Final Performance Report

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Laboratory assays for hypothesis testing and evaluation of candidate easy release and nontoxic deterrent surfaces.

PI: Professor Anthony S Clare
Co-PI: Dr John A Finlay

School of Natural & Environmental Sciences
Newcastle University
Newcastle upon Tyne
NE1 7RU
United Kingdom

Technical objectives

The objectives were to work with developers of coatings to: i) test hypotheses with experimental non-toxic formulations that are designed to interfere with the adhesion of marine fouling organisms (FR coatings) and/or deter the colonizing stages from attaching (AF approach); and ii) provide efficacy testing to facilitate the down-selection of candidate coatings for field testing.

Technical approach

As marine biofouling comprises a highly diverse assemblage of organisms with multiple colonization and adhesion strategies, the objectives were addressed with both micro- and macrofouling taxa and species with known differences in physico-chemical requirements for surfaces, specifically, the diatom *Navicula incerta*, the macroalga *Ulva linza*, and barnacles *Balanus amphitrite* (=*Amphibalanus amphitirite*) and *B. improvisus*. Experiments comprised settlement and/or adhesion (ease of removal) assays. Many of the protocols are described in detail in Callow et al. (2014) and briefly in Table 1. The selection of assays was informed by the nature of the coating and its intended end use.

Table 1. Laboratory assays of the efficacy of antifouling and fouling-release surfaces.

	Test organisms	Assay	Reference(s)
Attachment	Barnacle cyprids	24- to 48-h static assay. Adaptable to coating characteristics and format (e.g. slide or Petri dish). Expressed as percentage settlement.	Gatley-Montross et al. (2017); Kardela et al. (2019)
	Ulva linza zoospores	Microscope slide assay in quadriPERM dishes. After 45 min, counts of settled spores done by image analysis (Leica LASX software) or manually with fluorescence microscope.	Sundaram et al. (2011); Barry et al. (2019)
	Navicula incerta	Microscope slide assay in quadriPERM dishes. Initial attachment under gravity determined after removing unattached cells. Counts of settled spores done by image analysis (Leica LASX software) or manually with fluorescence microscope.	Barry et al. (2019)
Leachate assay of toxicity	*Ulva* zoospores/sporelings	Biomass of sporelings quantified as chlorophyll a (as relative fluorescence units; RFU) after 5-day exposure.	Xiao et al. (2013)
	Barnacle nauplii	24-h assay. Scored as swimming and non-swimming larvae. LC_{50} determined by probit analysis.	Rittschof et al. (1992); Clare et al. (1999)
Growth assay	*Ulva*	Sporelings cultured for 7 days. Biomass determined (as RFU) using a Tecan fluorescence plate reader.	Xiao et al. (2013); Barry et al. (2019)
Ease of removal	Barnacle cyprids and juveniles	Permanently attached cyprids or juveniles cultured for 4-6 days. Ease of removal	Aldred et al. (2010)

		determined using calibrated flow cell (~ 80 Pa) or waterjet.	
	Ulva spores	Attached spores tested in a fully turbulent water channel at max. 53 Pa for 5 min or with a waterjet, typically at 83 kPa. High-throughput screening uses the spin-jet (Fig. 1 B). Removal of spores attached in 24-well plates tested at 9 and 67 kPa.	Finlay et al. (2002); Cassie et al. (2007); Galhenage et al. (2016)
	Ulva sporelings	Sporelings cultured from settled spores for 7 d. Sporeling biomass (RFU) determined before and after exposure to impact pressure of 55 kPa from waterjet.	Barry et al. (2019)
	Navicula	Attached cells exposed to shear stress of ~ 20-40 Pa for 5 min in fully turbulent water channel. Counts of cells not exposed and after exposure are made to determine percentage removal.	Ventura et al. (2017); Benschop et al. (2018); Barry et al. (2019); Kardela et al. (2019)
Critical removal stress	Adult barnacles	Barnacles cultured on coating. Critical removal stress (adhesion strength) determined when a minimum basal diameter of 3.6 mm is attained using a fully automated instrument.	Beigbeider et al. (2008); Conlan et al. (2008); Martinelli et al. (2012)

Much of the instrumentation that was used for the assays is bespoke (Figure 1).

