Bacterial cell numbers and community structures of seawater biofilms depend on the attachment substratum

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Abstract

Seawater is increasingly being used as a source for various industrial applications. For such applications, biofilm growth creates various problems including but not limited to pipe biocorrosion. In this study, it is hypothesized that the material type is preferred by certain bacterial populations in the seawater to attach and establish biofilms. By comparing differences in the total cell counts and microbial communities attached to high density polyethylene (HDPE), polycarbonate, stainless steel (SS316) and titanium, the appropriate material can be used to minimize biofilm growth. All four materials have hydrophilic surfaces, but polycarbonate exhibits higher surface roughness. There were no significant differences in the cell numbers attached to polycarbonate, HDPE and titanium. Instead, there were significantly fewer cells attached to SS316. However, there was a higher relative abundance of genera associated with opportunistic pathogens on SS316. Copy numbers of genes representing Desulfobacteraceae and Desulfobulbaceae, both of which are sulfate-reducing bacteria (SRB), were approximately 10-fold higher in biofilms sampled from SS316. The enrichment of SRB in the biofilm associated with SS316 indicates that this material may be prone to biocorrosion. This study highlights the need for industries to consider the choice of material used in seawater applications to minimize microbial-associated problems.

1. Introduction

Seawater is increasingly being used as a source for various industrial applications in an attempt to alleviate the demand for freshwater. Examples of such industrial applications include seawater cooling tower systems and seawater injection for enhanced oil recovery. In most instances, seawater is usually recirculated at least once to minimize operating costs associated with continuous pumping of new seawater into the system. However, recirculating seawater allows for microbes, minerals and nutrients to accumulate within the system [1]. To tackle inorganic scaling problems arising from minerals, antiscalants are commonly used even though they generally include components of polymers, phosphates and phosphonates and can contribute nutrients that support microbial growth [2]. Hence, extensive biofilm growth is an inevitable problem in seawater pipelines.

Biofilm growth creates a suite of problems. For example, biofilms can serve as colonization platforms for pathogens to augment their environmental persistence [3, 4]. Biofilm formation is also correlated with the occurrence of biocorrosion [5], otherwise known as microbial-induced corrosion (MIC). This is shown by an increased rate of corrosion in the presence of biofilms [6]. Although total eradication of biofilms and the associated microbial populations would be impractical, efforts have been undertaken to minimize cell counts and bacterial populations that may be present in the biofilm by varying certain operational factors. These factors include maintaining the presence of residual disinfectant, optimizing water age, hydraulic regimes and pipe materials [7-15].

However, most of the existing studies utilized culture-based approaches or DNA-based fingerprinting techniques to determine potential impacts on the biofilm arising from these operational factors. Cultivation-based approaches to enumerating cell counts are subject to bias

and are not representative of bacterial species that are fastidious about growth conditions [16, 17]. When compared to high-throughput amplicon-based sequencing approaches, fingerprinting techniques, including denaturing gel electrophoresis or terminal restriction length polymorphisms, are not able to provide an in-depth characterization of the bacterial community structure. Neither are the fingerprinting techniques capable of providing quantitative measurements of certain bacterial populations when compared to the quantitative PCR (qPCR) approach. An earlier study used high-throughput sequencing to show that bacterial and/or eukaryotic community structures in drinking water biofilms can be affected by these factors. Specifically, pipe materials only affect the bacterial community structure but not the eukaryotic community [8]. However, similar studies on the variation of cell counts and bacterial community structures in seawater in relation to the pipe materials are not widely available.

A better understanding of whether material types result in differences in microbial populations, specifically, sulfate-reducing bacteria (SRB) and genera associated with opportunistic pathogens, is needed to assess the potential relevance to industrial stakeholders and public health. SRB possess organic filaments similar to nanowires [18], which may play a role in adhesion, biofilm formation and direct interspecies electron transfer [19]. Adherence to a conductive material may facilitate extracellular electron transfer and metal reduction, both of which are key metabolic traits required by the SRB [20]. As such, SRB are generally thought to be the protagonist in biocorrosion [21]. Biocorrosion is believed to account for 20% of the damage caused by corrosion [22], which can impart a tremendous financial burden on industrial stakeholders. In addition, the presence of opportunistic pathogens can also pose potential health threats to certain groups of individuals, for example, immunocompromised patients, the elderly and infants, upon exposure.

In this study, it is hypothesized that the material type is preferred by certain bacterial populations in the seawater to attach and establish biofilms. It is further hypothesized that the type of material plays a less significant role in affecting the total cell count and composition of the microbial community as the biofilm ages. The materials tested were stainless steel 316 (i.e., SS316), titanium, high density polyethylene (HDPE) and polycarbonate. SS316 and titanium are commonly used in the heat exchangers [23] for industrial seawater applications. HDPE and polycarbonate are included in this study to serve as non-metal controls and because they are plastic materials commonly used in industrial applications. Efforts were made to determine the effect of materials on the genera associated with opportunistic pathogens and sulfate-reducing bacteria (SRB). To address these issues, counts of cells adhering to the different type of materials were enumerated by flow cytometry, while both high-throughput sequencing and qPCR were utilized to characterize the microbial community structures.

2. Materials and methods

2.1. Experimental procedure

The materials tested in this study were stainless steel 316 (i.e., SS316), titanium, high density polyethylene (HDPE) and polycarbonate. These materials were characterized for their surface roughness and hydrophilicity as detailed in Section 2.2. The materials were then placed inside bioreactors and exposed to seawater over a period of four months (Section 2.3). Seawater biofilm established on the materials were harvested using sampling procedure described in Section 2.4. The harvested samples were divided into two portions. The first portion was determined for the total bacterial cell counts by flow cytometry (Section 2.5). The second portion was extracted for DNA (Section 2.6), and the DNA was used in two types of molecular-based analyses. The first molecular-based analysis was 16S rRNA gene amplicon sequencing to characterize the microbial community attached on the different materials (Section 2.7). The second molecular-based analysis was quantitative PCR (qPCR) to quantify for the copy numbers of *Acinetobacter baumannii* (Section 2.8), Desulfobacteraceae and Desulfobulbaceae (Section 2.9).

2.2. Material surface characterization

The materials tested in this study were purchased from Biosurface Technologies (Bozeman, MT, USA) in a circular disc coupon format, each with a diameter of approximately 1.2 cm. All the materials that were tested in this study were evaluated for their surface roughness and extent of hydrophobicity based on procedures described previously [24]. Briefly, atomic force microscopy (AFM) was used to characterize the surface topography of each material type based on the standard protocol defined by American Society of Mechanical Engineer (ASME) B46.1-2009. AFM imaging was performed using Bruker Dimension ICON equipment (Santa Barbara, CA, USA) in soft tap mode to scan images of 15 µm width by 15 µm length at three random locations

on each material type. The AFM images were analyzed on Pico Image Software (Keysight Technologies Inc., Santa Rosa, CA, USA). Specifically, surface roughness is represented as the root mean square (RMS) average of profile height deviations from the mean height observed for that particular material. A higher RMS value indicates more apparent surface peaks and valleys, thus representing a rougher surface. The contact angle was measured by an Easy Drop Shape Analyzer (Kruss, Hamburg, Germany) in static mode at ambient temperature. Ultra-pure water was used as the probing liquid, and the mean values were determined from three different independent specimens. Generally, if the contact angle is smaller than 90°, the solid surface is considered hydrophilic and vice versa [25].

