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Comparative study of biofilm formation on biocidal anti-fouling and fouling-release coatings using next generation DNA sequencing

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Abstract

The bacterial and eukaryotic communities forming biofilms on six different antifouling coatings, three biocidal and three fouling-release, on boards statically submerged in a marine environment were studied using next generation sequencing. Sequenced amplicons of bacterial 16S ribosomal DNA and eukaryotic ribosomal DNA internal transcribed spacer were assigned taxonomy by comparison to reference databases and relative abundances were calculated. Differences in species composition, bacterial and eukaryotic, and relative abundance were observed between the biofilms on the various coatings; the main difference was between coating type, biocidal compared to fouling-release. Species composition and relative abundance also changed through time. Thus, it was possible to group replicate samples by coating and time point, indicating that there are fundamental and reproducible differences in biofilms assemblages. The routine use of next generation sequencing to assess biofilm formation will allow evaluation of the efficacy of various commercial coatings and the identification of targets for novel formulations.

Keywords: Biofilms; biocidal anti-fouling; fouling-release; 16S; ITS; next generation sequencing

Abbreviations: BAF, biocidal anti-fouling; FR, fouling-release; ITS, internal transcribe spacer; NGS, next generation sequencing; NIS, non-indigenous species; OTU, operational taxonomic unit

Introduction

Surfaces submerged in a marine environment quickly become colonised by marine organisms. Although an oversimplification, this process can be seen as a progression that begins with the formation of a conditioning film as proteins, polysaccharides and glycoproteins accumulate on a clean surface within minutes of being immersed (Cooksey and Wiggleworth-Cooksey 1995; Dang and Lovell 2000). Bacteria and other microorganisms then begin to accumulate forming a microbial biofilm. This docking or primary adhesion phase is considered reversible and corresponds with the serendipitous meeting between a conditioned surface and planktonic microorganisms (Dunne 2002). This is followed by a locking phase where microorganisms, principally bacteria, become irreversibly anchored to the surface due to the production of exopolymers (An et al 2000; Toyofuku et al 2016). These biofilms (slimes) become colonised by fungi, micro-algae and protozoa and then by macroalgae and larvae of invertebrates. These latter stages occur over several days and weeks with rates dependent on biotic (propagule pressure) and abiotic factors including light and temperature (Cao et al 2011).

In the shipping industry, the fouling of ship hulls leads to a significant increase in frictional drag and the need to increase power to maintain a given speed (Shultz 2007). The resultant increase in fuel consumption has both a negative economic impact on the industry and an environmental cost due to increased emissions of greenhouse gases (e.g., CO_2) and pollutants (nitrogen and sulphur oxides) (Schultz et al. 2011). Fouling also facilitates the transport of species between environments creating the potential for the invasion by non-indigenous species (Sweat et al. 2014). Many vessels, therefore, require periodic cleaning resulting in costly down time.

To combat biofilm formation, antifouling coatings are applied to ship hulls. These coatings fall into two main categories: biocidal coatings and fouling-release coatings. Biocidal coatings function through the release of toxic chemicals to deter the settlement and growth of organisms. The most advanced of these coatings are based on self- or linear-polishing polymer technologies which facilitate the sustained delivery of biocide by seawater-mediated hydrolysis or ion exchange reactions. Since the ban on tributyltin in 2003 (Champ 2003; Yerba et al. 2004), copper is the most commonly used biocidal agent. Copper, which is an essential element for most organisms, can have adverse effects on survival, growth and reproduction when it accumulates above certain limits (https://www.epa.gov/wqc/aquatic-life-criteria-copper).

Although biocidal coatings still dominate the market, accounting for more than 90% of coating sales (Muthukrishnan et al. 2017), concerns over the environmental impact of antifouling biocides have

led to increased attention being paid to the development of biocide-free approaches to fouling control (Gittens et al. 2013). Foul-release coatings, the commercial development of which did not take off until after the first bans on TBT-based coatings (Yebra et al. 2004), rely on the modification of surface physical and chemical properties to both minimise adhesion of organisms and facilitate their removal by water flow (Finnie and Williams 2010; Bixler and Bhushan 2012).

In this study, in order to highlight potential differences between the fouling communities able to form on biocidal antifouling coatings (BAF) and fouling-release coatings (FR), we sampled biofilms from three different commercial coatings of each type in a static, time course experiment carried out at Hartlepool Marina in northeast England. The microorganisms that form biofilms are difficult or impossible to identify morphologically as most cannot be cultured - an estimated 85-99% of bacteria and archaea cannot be grown in the laboratory (Staley and Konopka 1985; Lok 2015; Solden et al. 2016). However, one can gain insight into the organisms present in a community through sequencing marker genes amplified from the DNA extracted from environmental samples (Leary et al. 2014; Muthukrishnan et al. 2014; Sathe et al. 2017). Such an approach circumvents the problems of identification and may even highlight the presence of 'unknown' organisms. Thus, identification of colonising microorganisms was performed using multiplexed, next generation sequencing (NGS) of bacterial 16S rRNA genes (Lee et al. 2008) and eukaryotic internal transcribed spacer (ITS) regions (Buckheim et al. 2011; Schoch et al. 2012; Stern et al. 2012; Guo et al. 2017). Given that the genes chosen are an essential component of the translational mechanism of all organisms they offer a broad, although not universal, barcode for taxonomic assignment: their previous characterisation in a very wide range of taxa allows the identification of most microorganisms in the biofilm.

Materials and methods

Experimental site and coatings

Six different coatings, three biocidal antifouling and three fouling-release, produced by AkzoNobel/International Paint Ltd, were used in this study.

Biocidal antifouling coatings:

1. Intersmooth® 7460HS (a high solids, high performance, low friction, self-polishing co-polymer)
2. Intersmooth® 7465Si (a high performance, low friction, self-polishing co-polymer coating based on silyl acrylate polymer technology)
3. Intercept® 8000 (a high performance, low friction, linear polishing polymer incorporating a unique patented Lubyon® technology)

Referred to, respectively, as BAF1, BAF2 and BAF3. The biocides in all three coatings are cuprous oxide and copper pyrithione.

Foul-release coatings:

1. Intersleek® 700 (a silicone-based coating)
2. Intersleek® 900 (a fluoropolymer-based coating)
3. Intersleek® 1100SR (an advanced fluoropolymer-based coating)

Referred to, respectively, as FR1, FR2 and FR3.

The product information cards for these six coatings can be obtained from the AkzoNobel web site: https://www.akzonobel.com/products/paints-and-coatings

The chosen test site was Hartlepool Marina in the UK (Latitude 54.69195, Longitude -1.20007). On the 2nd December 2014, twelve 61cm square, wooden boards were secured to metal frames and, in bays on a floating test facility, immersed vertically in the Marina to a depth of 1m. The water in this area is 7-8m deep and experiences low flow rates and minimal traffic (salinity and water temperature at Hartlepool Marina during the period December 2014 to June 2015 are given in Supplementary File 1). Each board supported six replicate, square panels (9cm x 9cm) of each coating following a Latin square design to control for spatial variation (Figure 1); prior to application of the coatings, the wooden boards were coated with a primer. All coatings were applied at

AkzoNobel's Marine Coatings Laboratory (AkzoNobel, International Paint Ltd, Felling, UK) as a full coating scheme comprising primer, tie coat and antifouling coating. The accumulated slime on the panels was sampled at four time points, 5th March, 30th March, 28th April and 8th June 2015, corresponding to 94, 119, 148 and 189 days of immersion, respectively; for each time point, the slime from three boards were sampled and analysed separately (biological replicates). Using a plastic scraper and flocked sterile swabs, all the slime from the six panels of a single coating type was removed and pooled in one sample tube.

In total, 72 slime samples were collected for analysis. Unfortunately, all samples from Board 1159 (harvested on March 5th 2015) were damaged in transport from the sample site to the laboratory and so couldn't be processed for either 16S or ITS analysis. Thus, only 66 slime samples were used in the study.

