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16S rRNA gene profiling of planktonic and biofilm microbial populations in the Gulf of Guinea using Illumina NGS

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ABSTRACT

16S rRNA gene profiling using a pipeline involving the Greengenes database revealed that bacterial populations in innermost (proximal to the steel surface) and outer regions of biofilms on carbon steel exposed 3 m below the surface at an offshore site in the Gulf of Guinea differed from one another and from seawater. There was a preponderance of gammaproteobacterial sequences, representing organisms known for hydrocarbon degradation. Total DNA from the innermost layer was 1500 times that recovered from the outermost. Stramenopiles (diatom) sequences were prevalent in the former. Rhodobacteriaceae, key biofilm formers, comprised 14.9% and 4.22% OTUs of inner and outer layers, respectively. Photo-synthetic anaerobic sulfur oxidizer sequences were also prominent in the biofilms. Analysis of data using a different pipeline with Silva111 allowed detection of 0.3e0.4% SRB in the biofilms. The high abundance of aerobic micro-algal sequences in inner biofilm suggests they are initial colonizers of carbon steel surfaces in a marine environment. This is the first time that the microbial population of the strongly attached inner layer of the biofilm on steel has been differentiated from the outer, readily removed layer. The accepted scraping removal method is obviously inadequate and the resulting microbial analysis does not offer complete information on the biofilm community structure.

Keywords: Marine biofilms Illumina NGS Planktonic seawater population 16S rRNA gene profile Carbon steel

1. Introduction

The introduction of Illumina high throughput DNA sequencing in the early 21st century has allowed the generation of a huge database of microbial genomic sequences and detailed investigations of microbial populations in previously studied and unstudied locations. One of the most investigated ecosystems has been the world's oceans. Comparisons of surface, subsurface and deeper seawater populations have been published (see, for example Friedline et al., 2012; Arumugam et al., 2013; Signori et al., 2014). There is considerable information on the microorganisms present in the open sea (for example, Yutin et al., 2007; Yooseph et al.,

2010). The open seawater (planktonic) microbial populations and those accumulating on surfaces immersed in the sea are considerably different and this subject has been recently been reviewed by Dang and Lovell (2016), but there is no published data on Gulf of Guinea microbiomes.

Steel offshore oil rigs of various types are economically important structures that are prone to corrosion in aggressive seawater environments. The site under study is located some 113 km offshore in water of depth 1463 m. Despite recent observations that microbiologically influenced corrosion (MIC) in this area was very high (Lee et al., 2015), there has, as yet, been no investigation of the microbial populations in the area. The present study was based on the hypotheses that there would be differences in microbial populations between biofilms on the metal surfaces and the local seawater, and that bacteria with the potential for MIC could be present. To investigate this, a sequencing study of the site was initiated using the Illumina MiSeq system. Populations in the local seawater were compared with those attached to carbon steel coupons, including both those microbial cells removed from the

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surface by the traditional scraping method, and those remaining on the metal surface after scraping (denominated “outer” and “inner” biofilm, respectively). The corrosion associated with these coupons is presented and discussed in another document (Witt et al., 2016).

2. Materials and methods

2.1. Metal coupons and exposure

Mild steel coupons ($0.5 \times 3 \times 1/16$ inches) were manufactured with a $3/16$ inch hole located 0.25 inches from one end. Twenty of these were attached to a synthetic fibre rope with plastic cable ties (Fig. 1).

In November 2014, coupons were exposed in the upper water column, 3 m below the surface of the sea, 113 km off the coast in the Gulf of Guinea. The pH of the water at sampling was 6.94, it contained $<0.001 \text{ mg l}^{-1}$ phosphate and total nitrate, 2960.3 mg l^{-1} sulfate and 28.45 mg l^{-1} ammonium ions (Witt et al., 2016). The system was originally designed to withstand exposure in the field for four to eight weeks (28–56 days). However, after a severe storm, all coupons were found to be missing from the rope and a second system was installed. After 5 weeks, only four coupons were found to remain attached to the rope. These coupons had suffered significant corrosion at all the edges; hence aggressive corrosion attack was proposed as the most likely cause of coupon loss. The 4 remaining coupons were collected for analysis under sterile conditions. Each coupon was allowed to dry in a laminar flow cabinet and then dipped into DNAzol[®] solution (Life Technologies, Carlsbad, CA, USA) to preserve DNA. DNAzol treated coupons were placed into sterile containers and shipped for analysis at the University of Oklahoma, where they were used for SEM and DNA analysis.

2.2. Seawater sampling

Seawater in the proximity of the coupons was collected, in a sterile manner, from the site and immediately sent to the Bio-corrosion laboratory at the University of Oklahoma, where they were stored at -20°C until analysis.

Fig. 1. Carbon steel coupon attached to the fiber rope prior to exposure, with protective plastic wrap.

2.3. Sample preparation for microbial analysis

Seven hundred ml of seawater were filtered through Nalgene Rapid-Flow sterile disposable polyethersulfone (PES) membrane filters, pore size $0.1 \mu\text{m}$ (Thermo Scientific, Waltham, MA, USA). The filter was transferred into a sterile 50 ml tube, 1 ml DNAzol[®] (Life Technologies, Carlsbad, CA, USA) added and the filter stored at -20°C for 12 days prior to DNA extraction. Metal coupons with their attached biofilms were also stored at -20°C , until DNA extraction.

2.4. DNA extraction, high-throughput sequencing and data processing

2.4.1. DNA extraction from seawater

Maxwell[®] 16 Tissue LEV Total RNA purification kit, with the Maxwell[®] 16 instrument, was used for DNA extraction, with slight modifications from the manufacturer's instructions. The filter was thawed, 1 ml nuclease-free water and $10 \mu\text{l}$ Proteinase K (Valencia, CA, USA) added, and the tube incubated at 55°C for 1 h. Five hundred μl RLA and $500 \mu\text{l}$ RDB buffers were added. The filter was bead beaten for 2 min using Lysing Matrix E beads (MP Biomedical, Santa Ana, CA, USA) and centrifuged at 6000 g for 5 min. Aliquots of the supernatant were transferred into 2 Maxwell cartridges. Extraction was carried out using the DNA and FFPE/Cells programme in the Maxwell[®] 16 instrument. DNA was eluted in $100 \mu\text{l}$ nuclease-free water.