2.3. Biofilm reactor operation

From February to June 2016, a total of four Communicable Disease Centre (CDC) bioreactors (BioSurface Technologies, Bozeman, MT, USA) were operated at 28 °C and at a stirring rate of 200 rpm in the KAUST Water Desalination and Reuse Center (WDRC) (Figure 1). Each bioreactor had eight coupon holders that could be fitted with a total of 24 coupons of the same material per reactor. Seawater fed into the bioreactors was sourced from the Red Sea through an approximately 1 km pipeline to the WDRC laboratory. The seawater tap was flushed for 10 minutes prior to collection of seawater in a sterile 20 L carboy container. Thereafter, seawater from the same container was fed at a continuous rate of 1.3 mL per minute to each bioreactor and replaced with fresh seawater every 10 days. The hydraulic retention time of seawater within the bioreactor was 6.4 h. The bioreactors were periodically checked for the hydraulic retention time, stirring rate, temperature and flow rate so as to ensure that the operating conditions among all four reactors are kept similar. Two L of seawater that was to be fed into the reactors were collected each month during the course of the experiment and evaluated for its total cell counts

and microbial community in accordance with protocols mentioned in the following subsections. All the bioreactors were covered with aluminum foil to limit exposure to light.

2.4. Sampling procedure

Biofilm was sampled at 1 through 4 months after the commencement of the bioreactors. Six coupons for each type of material were collected during each sampling month and provided two sets of biological replicates for that particular sampling month. Each biological replicate comprised the pooled biomass scraped down from three coupons. The six coupons were selected from two coupon holders placed at random locations within the bioreactor to minimize any sampling bias due to the location of the coupon. New holders containing coupons of the same material type were replaced after each sampling event to ensure that the shear force was kept constant within the bioreactor. The sampled coupons were placed into separate petri dishes, and 5 mL of 1X phosphate-buffered saline (PBS) solution was added. Autoclaved cotton swabs were then used to scrape down the loosely attached biofilm into the 1X PBS. The resulting suspension for each material was transferred to sterile 50 mL centrifuge tubes. The used cotton swabs and coupons were placed in a sterile centrifuge tube that contained 10 mL of 1X PBS. This tube was vortexed at high speed for 10 minutes with the aim of dislodging any attached biofilm on the cotton swab and coupons. Following this, the supernatant from each vortexed tube was transferred to the corresponding tube containing the loosely attached biofilm. A 20 μL sample of the combined supernatant was set aside for total cell enumeration by flow cytometry. The remaining supernatant was centrifuged at 8000 *g* for 10 minutes, and the cell pellet was used for DNA extraction.

2.5. Flow cytometry to obtain total cell counts

A 7 µL sample of the supernatant or seawater was diluted 100-fold with 1X PBS, and 10 µL of 500 mM ethylenediaminetetraacetic acid (EDTA) was added to chelate metals and other inhibitors that can affect flow cytometry measurements. The mixture was briefly vortexed and incubated at 35 °C for 20 minutes. Then, 7 µL of 100X SYBR Green I (Thermo Fisher Scientific, Carlsbard, CA, USA) was added, and the samples were incubated for 10 minutes. The number of cells in the resultant mixture was then quantified using a BD Accuri C6 (Thermo Fisher Scientific, Carlsbard, CA, USA) flow cytometer, based on protocol described by manufacturer [26].

2.6. DNA extraction

The microbial DNA from the biofilms was extracted using the UltraClean® PowerSoil DNA Isolation kit following the manufacturer's protocol with brief modifications as described previously [27]. To extract DNA from seawater, 2 L of seawater was first filtered through a 0.22 µm polycarbonate membrane filter (VWR, Radnor, PA, USA), and the retained biomass on the filter was extracted using a protocol similar to the one mentioned previously. The DNA concentration was then quantified using the Qubit Broad Range DNA Assay according to the manufacturer's protocol. The DNA was used for molecular-based analyses detailed in Section 2.7 to 2.9.

2.7. 16S rRNA gene-based amplicon sequencing and amplicon sequencing data analysis

16S rRNA genes were amplified from the DNA with primer pair 515F and 907R based on procedures described earlier [24]. Purified amplicons were pooled and submitted to the KAUST Genomics Core lab for sequencing on the Illumina MiSeq platform. All high-throughput sequencing data were deposited in European Nucleotide Archive under accession number

194 PRJEB20120.The two main hypotheses of this study are that first, certain bacterial populations
195 in the seawater prefer to attach to specific types of material prior to biofilm formation, and
196 second, the type of material plays a less significant role in affecting the total cell count and
197 composition of the microbial community as the biofilm ages. Amplicon sequences that denote
198 the microbial community were therefore analyzed to test these two hypotheses. Sequences were
199 sorted by the Bioinformatics Team at KAUST based on a Phred score of > 20. The sorted
200 sequences were then trimmed off for the primers, barcodes and adaptor sequences, and any
201 sequences > 300 nt in length were removed. Chimeras were identified and removed on UCHIME
202 [28] by comparison with a core reference FASTA file downloaded from Greengenes
203 (http://greengenes.lbl.gov/Download/). Chimera-free sequences were assigned to
204 bacterial/archaeal taxonomic hierarchy at the 95% classification reliability level using the
205 Ribosomal Database Project (RDP) Classifier [29]. The relative abundances of the bacterial and
206 archaeal genera were calculated, collated and then square-root transformed. Square-root
207 transformation was performed to down-weigh the dominant taxa and to achieve a better balance
208 of the abundant and rare species that are present in the samples. This allows the rare species,
209 which are common in the data generated from high-throughput sequencing, to exert some
210 influence on the calculation of similarity. The transformed data sets were then computed for their
211 Bray–Curtis similarities and represented graphically for relative differences in bacterial
212 community composition among samples aligned against either the materials or the duration of
213 operation as factors in a boot-strapped metric multidimensional scaling (mMDS) plot. That is,
214 samples that were further apart from each other shared less similarity than those that were closer
215 together. All mMDS plots were obtained using Primer-E version 7 [30]. Chimera-free sequences
216 were also submitted to the RDP pipeline for clustering analysis to identify the number of unique

operational taxonomic units (OTUs) with < 97% sequence similarity at each respective sequencing depth [31]. The number of unique OTUs identified at a sequencing depth of 2715 sequences were collated and used to compare the level of microbial richness among the individual samples. This sequencing depth was used because this was the lowest number of reads obtained for one particular sample, and the comparison of the microbial richness across samples, therefore, had to be standardized at this depth.

2.8. Quantitative PCR for *Acinetobacter baumannii*

Acinetobacter baumannii was chosen as the pathogenic bacterial species in the genus *Acinetobacter*, and its abundance was determined because this genus was detected in some of the biofilm samples. Copy numbers of the *ompA* gene representing *Acinetobacter baumannii* were determined using qPCR with a 7900 HT Applied Biosystems real-time PCR thermal cycler (Thermo Fisher Scientific, Carlsbad, CA, USA). Sequences of the primer pairs are listed in Table 1. The qPCR standards were prepared as described previously [32, 33]. To produce qPCR standard curves, plasmid DNAs were diluted in series to form concentrations ranging from 10^2 to 10^8 copies/µL. Each reaction volume of 20 µL contained 10 µL of Fast SYBR Green master mix, 0.4 µL of each primer (10 µM), 1 µL of DNA template and 8.2 µL H_2O. The reaction for amplification was run using an annealing temperature of 60 °C, and the melting curve analysis was performed with a dissociation cycle which included an increment of temperature from 60 °C to 95 °C with intervals of 0.5 °C for 5 s each. The threshold cycle (C_q) values for each dilution were plotted against the log-transformed concentration of each dilution. The amplification factor of the standards was 2.99, and the R-squared value was > 0.98. Amplifications to obtain standard curves were performed in triplicate, while test amplifications and negative non-template controls (NTCs) were run in duplicate. All NTCs had no determinable C_q values. The copy numbers

obtained from qPCR reactions were normalized against the total cell numbers obtained by flow cytometry for the corresponding sample.

