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(Principal Investigator) Dr. Teo Lay-Ming, Serena
(Team members) Chin Sing LIM, Gayathiri d/o SIVANANTHAN, Steve SUMMERS

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7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
NATIONAL UNIVERSITY OF SINGAPORE
21 LOWER KENT RIDGE ROAD
SINGAPORE 119077
REPUBLIC OF SINGAPORE

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
Office of Naval Research
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14. ABSTRACT
This report outlines results from field immersion tests of novel antifouling materials in Singapore tropical sea environment. In addition, an evaluation of techniques to characterize biofilms on marine coatings is presented.

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Antifouling performance, field immersion, Singapore, biofilms

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Biofouling Prevention in Tropical Southeast Asian Seas

Final Report

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Principal Investigator	Serena Lay-Ming TEO (Dr.)
Research team members	Chin Sing LIM, Gayathiri d/o SIVANANTHAN
Organization	Tropical Marine Science Institute, National University of Singapore
Collaborator:	Stephen SUMMERS (Dr.), Singapore Centre of Environmental Life Science and Engineering (SCELSE), Nanyang Technological University
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Executive Summary

A total of 11 assays comprising 315 panels and 60 small coupons were examined under static shallow submergence for biological settlement in a tropical coastal marine environment during the present Reporting Period 1 Jan 2020 to 31 May 2023. Four new arrays were launched during this Reporting Period. Strong coating performers from seven arrays from earlier reporting period continued immersion during this study period. As of 31 May 2023, a total of 162 panels from five assays remained under immersion. Of these, 40 panels were removed in June 2023. A summary of the performance of the materials against biofouling is provided in this report.

The study also conducted an evaluation of biofilms on test panels. Different approaches were evaluated to compare biofilm volume and community composition on panels in a field test array. Technique for sampling of biofilms from panels were developed, to minimize interference to biofouling accumulation. It was found that assessment of biofilm biovolume using crystal violet staining of samples was not useful. Molecular techniques such as TRFLP fingerprinting gave a useful preliminary separation of microbiomes amongst coatings. The Oxford Nanopore Minion provided useful insights into the communities on coatings, but Illumina sequencing was more effective for screening the large number of samples in a field test array.

A series of coatings from Adaptive Surface Technologies (AST) were used to develop protocols for comparing biofilms on different coatings in field static immersion tests. Our preliminary analyses indicated that coatings C, G, M, N and O had a higher absorbance/biofilm cover than the rest of the coatings in the array. Based on DNA fingerprinting of biofilm samples, Coating F (a commercial FR coating) had the highest richness and diversity of bacterial terminal restriction fragments. Coatings B (commercial biocidal coating) and P (FR/biocide hybrid) had significantly different bacterial community profiles from all other coatings in the array. Nanopore sequencing showed differences in taxonomic composition between coatings, with certain taxa such as *Erythrobacter* sp. and *Sphingomonas* sp. exhibiting higher abundance on coating B than other coatings. The panels remain in field tests. The results for macrofouling activity will be compared against the findings from the microfouling study in due course.

Preliminary laboratory experiments were conducted to understand the interaction of sediments on coating performance using *Halamphora coffeaeformis* diatoms and the barnacle *Amphibalanus Amphitrite.* The results showed that Silastic T2-coated substrates that were “pre-conditioned” in kaolinite sediment suspension had a higher settlement of diatoms than substrates that were not exposed to the sediments. However, there was no effect on barnacle settlement.

Section A. Field performance of novel antifouling materials

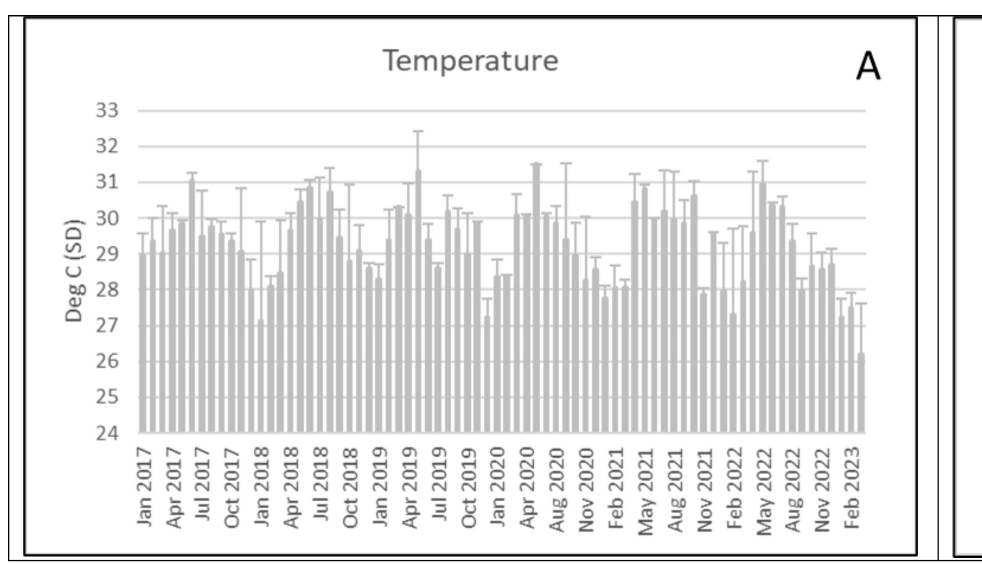
1. Field test site

Tropical Southeast Asian coastal seas are characterized by warm sea temperatures with monsoon-driven seasonality patterns, high nutrient levels and productivity, and a very rich organism biodiversity. The biotic factors coupled with warm year-round temperatures accelerate fouling growth and bio-deterioration. These conditions are useful for the purpose of antifouling tests (Swain et al., 2000). The TMSI field test site (Fig. 1) is located at the Republic of Singapore Yacht Club (RSYC) marina on the south-west coast of Singapore (1° 17' 40" N, 103° 45' 37.6" E). Surface water temperatures are relatively high for most part of the year, ranging between 27 to 31 °C (Fig. 2A). Salinities in the near-coastal areas are typically estuarine, and fluctuate between 20-30 ppt (Fig. 2B); with pH averaging around 8.2. Slime coverage was aggressive on all substrates especially during the NE monsoon months. The site experiences aggressive biofouling throughout the year, which makes it ideal for antifouling field tests.

1.1. Materials tested

Table 1 summarizes the assays that were examined under static shallow submergence for biological settlement in a coastal marine environment during the present Reporting Period 1 Jan 2020 to 31 May 2023. These included the novel coating systems employed, date started and ended. Known commercial standards employed included foul release (FR) Intersleek 900, Intersleek 1100SR and antifouling (AF) Interspeed 640 (AkzoNobel). An elastomer Silastic T2 (Dow Corning) was used as a FR control for a rapid assessment field test using small coupons prepared on microscope glass slide surfaces (i.e., Table 1, University of California). Strong coating performers identified from previous reporting periods were retained in the present immersion studies to further challenge coating performance. As of 31 May 2023, a total of 162 panels from five assays (Table 1) remained under immersion.

Fig. 1. Floating test platform located at a local marina for shallow submergence testing.



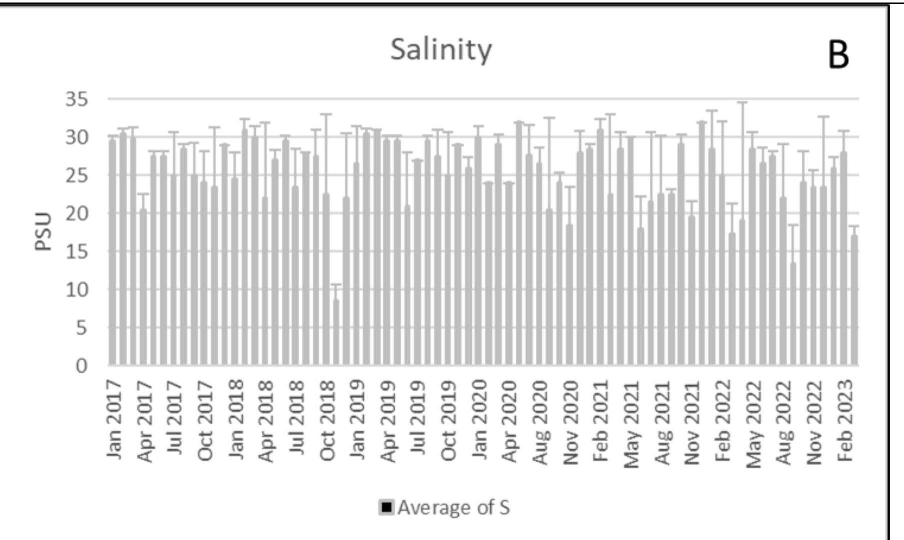


Fig. 2. Monthly temperature (A) and salinity (B) from Jan 2017 to Mar 2023.

Table 1. A total of 315 panels and 60 small coupons (inclusive of blank uncoated surfaces) were examined under static immersion during reporting period: 1 Jan 2020 to 31 May 2023.

No	Assay (PI)	Coating (formulation as received)	Commercial standards	Date Start	Date End	Number of panels/coupons tested
1	Harvard WYSS (Kolle S.)	• SM47i - 07	• Intersleek 1100SR	Jun 2016	Apr 2021	10
2	NatureCoat (Xu X.W.)	• NX1 (Zwitter with co-polymer 1, brush) • NX2 (Zwitter with co-polymer 1, spray) • NX3 (Zwitter with co-polymer 2, brush) • NX4 (Zwitter with co-polymer 2, spray) • NX5 (Zwitter with co-polymer 3, brush) • NX6 (Zwitter with co-polymer 1 & CPT, brush) • Blank PVC	• Intersleek 970	Mar 2017	Jan 2021	24
3	NatureCoat (Xu X.W.)	• NX7 • NX8 • NX9 • NX10 • NX11 • NX12 • Blank PVC	• Intersleek 970	Oct 2017	Oct 2021	24
4	NatureCoat (Xu X.W.)	• NX13 • NX14 • NX15 • NX16 • NX17 • NX18 • NX19 • NX20 • NX21 • Blank PVC	• Intersleek 970	Apr 2018	In progress	33

No	Assay (PI)	Coating (formulation as received)	Commercial standards	Date Start	Date End	Number of panels/coupons tested
5	Intersite Calibration (Holm E.)	• Selektope + co-biocides • Selektope + Copper • Selektope + co-biocides (Cu free) • Selektope + co-biocides (F) / Selektope-free (B) • PPG AF product 1 • PPG AF product 2 • PPG AF product 3 • Hempel Hempaguard X7 • Blank PVC	Not applicable	Jun 2018	In progress	27
6	Adaptive Surface Technologies (Khatri C.)	• AM-24Alt • AST_AFFR_Hybrid • SilCtrl • AM-14 • AM-24 • N1x • NegCtrl • LubCtrl • AM-26 • Blank PVC	• FRCtrl1 • FRCtrl2 • AFCtrl1	Feb 2019	In progress	52

No	Assay (PI)	Coating (formulation as received)	Commercial standards	Date Start	Date End	Number of panels/coupons tested
7	NDSU (Webster D.)	• Siloxane-polyurethane (SiPU) coating system with silicone oil additive • Amphiphilic SiPU coating system with surface modifying amphiphilic additive consisting of PDMS and PEG • SiPU coating system with suface modifying amphiphilic additive consisting of PDMS and PEG • SiPU coating system with suface modifying amphiphilic additive consisting of PDMS and PEG • SiPU coating system with suface modifying amphiphilic additive consisting of Zwitterionic groups and PDMS • SIloxane-polyurethane coating system with suface modifying amphiphilic additive consisting of Zwitterionic groups and PDMS • Blank PVC	• Self-polishing, copper containing, antifouling control • Intersleek 970 • Intersleek 1100SR	Aug 2019	Sep 2020	40
8	NatureCoat Sherwin Williams (Haslbeck E.)	• NatureCoat as provided to SW (NC1) • NatureCoat as optimized by SW (NC2)	• Commercial foul release control (NC3)	Apr 2021	In progress	9
9	Adaptive Surface Technologies (Khatri C.)	• 100342 • 100330 • 100153 • 100356 • 100329 • 100359 • 100375 • 100360 • 100331 • 100344	• BRA640 • Intersleek 1100SR	Apr 2021	In progress	60

No	Assay (PI)	Coating (formulation as received)	Commercial standards	Date Start	Date End	Number of panels/coupons tested
		• 100353 • 100363 FR/biocide hybrid • Blank PVC				
10	NDSU (Webster D.)	• Hydrophobic siloxane PU with hydrophilic additive (NDSU_1) • Amphiphilic PU with amphiphilic additive (NDSU_2) • Hydrophobic siloxane PU with amphiphilic additive (NDSU_3) • Amphiphilic PU with amphiphilic additive (NDSU_4) • Amphiphilic PU with amphiphilic additive (NDSU_5) • Blank PVC	• AkzoNobel Anti-fouling Interspeed 640 • AkzoNobel Fouling-release Intersleek 900 • AkzoNobel Fouling-release Intersleek 1100SR	Sep 2021	Feb 2022	36
11	University of California (Segalman R.)	• Peptoid with alternating ether and TEMPO-containing monomers on PS-P(DMS/VMS)-PS • Peptoid with three adjacent TEMPO-containing monomers and three ether monomers on PS-P(DMS/VMS)-PS • Peptoid with two adjacent TEMPO-containing monomers and four ether monomers on PS-P(DMS/VMS)-PS • Peptoid with one TEMPO-containing monomer and five ether monomers on PS-P(DMS/VMS)-PS • Control, polymer backbone	• Silastic T2 (Dow-Corning)	Oct 2021	Nov 2021	60

1.2. Coated Panels Submergence

Coated panels prepared on 10 cm by 20 cm substrates (D3623 − 78a *Standard Test Method for Testing Antifouling Panels in Shallow Submergence*) were typically mounted in a vertical orientation on 2-tier PVC frames using zip-ties (Fig. 3). The frames were then lowered and secured on wooden struts supported by metal brackets on the floating test platform. Larger ASTM-sized panels (e.g., width 150 to 250 mm by length 250 to 300 mm) were mounted on single tier frames. All coatings were randomized in a block fashion before securing onto frames. The panels were immersed at depths between 0.5 m to 0.8 m below sea surface.

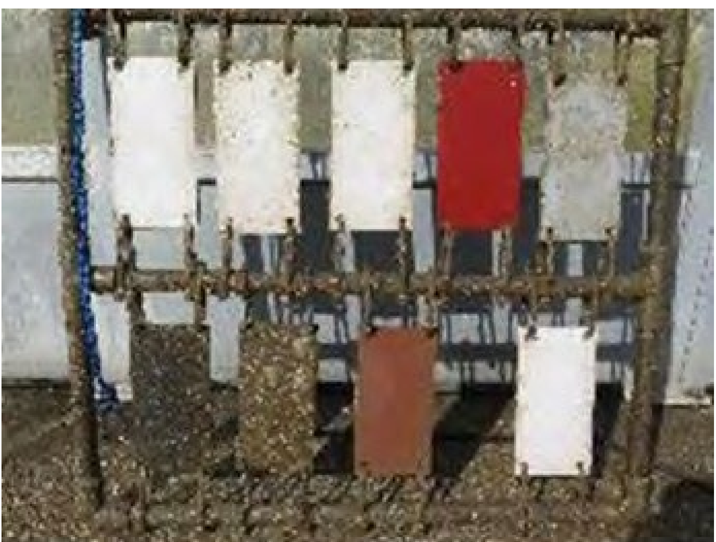


Fig. 3. Coated panels on PVC immersion frames.

1.3. Evaluation of Biofouling Settlement on Foul Release Coatings (Large Panels)

Biofouling settlement cover on coatings were assessed monthly. Panels were raised and gently shaken in surrounding seawater to remove loosely attached detritus.

For foul release (FR) coated panels, fouling on the top half of each panel was left undisturbed throughout immersion and quantified monthly for fouling settlement cover using a randomized point estimate of 50 points (Photogrid 1.0). To reduce edge effects, 10 percent of the panel edges (typically about half an inch from the panel edges) was not considered in the assessment (Fig. 4). Care is taken to minimize coating and biofouling organism exposure time to drying. Fouling composition was categorized following ASTM D6990 – 05 (*Standard Practice for Evaluating Biofouling Resistance and Physical Performance of Marine Coating Systems*), and only organisms directly attached to coated surfaces were recorded.

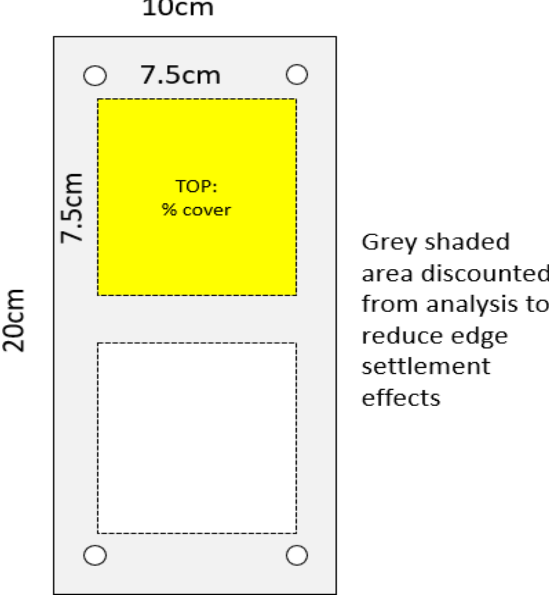


Fig. 4. For FR coatings, the top half of each panel (yellow highlight) is left undisturbed throughout immersion, and assessed monthly for fouling settlement cover. In the example shown above on a 10cm by 20cm coated panel, an area of 7.5cm by 7.5cm is quantified for settlement cover monthly.

1.4. Evaluation of Biofouling Settlement on Antifouling Coatings (Large Panels)

For Antifouling (AF) coated surfaces (e.g., biocide coatings), unlike FR coatings, the entire coated surface was assessed for biofouling settlement cover. Half an inch or roughly 1cm of the panel edges were discounted from assessment to reduce edge settlement effects. Fouling composition was similarly categorized following ASTM D6990 – 05.

