Barnacle cyprid motility and distribution in the water column as an indicator of the settlement-inhibiting potential of nontoxic antifouling chemistries

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Testing of new coatings to control fouling frequently involves single-species laboratory bioassays. Barnacle cyprids are among the most widely used model organisms in marine biofouling research, and surfaces that inhibit their settlement are considered to be promising candidates for new coating concepts. An analysis of motility parameters (mean velocity and swimming area coefficient) and distribution of cyprids of Balanus amphitrite in different swimming regions in the vicinity of model surfaces (self-assembled monolayers) is presented. The data are correlated with the settlement preferences of cyprids on these surfaces. Cyprids were predominantly found in interfacial regions and the transition frequencies between swimming regions of different depths were determined.

Keywords: cyprid; antifouling; settlement assay; 3D tracking; motility; swimming regions

Introduction

Marine biofouling affects maritime industries worldwide. The undesired accumulation of biomass on submerged underwater structures has adverse economic and environmental consequences (Callow & Callow 2011). Strategies to prevent the formation of biofouling have varied over time, ranging from using generally toxic materials eg tributyltin (Townsin 2003) to non-toxic fouling-release coatings (Adkins et al. 1996; Brady & Singer 2000; Finnie & Williams 2010). Current research aims to develop new coating concepts that avoid biofouling without the utilization of toxic agents.

To test new coating technologies, laboratory assays are frequently used as they allow rapid assessment of coatings under reproducible laboratory conditions (Briand 2009). Such assays evaluate antifouling (AF) efficacy and if fouling does occur, its ease of release from the surface is frequently determined (eg Yasani et al. 2014; Zhou et al. 2014). The aim is to downselect promising candidates for more costly and time-consuming field tests (eg Martinelli et al. 2012). As barnacles are one of the most widely distributed biofouling organisms (Aldred & Clare 2008) with major deleterious impacts on submerged structures, they have been widely adopted as a model test organism. Cyprids, the colonizing larval stage (Walley 1969; Maruzzo et al. 2011), have a striking ability to explore substrata for potential settlement and can discriminate between various surface characteristics, eg colour (Yule & Walker 1984; Swain et al. 2006; Dobretsov et al. 2013), surface chemistry (Roberts et al. 1991; Schmidt et al. 2009; Aldred et al. 2011; Petrone et al. 2011; Bauer et al. 2013), topography (Schumacher et al. 2007) and the presence of competitors/predators (Yule & Crisp 1983). Once a suitable site has been identified, permanent attachment is initiated and the cyprid metamorphoses into a juvenile barnacle. For the evaluation of the efficiency of new AF concepts, settlement preferences are an established indicator and now a standard tool in marine biofouling research (Rittschof et al. 1984, 1992; Maréchal et al. 2004; Petrone et al. 2011; Di Fino et al. 2013), albeit the true efficiency of coatings finally needs to be tested and verified in the field (Swain et al. 1992; Frederic et al. 2000; Rittschof et al. 2007; Prendergast et al. 2008).

The exploratory behaviour of cyprids has been studied by 2-D video tracking techniques, which provided insight into their behaviour prior to settlement (Matsumura et al. 2000; Maréchal et al. 2004; Prendergast et al. 2008; Chaw & Birch 2009; Pradhan et al. 2011). However, the missing z component in the analysis makes it difficult to identify motion on or close to the surface. Three-dimensional methods are thus a logical and useful extension. Digital in-line holographic microscopy allowed 3-D trajectories to be determined for motile zoospores of the alga Ulva linza and provided insight into exploration and surface selection mechanisms (Leal-Taixé et al. 2009; Heydt et al. 2009,

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While the technique is ideal to track small organisms down to the level of bacteria (Vater et al. 2014), stereoscopy is the technique of choice for larger organisms (Maleschlijski et al. 2012; Rosenhahn & Sendra 2012).

