Correlative assays of barnacle cyprid behaviour for the laboratory evaluation of antifouling coatings: A study of surface energy components

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Abstract

Laboratory evaluation of antifouling coatings is underpinned by settlement studies with specific fouling organisms. Established methods provide insight into the likelihood of failure of a particular coating system, but can neglect the process of surface selection that often precedes attachment. The present approach for quantifying the exploratory behaviour of barnacle cypris larvae suggested that inspection behaviour can be a rapid and predictive proxy for settlement. Two series' of xerogels with comparable total surface energy, but different dispersive and polar components, were evaluated. Settlement assays with 3-day-old cyprids of *Balanus improvisus* demonstrated that while attachment was not linked directly to dispersive free energy, the composition of the xerogel was nevertheless significant. Behavioural analysis provided insight into the mechanism of surface rejection. In the case of a 50:50 PH/TEOS (phenyltriethoxysilane-based) xerogel vs a 50:50 TFP/TEOS (3,3,3-trifluoropropyltrimethoxysilane-based) xerogel, wide-searching behaviour was absent on the former.

Keywords

Barnacle, antifouling, settlement behaviour, behavioural analysis, cyprid, xerogel.

Introduction

Biofouling in the marine environment is an important global challenge with serious environmental and economic consequences (Davidson et al. 2016). Although fouling affects most anthropogenic structures placed in the ocean, ships' hulls, heat exchangers, filtration systems and deployed sensors suffer particularly severe penalties. While these diverse platforms require bespoke antifouling solutions, which may also depend on the operational cycle, geographical location and the biological nature/species composition of the fouling challenge, there are still relatively few approaches being taken towards the development of, low-maintenance, fouling-resistant coatings.

Emphasis in the last decade has been on the design of materials that are inherently resistant to fouling, without the use of biocides. This research focus is not reflected in the shipping industry today, however, where biocidal coatings dominate the market with over 90% of sales (Muthukrishnan et al. 2017). The environmental impact of biocidal coatings, as well as concerns regarding freedom of operation and tightening regulation of biocidal formulations, has nevertheless maintained industrial and academic interest in identifying novel chemistries that prevent biofouling through passive means (e.g. Lejars et al. 2012).

The only non-biocidal coatings that are commercially available for ships' hulls are the so-called 'fouling-release' formulations. These silicone- or fluoropolymer-based elastomers allow the accumulation of fouling, but the physical, chemical and mechanical characteristics of the coating enable hard fouling to be removed under a moderate wall shear stress, equivalent to around 10-15 knots. Of greater interest, but technically more challenging, is the development of materials that resist initial attachment of fouling organisms altogether, due to the inherent non-stick properties of the coating. Amphiphilic chemistries (Patterson et al. 2017; Jakobie et al. 2018), poly-zwitterions (Jiang and Cao 2010), polyglycerols (Wanka et al. 2018) and poly(ethylene glycol)-based coatings (Galhenage et al. 2017) have all demonstrated the ability to deter or interfere with the adhesion of settling organisms in laboratory assays.

Beyond the complexity of the chemical formulations, however, there are other challenges relating to the development of practical materials based on non-fouling concepts. One such challenge is associated with their evaluation for rapid down selection in the laboratory. Field testing requires relatively large-scale production of materials and can often take weeks to months to produce meaningful results (Stafslien et al. 2016). Laboratory assays, although arguably less realistic than field exposure, are preferred in the early stages of coating development due to the precision of the results and rapid turnaround. Many of the propagules (larvae/spores) of fouling organisms used in routine laboratory assays have selective capability, however, so may not settle in the required timeframe on highly deterrent surfaces (Aldred et al. 2010). Degradation (e.g. oxidation) of some model coating formulations limits the maximum duration of experiments and thus presents a conundrum that is encountered with increasing frequency. How can several materials be separated in terms of their efficacy against fouling species, when all of the materials tested prevent settlement entirely over the course of a laboratory assay?

For selective organisms, analysis of settlement behaviour is a laboratory capability that could bridge the gap in our understanding of the mechanisms of surface rejection. 'Settlement behaviour' is taken here to encompass all the activities of settling organisms during surface exploration (if it occurs) and permanent adhesion to a surface. If rejection of surfaces is understood, this information clearly has greater value than an observation of whether or not permanent attachment occurs. However, behavioural analysis is a challenging discipline, and particularly so when applied to micro-scale organisms that are motile in three dimensions (Heydt et al., 2012; Vater et al., 2015) and that have complex settlement responses.

A second, related challenge pertains to the hypothesis-driven strategy for coating development. If the colonisation stage of a fouling species settles on one formulation but not on another, what is the mechanism of deterrence of the less-preferred coating? In most cases a standard settlement assay will not provide this information directly, although the question may be built into the experimental design. In either case, the absence of information regarding interactions of organisms with surfaces may lead to an encouraging assay result failing to provide clear direction for further development of the coating.

Relatively few attempts have been made to measure the settlement behaviour of fouling species, and even fewer have successfully linked specific behaviour to a settlement response, providing predictive capability. *Hydroides elegans* is a serpulid tubeworm that can dominate tropical fouling assemblages. The larvae of this species are known to respond positively to bacterial biofilms on surfaces, but using video analysis of their swimming and crawling behaviour Hadfield et al. (2014) demonstrated that larvae need to physically contact the biofilm in order to settle, and that water-soluble cues are not sufficient. However, the tracking method for *H. elegans* was not sufficiently developed to allow its use as a high-throughput indicator of likelihood to permanently attach. In a series of papers between 2009-2015, Rosenhahn and colleagues used holographic microscopy to reconstruct the swimming trajectories of spores of the green alga *Ulva linza*, and successfully correlated these data to settlement of spores on surfaces (Heydt et al., 2012; Vater et al., 2015). It was shown that spores decelerated as they approached a surface onto which higher settlement was observed, and that the proportion of various characteristic behaviours likewise changed in relation to subsequent settlement (Heydt et al. 2012). On surfaces functionalised with cationic peptides, for example, spores more often showed a 'hit and stick' motion, where the initial contact with the surface consummated the settlement process. However, given the data-intensive nature of the technique and the speed of the *U. linza* settlement process, embedding holographic microscopy in a coating evaluation pipeline would present challenges.