2.9. Quantitative PCR for SRB

Both the Desulfobacteraceae and Desulfobulbaceae are families comprised of genera associated with SRB. The copy numbers of 16S rRNA genes representing total Desulfobacteraceae and Desulfobulbaceae were, therefore, determined to discern the presence and abundance of certain types of SRB. The qPCR standards for Desulfobacteraceae and Desulfobulbaceae were prepared by first amplifying the gene fragment with the appropriate primer pairs using DNA extracted from *Desulfobacter hydrogenophilus* DSM3380 and *Desulfobulbus elongatus* DSM2908 as bacterial templates. The amplified products were cloned into vectors, and the inserted genes were sequenced to verify that the sequences were perfectly complementary to the primer target region. The qPCR reactions were carried out as described above. The amplification factor of the standards ranged from 1.98 to 2.13 with R-squared values > 0.99. Amplifications to obtain standard curves were performed in triplicate, while test amplifications and NTCs were run in duplicate. All NTCs had no determinable C_q values. Copy numbers obtained from qPCR were normalized against the total cell numbers obtained by flow cytometry for the corresponding sample.

2.10. Statistical analyses

Two-tailed t-tests were carried out to evaluate significant differences in surface roughness, cell counts and copy numbers of Desulfobacteraceae and Desulfobulbaceae among samples. To compare differences in the microbial communities, one-way unordered Analysis of Similarity (ANOSIM) was carried out on Primer-E version 7 for both material types and times. Similarity

percentage analysis (SIMPER) was also carried out using the Bray-Curtis similarity matrix generated from the high-throughput sequencing data and Primer-E version 7 to determine which microbial population contributed most to the differences between material types. All differences were determined to be significant at the 90% confidence level (i.e., $p < 0.10$). The confidence level was set at 90% on the basis that biological samples have a usual baseline variance that may vary with time, and can contribute to outliers. Hence, setting the confidence level at 90% would account for differences among treatment but at the same time, do not lower the comparison power into irrelevance.

3. Results

3.1. Surface characteristics of the different materials

Surface roughness and the extent of hydrophobicity were evaluated for the four types of materials. Polycarbonate exhibited a significantly higher surface roughness than stainless steel ($p = 0.10$) and titanium ($p = 0.08$). Although the root mean square (RMS) value representative of surface roughness for HDPE was higher than that for both metals, the values were not significantly higher (Table 2). All the materials were hydrophilic and had a liquid-surface droplet of contact angle < 90° (Table 2). However, it was observed that both HDPE and polycarbonate were relatively more hydrophilic than SS316 and titanium.

3.2. Cell counts on different materials

In general, the planktonic cell counts in seawater were lower than the cell counts attached to HDPE, polycarbonate and titanium but approximately the same as those attached to SS316. To illustrate, average planktonic cell counts in the seawater during the sampling months were $9.2 \times 10^5 \pm 7.4 \times$

10^5 cells/mL and were significantly lower than the cell counts attached to HDPE, polycarbonate and titanium ($p < 0.02$). However, the average planktonic cell counts in seawater were not significantly different from those attached to SS316 ($p = 0.20$) (Figure 2). Comparisons of the attached cell counts among the different materials suggested that 1 month was enough to appreciate statistically significant differences between SS316 and the rest of materials ($p < 0.01$). The average cell counts on HDPE, polycarbonate and titanium were $2.2 \times 10^6 \pm 9.0 \times 10^5$ cells/mL, $1.9 \times 10^6 \pm 7.6 \times 10^5$ cells/mL, and $7.9 \times 10^5 \pm 1.9 \times 10^4$ cells/mL, respectively. These counts were more than 1-log higher the one attached to SS316 ($7.2 \times 10^4 \pm 5.0 \times 10^3$) cells/mL. At 2nd and 4th month, the cell counts on SS316 remained significantly lower than those attached to the other three types of materials ($p < 0.01$, reaching a maximum value of 2.8×10^5 cells/mL in the 4 months of operation (Figure 2). There were no significant differences in the attached cell counts on HDPE, polycarbonate and titanium throughout the sampling months ($p > 0.10$).

3.3. Variations in microbial communities attached to different materials compared to those in planktonic seawater

The microbial community was assessed using 16S rRNA gene-based amplicon sequencing. The relative abundance of each genus and unclassified microbial group was used for multivariate analysis and represented on a metric multidimensional scaling (mMDS) plot (Figure 3A). Overall, the sample groupings indicate differences in the microbial communities attached to four types of materials compared to those in planktonic seawater. One-way ANOSIM revealed R statistic values of 0.171, 0.436, 0.382 and 0.357 between seawater and SS316, titanium, HDPE or polycarbonate, respectively ($p < 0.10$) (Table 3A).

The first difference between seawater and an attached biofilm microbial community was the number of unique operational taxonomic units (OTUs). To illustrate, at a sequencing depth of

2715 sequences, there was an average of 905 OTUs identified for SS316 throughout the study period, while the other materials had at least 1.2 times higher numbers of OTUs. However, the numbers of OTUs identified in the biofilms of all four materials were, on average, lower than the 1350 unique OTUs detected in the seawater.

The second difference between the microbial communities in seawater and in attached biofilms was the relative abundance of several microbial taxa. Unclassified Bacteria, unclassified Alphaproteobacteria and Bacillariophyta were the predominant groups present in a relative abundance of > 8% on all the materials. Both the unclassified bacteria and the unclassified Alphaproteobacteria were also present in relative abundances of 29.3% and 11.0% of the total microbial community, respectively, in the seawater (Figure 4). However, Bacillariophyta was present in an average relative abundance of only 0.8% in the seawater, and SIMPER analysis revealed that Bacillariophyta was the taxa (Figure 4) that accounted for an average of 6.3% of the difference between the microbial community in seawater and the communities attached to the materials (Supplementary S1). In addition, Candidatus *Pelagibacter* and unclassified Rhodobacteraceae were more abundant in the seawater than in the biofilms attached to all the materials (Figure 4), and they contributed to an additional percentage of approximately 3.2% of the difference between seawater and the attached biofilms.

3.4. Variations in the microbial communities attached to different materials

As observed in Figure 3A, the sample groups showed differences in the microbial communities attached to the HDPE and polycarbonate compared to those attached to titanium or SS316. The microbial communities on HDPE and polycarbonate showed a similarity of 65.2%, but they only shared an average of 53.7% similarity with the microbial communities attached to both types of metal. One-way ANOSIM analyses revealed significant differences between SS316 compared

with HDPE and polycarbonate at the 90% confidence level (Table 3A). To illustrate, the R statistic of SS316 against HDPE and polycarbonate were 0.163 ($p = 0.05$) and 0.133 ($p = 0.10$), while the R statistics of titanium against HDPE and polycarbonate were 0.128 ($p = 0.13$) and 0.149 ($p = 0.11$), respectively. There was also no significant difference between the microbial community attached to SS316 and titanium (One-way ANOSIM, $p = 0.31$).

SIMPER analysis revealed that Bacillariophyta and unclassified Planctomycetaceae were the predominant groups and contributed to a 13% cumulative difference in the microbial communities on metal and plastic (Supplementary S1). These taxa were present at relative abundances of 14.5% and 4.0% on the metals, respectively (Figure 4). In contrast, Bacillariophyta accounted for a relative abundance of 28.6% on the plastic materials while unclassified Planctomycetaceae accounted for < 1% of the total microbial communities attached to the plastics (Figure 4). SIMPER analysis also showed that titanium supported a higher diversity of different microbial populations, specifically, GpIV, *Kangiella*, unclassified Enterobacteriaceae, unclassified Cyanobacteria and *Lutaonella*, which were present in > 2% relative abundance on the titanium (Figure 4) and contributed to approximately 12% of the cumulative difference between the microbial communities on titanium and plastic materials (Supplementary S1).

