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Surface Sensing and Settlement Strategies of Marine Biofouling Organisms

A. Rosenhahn · G. H. Sendra

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Abstract This review article summarizes some recent insights into the strategies used by marine organisms to select surfaces for colonization. While larger organisms rely on their sensory machinery to select surfaces, smaller microorganisms developed less complex but still effective ways to probe interfaces. Two examples, zoospores of algae and barnacle larvae, are discussed and both appear to have build-in test mechanisms to distinguish surfaces with different physicochemical properties. Some systematic studies on the influence of surface cues on exploration, settlement and adhesion are summarized. The intriguing notion that surface colonization resembles a parallelized surface sensing event is discussed towards its complementarity with conventional surface analytical tools. The strategy to populate only selected surfaces seems advantageous as waves, currents and storms constantly challenge adherent soft and hard fouling organism.

This article is part of the Topical Collection "In Focus: Future of Biosensors".

The article is dedicated to Michael Grunze's 65th birthday.

A. Rosenhahn · G. H. Sendra
Institute of Functional Interfaces, Karlsruhe Institute of Technology, Eggenstein-Leopoldshafen, 76344 Karlsruhe, Germany

A. Rosenhahn · G. H. Sendra
Applied Physical Chemistry, University of Heidelberg, 69120 Heidelberg, Germany

A. Rosenhahn (✉)
Analytical Chemistry-Biointerfaces, Ruhr-Universität Bochum, 44780 Bochum, Germany
e-mail: axel.rosenhahn@rub.de

1 Biological Systems Respond Sensitively to External Cues

Living organisms typically respond sensitively to numerous cues. Probably the most severe responses occur upon their exposure to harmful substances. Hence, toxicology is one of the oldest disciplines and since evolution of mankind, living organisms are used to determine toxicity of substances [1]. Besides screening of drugs and active ingredients, response of small organisms is applied to monitor the existence of harmful substances in different water bodies. The conventional approach to monitor presence of toxins in rivers and estuaries applies physicochemical and analytical chemical techniques. However, the constantly increasing diversity of harmful substances and a range of potentially synergistic actions complicate a reliable assessment. Bioresponse-linked instrumental analytics bridges this gap to a certain extend as it links biomolecular recognition with chemical analysis [2]. Other approaches make use of the fact that harmful substances such as certain metals frequently accumulate in biological organisms. Thus, chemical analysis of accumulation levels can drastically increase sensitivity of detection [3].

Besides approaches involving expensive and technically demanding chemical analysis, biological activity provides another natural and sensitive evidence for the presence of harmful substances. Changes in behavior occur fast and are thus especially suited for continuous biological monitoring of sudden increments in the concentration of harmful substances [4]. Therefore, biological activity tests turned out to be complementary to trace analysis since water samples can not only be tested against known toxic contaminants, but also against other unknown substances. Within the framework for community action in the field of water policy of the European Commission such new tests are strongly encouraged [5].

In this sense, biological early warning systems are usually introduced to provide a "biological sum parameter" which points towards a possible pollution and thus triggers an extensive analytical program [6]. Modern tests use bacteria, algae, waterfleas, fish or bivalves. A reliable operation is required at least for 1 week, the test setup needs to be easy to handle with less than 3 h of maintenance per week, and it should automatically detect alarm situations [6]. *Daphnia* (water fleas) respond to toxins by changing their activity which is manifested in a change in motility [7, 8]. Such dynamical *Daphnia* tests were successfully applied at the Rhine river and seem to be especially useful to detect low concentrations of insecticides [9]. The mussel activity test uses the opening and closing cycles of bivalves as sensitive indicator for the presence of harmful substances [4, 10]. These cycles can remotely be followed either by strain gauges or by electromagnetic induction [4]. Since more than 10 years extensive data have been collected at different rivers (e.g. Rhine, Elbe, Danube) using the Dreissena-Monitor, an automated early warning system based on the opening and closing of the valves of 84 zebra mussels [6].

The fascinating selectivity and build-in sensory mechanisms of microorganisms do not only cause sensitive responses to toxins but also enables biofouling organisms to select surfaces and guide colonization. In this article some recent progress about the interaction of marine organisms with surfaces is reviewed. After a short outline describing the motivation to study cell-surface interactions in marine antifouling research, the behavior of zoospores of *Ulva linza* and barnacle cyprids are discussed in greater detail. For both species we summarize to which extend behavior and initial interactions correlate with surface properties. While biofouling organisms are frequently viewed as nuisance, this article highlights their intriguing skill to selectively colonize surfaces due to their ability to sense their properties.

2 Biofouling Research: The Quest for Environmentally Friendly Anti-fouling Coatings

Controlling the adhesion of marine biofouling organisms to surfaces is of great environmental and economic relevance as overgrown ship hulls can lead to an increase in drag, and thus in fuel consumption [11, 12]. Fouling pressure in the ocean is high as adhesion is a central stage in the life cycle of many micro- and macrofoulers [13, 14]. Three different approaches are commonly pursued against marine biofouling: toxic anti-fouling, fouling-inhibiting and fouling-release coatings (schematically depicted in Fig. 1a–c).

Toxic anti-fouling is achieved by killing the organisms upon contact with the surface (a). Such action is usually achieved by embedding biocides into paints. Besides the restricted tin containing formulations based on TBT (tributyltin) and TPT (triphenyltin), especially copper is applied (usually as oxides) [15, 16]. Most of such metal-containing biocides are embedded into ablative coatings, which were first described by Holzapfel in 1904 [17]. Even though ablative coatings [18] or self-polishing formulations [19] are able to limit the amount of deposited biocides into the environment, they cannot avoid the obvious environmental impact of the released toxins. Besides metal-based formulations, organic biocides are increasingly applied, either as active ingredient or as booster biocide. Some examples are zink pyrithiones (also used in anti-dandruff shampoos), isothiazolones, triazin-herbicides, dichloro-phenyl-dimethyl-urea (DCMU, Diuron), tetrachloro-isophthalonitrile, dichlorofuanid, zinc-ethylene-bis(dithio-carbamate), chlorothalonil, TCMS pyridine, and econea [13, 15, 16, 20].

A perfect alternative to ablative biocidal coatings would be inert foul-inhibiting surfaces (Fig. 1b). These anti-fouling surfaces are the most elegant, environmentally benign and desirable solution. Such inert coatings have been identified for a number of well-defined surfaces in short term, single species assays. Especially ethylene glycol $(EG)_x$-containing coatings have been used in the biomedical area [21–24] and have recently been investigated with respect to their marine anti-fouling potential [25–33]. However, the degradation of the ethylene-glycol-containing chemistries makes them unsuitable for long-term anti-fouling applications [34, 35]. Other promising approaches involve the use of amphiphilic [26, 27, 29, 36] or zwitterionic chemistries [37–40]. Even though fouling inhibition is the most desirable way of avoiding biofouling, the development of such inert, non-toxic, and long-term stable coatings remains to be the most challenging of the three approaches.