DNA extraction

DNA was extracted from each pooled sample using Power Biofilm® (Qiagen) extraction kits according to manufacturer's recommendations. For each pooled sample (the six replicate patches of a single board), the concentration of DNA was determined using a NanoDrop™ (Thermo Fisher). The DNA, which was taken up in elution buffer, was stored at -20°C prior to use.

Amplification of 16S rDNA gene

Before amplification, DNA samples were diluted to 10ng μl^{-1}. The primers used for the amplification were taken from Muyzer et al (1993) and were as follows:

forward 5'-CCTACGGGAGGCAGCAG-3'

reverse 5'-ATTACCGCGGCTGCTGG-3'

These primers correspond to positions 314 – 330 (forward primer) and 491 – 507 (reverse primer) in *E. coli* strain NBRC 102203. To both primers, a four base-pair barcode was added at the 5' end. Six different barcodes were used for the forward primer and six for the reverse primer. This allowed 36 different DNA samples to be multiplexed and run simultaneously during the sequencing reaction. All primers and their barcodes were synthesised by Eurofins Genomics (Ebersberg, Germany).

Amplification was carried out in a 20 µl reaction volume using 2X Phusion™ High-Fidelity Master Mix (ThermoFisher Scientific) according to manufacturer's recommendations. After amplification, 5 µl aliquots of sample were run on a 1.5% w/v agarose gel to ensure that amplification had been successful. The 16S amplicons were sequenced in the University of Bristol Genomics Facility on a 318 Chip using an Ion PGM Next Generation DNA Sequencer (ThermoFisher Scientific) according to manufacturer's standard protocol.

Amplification of ITS loci

Before amplification, DNA samples were diluted to 10ng μl^{-1}. For amplification of the ITS region, samples were PCR amplified using TruSeq-tailed primers (Illumina). The primers used for the amplification were taken from Ristaino et al. (1998) and are as follows:

forward 5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGTCCGTAGGTGAACCTGCGG-3'

reverse 5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGCTGCGTTCTTCATCGATGC-3'

In each case, after amplification, aliquots of samples were run on 1.5% agarose gels to ensure that amplification had been successful. Samples were Truseq barcoded then sequenced in the University of Bristol Genomics Facility using the Illumina MiSeq with a 2x300 cycle run.

Data analysis

The 16S sequences, with their custom four-base barcode combinations, were de-multiplexed using a PERL script to pool sequences according to their leading and trailing four bases. Sequences which did not match expected barcode combinations (due to errors) were discarded. The four base barcodes were designed such that a single sequence error would render the barcode ambiguous, but would never convert it into one of the alternatives used (i.e. an edit distance of at least two separated our primer barcodes). ITS sequences with TruSeq barcodes were automatically de-multiplexed by the Illumina RTA software at the end of the final cycle. Sequences were quality trimmed to PHRED 20 using the program fastq-mcf which is part of the ea-utils package (expressionanalysis.github.io/ea-utils/).

Sequences, both 16S and ITS, were analysed using the open source software package, QIIME (qiime.org; Caporaso et al. 2010). For the 16S samples, operational taxonomic units (OTUs) were identified using the USEARCH (Edgar 2010) option with minimum size set to 3 and reverse strand matching enabled; sequences were clustered using the default setting of 97% sequence similarity. OTUs were assigned a taxonomy by reference to the Greengenes 16S rRNA gene database (DeSantis et al. 2006). The steps used in the QIIME pipeline were as follows: a representative sequence was chosen for each OTU using the script *pick_rep_set.py*; taxonomy was assigned to the representative sequences using *assign_taxonomy.py*; the representative sequences were aligned to the Greengenes core dataset using PyNAST (*align_seqs.py*); alignments were filtered for gaps (*filter_alignment.py*); a phylogenetic tree was constructed using the resulting sequences (*make_phylogeny.py*); a table of OTUs and their abundance was produced (*make_otu_table.py*). Alpha rarefaction plots were produced using the script *alpha_rarefaction.py*. Finally, jackknifed beta diversity analysis was performed using the script *jackknifed_beta_diversity.py.*

There is no recognised rDNA-ITS database within QIIME. Thus, prior to analysis, an rDNA- ITS database was created from sequences down-loaded from the NCBI website using a text search for rDNA-ITS (Supplementary File 2). Sequences were obtained using naïve BLAST searches (i.e., no sequence information) with the search terms 'algae', 'protozoa' and 'plankton'. Essentially, the same QIIME pipeline as that for the 16S analysis was used but with the following modifications: taxonomy was assigned to the representative sequences using *assign_taxonomy.py* with the -m flag set to blast; and, given that no phylogenetic tree is available when taxonomy is assigned by BLAST, beta diversity was calculated using the script *beta_diversity_through_plots.py* with the metrics in the parameters file set to Bray-Curtis and Euclidean diversity.

Statistical analysis

Prior to statistical analysis, unassigned sequences were removed the data set. Also removed were taxa that were found on less than 4 panels, and those that had a mean relative abundance across all panels of < 0.1%. After unassigned sequences and rare taxa were removed, relative abundance was corrected so that the sum for each panel was 100%. On this reduced data set, two-way ANOVA was performed for taxa relative abundance at the highest (phylum) and the lowest (at best this was to genus, but not all OTU were identified to this level) taxonomic rank. Relative abundance scores were grouped by coating (FR1, FR2, FR3, BAF1, BAF2, BAF3), time in days (94, 119, 148, 189) and coating*time interaction. Bonferroni correction for multiple testing was performed for all scores.

Principle component analysis was carried out and plots produced using the workflow script *jackknifed_beta_diversity.py* in QIIME. To test for significant differences in biofilm communities between coatings, coating type (BAF vs FR) and time, ANOSIM analysis was carried using the script *compare_categories.py*; comparisons were made with groups based on coating, coating type and time. To identify the species that are most important in creating the observed pattern of dissimilarity between groupings, SIMPER analysis was carried out using the package VEGAN in R.

Results

The amplicons from the 16S rDNA gene were, as expected, approximately 200 base pairs in length. The ITS amplicons were of more variable length (approximate range 250 – 500 bp) with most samples having more than one band: this was not unexpected for mixed population samples where ITS spacer lengths exhibit significant length variation in our reference database. Of the 66 samples available for 16S analysis, only 58 samples gave adequate results from sequencing to perform QIIME analysis: 1 sample from 31st March (1162C) and 7 samples from 8th June (1018A and B; 1167A, C, D, E and F) failed, probably due to poor DNA amplification. Thus, results are based on 58 samples for the 16S analysis and 66 samples for the ITS analysis.

Number of taxa identified

We determined the level of alpha diversity in our samples using the QIIME workflow script *alpha_rarefaction.py*. The rarefaction curves plateaued indicating that we had sufficient sequence depth to capture most of the diversity in the samples. Within the QIIME analysis pipeline, amplicons derived from both bacterial 16S and eukaryotic ITS regions were clustered at 97% similarity and then assigned to OTUs. A small percentage of amplicons could not be assigned taxonomy (0.9% and 2.7% for 16S and ITS amplicons, respectively) and were removed from analysis. The remaining amplicons were assigned taxonomy. From the 16S analysis, 28 bacterial phyla were identified: 16 of these were recognised phyla (have representatives that can be grown in culture) and eight (Supplementary File 3) were candidate phyla (phyla containing bacteria that, to date, haven't been cultured and that are known solely through DNA sequence analysis). At the lowest taxonomic ranking (at best, classified to genus) 457 bacterial taxa were identified. From the analysis of ITS amplicons, 17 eukaryotic phyla and 243 taxa at the lowest taxonomic rank were identified. The average number of taxa on the six coatings (averaged over all four time points) and at each time point (averaged over all six coatings) is shown in Figure 2. A greater number of taxa, both bacterial and eukaryotic, were present on the FR coatings than on the BAF coatings. The average number of bacterial taxa per panel was 204 and 141 on the FR and BAF coatings, respectively. The average number of eukaryotic taxa was 123 and 104, respectively. Certain taxa were found exclusively on one coating type with more such taxa being found only on the FR coatings; the difference was more marked on the for bacterial taxa than eukaryotic taxa (Figure 2a and b). Fifty bacterial and 12 eukaryotic taxa were found exclusively on FR coatings and only 3 bacterial and 7 eukaryotic taxa were found exclusively

on BAF coatings. Other taxa showed a bias toward one or other of the coating types; in the majority of cases, the bias was in favour of presence on FR coatings (Figure 3 and Supplementary File 4).