3.5. Temporal variation of seawater biofilm

Cell counts attached as biofilm on HDPE and polycarbonate remained at the same level of ~10^6 cells/mL throughout the sampling months (Figure 2). In contrast, the 3-month-old biofilm attached to titanium increased by more than 1-log to $1.7 \times 10^7 \pm 9.3 \times 10^4$ cells/mL compared to 1-month-old biofilm ($7.9 \times 10^5 \pm 1.9 \times 10^4$ cells/mL). The mature biofilm from SS316 sampled at the 4^{th} month also had approximately 3-fold higher cell counts than the 1-month and 2-month biofilms from the same material (Figure 2). Multivariate analysis of the relative abundances of

the bacterial genera present in the biofilms sampled throughout the four-month period revealed a temporal succession in the microbial populations (Figure 3B). However, one-way ANOSIM revealed no significant difference in the overall microbial communities among the 4 sampling months (Table 3B).

3.6. Occurrence of opportunistic pathogen genera on different materials

Genera associated with opportunistic pathogens that were within the detection limits of the high-throughput sequencing approach included *Acinetobacter*, *Arcobacter*, *Coxiella*, *Legionella* and *Pseudomonas*. There were no apparent temporal changes in the relative abundance of *Arcobacter*, *Legionella* and *Pseudomonas*, and all were present in relative abundances of ≤ 0.35% of the total microbial community (Figure 5A). This is with the exception of a single instance of an exceedingly high relative abundance of *Pseudomonas* (35.1% of total microbial community) in the 2-month-old biofilm attached to SS316 (Figure 5A). *Acinetobacter* and *Coxiella* were also present at up to ca. 0.36% of the total microbial community on HDPE and SS316 (Figure 5A). In particular, the relative abundance of *Coxiella* remained higher in the biofilm attached to SS316 than in those attached to other materials throughout the 4-month sampling period.

3.7. Occurrence of SRB on different materials

Seawater contains a high sulfate content, which can favor the presence of SRB that are thought to be the main protagonists in biocorrosion. Emphasis was therefore placed on evaluating the presence of SRB attached to different materials using both high-throughput amplicon sequencing and qPCR. The main type of SRB detected in this study included unclassified Desulfobacteraceae, unclassified Desulfobulbaceae and unclassified Desulfovibrionaceae (Figure

5B). The qPCR analyses indicated that there was a 10-fold higher copy number of Desulfobulbaceae detected on SS316 than on the other materials ($p < 0.05$) (Figure 6). The average copy number of Desulfobulbaceae attached to SS316 was 2.1×10^{-3} copies/cell number and was similar to that detected in seawater (2.2×10^{-3} copies/cell number) ($p = 0.85$). In contrast, there was no significant difference in the abundance of Desulfobulbaceae attached to HDPE, polycarbonate and titanium ($p > 0.20$). Desulfobacteraceae was also present in ca. 10-fold higher abundance than Desulfobulbaceae across all samples, but the amounts attached to the different materials were significantly lower than those present in seawater (Figure 6) ($p < 0.10$). To illustrate, the copy number of Desulfobacteraceae present in seawater was 7.4×10^{-1} copies/cell number, while that attached to the SS316 coupons was highest at 9.5×10^{-2} copies/cell number, followed by that on titanium (6.3×10^{-2} copies/cell number), HDPE (3.9×10^{-2} copies/cell number) and polycarbonate (2.6×10^{-2} copies/cell number). The abundance of Desulfobacteraceae present on SS316 was significantly higher than that on HDPE and polycarbonate ($p < 0.05$) but was not significantly different from that on titanium ($p = 0.44$). Similarly, there were no significant differences in the abundances of Desulfobulbaceae on titanium compared with HDPE ($p = 0.51$) or polycarbonate ($p = 0.31$).

4. Discussion

The findings in this study demonstrated that different materials have varying extents of surface roughness and hydrophobicity/hydrophilicity. Unlike an earlier study, which showed that there was more microbial colonization on rougher surfaces [34], our study revealed no apparent differences in cell numbers attached to polycarbonate, which has the roughest surface (Table 2),

compared to HDPE and titanium. Earlier studies also demonstrated that relatively hydrophilic surfaces were less prone to cell attachment [35-37]. However, both HDPE and polycarbonate, which are more hydrophilic than titanium and SS316 (Table 2), had higher attached cell numbers than SS316 (Figure 2). In addition, the bacterial cell numbers attached to the different materials showed no significant differences as the biofilms matured with time (Figure 2). This observation is in agreement with that reported earlier [38, 39]. It is likely that as the biofilm matures, bacterial cells condition the surfaces of different materials through the production of extracellular polymeric substances and diminish the role of surface properties in cell attachment.

It is therefore likely that other factors, including the antibacterial effect associated with each type of material, would also affect cell attachment, colonization and biofilm development. To illustrate, in drinking water, plastic materials such as polyethylene were observed to have a higher number of attached total bacterial and viral-like particles than copper [14]. Copper is a heavy metal that was previously shown to inhibit the number of *L. pneumophila* cells in a biofilm matrix [40]. Similarly, stainless steel is an alloy comprising manganese, silicon, chromium, nickel and molybdenum, all of which are heavy metals that could possibly impede cell attachment and development. This likely accounts for the lower cell densities obtained on stainless steel for both the seawater microbial biofilm assessed in this study (Figure 2) and for the drinking water biofilm in other studies [11, 12]. Although titanium is also a metal, its effect contrasted with that of stainless steel. Instead, the total attached cell numbers on titanium were of the same range as those attached to HDPE and polycarbonate (Figure 2). Earlier studies found that pure titanium was non-mutagenic and non-cytotoxic [41, 42] and that comparable cell numbers adhered to titanium and HDPE [42].

Regardless, the microbial community structures attached to the two metallic materials were distinct from those on the two plastic materials. In particular, qPCR revealed that there were higher copy numbers of Desulfobulbacaeae and Desulfobacteraceae when normalized against the total attached cells number on the stainless steel compared to the other materials (Figure 6). Both Desulfobulbaceae and Desulfobacteraceae are members of SRB, which are thought to be the main protagonists for microbial-induced biocorrosion. Their presence on the stainless steel may have accounted for the occurrence of pitting on the SS316 (data not shown) but not on any of the other materials tested in this study. Numerous types of SRB, including *Desulfovibrio vulgaris* [43, 44] and *Desulfobulbus propionicus* [45], are capable of obtaining electrons from stainless or carbon steel by coupling with sulfate reduction to gain maintenance energy. This redox reaction, in turn, causes microbial-induced biocorrosion.

The higher abundance of Desulfobulbaceae and Desulfobacteraceae on stainless steel can be accounted for by the presence of sulfur contents present in the stainless steel alloy. The enrichment of SRB on a conductive matrix was also reported earlier in studies examining the microbial communities on granular activated carbons (GACs) [46, 47]. Similar to stainless steel, GACs are derived from lignite containing sulfur and are bound by van der Waals forces at the molecular level to permit free electron flow (i.e., they are good conductors of electricity). Similarly, the high relative abundance of *Pseudomonas aeruginosa* associated with the stainless steel can be accounted for by the fact that *P. aeruginosa* produces pyocyanins as electron transfer mediators [48, 49] and can, in turn, reduce thiosulfate for its metabolic needs on a conductive matrix such as stainless steel [50, 51]. In contrast, titanium is not a good conductor of electricity, with a relative conductivity of approximately 3% compared to that of copper [52]. Furthermore, based on thermodynamics and kinetics, SRB cannot corrode titanium to obtain energy. These

442 factors may have accounted for the lower abundance of SRB on titanium despite it being a
443 metallic material.