Recently, Aldred et al. (2018) presented progress in the development of an automated system for quantitatively analysing the settlement behaviour of barnacle cypris larvae, based on the algorithms of Alsaab et al. (2017). Barnacles are among the most problematic marine fouling species (Holm 2012), and the exploratory behaviour of their larvae prior to permanent attachment is among the most complex. Substantial previous work had identified characteristic behaviours of cyprids and used these, in some cases, to highlight differences in behaviour between experimental conditions (Prendergast et al. 2008; Chaw et al. 2011; Larsson et al. 2016). With the natural

settlement behaviour of cyprids largely uncharacterised and studies relying for comparison on short snapshots of specific behaviours, however, it was impossible to reliably implement any of the published methods for rigorous prediction of cyprid settlement in a given condition. The system outlined by Aldred et al. (2018) enabled this by computationally separating cyprid behaviour into inspection or walking, and then using combinations of these behaviours to identify the classic sequence of settlement behaviours outlined originally by Crisp (1976); namely swimming, wide searching, close searching and inspection. A long-term assay allowed for the tracking and automated behavioural classification of cyprids as they transition through their entire planktonic phase, from swimming through surface exploration to settlement. A simplified short-term assay complemented the detailed approach by providing larger data sets in a shorter time, that could then be compared statistically. One of the many significant issues that affected previous approaches, most of which relied on uninterrupted tracking of the cyprid, reconstruction of its trajectory and interpretation of that trajectory into recognisable behaviours, was that in a given experiment cyprids spent relatively little time exploring surfaces, but needed to be be tracked throughout for meaningful data to be produced. The recently published technique avoided this issue by focussing on the body movements of the larvae for detection of exploratory behaviours on surfaces, rather than the track trajectory. This minimised the error rate and the data processing burden, and theoretically enabled the application of the technique to long-term tracking of cyprid exploration on antifouling coatings. The efficacy of the technique for evaluation of coated surfaces was, however, not tested by Aldred et al (2018).

Xerogels have proven to be useful coating chassis in the laboratory-scale testing of biological hypotheses that require precise manipulation of chemistry and the physicochemical nature of the surface (Finlay et al. 2010). Xerogels produce dense, hard, thin films with relatively smooth surfaces (sub-nanometer roughness). Manipulations of the surface chemical groups alter properties such as surface energy and surface charge while maintaining a high elastic modulus and smooth topography (Bennet et al. 2008; Bennett et al. 2010). Here, they enabled production of a suite of coatings highly suited to the behavioural analysis method.

Recently, Gatley-Montross et al. (2017) used a range of xerogels with diverse surface chemical properties to exemplify the complexity of the settlement responses of several fouling organisms. Their data suggested that for the barnacle *Balanus improvisus* (= *Amphibalanus improvisus*), the dispersive component of free energy could be an important factor. While it is accepted that settlement of *B. improvisus* tends to be higher on hydrophobic surfaces, this trend is generally clear only at the extremes of the wettability spectrum. The variable response of *B. improvisus* cyprids to surfaces of intermediate wettability (Di Fino et al. 2014) has previously indicated that they respond to factors other than total surface free energy (Gibbs free energy), and the analysis of Gatley-Montross et al. (2017) suggested that variations in the dispersive component of free energy may be of particular importance.

To demonstrate the applicability of behavioural analysis to the evaluation of coating formulations, the role of dispersive free energy in surface selection by cyprids was investigated using a rational series of xerogel formulations. Both of the silanes used in production of the xerogels - phenyltriethoxysilane (PH) and 3,3,3-trifluoropropyltrimethoxysilane (TFP) - contributed equally to the hydrophobicity of the surface. Therefore,

xerogels containing increasing proportions of either silane showed a similar reduction in total surface free energy. However, those xerogels containing PH had a consistently higher dispersive component of surface energy compared to the equivalent TFP-containing xerogels, where the polar component was higher. A pair of surfaces that had ostensibly similar surface characteristics, but a markedly different settlement response, was selected for behavioural analysis using 3-day-old cypris larvae.

Methods

Preparation of xerogels

The xerogels were synthesized via the sol-gel process using organically-modified silane precursors, ethanol (EtOH) and water as solvents, and hydrochloric acid (HCl) as a catalyst. Four silane precursors were used to prepare the coatings: tetraethoxysilane (TEOS) provided the bulk in the coating compositions, while 3,3,3-trifluoropropyltrimethoxysilane (TFP) and phenyltriethoxysilane (PH) were used to adjust the surface chemical properties, and *n*-octyltriethoxysilane (C8) provided a hydrophobic reference surface when conducting settlement assays. All silanes were purchased from Gelest. EtOH was purchased from Decon Laboratories, Inc. and isopropanol, concentrated HCl, borosilicate glass microscope slides (25 mm × 75 mm) and borosilicate glass vials (22 ml; used in settlement assays) were obtained from Fisher Scientific. 12 mm-diameter open-ended glass tubes were acquired for tracking experiments from Cambridge Glassblowing, UK. The final compositions of the xerogel coatings were based on the molar ratios of silane precursors. For example, 50:50 PH/TEOS was composed of 50 mol-% PH and 50 mol-% TEOS. Xerogel sols were prepared by combining ethanol, the silane precursors and a solution of water and acid in a vial. The sols were stirred in ambient conditions until use. The amounts of reagents used in the production of each sol are compiled in Table 1.

[TABLE 1 HERE]

Coated vials were utilized for the settlement assays while open-ended coated tubes and microscope slides were used to collect tracking data. Prior to coating, all glass substrates were cleaned by immersion in aqueous piranha solution (1:4 30% hydrogen peroxide: sulfuric acid) for 24 hours. The substrates were then rinsed with deionized water and either dried in an oven (120°C) until the water had evaporated (vials and tubes) or stored in isopropanol until coating (microscope slides).

A variety of methods were used to coat the substrates. When preparing the vials, a xerogel sol was placed in the vial and gently rotated to cover the entire inside of the vial, then allowed one minute to bond to the glass. The sol was then removed and the vial was placed upside down for seven days in ambient conditions to allow the coating to cure. A similar procedure was used to coat the open-ended tracking tubes. The sol was micropipetted into the tube and the tube was rotated to allow the sol to coat the entire inside. The excess sol was drained from the tube and the coating was allowed to cure in ambient conditions for seven days. The sol was applied to microscope slides using a model P6700 spin coater. Slides were removed from isopropanol and dried with compressed air before loading onto the spin coater. 400 uL of sol was spun onto the slide at 100 rpm for 10

seconds, then cast onto the slide by spinning at 3000 rpm for 60 seconds. The slides were given 7 days to dry in ambient conditions to allow the coating to cure.

Characterisation of xerogels

The xerogels, applied on microscope slides, were characterized via contact angle analysis as described in Gatley-Montross et al. (2017). Deionized water was prepared to a specific resistivity of at least 18 MΩ-cm using a Barnstead NANOpure Diamond UV ultrapure water system. Diiodomethane was purchased from Sigma-Aldrich. The artificial sea water (ASW) used for soaking the slides before surface characterization was prepared based on a modification of the Marine Biological Laboratory (MBL) recipe. The modification involved adding 1.0 mM of sodium bromide and 1.0 µM of sodium iodide to give a more realistic representation of the halide ions present in natural seawater.

Contact angle analysis was performed on samples soaked in seawater for 24 h, following the procedure from Gatley-Montross et al. (2017). The Owens-Wendt method (Owens & Wendt 1969) was used to calculate the surface energy as well as the polar and dispersive components of surface energy, using water and diiodomethane as probing liquids. Xerogel coatings can be dynamic and rearrange upon immersion in an aqueous environment until an equilibrium has been achieved. For these coatings, this was accomplished within 24 hours. Surfaces left in a dry state eventually revert to their previous conformation, however this process takes 2-3 weeks and is not therefore relevant to the timeframe of this study (Evariste et al. 2013). Contact angles were recorded at 3 locations on each of 3 replicate coated surfaces. A droplet of probing liquid, between 10-15 µl, was placed on the surface of the film and the contact angle measured using a ramé-hart goniometer. This measurement, the 'static' contact angle, was used to determine the surface energy of the film.