Relative abundance

Across all panels, only three of the 28 bacterial phyla identified, Bacteriodetes (54.6%), Proteobacteria (38.5%) and Verrucomicrobia (6.2%), had a relative abundance of > 1.0% (Table 1). All other phyla had relative abundance of < 0.5%. At the lowest taxonomic ranking, only 16 taxa had relative abundance of > 1.0% (Table 2) whilst the majority (406 of 457) had relative abundance of < 0.1% (Table 1 and Supplementary File 4). The dominant OTUs were *Winogradskyella* (16.5%), an unidentified member of the order Methylococcales (15.8%), and two unidentified members of the family Flavobacteriaceae (15.3% and 9.3%).

Only 8 of the 17 eukaryotic phyla identified had relative abundancies > 1%. The dominant phyla were the Bacillariophyta (39.2%), Dinoflagellata (18.1%) and the Chlorophyta (14.2%) (Figure 4a). Of the 243 eukaryotic taxa identified to the lowest ranking, only 19 had relative abundancies of > 1% (Figure 4a and Supplementary File 4). The relatively most abundant taxa were the diatoms *Navicula* (20.3%), *Amphora* (8.6%) and *Sellophora* (4.4%), the dinoflagellate, *Amoebophyra* (15.9%) and, respectively, the green and red algae *Chlorothrix* (4.9%) and *Dilsea* (4.0%) (Figure 4b and Supplementary File 4).

Cluster analysis

Within the QIIME analysis pipeline, samples were clustered using principal coordinate analysis (PCO) based on taxa present and their relative abundances. This showed that, although the individual coatings didn't cluster (Figure 5a and d), panels clustered by coating type (Figure 5b and e). This was more pronounced for the bacterial components of the biofilm (Figure 5b) than for the eukaryotic components (Figure 5e). ANOSIM analysis of the bacterial data showed there to be a small difference between the individual coatings with an R value of 0.27 (p = 0.001), but a more distinct difference between coating type (the three biocidal coatings compared to the three fouling-release coatings) with an R value of 0.41 (p = 0.001). The ANOSIM values for the eukaryotic data were, by contrast, 0.13 and 0.15 (p = 0.001).

Significant differences between coatings

The majority of taxa were present at very low relative abundance and observed on a small number of panels and may represent serendipitous encounters with individual panels or false positives due to sequencing errors; these 'taxa' were not considered in the analysis. For both bacteria and eukaryotes, only those taxa with relative abundance of 0.1% or greater were carried forward for further analysis. Thus, 51 bacterial taxa and 56 Eukaryotes were used for statistical analysis (Supplementary File 5).

Bacteria

Some taxa had quite different levels of relative abundance on the two coating types. At the level of phylum, the taxa that were significantly different between the coatings were the Bacteroidetes (Bonferroni *p*-value 0.0005) and Proteobacteria (Bonferroni *p*-value 0.0085) (Supplementary File 5). The statistical differences with regard to the Bacteriodetes were confined to comparisons between coating BAF3 and all other coating either BAF or FR; this coating, of all six studied, had the highest relative abundance of taxa belonging to the Bacteriodetes; Table 1 shows the relative abundance of these phyla on the six coating types. At the lowest taxonomic ranking (at best classification to genus), 12 taxa were significantly different between coatings (Table 3). In most cases where there was a significant difference between coatings, relative abundance was higher on the FR coatings than the BAF coatings. The Gram-negative bacterium *Lewinella*, was an example of this pattern (Figure 6a). Only two taxa, an unclassified member of the family Flavobacteriaceae and *Winogradskyella*, had higher relative abundance on the BAF coatings than the FR coatings. In SIMPER analysis, the greatest dissimilarity between coatings was generated by Winogradskyella (15.7% of dissimilarity) and the unidentified member of the Flavobacteriaceae (14.0%) that had greater relative abundance on FR than BAF coatings (Tables 2 and Supplementary File 4).

Eukaryotes

At the level of phylum, the only taxon that proved to be statistically different in abundance between coatings was the Apusozoa (Bonferroni *p*-value 0.02). Although this was more abundant on the FR

coatings than on the BAF coatings it had a relative abundance of only 0.8% (Supplementary Files 4 and 5).

At the lowest taxonomic ranking, there were very few significant differences between the coatings. The only taxa that were significantly different were the pennate diatom, *Amphora*, which had a greater relative abundance on the BAF coatings than on the FR coatings, and the amoeba-like *Micronuclearia* (a member of the phylum Apusozoa), which, although present at very low relative abundance (0.15%), was more abundant on the FR coatings than on the BAF coatings. SIMPER analysis indicated that the most abundant taxa, and particularly *Amoebophyra*, *Navicula* and *Amphora*, were the major contributors to the differences between coating types explaining 15.2%, 14.0%, 9.5% of the difference, respectively.

Changes through time

The relative abundance of the bacterial phyla on the six different coatings showed significant changes over time for both the Bacteroidetes and the Proteobacteria. That is, while the relative abundance of the Bacteriodetes increased through time (Bonferroni *p*-value 0.00002) that of the Proteobacteria declined (Bonferroni *p*-value 0.002). There were no significant interactions between coating and time at this taxonomic level. At lower taxonomic ranking, fourteen taxa, all belonging to the Bacteriodetes and Proteobacteria, changed in relative abundance through time (Table 3 and Figure 6). For example, although the relative abundance of *Winogradskyella* at 94 days was high on the BAF coatings, it decreased gradually across the time course. At 189 days, the relative abundance of *Winogradskyella* was low on all coatings (Figure 6b). These changes in the bacterial community were evident in PCO analysis with samples clustering according to time of collection (Figure 5c). That is, for the 16S data, samples taken from the panels after 94 days and 119 days clustered together whilst the samples after 148 days and 189 days produced two separate clusters: ANOSIM R value of 0.41 (p = 0.001). SIMPER analysis indicated that a member of the Flavobacteriaceae, the largest family in the Bacteriodetes, was the major contributor to the difference between the panels sampled at 189 days and those sampled at earlier time points; it explained 20.0%, 21.2% and 17.1% of the difference between the samples collected at 94, 119 and 148 days, respectively..

The eukaryotic phyla showing significant differences across time were the Haptophyta (*p*-value 0.004), Cnidaria (*p*-value 0.006), the Rhodophyta (*p*-value 0.007) and the Ochrophyta (*p*-value 0.032). Apart from the Rhodophyta (7.3% relative abundance), these phyla represented a small

percentage of the relative abundance; 0.3, 2.5 and 3.6%, respectively (Supplementary File 4). No interaction between coating and time were observed. At lower ranking, the only significant taxa were the green algae *Caulerpa* (*p*_value 0.003) and *Codium* (*p*_value 0.017), and the pennate diatom *Pseudo-nitzschia* (*p*_value 0.021), all three showing a relative increase between 148 and 189 days (Figure 6c and d); Supplementary File 5 shows *p*-values for all taxa. These three taxa represented only 0.2, 1.6 and 0.5% of relative abundance. SIMPER analysis indicated that the main differences through time were driven by the relatively most abundant taxa, *Amoebophyra*, *Navicula* and *Amphora*, but none of these proved to be significantly different (ANOVA) in relative abundance. However, *Codium*, with a mean relative abundance across all panels of only 1.6%, contributed 3.8% of the difference between the samples taken at 189 days and all other samples (Figure 6d). The panels collected at different time points produced no distinct clusters in PCO analysis (Figure 5f). There was no significant interaction between coating and time (Supplementary File 5).