2.4.2. DNA extraction from biofilm

Biofilm on the coupon surface was removed using a sterile scalpel. Approx. 100 mg of biofilm was collected; this is referred to as the outer biofilm. A combination of the Power Soil DNA extraction kit (MO-BIO Laboratories, Carlsbad, CA) and the Maxwell[®] 16 method described above was used for DNA recovery.

After scraping off the outer biofilm, the entire coupon was subjected to DNA extraction using the larger tube of the PowerMax Soil DNA kit (MO-BIO Laboratories, Carlsbad, CA). The manufacturer's instructions were followed with the additional step of Proteinase K treatment. DNA was eluted in 5 ml of the eluent provided by the PowerMax Soil DNA kit.

DNA extracted from seawater and both types of biofilm was quantified using a Qubit 2.0 Fluorimeter with high sensitivity dsDNA reagents, according to the manufacturer's instructions (Invitrogen/Life Technologies, Carlsbad, CA, USA).

2.4.3. 16S rRNA gene amplicon library preparation

The bacterial 16S rRNA gene V4 region was amplified for high-throughput sequencing analysis using universal prokaryotic primers, 519F (5' CAGCMGCCGCGTAA 3') and 785R (5' TACNVGGGTATCTAATCC 3') (Klindworth et al., 2013), with the forward primer modified to contain the M13 sequence on its 5' end (M13-519F). 16S rRNA gene amplification took place in $50 \mu\text{l}$ PCR reactions containing 5–10 μl DNA, $25 \mu\text{l}$ of 2x Phusion master mix, 10 pmol of M13-519F and 785R primers. Amplification was performed in a Techne Prime thermal cycler, with an initial denaturation at 98°C for 1 min, followed by 25 cycles of denaturation at 98°C for 10 s, annealing at 52°C for 20 s, extension at 72°C for 10 s, and a final extension at 72°C for 5 min. PCR products were visualized on 1% (w/v) agarose gels pre-stained with SYBR Safe (Invitrogen, Carlsbad, CA, USA) and the images were recorded using a Gel Logic 112 Imaging System and Molecular Imaging Software v5 (Carestream, Woodbridge, CT, USA).

Triplicate reactions of each PCR product were pooled in order to minimize PCR biases; $2 \times 15 \mu\text{l}$ for each sample was run on a 1% (w/v) agarose gel and bands corresponding to the desired length

(approx. 280 bp) were cut out using sterile gel cutting tips and DNA eluted in 50 µl sterile nuclease-free water (Ambion®, Life Technologies, Carlsbad, CA, USA). Ten µl of PCR product eluted from the gel was added to a second PCR reaction containing specific 12 nt barcodes attached to M13. Duplicate samples were 'tagged' with different barcoded primers by re-amplification for ten cycles. Tagged PCR products were pooled in equimolar concentrations, corresponding to 1000 ng DNA/ml, and sequenced on an IlluminaMiSeq sequencer at Oklahoma Medical Research Foundation (Oklahoma City, OK, USA).

2.4.4. Sequence analysis

FastQC (Babraham Bioinformatics) was used for the quality control check of the raw sequencing data. The bacterial 16S rRNA gene libraries were pre-processed and analyzed using a combination of Trimmomatic 0.32 (Bolger et al., 2014), the USEARCH v7 (UPARSE) pipeline (Edgar, 2013), and the bioinformatics software package QIIME version 1.8.0 (Caporaso et al., 2010). Briefly, Illumina adapters, low quality regions of sequence reads (Phred score <25 over a 50 bp window), and sequences containing homopolymer regions (>6 bp) were eliminated from the paired-end sequence read files using Trimmomatic 0.32 (Bolger et al., 2014). Paired-end reads that passed this quality-filtering step were joined together using the EA-Utils package and fastq-join (Aronesty, 2011) and analyzed using two different data analysis pipelines. Pipeline 1 was based on the Greengenes database (McDonald et al., 2012) and Pipeline 2 on the Silva 111 database (Quast et al., 2013).

2.4.4.1. Pipeline 1 (Gutarowska et al., 2015). USEARCH v7 (UPARSE) was used for filtering the chimeric sequences and clustering OTUs (Edgar, 2013) and QIIME was used for beta-diversity analyses. No barcode mismatches were allowed. Sequences were demultiplexed using `split_libraries_fastq.py` script in QIIME (Caporaso et al., 2010). Duplicate sequences were removed with "derep_fulllength" command and single reads were eliminated by "sortbysize" command. Sequences were clustered based on 97% identity and an OTU table was created using the nonchimeric sequences. The Ribosomal Database Project classifier (Wang et al., 2007) against the Greengenes database (97% taxonomy) (2012 release: <http://greengenes.lbl.gov>; McDonald et al., 2012) was used for taxonomy assignment of bacteria. Beta diversity analyses were executed on rarefied data using `jackknifed_beta_diversity.py` script in QIIME (Caporaso et al., 2010) and Principal Coordinate Analyses (PCAs) were performed on weighted and unweighted unifracs calculations.

2.4.4.2. Pipeline 2. Unlike Pipeline 1, UCLUST algorithm (Edgar, 2010) and USEARCH v6.1 (Edgar, 2010) were used for clustering and eliminating chimeric sequences. Sequences were clustered based on 99% identity and an OTU table was created using the nonchimeric sequences. The Ribosomal Database Project classifier (Wang et al., 2007) against the Silva 111 database (99% taxonomy) (Quast et al., 2013) was used for taxonomy assignment of bacteria.