The third possibility consists not in preventing, but in removing all unwanted fouling by creating a "self-cleaning" surface. The removal of foulers from so-called fouling-release coatings is achieved by the hydrodynamic drag caused by the movement of the vessel (Fig. 1c). Such coatings are usually based on silicone elastomers or fluoropolymers which do not inhibit settlement of biofouling organisms and thus biomass accumulates. However, the weak attachment strength to these polymeric materials allows fast-moving ships to self clean simply by the shear force present during their movement through the ocean [13, 41–44]. Modern fouling-release coatings have self-cleaning ability even below 15 knots (e.g. Intersleek 900 requires >10 knots, Hempasil X3 87500 >8 knots) [45]. Especially the combination of fouling-release with mechanical cleaning techniques such as hull grooming seem to be promising hybrid approaches for the future [46, 47].

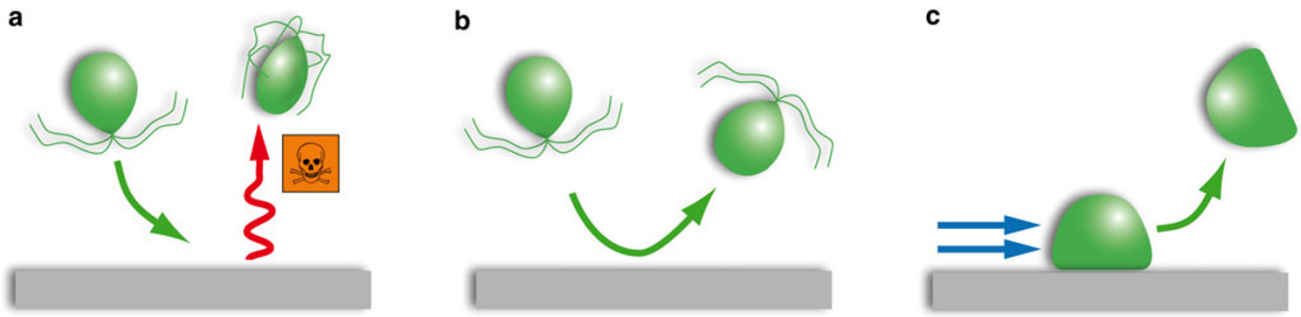

Fig. 1 Different approaches against biofouling: **a** toxic anti fouling, **b** foul inhibition, and **c** foul release

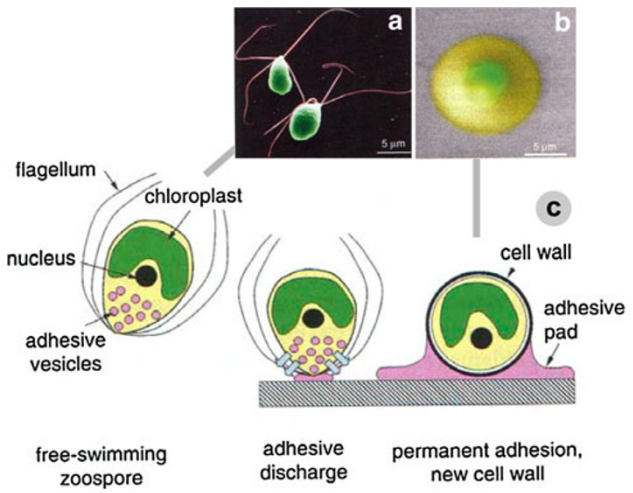

Fig. 2 **a** False color SEM image of zoospores of *Ulva linza*. **b** False color environmental-SEM image of a settled spore of *Ulva linza* showing the annular pad of adhesive surrounding the central spore body (reproduced from [51], reprinted by permission of Taylor and Francis Group Ltd, http://www.informaworld.com). **c** Cartoon depicting the course of events involved in the settlement and adhesion of Ulva spores (Whole figure adapted from Ref. [52] (Fig. 4.1, ©Springer-Verlag 2006] with kind permission from Springer Science and Business Media)

The development of any of these three types of antifouling coatings frequently involves laboratory assays to quantify the effectiveness of the coatings towards reduction of surface colonization and/or adhesion strength of single species under well-defined conditions. Many of such biofouling laboratory assays focus on the sessile spore or larvae stage rather than the adult, macroscopically visible biofouling organism [14, 48–50]. In general, the applied assays can be subdivided into biomass accumulation measurements (settlement assays), adhesion strength measurements, and tracking experiments to quantify exploration behavior. All three measurements reflect the responses or interactions of microorganisms and larvae with surfaces. Prior to permanent adhesion, motile and highly selective species are able to explore the surface and commitment to permanent adhesion only occurs if positive cues are sensed. Two remarkably selective species that have extensively been investigated with respect to their selection of surfaces are zoospores of the green algae *Ulva linza* and barnacle larvae.

3 Zoospores of the Green Alga *Ulva Linza*—Swimming and Selective Plants

The motile, quadriflagellated zoospores of the green algae *Ulva linza* with a spore body diameter of 4–5 μm have intensively been studied as a model biofouling organism (Fig. 2) [14, 52]. In order to complete their life cycle, zoospores are released from adult plants and must locate a surface to settle on (i.e. permanently attach to it). Once a spot suitable for settlement is identified, permanent settlement is initiated. At this stage, spores shed their flagella and secrete an adhesive for permanent attachment (Fig. 2b, c) [53]. Understanding the selectivity of spores is challenging as their motion is three-dimensional with mean velocities of ≈25 body lengths per second (150 μm/s) [54].

3.1 Holographic 3D Tracking of Zoospores Reveals How Spores Select a Suitable Location for Settlement

Digital in-line holographic microscopy (DIHM), as schematically shown in Fig. 3, is a technique capable to capture fast, three-dimensional motions by recording a movie of coherent far-field diffraction patterns of the sample volume [55–59]. A coherent laser beam is focused on a small pinhole to create a divergent light cone, which illuminates the volume of interest. The objects present in this volume scatter the laser light which interferes with the unscattered light to form a hologram. A series of such holograms, a holographic movie, is recorded using a single digital detector (camera). From these holograms, three-dimensional information can be retrieved by digital reconstruction algorithms [54, 60]. Image analysis of these

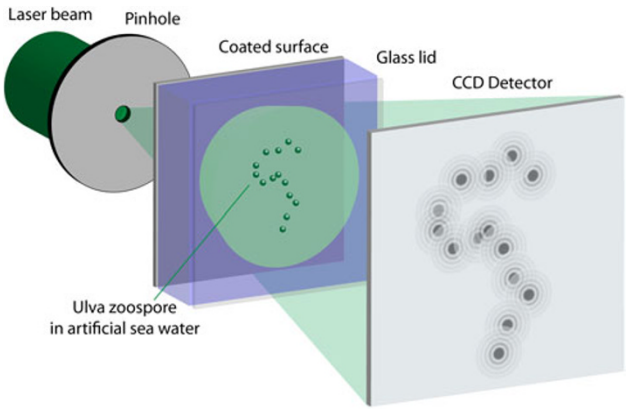

Fig. 3 Schematic representation of a digital in-line holographic microscope to track the motion of zoospores of *Ulva linza*