444 In addition to a higher abundance of Desulfobulbaceae and Desulfobacteraceae, there was
445 also high relative abundance of *Acinetobacter* and *Coxiella* associated with stainless steel. Both
446 genera contain species associated with opportunistic pathogens, namely *Acinetobacter*
447 *baumannii* and *Coxiella burnetii*. In an earlier study, bacterial species isolated from drinking
448 water were evaluated for their hydrophilicity and hydrophobicity, and it was observed that the
449 majority of the bacterial isolates, including *Acinetobacter,* were hydrophilic [53]. Since all the
450 materials evaluated in this study are hydrophilic surfaces, it is likely that *Acinetobacter* adheres
451 very well compared to the other isolates due to the hydrophilicity effect. However, qPCR did not
452 show *Acinetobacter baumannii* to be present in any of the samples (data not shown).

453 For the genus *Coxiella*, *C. burnetti* is the only member of this genus currently isolated
454 and characterized. This species is an obligate intracellular parasite that is commonly detected in
455 seawater by molecular methods [54], which may explain their occurrence in the seawater
456 biofilm. It has been postulated that being obligate intracellular parasites [55], species within this
457 genus may be protected from external environmental factors, including the antibacterial effect of
458 stainless steel, and may successfully establish itself as one of the bacterial populations in the
459 biofilms on all the tested materials. However, it remains unknown why this genus would attach
460 preferentially to stainless steel compared to the other materials.

461 **5. Conclusions**

462 Although this study did not assess the impact on cell numbers and microbial community
463 structure of pipe materials in combination with other factors (e.g., the presence of chlorine

disinfectant and/or varying shear force), it is one of the few studies providing a comprehensive evaluation of the differences in seawater microbial biofilms as a result of attachment to a material substratum. The findings from this study suggest that certain material types, for example polycarbonate and HDPE, are preferred by bacterial to attach and establish biofilm. This is evidenced from the higher cell numbers attached to both materials compared to SS316. Despite a lower number of cells attached on SS316, there was a selective enrichment of sulfate-reducing Desulfobacteraceae and Desulfobulbaceae on SS316 compared to the other materials. This may make the stainless steel piping network relatively prone to biocorrosion. The use of stainless steel as a piping material in most industrial applications involving seawater usage may not be as ideal compared to titanium.

6. Acknowledgements

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7. References

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631 **Table 1.** Primers used for quantitative PCR.

Target	**Primer name**	**Sequences (5'-3')**	**Reference**
Desulfobacteraceae (16S rRNA genes)	DSBAC357F	GTGAGGAATTTTGCGCAATGG	[56]
	519R	GWATTACCGCGGCKGCTG	[57]
Desulfobulbaceae (16S rRNA genes)	519F	CAGCMGCCGCGGTAATWC	[57]
	DSB706R	ACCGGTATTCCTCCCGAT	[58]
Acinetobacter baumannii (*ompA* gene)	*ompA*-F	TCTTGGTGGTCACTTGAAGC	[59]
	ompA-R	ACTCTTGTGGTTGTGGAGCA	

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633

634 **Table 2.** Surface characteristics of the tested materials.

	Average root mean square, RMS, of surface (n = 3) ± standard deviation	**Average liquid-solid contact angle (n = 6) ± standard deviation**
HDPE	0.24 ± 0.18 [a]	63.9° ± 1.8 [o]
Polycarbonate	0.53 ± 0.20 [b]	61.6° ± 1.7 [o]
Titanium	0.19 ± 0.11 [a]	80.2° ± 1.5 [o]
Stainless steel SS316	0.19 ± 0.06 [a]	75.6° ± 0.9 [o]

635 a, b Homogenous subgroups by two-tailed t-test method with a significance level of 0.10

636

Table 3. ANOSIM R statistic values and p-values between the **(A)** different materials, and **(B)** sampling months. Values in bold refer to significant differences between the sample pairs at the 90% confidence level.

A.	Seawater	HDPE	Polycarbonate	Titanium	SS316
Seawater		**0.382 (p = 0.008)**	**0.357 (p = 0.011)**	**0.436 (p = 0.016)**	**0.171 (p = 0.065)**
HDPE			-0.139 (p = 0.967)	-0.128 (p = 0.133)	**0.163 (p = 0.054)**
Polycarbonate				0.149 (p = 0.108)	**0.133 (p = 0.100)**
Titanium					0.037 (p = 0.305)
SS316					

B.	1-month	2-month	3-month	4-month
1-month		0.041 (p = 0.309)	-0.005 (p = 0.485)	0.125 (p = 0.115)
2-month			0.058 (p = 0.228)	0.087 (p = 0.146)
3-month				-0.009 (p = 0.404)
4-month				

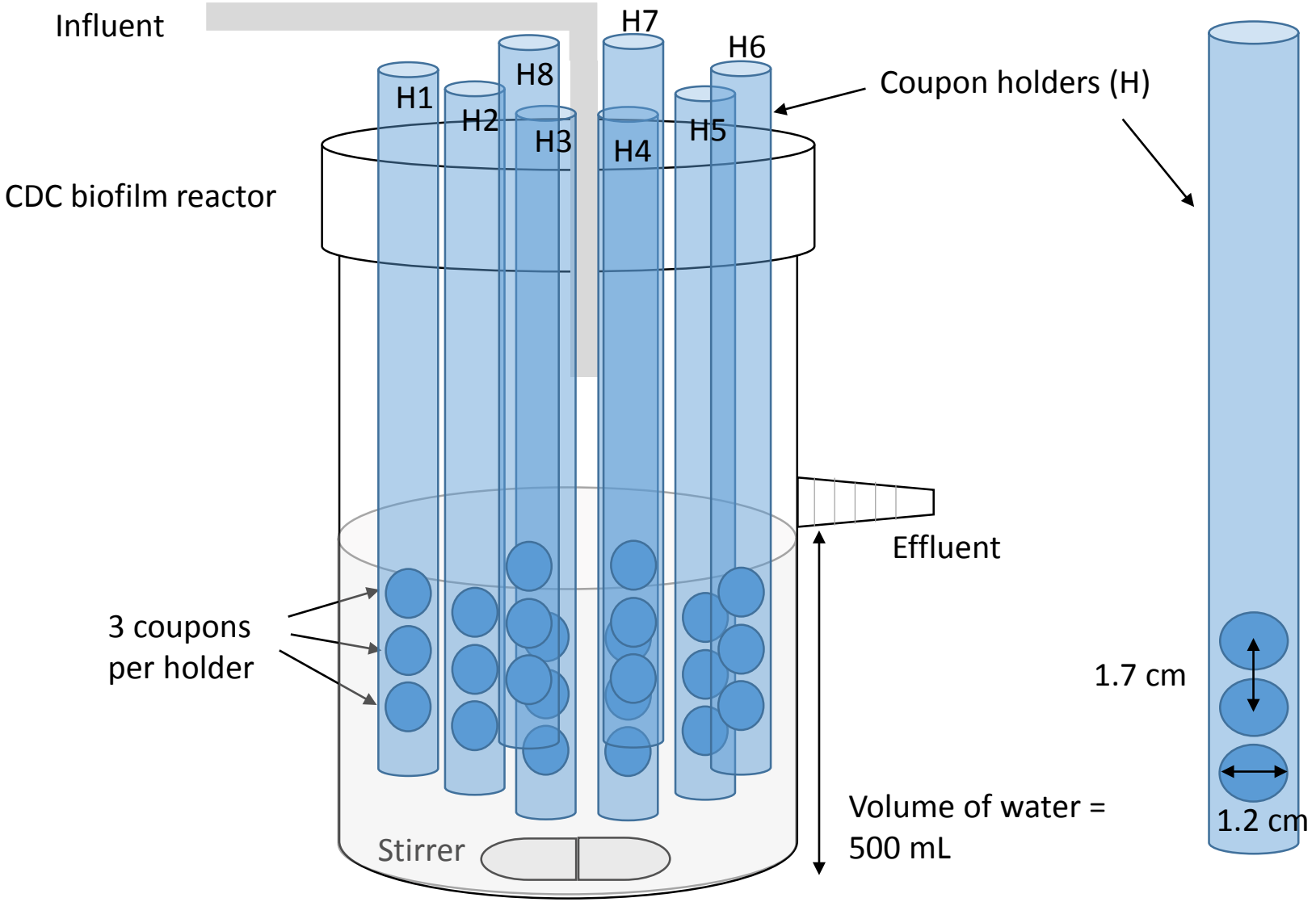

Figure 1. Illustration of the CDC biofilm reactor.