Raw data files were deposited in NCBI Sequence Read Archive (SRA) with accession number SRP069323 (Bioproject Accession Number: PRJNA309390, Biosamples SAMN04433034-SAMN04433039).

2.5. FESEM-EDX analysis

Field Emission Scanning Electron Microscopy (FESEM) images of the post-exposure iridium-coated metal surfaces were acquired with a Zeiss SUPRA 55VP Ultra-High Resolution Microscope in the Imaging and Chemical Analysis Laboratory (ICAL) of Montana State University. Energy-dispersive X-ray spectroscopy (EDS) was carried out as previously described (Lenhart et al., 2014).

3. Results and discussion

DNA concentrations recovered from seawater, outer and inner biofilms were 0.06 ng/µl (6 ng in 100 µl), 0.02 ng/µl (2 ng in 100 µl) and 0.60 ng/µl (60 ng in 100 µl), respectively. Considering the final eluent volumes, the amount of DNA recovered from the inner biofilm was 1500 times higher than that of the outer biofilm, emphasizing the immense importance of considering this removal-recalcitrant layer in all biofilm analyses.

For Pipeline 1, after quality checking and filtering, a total of 356,575 sequences were obtained from the duplicate water and biofilm samples, with 133,098 reads from the water sample, 96,199 from the inner biofilm and 127,278 from the outer biofilm. Clustering of these reads at 97% similarity threshold resulted in 2142 OTUs with 0.05% unassigned. The major group overall was the Bacteria (90.1%), with chloroplasts, representing Algae, present at 8.2% and Archaea at 1.7%. There were distinct differences between the microbial populations detected in the seawater, "inner" and "outer" biofilms (Fig. 2).

The statistical similarities and differences were confirmed by Principal Component Analysis (PCA), represented in Fig. 3.

For Pipeline 2, 291,603 sequences were obtained after quality checking and filtering, with 77,633 reads from the water sample, 64,307 from the outer biofilm and 75,010 from the inner biofilm. Clustering of these reads at 99% similarity threshold resulted in 9303 OTUs with 7.32–12.18% unassigned, considerably higher than the Pipeline 1 analysis, probably because of the use of a higher similarity threshold (99% rather than 97%). The major group was still the Bacteria (83.59%–92.63%), with chloroplasts, representing Algae, at 3.47%–18.99% in the biofilms and Archaea at 4.23%. As for Pipeline 1 analysis, there were distinct differences in the microbial populations detected in the seawater, inner and outer biofilms.

As there were almost no major differences between results of analyses by the two pipelines, the following discussions use data given by Pipeline 1, except where otherwise indicated.

3.1. Seawater

In seawater, clustering of the reads at 97% similarity threshold gave 0.05% Archaea, 98.52% Bacteria and 1.09% chloroplast OTUs. Gammaproteobacteria constituted the largest group (35.89%), followed by 30.05% alphaproteobacteria (see Fig. 2). These groups were mainly represented by the Oceanospirillales (16.02% of total OTUs) and Rickettsiales (15.39%), respectively. Acidimicrobiales, which are not included in the proteobacteria, comprised 19.17% of the total OTUs. This is a group that includes a number of iron-oxidizing bacteria, which could be important in the marine corrosion of steel structures (McBeth et al., 2011; Wang et al., 2014). Other groups present at over 1% of the total were Flavobacteriales (5.86%), Rhodobacterales (5.39%), Rhodospirillales (2.89%), Alteromonadales (4.6%), Methylococcales (5.78%), Vibrionales (2.7%) and *Pedospaerae* (1.18%). The latter belongs to the poorly characterized Verrucomicrobiales order.

These proportions differed from those reported by Agogu et al. (2011). They found that more than 80% of the most abundant OTUs in N. Atlantic seawater corresponded to proteobacteria, with 55% being alphaproteobacteria. Gammaproteobacteria included many OTUs belonging to Alteromonadales and Oceanospirillales, also detected earlier by Dang and Lovell (2002). The latter group was identified as a major component of the seawater population in the current study, but the Alteromonadales group was present in these samples at only 4.6% OTUs.

Elifantz et al. (2013) also detected mainly alphaproteobacteria, but only 7.6% gammaproteobacteria, in Eastern Mediterranean seawater. The difference from the present results lies in the fact that

Major groups and differences between seawater and inner and outer biofilms

Fig. 2. Major groups and differences between seawater and inner and outer biofilms at the offshore site, as analyzed using Pipeline 1.

Fig. 3. PCA plot obtained using data processing Pipeline1, demonstrating statistical differences between microbial populations in seawater and inner and outer biofilms on steel coupons at the offshore site.

these samples from the N. Atlantic and Eastern Mediterranean were taken from uncontaminated waters; gammaproteobacteria numbers rise both with increasing depth (Agogu et al., 2011; Friedline et al., 2012) and in areas of hydrocarbon presence (Rivers et al., 2013; King et al., 2015). Within the gammaproteobacteria, 1.26% *Oceanospirillales* were identified in the current study; these can be compared, for example, with the 3% identified by Friedline et al. (2012) in the deep water zone of the Eastern Atlantic and 4% by Agogu et al. (2011) in the N. Atlantic. This group is favoured by aliphatic hydrocarbons (Mason et al., 2012), which are likely present, at least sporadically, in oilfield areas. The gammaproteobacteria are known to play a role in the degradation of organic carbon compounds (Signori et al., 2014).

An important difference between the pipeline analyses of the water was the detection of the genus *Cycloclasticus* at 5% OTUs using Pipeline 2. This organism is able to degrade petroleum polycyclic aromatic hydrocarbons (Kasai et al., 2002) and it would be important to detect these bacteria in potentially hydrocarbon-contaminated waters.