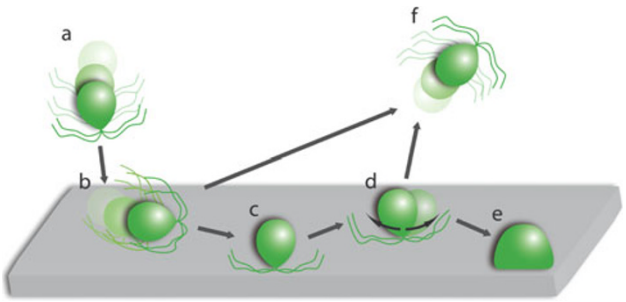

Fig. 4 Schematic representation of the settlement of spores of *Ulva linza*: (*a*) approach to the surface, (*b*) exploration of the surface, (*c*) initial adhesion, (*d*) spinning, which may lead to permanent adhesion (*e*) or to spores leaving the surface and continue exploration (*f*) (reproduced from Ref. [61] (Fig. 7, ©Springer 2012) with kind permission from Springer Science and Business Media)

reconstructions yields 3D trajectories with video frequency.

Recently, an extensive digital in-line holographic microscopy study on Ulva spores by Heydt et al. [61] revealed the mechanism by which spores select surfaces suited for settlement. The proposed mechanism is schematically depicted in Fig. 4. The first step in the selection process involves swimming of spores towards the surface (a). By swimming close to the surface (b) spores sense the suitability of a surface for settlement and only if positive cues are sensed, temporary adhesion via the apical papilla of the spore is initiated (c). Soon after this temporary 'sticking' event, spores initiate a spinning motion (d) that varies in duration, depending on the surface chemistry. Video evidence suggests that the spinning event may involve the secretion of a small amount of temporary adhesive as an elastic pad [53], although direct biochemical evidence for the existence of this adhesive has still to be proven. Spinning spores may then initiate permanent adhesion (e), however the majority of spores (>95 %—depending on the chemical termination of the surface) leave the surface to continue exploration (f). The mechanism involves two different ways of probing of the surface: (b) swimming close to the surface and (d) the spinning motion.

3.2 Swimming Zoospores Explore Surfaces and Respond to Surface Cues

Swimming close to the surface (b) is the first contact of the spore with an unknown interface. As pointed out by Heydt et al. [61], one important prerequisite for settlement is a deceleration of the spores. In Fig. 5a, the deceleration close to attractive fluorinated (FOTS) surfaces, moderately attractive glass surfaces (AWG), and inert ethylene glycol surfaces (PEG) are compared. The strongest deceleration is observed for FOTS (nearly 70 %), while it is a little bit weaker on AWG (nearly 40 %) and on PEG the velocity just barely changes (nearly 20 %). Interestingly the deceleration shows the same trend as the 45 min settlement assay (Fig. 5b) [61, 62].

However, the surface does not only influence the swimming speed of the spores, but also their behavior. Iken et al. [63] observed that the presence of a surface induces a motion behavior termed *gyration* of the brown algae *Hincksia irregularis* that differs from those ones observed in solution and is characterized by intense exploration and occasional surface contacts. Such patterns are also observed by holographic microscopy for the green algae *Ulva linza* and schematically depicted in Fig. 5c [61]. Within the *gyration* motion pattern (pattern 1), two extreme cases of motion can be subdivided: *hit and run* (pattern 2), which describes a single surface contact after which the spores immediately left the surface; and *hit and stick* (pattern 3), that describes the situation whereby, as soon as spores contacted the surface, they immediately stop swimming and stick to the surface. The term "sticking" means that the spores remain motionless at a distinct point on the surface, but they have not yet undergone the process of initiating irreversible settlement, i.e. shedding their flagella and discharge of adhesive. As shown in Fig. 5d, the occurrence of the different motion patterns depends on the surface chemistry. *Gyration* is detected as the dominant pattern on PEG and on AWG. However, on PEG the probability of observing a *hit and run* event is nearly twice as high (44 %) compared to AWG, indicating that the PEG surfaces are less attractive to the spores. The situation is different on FOTS and spores exploring the surface show predominantly the *hit and stick* behavior. A *hit and stick* pattern never occurred on PEG and AWG. The high probability of observing a *hit and stick* pattern indicates that the pristine and hydrophobic fluorinated surface attracted spores. The occurrence of the different motion patterns associated with attractive or repulsive properties of

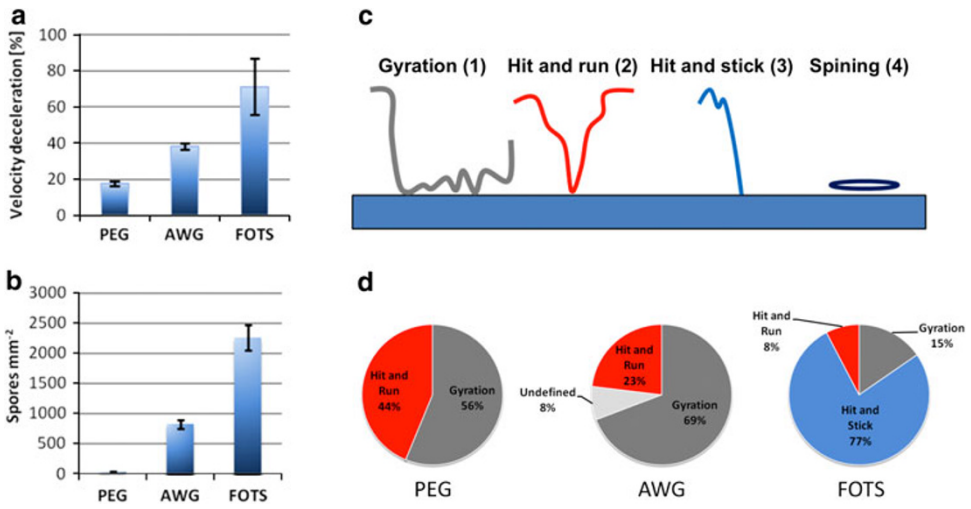

Fig. 5 Interaction of Ulva zoospores with polyethylene glycol coating (PEG), acid washed glass (AWG) and tridecafluoroctyl-triethoxysilane (FOTS) coated surfaces. **a** Velocity deceleration of exploring spores in close proximity of the surface (0–30 μm) as compared to the speed in bulk water. **b** Number of spores settled on the surface in a standard assay after 45 min. Analysis of motility patterns: **c** schematic sketch of the observed motion patterns. **d** Occurrence of motion patterns ≈40 s recordings (≈60 traces) immediately after injection in the vicinity of three different surfaces PEG, AWG, and FOTS, respectively (adapted from Ref. [61] (Fig. 3b–c, 4b, c, d, e, ©Springer 2012) with kind permission from Springer Science and Business Media)

the surface correlates well with the integral assay and the deceleration analysis.