1.5. Biofouling Removal using Monthly Waterjet Cleaning on FR Coatings (Large Panels)

For FR coatings, a biofouling removal test based on a previously described calibrated water jet test (Swain and Schultz, 1996) at 50 PSI nozzle pressure was conducted *in-situ* every month. The apparatus consisted of a pressure washer (RYOBI AJP 1600) receiving piped freshwater from source location, high pressure hose and a custom fitted nozzle with an attached pressure gauge. Desired water pressure was adjusted by a ball valve releasing excess pressure through a secondary hose. Fouling cover on the lower half of each coated panel was removed as much as possible by the incident waterjet at 50 mm away from the coating (Fig. 5). The top half of the panel was shielded from cleaning with a simple PVC sheet. The test was performed monthly, to monitor the efficacy of 'low pressure' cleaning or grooming (Tribou & Swain, 2010) to maintain a low-foul or foul-free condition on the coatings.

Fig. 5. Lower half of each coated panel was cleaned with (50 PSI) waterjet pressure from a pressure washer where desired pressure was achieved manually by valve control and nozzle gauge.

The cleaned surface (i.e., lower half of panel) was then quantified for fouling cover using a randomized point estimate of 50 points (Photogrid 1.0). To reduce edge effects, 10 percent of the panel edges was not considered in the assessment (Fig. 6). Care was taken to minimize coating and biofouling organisms exposure time to drying. Biofouling composition was categorized following ASTM D6990 – 05.

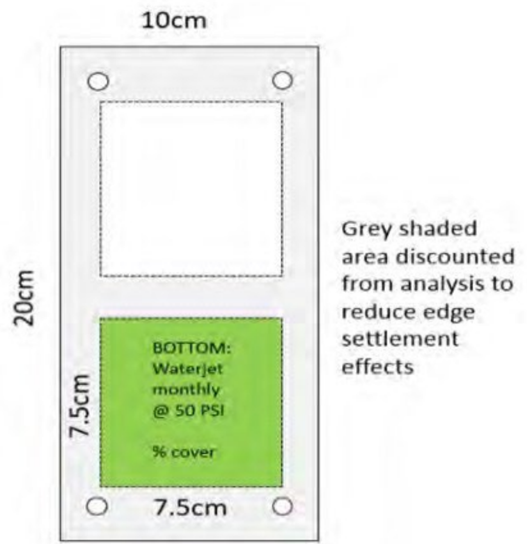


Fig 6. For FR coatings, the lower half of each coated panel was cleaned with 50 PSI waterjet pressure, and assessed monthly for biofouling settlement cover. In the example shown above on a 10cm by 20cm coated panel, an area of 7.5cm by 7.5cm was quantified for settlement cover monthly.

1.6. Rapid Assessment using Coated Microscope Slides (Small Coupons)

For the purpose of screening formulations to demonstrate an antifouling activity, rapid assessments may be deployed in sites with high biofouling pressure rates to gather information on antifouling

activity of developmental coatings. Short term assessments may be carried out in small coupons (e.g., microscope glass slide) with high sample replication for the purpose of down-selection of promising coatings for subsequent scale-up. In rapid assessment, coating physical integrity may additionally be challenged under natural seawater environment to study incompatible coating chemistries to minimize material cost investment (including logistical costs) associated with longer term studies. Short-term tests that challenge coatings with heavy fouling conditions, by employing small coupons, are desirable to coating developers as this method allows many formulations to be screened at lower material cost and time (Lim et al., 2015).

Coated coupons (e.g., glass slides) were deployed in standard microscope slide box that had been cut-out to allow seawater to flow freely (Fig. 7). The microscope slide box(es) was/were secured vertically onto PVC immersion frame(s) using zip-ties. Coated surfaces were faced downwards during immersion to minimize sediment build-up on the top surface. The materials were immersed at depth between 0.5 m to 0.8 m below sea surface. All coatings were randomized in a block fashion before securing onto frames/slide boxes.

Fig. 7. Coated coupons on a cut-out microscope slide box to allow seawater flow-through.

1.7. Evaluation of Biofouling Settlement on Coated Surfaces (Small Coupons)

For novel coating formulations prepared on small coupons, surfaces were inspected after one week immersion for physical damage, to identify as early as possible, any coating stability complications arising from seawater immersion e.g., delamination. Biofouling coverage was assessed within a month, to prevent heavy build-up of secondary biofouling settlement. The coupons were raised and gently shaken in surrounding seawater to remove loosely attached detritus. They were then inspected and digital photographs of the coupons were taken with a 10 megapixel camera. Assessment of biofouling cover of the coupons were carried out using enumeration of organisms. This was because

biofouling settlement at this stage comprised of discrete solitary organisms that might not be captured accurately by a point estimate method. Due to edge effects on biofouling settlement, the fouling cover within 2.5 mm from the width, and 7.5 mm from the length of the slide were excluded from the assessment (Fig. 8). No biofouling removal test were carried out for biocidal coatings. Biofouling settlement was categorized following ASTM D6990 – 05 (*Standard Practice for Evaluating Biofouling Resistance and Physical Performance of Marine Coating Systems*).

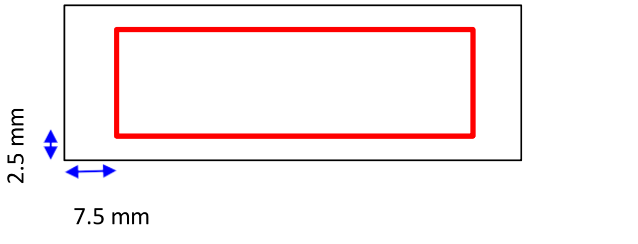


Fig. 8. Surface within the red rectangle marks the area scored for fouling coverage in a 1” by 3” small coupon.

1.8. Biofouling Removal using Waterjet Cleaning on FR coatings (small coupons)

A biofouling removal test based on a previously described calibrated water jet test (Swain and Schultz, 1996) at 50 PSI nozzle pressure was conducted *in-situ* within a month. The apparatus for the water jet test is described earlier in this report. Biofouling cover on the coating surface were removed as much as possible by the incident waterjet at 50 mm away from the coating. Coatings are enumerated for biofouling organisms settlement, before and after waterjet cleaning.

2. Results of Coating Field Immersion

A total of 11 assays comprising 315 panels and 60 small coupons were examined under static shallow submergence for biological settlement in a coastal marine environment during the present Reporting Period 1 Jan 2020 to 31 May 2023. In total, 68 novel formulations (i.e., excluding PVC blank controls and commercial standards) were tested. Four assays were launched during this Reporting Period. Strong coating performers from the remaining seven assays were identified in earlier reporting periods, and retained under present immersion studies to further challenge coating performance. As of 31 May 2023, a total of 162 panels from five assays remained under immersion (Table 1). Table 2 shows the images of commercial standards, best and least performing coatings in the assays deployed in the field. The assays were presented in chronological order by immersion i.e., launch date.

Most of the assays comprised of coatings that employed a Foul Release (FR) technology (Table 2). Biofouling settlement on the lower half of each FR coating panel was challenged with a monthly water jet cleaning procedure, while the top half was left undisturbed. On the FR coating images, a visible line across the centre of the panel may be observed, showing the difference in effect of water jet cleaning on the biofouling cover of the coatings. Coatings under the Intersite Calibration program, whose array included biocidal and copper-free formulations, had been monitored since June 2018, while Hempel Hempasil X7 (since July 2015) was assessed monthly with water jet cleaning for biofilm i.e., slime release efficacy. No water jet cleaning was performed on biocidal Antifouling surfaces under Intersite Calibration assay. Coating images and quantification of biofouling cover were shared with PIs in the respective programs.

Table 2. Images of commercial standards, best and least performing coatings in the assays deployed. Biofouling settlement on the lower half of each FR coating panel was challenged with a monthly water jet cleaning procedure, while the top half was left undisturbed.

Assay No	Assay (PI)	Technology type (Antifouling or Foul release)	Best performer identified in assay (duration)	Commercial Control (duration)	Poor performer identified in assay (duration)	Remarks
1	Harvard WYSS	Foul release	SM47i – 07 (46 months)	IS1100SR (46 months)	KM32t – 0011 (3 months)	• Best performer SM47i-07 retained in current immersion program; strong foul release effect up to 46 months. • Delamination of SM47i-07 from substrate occurred after 48 months. Coating failed after 58 months.

Assay No	Assay (PI)	Technology type (Antifouling or Foul release)	Best performer identified in assay (duration)	Commercial Control (duration)	Poor performer identified in assay (duration)	Remarks
2	NatureCoat	Foul release	NX6 (41 months)	IS970 (41 months)	NX3 (24 months)	• Best performer NX6 retained in current immersion program; strong foul release effect up to 41 months. • Terminated due to heavy fouling cover after 46 months. • Edges of panels with novel coatings were not coated, which contributed to encroachment of biofouling from edges

Assay No	Assay (PI)	Technology type (Antifouling or Foul release)	Best performer identified in assay (duration)	Commercial Control (duration)	Poor performer identified in assay (duration)	Remarks
3	NatureCoat	Foul release	NX10 (36 months)	IS970 (36 months)	NX8 (18 months)	• Best performer NX10 retained in current immersion program; strong foul release effect up to 36 months. • Terminated due to heavy fouling cover after 48 months.

Assay No	Assay (PI)	Technology type (Antifouling or Foul release)	Best performer identified in assay (duration)	Commercial Control (duration)	Poor performer identified in assay (duration)	Remarks
4	NatureCoat	Foul release	NX21 (59 months)	IS970 (59 months)	NX15 (36 months)	• Best performer NX21 retained in current immersion program; strong foul release effect up to 59 months.

Assay No	Assay (PI)	Technology type (Antifouling or Foul release)	Best performer identified in assay (duration)	Commercial Control (duration)	Poor performer identified in assay (duration)	Remarks
5	InterSite Calibration	Antifouling and Foul release	Antifouling Selektope + Copper (57 months)	Foul release Hempel Hempaguard X7 (92 months)	Antifouling Selektope + co-biocides (57 months)	• Hempel Hempaguard X7 retained in current immersion program from Jun 2015; retained effective biofilm removal after 92 months. • Heavy slime fouling on Antifouling coatings.

Assay No	Assay (PI)	Technology type (Antifouling or Foul release)	Best performer identified in assay (duration)	Commercial Control (duration)	Poor performer identified in assay (duration)	Remarks
6	Adaptive Surface Technologies	Foul release	AM-26 (49 months)	FRCtrl2 (49 months)	AM-24Alt (49 months)	

Assay No	Assay (PI)	Technology type (Antifouling or Foul release)	Best performer identified in assay (duration)	Commercial Control (duration)	Poor performer identified in assay (duration)	Remarks
7	NDSU	Foul release	AR-6 (12 months)	IS970 (12 months)	JB_AMP_SiPU_F9 (12 months)	• Delamination on Coating JB_AMP_SiPU_F9 and JB_SMAA_#54 after 2 months. • Heavy fouling on un-washed surface • Waterjet pressure of 150PSI was used

Assay No	Assay (PI)	Technology type (Antifouling or Foul release)	Best performer identified in assay (duration)	Commercial Control (duration)	Poor performer identified in assay (duration)	Remarks
8	NatureCoat Sherwin Williams	Foul release	NatureCoat as optimized by Sherwin-Williams (23 months)	NatureCoat as provided to Sherwin-Williams (23 months)	Commercial foul release control (23 months)	

Assay No	Assay (PI)	Technology type (Antifouling or Foul release)	Best performer identified in assay (duration)	Commercial Control (duration)	Poor performer identified in assay (duration)	Remarks
9	Adaptive Surface Technologies	Foul release	100363 FR/biocide hybrid (23 months)	IS1100SR (23 months)	100331 (23 months)	• Biofouling cover on BRA640 exceed 20% after 12 months. • Gradual loss of pigmentation/coating color on 100363 FR/biocide hybrid.

Assay No	Assay (PI)	Technology type (Antifouling or Foul release)	Best performer identified in assay (duration)	Commercial Control (duration)	Poor performer identified in assay (duration)	Remarks
10	NDSU	Foul release	A4-SMAA-54 (2 months)	IS1100SR (2 months)	F5 (2 months)	• Incomplete fouling removal by monthly waterjet cleaning on novel coatings. • Set terminated after 6 months.

Assay No	Assay (PI)	Technology type (Antifouling or Foul release)	Best performer identified in assay (duration)	Commercial Control (duration)	Poor performer identified in assay (duration)	Remarks
11	University of California	Foul release	1-TEMPO	Silastic-T2	3-TEMPO	• No major coating damage identified • Little fouling removal achieved from waterjet cleaning on test surfaces, compared to Silastic T2 • Images shown are after waterjet cleaning at 50 PSI.

Tables 3–12 compared the total macrofouling cover with immersion duration of all novel coatings, commercial standards, and PVC Blanks in each assay. Coating codes or numbers were listed as supplied. For FR coatings, macrofouling cover were categorized into 1) surface cover that were cleaned with water jet monthly; and 2) surface cover that were left uncleaned for the duration of the immersion. Strong coating performers were identified as coatings with <5% macrofouling cover after water jet cleaning. Similarly, coatings with high settlement cover (≥20% macrofouling cover) were also listed to identify coatings that were not compatible with demonstrating a strong field performance. For ease of comparison, four colour codes were used to designate different levels of macrofouling cover on coatings i.e., green: <5%; yellow: 5 to <10% amber: 10 to <20%; and red: ≥20%. Macrofouling cover on coatings after one year were shown in half-yearly duration for ease of comparison across coatings in long term immersion assays. The foul release strength of coatings were estimated by the efficacy of monthly water jet cleaning on maintaining low macrofouling cover. In general, macrofouling cover on strong performing FR surfaces with monthly water jet cleaning were lower than on surfaces that were uncleaned. The results are briefly discussed below.

2.1. Harvard WYSS

Only two coatings from this assay were tested in this Reporting Period i.e., SM47i-07 and IS1100SR (commercial FR standard). SM47i-07 was identified as the best coating performer in the assay. Efficacy in biofouling removal by water jet was demonstrated up to 46 months (Assay 1, Table 2). However, delamination of SM47i-07 appeared to occur after 48 months, leading to progressive coating failure. Three coatings i.e., MM47i - 02 - 3GSa, SM47i – 02, SM47i – 04 maintained <5% macrofouling cover with monthly water jet between 12 to 24 months, but other coatings in the assay were heavily fouled (≥20%) by three months with water jet cleaning (Table 3).

2.2. NatureCoat

Three sets of coatings from NatureCoat were progressively carried out in Mar 2017, Oct 2017 and Apr 2018. Novel coatings that were tested in the first batch of coatings (Mar 2017) were not fully coated i.e., uncoated substrate were exposed along the edges. Macrofouling encroachment from uncoated panel edges contributed to overall settlement cover. Coatings from subsequent sets i.e., Oct 2017 and Apr 2018, were fully coated along the panel edges.

Coatings NX1 to NX6 were efficacious to biofouling removal by water jet cleaning up to 18 months (Table 4), with best performer NX6 (Zwitter with co-polymer 1 & CPT), performing up to 41 months.

Coatings NX7 and (best performer) NX10 (Table 5) were efficacious to biofouling removal by water jet cleaning up to 24 and 36 months respectively. Coatings NX13 to NX20 were efficacious to biofouling removal by water jet cleaning between 24 and 30 months, while best performer NX21 remained effective up to 42 months (Table 6).

2.3. Intersite Calibration

Macrofouling cover on 1) Selektope + co-biocides, 2) PPG AF product 2, and 3) Selektope + co-biocides (Cu free) exceeded 20% after 24, 36 and 54 months respectively (Table 7). Four coatings, 1) Selektope + Copper, 2) Selektope + co-biocides/Selektope-free, 3) PPG AF product 1, 4) PPG AF product 3 were generally covered by heavy slime. The best performer Selektope + Copper, continued to maintain low macrofouling cover after 57 months.

2.4. Adaptive Surface Technologies (AST)

Two immersion sets were carried out separately in Feb 2019 and Apr 2021. In the first set of coatings, monthly water jet cleaning was efficacious in maintaining a low macrofouling cover on novel coatings for at least 12 months, compared to six months without cleaning (Table 8). SilCtrl and AM-26 were identified as the best performers after 48 months. Two commercial standards used i.e., FRCtrl1 and FRCtrl 2 sustained heavy macrofouling cover after 12 months despite monthly cleaning.

For the second set of coatings, monthly water jet cleaning was efficacious in maintaining lower macrofouling cover on all novel coatings up to 21 months, compared to surfaces without cleaning (Table 9). The best performer was 100363 FR/biocide hybrid.

2.5. NDSU

Two immersion sets were carried out separately in Aug 2019 and Sep 2021. The coatings from the Aug 2019 set were washed monthly at a water jet pressure of 150 PSI to test for coating durability at elevated pressure. The set launched in Sep 2021 were cleaned at 50 PSI. Extensive coating damage (delamination) due to water jet cleaning were observed on some coatings on both sets of coatings.

In the earlier set (Aug 2019), water jet cleaning likely exacerbated coating delamination in JB_AMP_SiPU_F9, JB_SMAA_#50 and JB_SMAA_#54. Slime removal was not effective by waterjet (except on commercial standards IS970 and 1100SR). The set was terminated after 12 months, after consultation with NDSU.

In the Sep 2021 set, three coatings A4-SMAA-54, SMAA-33_10% and SMAA-33_5% exhibited coating delamination in approximately 5-10% of the area on the lower half of the panels, possibly as a result of damage caused by monthly pressure cleaning. The set was terminated after 5 months, after consultation with NDSU.

2.6. NatureCoat Sherwin-Williams

Both novel formulations, System 1 (NatureCoat coating as provided) and System 2 (optimized NatureCoat) outperformed System 3 (Commercial FR control). Macrofouling cover on Commercial FR control exceeded 20% after 9 months (without water jet cleaning) and 18 months (with water jet cleaning).

2.7. University of California (Santa Barbara)

This test comprised of novel FR coatings carried out on small glass (microscope) coupons in a rapid field assessment. After 18 days immersion, Silastic T2 has significantly greater abundance of macrofouling than all test coatings. After waterjet at 50 PSI, there was incomplete removal of fouling on novel coatings, while most fouling on Silastic T2 were removed. Percent fouling removal on Silastic T2 was significantly greater than all test coatings.