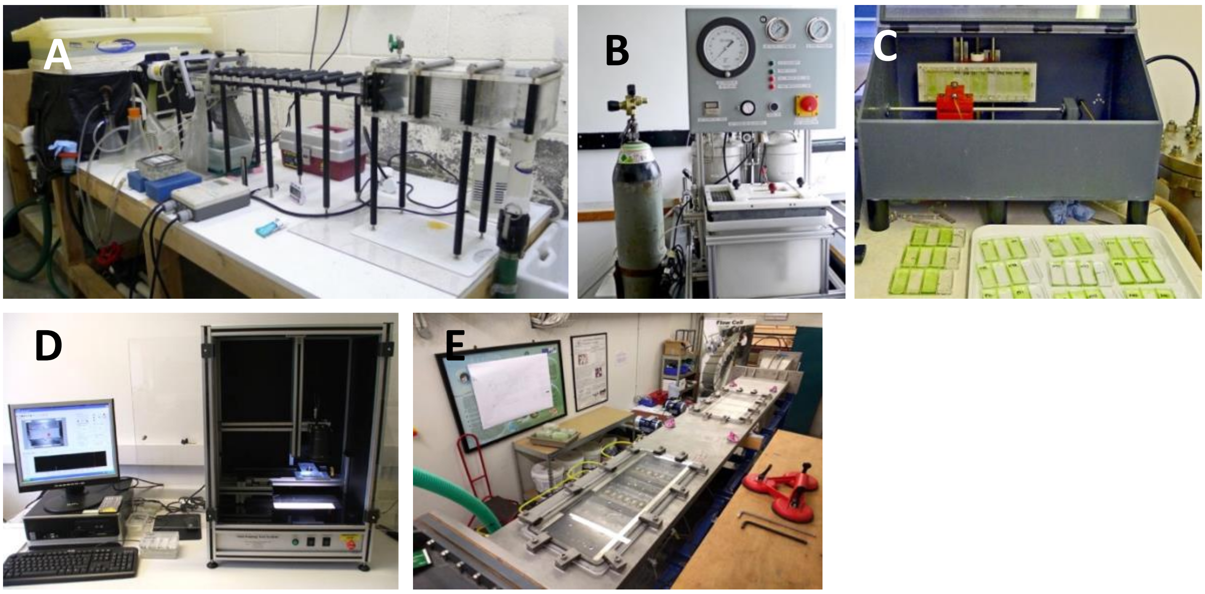


Figure 1. Bespoke equipment for antifouling/fouling release testing. A) Fully turbulent water channel used for algae testing; B) NDSU-designed spin jet for *Ulva* spore removal assays; C) water jet; D) fully automated instrument for strength of adhesion testing of adult barnacles; E) fully turbulent flow cell.

Accomplishments

During this award, 1946 individual samples were tested covering 285 different surface formulations (Table 2). Internal laboratory standards (glass, polystyrene and PDMS) are not included in the figures. Annual reports have been submitted on progress against the objectives. Nine articles have been published, supported wholly or in part by this award, with one in press (see publication list at end of report).

Table 2. Samples tested in the barnacle, diatom and *Ulva* assays.

Supplier	**Barnacles**		**Diatoms**		***Ulva***	
	Formulations	Slides	Formulations	Slides	Formulations	Slides
Brennan/Tonks			5	30	15	35
Jiang			8	39		
Ober	23	204	13	78	23	138
Rosenhahn					14	84
Segalman	11	102	4	24	4	40
Silberstein			12	76	17	73
Webster	27	288			46	184
NatureCoat/Imperion Coatings	8	96	16	96	12	104
Adaptive Surfaces	6	72			19	164
Schlenoff			1	10	1	9
Total	**75**	**762**	**59**	**353**	**151**	**831**

From the surfaces tested, three examples have been selected below to illustrate the approaches examined to control fouling in this program:

Nitroxide-containing amphiphilic random terpolymers

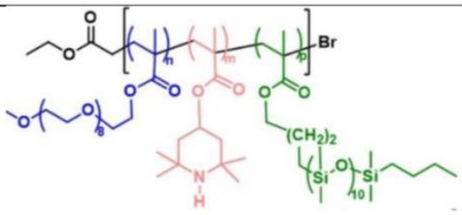
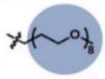
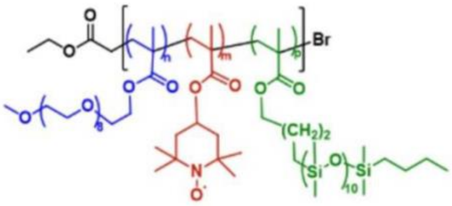
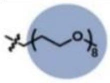
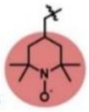
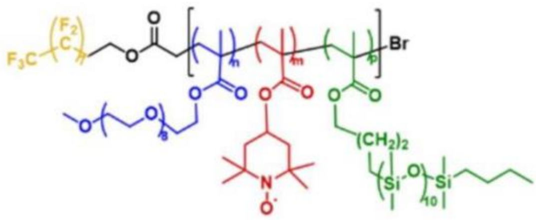
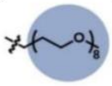
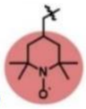
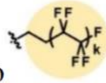
The first example is work carried out in conjunction with the Ober laboratory (Cornell) on nitroxide-containing amphiphilic random terpolymers and their use as antifouling and fouling-release coatings.

The potential activity of nitroxides, such as the stable radical TEMPO (2,2,6,6-tetramethylpipiderine-1-oxyl), against fouling organisms has previously been established (Leonardi 2021). The action of TEMPO is believed to be through antioxidant activity and its ability to quench oxidative processes that are involved in adhesive curing.

In this study, methacrylate random terpolymers consisting of amphiphilic polyethylene glycol/polydimethyl siloxane (PEG/PDMS) pendant groups and TEMPO moieties were synthesized via atom transfer radical polymerization (ATRP). These coatings combined amphiphilic characteristics, which are well known to reduce the settlement and adhesion strength of fouling organisms, with TEMPO groups that have the potential to interfere with the curing of adhesives.

Three sets of coatings were produced, each containing a mixture of either TEMPO or an inactive amine form of TEMPO, with PEG and/or fluoroalkyl groups. It has previously been demnstrated that mixing PEG with TEMPO on a polystyrene-PDMS (PS-PDMS) backbone promotes the expression of TEMPO at the surface of the coating, rather than it remaining buried within the bulk of the coating and biologically unavailable. Under certain conditions, fluoroalkyl chains reduce barnacle settlement and these were supplied in these studies in an end-capping arrangement. The functional groups are shown in Table 3 alongside a color-coding system that denotes the relative proportion of each group in the coating.

Table 3. Functional groups with color coding.

Key	Structure	Colour Coding as used in the plots
Set 1 contained the inert amine precursor to TEMPO		Ether  Amine 
Set 2 contained TEMPO		Ether  TEMPO 
Set 3 contained TEMPO and was fluoro end-capped		Ether  TEMPO  Fluoro 

The coatings were assayed for antifouling and fouling-release activity using the green alga *Ulva linza* and the barnacle *Balanus improvisus*. Two experiments were run with *Ulva linza*, the first with Sets 1 and 2 and the second with Set 3, but with a glass reference surface in each experiment.