3.3 The Spinning Motion Tests the Strength of Temporary Adhesion

The accumulation kinetics of spores is not only a consequence of deceleration and exploration patterns as both can change with time. Such changes are most obvious on very hydrophobic surfaces (i.e. FOTS) as the probability to observe patterns indicative of an attractive surface vanish over time [61]. On such hydrophobic surfaces, conditioning films are formed within hours and Thomé et al. [35] revealed that the presence of such overlayers decreases the settlement rates of *Ulva* zoospores by ≈50 %. Thus it is likely that surface conditioning affects the deceleration and the probability to observe certain motion patterns. However, *Ulva* zoospores have a second build-in sensory mechanism that involves the *spinning* motion (Fig. 4d).

Irrespectively of whether spores got stuck on the surface as a result of *hit and stick* motion or *gyration*, soon after the surface contact a *spinning* motion is started. This motion involves a rapid spinning of the spores around a temporary anchoring point on the surface (Fig. 4d). This spinning motion can take up to several minutes, but most spores (>95 %) leave the surface soon after spinning is initiated and continue exploration. Only a minority of spores (<5 %) spins for a longer time and finally settles permanently, which involves secretion of adhesive and shedding of the flagella [52, 53]. The duration of the spinning phase depends on the surface chemistry and spinning is longer on FOTS than on the less attractive AWG surface [61]. Only those spores that spin long enough initiate permanent settlement. It appears as if the spinning motion exerts a force on the temporary surface contact and only if the spore-surface contact is strong enough, the spinning process reaches the required critical duration to trigger the permanent secretion of adhesive. The duration of the spinning phase may thus reflect the strength of the initial temporary bond to the surface. This strategy seems advantageous since it may reduce the likelihood of spores committing to permanent settlement on surfaces to which they adhere weakly, as they immediately leave such surfaces after initiation of spinning. Therefore, spores use a sophisticated spinning mechanism to probe the stability of the cell-surface contact in order to restrict permanent settlement to those surfaces providing a stable anchoring point. This mechanism complements surface selection by exploration behavior.

3.4 Surface Cues Can Trigger Permanent Adhesion of Zoospores of *Ulva linza*

The deceleration, the behavioral response, and the spinning phase finally determine the kinetics by which spores colonize a surface. This, in turn, is affected by the chemical termination of the surface [35, 64]. The Callow group established an assay that allows spores to settle within 45 min to surfaces in order to compare the spore accumulation rate on different surfaces and thus to discriminate their non-fouling potential [62]. A vast number of experiments demonstrated that the settlement kinetics of zoospores of *Ulva* is affected by a number of physical and

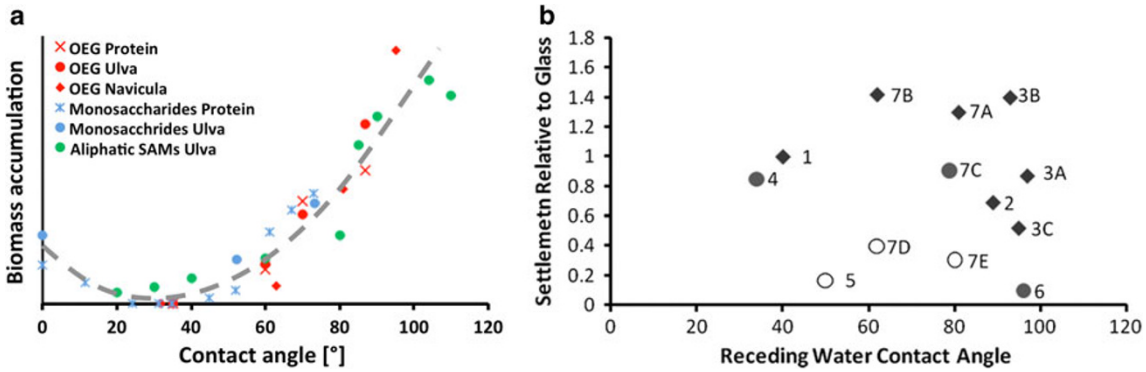

Fig. 6 The effect of wettability on spore and algae accumulation. **a** Compiled data for accumulation of algae (Ulva: *filled circles*; Navicula: *diamonds*) and proteins (*crosses*) on three chemically different SAMs: Ulva settlement on mixed hydroxyl and methyl terminated alkylthiols (*green*, data kindly provided by M. Callow) [64], Ulva and Navicula settlement and protein adsorption on hexaoligoethylene glycol SAMs with different aliphatic termination (*red*, Schilp et al. [31].) and protein adsorption and Ulva settlement on oligosaccharides with different degrees of methylation (blue, Hederos et al. [48, 65], data kindly provided by B. Liedberg). **b** Receding water contact angle of amphiphilic and non-amphiphilic surfaces along with references collected by Grozea and Walker [66] [reproduced by permission of The Royal Society of Chemistry (http://dx.doi.org/10.1039/b910899h)]

chemical surface cues, such as wettability [31, 64, 67, 68], topography [69–72], and charge [73, 74].

Especially self-assembled monolayers (SAM) [75, 76] are a versatile class of functional interfaces that are frequently applied in biofouling research, since their mechanical properties are determined by the substrate and thus biological response is solely caused by the surface chemistry. Application of SAM in biofouling research was pioneered by the Lopez group [25, 64]. Extensive studies followed, focusing on various surface properties including wettability, hydration, and lubricity [31, 33, 64, 67, 77, 78]. Figure 6a shows a compiled viewgraph of *Ulva linza* zoospore settlement, *Navicula perminuta* settlement, and protein resistance on different aliphatic SAMs [64], methyl terminated oligosaccharide SAMs [48, 65], and oligoethyleneglycol based SAMs with aliphatic termination [31]. The individual data sets have been rescaled so that they can directly be compared. The common trend shows that the wettability of the surfaces apparently determines the accumulation kinetics of zoospore and diatoms, even though the terminating chemistries are entirely different. The general trend confirms the Berg limit which predicts inertness for contact angles below 65° [79, 80]. Furthermore, the curve shows a minimum for the settlement of Ulva and Navicula on different SAMs at a contact angle of $\approx 35°$. The presence of such a minimum follows the early notion of Baier that bioadhesion shows a minimum at a surface energy of ≈ 25 dyn/cm [81].