In this work a stereoscopic setup (Maleschlijski et al. 2012) was used to study the exploratory behaviour of cyprids in the vicinity of surfaces with different chemical terminations were compared. The advantage of this apparatus is that 3-D data can readily be extracted and the system provides a relatively broad field of view that allows the observation of a large part of the swimming arena. The 3-D swimming trajectories of cyprids were studied in an assay format and surfaces with different chemical termination were compared. The probability of settlement on these surfaces was correlated with motility parameters, such as the velocities and the area cyprids passed during swimming.

**Materials and methods**

**Surface preparation and characterization**

Ethanol p.a., 1-dodecanethiol (DDT), 11-mercapto-1-undecanol (HUDT) and 12-mercaptoundecanoic acid (MUDA) were purchased from Sigma Aldrich (Munich, Germany). N,N,N-trimethyl-(11-mercaptoundecyl) ammonium chloride (TMA) was purchased from Prochimia (Sopot, Poland). Thin films of polycrystalline gold were prepared by thermal evaporation of a 5 nm Ti adhesion layer and subsequent deposition of 30 nm Au (99.99 % purity) on Si wafers or quadriPERM dishes (PVD-Beschichtungen, Silz, Germany). Preparation of the self-assembled monolayers (SAMs) followed earlier protocols (Thome et al. 2012; Bauer et al. 2014). Substrata were cleaned under an ozone-generating UV lamp for 90 min, subsequently ultrasonicated in ethanol p.a. for 3 min, rinsed with ethanol and dried in a stream of N₂. DDT and HUDT were assembled by immersing the cleaned substrata in thiol solutions at a concentration of 1 mM in ethanol p.a. (Thome et al. 2012). TMA was also prepared from 1 mM ethanol solutions following literature protocols (Petrone et al. 2011; Shen & Lin 2012). Mixed SAMs were prepared from a solution containing 50% TMA and 50% MUDA at a total concentration of 1 mM (Shen & Lin 2012; Bauer et al. 2014). The SAMs were allowed to assemble under ambient conditions for 24 h, subsequently rinsed with ethanol, ultrasonicated for 3 min in ethanol p.a., rinsed with ethanol again and dried in a stream of N₂.

All samples were stored under Ar until they were used; SAMs prepared on Au-coated Si wafers were used as references for characterization. Surface analysis by spectroscopic ellipsometry, contact angle goniometry (Woollam M44, J.A. Woollam Co. Inc., Lincoln, NE, USA) (Table 1) and XPS revealed identical surface properties as in previous publications (Petrone et al. 2011; Thome et al. 2012; Bauer et al. 2014). HUDT (static contact angle of 34°), TMA (static contact angle of 36°), and TMA+MUDA (static contact angle of 19°) are all rather hydrophilic. DDT in turn is hydrophobic (static contact angle of 105°).

**Settlement of barnacle cyprids**

The experiments were performed with cyprids of the species *Balanus amphitrite (= Amphibalanus amphitrite)*, which were cultured and harvested at Newcastle University, UK, following established protocols (Imbesi et al. 2012). The cyprids were shipped in a cool box overnight to Karlsruhe Institute of Technology, Germany where the experiments were carried out the following day. The age of the cyprids at the time of recording was three to four days. All experiments were performed in filtered (0.22 µm pore size) and sterilized (autoclaved) natural seawater. The experiments were repeated with two different batches of cyprids in order to verify reproducibility. Settlement assays were conducted in coated quadriPERM® culture dishes (Greiner Bio-one Ltd, Kremsmuenster, Austria), which were filled with seawater and to which ∼30 cyprids were added per compartment. Subsequent to the 3-D tracking experiment, the same culture dishes were used to quantify settlement and thus to directly link settlement and behaviour for the same batch of cyprids. The proportion of settled cyprids was determined after 48 h using light microscopy.