Table 3. Harvard WYSS coatings. Immersion duration: 59 Months (May 2016 – Apr 2021). : macrofouling cover <5%; : macrofouling cover 5 to <10%; : macrofouling cover 10 to <20%; : macrofouling cover ≥20%; : coating terminated due to heavy biofouling cover or damage. All the commercial standards are listed with an Asterix.

Coatings	**Duration of immersion (months)** Surface without waterjet									**Duration of immersion (months)** Surface with monthly waterjet							
	1	3	6	9	12	18	24	30		1	3	6	9	12	18	24	30
IS1100SR*																	
PVC Blank																	
Surface slips FH																	
Surface slips FL																	
KM32t - 0011																	
MM47i - 02 - 15Ta																	
MM47i - 02 - 3GSa																	
SM47i - 02																	
SM47i - 04																	
SM47i - 07																	

Table 4. NatureCoat coatings. Immersion duration: 30 Months (Mar 2017 – Sep 2019). : macrofouling cover <5%; : macrofouling cover 5 to <10%; : macrofouling cover 10 to <20%; macrofouling cover ≥20%.

Coatings	**Duration of immersion (months)** Surface without waterjet										**Duration of immersion (months)** Surface with monthly waterjet								
	1	3	6	9	12	18	24	30	36		1	3	6	9	12	18	24	30	36
NX1																			
NX2																			
NX3																			
NX4																			
NX5																			
NX6																			
IS970*																			
PVC Blank																			

Table 5. NatureCoat coatings. Immersion duration: 24 Months (Oct 2017 – Oct 2019). : macrofouling cover <5%; : macrofouling cover 5 to <10%; : macrofouling cover 10 to <20%; macrofouling cover ≥20%.

Coatings	**Duration of immersion (months)** Surface without waterjet										**Duration of immersion (months)** Surface with monthly waterjet								
	1	3	6	9	12	18	24	30	36		2	4	6	9	12	18	24	30	36
NX7																			
NX8																			
NX9																			
NX10																			
NX11																			
NX12																			
IS970*																			
PVC Blank																			

Table 6. NatureCoat coatings. Immersion duration: 36 Months (Apr 2018 – Apr 2021). : macrofouling cover <5%; : macrofouling cover 5 to <10%; : macrofouling cover 10 to <20%; macrofouling cover ≥20%.

Coatings	**Duration of immersion (months)** Surface without waterjet												**Duration of immersion (months)** Surface with monthly waterjet										
	1	3	6	9	12	18	24	30	36	42	48		1	3	6	9	12	18	24	30	36	42	48
NX13																							
NX14																							
NX15																							
NX16																							
NX17																							
NX18																							
NX19																							
NX20																							
NX21																							
IS970*																							
PVC Blank																							

Table 7. Intersite Calibration 5 coatings. Immersion duration: 54 Months (Jun 2018 – Dec 2022). : macrofouling cover <5%; : macrofouling cover 5 to <10%; : macrofouling cover 10 to <20%; macrofouling cover ≥20%.

Coatings	Duration of immersion (months)											
	1	3	6	9	12	18	24	30	36	42	48	54
Selektope + co-biocides												
Selektope + Copper												
Selektope + co-biocides (Cu free)												
Selektope + co-biocides (F) / Selektope-free (B)												
PPG AF product 1												
PPG AF product 2												
PPG AF product 3												

Table 8. AST coatings. Immersion duration: 48 Months (Feb 2019 – Feb 2023). : macrofouling cover <5%; : macrofouling cover 5 to <10%; : macrofouling cover 10 to <20%; macrofouling cover ≥20%.

Coatings	**Duration of immersion (months)** Surface without waterjet											**Duration of immersion (months)** Surface with monthly waterjet										
	1	3	6	9	12	18	24	30	36	42	48	1	3	6	9	12	18	24	30	36	42	48
AM-24Alt																						
FRCtrl2*																						
Blank																						
AST_AFFR Hybrid																						
SilCtrl																						
AM-14																						
FRCtrl1*																						
AM-24																						
N1x																						
NegCtrl																						
LubCtrl																						
AFCtrl*																						
AM-26																						

Table 9. AST coatings. Immersion duration: 21 Months (Apr 2021 – Jan 2023). : macrofouling cover <5%; : macrofouling cover 5 to <10%; : macrofouling cover 10 to <20%; macrofouling cover ≥20%.

Coatings	Duration of immersion (months) Surface without waterjet									Duration of immersion (months) Surface with monthly waterjet							
	1	3	6	9	12	15	18	21		1	3	6	9	12	15	18	21
100342																	
BRA640*																	
100330																	
100153																	
100356																	
IS1100SR*																	
100329																	
Bare PVC Control																	
100359																	
100375																	
100360																	
100331																	
100344																	
100353																	
100363 FR/biocide hybrid																	

Table 10. NDSU coatings. Immersion duration: 12 Months (Aug 2019 – Aug 2020). : macrofouling cover <5%; : macrofouling cover 5 to <10%; : macrofouling cover 10 to <20%; macrofouling cover ≥20%. Waterjet pressure of 150PSI was used.

Coatings	**Duration of immersion (months)** Surface without waterjet						**Duration of immersion (months)** Surface with monthly waterjet				
	1	3	6	9	12		1	3	6	9	12
A4-SO-0021											
JB_AMP_SiPU_F9											
JB_SMAA_#50											
JB_SMAA_#54											
AR-6											
AR-26											
Micron CSC HS*											
IS970*											
IS1100SR*											
PVC Blank											

Table 11. NDSU coatings. Immersion duration: 5 Months (Sep 2021 – Feb 2022). : macrofouling cover <5%; : macrofouling cover 5 to <10%; : macrofouling cover 10 to <20%; macrofouling cover ≥20%.

Coatings	**Duration of immersion (months)** Surface without waterjet						**Duration of immersion (months)** Surface with monthly waterjet				
	1	2	3	4	5		1	2	3	4	5
Hydrophobic siloxane PU with hydrophilic additive (F5)											
Amphiphilic PU with amphiphilic additive (R5)											
Hydrophobic siloxane PU with amphiphilic additive (A4-SMAA-54)											
Amphiphilic PU with amphiphilic additive (SMAA-33_10%)											
Amphiphilic PU with amphiphilic additive (SMAA-33_5%)											
AkzoNobel Anti-fouling Interspeed 640 (Control)*											
AkzoNobel Fouling-release Intersleek 900 (Control)*											
AkzoNobel Fouling-release Intersleek 1100SR (Control)*											
PVC Blank											

Table 12. NatureCoat Sherwin-Williams coatings. Immersion duration: 21 Months (Apr 2021 – Jan 2023). : macrofouling cover <5%; : macrofouling cover 5 to <10%; : macrofouling cover 10 to <20%; macrofouling cover ≥20%.

Coatings	**Duration of immersion (months)** Surface without waterjet									**Duration of immersion (months)** Surface with monthly waterjet							
	1	3	6	9	12	15	18	21		1	3	6	9	12	15	18	21
System 1: NatureCoat as provided to Sherwin-Williams																	
System 2: NatureCoat as optimized by Sherwin-Williams																	
System 3: Commercial foul release control*																	

Section B. Assessment of Biofilms on Field Static Immersion Panels

1. Introduction

Biofilms are three-dimensional communities that develop on surfaces immersed in an aquatic environment and consist of a consortium of micro-organisms, including bacteria, diatoms, protozoa, fungi and viruses (Railkin 2004), held together by a self-produced matrix of extracellular polymeric substances (EPS) such as polysaccharides, lipids and proteins (Watnick & Kolter 2000; Flemming & Wingender 2010). Biofilm development, also known as microfouling, occurs when prokaryotes and unicellular eukaryotes colonize surfaces typically after a conditioning film, or an organic layer is formed by the initial adherence of macromolecules to surfaces immersed in seawater. Although the different phases of biofouling can overlap or occur in parallel (Dobretsov 2010), biofilms have been demonstrated in many cases to be a pre-requisite for the later stages of biofouling, particularly macrofouling settlement, by exuding cues that encourage their settlement and/or metamorphosis (e.g. see review by Salta et al., 2013; Dobretsov & Rittschof, 2020; Agostini et al., 2021). This link between biofilms and macrofouling, renders biofilm development an important target for antifouling technologies. Heavy slime without hard fouling on underwater hulls of ships has been estimated to increase fuel consumption by 10.3% and annual fuel costs by approximately $1.15M per year, relative to a hydraulically-smooth hull condition (Schultz et al. 2010).

Tenacious forms of biofilms prove to be a challenge to antifouling coatings, even on commercial coatings that are able to achieve low macrofouling rates (Atlar & Callow, 2003; Molino et al., 2009a, b; Salta et al., 2016), as they adhere strongly to surfaces and are resistant to removal pressures such as waterjet cleaning and mechanical grooming. In a study evaluating the effectiveness of weekly grooming on a large-scale foul-release test panel, Hearin et al. (2015) found no significant difference in biofilm coverage between weekly groomed and ungroomed (control) IS900 surfaces due to the permanent establishment of low-profile (<1mm thick) tenacious biofilms. Factors that can affect the strength of biofilm adhesion include the mechanism and/or structure of attachment of microfouling organisms. For example, *Amphora* sp. diatoms have two raphes that lie on the same side of the cell, enabling them to have two points of attachment to surfaces, which in turn allow them to adhere strongly to foul-release coatings; they have also been isolated from areas of ship hulls that are subjected to turbulent flow (Hunsucker et al., 2014; Zargiel & Swain 2014). The chemical and physical properties of antifouling coatings have also been demonstrated to impact the adhesion strength of microfouling organisms. Generally, diatoms are expected to adhere more strongly to hydrophobic foul-release surfaces than hydrophilic surfaces like glass (Holland et al., 2004; Molino & Wetherbee

2008). However, Faria et al. (2021) reported that cyanobacterial biofilms formed on glass and epoxy-coated surfaces were more developed than those on silicone hydrogel surfaces. They attributed this to the formation of a conditioning film which may have altered the surface properties presented to microorganisms, and the physicochemical properties of the microorganisms and inherent biofilm capacity of microorganisms.

As biofilm development remains a key area of interest in antifouling technology development, there is practical interest in the establishment of methods to quantify and characterise biofilms in order to assess the efficacy of antifouling technologies in reducing biofilm settlement or facilitating their removal. Moreover, understanding the interactions between biofilms and the marine environment, e.g. suspended solid particles, can lead to a better understanding of factors that contribute to the failure of novel coatings in field tests, which otherwise may not be appreciated under laboratory settings (Koc et al., 2019).

The objectives of this study are three-fold:

1. To develop and optimize protocols for sampling and quantification of tropical slimes on novel antifouling coatings using a crystal violet multi-well plate assay
2. To characterise the composition of biofilm communities on antifouling coatings by DNA fingerprinting and Next-generation sequencing.
3. To determine if sediments interfere (e.g. impair) with substrate properties and modify the settlement of marine diatoms and larvae.

The study focuses on evaluation of biofilms on coated panels in field static immersion. Typically, these panels would be maintained in static immersion for up to 3 years for macrofouling assessment. The study aims to evaluate if assessments of microfouling in early stage of immersions may provide early indicators of its longer term coating performance. Such information would allow further work on formulation improvement to be pursued while field testing is still in progress.

2. Materials and Methods

The coatings developed by Adaptive Surface Technologies (AST) were sampled for quantification of the amount of biofilm present and analysis of community composition. The AST array consists of 15 treatments, with four replicate panels for each coating (Table 13). A commercial biocidal coating and a commercial foul-release coating are included in the array as positive controls (Table 13). PVC is used as a negative control.

Table 13. Array of coatings developed by Adaptive Surface Technologies.

Coating formulation	Code (as provided)	Number of replicates
100342	A	4
BRA640 (AF control)	B	4
100330	C	4
100153	D	4
100356	E	4
IS1100SR (FR control)	F	4
100329	G	4
Bare PVC Control	I	4
100359	J	4
100375	K	4
100360	L	4
100331	M	4
100344	N	4
100353	O	4
100363 (FR/biocide hybrid)	P	4

2.1. Crystal violet multi-well plate assay

2.1.1. Biofilm sampling methodology

The AST coatings were subjected to biofilm sampling for crystal violet assay 13 months after their immersion. All four replicate panels of the AST coating formulations, except coating I (PVC control), were sampled approximately two and a half weeks after the bottom half of each panel was subjected to waterjet cleaning. Coating I panels were excluded from the sampling as they had heavy macrofouling on their surfaces making it almost impossible to obtain clean biofilm samples. A pre-cut transparent plastic sheet was used as a template to define a fixed sampling window of 8 cm^2 (2 × 4 cm). This ensured consistency in sampling area across all panels of each set of coatings.

The sampling area was rinsed with 0.22µm-filtered sterile aged seawater (FSW) before collection of biofilm to remove any loosely attached sediments, debris and biofilm material. During the sampling process, the plastic template was secured in place along the bottom edge of each panel with plastic clips. This hands-free method of securing the plastic template in place allowed the sampling process to be carried out with ease and stability. Sterile, individually packaged 20mm cell scrapers were used to scrape biofilm from the sampling area as defined by the plastic template. If the intended sampling area had macrofouling cover (e.g. tubeworms, oysters), the plastic template was shifted to an alternative area within the bottom half of the panel to define a sampling window that was free from macrofouling. The cell scraper blades with biofilm samples were detached and placed in individual 20

ml scintillation vials. The plastic template was wiped down with a paper towel to remove any visible biofilm material before repeating the sampling process for the next panel. All vials with the biofilm samples were kept on ice in a cooler box till they were transported to the laboratory within the same day and stored at −20 °C.

2.1.2. Preparation of crystal violet staining suspension

Crystal violet staining suspension was prepared by diluting 6.5 ml of crystal violet stock suspension (BD BBL Gram Crystal Violet) in 390 ml of Milli-Q water. Prior to use, the suspension was filtered through a 50µm nylon mesh to remove any dye coagulates.

2.1.3. Crystal violet assay protocol

The vials containing the biofilm samples from their respective coatings were filled with 5 ml of FSW and vortexed for 10–20 sec to dislodge the samples from the cell scraper blades. The scraper blades were removed from the vials after the biofilms were dislodged. A 2 ml sub-sample of the biofilm suspension in each vial was drawn using a 5 ml syringe and filtered through a 0.22 µm syringe filter (PES membrane). In addition to four replicate vials, a negative control was established for each of the AST coating formulation (Table 14). Blanks were also included to account for any background absorbance of the crystal violet dye (Table 14). For blank samples, 2 ml of FSW, instead of the biofilm suspension, was filtered through the 0.22 µm syringe filter. This step was repeated thrice to establish four replicates of blank samples.

Table 14. Summary of biofilm and blank samples used for crystal violet assay.

Coating treatment	Number of replicates	Negative control
Blank (ASW only)	4	-
A	4	✓
B	4	✓
C	4	✓
D	4	✓
E	4	✓
F	4	✓
G	4	✓
J	4	✓
K	4	✓
L	4	✓
M	4	✓
N	4	✓

O	4	✓
P	4	✓

*FSW = 0.22µm-filtered sterile aged seawater

A 3 ml volume of the crystal violet staining suspension (See section 2.1.2. for preparation methods) was filtered through the syringe filters of all biofilm samples and blanks. For the negative controls, 3ml of Milli-Q water, instead of crystal violet suspension, was passed through the syringe filters.

To wash off excess crystal violet dye, 10 ml of Milli-Q water was slowly filtered through the syringe filters for all biofilm samples, blanks and negative controls. Bound crystal violet dye was eluted directly into a 12-well plate by passing 3 ml of 30% acetic acid through the syringe filters for all biofilm samples, blanks and negative controls. A 100 µl subsample of the eluted dye for each treatment was pipetted to a flat-bottomed 96-well plate. The absorbance of all samples, blanks and negative controls were measured at 600 nm using a microplate reader (Synergy H1, BioTek). Figure 9 summarises the work flow for the crystal violet assay protocol.

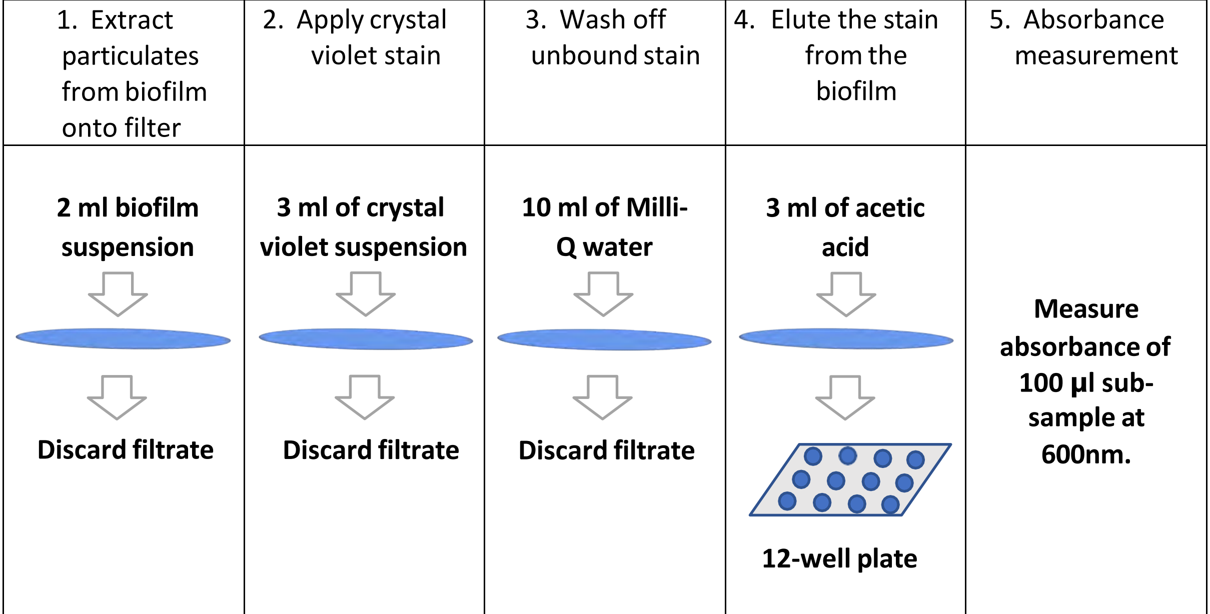


Fig. 9. Workflow for crystal violet assay protocol.