A comparison of receding contact angle against spore settlement has been done for a number of amphiphilic and non-amphiphilic polymer surfaces by Grozea and Walker [66]. The study clearly shows that receding contact angle is not the only surface property that mediates spore settlement and there exist classes of surfaces where such a correlation is not valid. A similar observation has been made by the Grunze group, who found that surfaces with similar wettability can show different settlement of zoospores [33, 82]. In this study, ethylene glycols with different chain length and thus decreasing packing density were used [82, 83]. Monte Carlo simulations revealed that such a decreased packing density facilitates penetration of water into the thin films, providing the necessary steric freedom for a stable binding of water [84, 85]. Despite the different hydration, all of the tested surfaces have a similar water contact angle [82, 83]. *Ulva* zoospore experiments show that spores adhere much weaker on well hydrated surfaces [33], an observation in line with the protein resistance of the surfaces [83]. The fact that changing hydration continuously alters the inertness of a surface was finally proven by Christophis et al. [86] who used a microfluidic experiment to show that the adhesion strength of cells gradually decreases with increasing ethylene glycol chain length.

The selected overview on settlement data shows that the accumulation rate of spores on surfaces is not determined by one surface property alone but results as a combination of different properties. The sensory mechanism of spores thus responds to each surface property in a different way. When viewing colonization of surfaces by spores as highly parallelized surface sensing event, the relative contribution of the different physicochemical properties on the sensing process seems to be of major relevance, but yet needs to be fully understood. In a way, the situation is similar to protein affinity assays that also not always correlate with single surface properties. It seems to be rather the interaction strength that results from the combined physicochemical properties of the surface that finally determines adhesion and potential degeneration of proteins or, in the discussed example, settlement of algal spores.

3.5 Settlement and Adhesion Strength

As revealed by the holography study summarized above, spores select surfaces by different ways of active probing. This involves a spinning phase which is used to test adhesion strength to a surface. The accumulation kinetics that leads to the spore biomass data in Fig. 6 should be a direct consequence from this selection process. One could now ask, how well is the spinning phase capable to predict adhesion strength of settled spores, i.e. how reliable is this mechanism. Experiments in calibrated flow channels allow to measure the removal of spores from surfaces and thus to discriminate between weakly and strongly sticking spores [87, 88]. For the series of alkyl terminated OEG SAMs with different wettability (used in Fig. 6a), the removal is easier from surfaces with low settlement [31]. This means that spores accumulate only on those surfaces where stable anchoring is possible. A similar correlation has been found comparing amphiphilic and other polymeric materials by the Ober group [89]. However, in other examples such as on mixed aliphatic SAMs, opposite trends are observed [90]. In some cases such as hexaethylene glycols, even highly gregarious behavior is observed, although attachment strength is extremely weak [31]. The contrary examples show that the rate of spore accumulation does not always correlate with adhesion strength. One of the many possible reasons for the observed discrepancies could be a different composition of the temporary and the permanent adhesive.

Summarizing, despite their small size and their limited sensory abilities, spores show a surprisingly sophisticated mechanism for selecting surfaces. Although they might be viewed as living surface analytical tool, further research is required for a more detailed interpretation of the obtained data and a better understanding of the correlation between interface properties, spore behavior, settlement and adhesion strength.

4 Barnacle Cyprids: Motile and Selective Larvae as Early Stage of Hard Fouling

Especially hard foulers, such as barnacles and mussels, have a major impact on the hydrodynamic properties of ship hulls, namely increased hydrodynamic drag and thus increased fuel consumption [11, 12]. As for the zoospores of algae, antifouling tests targeting barnacles frequently focus on the sessile stage, which are cyprids that hatched from the adult organisms [91–93]. The two conventional approaches to study the interaction of these microorganisms with a surface involve settlement assays of competent larvae [97] and/or the mechanical removal of adults organisms in order to estimate the adhesion strength [42, 44, 94, 95]. A simplified life-cycle of barnacles (Fig. 7)

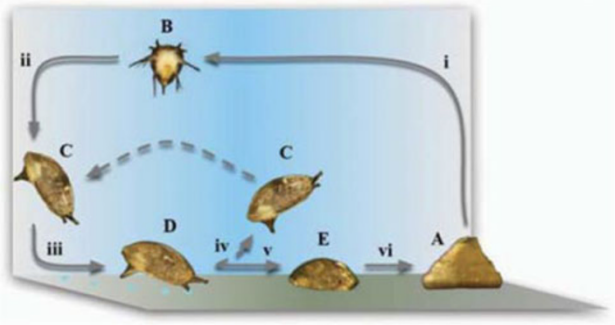

Fig. 7 A simplified life cycle for a generalized thoracican barnacle, illustrating site selection and settlement by a cyprid followed by metamorphosis. Letters (A–E) indicate stages in the development of a barnacle and numerals (i–vi) indicate behaviors. A is a juvenile barnacle. Within a few months this barnacle will be sexually mature and able to release nauplii, (B) into the water column from eggs brooded within the mantle cavity. After feeding in the water column for days to weeks (i–ii) the nauplii metamorphose into cyprids (C). When competent, the cyprids migrate to the benthos (iii) and explore surfaces, depositing footprints as they explore (D). Cyprids may re-enter the water column if the surface is not satisfactory, thus delaying settlement (iv), or settle immediately (v) if stimulated to do so. (E) A permanently attached (settled) cyprid. Within 12 h of permanent attachment, a cyprid will complete metamorphosis (vi) into a juvenile barnacle (A) (reproduced from [91], reprinted by permission of Taylor and Francis Group Ltd, http://www.informaworld.com)

starts with nauplii (B) released by adult barnacles (A) [91]. After some time of feeding (from days to months), the naupli metamorphose into cyprids (C). These cyprids explore surfaces (D) and sometimes deposit footprints during exploration. If the surface is not satisfactory, they leave into the water column (C), otherwise they settle permanently (E). Within 12 h of permanent attachment they complete the metamorphosis into a juvenile barnacle (A). Hence, the phase D is the stage when cyprids actively probe the surface.

4.1 Settlement of Barnacle Cyprids

The probability that cyprids initiate the adhesion process and metamorphose into a juvenile barnacle depends on the properties of the surface. The influence of surface energy and surface charge on the settlement of cyprids of *Balanus amphitrite* was recently studied by Petrone et al. [93]. SAMs on gold-coated Polystyrene (PS) surfaces were used to change the surface properties. Experiments were conducted over CH_3–, OH–, COOH–, $N(CH_3)_3^+$– and NH_2-terminated SAMs, as well as Thiosalicyclic acid and Thioglycolic acids. The negatively charged surfaces (COOH, Thiosalicylic acid and Thioglycolic acid) enhance settlement compared to the neutrally and positively charged surfaces (OH, NH_2, $N(CH_3)_3^+$) (Fig. 8). If the response towards surface energy is analyzed, settlement on $–CH_3$, –OH, and amino-terminated surfaces indicate an