Discussion

An understanding of the biofilm communities that accumulate on ship hulls in marine habitats is important to manufacturers of antifouling coatings. Biofilms can significantly impact the drag experienced by vessels as they travel through the water and thus increase both service costs and the production of greenhouse gases and other pollutants. A further concern arises from the unwitting transport of species around the globe and thus the potential problem of the introduction of non-indigenous species the control of which has significant economic impact (Ralston and Swain 2014; Sweat et al. 2017). The bacterial and algal species that form biofilms may also have an impact on the macrofoulers that eventually accumulate (Lau et al. 2005; Dash et al. 2011) as they are thought to provide cues and a modified surface for settlement (Hadfield 2011). However, work by Sweat et al (2017) suggests that macrofoulers are able to colonise a variety of biofilms so that it may not be possible to predict the species of macrofoulers on the basis of the biofilm community.

In this paper, we report the study of biofilm formation on six commercially available anti-fouling coatings, three biocidal and three fouling-release, over a six-month period from December 2014 to June 2015. Using sequenced amplicons of both a bacterial 16S rRNA genes (Lee et al. 2008) and eukaryotic ITS regions (Buckheim et al. 2011) we were able to identify the bacterial and algal taxa involved in biofilm formation on these contrasting surfaces. We chose rDNA-ITS as our eukaryotic marker due to its ability to differentiate closely related bacillariphyta (Guo et al. 2017), anticipated to be key biofouling taxa, and other microbial eukaryotes including the paraphyletic grouping, “fungi” (Schoch et al. 2012) and apicomplexan (Stern et al. 2012). We discounted plastid 23S rDNA barcoding and *rbcL-rbcS* region markers as we did not wish to focus solely on photosynthetic microbes.

A large number of taxa, both bacterial and eukaryotic, were identified in this study, although the majority of these were present at very low relative abundance. No clear difference between the communities that developed on the three coatings of a particular type were found. This corresponds with the findings of Muthukrishnan et al. (2014) who studied biofilm formation on several commericial biocidal coatings including BAF1 and BAF2 used in our study; they found little difference between the biofilm communities on two coatings. All three biocidal coatings used in our study contain the same combination of biocides, albeit at different amounts, and may under the static conditions used here perform similarly. Differences were observed, however, between the coating types with a greater number of taxa on the FR than the BAF coatings. This might account for the separation of the FR and BAF clusters in PCO analysis. Cluster analysis, particularly in the case of

the 16S data, showed there to be a difference between coating types, BAF compared to FR coatings, with regard to biofilms formation. This separation, presumably, is explained by the biocides inhibiting the growth of many taxa on BAF coatings. Indeed, in most cases, if an organism grew on the BAF coatings it also grew on the FR coatings. The reverse, ie an organism that grew on the BAF coatings but not the FR coatings, was much less common. However, a small number of apparently biocide-tolerant taxa, such as *Winogradskyella* and *Amphora* and *Codium*, were able to grow better on this surfaces.

There was also a general trend for the number of taxa to increase through time, as reported in other studies (Huggett et al 2009). That is, the largest number of taxa, both bacterial and eukaryotic, was found after the longest immersion time (189 days). This suggests that, throughout the period of immersion, the biofilm community continued to change and explains the clear separation of the 189 day cluster on the PCO plot of bacterial taxa. This difference reflects a succession within the biofilms where early settlers, such as *Winogradskyella*, decrease in relative abundance as late appearing taxa, such as *Lewinella*, begin to accumulate. A change through time with regard to the eukaryotic community was not evident, although some taxa showed changes in relative abundance. The appearance of the green alga, *Codium*, at 189 days might indicate that, after 6 months of static exposure, the communities on the panels are moving beyond the initial stages of microfouling and are entering the stage of macrofouling. This result is in contrast to the findings of Muthukrishnan et al. (2014) which indicate that biocidal coatings remained 100% free of macrofouling after one year of static immersion. However, their report is based on visual inspection of the surface rather than the uses of molecular markers. The appearance of *Codium* principally on the BAF coatings would indicate either that this species is able to tolerate the biocides they release or that the established bacterial biofilm is shielding late-arriving taxa from exposure to the biocides (Zobell and Allen 1934; Chen et al. 2013). Conversely, the relative absence of *Codium* on the FR coatings could be explained simply by its inability to remain attached to these surfaces.

It has been reported that biofilms that develop on artificial surfaces in marine environments are dominated by bacteria and diatoms (Briand et al. 2012). In this study, the main bacterial phyla observed, Bacteriodetes and Proteobacteria, are those that have been reported by others (Dang and Lovell 2000; Salta et al. 2013). However, rather than Proteobacteria being the dominant phyla, as reported in a number of articles (Dang and Lovell 2000; Jones et al. 2007; Huggett et al. 2009; Chung et al. 2010, Sathe et al., 2017) in this study, Bacteriodetes were found to dominate: this dominance was more pronounced on the BAF coatings than on the FR coatings. Dang and Lovell (2000), as in this study, used a 16S approach to study the presence of bacterial species on submerged surfaces

and reported α-proteobacteria to be the dominant phyla. However, their study was of very early colonisation (up to 72 hours) whereas our study was over a period of six months with the first sample being taken after three.

The relative abundance levels for eukaryotic taxa observed in this study is in line with the studies of Camps et al. (2014) and Hunsucker et al. (2014); that is, the most abundant eukaryotic taxa belonged to the Bacillariophyceae (diatoms) with the pennate genera, in this case *Navicula*, *Amphora*, *Sellaphora* and *Haslea*, being the most dominant. A clear distinction between the coating types with regard to the genus *Amphora* was observed, with a greater relative abundance on the BAF coatings. It has been known for a long time that *Amphora* shows resistance to copper-based coatings (Daniel et al. 1980) and this probably accounts for the greater relative abundance of this species on the BAF coatings. It should be taken into account that this study was carried out under static conditions and it has been reported that diatom populations within biofilms may be fewer under static rather than dynamic conditions (Zargiel and Swain 2014). The relative abundances of the observed diatoms might prove to be quite different under service conditions.

Some taxa showed very wide variability in relative abundance between panels. The jellyfish, *Drymonema*, was a good example of this; it had a low relative abundance on most panels, both FR and BAF. However, on the FR panels at 189 days, there was very high relative abundance on six of the nine panels and very low relative abundance on the other three. Settlement of propagules is an inherently stochastic process, and the establishment of a significant community of a given taxa may be due to local spread after initial recruitment, rather than as a result of a purely Poisson process. The settlement of *Drymonema* on the FR rather than the BAF coatings might, however, be indicative that propagules from this species are sensitive to the biocides in the latter.

Once species have begun to accumulate, chemical antagonism between members of the developing biofilm community is likely to influence, either positively or negatively depending on the precise interactions, recruitment of additional species. For example, the taxa that constitute the biofilm are thought to play an important role in mediating settlement and metamorphosis of macrofoulers (Egan et al. 2001; Hadfield 2011; Maki et al. 1988). The bacteria, *Winogradskyella* and *Alteromonas*, for example, have been shown to produce biocidal compounds that might interfere with the settlement of other organisms. Some members of the genus *Alteromonas* have been shown to produce ubiquinones that inhibit the settlement of barnacle larvae (Kon-ya et al. 1994), whilst some *Winogradskyella* species produce poly-ethers that are known to have a biocidal activity (Dash et al. 2011). Indeed, the organisms found in biofilms are being studied as a potential source of antifouling agents to be used in antifouling applications (Dobretsov et al. 2006).

Limitations of the study

About 4,000 species have been identified as fouling organisms but this is a very small proportion of the known marine species (Yebra et al. 2004). The taxa found on the hulls of in-service ships are those able to attach to surfaces designed to inhibit their attachment and growth and, at the same time, tolerate wide fluctuations in environmental conditions such as temperature, water flow and salinity. Our experimental boards were fixed in a single environment (Hartlepool Marina) and so the species present and their relative abundance might not reflect those that would be seen were the same analysis to be carried out with coatings applied to an active ship. In future experiments, we wish to address this issue.