Culture of barnacle larvae

Barnacle larvae were obtained from stocks of *B. improvisus* that are maintained in semi-continuous culture at Newcastle University, UK. These stocks were originally sourced from the Sven Lovén Centre for Marine Sciences, Tjärnö, Sweden. Adult *B. improvisus* were maintained in a 19 °C recirculating aquarium in brackish conditions (25 ppt) and fed daily with *Artemia* sp. and *ad libitum* with the chlorophyte *Tetraselmis suecica*. To collect larvae, the adult barnacles were removed from water overnight. On re-immersion, nauplius larvae were released into the water column and were collected by attraction to a light source. Nauplii (~10,000) were transferred into 10 L buckets containing aerated ASW at a salinity of 25 ppt. Nauplii of *B. improvisus* were initially fed a 50:50 mixture of *T. suecica* and *Thalassiosira pseudonana* with the proportion of *T. pseudonana* reduced to zero by the third day of culture. The nauplii took between 4-5 days to metamorphose into cyprids, at which point they were collected by filtration and stored in the dark at 6 °C for 3 days.

Cyprid settlement assays

Twenty cypris larvae were added to each coated experimental tube using a glass pipette. Due to the strong tendency of cyprids of *B. improvisus* to become trapped at the air-water interface, the assay vials were positioned,

once filled, at an angle of approximately 45° from vertical. While this did not reduce the tendency of the larvae to 'float', it presented a more equal opportunity for larvae to encounter the walls of the vials and attach, regardless of surface hydrophobicity. In the vertical orientation, the differences in surface contact angle would have significantly biased access to the surface towards hydrophobic materials. The vials were placed in a dark incubator at 28 °C for 24 h. After 24 h, the cyprids were counted and response expressed as the proportion of larvae having permanently attached.

Design of the tracking system

The physical design of the tracking system was as described in Alsaab et al. (2017), but with the addition of a second camera. Briefly, two Basler scS1300-32gm monochrome, high-definition cameras were connected to a computer using Gigabit Ethernet and controlled using a simultaneous trigger. Each camera was fixed above an experimental aquarium, 10 L in volume, allowing for simultaneous experimentation in two conditions. Cyprids were contained within hollow 70 mm x 12 mm quartz glass tubes with 1mm wall thickness (Cambridge Glass Blowing) for the duration of the experiments. The inner surface of each tube was coated with a xerogel. The tube was orientated vertically and the cyprids were contained within it by closing the open ends of the tube with glass microscope slides, which were also xerogel-coated. A camera was fixed above the tube with a bespoke infrared LED light source beneath, allowing for visualization of cyprids as silhouettes against a light background. The tube, with cyprids inside, was placed inside a 10-L aquarium filled with artificial seawater. The large volume acted as an efficient temperature buffer to maintain experiments at a constant 23 ^{o}C. Once the cyprid-containing tube was within the filled aquarium, a thin sheet of transparent polystyrene was floated on the surface of the water to stabilize the image. When recording commenced, live video was retrieved by custom frame-grabbing software to a solid-state 1TB hard disk, housed within the high-performance desktop PC (Intel Xeon E5-2660v3 – 20x CPU @ 2.60 GHz, 32 GB RAM) running Ubuntu Linux v12.0.4. The footage was stored as single frames at 33 s^{-1} and all recordings took place in darkness using infrared illumination.

Cyprid tracking experiments

The algorithms for identification of cyprid exploratory behaviours, as well as the acquisition, filtering and processing pipelines, have all been the subject of previous publications (Alsaab et al. 2017; Aldred et al. 2018) and will not be covered in detail here. Briefly, recordings of cyprids in experimental tubes were fed into bespoke tracking software, which recorded the body movement of all cyprids in the field of view for the duration of the recording. The coordinate data were then analysed by a classification system that, once briefly calibrated, identified movements associated with inspection and walking behaviours with considerable accuracy. Following a 'light-touch' filtering step, in which large numbers of very minor body movements were removed as noise, the classification data for walking and cumulative inspection-related body movements were simply plotted against time or statistically compared between treatments.

Two experimental designs were implemented in this study, as described previously (Aldred et al. 2017):

Short-term experiments – The purpose of the short-term assay was to provide a high throughput experimental design with greater replication and statistical power than the long-term assays. Repeated 1-h recordings of approx. 10 cyprids per replicate were conducted, during which total behaviour was quantified per trial, rather than per cyprid. Cyprids were 3 days old and from 4 independent larval cultures for each experimental condition. Therefore, the unit of replication, in this case, was the culture not the cyprid.

Experiments were conducted at 23 ^{o}C using cyprids raised gradually to room temperature and allowed 5 minutes to acclimate prior to recording. Inspection or walking data recorded in the 1-h period were simply taken as a total for each recording/replicate and divided by the number of cyprids in the tube, providing a measure of average inspection events, movements and walking events per cyprid, per hour. These values were then averaged for the 4x replicate cultures to produce means.

Long-term experiments – In contrast, long-term experiments were designed to provide the maximum information and behavioural detail relating to a small number of cyprids on each surface. A long-term experiment involved addition of 5 or 6 cyprids, 3 days old, to an experimental tube. Recordings were then made as in the short-term experiments, but for up to 18 h. Any cyprids that permanently attached during this period were then tracked through their final exploratory period. This was defined as the last period of exploratory behaviour before attachment that was not interrupted by returns to the water column. The only exceptions to this were when many inspection events were linked by very short returns to the water column (for example in Figure 5 iv). Two repeat experiments were performed for each surface evaluated (two different cyprid batches) and, for each experiment, two cyprids were tracked all the way to settlement on each surface. Therefore, n = 4 for all long-term experiments.

Statistics

Due to the similarly-shaped, but non-normal, distributions of data for formulations that attracted low settlement, statistical analysis was performed using a Mann-Whitney test in Minitab version 17 at an α level of 0.05. All error values cited in the text and figures are standard errors of the mean.

Results

Cyprid settlement on a range of PH- and TFP-based xerogels

Initial experiments investigated cyprid settlement on a range of phenyltriethoxysilane (PH)- and 3,3,3-trifluoropropyltrimethoxysilane (TFP)-based xerogels, with varying proportions, in order to identify correlations between settlement and the total surface free energy of the xerogels, as well as the dispersive and polar components of free energy.

[FIGURE 1 HERE]

The internal standard materials of glass and a 50:50 C8:TEOS xerogel received settlement at the levels anticipated from previous assays (Figure 1). Settlement on glass was negligible over the course of a 24-h assay. On the other hand, settlement on the 50:50 C8:TEOS positive standard reached 50% over the same period.