**Table 1.** Surface characterization (static water contact angle and thickness as determined by spectroscopic ellipsometry using a Cauchy model for the organic layer with A = 1.45 and B = 0.01) of the SAMs.

| Chemical formula | Short name | Water contact angle (°) | Thickness (Å) |
|------------------|------------|-------------------------|--------------|
| HS-(CH₂)₁₁-CH₃  | DDT        | 105 ± 3                 | 11 ± 2       |
| HS-(CH₂)₁₁-OH   | HUDT       | 34 ± 3                  | 11 ± 2       |
| HS-(CH₂)₁₁-NMe₃⁺ | TMA        | 36 ± 3                  | 15 ± 2       |
| 50% HS-(CH₂)₁₁-NMe₃⁺ /50% HS-(CH₂)₁₁-COO⁻ | TMA+MUDA  | 19 ± 3                  | 15 ± 2       |

All values reflect the average of three measurements and the corresponding SE.
Stereoscopic 3-D tracking

The SAM-coated culture vessels (75 mm × 25 mm × 15 mm; 1 × w × h) were filled with filtered seawater. The depth of the water column was 10 mm (20× larger than the cyprid body size), thus providing a water volume of 18.75 ml available for swimming. After removing the cyprids from a refrigerator (6°C) they were allowed to warm up to room temperature (20°C) for 60 min before ≈30 larvae were added to the SAM-coated containers. The cyprids were allowed to equilibrate for an additional 5 min before stereoscopic video recording began in order to minimize the convection from the inoculation procedure. The stereoscopy setup consisted of two synchronized consumer HD camcorders (Sony HDR-XR550, Sony Corp., Tokyo, Japan) (Maleschlijski et al. 2012). Both camcorders imaged the dish from above at a relative angle of ≈80° and 40 cm from the water surface. The whole arena was illuminated from above under normal incidence. The 2-D field of view (FOV) of the camcorders was ≈47 mm × 35 mm. As the width of the FOV was larger than the width of the container, image analysis was restricted to the relevant area. Furthermore, in order to restrict effects originating from the walls of the container, cyprids were tracked only in the middle, at a distance of at least 2 mm from the walls, thus imaging an effective arena size of approximately 60% of the container base. Movements within each container were recorded for ≈5–7 min at a frame rate of 25 fps. Further video sequences were recorded after 4 h and 12 h to reveal the time dependence of the obtained data. A computer-aided tracking algorithm was applied to extract trajectories from the movies. Therefore, for each object of interest, the xy coordinates were analysed in both camera perspectives and merged into 3-D coordinates. Here, the z values represented the distance to the surface, while the x and y values represented the position parallel to the bottom of the culture dishes. To accomplish the epipolar transformation, a previously applied calibration procedure was used (Maleschlijski et al. 2012). If all 3-D coordinates of a single object of interest are connected in time, trajectories are obtained from which quantitative information on swimming speed, direction of motion, swimming angle, distance to surface and swimming area can be calculated. For each of the surfaces of interest, three separate replicates were analysed. For each replicate ≈20 different cyprids were identified and tracked throughout the observation period. This provided ≈0.2 million data points per replicate, resulting in ≈0.6 million cyprid positions available for analysis for each surface. Three specific parameters were calculated for the analysis: area visited by the cyprid within the field of view (swimming area coefficient, CSA), probability of finding a cyprid at a defined swimming depth (occurrence value, OV) and the number of transitions per time unit between regions of different swimming depths (transition frequency). The numerical calculation of these three parameters is described in detail in Figure S1 in Supplementary information. [Supplementary information is available via a multimedia link on the online article webpage].

Statistical analysis

To determine significant differences (p < 0.05) between two groups of data representing the same variable (e.g. settlement on different surfaces, and velocity on different surfaces), a Student’s t-test was used.