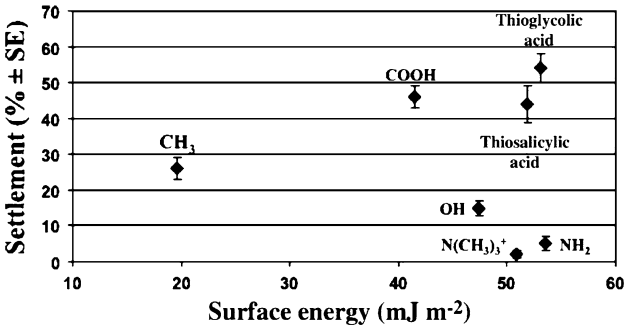

Fig. 8 Settlement of *B. amphitrite* cyprids on different SAMs (reproduced from [93], reprinted by permission of Taylor and Francis Group Ltd, http://www.informaworld.com)

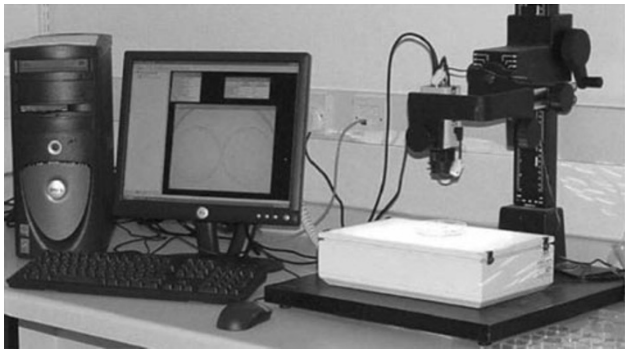

Fig. 9 Video-tracking equipment with computer, camera, and backlighting box (adapted from [96], reprinted by permission of Taylor and Francis Group Ltd, http://www.informaworld.com)

inverse relation between settlement and surface energy. However, all anionic surfaces, such as COOH, Thiosalicyclic acid, and Thioglycolic acid exhibit higher settlements than CH_3, even if the surface energy is also higher [93]. As no clear correlation between surface energy and settlement can be seen, it appears as if both, surface charge and surface energy influence the settlement process.

4.2 Behavior of Barnacle Cyprids on Surfaces and Response to Surface Cues

The surface cues responsible to induce settlement and adhesion of barnacle cyprids are still not fully understood. A systematic understanding is complicated by the fact that different barnacle species show different responses. To observe settlement behavior, tracking techniques are well suited as they allow visualizing how the exploration process and the sensing of the surface are influenced by the surface properties. Marechal et al. [96] described a tracking system (Fig. 9) capable of recording 2D traces and characterizing the swimming behavior of cyprids based on the observed motion patterns. Additionally, the motility data allows extraction of quantitative measures such as velocity, angular velocity, and turning angle.

2D tracking was applied by Aldred et al. [97] to compare the exploration behavior of cyprids of the barnacle species *Balanus amphitrite* over glass (AWG) and the zwitterionic polymers poly(sulfobetaine methacrylate) (polySBMA) and poly(carboxybetaine methacrylate) (polyCBMA). The latter two zwitterionic chemistries show no settlement during a 48 h settlement assay, while 48 % of the cyprids settle on glass, indicating their settlement-inhibiting character. Despite major changes in settlement after 48 h, the mean larvae velocities are similar on glass and polyCBMA (0.07 cm s^{-1}). Even though polyCBMA and polySBMA have equally low settlement, the velocity is 20 % lower on polySBMA (0.056 cm s^{-1}). The mean angular velocity, however, is 10 % lower on polyCBMA (749° s^{-1}) compared to AWG (820° s^{-1}), while on polySBMA (851° s^{-1}) velocities are only 3 % higher and thus comparable to glass. Finally, glass and polySBMA show similar exploration behaviour and swimming patterns on the surface while in the case of polyCBMA the cyprids swim at a certain distance from the surface showing only little interaction (Fig. 10). It is interesting to note that surfaces with similar settlement inhibition show different exploration while similar motility is observed despite different probabilities for settlement. It is yet an open question which surface properties cause the different motion patterns.

4.3 Field Studies of Surface Exploration and Settlement Behavior

The behavior of wild cyprid larvae of *Semibalanus balanoides* in situ in the ocean close to different surface textures treated and untreated with crude conspecific adult extract (AE) has been studied by Prendergast et al. [98]. The treatment with AE produces an increase in the number of cyprids arriving on the surface both, within the first minute and after a longer time. Results furthermore suggest that cyprids tend to explore smooth surfaces longer and leave rough surfaces earlier. This means that during the exploration phase cyprids were not only sensitive to the surface chemistry but also to the surface topography as they directly respond by changing their behavior. As pointed out by Aldred et al. [99], those topographies reducing adhesion are less likely to be selected for settlement and metamorphosis. Probably, the sensing during the exploration phase and the observed responses are directly connected with the strong influence of surface morphology on the probability of cyprids to settle and metamorphose [99–101].

4.4 3D Tracking of Barnacle Cyprids

Two-dimensional tracking proves to be a versatile technique to understand surface exploration and surface

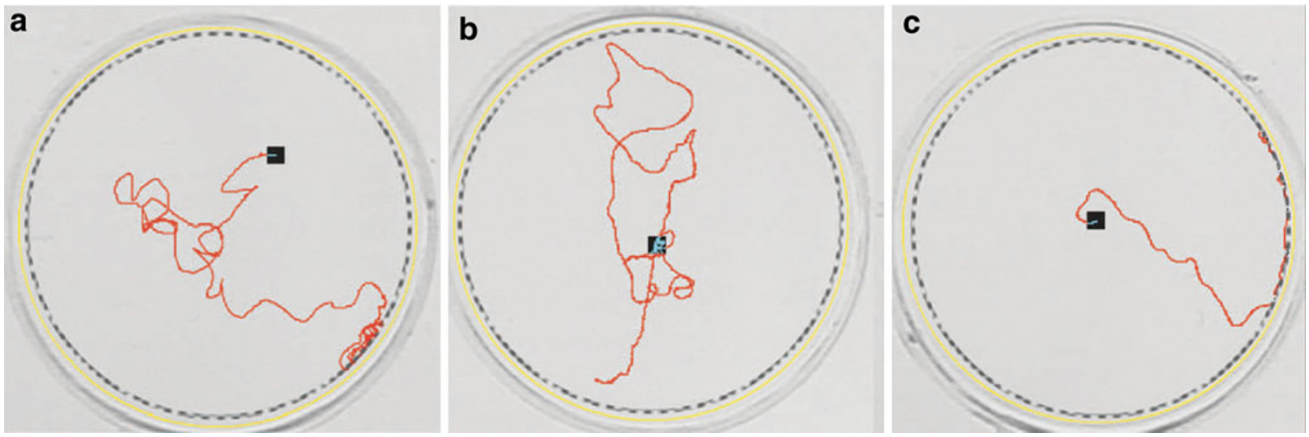

Fig. 10 Selected cyprid tracks over AWG (**a**), polySBMA (**b**) and polyCBMA (**c**). The irregular, *red solid line* is the cyprid track, the *solid outer circle* is the petri dish, and the inner textured circle represents the test surface. On the AWG control surface (**a**) and on polySBMA (**b**) broad and sweeping trajectories are observed. On polyCBMA, many cyprids spent little time either swimming over the surface or exploring it and swam immediately to the edge of the surface (reproduced from [97], reprinted by permission of Taylor and Francis Group Ltd, http://www.informaworld.com)

selection. However, the missing third spatial component complicates an accurate quantitative data analysis since there are important characteristic values such as swimming velocities that cannot accurately be calculated. Recent work showed that 3D tracking of cyprids provides more detailed information, since it allows a clear distinction between sinking and swimming phases (Fig. 11) [102]. The data reveals that positively charged surfaces seem to cause longer periods of close surface inspection of cyprids of the barnacle *Semibalanus balanoides* than glass or PEG.