Cyanobacteria were found in low proportion in the Gulf of Guinea samples (0.31%), similar to the results of Agogu et al. (2011). Their samples, however, were taken from the bathypelagic zone of the N. Atlantic, where conditions are unsuitable for light-dependent phototrophs. Surface and subsurface waters may normally contain 1.7–27% of these OTUs, depending on the season

(Agogu et al., 2011; Friedline et al., 2012) and so the Gulf of Guinea levels were lower than expected, possibly caused by primer/PCR bias.

3.2. Biofilms

The clear differences in microbial community structure between planktonic and biofilm organisms (Fig. 2) were consistent with previous studies on various types of abiotic immersed substrates (Araya et al., 2003; Jones et al., 2007; Salta et al., 2013; Pepe-Ranney and Hall, 2015; Dang and Lovell, 2016, and references therein). There was also a clear distinction between the microbial OTU groups detected in the two biofilm layers (Fig. 2).

The outer biofilm (that removed by the standard scraping method) contained 0.22% unclassified OTUs. There were 3.7% OTUs of Archaea, 68.5% of Bacteria and 20.0% of Cyanobacteria. Chloroplasts, representing algae, comprised 5.65% of OTUs. The most abundant in the Archaea group was the Thermoplasmata, at 3.6%; this should be compared with their extremely low proportion in the inner biofilm (<0.1%) and their apparent absence in the seawater. Marine group II euryarchaeota, identified predominantly in the outer biofilm samples (3.1% compared with 0.07% OTUs in inner biofilm and 0% in water), are photoheterotrophs capable of xenobiotics degradation and considered to be associated with marine particles (Martin-Cuadrado et al., 2014, and references therein). Their preferred environmental niche is, in fact, on surfaces and it is not surprising that they are present at higher levels in sessile than in planktonic populations.

Unclassified proteobacteria constituted 0.68% of the outer biofilm OTUs; 13.75% were alphaproteobacteria, 0.95% betaproteobacteria, 17.24% gammaproteobacteria and 3.07% deltaproteobacteria. Elifantz et al. (2013) noted that gammaproteobacteria OTUs increased in overall proportion on their glass slides inserted in the inlet of a desalination plant in Israel with the age of the biofilm, eventually becoming equal to the alphaproteobacteria in number. As with the seawater, the greatest group in the outer biofilm in the current study was the gammaproteobacteria, but in this case the Chromatiales, Oceanospirillales, Vibrionales and Alteromonadales, in that order, predominated, different from the seawater population. The most abundant group in the alphaproteobacteria was the Rhodobacteriales, at 4.8% OTUs, with Rickettsiales next at 2.9%. Flavobacteriales, one of the Bacteroidetes

group, comprised 10.5%. Phototrophs were rather prominent in this layer, with the cyanobacterial family Synechococcophycideae present at 19.8% and the algal Stramenopiles at 1.7% OTUs.

An important difference detected by Pipeline 2 analysis was the presence of the phylum Deferribacteres, which can respire anaerobically using Fe(III), Mn(IV) and nitrate as electron acceptors (Garrrity and Holt, 2001). This group was found at 0.37% OTUs in inner and 1.57% in the outer biofilm using the Silva database.

In the inner biofilm, which remained attached to the steel coupons after scraping, the OTUs identified as Archaea, Bacteria, Cyanobacteria and chloroplasts comprised 0.1%, 80%, 1.28% and 18.8%, respectively, with 0.025% unclassified. The groups detected in the inner, but not the outer biofilm are listed in Table 1. It must be remembered that the total weight of DNA extracted from the inner biofilm was one and a half thousand times that of the outer biofilm. Hence these percentages represent much higher numbers of OTUs.

The inner and outer biofilm percentage OTUs for the phototrophs could reflect the suggestion by Gaylarde and Gaylarde (1999) that algae precede cyanobacteria in biofilm formation in terrestrial habitats. The presence of more algae than cyanobacteria in the Gulf of Guinea inner biofilm may indicate that the algae have attached to the metal surface before the cyanobacteria, the latter being present in much greater proportion in the outer biofilm. This could differentiate this particular environment from that of Dona Paula Bay, in Goa, where cyanobacteria were shown to be the first phototrophs attaching to glass slides immersed in the tropical seawater (Mitbavkar et al., 2012). The difference in substrate (glass

versus metal) could, of course, alter initial adhesion properties, and the colonization sequence is doubtless better defined by time course studies, which should be carried out to confirm the hypothesized sequence in the Gulf of Guinea. The layer of first-attached cells showed other differences from both the seawater and the outer biofilm layer. In particular, the alphaproteobacteria were the predominant group, at 24.56% OTUs. Gammaproteobacteria constituted only 18.2%, with delta-, beta- and epsilon-proteobacteria present at 2.51%, 0.025% and 0.005% OTUs, respectively. Of the alphaproteobacteria, the Rhodobacterales were by far the most abundant, at 14.2%, and the Rickettsiales second, at 6.3% OTUs. The gammaproteobacterial group contained Chromatiales, the purple sulfur bacteria, at a lower proportion than those found in the outer biofilm (2.99% compared with 5.3%), but this group was found at <1% in the seawater. The presence of sulfur, shown by EDS analysis (Table 2), can be correlated to the metabolic function of this group, which can oxidise hydrogen sulfide to elemental sulfur or sulfuric acid.

Of the 2.51% OTUs of deltaproteobacteria, the Myxococcales proportion in the inner layer was above that in the outer biofilm (1.55% compared with 0.9%). Flavobacteriales (mainly Flavobacteriaceae and *Tenacibaculum*) showed a strong presence, with 14.5% OTUs; however, the most abundant group overall in the inner biofilm was the algae, which included Ulvophyceae, Haptophyceae and Rhodophyta at low levels, but with Stramenopiles making up 17.8% of total OTUs. FE-SEM of the biofilms showed the presence of many pennate diatoms (Fig. 4), which certainly comprised the

Table 1

OTUs that were identified in the inner, but not the outer biofilm layers on steel coupons immersed for 5 weeks in seawater at the offshore Gulf of Guinea site, using Pipeline 1 analysis with the Greengenes 97 database.