Swimming regions

During analysis it turned out that those regions within the water column close to the surface of the sample and close to the water surface were preferred by the cyprids. In consequence, three ‘swimming regions’ were identified and defined as lower, bulk and upper swimming regions (LSR, BSR, and USR) denoting the distance of the cyprids from the interface studied. The borders of the regions were determined by analysis of the distribution of the z position of the cyprids. For this purpose the cumulative distribution function (CDF) of the histograms was calculated. In the case of the LSR, as soon as the function reached a plateau and the occurrence values became lower than 5% (depleted bulk region), the corresponding z position was defined as the border of the region. For the USR, the CDF was analysed from higher to lower values, and as soon as the plateau was reached and occurrences were lower than 5% the z value was used for a lower border of the region. A graphical visualization of this process is schematically illustrated in Figure S2.

Results and discussion

The behaviour and distribution of cyprids close to the SAMs (water depth ≈9–10 mm) was evaluated using 3-D video stereoscopy. Using two synchronized cameras that record the motion from different angles, the 3-D positions of the cyprids were determined over time (Maleschlijski et al. 2012). Evaluating the distribution of the positions of cyprids within the container during swimming revealed three distinct regions: the LSR; the USR; and the region in between (Figure 1a). A high density of data points implies that cyprids spent much of their swimming time in the corresponding region. Figure 1b illustrates the occurrence of cyprids in the water column above the DDT surface. For low values of the z position (vicinity of the coated surface) and for
high z positions (close to the water surface), the probability of observing cyprids was strongly enhanced. In between these regions, the occurrence was much lower.

The USR extended from the water surface down to \( \approx 1.5 \) mm (for details on border definition see Figure S2) swimming depth. In Figure 2 (top panel, in light grey), the distribution of the z velocities within the USR has been calculated. These values represent only the z component of the velocity vector describing the velocity of swimming up (positive velocities) and diving down (negative velocities). In general, the velocity distribution was characterized by narrowly distributed values in the proximity of 0 mm s\(^{-1}\) (representing \( \approx 70\% \) of all values), indicating movements parallel to the water surface. It is worth mentioning that there were still a substantial number of changes in the z position (velocities \( \approx +1 \) mm s\(^{-1}\) or \( -1 \) mm s\(^{-1}\)), indicating that the cyprids in the USR moved freely and were clearly distinguishable from those captured in the meniscus. Furthermore in Figure 2 (the histograms in the darker colour), the xy component of the velocity vector showed that cyprids were still active in the xy plane of the USR and LSR. At this point it should be noted that cyprids trapped at the liquid/air interface were excluded from the analysis.

The BSR started below the USR. The transition into the BSR occurred gradually as soon as the distribution of the swimming positions in USR decayed to very low values. Those parts of trajectories found in the BSR mainly originated from transitions between the LSR and the USR, thus the probability of finding cyprids in this region was very low. The values of the z component of the velocity in this region were broadly distributed and no peak was detectable at values \( v_z \approx 0 \) mm s\(^{-1}\) (Figure 2 in light grey, middle panel). Interestingly, velocities in the xy plane higher than 0 mm s\(^{-1}\) were also present (Figure 2 dark histogram, middle panel).

The LSR was located between the BSR and the chemically functionalized surface. Similar to the USR, it extended from the surface \( \approx 1.5 \) mm into the solution with a high probability of finding cyprids. In this region,
the cyprids swam slowly \((v_{xy} \approx 0.3 \text{ mm s}^{-1})\); dark histogram in bottom panel of Figure 2) and mostly parallel to the surface \((v_z \approx 0 \text{ mm s}^{-1})\), light grey histogram. It can be speculated that the slow velocity is associated with a transition into surface interaction (Crisp 1984; Maréchal et al. 2004; Chaw & Birch 2009; Aldred et al. 2013).