Spore settlement densities were lower on all the TEMPO- containing coatings of Set 2 than on the amine-containing samples in Set 1 (Figure 2). The outer layer of settling spores is known to carry a negative charge and consequently they tend to attach in large numbers to positively charged surfaces, such as those containing amine groups. Settlement was also lower on the TEMPO samples than on sample 4 that contained no amine.

Settlement densities on the fluoro end-capped samples of Set 3 (Figure 3) were all lower than those on the amine-containing Set 1, although this may not have been entirely due to the presence of TEMPO as settlement was also reduced on coating 11 in which TEMPO was absent. The settlement densities on Set 2 and 3 were all broadly similar to that on the PDMS reference coating.

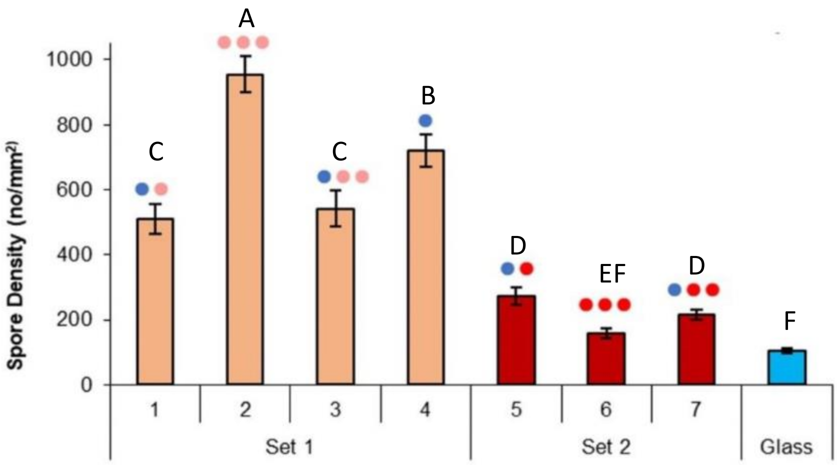


Figure 2. The density of attached spores on stable radical coatings after 45 minutes of settlement for Set 1 containing the amine form of TEMPO and Set 2 containing TEMPO itself. Each point is the mean from 90 counts on 3 replicate slides. Bars show 95% confidence limits. Bars that do not share the same letter are significantly different (ANOVA and Tukey test). Refer to Table 1 for sample IDs and color labels.

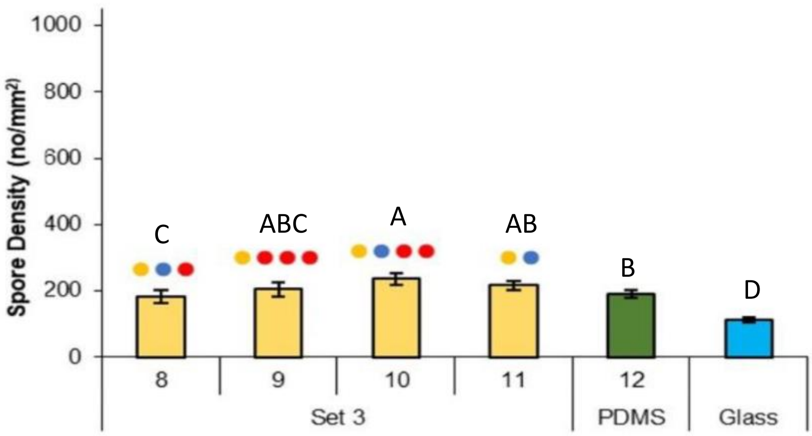


Figure 3. The density of attached spores on stable radical coatings after 45 minutes of settlement for the fluoro end-capped Set 3. Each point is the mean from 90 counts on 3 replicate slides. Bars show 95% confidence limits. Bars that do not share the same letter are significantly different (ANOVA and Tukey test). Refer to Table 1 for sample IDs and color labels.

Removal experiments were carried out using a custom-built turbulent flow water channel providing a shear stress of 52Pa (see Figure 1). Removal from the TEMPO-containing samples (Sets 2 and 3) was similar or lower to that from the coatings containing the amine precursor (Set 1) (Figures 4 and 5), which implies that TEMPO was not influencing attachment strength in this format. However, within Set 2, there was a much higher removal from samples 6 and 7, which contained medium to high levels of TEMPO, than from sample 5 with the lowest level of TEMPO. Indeed, removal from sample 5 was negligible. This suggests that the inclusion of TEMPO increased removal within the series. In the fluoro end-capped samples (Set 3) removal was higher from all the coatings containing TEMPO than from the control (11) without TEMPO.

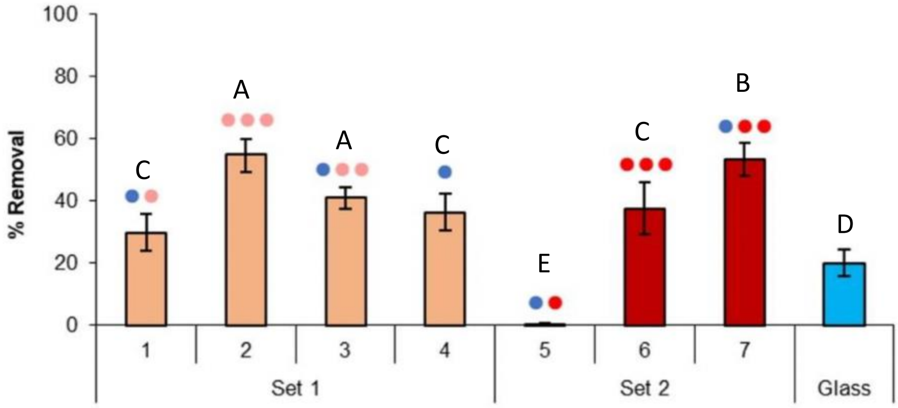


Figure 4. The removal of spores from the stable radical coatings due to exposure to a shear stress of 50 Pa, for Set 1 containing the amine form of TEMPO and Set 2 containing TEMPO itself. Bars show 95% confidence limits derived from arc-sine transformed data. Bars that do not share the same letter are significantly different (ANOVA and Tukey test). Refer to Table 1 for sample IDs and color labels.