[FIGURE 2 HERE]

The xerogels with low ratios of PH:TEOS and TFP:TEOS (2% and 5%) received settlement of between 10 and 20%, with no difference in settlement between PH and TFP xerogels with the same proportion of silane. At 10% PH/TFP, there was a slight increase in settlement for both formulations, relative to 2 and 5%. From 20% upwards, however, the difference in settlement between PH and TFP became highly significant (Table 2) with more settlement, in all cases, on the TFP formulations at 20, 35 and 50%. The pattern of settlement in response to increasing % of PH and TFP bore no clear relationship to the trend in dispersive surface energy, or the polar component of surface energy (Figure 2 i-iii). In particular, there was no indication from these data as to why such large differences in settlement should occur between PH and TFP-based xerogels at 20%, 35% and 50% formulations, but not for the lower ratios. As the proportion of the experimental silane increased in TFP-based xerogels, making them more hydrophobic (Figure 2 i), settlement increased as expected for *B. improvisus* (Figure 1). On the PH-based xerogels, however, settlement decreased on the more hydrophobic surfaces with larger proportions of PH. Despite differences in dispersive free energy (Figure 2 ii) and polar free energy (Figure 2 iii) between PH and TFP formulations, these differences remained relatively constant across the range of xerogel ratios (Figure 2 i-iii), while differences in settlement varied between 0 at the low ratios to >70% at the higher ratios (Figure 1). If individual components of surface free energy do influence settlement of *B. improvisus*, therefore, they must interact with other factors to do so.

[TABLE 2 HERE]

Short-term behavioural analysis on 50:50 PH and TFP xerogels

The short-term, fully automated, behavioural analysis method (Aldred et al. 2018) was applied to recordings of cyprids that were contained within xerogel-coated tubes. Figure 3i presents the settlement data for *B. improvisus*, replotted from Figure 1, on only the 50:50 PH:TEOS and 50:50 TFP:TEOS xerogels, as well as five measures of walking and inspection behaviour derived from the behavioural analysis (Figure 3ii-vi). While settlement on these two surfaces was the inverse of the dispersive component of free energy, this was not the case for all xerogels (see Figure 2 and discussion above). All behavioural measures calculated from the tracking assay (Figure 3ii-iv) reflected the significant difference in settlement ($F = 19.63$ $P = <0.001$; Figure 3i) between the formulations, with a significant reduction in the number of inspection-related movements (Figure 3ii; $F = 15.17$ $P = 0.011$) and all elements of walking behaviour; namely the walking rate (Figure 3iii; $F = 63.37$ $P = <0.001$), the duration of walking (Figure 3iv; $F = 23.07$ $P = 0.003$), the number of walking-related interactions with the surface (Figure 3v; $F = 120.55$ $P = <0.001$) and the length of those walking periods (Figure 3vi; $F = 15.53$ $P = 0.008$).

It is noteworthy that the average number of nodes, or number of inspection-like interactions, per cyprid was similar for both surfaces (16.2 h^{-1} ± 3.4 for PH and 18.5 h^{-1} ± 3.5 for TFP). Therefore, the doubling of 'movements

per node' on TFP compared to PH (Figure 3ii) was a result of each inspection event being sustained for longer and containing more movements. It can thus be concluded that while the surface encounter rate of cyprids was similar for both xerogels, they detached quickly from the PH and inspected for longer on the TFP. The difference in total walking period (Figure 3iv) was stark, with practically no walking recorded from cyprids exposed to a PH xerogel, compared to an average of over 4 minutes of walking cyprid^{-1} h^{-1} on TFP.

[FIGURE 3 HERE]

The short-term assay indicated that cyprids probed more extensively on the TFP surface, during both walking and inspection behaviour. However, no cyprids settled over the course of this 1-h assay and the identities of the cyprids tracked were not maintained (Aldred et al. 2018). Thus, the correlation of behavioural measures to settlement remained indirect.

Long-term behavioural analysis on 50:50 PH and TFP xerogels

A second experiment employed a long-term assay where a small number of cyprids were tracked all the way through their planktonic phase and through surface exploration to settlement, maintaining their identities throughout. While this approach is more time-consuming, it provided detailed insight into cyprid behaviour prior to attachment.

Figures 4 and 5 present long-term tracks of four individual cyprids on 50:50 PH/TEOS and 50:50 TFP/TEOS xerogels. The absence of walking behaviour on the PH surface was immediately striking and supported the results of the short-term assay. Figures 4 and 5 show movement data and track trajectories for cyprids *only* while engaged in surface exploration (i.e. no swimming or resting). The only exceptions to this were when many inspection events were linked by very short returns to the water column (typically < 2s; for example in the bottom panel of Figure 5iv). The trajectories are colour coded according to the dominant behaviour (either walking (blue) or inspection (red)) in the period of time illustrated for each letter.

It was apparent from the plots in Figure 5 that wide searching (long stretches of linear walking, uninterrupted by inspection pauses, or returns to the water column) was entirely absent on the PH xerogel, and that close searching (a mixture of wide searching and inspection), if it occurred on that surface, was inseparable from inspection (surface probing in one location, while attached by one or both antennules). On the other hand, the tracks on the TFP xerogel (Figure 4) contained all of the classic behaviours. While the mean time spent walking on 50:50 PH/TEOS was only 1 minute cyprid^{-1} (± 1) there was an average of 69 (± 36) minutes of walking cyprid^{-1} on the 50:50 TFP/TEOS xerogel. In contrast, the number of extended periods of inspection cyprid^{-1} was similar on the two materials (4 for PH, ± 0 and 3 for TFP ± 1). Interestingly, however, the small proportion of cyprids that did settle on the 50:50 PH/TEOS commenced settlement at a similar time to those that settled on the much preferred 50:50 TFP/TEOS (590 minutes ± 24, versus 528 ± 59 respectively).

[FIGURE 4 HERE]

[FIGURE 5 HERE]

Discussion

The principle objective of this study was to demonstrate the utility of the tracking system for quantitative evaluation of antifouling coatings. The series of coatings chosen also provided insight into the importance of the dispersive component of free energy during cyprid settlement behaviour. Recent studies indicated that the dispersive component of surface energy was likely to be an important factor in surface selection by cyprids. Based upon the present results, however, it would seem that the influence of dispersive free energy is not paramount in the significant differences between higher-ratio PH- and TFP-based xerogels with regard to cyprid settlement. Indeed, it remains unknown why settlement should differ so starkly on the pairs of xerogels that would be considered similar based upon the results of standard surface evaluation methods for determining roughness, charge and free energy. A possibility not addressed in this study is the potential for coating porosity to effect the measurement of surface energy, as well as the possible influence of xerogel porosity towards cyprid surface selection. The porosity of xerogels can be affected by the sol preparation as well as the drying conditions (Elferink et al. 1996; Meixner et al. 1998), however xerogels that are cast as thin films and dried at ambient temperatures, as was done here, generally have reduced pore size and volume (Brinker et al. 1994). While these xerogels are relatively smooth (nanometer scale roughness), therefore, the unknown pore size could affect the surface area of the coating and, therefore, the measured surface energy and dispersive component. While the results of this study do not support a correlation between the dispersive component of surface energy and cyprid settlement behaviour, therefore, this finding should be interpreted within the context of the specific coatings used.