4.5 A Closer View on Surface Exploration: "Walking" Cyprids

Tracking of cyprids under flow allowed to understand the influence of the surface properties on exploration under dynamic conditions [103]. As cyprids use antennules to attach, detach, and reattach to surfaces, Chaw and Birch evaluated the "walking" behavior of *Amphibalanus amphitrite* by measuring the step length and the duration required to carry the steps out. In the absence of water flow, both parameters are significantly influenced by the surface properties. The mean step length on hydrophilic surfaces (bare glass and $–NH_2$ functionalized glass) is larger than on hydrophobic surfaces ($–CH_3$ functionalized glass). In turn, the step duration is longer on the hydrophilic surfaces than on the hydrophilic ones. Consequently, the longer step duration and shorter steps leads to a slower motion on the hydrophobic surfaces, while the opposite is observed for the hydrophilic coatings [103].

If a water flow is applied and shear forces are present, cyprids actively respond by altering their exploration behavior [103]. On hydrophilic surfaces, the step length remains unchanged, but the step duration increases. In contrast, larger step lengths and shorter step durations are observed on hydrophobic surfaces. Especially the shorter steps can be connected with the requirement to re-generate surface contacts more frequently as the temporary anchoring point is challenged by the presence of shear. It was also found that behavior of cyprids depended on the age and discrimination power between hydrophilic and hydrophobic surfaces is lost when older cyprids are used [103].

As already observed by Schumacher et al. [100], Aldred et al. [99] and in field experiments by Prendergast et al. [98], surface morphology affects exploration and settlement. Chaw et al. [104] described the behavior of *Amphibalanus amphitrite* over a pattern of cylindrical micropillars with heights of 5 and 30 μm, a separation of 10 μm and diameters ranging from 5 to 100 μm. Only the higher pillars significantly influence cyprid exploration. Temporary attachment mainly occurs in the voids or at the sides of the pillars rather than on their top. The 30 μm high and 5 μm thin pillars offer a very small contact area for the attachement discs of the cyprids, resulting in a strong reduction of the step length and a large increase of the step duration (at least 50 % compared to other diameters and smooth surfaces).

4.6 Footprints of Walking Cyprids Visualized by Imaging Surface Plasmon Resonance

During the "walking phase", temporary contacts with the surface are established by the two antennules [91]. The antennules touch the surface via attachment disks that facilitate bipedal walking over the surface. The attachment disk itself is covered with small cuticular villi and pores allow the secretion of a 'temporary adhesive' composed

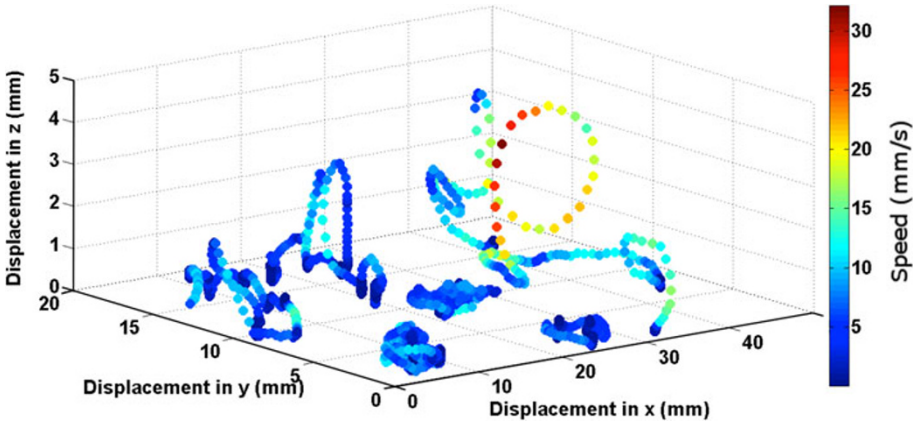

Fig. 11 3D Trajectories of swimming and exploring cyprids of the barnacle *Semibalanus balanoides* close to a glass surface (reproduced from Ref.[102] (Fig. 5a, ©Springer 2012) with kind permission from Springer Science and Business Media)

majorly of proteins [91, 105]. The antennules do not only moderate adhesion but also serve as sensory organ. Small setae are present as mechanosensors to perceive surface properties. During exploration and intense inspection, cyprids can use a 'temporary adhesive' to interact with the surface. As consequence, exploring barnacle cyprids may leave footprints that contain pheromones [106].

Recently, imaging Surface Plasmon Resonance (iSPR) was applied by the Liedberg group to observe and quantify the adhesive deposition during "walking" of the cyprids (Fig. 12) [107]. Touchdowns of the antennules can be visualized and it is possible to observe whether or not adhesive remains on the surface. Cyprids of the species *Semibalanus balanoides* left footprints on bare gold surfaces while on mOEG only touchdown events were detected [107]. Especially noteworthy from a surface science point of view is the mechanism of attachment and detachment of the antennules, which has similarities with the detachment of protein loaded AFM tips. Deposited amounts of footprint adhesive should therefore increase with enhanced interaction strength with the interface.

Aldred et al. [108] compared the effect of four SAMs and an ultrathin hydrogel coating on the footprint/touchdown frequency and the amount of deposited material of cyprids of the barnacle *Semibalanus balanoides*. The chemistries used to form the SAMs were $HS(CH_2)_{15}CH_3$ (named CH_3), $HS(CH_2)_{15}COOH$ (named COOH), $HS(CH_2)_{11}NH_2{\cdot}HCl$ (named NH_2), and $HS(CH_2)_{11}CONH(C_2H_4O)_{11}CH_3$ (named mPEG), while the hydrogel chemistry was $PEG_{10}MA/HEMA$. Figure 13a shows the occurrence of touchdowns and footprints on each surface during an experiment of 15 min duration. The touchdown frequency does not vary significantly with surface chemistry, indicating that the probing frequency is not affected by the chemical termination of the surface [108]. However, surface chemistry affects the probability that a footprint remains on the surface after touchdown (Fig. 13a). Especially on the negatively charged –COOH terminated SAM, the probability that a footprint remains after touchdown is comparably high compared to the other coatings. The amount of adhesive deposited on the surface can be estimated by analyzing the frequency shifts in the iSPR data (Fig. 13b) [108]. $–NH_2$, –COOH, and $–CH_3$ terminations leads to thick footprints, while hydrophilic mPEG and PEG-methacrylate hydrogel surfaces show only low amounts of adhesive in each footprint. Both, the high probability of footprint deposition, and the large amount of deposited material led Aldred et al. [91] to conclude that