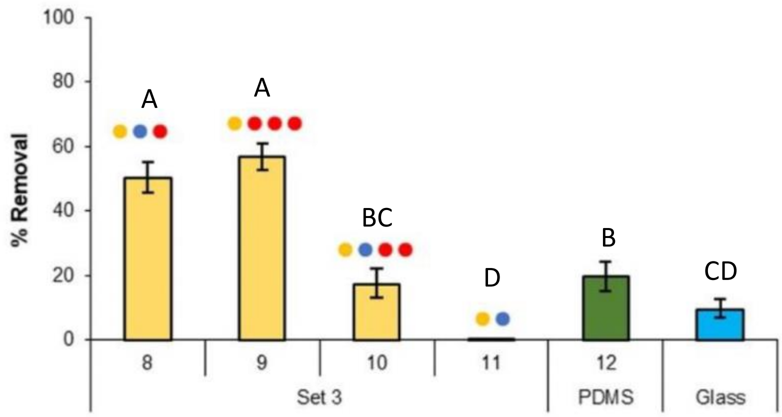


Figure 5. The removal of spores from the stable radical coatings due to exposure to a shear stress of 50 Pa, for the fluoro end-capped Set 3. Bars show 95% confidence limits derived from arc-sine transformed data. Bars that do not share the same letter are significantly different (ANOVA and Tukey test). Refer to Table 1 for sample IDs and color labels.

Settlement of 3-day-old barnacle cypris larvae was carried out over a 72-hour period. The percentage settlement of cyprids was generally higher on the amine-containing coatings of Set 1 than on the TEMPO-containing coatings of Set 2. The highest settlement was on the two coatings containing the highest amine content (samples 2 and 3). Settlement on all the TEMPO-containing coatings was lower than on coating 4 which contained no amine or TEMPO (Figure 6).

Settlement on the TEMPO-containing coatings of the fluoro end-capped Set 3 was lower than on the non-TEMPO containing control (11) (Figure 6). Overall, this suggests that the inclusion of TEMPO results in reduced settlement densities compared to unmodified controls.

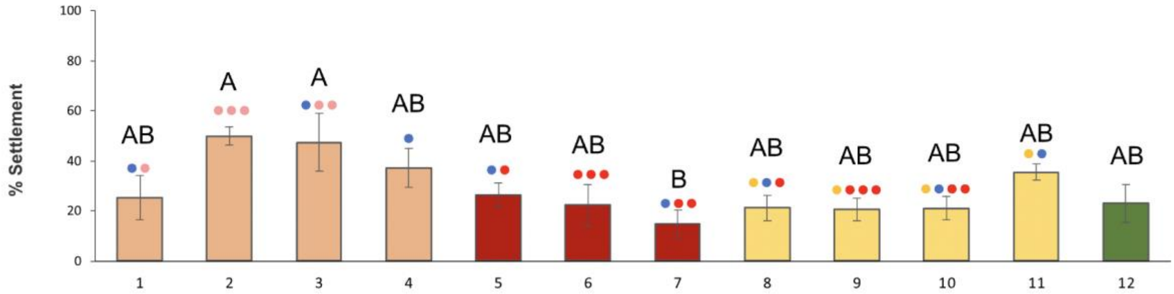


Figure 6. Percent settlement of 3-day-old *B. improvisus* cyprids on the test coatings after 3 days. Mean from 6 replicates with error bars showing 95% confidence limits derived from arc-sine transformed data. Bars that do not share the same letter are significantly different (ANOVA and Tukey test). Refer to Table 1 for sample IDs and color labels. 12 = PDMS.

Removal studies on juvenile barnacles were carried out using a water jet with an impact pressure of 65 kPa (Figure 1C). Removal was no higher from the TEMPO containing coatings than from coatings from which it was absent (Figure 7).

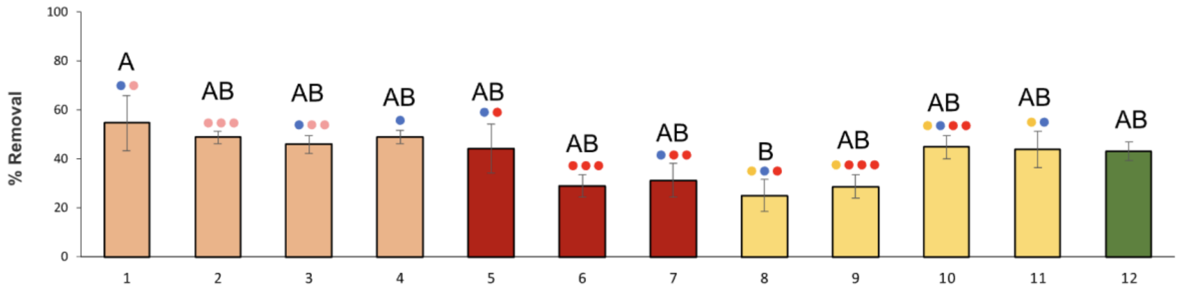


Figure 7. Percent removal of *B. improvisus* barnacles from test coatings. Mean from 6 replicate coatings with error bars showing 95% confidence limits derived from arc-sine transformed data. Bars that do not share the same letter are significantly different (ANOVA and Tukey test). Refer to Table 1 for sample IDs and color labels. 12 = PDMS.