While the predilection of *B. improvisus* for hydrophobic surfaces (low total free energy) was broadly apparent on the series of TFP xerogels (Figure 1) this did not hold for the PH series where higher PH ratios clearly had a repellent effect on settlement. One explanation may be related to intermolecular forces of the phenyl ring on this particular silane, and the cations present in seawater. A cation-pi interaction between the two may cause adsorbance of cations to the surface of the PH/TEOS coatings. This would produce a 'glass-like' surface with negatively charged functionality, onto which the positively charged cations in seawater could themselves adsorb. In this scenario, as the ratio of PH increases, the surface would present a more negative charge with an adsorbed layer of cations. Cationic surfaces have been demonstrated in previous studies to be repellent to this barnacle species (Di Fino et al. 2014). Evidence in the literature supports the presence of negative charges at some interfaces between hydrocarbons, or fluorocarbons, and water (Marinova, et al 1995; Beattie et al 2004), which could plausibly include the xerogels tested here. Measurements of zeta potential for the 20:80 and 50:50 formulations have been reported previously (Gatley-Montross, et al. 2017). Those measurements indicated that both the TFP and PH surfaces possess a surface charge, but that the zeta potential was not strongly correlated with attachment. As the 2017 study investigated the surface properties influencing the attachment of multiple organisms simultaneously,

a future study investigating the influence of surface charge on *A. improvisus* attachment specifically would be beneficial.

It was nevertheless demonstrated quite clearly that settlement behaviour, in addition to settlement rate, could provide useful insight into the 'decision making' process of cyprids that are presented with favourable and less-favourable surfaces to explore. Cyprid behaviour was shown to differ markedly between the 50:50 TFP/TEOS and 50:50 PH/TEOS xerogels in both short- and long-term behavioural assays. Short-term analysis using a large number of cyprids demonstrated significant reductions in all walking-related statistics on the PH xerogel, suggesting little, if any, wide- or close-searching behaviour on that surface. This was borne out in long-term experiments that tracked a small number of cyprids through the complete settlement process. No wide- or close-searching behaviour of note was observed on the 50:50 PH/TEOS xerogel. More importantly, inspection behaviour was demonstrably less frequent on the 50:50 PH/TEOS xerogel, which supported previous observations from experiments using cyprids of varying age and exposure to a chemical settlement inducer; namely that there is consistently less inspection behaviour in conditions where cyprids are less likely to permanently attach. These results therefore support the use of inspection behaviour as a predictive measure of propensity to attach and metamorphose, not only in aqueous treatments, but also when cyprids are exposed to novel coating formulations. In the future, this approach could potentially be developed into a robust assay for discrimination between surfaces that are inseparable by standard settlement experiments, or on surfaces to which cyprids will not settle readily in the laboratory. Similarly, the technique may enable future assay development for barnacle species that will not settle readily in the laboratory, but which are nonetheless problematic foulers.

It was evident from the behavioural assays that cyprids were able to perceive differences in the surface characteristics of the xerogels instantly at the point of temporary adhesion, and respond accordingly. On The TFP-based xerogel, exploratory behaviour progressed through the 'classic' process described by Crisp (1976) of wide-searching, close-searching, inspection and, finally, irreversible attachment. On the 50:50 PH/TEOS, however, the cyprids most often detached without exploring. Occasionally a cyprid would attach on 50:50 PH/TEOS, but without engaging in the expected exploratory sequence. This observation, although seemingly incidental, had a number of interesting implications that require dedicated investigation. First, the exploratory process can clearly be 'short-cut', and this was also observed by Aldred et al. (2018) in experiments using cyprids that had been subject to cold storage for 5 days. It was presumed that so-called 'hit and stick' behaviour (i.e. eliminating wide and close-searching) would usually indicate a desirable surface, or a 'desperate' larva. However, it is clear from the present data that this response can be observed in young cyprids on surfaces that are not preferred for settlement by the majority in the population under investigation. Further, the average point in the experiment at which cyprids settled on the 50:50 PH/TEOS was only an hour after those settling on 50:50 TFP/TEOS. This is astonishing given the large difference in total settlement after 24 h, and suggests that the difference in the 24-h settlement results must be a consequence of continued settlement on the 50:50 TFP/TEOS xerogel and the cessation of settlement on the 50:50 PH/TEOS after the initial, rapid, settlement of a few 'pioneer' cyprids on both surface types. If it is true that there are a small proportion of cyprids per batch that are genetically predisposed

to promiscuous settlement, as has been suggested for other marine larvae (Toonen and Pawlik 2001), then it is perhaps these that are the central problem for antifouling technologies, as opposed to the larger number of 'choosy' cyprids that will settle only on surfaces that meet their innate selection criteria. In the context of the present data this remains conjecture, but a reasonable direction for future work.

Acknowledgements

We extend our gratitude to the Office of Naval Research (ONR) for funding support to develop our larval tracking system (N00014-13-1-0633 & N00014-16-1-2988 to ASC and N00014-13-1-0634 & N00014-16-1-3125 to ASC & NA, and to the European Commission FP7 SEAFRONT project (ASC). MRD would also like to thank the ONR for funding support (N0014-16-1-3065 & N0014-16-1-2143) and all of the authors are grateful to Dr Eric Holm of the Naval Surface Warfare Centre for his helpful comments on the experiments and draft manuscript.

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512
513 **Table legends**
514 **Table 1:** The quantities of each reagent used to prepare the xerogel sols - 200 proof ethanol, deionized water,
515 and 12 M HCl were used for coating production. The silane precursors were used as purchased.
516
517 **Table 2:** Statistical comparison (Mann-Whitney) of cyprid attachment to pairs of PH and TFP-based xerogels with
518 the same relative proportions. Probabilities (P) of <0.05 are considered to indicate significant differences between
519 the pair of treatments being compared.
520
521 **Figure legends**
522 **Figure 1:** Settlement of 3-day-old *Balanus improvisus* cypris larvae on a range of xerogels based on two different
523 silane functionalities, (phenyltriethoxysilane (PH) or 3,3,3-trifluoropropyltrimethoxysilane (TFP)), at a range of
524 ratios to TEOS. Asterisk (*) above a pair of bars indicates a significant difference at $\alpha = 0.05$ (Table 2).
525
526 **Figure 2: (i)** Total surface free energy of phenyltriethoxysilane (PH)- and 3,3,3-trifluoropropyltrimethoxysilane
527 (TFP)-based xerogels at the ratios used in bioassays. White bars = PH, black bars TFP. **(ii)** Dispersive and **(iii)** polar
528 free energy components on the same surfaces. The total surface energy, dispersive, and polar free energies are
529 from data collected after soaking the coatings for 24 h in artificial sea water.
530
531 **Figure 3:** Data collected from **(i)** a settlement experiment with 3-day-old larvae of the barnacle *Balanus improvisus*
532 and **(ii-vi)** short term tracking experiments. All differences were significant; **(i)** F = 19.63 P = <0.001 **(ii)** F = 15.17
533 P = 0.011 **(iii)** F = 63.37 P = <0.001 **(iv)** F = 23.07 P = 0.003 **(v)** F = 120.55 P = <0.001 **(vi)** F = 15.53 P = 0.008. Red
534 circles on '**i**' represent the dispersive free energy values for the two surface types. For **ii – vi** each bar is a mean
535 of 4 replicate experiments/cyprid cultures.
536
537 **Figure 4: (i-iv)** Four independent tracks of cyprid walking (blue) and inspection (red) behaviours prior to
538 permanent attachment on 50:50 TFP/TEOS xerogels. The circular boundary is the inner edge of the tube where

most activity will usually be concentrated. Plots of cumulative inspection movements and walking stretches versus time are presented alongside, with letters correlating between the trajectory and data plot for each of i-iv.