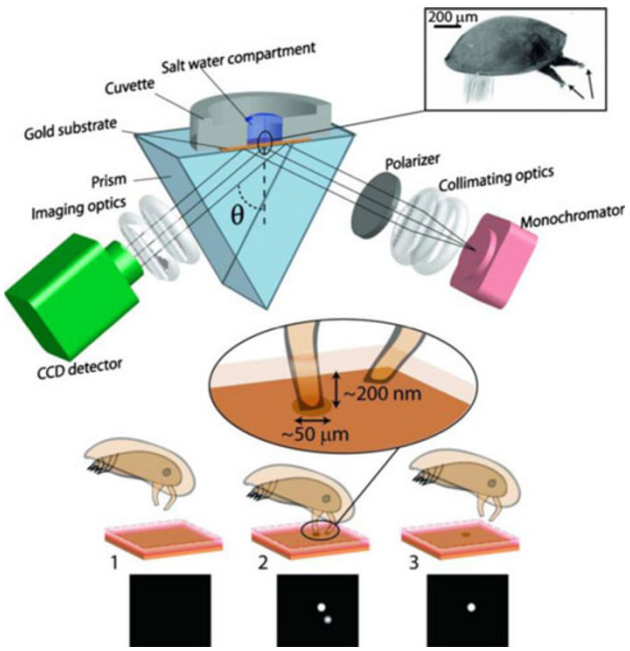

Fig. 12 Cartoon of the setup of the imaging SPR experiment. The *inset* shows the cyprids of *Semibalanus balanoides* and the arrows indicate their antennules. When the antennules get in contact with the surface, a bright spot appears in the SPR image. On "sticky" surfaces, adhesive material and thus the bright spot in SPR will remain as a footprint in the image. The magnified view of the contact point illustrates schematically the probing depth of SPR of a few hundreds of nanometers (reproduced from Ref.[107] (Fig. 1, ©Springer 2009) with kind permission from Springer Science and Business Media)

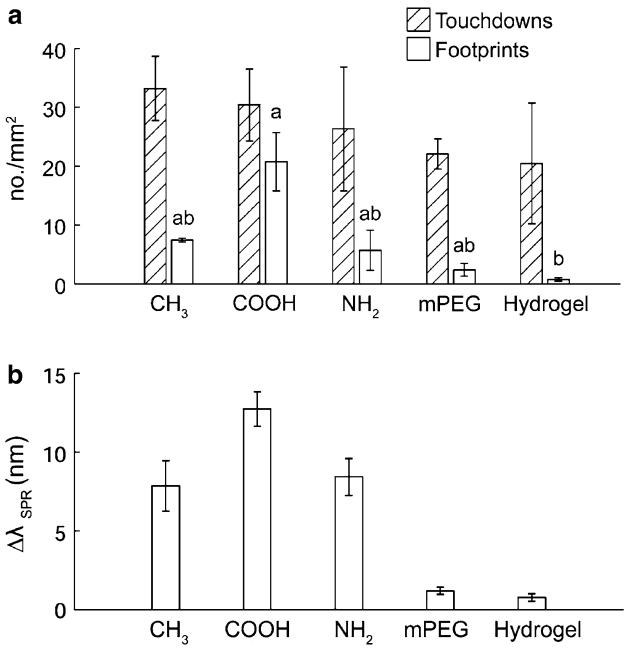

Fig. 13 **a** Number of touchdowns made by cyprids and **b** number of footprints deposited on chemically differently terminated surfaces. Mean SPR wavelength shift for the deposited footprints with large wavelength shifts indicating large amounts of deposited material (eeprinted (adapted) with permission from [108]. Copyright 2011 American Chemical Society)

electrostatic interactions between the footprint material and the negatively charged surfaces occur.

Summarizing, barnacle cyprids exhibit a selective mechanism to determine where to settle. This selection process involves initial contacts by walking and exerting of local forces on the temporary adhesive. As consequence, one observes behavioral responses and eventually commitment to settlement. As in the case of Ulva spores, more studies are needed for a better understanding of the surface properties involved, but also surface colonization by cyprids of barnacles can be viewed as collective surface sensing event.

5 Summary and Outlook

Some recent results on the interaction of algal zoospores and barnacle cyprids with well-characterized surfaces were summarized with examples of how the sessile stages of marine organisms respond to the properties of surfaces by changing their exploration behavior. *Ulva* zoospores established a remarkable strategy to test surfaces, involving deceleration close to the surface and a subsequent spinning behavior to probe cell-surface contact. Both, the deceleration and the duration of the spinning phase depend on the surface properties. This surprisingly sophisticated mechanism leads to different accumulation kinetics on chemically different surfaces. Interestingly, hydrophilic, well-hydrated surfaces seem to reduce settlement and adhesion strength, while hydrophobic, weakly hydrated surfaces encourage settlement with strong spore adhesion. However, this picture is merely a black and white picture as e.g. trends in amphiphilic coatings are more challenging to understand. Especially the combination of behavioral studies, spinning phase analysis, and potential future combination with SPR could serve to understand this open question. Surface colonization can be considered as parallelized surface sensing event and the general perspective to have many little and independent surface sensors is intriguing as each of them is capable of attaching and exerting a force on a temporary adhesive. However, for a future application, e.g. for multiplexed testing of interaction forces, more knowledge about the complementarity of spore settlement, protein affinity and physicochemical surface properties need to be derived.

A similar conclusion can be drawn for exploration behavior of barnacle cyprids. Settlement preferences are different for different species and are guided by the physicochemical properties of the surfaces. Cyprids distinguish surface topographies and select those morphologies for settlement and metamorphosis that allow thorough adhesion. Tracking reveals behavioral responses and velocities close to chemically and morphologically different surfaces. Especially 3D tracking has great potential as it allows direct imaging of larvae responses not only in the laboratory but also in the natural habitat, the real ocean environment. In particular, the correlative analysis of the "walking" behavior and footprint deposition as accessible with iSPR seems to be very promising and can be expected to contribute to understand surface selection, settlement and adhesion of cyprids.

We expect three research and application fields to be relevant in the future: First of all, 3D tracking and time resolved, interface sensitive surface analysis techniques will allow to understand surface selection strategies of marine biofouling organisms. Secondly, the highly parallelized surface selection that involves active surface sensing could be applied to test surfaces with respect to their inert properties. However, this application requires more knowledge about which physicochemical surface properties are probed in such an experiment. The third point is rather a consequence as these new techniques will help to identify novel surface coatings that aim on reduced adhesion and thus enhanced foul released properties.

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