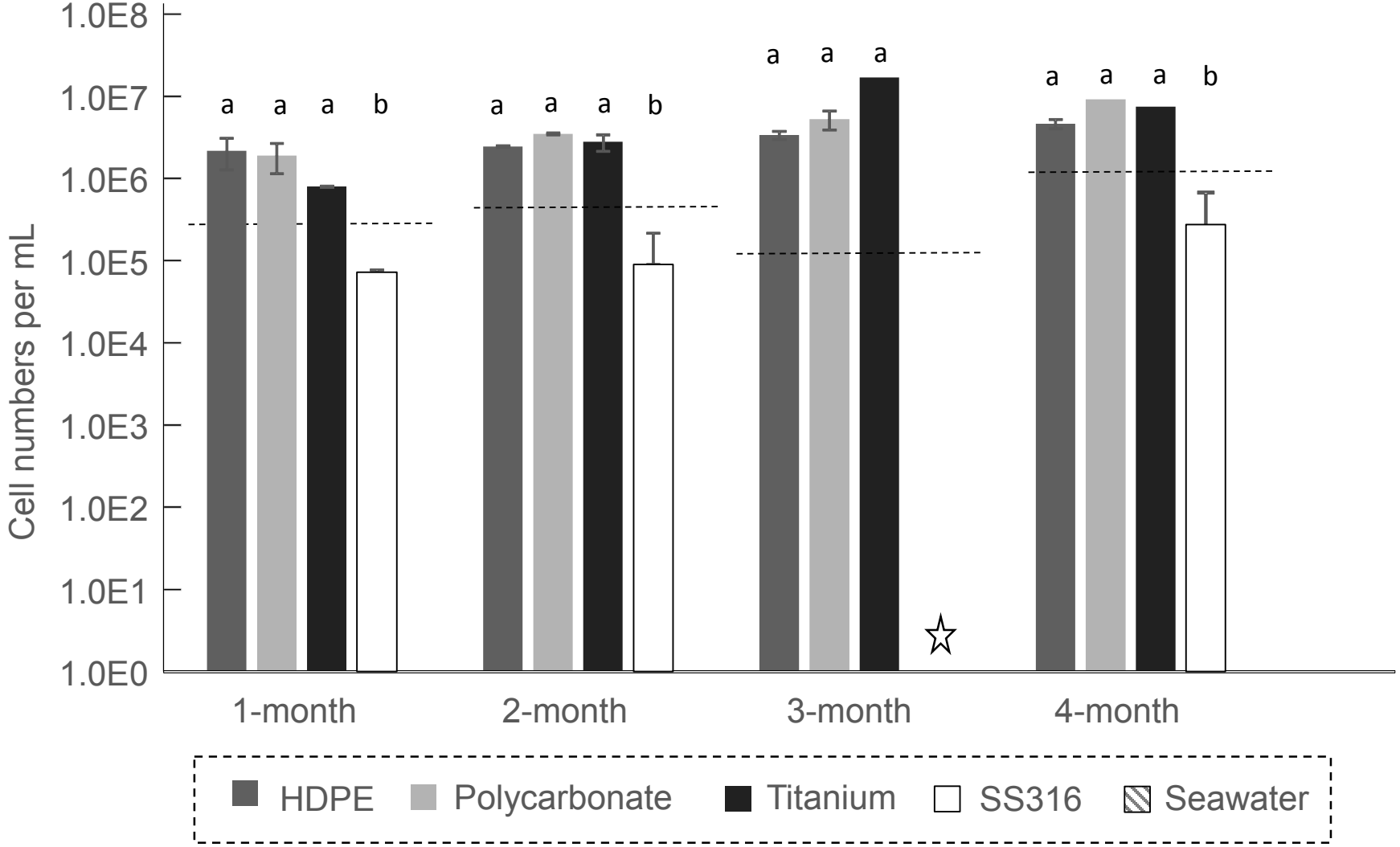

Figure 2. Enumeration of total cell counts attached to different types of materials over a duration of four months. The star denotes no measurements obtained for that sampling month for SS316 due to sampling error. a, b denote homogenous subgroups by two-tailed t-test method with a significance level of 0.10. Dash lines correspond to the cell numbers per mL of seawater at that month. Star denotes no measurements were obtained for that sampling month for stainless steel.

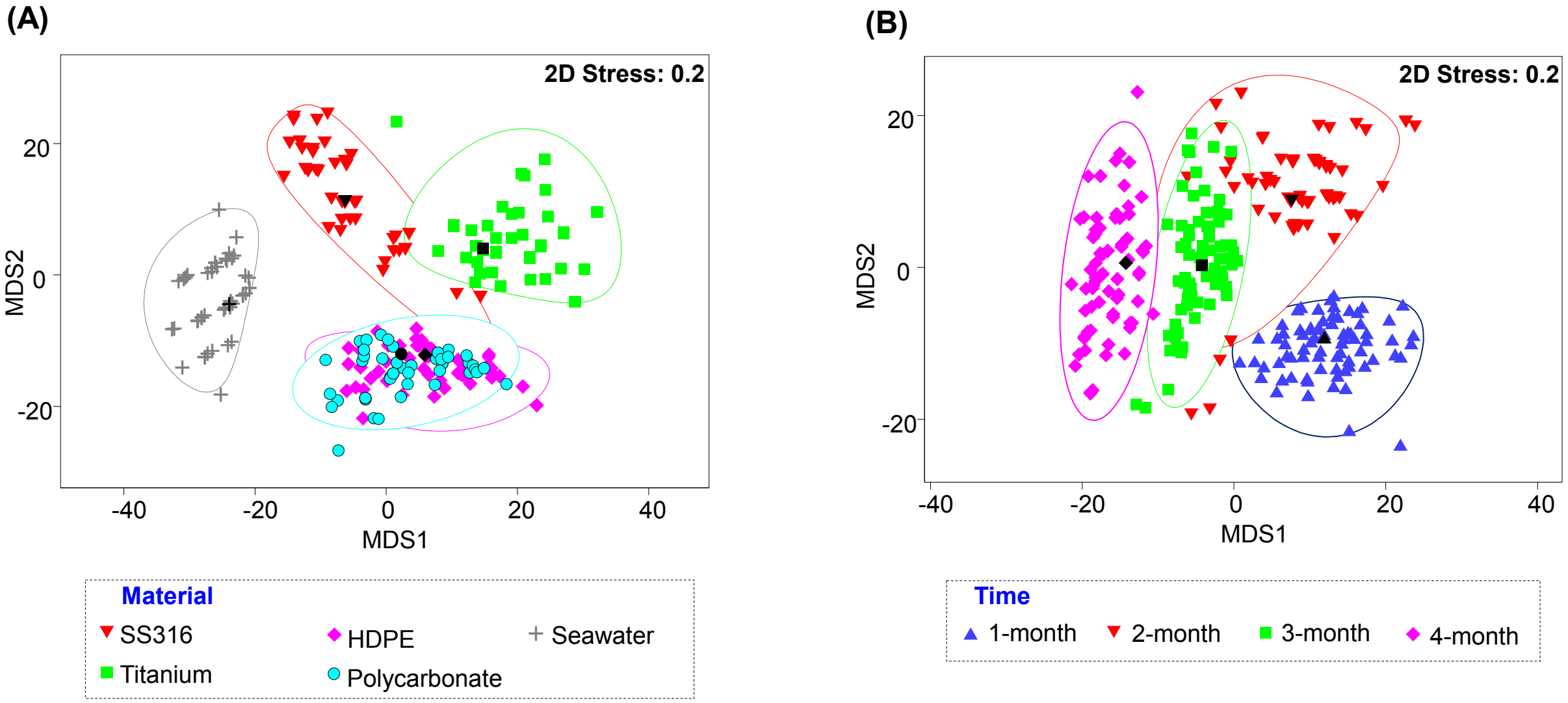

Figure 3. Bootstrapped metric multidimensional scaling (MDS) plots of microbial communities in relation to **(A)** the type of material and **(B)** the temporal effect.