It has been reported that antifouling coatings perform quite differently under static rather than dynamic conditions (Shultz et al. 2011; Zargiel and Swain 2014). Indeed, it has been reported that many fouling organisms that are normally encountered on a ship's hull cannot colonise at velocities above 4-5 knots (Lindholdt et al. 2015). Thus, a single coating formulation might perform quite differently depending on the type of ship, and it operational profile (speed, time spent in port), to which it is applied (Davidson et al. 2009; Sweat et al. 2017). Thus, many of the organisms that were identified on the BAF and FR coatings in this study might not have appeared under dynamic/in-service conditions or their relative abundances might have been quite different (Sweat et al. 2017). Additionally, microbial communities and, in particular, the dense extracellular polymeric substances produced by them have been known to interfere with the performance of biocidal coatings by altering the release rate of compounds in the coatings (Chen et al. 2013; Yebra et al. 2006); this can potentially results in impaired anti-fouling activity.

Environmental factors can influence both coating performance and colonisation of the surface. Temperature (see below) and other physical characteristics of the marine environment (pH and sea water ions (salinity)) will influence the efficacy of the various coatings (Yebra et al. 2004; Briand et al. 2017). During their development, coatings are tested under a range of conditions in the laboratory and at field sites. However, as in this static experiment in Hartlepool Marine, these test conditions won't truly replicate in-service conditions and so performance may be misleading. Seasonality, (eg colder vs warmer periods, Bernbom et al. 2011; Briand et al. 2017), also likely influences the species that first colonise a surface, so starting the experiment at a different time of year might well affect the colonisation process significantly.

It is important to note that the results presented here are relative abundance of taxa, not absolute abundance. Thus, although the FR coatings appear to harbour a greater diversity of taxa than the BAF coatings they may have had less visible fouling; unfortunately, we did not estimate this. In future studies, we hope to quantify absolute fouling levels by various means. Similarly, the number of sequences identified to a particular taxon does not necessarily correlate perfectly with the abundance of that taxon, due to differential lysis of different taxa and variations in copy number of our chosen marker genes. Nonetheless, shifts in relative abundance between treatment levels are likely to be robust as DNA extraction efficiencies and copy number variation will affect taxa equally across treatments.

Finally, the taxonomy in our 'algal' database is drawn from various authorities that give different taxonomic rankings or entirely different taxonomies. Our rDNA-ITS database follows the NCBI taxonomy contained in the taxonomy-related files downloaded from their database. Errors and inconsistencies within this taxonomy were found and, whilst we have tried to present a consistent usage of terms, we recognise that we might not be using the most accepted authorities; the task of re-mapping the NCBI algal taxonomy was beyond the scope of this study. For example, the genus *Micronuclearia* that was identified as significantly different between FR and BAF coatings, belongs to a polyphyletic grouping, the Apusozoa, that has undergone several revisions. In addition, there is the difficulty of assigning names to unculturable samples because of the rules of taxonomy (Hibbett 2016). Therefore, the classification given in this paper should only be considered as a guide. Further work in improving the NCBI taxonomy would be useful.

In future work, we wish to assess the potential biological activity of the identified organisms through full metagenome sequencing. Using these data we will be able to test associations between coatings and specific gene abundances. Analysis of samples from multiple coatings, time points and locations will show which factors explain the greatest amount of variance in taxonomic and gene composition of samples. We have also instigated immersion tests under a variety of dynamic regimes to extend our understanding of biofilm dynamics under service conditions.

In conclusion, a better understanding of the bacterial communities colonising marine, anti-fouling coating, whether biocidal or fouling release, is of great interest for both the marine coating industry and marine microbiologists. Information of these early adherent marine bacteria and eukaryotes, which proved to be different between BAF and Fr coatings, will serve as a basis for further development of antifouling strategies, and extend our understanding of marine biofilm diversity.

Aknowledgements

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Disclosure Statement

This research is sponsored by European Union and and we believe that there are no conflicts of interest to report. Although the coatings and test site were provided by AkzoNobel, the publications of this article will provide no financial benefit to the authors.

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Table legends

Table 1. Average relative abundance of bacterial phyla on the six coatings ranked by abundance averaged across all coatings (last column of table); only phyla with relative abundance of > 1% are listed. FR1 = Intersleek 700; FR2 = Intersleek 900; FR3 = Intersleek 1100SR; BAF1 = Intersmooth 7460HS; BAF2 = Intersmooth 7465Si; BAF3 = Intercept 8000. FR are fouling-release coatings; BAF are biocidal antifouling coatings.

Table 2 Average relative abundance of bacterial taxa on six different coatings ranked by abundance averaged across all coatings (last column of table); only taxa with relative abundance of > 1% are listed. FR1 = Intersleek 700; FR2 = Intersleek 900; FR3 = Intersleek 1100SR; BAF1 = Intersmooth 7460HS; BAF2 = Intersmooth 7465Si; BAF3 = Intercept 8000. FR are fouling-release coatings; BAF are biocidal antifouling coatings.

Table 3. Statistically significant taxa. HTCC = high throughput culture clades that belong to the oligotrophic marine Gammaproteobacteria (Cho and Giovannoni, 2004). FR1 = Intersleek 700; FR2 = Intersleek 900; FR3 = Intersleek 1100SR; BAF1 = Intersmooth 7460HS; BAF2 = Intersmooth 7465Si; BAF3 = Intercept 8000. FR are fouling-release coatings; BAF are biocidal antifouling coatings.

Figure Legends

Figure 1. Sample site and sample boards for biofilm collection: a) position of Hartlepool Marina on the east coast of the UK; b) latin square design for sample boards; the different coloured squares represent the panels and their coatings: BAF = biocidal antifouling; FR = fouling-release.

Figure 2. Average number of taxa identified on the six coatings averaged over the four time points of collection (a and b) and for the four immersion times average over the three FR and three BAF coatings (c and d).

Figure 3. a) Venn diagrams of number of taxa species found on each coating type (pale blue = FR coatings; orange = BAF coatings). b) Taxa tended to be found on more panels with FR coatings than those with BAF coatings: ratio (number of FR panels divided by number of BAF panels) of number of panels on which taxa were found. For each bar, the lower darker shade represents bacterial taxa, the upper lighter shade represents eukaryotes.

Figure 4. Relative abundance of eukaryotic taxa on the six coatings. a) Eukaryotic phyla with relative abundance of > 1%. b) Eukaryotic genera with relative abundance > 2%. The taxon Tintinnida was identified only to the level of Order.

Figure 5. Principal co-ordinate plots of bacterial OTU relative abundance on each of six different coatings; three biocidal coatings and three fouling-release coatings. There were three biological replicates for each coating at each of four time points: a) and d) samples coloured by coating; b) and e) samples coloured by coating type; c) and f) samples coloured by date of collection.

Figure 6. Statistically significant differences between coatings and length of immersion in days. a) *Lewinella*, a Gram-positive bacterium and b) *Winogradskyella*, a Gram-negative bacterium, showed significant differences in relative abundance between coatings and through time. The eukaryotic taxa c) *Pseudo-nitzschia*, a pennate diatom, and d) *Codium*, a green alga, showed significant difference through time.

Supplementary Files

***Supplementary File 1*:** Hartlepool Marina salinity and temperature December 2014 to June 2015

***Supplementary File 2*:** ITS taxonomy database

***Supplementary File 3*:** Taxa found exclusively of FR or BAF coatings

***Supplementary File 4*:** Relative abundance

***Supplementary File 5*:** Statistically significant taxa

Table 1. Average relative abundance of bacterial phyla.

Phylum	FR1	FR2	FR3	BAF1	BAF2	BAF3	FR	BAF	All
Bacteriodetes	45.4	51.1	54.3	51.8	54.2	69.9	50.3	58.7	54.6
Proteobacteria	48.3	45.2	41.4	37.3	34.0	25.9	45.0	32.4	38.5
Verrucomicrobia	5.3	2.9	3.2	10.3	11.4	3.8	3.8	8.5	6.2

Table 2. Average relative abundance of bacterial taxa.