The aim of this study was to produce coatings with amphiphilic surface chemistry that also contained nitroxide functionalities that inhibit oxidative curing of adhesives. To function effectively, coatings need activity against a range of organisms. These studies did not indicate that TEMPO in these formulations disrupted the adhesion strength of either the spores of *U. linza* or juvenile *B. improvisus*. However, there is evidence that it did affect their settlement and, in some cases reduced the numbers attaching. The reason for this is unclear, but it is possible that the antioxidant properties of TEMPO may interfere with surface sensing mechanisms of the settling organisms. Overall, the low level of barnacle settlement on coating 7 is promising. This correlates with other ongoing studies and therefore deserves further investigation.

This study has recently been published (Medhi et al. 2023).

Zwitterglass

Zwitterglass samples were supplied on glass slides by the Schlenoff laboratory (Florida State University). The coatings were transparent, hard and extremely thin. Assays were done with the diatom *Navicula incerta* and spores/sporelings of the green alga *Ulva linza*.

Each slide was divided into two regions: two thirds were covered by the zwitterglass coating and one third left with unmodified glass. Polydimethyl siloxane (PDMS)-coated slides (Silastic T2) and acid-washed glass were included as reference surfaces.

The initial attachment of diatoms on the zwitterglass surfaces was approximately 40% lower than on the unmodified glass region of the test slides and also lower than on the reference surfaces (Figure 8). The ability of the cells to form a strong attachment to the zwitterglass surface was measured in a flow channel (see Figure 1A) using a wall shear stress of 32Pa.

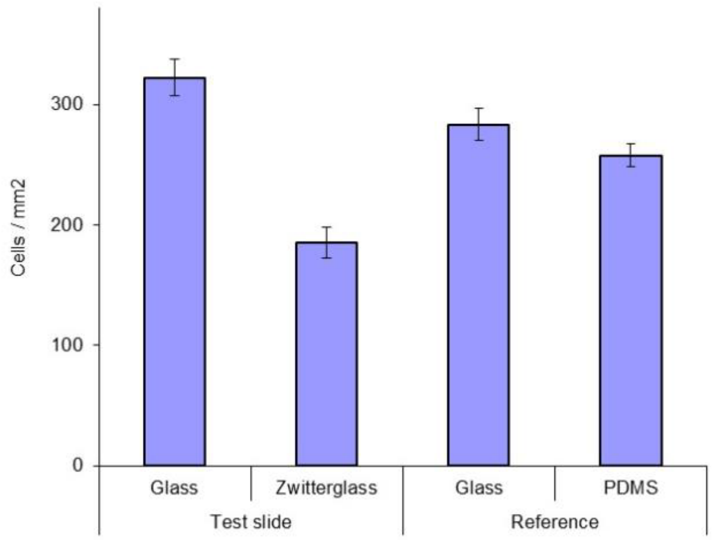


Figure 8. The density of attached diatoms on the test coating and reference surfaces after 2 hours followed by washing. Each point is the mean from 90 counts from 3 replicate slides. Bars show 95% confidence limits.

The percentage removal of diatoms from the zwitterglass was higher than from the uncoated section of the glass slides (Figure 9). The density of diatoms remaining on the coating was much lower than on the other surfaces (approximately 20% of that on the PDMS reference surface) (Figure 10).

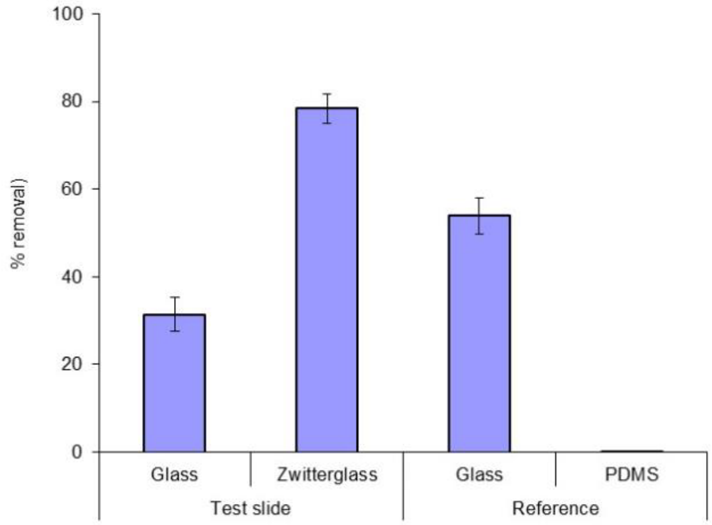


Figure 9. Percent removal of diatoms from the test coating and reference surfaces after exposure to a shear stress of 32Pa. Each point is the mean from 90 counts from 3 replicate slides. Bars show 95% confidence limits derived from arc-sine transformed data.

