoidable artifact of the assay design, may also be operating. Settlement assays are conducted on coated glass slides immersed in the spore suspension in individual compartments of polystyrene ‘quadrimer’ dishes. The slide (75 \( \times \) 25 mm) only partially covers the polystyrene surface (80 \( \times \) 28 mm) so spores are able to settle on the uncovered base of the dish and the sidewalls. Since polystyrene is attractive for spore settlement, during long assay periods the spore suspension becomes progressively depleted with spores, especially as spores are attracted to settle in the angle between the bottom and side walls of the dish. Callow et al. (1997) used Scatchard analysis to demonstrate that at high levels of spore settlement negative cooperative effects may be detected, ie the settlement of spores is inhibited by previously settled spores in a concentration-dependent manner. The basis for negative cooperativity may lie in the secretion of chemical anti-settlement cues as an adaptive mechanism to limit competition for resources between settled spores.

Figure 6 compares the thickness of the conditioning layers and the number of spores on the differently terminated surfaces after 1 h, a time point at which the accumulation rate is high and thus allows a comparison of the accumulation of the conditioning film (a) as well as the settlement density on the different self-assembled monolayers. The thickness of the conditioning film (Figure 6a) broadly increases with increasing water contact angle, which is consistent with the published literature (Kirchman et al. 1989). Figure 6b shows the spore density on pristine surfaces after a 1 h assay, ie without preincubation. It shows that the rate of spore settlement also broadly scales with the wettability of the surfaces, which is in line with previous findings (Callow et al. 2000; Schilp et al. 2007; Bennett et al. 2010).

The fact that conditioning and colonization by spores occurred over a similar timescale and that both varied on the surface chemistries investigated raises the question to what extent the increased conditioning of the surface changes the density of spores that settle. Therefore the density of settled spores in a 45 min assay was investigated as a function of different lengths.
of surface pre-conditioning. DDT was used as a hydrophobic, non-protein-resistant test surface. This surface was pre-immersed in SP or filtered (0.22 μm) ASW (TM) for different durations (30 min, 60 min, 13 h or 24 h). After the different lengths of conditioning, a conventional 45 min spore settlement assay was carried out on all surfaces simultaneously in order to investigate if the pre-immersion caused any differences in the density of settled spores. Pristine samples as well as samples pre-incubated in seawater (TM) were included as controls. The results (Figure 7) show that preincubation with TM reduced the settlement of zoospores by ~10% at short time intervals (30, 60 min), compared with the pristine, non-preconditioned control. This effect was reduced at longer time periods. Pre-incubation in SP for <60 min caused a small (~11%) reduction in spore settlement density compared with TM, and approximately a 17% reduction compared with pristine controls. Interestingly, even after 1 h, although the conditioning film was not fully developed at this stage and was still very thin (<1 nm), changes in the settlement density could be observed. The contact angles also changed on a similar timescale. Preincubation in SP for >1 h caused a more substantial decrease in the number of settled zoospores (~50% compared with the controls).

Epifluorescence micrographs (Figure 8) reveal that on pristine samples and at short incubation times, spores settled in small groups on the surface. This pattern is typically observed on hydrophobic surfaces. After preconditioning in SP for 13 h and 24 h, a clear trend can be observed towards fewer spores settling, mainly as individuals on the surface.

Figure 7. Settlement density of spores on DDT SAMs after varying durations of pre-incubation in either spore water (SP) and Tropic Marin (TM). Separate blanks were also included in the assay at each of the time points. Error bars are 2 × SE of the mean (SEM).

Figure 8. Epifluorescence micrographs of settled spores of U. linza on DDT SAMs after varying durations of pre-incubation. TM and blank samples at the different time points were visually similar to the 30 min samples (top row). Image width is 450 μm.
where the spore density stayed almost as low as it was without preincubation.

Summary and conclusions

Standard spore settlement assays are used extensively as laboratory assays to explore the influence of physico-chemical surface properties on settlement, and to evaluate potential AF coatings. The linear kinetics at typical settlement periods of 45 min are suitable for comparing spore settlement rates and thus comparing the ‘attractiveness’ of different surfaces. The assay time of 45 min furthermore minimizes complications due to saturation and conditioning. In the present study the influence of such a conditioning film on the spore settlement process was investigated using SP (commercial seawater in which spores were allowed to swim for 1 h before filtering out). A protein film was formed on different surfaces independent of the surface chemistry. Furthermore, the settlement density of spores on the test surfaces preincubated in SP was reduced for the hydrophobic surfaces or increased for the hydrophilic ones, for preincubation times in the range of 24–48 h.

The maximum effect on spore settlement of preconditioning a test surface with SP for 30–60 min followed by a 45 min settlement period was up to 17%. However, this is a worst-case estimate of the potential effect since in a standard bioassay, test surfaces are not preconditioned, ie both spores and conditioning molecules are presented to the test surface at the same time. While for a standard spore assay of 45 min the effect of conditioning is likely to be rather slight, this work also suggests that care must be taken for any laboratory study on longer time scales as surface conditioning can contribute to the outcome of the assay. Furthermore, it should be noted that filter-sterilized commercial ASW contains macromolecules that will condition the surface if assays are of long duration. Surface conditioning can be reduced by the use of self-made ASW.

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