Upper rank	Lower rank	Genus/denomination	%OTUs
Holophagales			0.09
Acidimicrobiales		TK06	0.025
Bacteroidetes		BME43	1.51, compared with 0.015 in outer biofilm
	Flavobacteriaceae	<i>Aquimarina</i>	0.19
		<i>Olleya</i>	1.1
Cytophagales	unclassified		0.165
		<i>Microscilla</i>	1.325
		<i>Reichenbachella</i>	0.01
	Amoebophilaceae		0.01
	Amoebophilaceae	SC3-56	0.105
Rhodothermales	Rhodothermaceae		0.34
Caldithrix	Caldithrixae: unclassified		0.015
		BA059	0.305
Cyanobacteria		SM2 F09	0.01
		ML635j-21	0.01
Lentisphaeriae:	Unclassified		0.02
Planktomycetes	Unclassified		0.305
	Planktosphaerae	Mle1-8	0.02
Rhizobiales		<i>Devosia</i>	0.01
Rickettsiales	Unclassified		0.03
Deltaproteobacteria	Bacteriovorax		0.055
Gammaproteobacteria	Unclassified		0.055
	Aeromonadaceae		0.06
	Unclassified		
	Alteromonadales		0.18
	Unclassified		
		BD2-13	0.035
		<i>CandidatusEndobium</i>	0.045
		<i>Thalassomonas</i>	0.015
		HTCC 2089	0.09
		<i>Francisella</i>	0.09
		<i>Nebulobacter</i>	0.065
		<i>Marinomonas</i>	0.035
	Oleiphilaceae		0.255
Spirochaetes		<i>Leptonema</i>	0.055
Mollicutes		<i>Mycoplasma</i>	0.02

Note: Using Pipeline 2, only 2 major phyla were detected in inner but not outer biofilm: Spirochaetes (0.1%) and Tenericutes (0.03%). The highest percentage bacterial OTUs in inner that were not detected in outer biofilm are the Cytophagia (Order II Incertae_ Sedis at 0.335%).

Table 2

EDS analysis of surface of mild steel coupon after exposure to Gulf of Guinea seawater at 3 m depth for 5 weeks.

Element	KRatio	Wt%	At%
O	0.1045	23.04	43.17
S	0.2321	20.63	19.29
Cl	0.0266	2.70	2.28
Fe	0.6301	50.22	26.96
Mn	0.0000	0.00	0.00
C	0.0056	3.23	8.08
Si	0.0004	0.05	0.05
Mg	0.0007	0.14	0.18
Total	1.0000	100.00	100.00

majority of the Stramenopiles detected by sequencing. These algae have been stated to dominate in biofilms, whilst centric diatoms are more frequent in the water column (Patil & Anil, 2005).

Elifantz et al. (2013) found no cyanobacteria in the biofilms on their glass slides immersed in the seawater entry of a desalination plant off the coast of Israel, although these phototrophs were present in quantity in the water itself. Again, this echoes the data acquired from terrestrial studies. The sub-aerial biofilms formed on glass solar panels in Brazil showed the presence of few algae and no cyanobacteria (Shirakawa et al., 2015), even though these organisms were present in the tree-shaded environment and had been found in high numbers on building materials at this site. It was suggested that the phototrophs are less able to adhere to glass surfaces than to other substrates. Saikia and Das (2011) also indicated the relatively low adherence in a wetland environment of cyanobacteria to glass in comparison with plant matter; they also indicated that the Bacillariophyceae (diatoms) preferred the glass substrate.

The results of the inner biofilm analyses suggested that algae are among the first colonisers of metal surfaces immersed in the sea and that they attach sufficiently well to resist removal by scraping. Interestingly, the cyanobacterial groups 4Cod-2 and ML635J-21 were detected at 0.01% OTUs each in the inner biofilm, but were not found in any of the other samples. These are members of the non-photosynthetic class, Melainabacteria, considered to represent an ancient lineage of the Cyanobacteria (Soo et al., 2014).

The Rhodobacteriaceae were also more abundant in the inner than the outer biofilm (14.2% compared with 4.22% OTUs). Elifantz et al. (2013) noted that these prosthecate bacteria are key members

of the initial stages of biofilm formation on glass slides immersed in seawater and Dang and Lovell (2002) and Dang et al. (2008) suggested that the whole order of Rhodobacteriales are important primary colonisers of surfaces. This is apparently true also for steel surfaces.

The Saprospiraceae were concentrated in the inner biofilm (6.69% compared to 3.22% OTUs in the outer biofilm). These organisms have been associated with the ability to utilize complex carbon sources, as have other members of the Bacteroidetes (Xia et al., 2008). Some families of the Oceanospirillales were also more abundant in the inner biofilm; the Endozoicimonadaceae, Oceanospirillaceae and Oleiphilaceae OTUs were all increased by at least 0.15% and the last group was completely absent from other samples, but present as 0.255% OTUs in the layer adjacent to the metal surface. It could be speculated that these bacteria attached during the early stages of exposure, a supposition that could also be applied to the Marinicellaceae, found at 1.9% in the inner, but only 0.4% in the outer biofilm layers.

Elifantz et al. (2013) found that Actinobacteria were absent from their biofilms on glass, even though they were present at substantial levels in the water. In the Gulf of Guinea analyses, although Actinobacteria OTUs constituted 19.27% of seawater totals, they were only 3.37% and 0.68% of outer and inner biofilms, respectively, confirming the apparent inability of Actinobacteria to participate in biofilms on abiotic solid surfaces in a liquid medium. In fact, some Actinobacteria have actually been shown to inhibit biofilm formation by other organisms (Nithyanand et al., 2010; Park et al., 2012).