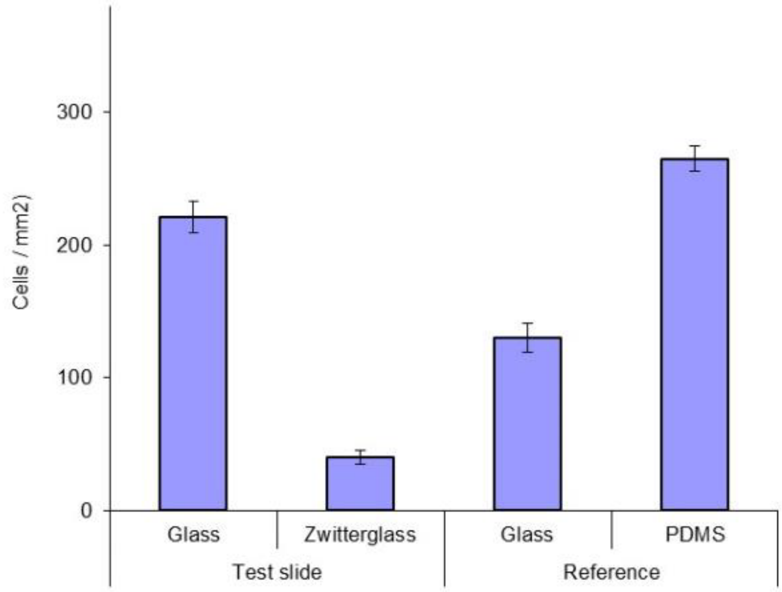


Figure 10. The density of attached diatoms remaining on the test coating and reference surfaces after exposure to a shear stress of 32Pa. Each point is the mean from 90 counts from 3 replicate slides. Bars show 95% confidence limits.

Although diatom cells settle on a surface as individual cells, they soon multiply to produce multicellular biofilms that are held together by extracellular polymeric substances (EPS). The interaction between these three-dimensional structures and hydrodynamic removal forces can shed light on the likely robustness of diatom slime films on the hulls of ships. To investigate this, diatom biofilms were cultured on the zwitterglass surfaces for 7 days. The development of the biofilm was broadly similar on all surfaces. This was expected, as there was no washing stage after settlement and therefore each sample had the same initial density of diatoms on its surface.

The percentage removal of the diatom biofilm from the zwitterglass coating at a shear stress of 26Pa was higher than from the uncoated section of the glass slides and the glass reference (Figure 11). Taken together, the results demonstrate that the zwitterglass is an effective antifouling and fouling-release coating against diatoms.

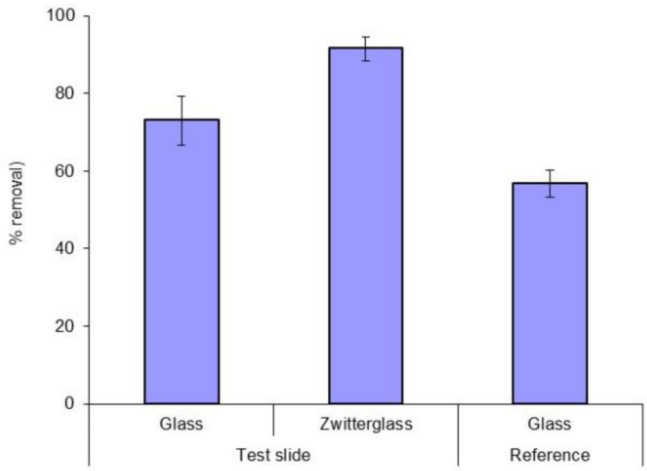


Figure 11. Percent removal of diatom biofilm from the test coating and reference surfaces due to a shear stress of 26Pa. Bars show 95% confidence limits derived from arc-sine transformed data.

The spores and sporelings (young plants) of *U. linza* are frequently found on the hulls of fouled ships. There preferences for surfaces and adhesion strengths are often the opposite to those of diatoms making them complimentary test organisms. In a 45-minute settlement assay, the spore densities on the zwitterglass were approximately 14% of that on the glass part of the test slide (Figure 12). Settlement was also lower than on the glass and PDMS reference surfaces.

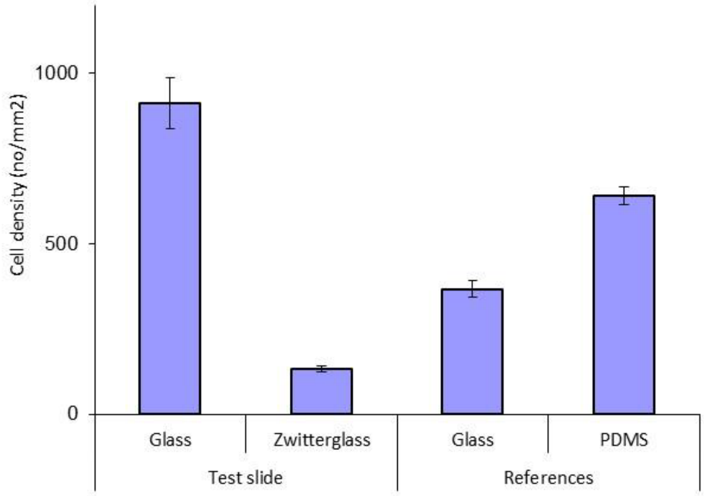


Figure 12. The density of attached spores on the test and reference surfaces after 45 minutes of settlement. Each point is the mean from 90 counts on 3 replicate slides. Bars show 95% confidence limits.

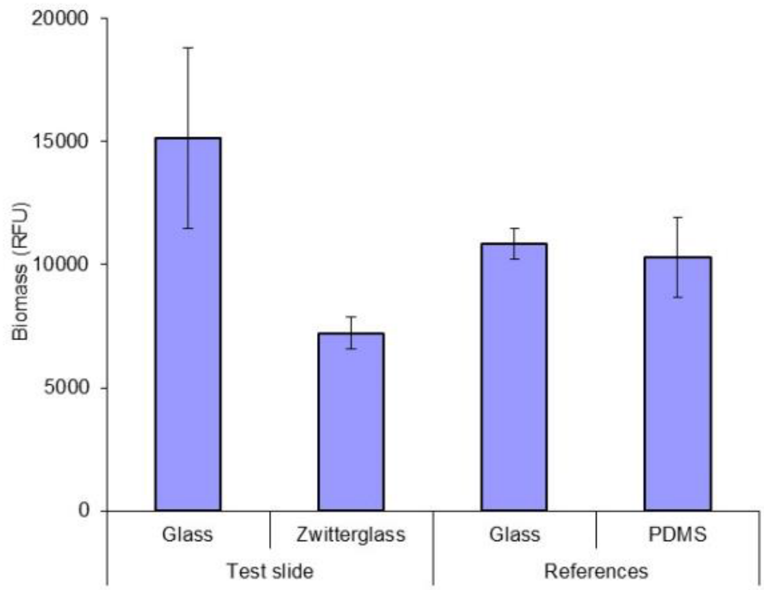


Figure 13. The biomass of sporelings on the test and reference surfaces after 8 days. Each point is the mean biomass from 5 replicate slides measured using a fluorescence plate reader (RFU; relative fluorescence unit). Bars show 95% confidence limits.