Figure 5: (i-iv) Four independent tracks of cyprid walking (blue) and inspection (red) behaviours prior to permanent attachment on 50:50 PH/TEOS xerogels. The circular boundary is the inner edge of the tube where most activity will usually be concentrated. Plots of cumulative inspection movements and walking stretches versus time are presented alongside, with letters correlating between the trajectory and data plot for each of i-iv.

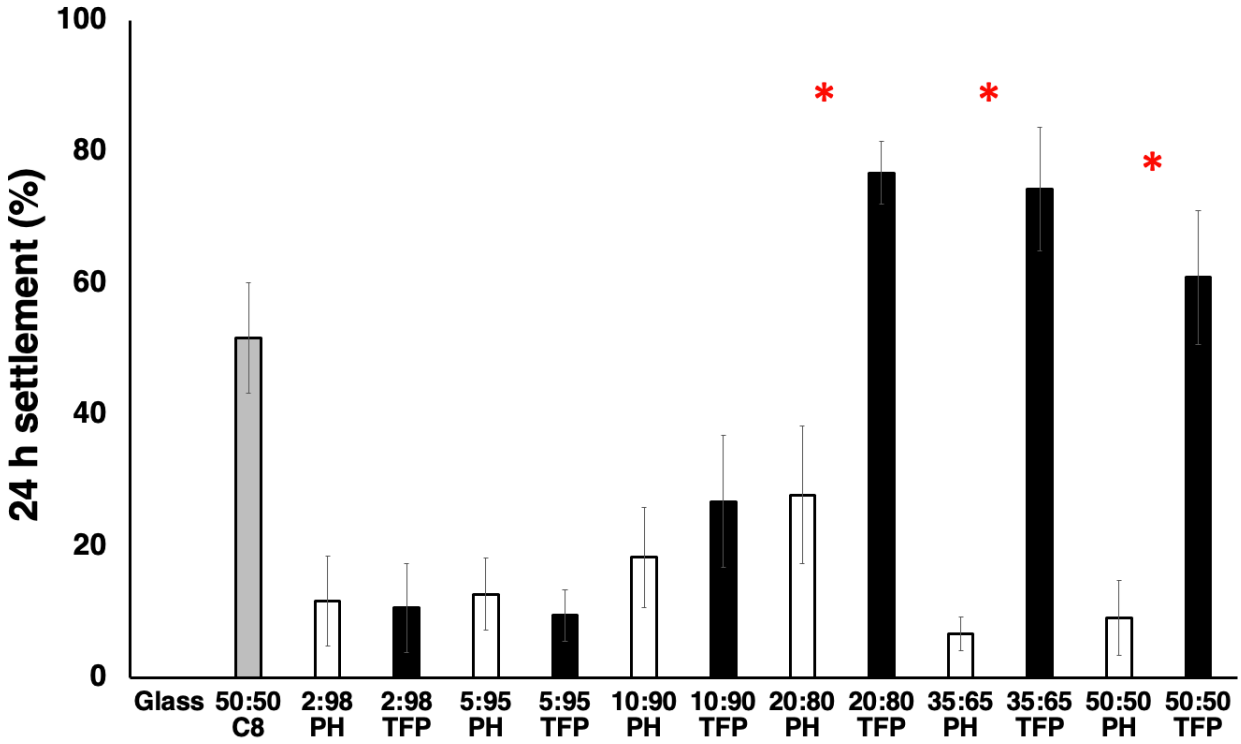

Figure 1

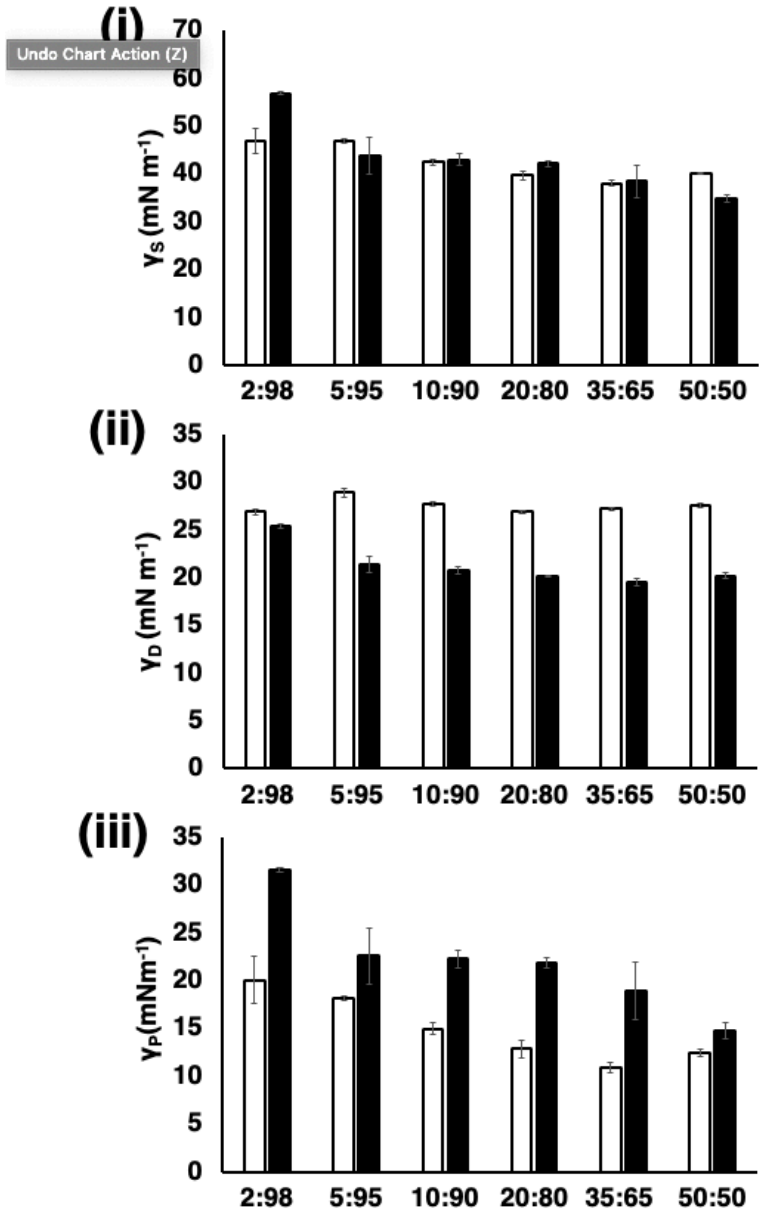
(i)
Undo Chart Action (Z)
70
60
50
40
30
20
10
0
γ_S (mN m^{-1})
2:98
5:95
10:90
20:80
35:65
50:50
(ii)
35
30
25
20
15
10
5
0
γ_D (mN m^{-1})
2:98
5:95
10:90
20:80
35:65
50:50
(iii)
35
30
25
20
15
10
5
0
γ_P(mNm^{-1})
2:98
5:95
10:90
20:80
35:65
50:50