2.1.4. Data analysis

The crystal violet absorbance data was non-parametric based on the Shapiro-Wilk test of normality (W = 0.89285; *p*-value < 0.01). Differences in crystal violet absorbance values between the AST coatings were analysed using Kruskal-Wallis rank sum test, followed by Dunn's post-hoc test with *p*-values corrected by Benjamini-Hochberg method.

2.2. Molecular analysis of biofilm bacterial communities

2.2.1. Biofilm sampling methodology

The AST coatings were subjected to biofilm sampling for an analysis of their bacterial communities after the 1st, 3rd and 6th month of immersion. Across all three sampling time points, the bottom half of all four replicate panels of the 15 AST coating formulations, were sampled before waterjet cleaning. Additionally, at the 6th month time point, biofilm sampling was also carried out after waterjet cleaning. The AST panels were overlaid with a transparent plastic sheet pre-cut to their dimensions, with a single 2 × 2 cm perforation on the bottom half to define the sampling area on each panel. The plastic sheet positioned the sampling window within the central area of the bottom half of the panel, which is approximately 2 cm from the bottom and vertical edges of each panel (Fig. 10).

The sampling area was rinsed with 0.22µm-filtered sterile aged seawater (FSW) before collection of biofilm to remove any loosely attached sediments, debris and biofilm material. Sterile viscose swabs were used to collect biofilms from the sampling area as defined by the plastic sheet. If the intended sampling area had macrofouling cover, the plastic sheet was shifted to an alternative area within the bottom half of the panel to define a sampling window that was free from macrofouling.

After collecting the biofilm samples, the swabs were placed back into their respective storage tubes. All storage tubes with biofilm samples were kept on ice in a cooler box till they were transported to the laboratory within the same day and stored at −20°C. The plastic sheet was sprayed with 70% ethanol and wiped down with a paper towel to remove any visible biofilm material each time before sampling a new panel.

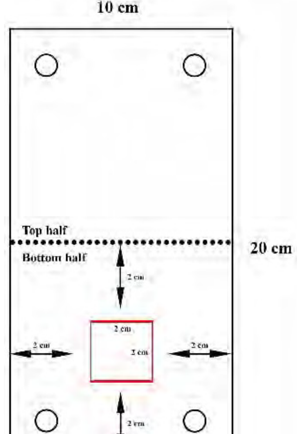


Fig. 10. Diagram showing location of sampling area (red square) between the two mounting holes, on the bottom half of each panel which is subjected to monthly waterjet cleaning.

2.2.2. DNA extraction

The biofilm swabs from only the 1st and 6th month time points were processed for molecular analysis in this study. The swabs were fitted entirely into 2 mL microcentrifuge tubes by snapping off part of their handles. Biofilm samples were dislodged from the swabs by vortex mixing in ~700 µL of Tris-EDTA buffer for 1−3 minutes until most of the material was visibly dislodged from the swab. After removing the swab from the microcentrifuge tube, the biofilm suspension was centrifuged at 7,500 rpm for 10 minutes to concentrate the biofilm material into a pellet. The resulting supernatant was discarded as much as possible while making sure that the biofilm pellet was not disturbed.

Genomic DNA was extracted from the biofilm pellets sampled from the 60 panels in the AST coating array using DNeasy Blood and Tissue Kit (Qiagen, Dusseldorf, Germany) according to the manufacturer's protocol. An additional enzymatic lysis step was added according to the pre-treatment protocol recommended in the DNeasy Blood and Tissue Handbook for Gram-Positive bacteria. An enzymatic lysis buffer was added to re-suspend the pellets and the mixture was incubated for 1 hour at 37°C. Then 25 µL of Proteinase K and 200 µL of AL buffer were added and the mixture was incubated for an additional 30 minutes at 56°C. The rest of the extraction protocol was performed based on the DNeasy Blood & Tissue Kit. Yield and purity of extracted DNA were checked using a Nanodrop spectrophotometer.

2.2.3. Terminal Restriction Fragment Length Polymorphism (tRFLP)

Biofilm communities sampled from the AST coatings after the 1st month of immersion were fingerprinted by tRFLP analysis of the V3-V4 region of the bacterial 16S rRNA gene (Fig. 11).

After DNA extraction was carried out according to the methods detailed in Section 2.2.2., Polymerase Chain Reaction (PCR) amplification of the V3–V4 region of the 16S rRNA gene (~460 bp) was performed using the primers 341F (CCTACGGGNGGCWGCAG) and 806R (GGACTACHVGGGTWTCTAATCC). The 5' end of the forward primer (341F) was labelled with 6-FAM dye (fluorescein) for downstream fragment analysis by capillary electrophoresis sequencing (CES). The region of interest was amplified with the following thermocycling conditions: initial denaturation at 95°C, 3 min; 30 cycles: 95°C, 30 sec; 45°C, 30 sec; 72°C, 30 sec; final extension at 72°C, 5 min. Following amplification, 5 µL aliquots of PCR products were run on 1.5% agarose gels to verify that the amplification was successful. In addition, yield of PCR products was also quantified using Qubit 3.0 fluorometer.

PCR products were purified using the Qiaquick PCR purification kit (Qiagen, Dusseldorf, Germany) and subsequently digested with 10U of *MspI* (Thermofisher Scientific) at 37°C for 2 hours. Fluorescently-labelled fragments were sent to Macrogen Asia-Pacific Pte Ltd for fragment analyses by CES. Electropherograms produced by CES were analysed with GeneMarker software, using manually created bins. Individual peaks, corresponding to the terminal restriction fragments (tRFs), which represented <50 nucleotides in length and had an intensity of <50 units were excluded from subsequent analyses.

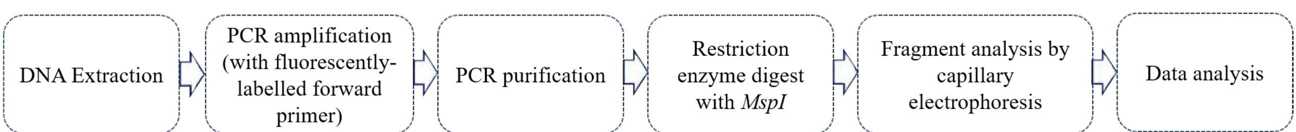


Fig. 11. Workflow for fingerprinting biofilm communities of each treatment by Terminal Restriction Fragment Length Polymorphism. Quality control steps for DNA extraction, PCR and restriction enzyme digest are not included in this flowchart (e.g. yield quantification by Nanodrop and Qubit and verification of successful PCR amplification and restriction enzyme digest by agarose gel electrophoresis).

2.2.4. Nanopore sequencing

The taxonomic composition of biofilm communities sampled from the AST coatings after the 6th month of immersion was characterised by sequencing the full length of the bacterial 16S rRNA gene using Oxford Nanopore MinION sequencing platform. Biofilm samples from only one replicate panel of the following AST coatings were processed for Nanopore sequencing: coatings A, B, C, D, J, K, L, N, O and P.

After DNA extraction was carried out according to the methods detailed in Section 2.2.2., PCR amplification of the 16S rRNA gene (~1500 bp) was performed using the Oxford Nanopore 16S Barcoding kit (SQK-RAB204). A 40 µl PCR reaction mix consisting of 10 µl of DNA, 25 µl of of LongAmp HotStart Taq 2X master mix and 5 µl of nuclease-free water was prepared.

The 16S rRNA gene was amplified with the following thermocycling conditions: initial denaturation at 95 °C, 1 min; 25 cycles: 95 °C, 20 sec; 55°C, 30 sec; 65°C, 2 min; final extension at 65 °C, 5 min. PCR products were cleaned up using AMPure XP magnetic beads and eluted in 10 µL of 10 mM Tris-HCl with 50mM NaCl at pH 8. Yield of PCR products was quantified using Qubit 3.0 fluorometer before pooling the DNA barcoded libraries. Platform quality control (QC) was carried out using MinKNOWTM on a brand new R9.4 chemistry MinIONTM flow cell before the flow cell was washed and primed. Nanopore sequencing was performed over a 48 hr duration.

2.2.5. Data analysis

The number of terminal restriction fragments (tRFs) generated from tRFLP fingerprinting of the first month biofilm bacterial communities was not normally distributed (Shapiro-Wilk: W = 0.95112; *p*-value < 0.05). Differences in the richness of tRFs were analysed using Kruskal-Wallis rank sum test, followed by Dunn's post-hoc test with *p*-values corrected by Benjamini-Hochberg method.

Diversity of biofilm communities was determined by computing the Shanon-Weiner diversity index based on the relative abundance of tRFs for each coating. The diversity index values conformed to a normal distribution (Shapiro-Wilk: W = 0.98248; *p*-value > 0.05) and were further analysed with ANOVA, followed by Tukey HSD post-hoc test.

Differences in bacterial composition of AST coatings were visualised using non-metric multidimensional scaling (nMDS) and tested by PERMANOVA based on the relative abundance of tRFs. All statistical analyses were carried out with R software (R Core Team 2019).

2.3. Interaction between coatings, biofilms and sediments

Settlement assays were conducted to determine the effects of sediments and biofilms on the settlement of two model organisms widely used in anti-fouling studies: diatoms (*Halamphora coffeaeformis*) and barnacles (*Amphibalanus amphitrite*).

Kaolinite (Sigma-Aldrich) was used in the diatom and barnacle settlement assays as a proxy for inorganic sediment particles (Alam et al., 2022). Kaolinite is a relatively inert natural particulate clay material which has been used to study mussel response to inorganic particles in the water (Baldwin et al., 2002; Lim et al., 2020).

2.3.1. Diatom settlement assays

Diatom settlement assays were carried out on coverslips coated with polydimethylsiloxane (PDMS) and exposed to kaolinite particles for ten minutes. During preliminary trials, it was briefly noted that PDMS surfaces were able to retain the kaolinite particles, even after undergoing a freshwater rinse. This observation led us to run the diatom assays on PDMS-coated surfaces to investigate if the accumulated particulate matter could affect the settlement of diatoms.

2.3.1.1. Preparation of polydimethylsiloxane-coated coverslips

Silastic T2 silicone rubber was prepared according to manufacturer instructions by mixing Silastic T2 Translucent base with Silastic T2 Curing Agent in a 10:1 mix ratio by weight. The two components of the Silastic T2 rubber were mixed uniformly by stirring the mixture with a glass rod in a zig-zag fashion for at least one minute.

The mixture was applied as a thin layer to completely coat the surface of 22 x 22mm glass coverslips using the glass rod. A thin layer of the Silastic T2 rubber coating was applied to allow air bubbles that were formed during the polymerisation stage to escape from the coating during the curing stage. The rubber coating on the coverslips was left to cure over 24hr in a fume hood. After curing, the coverslips were rinsed and soaked in distilled water for 48hr to allow for leaching of any chemicals, following which, the coverslips were dried in a fume hood and kept aside till use.

2.3.1.2. Assay methodology

Silastic T2-coated coverslips were exposed to sediment loading using kaolinite as model inorganic sediments. A stock suspension of kaolinite was prepared by mixing 20mg of kaolinite powder in 200ml of 0.22µm-filtered sterile aged seawater (FSW) to form a 100mg/l suspension. From the stock suspension, 50ml of 25mg/l, 50mg/l and 75mg/l kaolinite suspensions were prepared in 0.22µm FSW.

Silastic T2-coated coverslips were randomly immersed in 5ml of one of the four kaolinite concentrations (25mg/l, 50mg/l, 75mg/l, 100mg/l), or 5ml of 0.22µm FSW (no sediments) in untreated polystyrene six-well plates (ThermoFisher Scientific) for 10min on an orbital shaker at 60rpm. After "pre-conditioning" the coverslips with kaolinite, the suspension was removed from each well by pipetting. The wells were then gently rinsed with distilled water while ensuring that the coverslips remain within their respective wells.

A 5ml suspension of *H. coffeaeformis* diatoms prepared at a cell density of 10, 000cells/ml and salinity of 30psu was added to each well. The experiment was allowed to incubate for 24hr in a 12hr light: 12hr dark cycle at 23°C. At the end of the incubation period, all slides were gently dipped in a beaker of 0.22µm FSW (30psu) to rinse off any unattached cells. This rinse step was repeated three times. The coverslips were then fixed in 2.5% glutaraldehyde in 0.22µm FSW. The coverslips were examined under an epi-fluorescence microscope (Olympus DP74) at 20x magnification and the number of diatom cells were enumerated in ten random fields of view (0.916mm^2 per field of view) per slide.

2.3.2. Barnacle cyprid settlement assays

Barnacle cyprid settlement assays were designed to determine the effects of two factors on cyprid settlement: (1) kaolinite loading at increasing concentrations (0-100 mg/L) and (2) biofilms developed in the presence of different combinations of sand-filtered seawater, kaolinite particles and F/2 media (Table 15). Barnacle assays were conducted in untreated polystyrene 12-well plates (ThermoFisher Scientific). During a preliminary trial, cyprid settlement was initially tested in 12-well plates which were coated with PDMS. However, the cyprids had a tendency to get "stuck" onto the surfaces of the wells. This could be due to hydrophobic interactions between the hydrophobic carapace of the cyprids and the PDMS-coated well surfaces. The "stucked" cyprids were probably less able to explore the surfaces presented to them in the settlement assay, which would render the assay invalid. Therefore, barnacle assays were conducted on bare polystyrene surfaces instead of PDMS-coated surfaces like in the diatom assays, to enable a more accurate assessment of their settlement behaviour in the presence of sediments.

For both assays, *Amphibalanus amphitrite* barnacle larvae were spawned from adults collected from Kranji mangroves, Singapore. The nauplius larvae were fed an algal mixture of *Tetraselmis suecica* and *Chaetoceros muelleri* at a density of approximately 2.5 $\times 10^5$cells/mL and 5 $\times 10^5$cells/mL respectively. They were reared at 28°C in 0.22µm FSW adjusted to a salinity of 27psu. Nauplii metamorphosed into cyprids within 5 days, and the cyprids were aged for 1—2 days at 4−6 °C prior to use in the settlement assays.

2.3.2.1. Assay 1 Methodology: Effects of kaolinite loading at increasing concentrations on cyprid settlement

The effects of kaolinite loading on barnacle cyprid settlement were examined at the following five concentrations of kaolinite suspensions: 0 (control), 25, 50, 75 and 100 mg/L. A stock suspension of kaolinite was prepared by mixing 20mg of kaolinite powder in 100ml of 0.22µm FSW to form a 200mg/L suspension. From the stock suspension, 10mL of 50, 100 and 150mg/L kaolinite suspension were prepared in 0.22µm FSW, and 1mL of each suspension was then added to four replicate wells for each treatment to achieve a final concentration of 25, 50, 75 and 100 mg/L respectively, in a final assay volume of 2mL. For control wells, 1mL of 0.22µm FSW was added per replicate well.

Cyprid larvae were introduced to each well at a loading density of 20 larvae/mL in 1mL of 0.22µm FSW. The well plates were maintained in an environmental chamber under a 12hr light: 12hr dark cycle at 28°C. Cyprid settlement and mortality were scored after 24hr and 48hr of incubation. The assay was repeated thrice with different cohorts of larvae spawned from the same parent batch of barnacles.

2.3.2.2. Assay 2 Methodology: Effects of biofilm development on cyprid settlement

The effects of biofilm formation on barnacle cyprid settlement were examined by "pre-conditioning" individual wells of 12-well plates with one of the following six treatments:

1. Sand-filtered seawater,
2. Sand-filtered seawater enriched with F/2 nutrient media (encourage biofilm growth),
3. Sand-filtered seawater inoculated with 50mg/L kaolinite suspension,
4. Sand-filtered seawater enriched with F/2 media and inoculated with 50mg/L kaolinite suspension,
5. Sand-filtered seawater inoculated with 100mg/L kaolinite suspension and
6. Sand-filtered seawater enriched with F/2 media and inoculated with 100mg/L kaolinite suspension.

F/2 media, commonly used as a nutrient growth medium for marine microalgae, was incorporated in this assay to investigate its effect on cyprid settlement to encourage the growth of microbial community in the sand-filtered seawater. Similarly, kaolinite, which was used as a proxy for inorganic sediment particles in this assay, were included in abovementioned experimental treatments to assess their impacts on cyprid settlement.

A stock suspension of kaolinite was prepared by mixing 5mg of kaolinite powder in either 50mL of sand-filtered seawater or sand-filtered seawater enriched with F/2 media to form a 100mg/L suspension. From the stock suspension, 20mL of 50mg/L kaolinite suspension was prepared in either sand-filtered seawater or sand-filtered seawater enriched with F/2 media. Subsequently, 2mL of each suspension was added to four replicate wells for each treatment. For control wells, 2mL of either sand-filtered seawater or sand-filtered seawater enriched with F/2 media was added per replicate well. The inoculated well plates were incubated in an environmental chamber under a 12hr light: 12hr dark cycle at 28°C for 24hr to encourage biofilm formation. After 24hr, the seawater suspension was carefully pipetted from each well.

Cyprid larvae were introduced to each well at a loading density of 10 larvae/mL in 2mL of 0.22µm FSW. The well plates were maintained in an environmental chamber under a 12hr light: 12hr dark cycle at 28°C. Cyprid settlement and mortality were scored after 24hr and 48hr of incubation. The assay was repeated thrice, with the first two runs using different cohorts of larvae spawned from the same parent batch of barnacles, and the third run using larvae spawned from a different parent batch.

Table 15. Summary of settlement assay methodologies.