Culture of the spores produced sporelings which were approximately 100µm long at 7 days. Total biomass for the test slides broadly followed the pattern of spore settlement, with lower amounts on the zwitterionic coating than on the glass (Figure 13). The large error bar for the glass part of the test slide was caused by patchy growth. Biomass levels on the PDMS were also variable and hence the overall mean was similar to that on the glass.

Removal was only measured for the zwitterglass-coated section of the slide, as the area of the glass part was too small to be tested accurately. Sporelings were attached to the zwitterionic surface more strongly than to the PDMS reference coating (Figure 14).

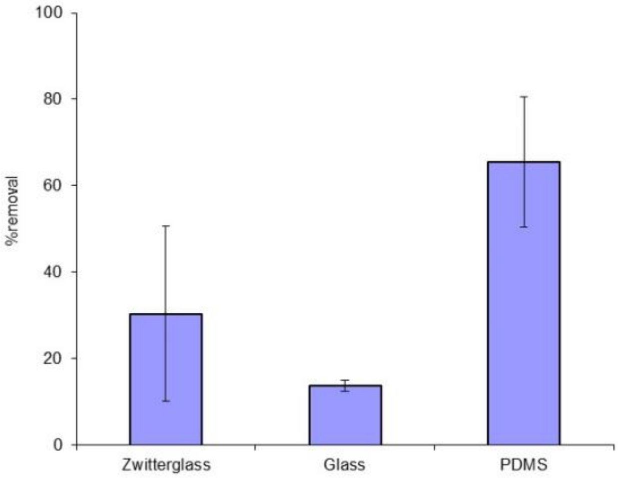


Figure 14. Percent removal of 8-day-old sporelings from the zwitterion coating and the two reference surfaces due to a water jet of impact pressure 110 kPa. Each point is the mean removal of biomass from 5 replicate slides measured using a fluorescence plate reader. Bars show 95% confidence limits derived from arc-sine transformed data.

Overall, the zwitterglass samples performed well against both species of algae. Single diatom cells of *Navicula incerta*, as well as biofilms, were relatively weakly attached to this surface compared to PDMS. Settlement densities of spores of *U. linza* were also lower on the zwitterglass than on the unmodified glass section of the test slides, though ease of removal was inferior PDMS. The results are sufficiently encouraging to evaluate different formulations of zwitterglass in the future.

Metal-ligand coordinated polydimethyl siloxane (PDMS) polymers

The coatings were supplied by the Silberstein laboratory at Cornell University and were composed of metal-ligand coordinated polydimethyl siloxane (PDMS) polymers. The metal ions were either copper or cobalt. The PDMS had a molecular weight of either 1000 or 3000 and was either multi-functionalised or end-fuctionalised with the metal ions (Figure 15). The metal ions were strongly coordinated with the PDMS matrices resulting in a coating of high mechanical strength with a non-leaching surface. There is interest in these coatings both in their natural state, as the presence of the metal ions within the PDMS makes the coatings more hydrophilic than regular PDMS and also under applied force.

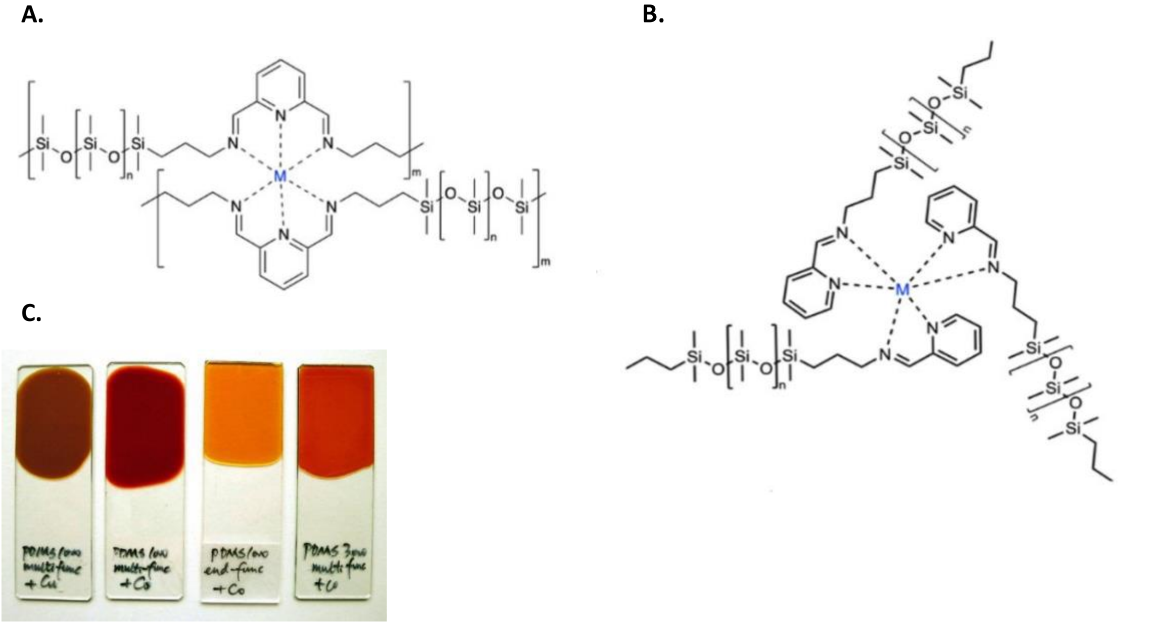


Figure 15. Coating chemistry and appearance: A. Multi-functionalized PDMS; B. End-functionalized PDMS; and C. coatings on glass slides.