The Pseudomonadaceae were found at surprisingly low levels in all samples, with 0.01% and 0.005% OTUs in seawater and inner biofilm, respectively. In the outer biofilm, however, they comprised 0.255% of OTUs. Members of this group are regarded as archetypal biofilm formers, known to attach to metal surfaces (Beech and Gaylarde, 1992; Vu et al., 2009; Harimawan et al., 2011) and these results can only be explained through the unexpectedly low seawater levels of these bacteria.

Although pseudomonads were found in low abundance, the Vibrionales were present in all samples, 2.7%, 2.5% and 3.23% OTUs in seawater, outer and inner biofilms, respectively. Like the pseudomonads, these organisms are producers of extracellular polysaccharides, which facilitate attachment to surfaces (Kavita et al., 2011, and references therein). The genus *Vibrio*, found in all samples, has been associated with metal corrosion (Gaylarde and Videla, 1987), but no groups specifically involved with this process (sulfate-reducing bacteria, zetaproteobacteria) were detected at significant levels in the samples when the Pipeline 1 (Green-genes database) was used in the analysis. When the Silva database (Pipeline 2) was employed, however, SRB OTUs were found in the inner (0.365%) and outer (0.39%) biofilms and in the water (0.015%), showing the importance of using more than one type of analysis of DNA sequencing data. The relatively low exposure time (5 weeks) may have led to biofilms of insufficient thickness to allow the development of large numbers of strict anaerobic bacteria; the highest OTU percentage for such organisms was below 0.5, for Peptostreptococcaceae. Sulfate reducing genera such as *Desulfobacter*, *Desulfovibrio*, *Desulfosporosinus* and *Thermoanaerobacter* and Syntrophobacteraceae were observed with a total abundance of less than 0.05% OTUs in the Pipeline 1 analysis.

At relatively low depths in coastal seawater sites, accelerated low water corrosion (ALWC), often associated with zetaproteobacteria iron-oxidizing bacteria, is considered to be important (Dang et al., 2011). The Gulf of Guinea samples showed <0.005% zetaproteobacteria OTUs, suggesting that, at this particular site, ALWC was not a major concern. The low levels of nitrate in the water (Witt et al., 2016) provided further evidence for this suggestion. Recent work has indicated that steel structures positioned

Fig. 4. FE-SEM image of biofilm surface, showing large numbers of pennate diatoms of various sizes (arrows).

on the sediment layer may be more likely to develop biofilms containing iron-oxidizing bacteria (McBeth and Emerson, 2016).

The overall results suggested the importance of the groups Stramenopiles and Rhodobacterales in initial biofilm formation on steel surfaces at the investigated site. The low adherence of Actinobacteria to inert surfaces was confirmed. It was also confirmed that the industrial practice of extrapolating planktonic community structure to that of the biofilm is incorrect. It is emphasized that studies on biofilms must consider the detection of organisms comprising the inner biofilm. Most studies on marine biofilms on metallic materials focus on information based on DNA extracted from surface deposits removed by physical means. It is well recognized that biofilms are never completely removed by these methods. To our knowledge, the extent of DNA remaining on the surface has never been reported but it is widely underestimated. Whether this DNA originates from lysed cells of primary colonizers, or originates from free, extracellular DNA (e-DNA) present in the marine environment, is unclear. Certainly, it has been documented that this e-DNA reacts strongly with minerals, which are also present in steel corrosion products (Torti et al., 2015, and references therein). Because of its known ability to interact with iron, e-DNA is very likely to be present on corroding metal surfaces in a biotic environment.

This is the first investigation demonstrating the abundance of DNA directly associated with the metal surface and showing that the population associated with this DNA is different from that forming the outer biofilm layer. Whether this DNA represents the first colonizers on the metal surface, or whether it is e-DNA adsorbed from seawater or released from dead cells, it is an essential component of the biofilm and must not be ignored.