Assay	Diatom Assay	Barnacle assay 1	Barnacle assay 2
Aim of assay	To determine if kaolinite accumulation on PDMS substrates can affect settlement	To determine the effects of kaolinite loading at increasing concentrations on settlement	To determine the effects of biofilm formation on settlement
Model organism	Diatom	Barnacle	Barnacle
Type of substrate	PDMS-coated coverslips	Uncoated polystyrene 12-well plates	Uncoated polystyrene 12-well plates
Species	*Halamphora coffeaeformis*	*Amphibalanus Amphitrite*	*Amphibalanus Amphitrite*
Proxy for inorganic sediment	Kaolinite		
Method of kaolinite particle exposure	Pre-conditioning substrate for 10 minutes with one of five concentrations of kaolinite	As a suspension	Pre-conditioning substrate for 24hr with one of six combinations of seawater, kaolinite suspension and F/2 media
Kaolinite concentrations tested (mg/L)	0, 25, 50, 75, 100	0, 25, 50, 75, 100	0, 50, 100

2.3.3. Data analysis

The effects of kaolinite exposure on *H. coffeaeformis* settlement were analysed using one-way ANOVA test with Tukey HSD post-hoc test. The diatom settlement data fulfilled the test requirements of normality (Shapiro-Wilk test: W = 0.93053; p-value > 0.05) and homogeneity of variances (Bartlett test of normality: Bartlett's K-squared = 3.3725; p-value > 0.05).

The effects of kaolinite loading on *A. amphitrite* settlement at 24hr and 48hr were analysed using one-way ANOVA test with Tukey HSD post-hoc test. Both 24hr and 48hr cyprid settlement data collected

during the three replicate runs of the assay fulfilled the test requirements of normality (Shapiro-Wilk test: *p*-value > 0.05) and homogeneity of variances (Bartlett test of normality: p-value > 0.05).

To understand the effects of biofilm formation on *A. amphitrite* settlement at 24hr and 48hr, one-way ANOVA followed by Tukey HSD post-hoc test were employed for data sets that fulfilled the test requirements of normality (Shapiro-Wilk test: *p*-value > 0.05) and homogeneity of variances (Bartlett test of normality: p-value > 0.05). For datasets that did not conform to a normal distribution, Kruskal-Wallis test was applied for analysis. All aforementioned statistical analyses were carried out with R software (R Core Team 2019).

3. Results

3.1. Crystal violet multi-well plate assay

The amount of biofilms sampled from the AST coatings was quantified in terms of absorbance values using a crystal violet multi-well plate assay. Coating I i.e., PVC blank was not sampled due to heavy macrofouling cover. The absorbance of the biofilm samples, as measured at 600 nm, are presented in Figure 12, after correction for blank sample absorbance. Since absorbance is a measure of the amount of light that is absorbed by the sample in question, higher absorbance values will indirectly indicate a greater amount of biofilm.

Coating M had the highest average absorbance value. This indicated that it had the highest amount of biofilm of the array (0.763 ± 0.187; Fig. 12). Coating P had the lowest (0.172 ± 0.009; Fig. 12). However, differences between the absorbance of the biofilm samples from the AST coatings were not statistically significant when analysed by multiple pairwise comparisons in Dunn's post-hoc test with Benjamini-Hochberg correction. This could have been a result of the biofilm communities reaching a point of maturity in biomass and composition (e.g., "climax community") after 13 months of immersion.

In comparison, the surface cover of tenacious biofilms (i.e., biofilms remaining on the surfaces of the coatings after waterjet cleaning) was quantified (Fig. 13). Coating M had the highest average surface cover of tenacious biofilms (74 ± 3.56%; Fig. 13). On the other hand, several coatings were found with low surface cover. These include Coatings P, F, J, K and L. Based on both 1) tenacious biofilm cover and 2) absorbance measurements, Coating M had the largest amount of biofilm of the array, while Coating P was associated with a lower amount of biofilm.

The results from crystal violet staining and tenacious biofilm cover measurements indicated that in general, Coatings C, G, M, N and O had higher amount of absorbance and surface cover (Figs 12 & 13 respectively) than the rest of the coatings. This suggests that these few coatings may be less effective in 1) inhibiting microfouling settlement or 2) releasing biofilms after waterjet cleaning. A summary of the 13-month old panels with a representative image of each coating after waterjet cleaning is presented in Table 16.

It is worthwhile to note the fundamental differences between both methods of biofilm assessment. While surface cover provides a visual estimate of the extent of biofilm cover on the coating surfaces, it does not account for the three-dimensionality of the biofilms in terms of its thickness or biovolume. On the other hand, crystal violet dye is able to bind to negatively charged particles associated with cells in the biofilm and its matrix of extracellular polymeric substances (EPS). As a result, the crystal violet assay is able to provide an indirect estimate of the amount of biofilm present based on the absorbance of eluates of the crystal violet stained samples. In previous works, the efficacy of novel antifouling surfaces to inhibit settlement or facilitate release of model biofilm-forming microbes was commonly ascertained through crystal violet staining and absorbance measurements (e.g., *Cellulophaga lytica* and *Navicula incerta:* Sokolova et al., 2012; *Staphylococcus epidermidis*: Papa et al., 2013; *Halomonas pacifica*: Yee et al., 2016; *Pseudoalteromonas tunicate*: Silva et al., 2019). However, although crystal violet staining has demonstrated potential in identifying the best and worst performers in an array of novel coatings, it may still be limited in its ability to meaningfully differentiate foul-release efficacy among “mid-range” performers. Therefore, crystal violet staining does not currently appear to provide a major advantage over visual surface cover assessment of the panels. We cannot also discount the possibility that “mid-range” performers may be virtually indistinguishable in terms of their foul-release efficacy at present, but may present differences in efficacies with time.

Through this study, we have been able to develop and optimise a crystal violet assay protocol that can be applied to tropical biofilm samples from antifouling surfaces in a field environment. Current protocols predominantly quantify model biofilms that are cultured in the laboratory on multi-well plates. Overall, crystal violet staining and absorbance measurements of natural biofilms on antifouling surfaces broadly identified surfaces with varying amounts of biofilm; these results were generally in agreement with a traditional test using visual assessment of biofilm surface cover.

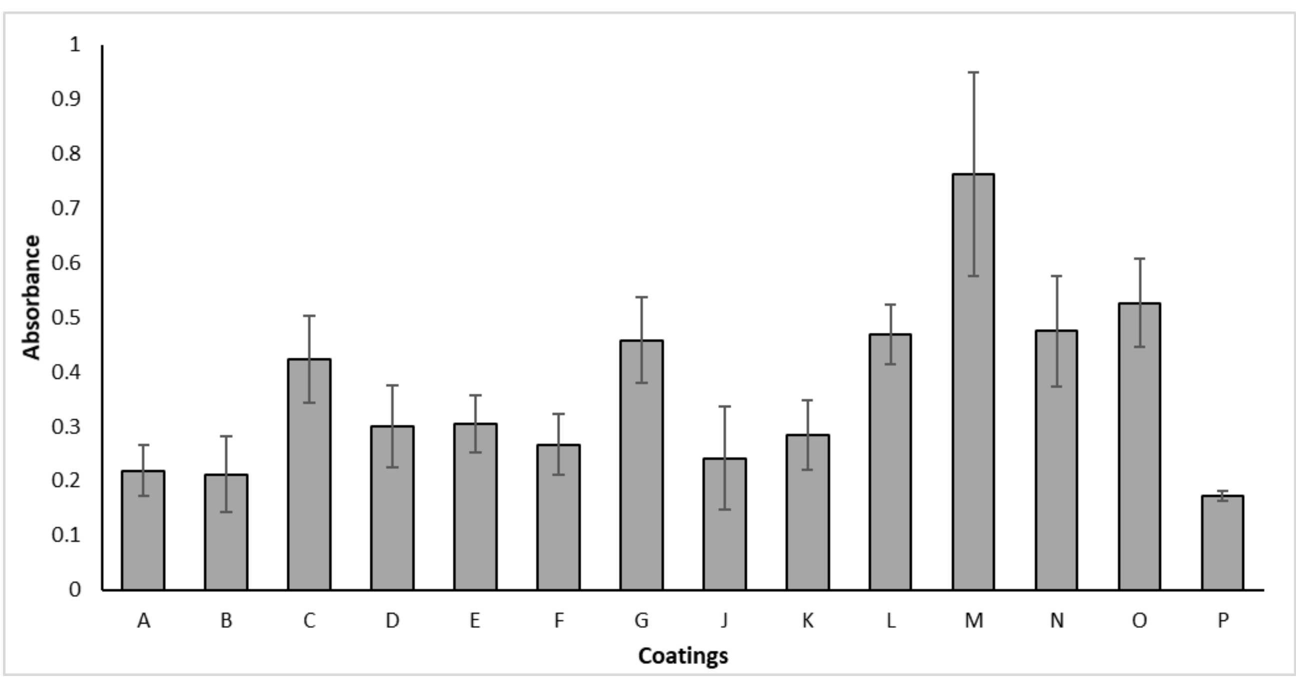


Fig. 12. Amount of biofilms sampled from AST coatings approximately two and a half weeks after waterjet cleaning in May 2022 (total immersion duration of coatings=13 months) as measured by crystal violet absorbance readings (600 nm). The readings shown in this plot have been corrected for blanks. Error bars indicate standard error.

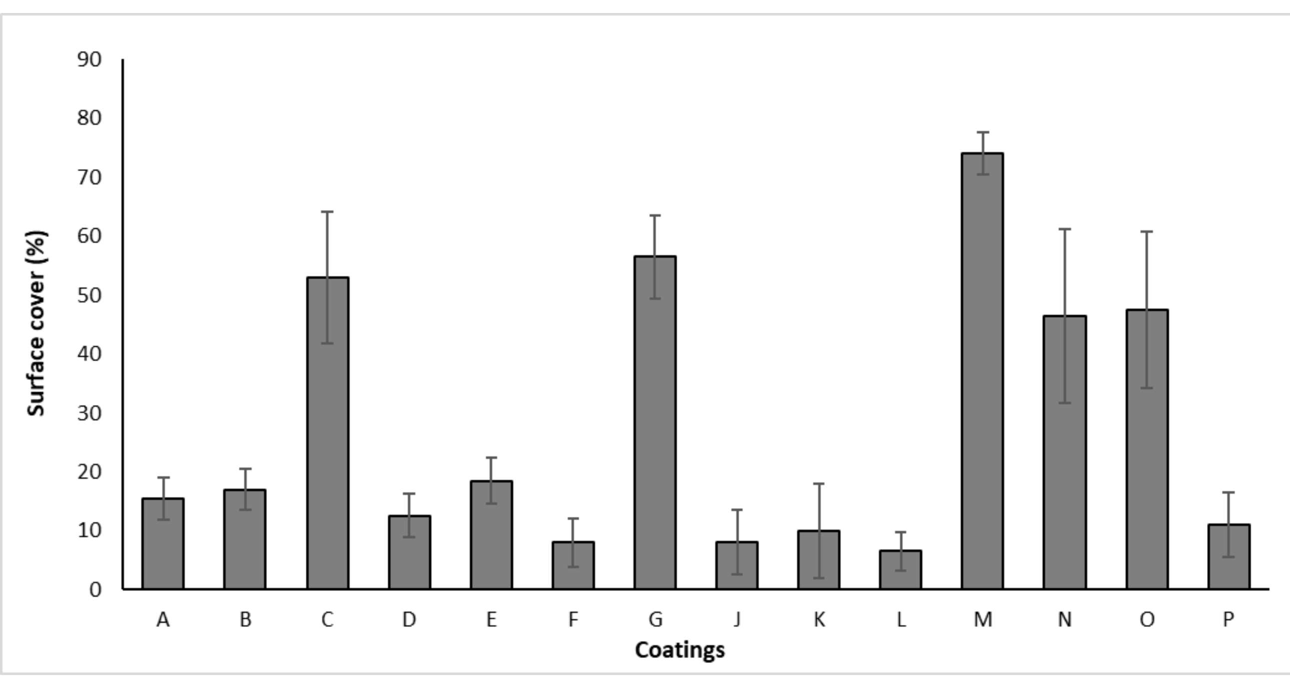


Fig. 13. Surface cover of biofilm on AST coatings after waterjet cleaning as of May 2022 (total immersion duration of coatings=13 months). Macrofouling cover (hard and soft fouling) is not shown here as it accounted for a small percentage (<10%) of biofouling cover after waterjet cleaning across all coatings. Error bars indicate standard error.

Table 16. Summary of 13-month old AST panels with a representative image of each coating after waterjet cleaning.

Code	A	B	C	D	E	F	G	I
Formulation	100342	BRA640 (commercial biocidal control)	100330	100153	100356	IS1100SR (commercial FR control)	100329	PVC Blank (negative control)
Panel								

Code	J	K	L	M	N	O	P	
Formulation	100359	100375	100360	100331	100344	100353	100363 FR/biocide hybrid	
Panel								

3.2. Terminal Restriction Fragment Length Polymorphism (TRFLP) analysis

3.2.1. Alpha diversity

Biofilm communities sampled from the AST coatings after the first month of immersion were characterised by DNA fingerprinting using TRFLP analysis of the V3-V4 region of the 16S rRNA gene. Samples were obtained before subjecting the coatings to waterjet exercise.

The richness, or number of terminal restriction fragments (TRFs), was quantified for each coating. Coating F, with an average of 39.3 TRFs, had the highest number of TRFs of the set (Fig. 14). Dunn's post-hoc test with Benjamini-Hochberg correction showed that TRF richness of coating F was significantly higher than coatings A, E and P (Fig. 14). The lowest average number of TRFs was found in coatings J, L and P which had 17.3 TRFs (Fig. 14). No significant differences in TRF richness were observed among the novel coatings (Fig. 14).

The Shannon-Wiener diversity index was computed based on the relative abundance of TRFs for each coating. As with TRF richness, Coating F had the highest diversity of TRFs in the set (2.74 ± 0.17; Fig. 15). Coating L had the lowest diversity (1.58 ± 0.12; Fig. 15). The diversity of TRFs was significantly different between coatings (ANOVA; $F = 5.62$; p-value < 0.001). A post-hoc Tukey HSD test showed that coating F had significantly higher diversity than most coatings in the set, apart from coatings B, C, G, M and O (Fig. 15). Coating L had significantly lower diversity than coatings G and M (Fig. 15).

Overall, based on the TRF diversities of their first-month bacterial communities, separation was achieved between a few of the novel coatings in the AST array. The diversity of bacterial TRFs of different fragment lengths can be taken as an estimate of the diversity of bacterial species on the coating in question. Coating L had significantly lower TRF diversity than coatings G and M. This could be indicative of underlying differences in the chemical and/or structural composition between the coatings, which could be causing coating L to inhibit the settlement of taxa that are otherwise able to settle on coatings G and M.

Coating F (commercial foul-release control) showed greater TRF richness than coatings A, E and P and a higher diversity than most coatings in the array. As a foul-release coating, coating F does not prevent the accumulation of fouling, but rather facilitates the 'release' of fouling organisms and therefore, likely does not exert selective pressure on fouling organisms. On the other hand, the alpha diversity results could also be indicative that coatings which had lower richness and diversity than coating F, may be inhibiting the settlement of taxa that are not tolerant of their chemical and/or structural composition.

Most of the AST novel coatings had similar richness and diversity of TRFs. Likewise, a lack of statistically significant separation is also seen here between the richness and diversity of coating B (commercial biocidal control) and coating F which represent the two broad classes of antifouling coatings. This observation is in contrast to the findings of several studies, including Papadatou et al. (2020) and Winfield et al. (2018), who showed that foul-release coatings generally exhibited higher biofilm diversity than biocidal antifouling coatings. Winfield et al. (2018) found that in most cases, if an organism grew on a biocidal antifouling coating, it likely also grew on the foul-release coating, apart from a few biocide-tolerant taxa, which were able to grow better on biocidal surfaces. Likewise, Papadatou et al. (2020) demonstrated that biocidal antifouling coatings were characterised by lower diversity and a higher relative abundance of the specific taxa they harboured, compared to foul-release and polydimethylsiloxane coatings, due to the presence of copper biocides that select for biocide-tolerant taxa. In explaining observed differences in bacterial community structures between different types of coatings, Muthukrishnan et al. (2014) suggested that even differences in the composition of biocides present in the coatings might create a selective chemical environment for the growth and development of specific bacterial genera. Findings from these studies therefore raise the question of why significant differences were not observed between the richness and diversity of most of the novel and commercial coatings. Notably, the abovementioned studies were conducted over a longer immersion period (Muthukrishnan et al., 2014: 1 year; Papadatou et al., 2020: ~4 months (119 days); Winfield et al., 2018: minimum of ~3 months (94 days)) than our study (1 month). Immersion duration has been shown to affect the alpha diversity of biofilm communities. Karačić et al. (2022) showed that microbial alpha diversity increased with time. Therefore, it is likely that a longer immersion period will allow differences in community diversity to build up, leading to significant separation among the novel and commercial coatings in the AST array.

Alpha diversity quantification is useful for separating coatings based on community composition in terms of the number of taxa and evenness of taxa. We may be able to compare the performance of coatings based on the number of taxa or in the case of this study, the number of TRFs, they have accumulated after a suitable immersion duration, or by comparing the number of TRFs before and after waterjet exercise. However, it is crucial to note that a higher number of taxa or TRFs may not necessarily indicate poorer antifouling efficacy.