Figure 2

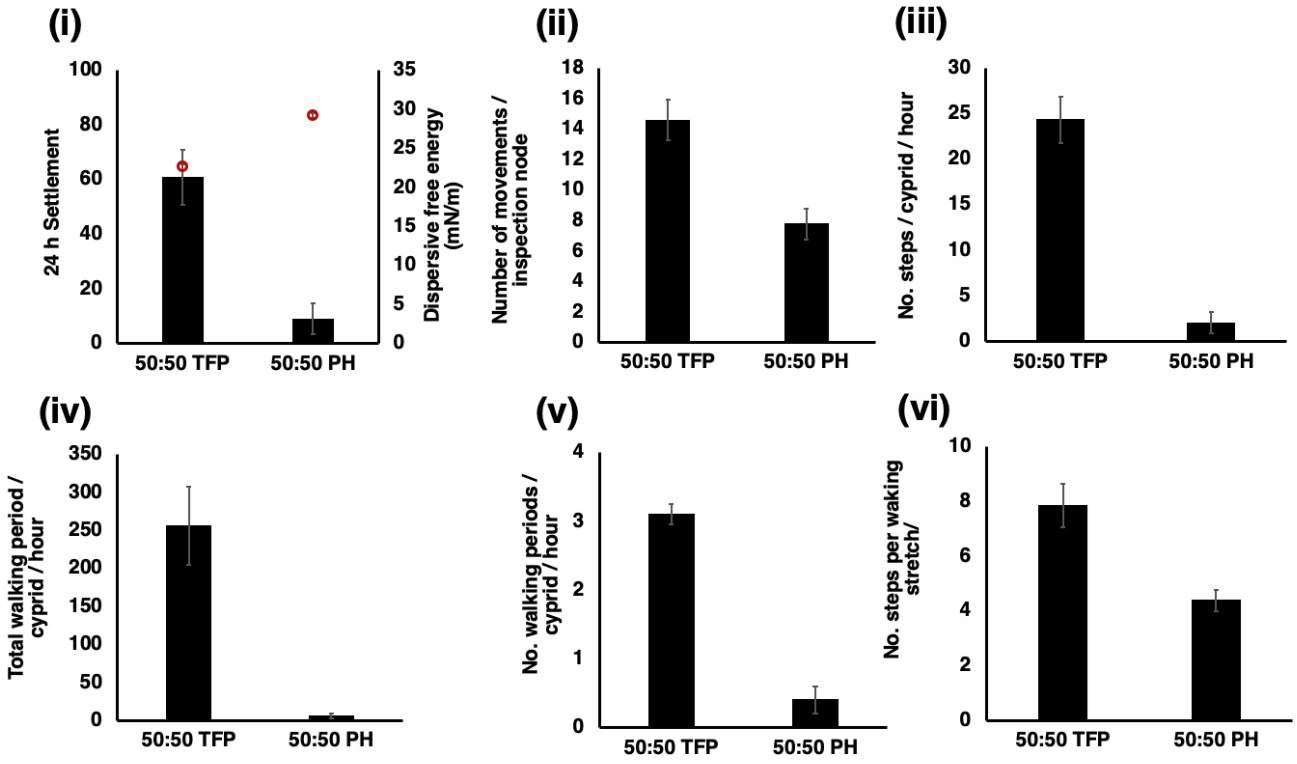
(i)
24 h Settlement
Dispersive free energy (mN/m)
50:50 TFP
50:50 PH
(ii)
Number of movements / inspection node
50:50 TFP
50:50 PH
(iii)
No. steps / cyprid / hour
50:50 TFP
50:50 PH
(iv)
Total walking period / cyprid / hour
50:50 TFP
50:50 PH
(v)
No. walking periods / cyprid / hour
50:50 TFP
50:50 PH
(vi)
No. steps per waking stretch/
50:50 TFP
50:50 PH

Figure 3

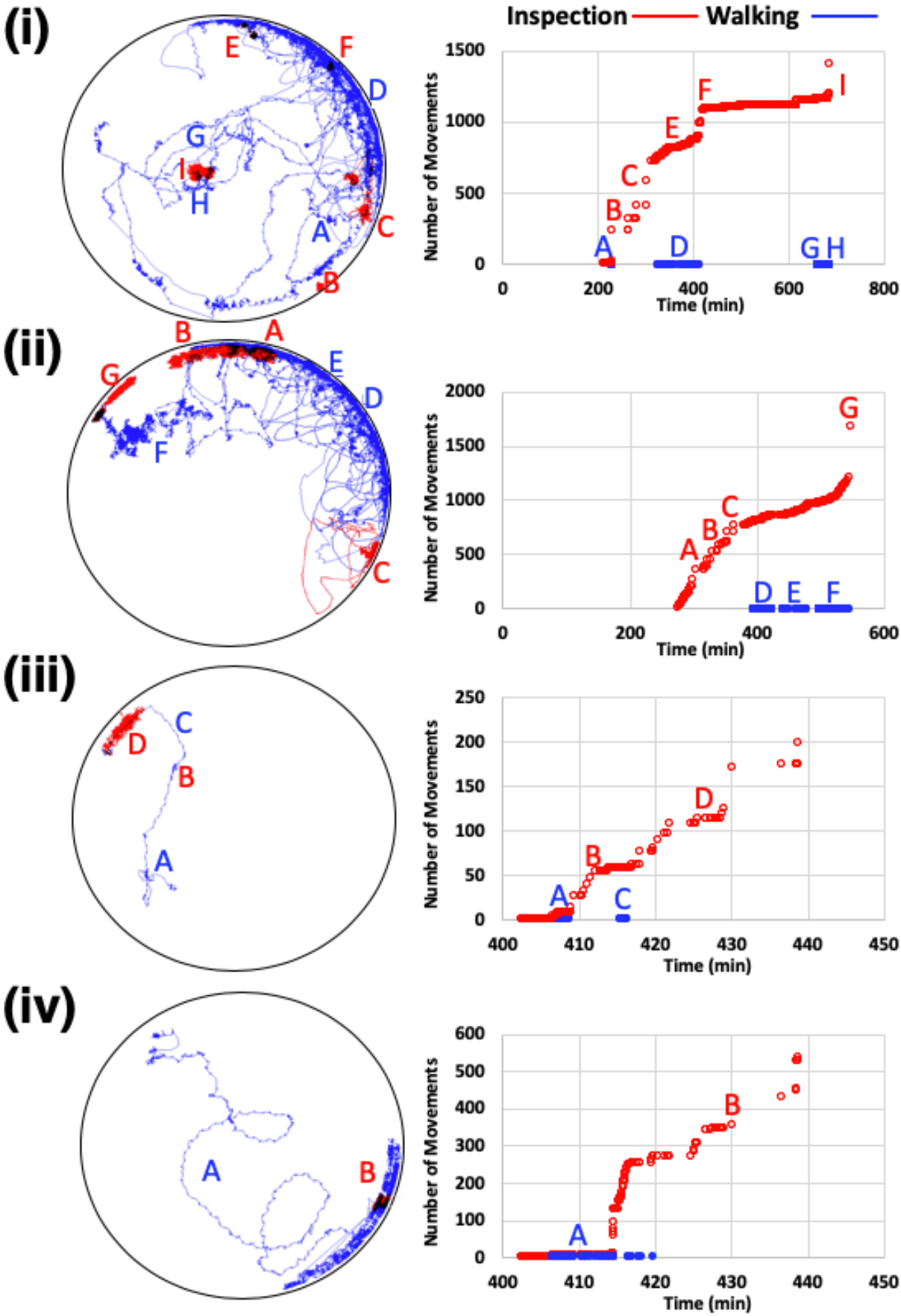
(i)
E
F
D
G
I
H
A
C
B
Inspection
Walking
Number of Movements
Time (min)
(ii)
B
A
G
E
D
F
C
(iii)
D
C
B
A
(iv)
A
B