Order	Family	Genus	FR1	FR2	FR3	BAF1	BAF2	BAF3	FR	BAF	All
Flavobacteriales	Flavobacteriaceae	*Winogradskyella*	9.1	7.5	11.1	18.8	21.7	29.3	9.2	23.3	16.5
Methylococcales	----	----	18.8	18.9	17.2	11.8	19.3	9.3	18.3	13.5	15.8
Flavobacteriales	Flavobacteriaceae	----	16.9	21.3	20.0	12.8	9.9	11.5	19.4	11.4	15.3
Flavobacteriales	Flavobacteriaceae	----	5.8	5.9	6.6	7.7	11.3	18.1	6.1	12.4	9.3
Flavobacteriales	Flavobacteriaceae	*Polaribacter*	3.4	5.0	4.3	4.9	3.0	3.1	4.3	3.7	4.0
Flavobacteriales	Flavobacteriaceae	*Ulvibacter*	2.5	2.8	4.7	4.1	4.2	2.1	3.3	3.5	3.4
Puniceicoccales	Puniceicoccaceae	*Coralimargarita*	0.6	0.3	1.2	3.7	9.2	3.3	0.7	5.4	3.1
Flavobacteriales	Flavobacteriaceae	*Maribacter*	2.9	2.6	2.7	1.8	2.8	3.4	2.7	2.7	2.7
Alteromonadales	Alteromonadaceae	----	2.1	2.1	1.7	4.4	1.8	2.0	2.0	2.8	2.4
Alteromonadales	----	----	3.5	3.3	3.3	1.6	1.2	0.6	3.4	1.1	2.2
Alteromonadales	Colwelliaceae	----	2.2	1.2	1.3	3.7	1.4	2.2	1.6	2.4	2.0
Alteromonadales	OM60	----	2.5	3.1	3.4	1.4	1.0	0.7	3.0	1.1	2.0
Verrucmicrobiales	Verrucomicrobiaceae	*Luteolibacter*	0.6	0.7	0.7	3.9	1.9	0.5	0.7	2.1	1.4
Alteromonadales	Alteromonadaceae	----	0.9	1.3	1.2	1.4	1.1	1.3	1.1	1.3	1.2
Methylophilales	Methylophilaceae	*Methylotenera*	1.3	2.0	1.7	0.8	0.7	0.3	1.7	0.6	1.1
Thiohalorhabdales	----	----	1.4	1.0	1.0	1.7	0.7	0.5	1.1	1.0	1.0

Table 3. Statistically significant taxa.

Bacteria

Phylum	Class	Order	Family	Genus	Coating	Time	Coating*Time
Bacteriodetes	Saprospirae	Saprospirales	Saprospiraceae	*Lewinella*	0.000	0.000	0.000
Proteobacteria	Gammaproteobacteria				0.000	0.001	
Bacteriodetes	Saprospirae	Saprospirales	Saprospiraceae		0.000	0.000	0.003
Bacteriodetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae		0.000		
Bacteriodetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	*Winogradskyella*	0.000	0.000	
Proteobacteria	Gammaproteobacteria	Alteromonadales	Alteromonadaceae	*Glaciecola*	0.004		
Proteobacteria	Gammaproteobacteria	Alteromonadales	HTCC2188	HTCC	0.004		
Proteobacteria	Gammaproteobacteria	Oceanospirillales	Halomonadaceae	Candidatus Portiera	0.008		
Proteobacteria	Gammaproteobacteria	Alteromonadales	Alteromonadaceae		0.008	0.014	
Bacteriodetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae		0.013	0.000	
Bacteriodetes	Flavobacteriia	Flavobacteriales			0.038		
Proteobacteria	Gammaproteobacteria				0.041		
Bacteriodetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	*Maribacter*		0.000	
Proteobacteria	Gammaproteobacteria	Alteromonadales	OM60			0.000	
Proteobacteria	Gammaproteobacteria	Alteromonadales	HTCC2188			0.000	0.002
Proteobacteria	Gammaproteobacteria	Thiohalorhabdales				0.001	
Proteobacteria	Gammaproteobacteria	Marinicellales	Marinicellaceae	*Marinicella*		0.001	
Proteobacteria	Gammaproteobacteria	Alteromonadales	OM60			0.005	
Proteobacteria	Gammaproteobacteria	Alteromonadales	Alteromonadaceae			0.027	
Proteobacteria	Deltaproteobacteria	Bdellovibrionales	Bacteriovoracaceae			0.049	

Eukaryota

Phylum	Class	Order	Family	Genus	Coating	Time	Coating*Time
Bacillariophyta	Bacillariophyceae	Thalassiophysales	Catenulaceae	*Amphora*	0.020		
Apusozoa	Hilomonadea	Rigifilida	Micronucleariidae	*Micronuclearia*	0.023		
Chlorophyta	Ulvophyceae	Bryopsidales	Caulerpaceae	*Caulerpa*		0.003	
Chlorophyta	Ulvophyceae	Bryopsidales	Codiaceae	*Codium*		0.017	
Bacillariophyta	Bacillariophyceae	Bacillariales	Bacillariaceae	*Pseudo-nitzschia*		0.021	

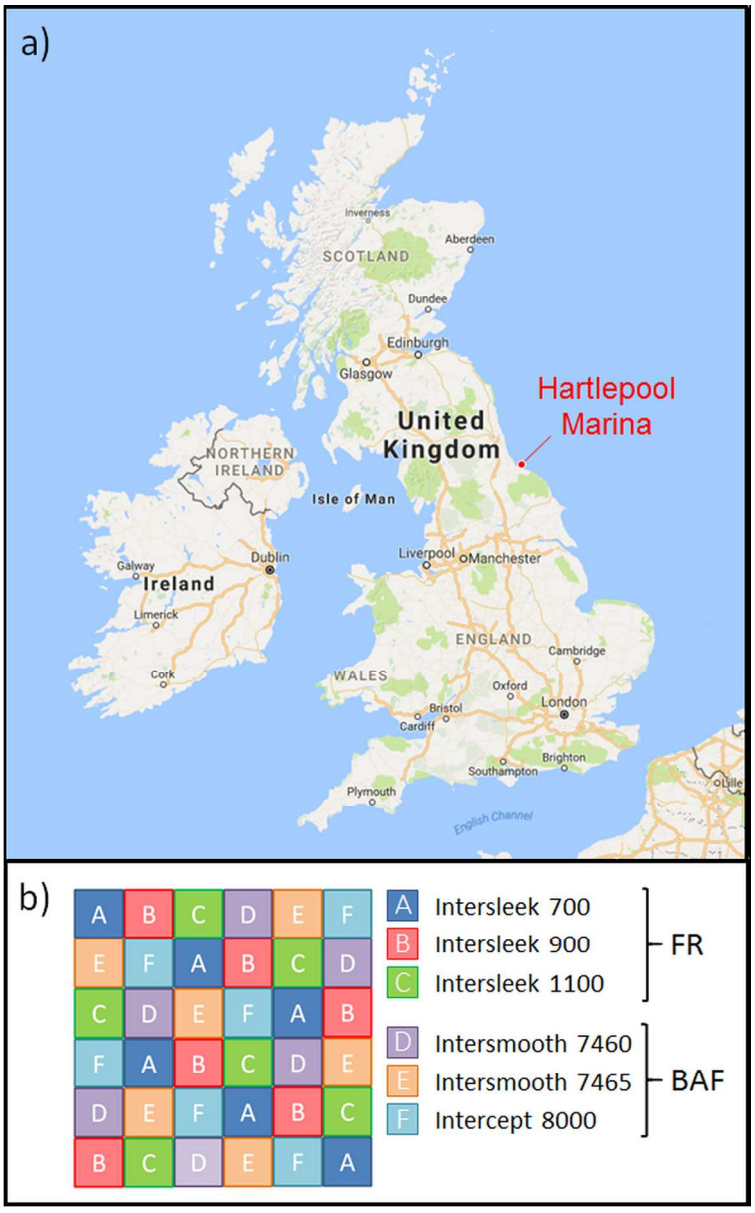

Figure 1. Sample site and sample boards for biofilm collection: a) position of Hartlepool Marina on the east coast of the UK; b) latin square design for sample boards; the different coloured squares represent the panels and their coatings: BAF = biocidal antifouling; FR = fouling-release.