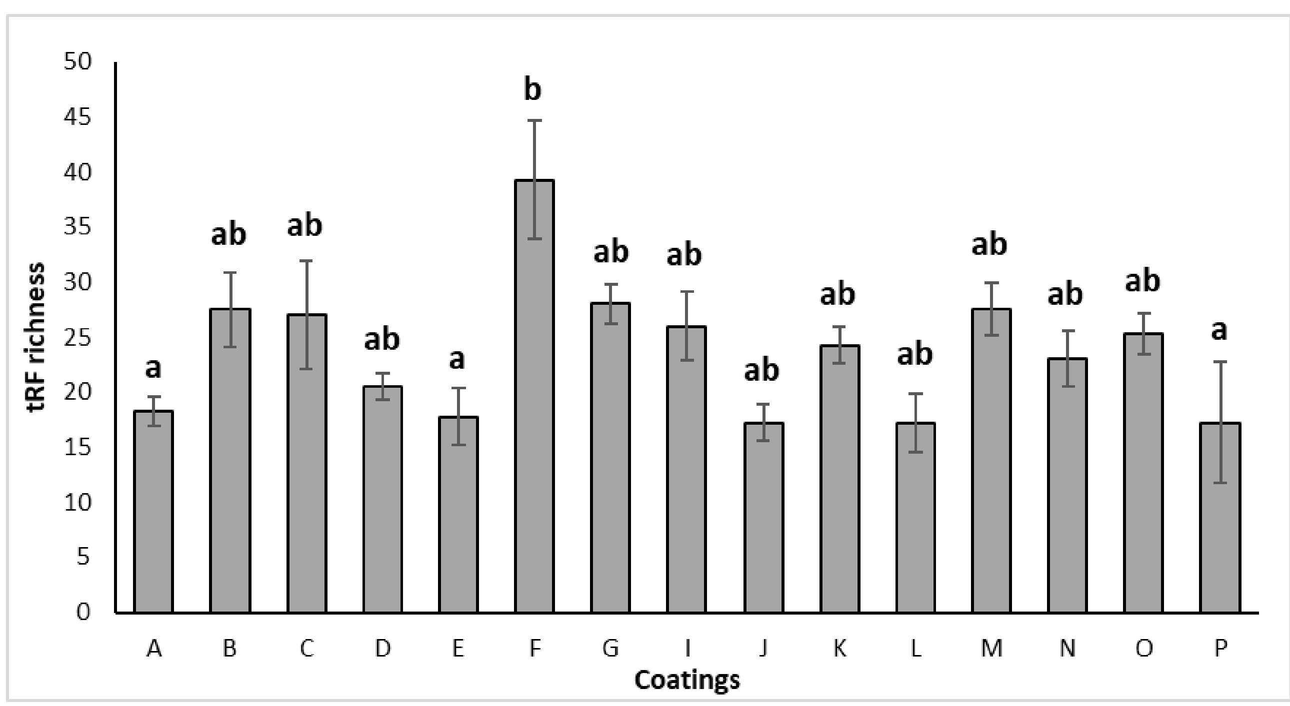


Fig. 14. Average number of terminal restriction fragments (tRFs) of AST coatings after the 1st month of immersion. Average values denoted by a different letter indicate significant differences between coatings (*p*-value < 0.05). Error bars indicate standard error.

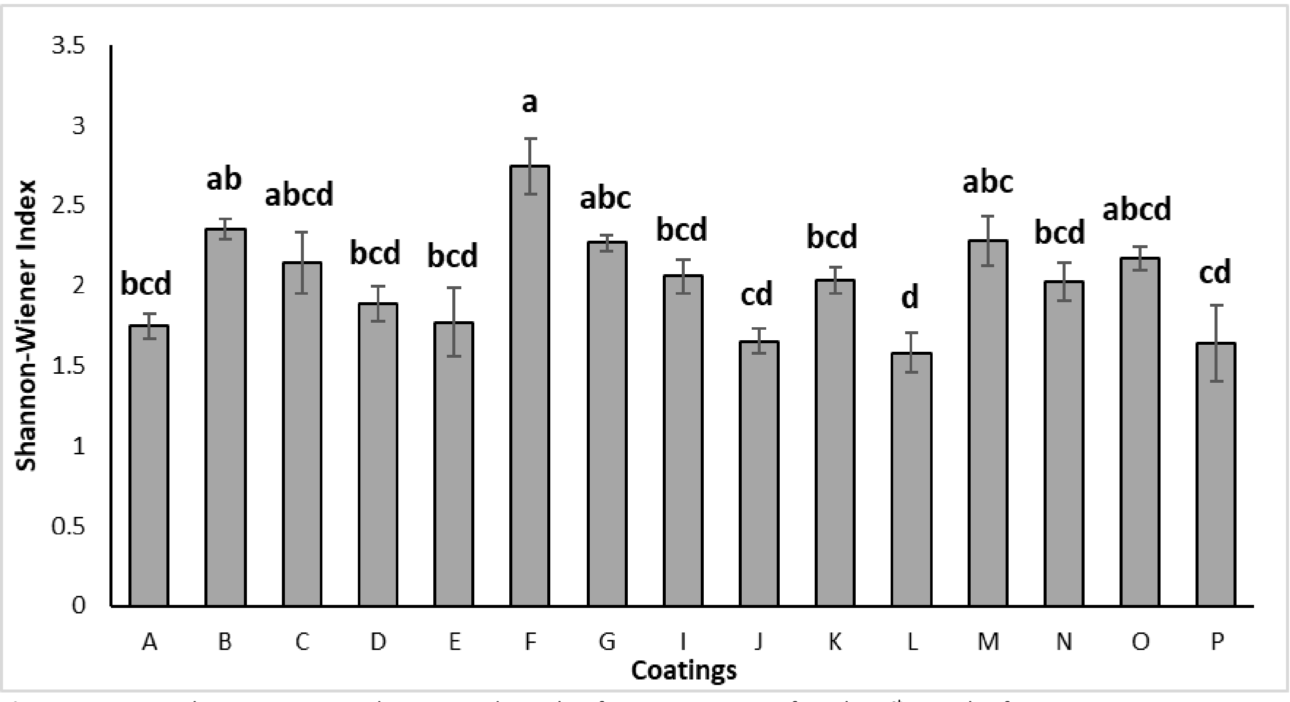


Fig. 15. Average Shannon-Wiener diversity index value for AST coatings after the 1st month of immersion. Average values denoted by a different letter indicate significant differences between coatings (*p*-value < 0.05). Error bars indicate standard error.

3.2.2. Beta diversity

Differences between the first month biofilm bacterial composition of the AST coatings based on the relative abundance of TRFs were visualised using a non-metric multidimensional scaling (NMDS) plot (Fig. 16).

The plot showed distinct separation of the community fingerprints of coating B (commercial biocidal coating) and P from other treatments (Fig. 16). This is further confirmed by pairwise comparisons performed using permutation multivariate analysis of variance (PERMANOVA; adjusted p-value < 0.05) which found significant differences between the diversity of treatments B and P and the other coatings. Coating A formed a separate cluster from other coatings in the AST coating array (Fig. 16). Statistical analysis by PERMANOVA showed that coating A had a significantly different composition from most coatings in the array, apart from coatings D, I and J. The tighter clustering of the data points of most of the coatings in the NMDS plot (Fig. 16) indicate that the bacterial TRF composition of most coatings were similar to one another as of one month of immersion.

TRFLP serves as an inexpensive and quick method for profiling the microbial communities on antifouling coatings. However, using the relative abundance of TRFs as a proxy for microbial species composition has been reported to lead to an underestimation of true community diversity firstly because multiple taxa can generate TRFs of the same length (Blackwood et al., 2007). Secondly, TRFs may be excluded if they fall outside of the size range that can be resolved by capillary electrophoresis, and thirdly, TRFs may not be detected if they fall below a certain fluorescence threshold (Blackwood et al., 2007). As such, while TRFLP may be used as a suggestion of the differences between community profiles of antifouling coatings, it should not be taken as the sole indicator of community composition.

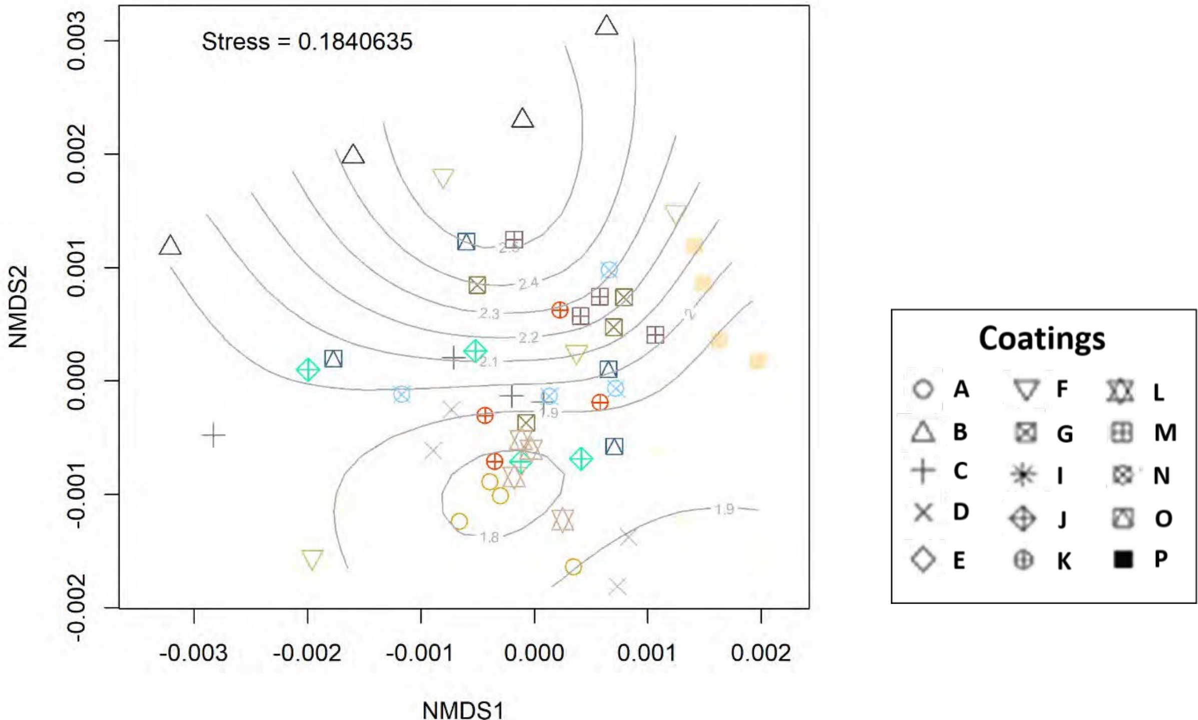


Fig. 16. Non-metric multidimensional scaling (NMDS) plot based on relative abundance of terminal restriction fragments from TRFLP analysis of biofilms sampled from AST coatings after the 1st month of immersion. Symbols denoting each coating are defined in the legend; they are colour-coded in the NMDS plot for easy distinction between treatments.

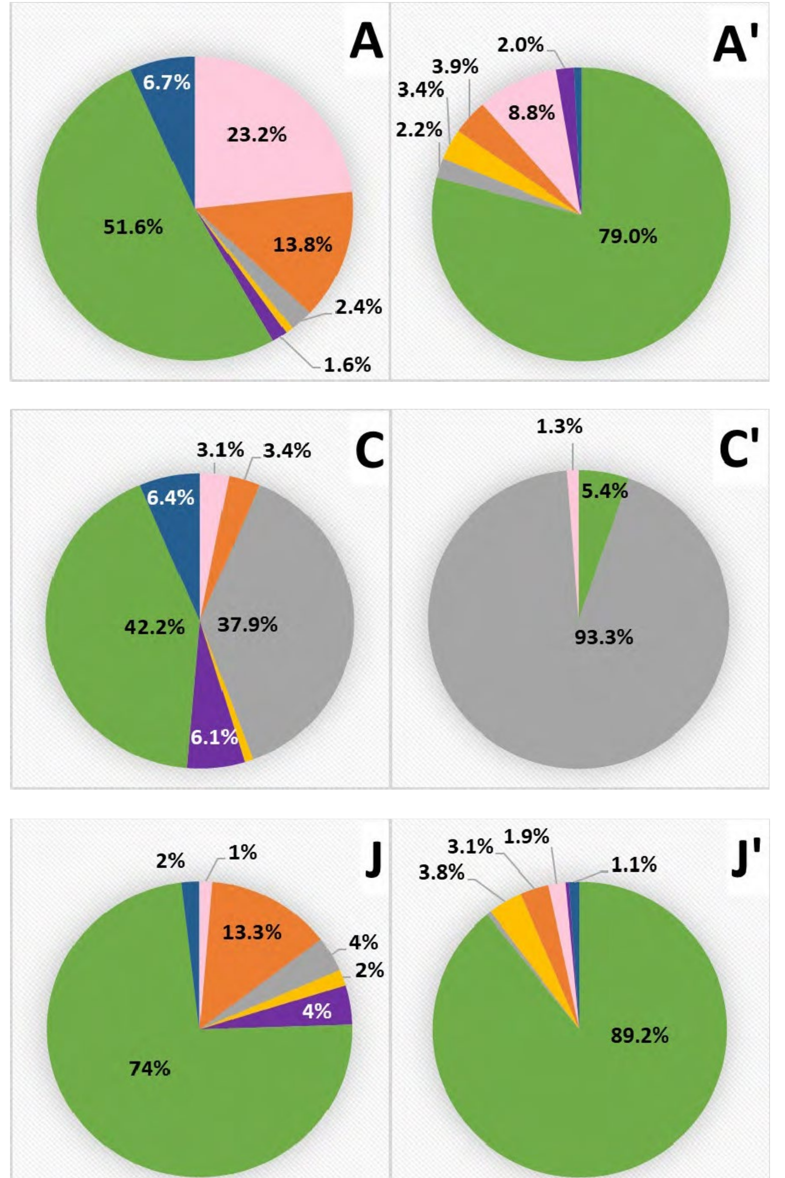
3.3. Nanopore sequencing

The bacterial communities of biofilms sampled from selected AST coatings were sequenced using the Nanopore MinION sequencing platform. The taxonomic profile of the bacteria found on each coating before and after waterjet cleaning was analysed at phylum and genus level based on relative abundance (Figs 17–19). It is worthwhile to note that the composition data presented here are representative of only one replicate of each of the selected AST coating.

Proteobacteria accounted for more than 50% of bacterial community composition on most coatings, except in coatings C and N (before and after waterjet), and O (after waterjet) (Fig. 17). Coating P, in particular, composed of more than 95% of Proteobacteria before waterjet (Fig. 17). Cyanobacteria contributed to more than 20% of community composition on coatings C, N and O before waterjet (Fig. 17). Compared to the rest of the coatings, C, N and O had higher relative abundance of the cyanobacterium *Schizothrix* sp. (Figs 18–19). Cyanobacteria that were not removed by the waterjet exercise accounted for 40% of community composition on coating N, and ≥90% on C and O (Fig. 17).

Based on the community composition before waterjet, coating B (commercial biocidal control) was observed to have a greater relative abundance of *Erythrobacter* sp., *Pseudomonas* sp., *Sphingomonas* sp. and *Novosphingobium* sp. bacteria than other coatings (Fig. 18). It is likely that the bacteria from these genera have a higher tolerance to biocidal compounds than other taxa recorded in this study. In particular, *Erythrobacter* sp. has been found to be associated with biofilms sampled from copper-based Interspeed BRA640 two weeks after immersion (Chen et al., 2013). Likewise, Papadatou et al., (2021) reported that *Erythrobacter* sp. bacteria was one of the most abundant genus on Intersmooth® 7460HS SPC coating.

Genus *Mesorhizobium*, which was found in highest abundance on Coating P before waterjet cleaning, showed a sharp decline of approximately 18% (141 reads), after waterjet cleaning. Since waterjet cleaning effectively removes any PCR inhibitors picked up in the biofilm sample, it is not surprising that certain bacterial genera show increases in relative abundance after waterjet cleaning (Fig. 19) as template strength and therefore, PCR success is improved after waterjet cleaning.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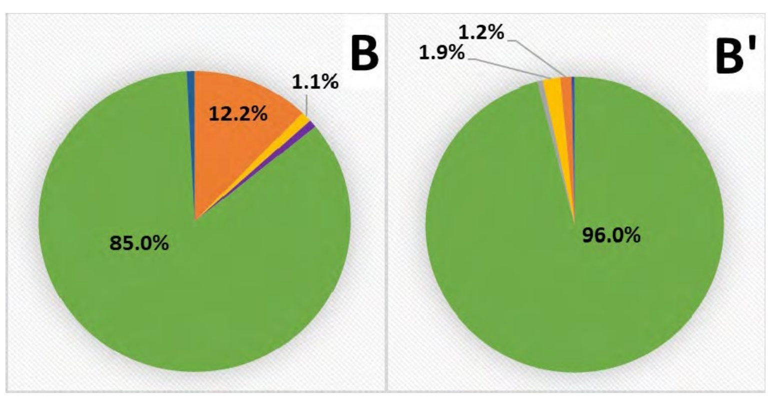
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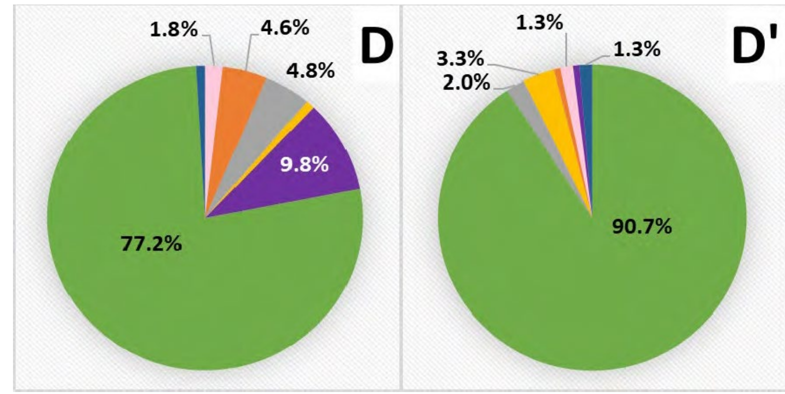
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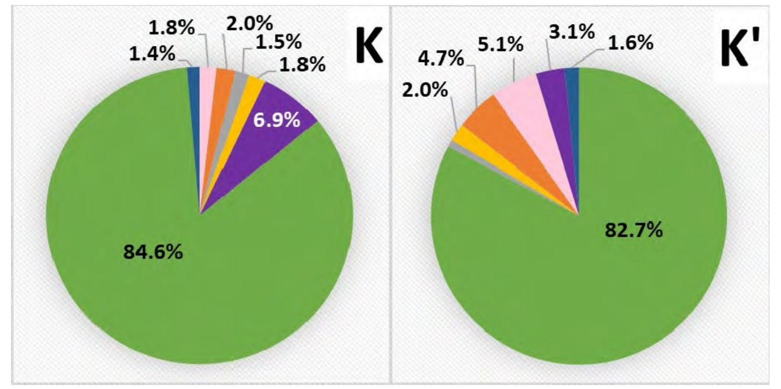