The presence of two preferred regions where cyprids accumulated raised the question of whether the height of the water column affected the position of the two swimming regions. Therefore, experiments were conducted to investigate the influence of different water depths on the USR. Figure 3 shows the occurrence of cyprids as a function of their swimming depth (\(z\) position) in culture vessels coated with DDT for water depths of 3.5, 6.5 and 8 mm. It was immediately obvious that the position of the USR was determined by the position of the water surface and increased with increasing water depth. In all three cases, the USR extended \(\approx 1.5\) mm beneath the water surface. This finding combined with the observation that the occurrence value for BSR remained very low \((\text{OV}_{\text{LSR}} = 84\% \pm 4\%, \text{OV}_{\text{USR}} = 13\% \pm 4\% \text{ compared to OV}_{\text{BSR}} = 4\% \pm 1\%}\) for all examined depths supported the notion that for the assay geometry employed, cyprids expressed a clear preference for interfacial regions and spent most of their time swimming either close to the air/water or close to the water/solid interface. In the literature, different mechanisms involved in sensing of the two interfacial regions have been discussed, viz. phototaxis, rheotaxis and barokinesis (DiBacco et al. 2011).

To find a correlation between the probability of settlement and motility parameters such as distribution in the water column, velocity and swimming area coefficient, different surface chemistries were compared. Settlement after 48 h (Figure 4a) showed that the DDT surface was attractive \((\approx 10\%)\) for settlement compared to the HUDT surface on which much lower settlement \((\approx 0.2\%)\) was observed. This trend was in good agreement with settlement values reported by Petrone et al. (2011) where on DDT \(\approx 25\%\) settlement was observed compared to \(\approx 12\%\) on HUDT. While the general reduction in settlement agreed in the two studies, total settlement values were lower in the present work. One possible reason could be the effect of the experimental geometry (see Qiu et al. 2008). While coated dishes were used as substrata in the present study, Petrone et al. (2013) used either homogeneously coated wells or a novel experimental geometry that eliminated the air–liquid interface. In addition, the cyprid density for the experiments in the presented work was lower, which probably reduced the settlement increasing cyprid–cyprid interactions as described in Clare et al. (1994) and Elbourne et al. (2008).

Analysis of the occurrences in the LSR over the DDT and the HUDT surfaces revealed that in the case of the attractive, hydrophobic surface (DDT) the values \((75\% \pm 1\%)\) were more than twice as high as the values \((33\% \pm 3\%)\) for the less attractive, hydrophilic surfaces (HUDT). Moreover, in both cases, the occurrence in the BSR was low, with somewhat lower values for DDT \((6\% \pm 1\%)\) compared to HUDT \((23\% \pm 6\%)\). To further quantify behaviour, the mean velocities of cyprids were investigated over both surfaces. This parameter represented the averaged value of the locomotion velocities of all observed cyprids swimming in the water volume (including the three regions; USR, BSR and LSR). The velocities on the attractive surface (DDT) were about two times lower \((0.5 \text{ mm s}^{-1} \pm 0.1 \text{ mm s}^{-1})\) than the velocities over the HUDT \((1.1 \text{ mm s}^{-1} \pm 0.2 \text{ mm s}^{-1})\) surface (Figure 4c).

The third parameter described the extent of surface area visited by the cyprid in the field of view during the measurement, either by surface inspection or swimming including trajectories from the whole water volume (USR, BSR and LSR). A lower value for the swimming area coefficient originated from a more localized and convoluted path, while higher values represented an extended and straighter path. The swimming area coefficient on DDT \((0.47 \pm 0.06)\) was only half of the value for HUDT \((0.94 \pm 0.01)\) (Figure 4d), indicating that cyprids on the DDT spent more time in a specific area and moved at lower speeds \((0.5 \text{ mm s}^{-1}\) compared to \(1.1 \text{ mm s}^{-1}\)). It is likely, though not examined in detail here, that these cyprids were engaged in inspection of the surface. The general trend that cyprids expressed lower speeds and more focused swimming in the vicinity of attractive surfaces was in a good agreement with literature reports. Matsumura et al. (2000) showed that cyprids spent more time, traversed more slowly and took

Figure 3. Histogram of the occurrence of active \((v > 0 \text{ mm s}^{-1})\) cyprids as a function of their \(z\) position in the water column. Cyprids in containers with different water depths showed a clear preference for interfacial regions.
a more convoluted path on attractive surfaces. Chaw & Birch (2009) noted that cyprids explored attractive surfaces (CH$_3$-treated glass) more thoroughly and the duration of the steps was much longer than the step duration on surfaces of no particular interest (eg a NH$_2$-treated glass slide). In turn, on highly hydrophilic zwitterionic polymers, a fast motion across large areas has been found (Aldred et al. 2010).