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References

- Agogué, H., Lamy, D., Neal, P.R., Sogin, M.L., Herndl, G.J., 2011. Water mass-specificity of bacterial communities in the North Atlantic revealed by massively parallel sequencing. *Mol. Ecol.* 20, 258–274. <http://dx.doi.org/10.1111/j.1365-294X.2010.04932.x>.
- Araya, R., Tani, K., Takagi, T., Yamaguchi, N., Nasu, M., 2003. Bacterial activity and community composition in stream water and biofilm from an urban river determined by fluorescent *in situ* hybridization and DGGE analysis. *FEMS Microbiol. Ecol.* 43, 111–119.
- Aronesty, E., 2011. *ea-utils*: Command-line Tools for Processing Biological Sequencing Data. Accessed on 28th April, 2016. <http://code.google.com/p/ea-utils>.
- Arumugam, R., Chan, X.-Y., Yin, W.F., Choo, S.W., Chan, K.G., 2013. Metagenomic analysis of microbial diversity of tropical sea water of Georgetown coast, Malaysia. *Life Sci. J.* 10, 2392–2396.
- Beech, I.B., Gaylarde, C.C., 1992. Attachment of *Pseudomonas fluorescens* and *Desulfovibrio* mild steel and stainless steel—first step in biofilm formation. In: Sequeira, A.C., Tiller, A.K. (Eds.), *Proceedings of the 2nd European Federation of Corrosion. European Federation of Corrosion Publication number 8*, The Institute of Materials, Lisbon, Portugal, pp. 61–66.
- Bolger, A.M., Lohse, M., Usadel, B., 2014. Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics* 30, 2114–2120.
- Caporaso, J.G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F.D., Costello, E.K., Fierer, N., Gonzalez Peña, A., Goodrich, J.K., Gordon, J.I., et al., 2010. QIIME allows analysis of high-throughput community sequencing data. *Nat. Meths* 7, 335–336.
- Dang, H.Y., Lovell, C.R., 2002. Numerical dominance and phylotype diversity of marine *Rhodobacter* species during early colonization of submerged surfaces in coastal marine waters as determined by 16S ribosomal DNA sequence analysis and fluorescence *in situ* hybridization. *Appl. Environ. Microbiol.* 68, 496–504.
- Dang, H.Y., Lovell, C.R., 2016. Microbial surface colonization and biofilm development in marine environments. *Microbiol. Mol. Biol. Rev.* 80, 91–138. <http://dx.doi.org/10.1128/MMBR.00037-15>.
- Dang, H., Li, T., Chen, M., Huang, G., 2008. Cross-Ocean distribution of *Rhodobacterales* bacteria as primary surface colonizers in temperate coastal marine waters. *Appl. Environ. Microbiol.* 74, 52–60.
- Dang, H., Chen, R., Wang, L., Shao, S., Dai, L., Ye, Y., et al., 2011. Molecular characterization of putative biocorroding microbiota with a novel niche detection of Epsilon- and Zetaproteobacteria in Pacific Ocean coastal seawaters. *Environ. Microbiol.* 13, 3059–3074.
- Edgar, R.C., 2010. Search and clustering orders of magnitude faster than BLAST. *Bioinformatics* 26, 2460–2461.
- Edgar, R.C., 2013. UPARSE: highly accurate OTU sequences from microbial amplicon reads. *Nat. Meths* 10, 996–998.
- Elifantz, H., Horn, G., Ayon, M., Cohen, Y., Minz, D., 2013. *Rhodobacteraceae* are the key members of the microbial community of the initial biofilm formed in Eastern Mediterranean coastal seawater. *FEMS Microbiol. Ecol.* 85, 348–357.
- Friedline, C.J., Franklin, R.B., McCallister, S.L., Rivera, M.C., 2012. Bacterial assemblages of the Eastern Atlantic Ocean reveal both vertical and latitudinal biogeographic signatures. *Biogeosciences* 9, 2177–2193.
- Garrity, M., Holt, J.M., 2001. *Bergey's manual of systematic bacteriology*. In: Boone, D.R., Castenholz, R.W., Garrity, G.M. (Eds.), *The Archaea and the Deeply Branching and Phototrophic Bacteria*, Volume One. Springer, New York, pp. 465–471.
- Gaylarde, P.M., Gaylarde, C.C., 1999. Colonization sequence of phototrophs on painted walls in Latin America (abstract). *Int. Biodeter. Biodeg.* 44, 168.
- Gaylarde, C.C., Videla, H.A., 1987. Localised corrosion associated with a marine *Vibrio*. *Int. Biodeter. Biodeg.* 23, 91–104.
- Gutierrez, J., Gudiño, K., Maldonado, S., Benítez, S., Macías, A., 2015. Old bacterial A.L. and high-throughput sequencing analysis—modern approach for the assessment of biodegradation of materials from historic buildings. *Front. Microbiol.* 6, Article 979.
- Harimawan, A., Rajasekar, A., Ting, Y.-P., 2011. Bacteria attachment to surfaces—AFM force spectroscopy and physicochemical analyses. *J. Coll. Interf. Sci.* 364, 213–218.
- Jones, R.D., Ruppel, M., Kirchner, J., Macdonald, S., 2007. *Bacterial* 53, 151–162.
- Kasai, Y., Kishira, H., Harayama, S., 2002. Bacteria belonging to the genus *Cycloclasticus* play a primary role in the degradation of aromatic hydrocarbons released in a marine environment. *Appl. Environ. Microbiol.* 68, 5625–5633.
- Kavita, K., Mishra, A., Jha, B., 2011. Isolation and physico-chemical characterisation of extracellular polymeric substances produced by the marine bacterium *Vibrio parahaemolyticus*. *Biofouling* 27, 309–317.
- King, G.M., Kostka, J.E., Hazen, T.C., Sobczyk, P.A., 2015. Microbial responses to the Deepwater Horizon oil spill: from coastal wetlands to the deep sea. *Annu. Rev. Mar. Sci.* 7, 377–401.
- Klindworth, A., Priesse, E., Schweer, T., Peplies, J., Quast, C., Horn, M., Glockner, F.O., 2013. Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. *Nucleic Acids Res.* 41, e1.
- Lee, T.M., Melchers, R.E., Beech, I.B., Potts, A.E., Kilner, A.A., 2015. Microbiologically influenced corrosion (MIC) of mooring systems: diagnostic techniques to improve mooring integrity. In: *Proc. of the 20th Offshore Symposium*, Feb. 2015. Society of Naval Architects and Marine Engineers (SNAME), Houston, TX.
- Lenhart, T.R., Duncan, K.E., Beech, I.B., Sunner, J.A., Smith, W., Bonifay, V., Biria, B., Suffita, J.M., 2014. Identification and characterization of microbial biofilm communities associated with corroded oil pipeline surfaces. *Biofouling* 30, 823–835.
- Martin-Cuadrado, A.-B., Garcia-Heredia, I., Moltó, A.G., López-Úbeda, R., Kimes, N., López-García, P., Moreira, D., Rodríguez-Valera, F., 2014. A new class of marine Euryarchaeota group II from the Mediterranean deep chlorophyll maximum. *ISME J. Adv. online Publ.* <http://dx.doi.org/10.1038/ismej.2014.249>, 23 December 2014.
- Mason, O.U., Hazen, T.C., Borglin, S., Chain, P.S., Dubinsky, E.A., Fortney, J.L., Han, J., Holman, H.Y., Hultman, J., Lamendella, R., et al., 2012. Metagenome, meta-transcriptome and single-cell sequencing reveal microbial response to Deep-water Horizon oil spill. *ISME J.* 6, 1715–1727.
- McBeth, J.M., Emerson, D., 2016. *In Situ* microbial community succession on mild steel in estuarine and marine environments: exploring the role of iron-oxidizing bacteria. *Front. Microbiol.* 7, 767. <http://dx.doi.org/10.3389/fmicb.2016.00767>.
- McBeth, J.M., Little, B.J., Ray, R.I., Farrar, K.M., Emerson, D., 2011. Neutrophilic iron-oxidizing “Zetaproteobacteria” and mild steel corrosion in nearshore marine environments. *Appl. Environ. Microbiol.* 77, 1405–1412.
- McDonald, D., Price, M.N., Goodrich, J., Nawrocki, E.P., DeSantis, T.Z., Probst, A., Andersen, G.L., Knight, R., Hugenholtz, P., 2012. An improved greengenes taxonomy with explicit ranks for ecological and evolutionary analyses of bacteria and archaea. *ISME J.* 6, 610–618.
- Mitbavkar, S., Raghu, C., Rajaneesh, K.M., Pavan, D., 2012. Picophytoplankton community from tropical marine biofilms. *J. Exp. Mar. Biol. Ecol.* 426/7, 88–96.
- Nithya, S., Sathya, S., Sathya, S., Sathya, S., Sathya, S., Sathya, S., 2016. *Current Microbiol.* 60, 454–460.
- Park, J.-H., Lee, J.-H., Kim, C.J., Lee, J.-C., Cho, M.H., Lee, J., 2012. Extracellular protease in Actinomycetes culture supernatants inhibits and detaches *Staphylococcus aureus* biofilm formation. *Biotechnol. Lett.* 34, 655–661.
- Patil, J., Anil, A., 2005. Biofilm diatom community structure: influence of temporal and temporal variability. *Biofouling* 21, 189–206.
- Pepe-Ranney, C., Hall, E., 2015. The effect of carbon subsidies on planktonic niche