156x250mm (200 x 200 DPI)

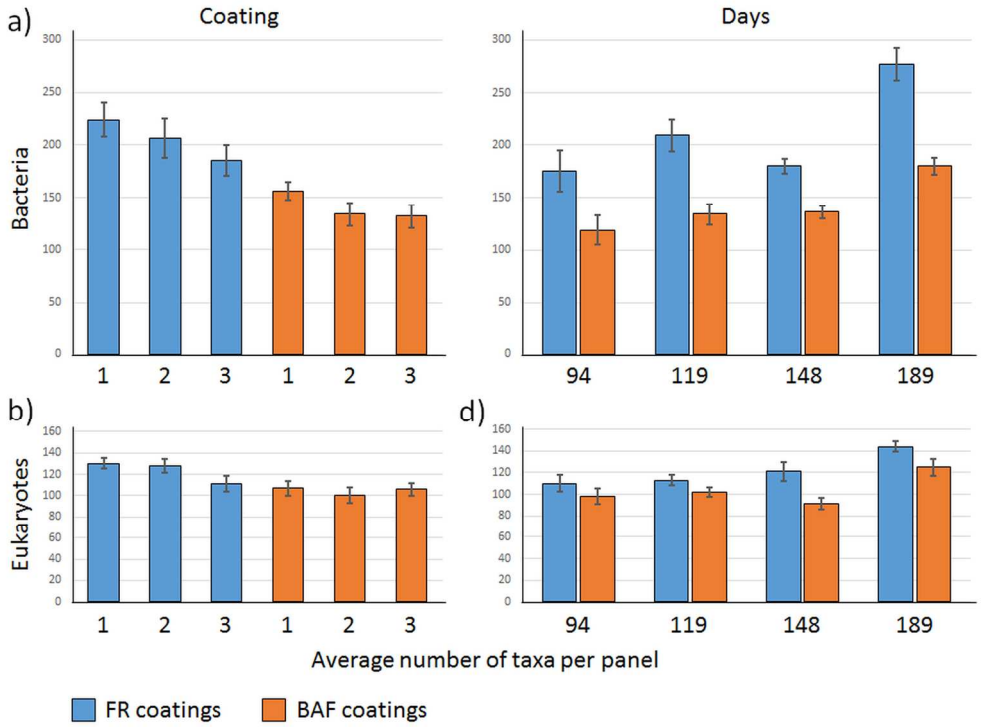

Figure 2. Average number of taxa identified on the six coatings averaged over the four time points of collection (a and b) and for the four immersion times average over the three FR and three BAF coatings (c and d).

253x189mm (200 x 200 DPI)

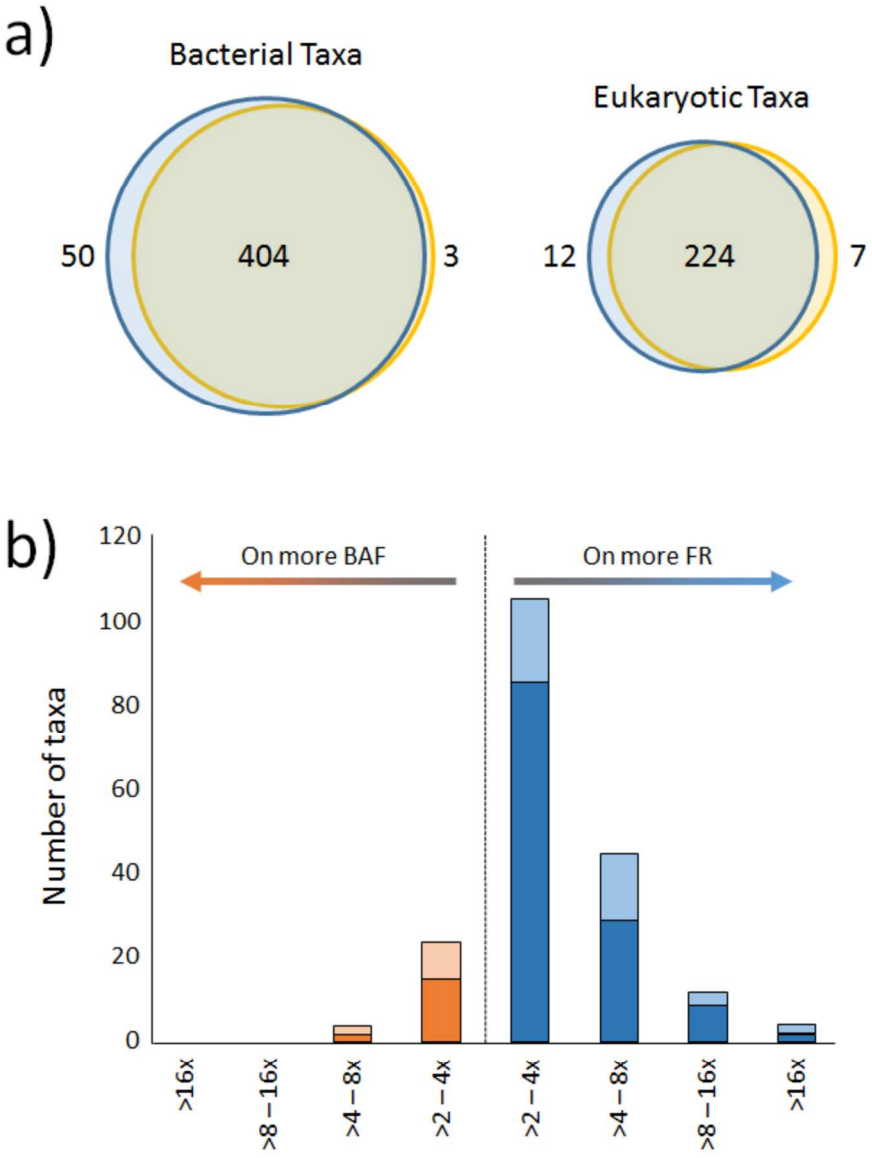

Figure 3. a) Venn diagrams of number of taxa species found on each coating type (pale blue = FR coatings; orange = BAF coatings). b) Taxa tended to be found on more panels with FR coatings than those with BAF coatings: ratio (number of FR panels divided by number of BAF panels) of number of panels on which taxa were found. For each bar, the lower darker shade represents bacterial taxa, the upper lighter shade represents eukaryotes.