Bacterial cell numbers and community structures of seawater biofilms depend on the attachment substratum

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Running title: Seawater biofilm on plastic and metal surfaces

Abstract

Seawater is increasingly being used as a source for various industrial applications. For such applications, biofilm growth creates various problems including but not limited to pipe biocorrosion. In this study, it is hypothesized that the material type is preferred by certain bacterial populations in the seawater to attach and establish biofilms. By comparing differences in the total cell counts and microbial communities attached to high density polyethylene (HDPE), polycarbonate, stainless steel (SS316) and titanium, the appropriate material can be used to minimize biofilm growth. All four materials have hydrophilic surfaces, but polycarbonate exhibits higher surface roughness. There were no significant differences in the cell numbers attached to polycarbonate, HDPE and titanium. Instead, there were significantly fewer cells attached to SS316. However, there was a higher relative abundance of genera associated with opportunistic pathogens on SS316. Copy numbers of genes representing Desulfobacteraceae and Desulfobulbaceae, both of which are sulfate-reducing bacteria (SRB), were approximately 10-fold higher in biofilms sampled from SS316. The enrichment of SRB in the biofilm associated with SS316 indicates that this material may be prone to biocorrosion. This study highlights the need for industries to consider the choice of material used in seawater applications to minimize microbial-associated problems.

1. Introduction

Seawater is increasingly being used as a source for various industrial applications in an attempt to alleviate the demand for freshwater. Examples of such industrial applications include seawater cooling tower systems and seawater injection for enhanced oil recovery. In most instances, seawater is usually recirculated at least once to minimize operating costs associated with continuous pumping of new seawater into the system. However, recirculating seawater allows for microbes, minerals and nutrients to accumulate within the system [1]. To tackle inorganic scaling problems arising from minerals, antiscalants are commonly used even though they generally include components of polymers, phosphates and phosphonates and can contribute nutrients that support microbial growth [2]. Hence, extensive biofilm growth is an inevitable problem in seawater pipelines.

Biofilm growth creates a suite of problems. For example, biofilms can serve as colonization platforms for pathogens to augment their environmental persistence [3, 4]. Biofilm formation is also correlated with the occurrence of biocorrosion [5], otherwise known as microbial-induced corrosion (MIC). This is shown by an increased rate of corrosion in the presence of biofilms [6]. Although total eradication of biofilms and the associated microbial populations would be impractical, efforts have been undertaken to minimize cell counts and bacterial populations that may be present in the biofilm by varying certain operational factors. These factors include maintaining the presence of residual disinfectant, optimizing water age, hydraulic regimes and pipe materials [7-15].

However, most of the existing studies utilized culture-based approaches or DNA-based fingerprinting techniques to determine potential impacts on the biofilm arising from these operational factors. Cultivation-based approaches to enumerating cell counts are subject to bias

and are not representative of bacterial species that are fastidious about growth conditions [16, 17]. When compared to high-throughput amplicon-based sequencing approaches, fingerprinting techniques, including denaturing gel electrophoresis or terminal restriction length polymorphisms, are not able to provide an in-depth characterization of the bacterial community structure. Neither are the fingerprinting techniques capable of providing quantitative measurements of certain bacterial populations when compared to the quantitative PCR (qPCR) approach. An earlier study used high-throughput sequencing to show that bacterial and/or eukaryotic community structures in drinking water biofilms can be affected by these factors. Specifically, pipe materials only affect the bacterial community structure but not the eukaryotic community [8]. However, similar studies on the variation of cell counts and bacterial community structures in seawater in relation to the pipe materials are not widely available.

A better understanding of whether material types result in differences in microbial populations, specifically, sulfate-reducing bacteria (SRB) and genera associated with opportunistic pathogens, is needed to assess the potential relevance to industrial stakeholders and public health. SRB possess organic filaments similar to nanowires [18], which may play a role in adhesion, biofilm formation and direct interspecies electron transfer [19]. Adherence to a conductive material may facilitate extracellular electron transfer and metal reduction, both of which are key metabolic traits required by the SRB [20]. As such, SRB are generally thought to be the protagonist in biocorrosion [21]. Biocorrosion is believed to account for 20% of the damage caused by corrosion [22], which can impart a tremendous financial burden on industrial stakeholders. In addition, the presence of opportunistic pathogens can also pose potential health threats to certain groups of individuals, for example, immunocompromised patients, the elderly and infants, upon exposure.

In this study, it is hypothesized that the material type is preferred by certain bacterial populations in the seawater to attach and establish biofilms. It is further hypothesized that the type of material plays a less significant role in affecting the total cell count and composition of the microbial community as the biofilm ages. The materials tested were stainless steel 316 (i.e., SS316), titanium, high density polyethylene (HDPE) and polycarbonate. SS316 and titanium are commonly used in the heat exchangers [23] for industrial seawater applications. HDPE and polycarbonate are included in this study to serve as non-metal controls and because they are plastic materials commonly used in industrial applications. Efforts were made to determine the effect of materials on the genera associated with opportunistic pathogens and sulfate-reducing bacteria (SRB). To address these issues, counts of cells adhering to the different type of materials were enumerated by flow cytometry, while both high-throughput sequencing and qPCR were utilized to characterize the microbial community structures.

2. Materials and methods

2.1. Experimental procedure

The materials tested in this study were stainless steel 316 (i.e., SS316), titanium, high density polyethylene (HDPE) and polycarbonate. These materials were characterized for their surface roughness and hydrophilicity as detailed in Section 2.2. The materials were then placed inside bioreactors and exposed to seawater over a period of four months (Section 2.3). Seawater biofilm established on the materials were harvested using sampling procedure described in Section 2.4. The harvested samples were divided into two portions. The first portion was determined for the total bacterial cell counts by flow cytometry (Section 2.5). The second portion was extracted for DNA (Section 2.6), and the DNA was used in two types of molecular-based analyses. The first molecular-based analysis was 16S rRNA gene amplicon sequencing to characterize the microbial community attached on the different materials (Section 2.7). The second molecular-based analysis was quantitative PCR (qPCR) to quantify for the copy numbers of *Acinetobacter baumannii* (Section 2.8), Desulfobacteraceae and Desulfobulbaceae (Section 2.9).

2.2. Material surface characterization

The materials tested in this study were purchased from Biosurface Technologies (Bozeman, MT, USA) in a circular disc coupon format, each with a diameter of approximately 1.2 cm. All the materials that were tested in this study were evaluated for their surface roughness and extent of hydrophobicity based on procedures described previously [24]. Briefly, atomic force microscopy (AFM) was used to characterize the surface topography of each material type based on the standard protocol defined by American Society of Mechanical Engineer (ASME) B46.1-2009. AFM imaging was performed using Bruker Dimension ICON equipment (Santa Barbara, CA, USA) in soft tap mode to scan images of 15 µm width by 15 µm length at three random locations

on each material type. The AFM images were analyzed on Pico Image Software (Keysight Technologies Inc., Santa Rosa, CA, USA). Specifically, surface roughness is represented as the root mean square (RMS) average of profile height deviations from the mean height observed for that particular material. A higher RMS value indicates more apparent surface peaks and valleys, thus representing a rougher surface. The contact angle was measured by an Easy Drop Shape Analyzer (Kruss, Hamburg, Germany) in static mode at ambient temperature. Ultra-pure water was used as the probing liquid, and the mean values were determined from three different independent specimens. Generally, if the contact angle is smaller than 90°, the solid surface is considered hydrophilic and vice versa [25].