In addition to the two surfaces with different wettability, positively charged trimethylammoniumundecanethiol (TMA) and mixed SAMs (consisting of 50 % TMA and 50 % mercaptoundecanoic acid, MUDA) (TMA + MUDA) with a net neutral charge (Figure 4e–g) were compared. Figure 4a shows that more cyprids settled on TMA compared to the TMA + MUDA SAM. However, the difference was not as pronounced as between DDT and HUDT. Figure 4e compares the occurrences of cyprids in the respective swimming regions. The probability of finding cyprids in the LSR close to the positively charged surface (TMA, $\approx$67%) was nearly as high as for DDT ($\approx$75%). Additionally, cyprids moved with low mean velocity on the positively charged TMA ($\approx$0.55 mm s$^{-1}$). The corresponding surface area coefficient on this surface was comparable to the DDT value and a smaller area was explored in a more convoluted path ($C_{SA} = 0.5$). These observations combined with the high occurrence in the LSR region correlated well with high settlement on this more attractive surface. Conversely, the time spent in the

Figure 4. (a) Settlement values, (b, e) distribution of the occurrence values for the swimming regions, (c, f) velocities and (d, g) swimming area coefficients over surfaces with different wettability and surface charge of active ($v > 0$ mm s$^{-1}$) cyprids. ($N = 4$, error bar = SE). Asterisks represent statistically different groups (Student’s $t$-test, **$p < 0.001$; *$p < 0.05$; ns: not significantly different).
LSR over the TMA + MUDA surface was, as expected, lower (< 50%). The trend was more apparent when the occurrence of cyprids in the USR was compared. The probability of finding cyprids in the USR above the attractive surface (≈20%) was only around half of that for the TMA + MUDA SAM (≈37%). Also here, occurrence in the BSR was very low. Furthermore, cyprids moved with higher mean velocity on the TMA + MUDA SAM (≈0.85 mm s⁻¹) compared to the TMA SAM and the corresponding surface area coefficient was CSA = 0.75 on the mixed SAM, denoting a broader and more linear way of exploration of this surface. These findings, combined with the fact that cyprids moved into the USR away from the TMA + MUDA SAM, correlated positively with reduced settlement.

There have been several reports correlating settlement preferences of cyprids and their interaction with the substratum via the temporary adhesive located on the end of the antennules (Berglin & Gatenholm 2003; Andersson et al. 2009). Strong adhesion could be related to high settlement. Imaging surface plasmon resonance (iSPR) allowed direct imaging of this initial interaction and revealed that the temporary adhesive adhered less well to protein resistant surfaces, such as ethylene glycols, which also showed low settlement (Aldred et al. 2011). In a similar way charge equilibrium at zwitterionic interfaces facilitated protein resistance and low algal spore settlement, while charge excess facilitated binding (Holmlin et al. 2001; Chen et al. 2006; Ekblad et al. 2009; Bauer et al. 2014). Recent studies have shown that cyprid settlement is also influenced by surface charge (Petrone et al. 2011; Di Fino et al. 2013). Here, settlement on the charged TMA surfaces was higher compared to the mixed TMA + MUDA SAMs. The stronger interaction with the charged surface is furthermore reflected in the lower velocity, the more localized exploration and the more frequent occurrence in the LSR. The high velocity and the high exploration coefficient on surfaces with mixed zwitterionic charges correlated well with previous findings on zwitterionic polymers which revealed extended swimming motion at high velocities (Aldred et al. 2010). In both cases, ie DDT/HUDT and the TMA/ TMA + MUDA, a lower velocity (Figure 4c, f) correlated with a smaller swimming area coefficient (Figure 4d, g), higher occurrence in the LSR and higher settlement.