170x220mm (200 x 200 DPI)

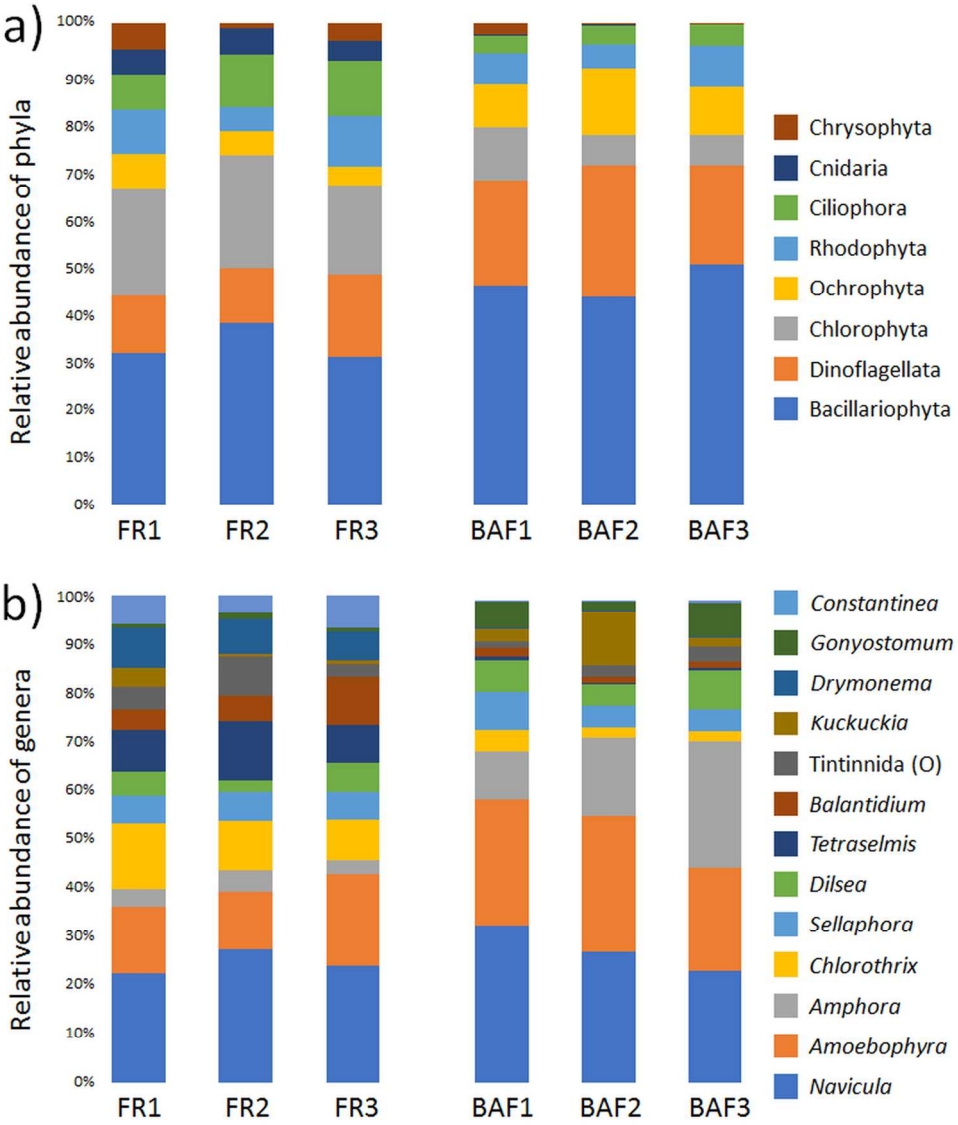

Figure 4. Relative abundance of eukaryotic taxa on the six coatings. a) Eukaryotic phyla with relative abundance of > 1%. b) Eukaryotic genera with relative abundance > 2%. The taxon Tintinnida was identified only to the level of Order.

210x246mm (200 x 200 DPI)

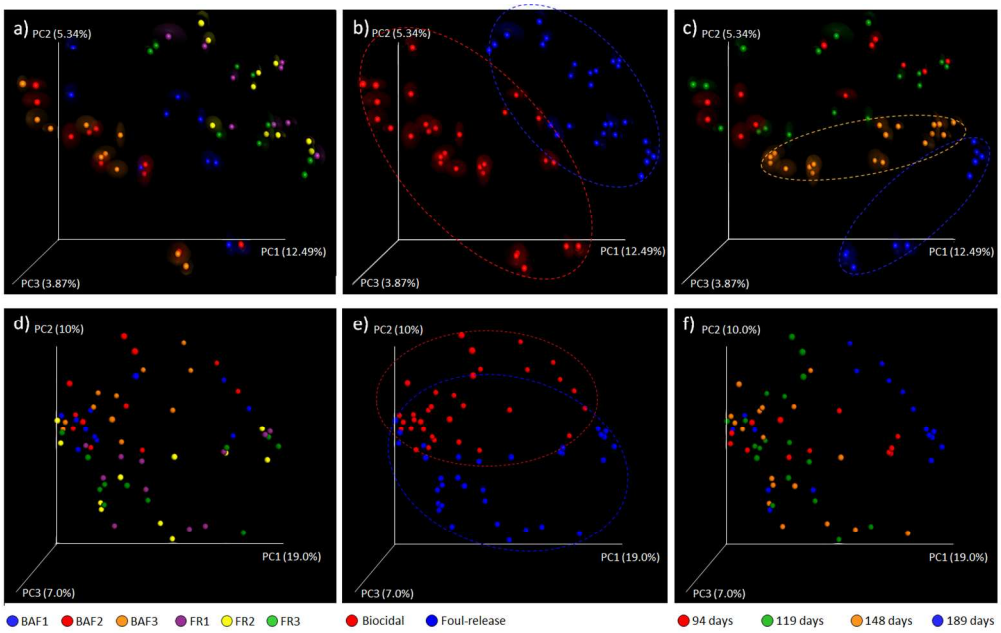

Figure 5. Principal co-ordinate plots of bacterial OTU relative abundance on each of six different coatings; three biocidal coatings and three fouling-release coatings. There were three biological replicates for each coating at each of four time points: a) and d) samples coloured by coating; b) and e) samples coloured by coating type; c) and f) samples coloured by date of collection.

420x266mm (96 x 96 DPI)

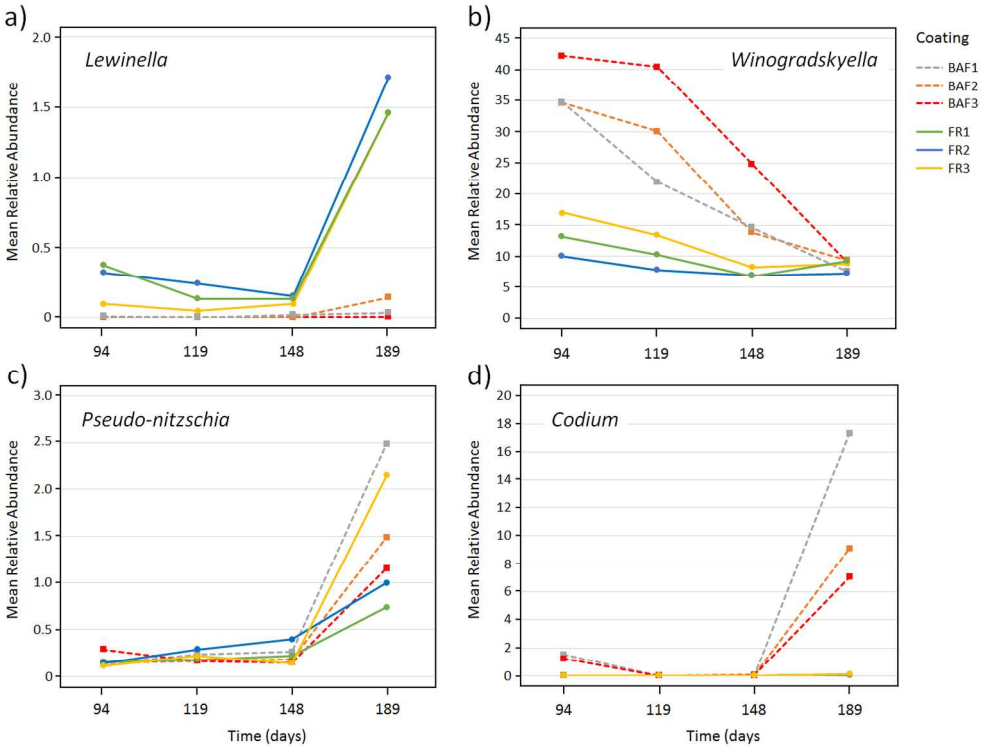

Figure 6. Statistically significant differences between coatings and length of immersion in days. a) Lewinella, a Gram-positive bacterium and b) Winogradskyella, a Gram-negative bacterium, showed significant differences in relative abundance between coatings and through time. The eukaryotic taxa c) Pseudo-nitzschia, a pennate diatom, and d) Codium, a green alga, showed significant difference through time.

380x292mm (200 x 200 DPI)