- partitioning and recruitment during biofilm assembly. *Front. Aquat. Microbiol. Res.* Article 13 January 2015.
- Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., Peplies, J., Glöckner, F.O., 2013. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucl. Acids Res.* 41 (D1), R59–R66.
- Rivera-Soto, S., Tringe, S.G., Martin, J., Joye, S.B., Moran, M.A., 2013. Transcriptional response of bathypelagic marine bacterioplankton to the Deepwater Horizon oil spill. *ISME J.* 7, 2315–2329.
- Saikia, S.K., Das, D.N., 2011. Diversity and productivity (Chlorophyll-a and Biomass) of periphyton on natural and artificial substrates from wetland ecosystem. *J. Wetl. Ecol.* 5, 1–9.
- Salta, M.S., Wharton, J.A., Blache, Y., Stokes, K.R., Briand, J.-F., 2013. Marine biofilms on artificial surfaces: structure and dynamics. *Environ. Microbiol.* 15, 2879–2893.
- Shirakawa, M.A., Zilles, R., Mocelin, A., Gaylarde, C.C., Gorbushina, A., Heidrich, G., Giudice, M.C., Del Negro, G.M.B., John, V.M., 2015. Microbial colonization affects the efficiency of photovoltaic panels in a tropical environment. *J. Environ. Manage* 157, 160–167.
- Signori, C.N., Thomas, F., Enrich-Prast, A., Pollery, R.C.G., Sievert, S.M., 2014. Microbial diversity and community structure across environmental gradients in Bransfield Strait, Western Antarctic Peninsula. *Front. Microbiol.* <http://dx.doi.org/10.3389/fmicb.2014.00647>.
- Soo, R.M., Skennerton, C.T., Sekiguchi, Y., Imelfort, M., Paech, S.J., Dennis, P.G., Steen, J.A., Parks, D.H., Tyson, G.W., Hugenholtz, P., 2014. An expanded genomic representation of the phylum Cyanobacteria. *Genome Biol. Evol.* 6, 1031–1045. <http://dx.doi.org/10.1093/gbe/evu073>.
- Torti, A., Lever, M.A., Jørgensen, B.B., 2015. Origin, dynamics, and implications of extracellular DNA pools in marine sediments. *Mar. Genomics* 24, 185–196.
- Vu, B., Chen, M., Crawford, R.J., Ivanova, E.P., 2009. Bacterial extracellular polysaccharides involved in biofilm formation. *Molecules* 14, 2535–2554.
- Wang, Q., Garrity, G.M., Tiedje, J.M., Cole, J.R., 2007. Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Appl. Environ. Microbiol.* 73, 5261–5267.
- Wang, H., Ju, L.-K., Castaneda, H., Cheng, G., Newby, B.-M.Z., 2014. Corrosion of carbon steel C1010 in the presence of iron oxidizing bacteria *Acidithiobacillus ferrooxidans*. *Corr. Sci.* 89, 250–257.
- Witt D, Ma K-T, Lee T, Gaylarde C, Celikkol S, Makama Z, Beech I. 2016. Field Studies of Microbiologically Influenced Corrosion of Mooring Chains. Paper to be presented at: Offshore Technology Conference, 2–5 May 2016, Houston, Texas, USA, Paper no. OTC-27142-MS.
- Xia, Y., Kong, Y., Thomsen, T.R., Nielsen, P.H., 2008. Identification and ecophysiological characterization of epiphytic protein hydrolyzing *Septosporaceae* (“*Candidatus Epiflobacter*” spp.) in activated sludge. *Appl. Environ. Microbiol.* 74, 2229–2238.
- Yooseph, S., Nealson, K.H., Rusch, D.B., McCrow, J.P., Dupont, C.L., Kim, M., Johnson, J., Montgomery, R., Ferriera, S., Beeson, K., et al., 2010. Genomic and functional adaptation in surface ocean planktonic prokaryotes. *Nature* 468, 60–66.
- Yutin, N., Suzuki, M.T., Teeling, H., Weber, M., Venter, J.C., Rusch, D.B., Bèjà, O., 2007. Assessing diversity and biogeography of aerobic anoxygenic phototrophic bacteria in surface waters of the Atlantic and Pacific Oceans using the Global Ocean Sampling expedition metagenomes. *Env. Microbiol.* 9, 1464–1475.