Figure 4

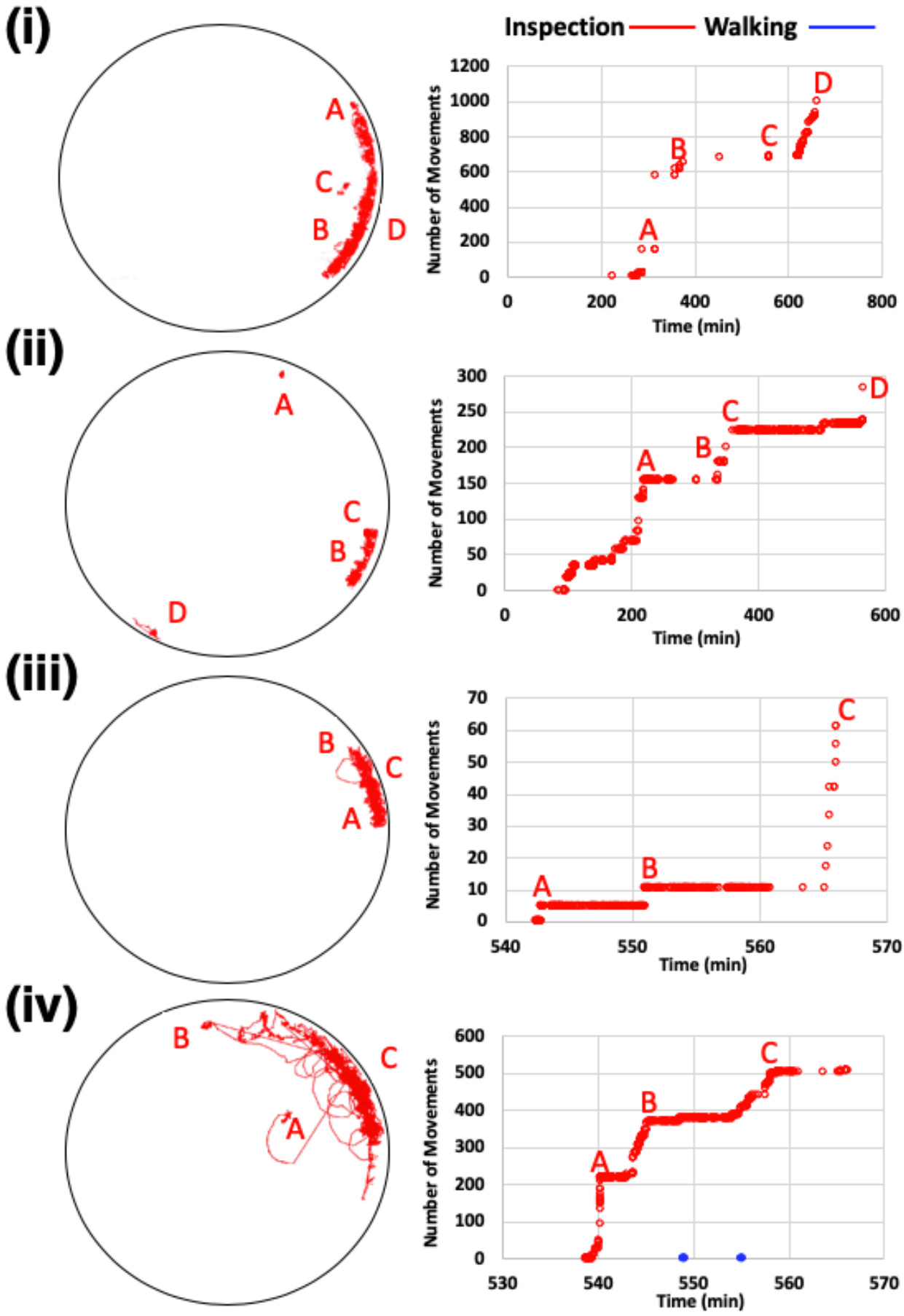
(i)
A
C
B
D
Inspection
Walking
Number of Movements
Time (min)
(ii)
A
C
B
D
(iii)
B
C
A
(iv)
B
C
A

Figure 5

Table 1

	Functionalized silane	TEOS	EtOH	H_2O	HCl
50:50 C8/TEOS	6.00 mmol	6.00 mmol	4.90 ml	1.13 ml	25.0 µl
50:50 PH/TEOS	6.00 mmol	6.00 mmol	4.90 ml	1.13 ml	25.0 µl
50:50 TFP/TEOS	6.00 mmol	6.00 mmol	4.90 ml	1.13 ml	25.0 µl
35:65 PH/TEOS	6.50 mmol	3.50 mmol	5.31 ml	1.23 ml	27.0 µl
35:65 TFP/TEOS	6.50 mmol	3.50 mmol	5.31 ml	1.23 ml	27.0 µl
20:80 PH/TEOS	9.60 mmol	2.40 mmol	7.84 ml	1.81 ml	40.0 µl
20:80 TFP/TEOS	9.60 mmol	2.40 mmol	7.84 ml	1.81 ml	40.0 µl
10:90 PH/TEOS	9.00 mmol	1.00 mmol	7.35 ml	1.70 ml	37.5 µl
10:90 TFP/TEOS	9.00 mmol	1.00 mmol	7.35 ml	1.70 ml	37.5 µl
5:95 PH/TEOS	9.50 mmol	0.50 mmol	7.75 ml	1.80 ml	40.0 µl
5:95 TFP/TEOS	9.50 mmol	0.50 mmol	7.75 ml	1.80 ml	40.0 µl
2:98 PH/TEOS	9.80 mmol	0.20 mmol	8.00 ml	1.85 ml	41.0 µl
2:98 TFP/TEOS	9.80 mmol	0.20 mmol	8.00 ml	1.85 ml	41.0 µl

Table 2

Compared formulations	**n**	**W**	**P**
2:98 TFP/TEOS vs 2:98 PH/TEOS	10	105.5	1.00
5:95 TFP/TEOS vs 5:95 PH/TEOS	10	109.0	0.79
10:90 TFP/TEOS vs 10:90 PH/TEOS	10	100.0	0.72
20:80 TFP/TEOS vs 20:80 PH/TEOS	10	65.0	0.0026
35:65 TFP/TEOS vs 35:65 PH/TEOS	10	62.0	0.0013
50:50 TFP/TEOS vs 50:50 PH/TEOS	10	61.0	0.0008