The low occurrence of cyprids swimming in the bulk region for all test surfaces suggested that this region was mainly used for transitions between the interfacial regions. In order to investigate this observation, the transition frequency into and out of the BSR over DDT and HUDT was determined (Figure 5a). It can be seen that in both cases the transitions out of the bulk swimming region were more frequent (15.2 ± 1.8 min⁻¹ for DDT and 14.1 ± 1.8 min⁻¹ for HUDT) than the transitions into the BSR (1.0 ± 0.1 min⁻¹ for DDT and 3.4 ± 0.1 min⁻¹ for HUDT). It was concluded that the distribution shown in Figure 4b was a consequence of an active process in which cyprids left the BSR and accumulated at the interfaces, rather than accidental inhomogeneous distributions. The fact that the experimental groups containing ≈20 cyprids each demonstrated a summed transition frequency into and out of the BSR of nearly 17 transitions per minute indicated highly dynamic behaviour and that the distributions in Figure 4b were a consequence of active redistribution and not a simple static situation. Interestingly, no significant differences (p > 0.05) were detected for the frequencies out of BSR for DDT and HUDT.

While the transition frequencies out of the BSR explained the accumulation at the interfaces, the transition frequencies into and out of the lower swimming region (Figure 5b) and the upper swimming region (Figure 5c) were calculated in order to analyse the

![Figure 5. Transition frequencies on DDT and HUDT samples for in and out of the (a) bulk swimming region, (b) lower swimming region and (c) upper swimming region (Mean values shown, error bars represent SE, N = 42). Student’s t-test, *p < 0.05; ns: p > 0.05, not significantly different groups.](image-url)
response to the chemical surface cues in greater detail. As shown for the DDT surface in Figure 5b, fewer transitions occurred out of the LSR (1.3 ± 0.2 min⁻¹) and a higher frequency was observed for cyprids that entered the LSR (14.0 ± 1.0 min⁻¹). On HUDT, these two probabilities were nearly identical (3.9 ± 0.5 min⁻¹ for DDT and 4.2 ± 1.0 min⁻¹ for HUDT). In particular, the high probability of cyprids entering the LSR of DDT was the reason for the observed accumulation in Figure 4b. In turn, the transition frequencies of cyprids into or out of the USR of the well containing the DDT-coated surface was nearly identical (1.5 ± 0.3 min⁻¹ for swimming out of the USR and 3.5 ± 0.5 min⁻¹ for swimming into it). However, the transition frequency of cyprids entering the USR in the wells with the HUDT coatings was much higher (10.9 ± 1.8 min⁻¹). Thus, the attractive DDT surface showed a high transition frequency of cyprids entering the LSR with no marked preference for the USR, the unattractive HUDT surfaces obviously stimulated an accumulation in the USR as indicated by the high frequency of cyprids entering this region. Thus, the different distributions in the water column above the DDT and the HUDT surfaces were caused by dynamic transitions and the different transition frequencies for USR and LSR were guided by the surface chemistry.

Since most of the trajectories were extracted from videos recorded shortly after the initial contact of cyprids with the test surfaces, it was important to investigate how the existing values for occurrences in the LSR, mean velocity and swimming area coefficient change with time and whether the correlation with probability of settlement remains. Again, the hydrophobic DDT and the hydrophilic HUDT were compared as shown in Figure 6. Cyprid occurrence in the LSR of DDT remained high throughout the whole measurement, confirming that the larvae were ‘interested’ in this surface. Conversely, few cyprids were present in the LSR for the HUDT surface at time point 0 h (≈33%), but the number increased with time. This increase implied that what were statistically significantly different values (p < 0.001, Student’s t-test) for the first experiment with HUDT and DDT surfaces, were statistically indistinguishable after 4 h and remained the same also after 12 h. It seemed that the initial correlation of occurrences in the LSR with the overall settlement preference disappeared after 4 h. While it is currently only possible to speculate whether conditioning effects (Garg et al. 2009; Thome et al. 2012) or footprints deposited on the surface (Elbourne & Clare 2010) might have changed the occurrence of cyprids in the different parts of the water column, it is important to keep the time dependence in mind if a similar probability analysis is anticipated.