The surfaces were evaluated for antifouling efficacy against diatoms, *Navicula incerta*. Bluesil PDMS-coated slides and acid-washed glass were included in the assays as reference surfaces. Initial attachment densities on the metal-ligand coordinated PDMS coatings were all broadly similar (Figure 16). The attachment density was slightly lower on the glass reference surface due to the initial washing procedure, which removed cells that had not adhered strongly to its hydrophilic surface.

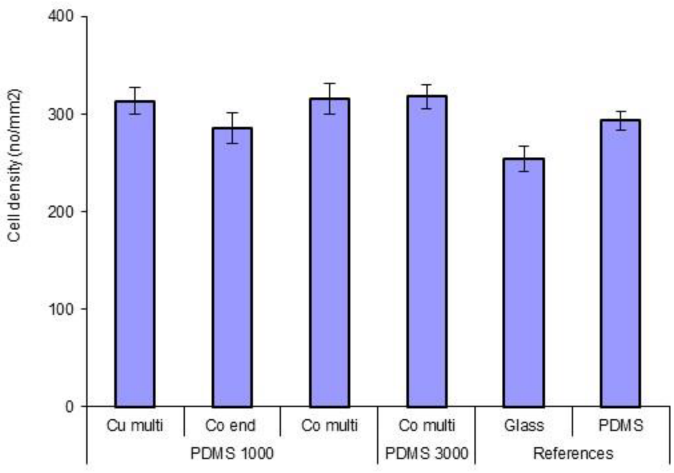


Figure 16. The density of attached diatoms on the metal-ligand polymer coatings after 2 hours followed by washing. Multi = multi-functionalised PDMS. End = end-functionalised PDMS. Molecular weights of PDMS were either 1000 or 3000. Reference PDMS is Bluesil. Each point is the mean from 90 counts from 3 replicate slides. Bars show 95% confidence limits.

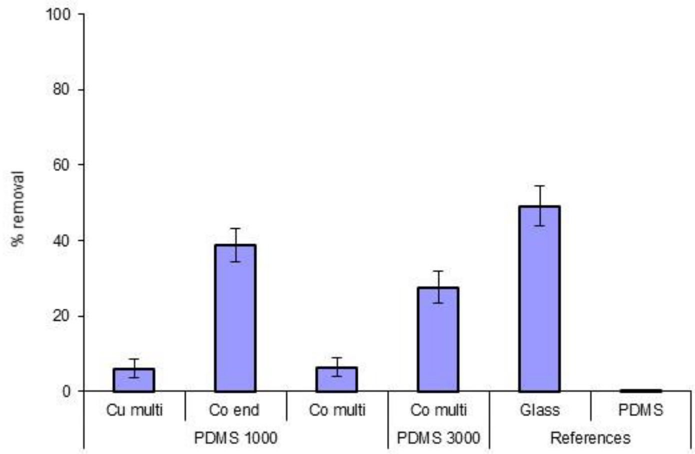


Figure 17. Percent removal of diatoms from the metal-ligand polymer coatings due to a shear stress of 38Pa. Multi = multi-functionalised PDMS. End = end-functionalised PDMS. Molecular weights of PDMS were either 1000 or 3000. Reference PDMS is Bluesil. Each point is the mean from 90 counts from 3 replicate slides. Bars show 95% confidence limits derived from arc-sine transformed data.

The release data showed clear differences in the fouling-release performance of the coatings (Figure 17). The greatest removal was from the 1000 MW PDMS end-functionalised cobalt-containing coating, and lower than from the hydrophilic glass reference surface. The multi-functionalised coating containing the higher MW (3000) PDMS had higher removal than the lower MW (1000) equivalent coating. Although there was an issue with dispersion and aggregation of the particles in the 3000 MW coating, which may have influenced removal, the result suggests that the MW of the PDMS could influence the performance of the coating. Removal from the 1000 MW PDMS multi-functionalised cobalt and copper coatings was substantially lower.

Overall, the data indicate that the different functionalities and molecular weight of PDMS influence the fouling-release properties of the coatings. Although a direct comparison with the same PDMS material as the metal-ligand cross-linked coatings were made from was not possible, the release from all the polymers was higher than from the Bluesil PDMS reference coating. Further studies are required to identify performance against other fouling organisms before the mechano-responsive properties are investigated.

References

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14. ABSTRACT
The objectives were to work with developers of coatings to: i) test hypotheses with experimental non-toxic formulations that are designed to interfere with the adhesion of marine fouling organisms (FR coatings) and/or deter the colonizing stages from attaching (AF approach); and ii) provide efficacy testing to facilitate the down-selection of candidate coatings for field testing. Over the course of the award, 1946 individual samples, covering 285 different coating formulations, were evaluated in the laboratory with a range of marine biofouling organisms and bespoke testing equipment. AF and FR approaches evaluated included amphiphilic surfaces, zwitterions, and surface lubricity.

15. SUBJECT TERMS
Marine biofouling; antifouling; fouling release; coating evaluations

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