K
1.8%
2.0%
1.5%
1.4%
1.8%
6.9%
84.6%
K'
4.7%
5.1%
3.1%
1.6%
2.0%
82.7%

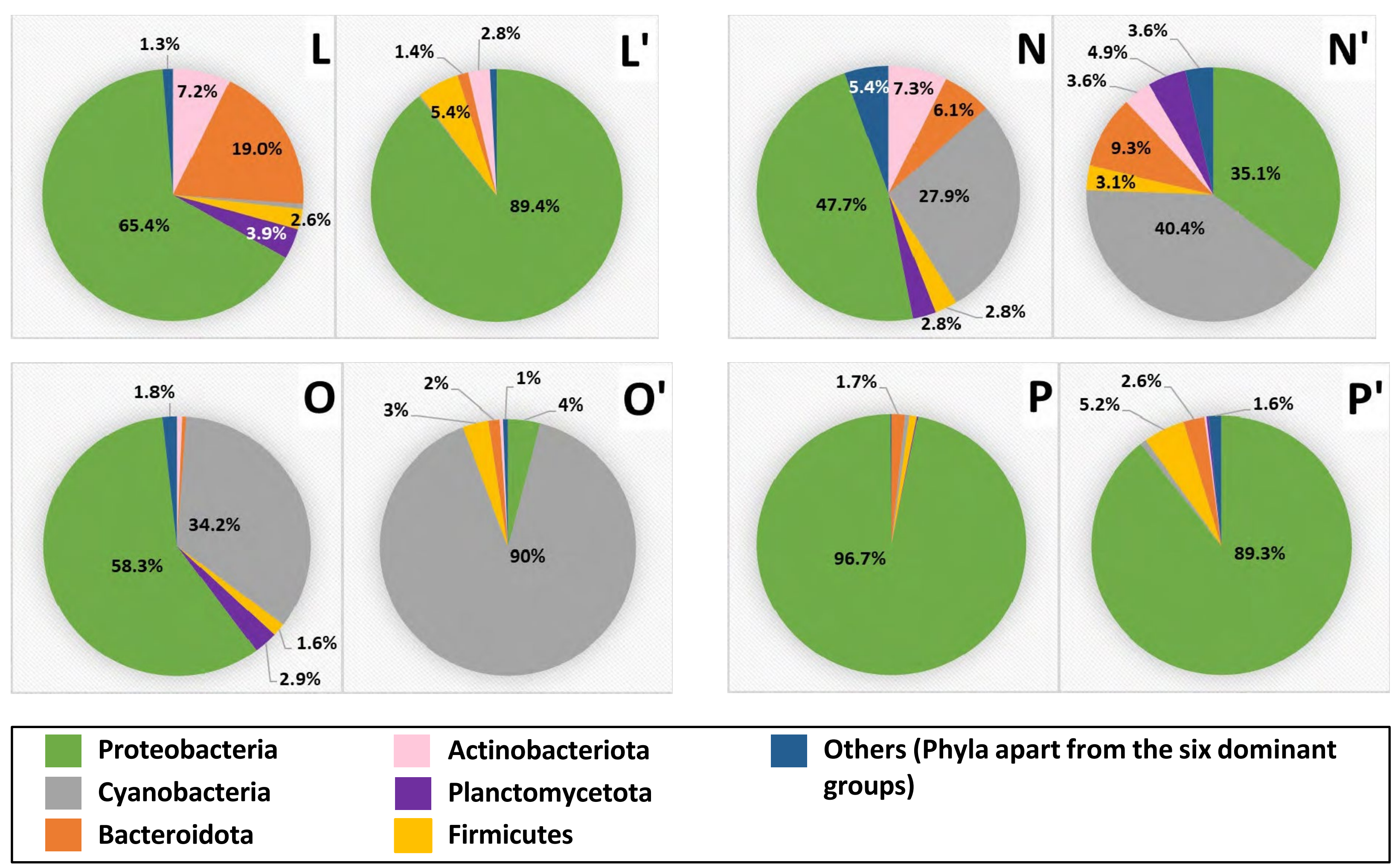


Fig. 17. Piecharts showing an overview of the contribution of the six most dominant bacterial phyla to the overall composition of the "before" (code) and "after" (code') waterjet biofilm communities. Other phyla apart from the six dominant groups are categorised as "Others".

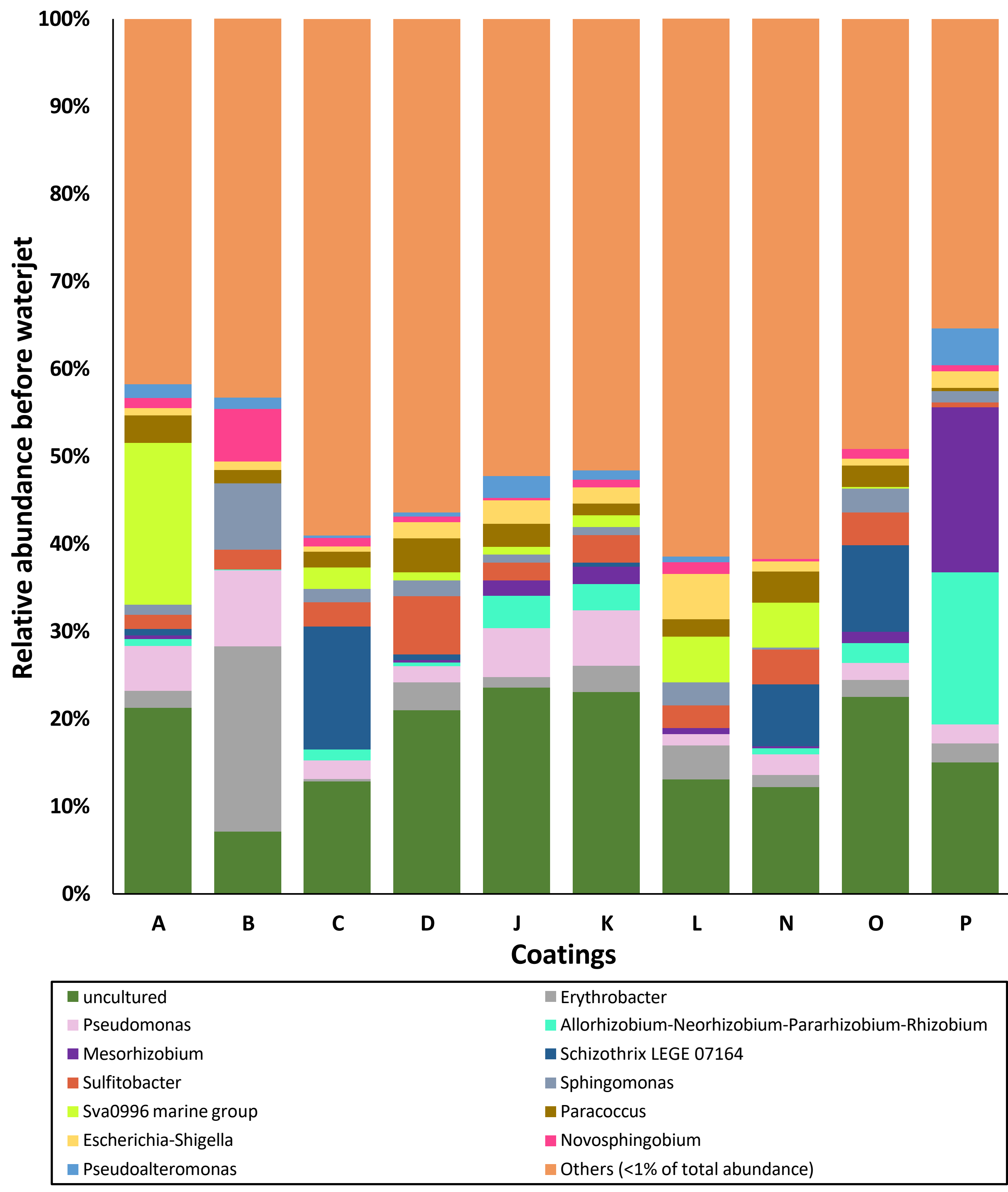


Fig. 18. Stacked bar chart showing the relative abundance of bacterial genera on each coating before waterjet cleaning based on Nanopore sequencing.

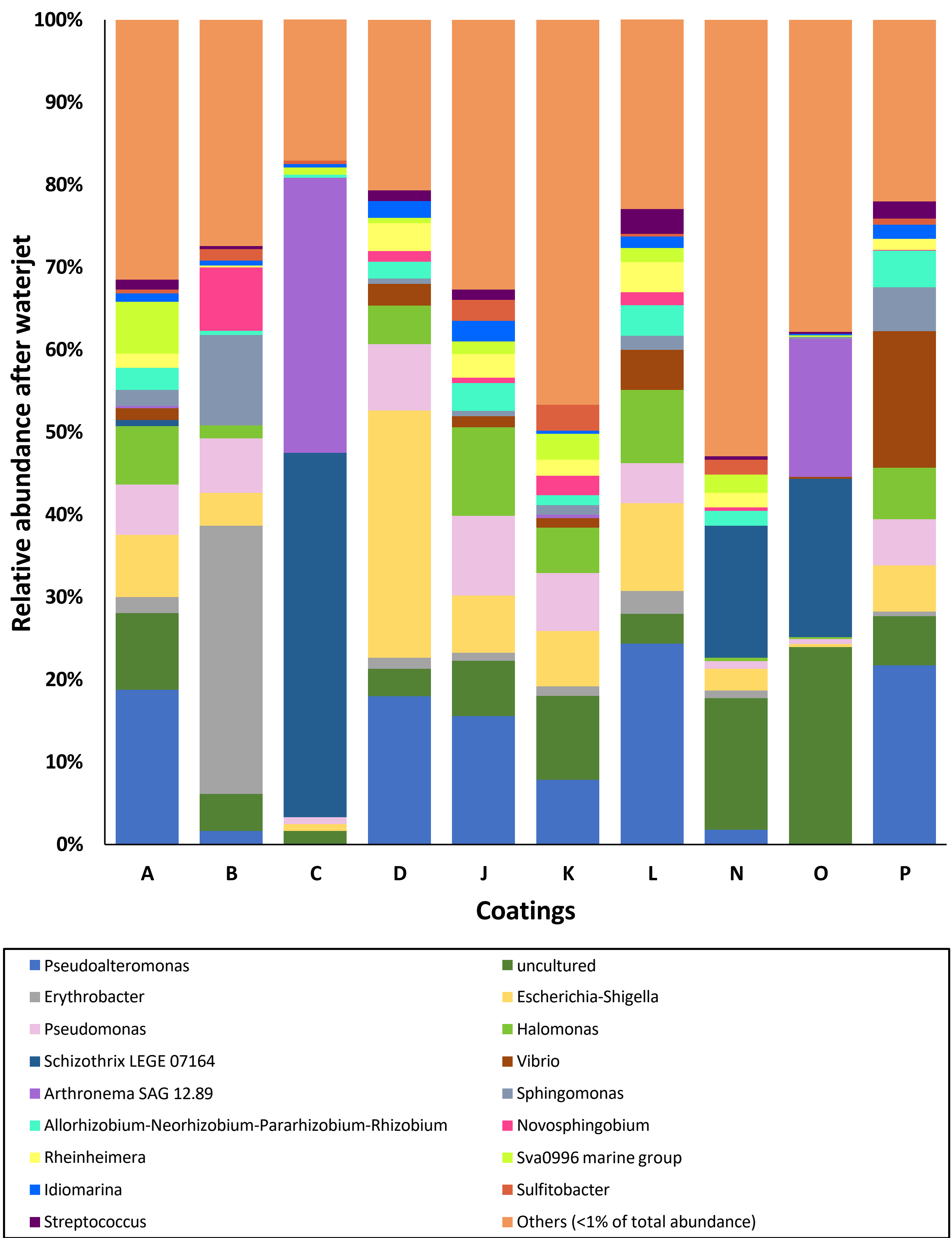


Fig. 19. Stacked bar chart showing the relative abundance of bacterial genera on each coating after waterjet cleaning based on Nanopore sequencing.

3.4. Settlement assays—effects of sediments on diatom and barnacle cyprid settlement

3.4.1. Diatom settlement assay

Halamphora coffeaeformis was used as a model organism in a settlement assay to evaluate the effect of exposing test substrates to sediments. Silastic T2 coverslips were used as the test substrate, with kaolinite (Sigma-Aldrich) used as model inorganic sediment particles.

The settlement of *H. coffeaeformis* on control Silastic T2 coverslips that were not exposed to kaolinite sediments (78.0 ± 9.3 diatoms/mm^2) was lower than the rest of the coverslips that were exposed to the sediment suspensions (Fig. 20). However, there were no significant differences observed in the number of settled diatoms among the treatments (ANOVA; $F = 3.37$; p-value > 0.05).

During preliminary trials, examination of Silastic T2 substrates that were "pre-conditioned" in kaolinite suspensions under a stereo microscope showed the adsorption of kaolinite particles on the substrates. Therefore, the adsorption of kaolinite particles onto the surface of the Silastic T2 coverslips may have promoted the attachment of *H. coffeaeformis* diatoms. The control coverslips, on the other hand, were not exposed to kaolinite particles and hence did not present an adsorbed layer of particles over which the diatoms seem to preferentially settle on, resulting in a lower number of settled diatoms. The results of our diatom adhesion assay are in line with the observations of Koc et al. (2019) who showed that particulate matter that accumulated on foul-release coatings functioned as attachment points for *Navicula perminuta* diatoms, thus promoting their settlement.

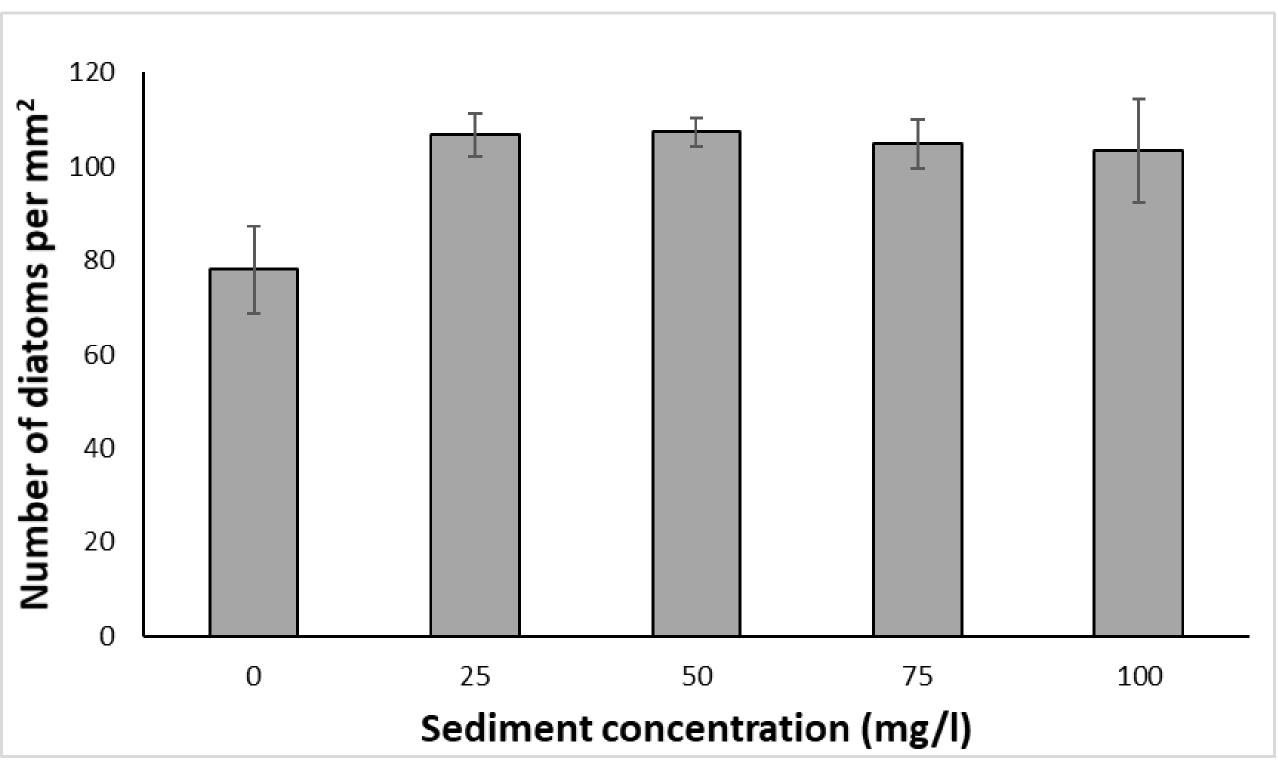


Fig. 20. Total number of *Halamphora coffeaeformis* cells settled per square millimeter on Silastic T2-coated coverslips "pre-conditioned" in 0 (no sediments; 0.22µm FSW only), 25, 50, 75 and 100mg/l kaolinite suspensions.

3.4.2. Barnacle cyprid settlement assays

3.4.2.1. Assay 1 Methodology: Effects of kaolinite loading at increasing concentrations on cyprid settlement

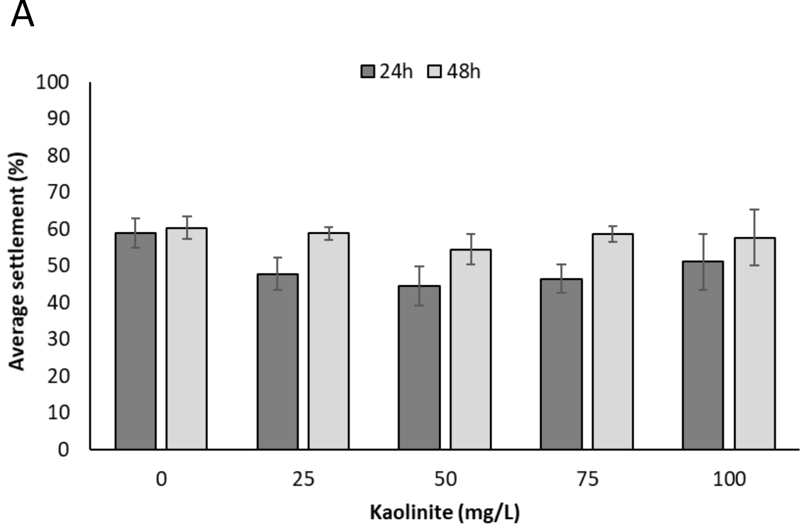
The effects of increasing concentrations of kaolinite loading (0, 25, 50, 75 and 100 mg/L) on *Amphibalanus amphitrite* cyprid settlement were examined. The assay was conducted in uncoated polystyrene 12-well plates and kaolinite (Sigma-Aldrich) was used as model inorganic sediment particles. The assay was repeated thrice.