In addition to the analysis of occurrence within the water column, changes in the mean velocities were compared. Figure 7a shows the comparison of all velocities obtained for the DDT and HUDT surfaces for the three time points (0, 4 and 12 h after initial exposure) in all three regions together. In general, cyprid velocity over HUDT was more than twice as high as over the attractive DDT and this ratio increased slightly with time. The increase was mainly caused by higher velocities over HUDT. Faster swimming over HUDT correlated with a reduced occurrence near the surface and lower settlement. Chaw & Birch (2009) demonstrated that cyprids expressed longer steps with shorter duration, thus higher locomotion velocities, when engaging in close surface inspection on hydrophilic surfaces. This observation is in agreement with the higher velocities in the present experiment close to the HUDT surfaces. It is important to note that the resolution of the setup was not sufficient to differentiate between surface inspection interactions and swimming movements close to the surface. In addition to the velocity, it was analysed whether cyprids swam across large areas or if the movements were localized. The corresponding ‘swimming area coefficient’ tended to decrease with time (Figure 7b), indicating that swimming became more localized, probably including close surface inspection. The effect of the decreasing swimming area was observed on both DDT and HUDT surfaces. After a longer time, the swimming areas were still different, though the differences were found to be reduced.

Combining these observations with the distribution of cyprids within the water column revealed that on DDT, which had the highest occurrence of cyprids in the LSR at all three time points, the velocity remained low and the swimming area coefficient decreased. This indicates that the surface was highly attractive to cyprids, causing a more focused and localized swimming motion. After 48 h, this attractiveness was confirmed by the
higher settlement values found on these surfaces. On HUDT, cyprids were initially less frequently observed in the LSR (less than half of the occurrence compared to DDT). However, after 4 h there was a higher probability of finding them in the LSR; indeed, values were equal to those for DDT. At later time points, the velocities close to HUDT were more than twice as high as for DDT, with an increasing ratio. Obviously, the higher velocities correlated with lower settlement on the HUDT surfaces. Interestingly, the swimming area coefficient over HUDT also decreased with time, which could be caused by increasing surface inspection. The relative differences in swimming area coefficient between DDT and HUDT remained for the different time points, and correlated with the different settlement values after 48 h. Despite the clear trends observed, it is important to emphasize that the volume of the test vials was limited to 18.75 ml due to geometric restrictions of the laboratory tracking assay. Compared to conventional droplet settlement assays the volume is relatively large, but of course it does not reflect the natural situation in the ocean. It would be interesting to investigate if similar selection mechanisms occur in the ocean and to include the walking motions for a more detailed and complete understanding of cyprid–surface interactions in the future. As the method has no specific requirements regarding the surfaces, all coating types from monolayers, surface morphologies to technical coatings can easily be analysed. The size of the organisms investigated is solely dependent on the imaging optics, but cyprids seem to be at the lower end for consumer camcorders.

In summary, a new method to monitor cyprid exploration is presented for evaluating surface attractiveness under laboratory conditions based on the mean velocity, the swimming area coefficient and the occurrence zones within the water column. These motility parameters indicate settlement preference as high number of cyprids in the lower swimming region, lower velocities and more localized swimming (lower swimming area coefficient) correlated positively with higher settlement values (DDT SAMs or positively charged TMA SAMs). Conversely, less attractive surfaces (HUDT SAMs or uncharged mixed SAMs) had a lower number of cyprids in the LSR, the mean velocities were higher and the swimming area coefficient of the more directed trajectories was higher. Analysis of different SAMs revealed that measuring differences in motion parameters provides a sensitive tool to determine cyprid responses to surfaces and might serve as an early indicator for settlement preferences.

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