Across all three runs of the assay, there was no clear relationship observed between increasing kaolinite concentration and average percentage settlement of *A. amphitrite* cyprids (Figs 21A—C). During the first run of the assay, cyprid settlement in the presence of kaolinite loading at 25 to 100 mg/L concentrations was not significantly different from cyprid settlement without kaolinite loading (0 mg/L; control) at both 24 and 48hr (Fig. 21A). Similarly, during the third run of the assay, no significant differences in cyprid settlement were observed between the different concentrations of kaolinite loading at 24 and 48hr (Fig. 21C).

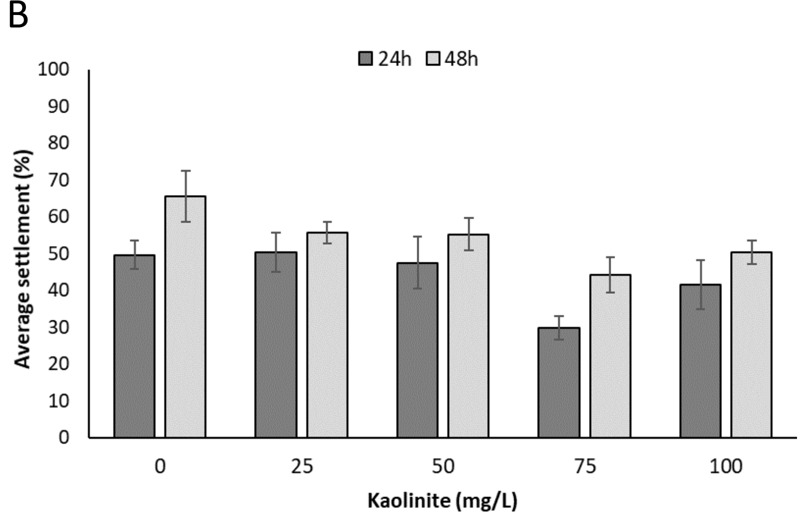
However, at the 48hr end point of the second run of the assay, cyprid settlement in 75 mg/L kaolinite suspension (44.2%) was found to be significantly lower than that in the control (65.4%; ANOVA; F = 7.83; p-value < 0.05; Fig 21B). It is worthwhile to note that total cyprid mortality at 48hr was highest across all treatments in the second run of the assay (~29—46%). In particular, cyprids exposed to kaolinite at 75 mg/L concentration (45.9%) experienced the highest mortality rate of all treatments in the second run (45.9%). The higher mortality of cyprids in the 75 mg/L kaolinite treatment would have inevitably led to a reduction in the number of surviving cyprids, thus resulting in significantly lower settlement at 75 mg/L during the second run. Higher cyprid mortality in the second run of the assay compared to the other two runs could be indicative of batch differences in the health of larvae spawned from the same parents.

In general, the results of the assay suggest that kaolinite loading does not affect the settlement of *A. amphitrite* cyprids (Figs 21A—C; Table 17). Cyprids were likely to have been exposed to kaolinite particles as a combination of suspended and deposited particles, given that the assays were conducted in well plates in the absence of aeration, which would have caused most of the kaolinite particles to settle out of suspension over the 24 to 48hr assay period. Most cyprids were observed to have settled at the corners and along the walls of the well plates. The walls of the well plates would expectedly have little to no sediments unlike the corners and bottom centre of the well plates. Since the cyprids were also found to have settled at the corners of the well plates, and occasionally around the bottom centre of the well plates, there is insufficient evidence to suggest that the deposited cyprids actively avoided settling on the kaolinite particles. On the other hand, the *A. amphitrite* cyprids also did not exhibit a preference to settle on deposited kaolinite particles. This contrasts with the results of Koc et al. (2019) who demonstrated the preference for *Navicula perminuta* diatoms to settle on particulate matter that accumulated on foul-release surfaces.

A



B



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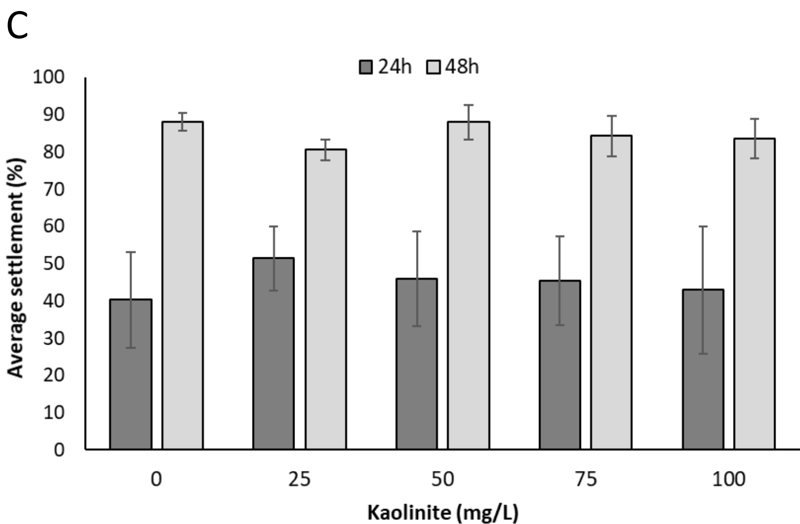


Fig. 21. Average percentage settlement of barnacle cyprids in the presence of increasing concentrations (0, 25, 50, 75 and 100 mg/L) of kaolinite loading at 24h and 48h. The settlement results of the first (A), second (B) and third (C) runs of this assay are presented here. Error bars indicate standard error.

Table 17. Summary of *Amphibalanus amphitrite* cyprid settlement assay results at 24 and 48hr.

Kaolinite concentration (mg/L)	**Average settlement ± standard error (%)**		
	Assay run 1	Assay run 2	Assay run 3
24hr			
0	59.0 ± 3.9	49.6 ± 3.9	40.3 ± 12.9
25	47.8 ± 4.4	50.3 ± 5.4	51.4 ± 8.6
50	44.6 ± 5.3	47.4 ± 7.1	45.9 ± 12.6
75	46.5 ± 3.9	29.7 ± 3.2	45.4 ± 11.9
100	51.1 ± 7.5	41.6 ± 6.7	43.0 ± 17.0
48hr			
0	60.4 ± 3.0	65.4 ± 6.9	88.0 ± 2.4
25	58.8 ± 1.8	55.6 ± 3.0	80.5 ± 2.8
50	54.5 ± 4.0	55.1 ± 4.4	87.9 ± 4.7
75	58.6 ± 2.1	44.2 ± 4.8	84.3 ± 5.4
100	57.7 ± 7.6	50.3 ± 3.3	83.6 ± 5.3

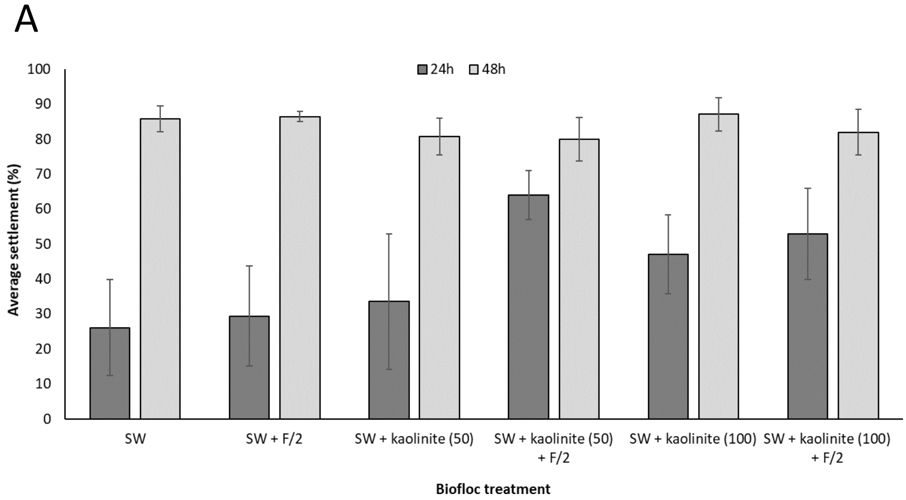
3.4.2.2. Assay 2 Methodology: Effects of biofilm formation on cyprid settlement

The effects of biofilm formation on *Amphibalanus amphitrite* cyprid settlement were examined by "pre-conditioning" individual wells of uncoated polystyrene 12-well plates with one of six treatments comprising different combinations of sand-filtered seawater (SW), kaolinite (50 or 100 mg/L) and F/2 media for 24hr. The assay was repeated thrice.

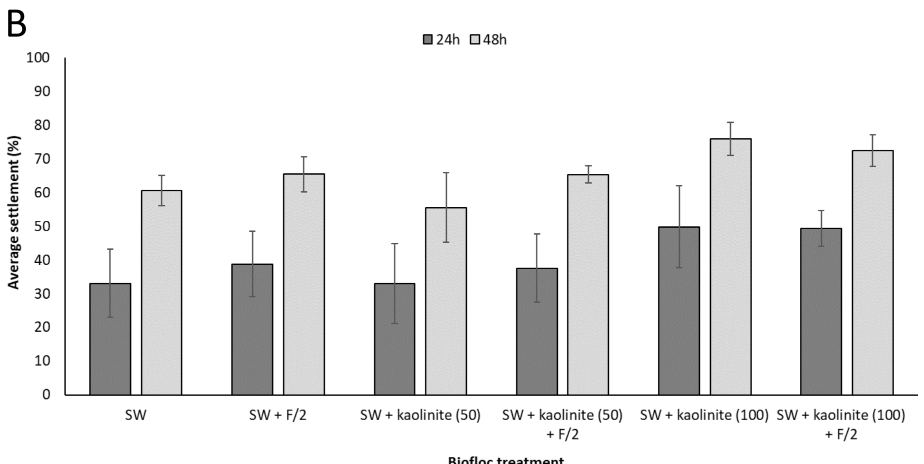
Cyprid settlement in wells pre-conditioned with sand-filtered seawater enriched with F/2 media did not differ significantly from those pre-conditioned with only sand-filtered seawater at 24 and 48hr, across all three runs of the biofilm assay (Figs 22A—C; Table 18). Likewise, F/2 media enrichment did not cause a significant difference in cyprid settlement in wells inoculated with kaolinite suspension at 50 and 100 mg/L concentrations across all assays, at both timepoints (Figs 22A—C; Table 18). Overall, there were also no clear patterns in the effects of the addition of kaolinite and F/2 media on cyprid settlement as opposed to conditioning the wells with only sand-filtered seawater (Figs 22A—C; Table 18).

The effects of mixed species, natural biofilms on the settlement of various barnacle species were summarised in a review paper by Rajitha et al. (2020). Previous studies have demonstrated that natural biofilms have contrasting effects on *Amphibalanus amphitrite* settlement, either promoting or inhibiting their settlement (Rajitha et al. 2020). This study however has showed that biofilms developed from different starting consortia comprising F/2 media and kaolinite particles have no effect on the settlement of *A. amphitrite* settlement. Tsurumi and Fusetani (1998) showed that cyprid metamorphosis increased with the age of biofilm, and dropped with increasing biofilm volume after reaching a peak. Cyprid settlement peaked at a minimum biofilm age of two days in their study (Tsurumi & Fusetani 1998). It is likely that the 24hr "pre-conditioning" duration that was incorporated in this assay was not sufficient for allowing the development of biofilms with significantly different microbial compositions among the treatments, that would be capable of generating an inductive or inhibitory effect on the settlement of *A. amphitrite* cyprids. A longer conditioning period of the wells may be needed.

A



B



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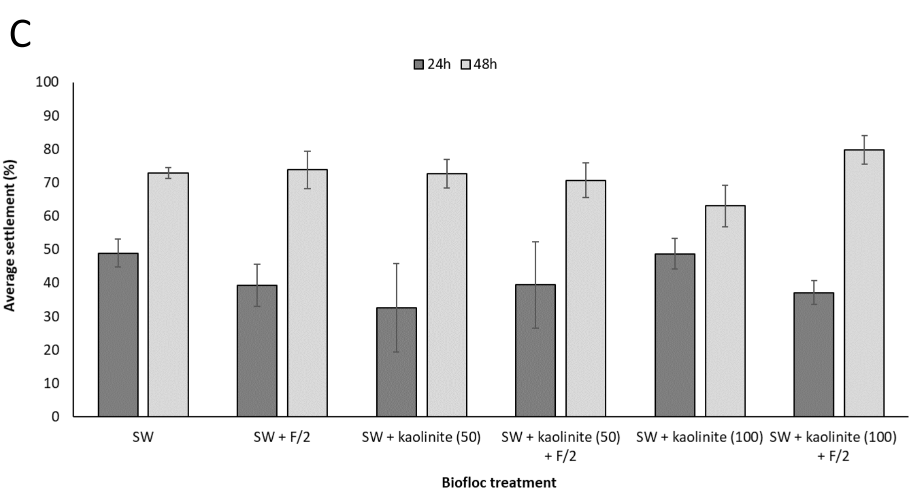

Fig. 22. Average percentage settlement of barnacle cyprids at 24h and 48h in response to six treatments that were used to pre-condition the 12-well plates for biofilm formation. SW=sand-filtered seawater only; SW + F/2=SW enriched with F/2 media; SW + kaolinite (50)=50 mg/L kaolinite suspension prepared in SW; SW + kaolinite (50) + F/2=50 mg/L kaolinite suspension prepared in SW enriched with F/2 media; SW + kaolinite (100)=100 mg/L kaolinite suspension prepared in SW; SW + kaolinite (100) + F/2=100 mg/L kaolinite suspension prepared in SW enriched with F/2 media.

Table 18. Summary of *Amphibalanus amphitrite* cyprid biofilm settlement assay results at 24 and 48hr. SW=sand-filtered seawater only; SW + F/2=SW enriched with F/2 media; SW + kaolinite (50)=50 mg/L kaolinite suspension prepared in SW; SW + kaolinite (50) + F/2=50 mg/L kaolinite suspension prepared in SW enriched with F/2 media; SW + kaolinite (100)=100 mg/L kaolinite suspension prepared in SW; SW + kaolinite (100) + F/2=100 mg/L kaolinite suspension prepared in SW enriched with F/2 media.

Biofilm treatments	Average settlement ± standard error (%)		
	Assay run 1	Assay run 2	Assay run 3
24hr			
SW	26.1 ± 13.7	33.1 ± 10.1	49.0 ± 4.1
SW + F/2	29.4 ± 14.3	38.8 ± 9.6	39.3 ± 6.3
SW + kaolinite (50mg/L)	33.6 ± 19.4	33.0 ± 11.8	32.6 ± 13.1
SW + kaolinite (50mg/L) + F/2	64.0 ± 7.0	37.6 ± 10.1	39.4 ± 12.9
SW + kaolinite (100mg/L)	47.1 ± 11.3	49.8 ± 12.2	48.8 ± 4.5
SW + kaolinite (100mg/L) + F/2	52.9 ± 13.0	49.4 ± 5.3	37.1 ± 3.6
48hr			
SW	85.8 ± 3.7	60.6 ± 4.5	72.8 ± 1.6
SW + F/2	86.5 ± 1.4	65.4 ± 5.2	73.8 ± 5.5
SW + kaolinite (50mg/L)	80.8 ± 5.2	55.6 ± 10.4	72.6 ± 4.3
SW + kaolinite (50mg/L) + F/2	80.0 ± 6.2	65.3 ± 2.6	70.7 ± 5.2
SW + kaolinite (100mg/L)	87.1 ± 4.7	75.9 ± 4.8	63.0 ± 6.3
SW + kaolinite (100mg/L) + F/2	82.0 ± 6.6	72.4 ± 4.8	79.8 ± 4.2

3.5 Discussion

The aim of the biofilm studies was to determine if assessment of biofilms would be useful for predicting the long term performance of novel antifouling coatings. As noted in Section A, as technology development matures, the time taken for different coating formulations to separate out in terms of preventing macrofouling growth may take months to years. Biofilm communities vary hugely in time and space. At the same time, it is known that different substrates may harbour unique microbiomes, depending on the environmental/ecological circumstances. This preliminary study sought to assess if there may be clues in the biofilms that may provide some insight into the long term performance of the different coatings.

Sampling of biofilms from the Adaptive Surface Technologies (AST) field test array was conducted after the 1st, 3rd and 6th month of its immersion. Sampling of biofilms was done before water jet cleaning at all three timepoints. Additionally, at the 6th month time point, sampling was also done after water jet cleaning. With 60 panels in the array, this activity yielded a very large number of DNA samples. To date, we were only able to sequence and analyse a subset of samples. The sampling effort highlighted the logistical challenges in sampling biofilms from large numbers of panels in the field.

It was observed that the different coatings were covered with different biofilms, both in terms of thickness and quality (color, texture). An initial attempt was made to quantify the biofilm over a fixed area using crystal violet staining. Due to the large amount of water in the slimes removed, there was too much variation between replicates. We concluded that biovolume did not provide an efficient way to separate coatings.

Molecular tools (DNA fingerprinting and Next-Generation Sequencing) were employed to evaluate whether an analysis of the microfouling community may provide useful indicators of its antifouling properties. In general, the molecular tools such as TRFLP fingerprinting was able to distinguish the coatings based on their microbial composition, which may arise from differences in its chemistry. The Oxford Nanopore Minion approach provided information on the community. The results are reported above. However, due to the maximum capacity of the barcoding kits and MinION flowcell we used, we were not able to accommodate too many samples at once. There was insufficient information to draw an association with macrofouling. Subsequently, we pursued Illumina HiSeq platform which is a short read approach, but we were able to run the entire array samples. The analysis of this very large data set is in progress.

Based on the above, we believe that the biofilms may provide indicators of the biological activity of the materials. The next steps may be to apply functional metagenomics to study the relationships between the micro- and macro-fouling.

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