2.3. Biofilm reactor operation

From February to June 2016, a total of four Communicable Disease Centre (CDC) bioreactors (BioSurface Technologies, Bozeman, MT, USA) were operated at 28 °C and at a stirring rate of 200 rpm in the KAUST Water Desalination and Reuse Center (WDRC) (Figure 1). Each bioreactor had eight coupon holders that could be fitted with a total of 24 coupons of the same material per reactor. Seawater fed into the bioreactors was sourced from the Red Sea through an approximately 1 km pipeline to the WDRC laboratory. The seawater tap was flushed for 10 minutes prior to collection of seawater in a sterile 20 L carboy container. Thereafter, seawater from the same container was fed at a continuous rate of 1.3 mL per minute to each bioreactor and replaced with fresh seawater every 10 days. The hydraulic retention time of seawater within the bioreactor was 6.4 h. The bioreactors were periodically checked for the hydraulic retention time, stirring rate, temperature and flow rate so as to ensure that the operating conditions among all four reactors are kept similar. Two L of seawater that was to be fed into the reactors were collected each month during the course of the experiment and evaluated for its total cell counts

and microbial community in accordance with protocols mentioned in the following subsections. All the bioreactors were covered with aluminum foil to limit exposure to light.

2.4. Sampling procedure

Biofilm was sampled at 1 through 4 months after the commencement of the bioreactors. Six coupons for each type of material were collected during each sampling month and provided two sets of biological replicates for that particular sampling month. Each biological replicate comprised the pooled biomass scraped down from three coupons. The six coupons were selected from two coupon holders placed at random locations within the bioreactor to minimize any sampling bias due to the location of the coupon. New holders containing coupons of the same material type were replaced after each sampling event to ensure that the shear force was kept constant within the bioreactor. The sampled coupons were placed into separate petri dishes, and 5 mL of 1X phosphate-buffered saline (PBS) solution was added. Autoclaved cotton swabs were then used to scrape down the loosely attached biofilm into the 1X PBS. The resulting suspension for each material was transferred to sterile 50 mL centrifuge tubes. The used cotton swabs and coupons were placed in a sterile centrifuge tube that contained 10 mL of 1X PBS. This tube was vortexed at high speed for 10 minutes with the aim of dislodging any attached biofilm on the cotton swab and coupons. Following this, the supernatant from each vortexed tube was transferred to the corresponding tube containing the loosely attached biofilm. A 20 μL sample of the combined supernatant was set aside for total cell enumeration by flow cytometry. The remaining supernatant was centrifuged at 8000 *g* for 10 minutes, and the cell pellet was used for DNA extraction.

2.5. Flow cytometry to obtain total cell counts

A 7 µL sample of the supernatant or seawater was diluted 100-fold with 1X PBS, and 10 µL of 500 mM ethylenediaminetetraacetic acid (EDTA) was added to chelate metals and other inhibitors that can affect flow cytometry measurements. The mixture was briefly vortexed and incubated at 35 °C for 20 minutes. Then, 7 µL of 100X SYBR Green I (Thermo Fisher Scientific, Carlsbard, CA, USA) was added, and the samples were incubated for 10 minutes. The number of cells in the resultant mixture was then quantified using a BD Accuri C6 (Thermo Fisher Scientific, Carlsbard, CA, USA) flow cytometer, based on protocol described by manufacturer [26].

2.6. DNA extraction

The microbial DNA from the biofilms was extracted using the UltraClean® PowerSoil DNA Isolation kit following the manufacturer's protocol with brief modifications as described previously [27]. To extract DNA from seawater, 2 L of seawater was first filtered through a 0.22 µm polycarbonate membrane filter (VWR, Radnor, PA, USA), and the retained biomass on the filter was extracted using a protocol similar to the one mentioned previously. The DNA concentration was then quantified using the Qubit Broad Range DNA Assay according to the manufacturer's protocol. The DNA was used for molecular-based analyses detailed in Section 2.7 to 2.9.

2.7. 16S rRNA gene-based amplicon sequencing and amplicon sequencing data analysis

16S rRNA genes were amplified from the DNA with primer pair 515F and 907R based on procedures described earlier [24]. Purified amplicons were pooled and submitted to the KAUST Genomics Core lab for sequencing on the Illumina MiSeq platform. All high-throughput sequencing data were deposited in European Nucleotide Archive under accession number

194 PRJEB20120.The two main hypotheses of this study are that first, certain bacterial populations
195 in the seawater prefer to attach to specific types of material prior to biofilm formation, and
196 second, the type of material plays a less significant role in affecting the total cell count and
197 composition of the microbial community as the biofilm ages. Amplicon sequences that denote
198 the microbial community were therefore analyzed to test these two hypotheses. Sequences were
199 sorted by the Bioinformatics Team at KAUST based on a Phred score of > 20. The sorted
200 sequences were then trimmed off for the primers, barcodes and adaptor sequences, and any
201 sequences > 300 nt in length were removed. Chimeras were identified and removed on UCHIME
202 [28] by comparison with a core reference FASTA file downloaded from Greengenes
203 (http://greengenes.lbl.gov/Download/). Chimera-free sequences were assigned to
204 bacterial/archaeal taxonomic hierarchy at the 95% classification reliability level using the
205 Ribosomal Database Project (RDP) Classifier [29]. The relative abundances of the bacterial and
206 archaeal genera were calculated, collated and then square-root transformed. Square-root
207 transformation was performed to down-weigh the dominant taxa and to achieve a better balance
208 of the abundant and rare species that are present in the samples. This allows the rare species,
209 which are common in the data generated from high-throughput sequencing, to exert some
210 influence on the calculation of similarity. The transformed data sets were then computed for their
211 Bray–Curtis similarities and represented graphically for relative differences in bacterial
212 community composition among samples aligned against either the materials or the duration of
213 operation as factors in a boot-strapped metric multidimensional scaling (mMDS) plot. That is,
214 samples that were further apart from each other shared less similarity than those that were closer
215 together. All mMDS plots were obtained using Primer-E version 7 [30]. Chimera-free sequences
216 were also submitted to the RDP pipeline for clustering analysis to identify the number of unique

operational taxonomic units (OTUs) with < 97% sequence similarity at each respective sequencing depth [31]. The number of unique OTUs identified at a sequencing depth of 2715 sequences were collated and used to compare the level of microbial richness among the individual samples. This sequencing depth was used because this was the lowest number of reads obtained for one particular sample, and the comparison of the microbial richness across samples, therefore, had to be standardized at this depth.

2.8. Quantitative PCR for *Acinetobacter baumannii*

Acinetobacter baumannii was chosen as the pathogenic bacterial species in the genus *Acinetobacter*, and its abundance was determined because this genus was detected in some of the biofilm samples. Copy numbers of the *ompA* gene representing *Acinetobacter baumannii* were determined using qPCR with a 7900 HT Applied Biosystems real-time PCR thermal cycler (Thermo Fisher Scientific, Carlsbad, CA, USA). Sequences of the primer pairs are listed in Table 1. The qPCR standards were prepared as described previously [32, 33]. To produce qPCR standard curves, plasmid DNAs were diluted in series to form concentrations ranging from 10^2 to 10^8 copies/µL. Each reaction volume of 20 µL contained 10 µL of Fast SYBR Green master mix, 0.4 µL of each primer (10 µM), 1 µL of DNA template and 8.2 µL H_2O. The reaction for amplification was run using an annealing temperature of 60 °C, and the melting curve analysis was performed with a dissociation cycle which included an increment of temperature from 60 °C to 95 °C with intervals of 0.5 °C for 5 s each. The threshold cycle (C_q) values for each dilution were plotted against the log-transformed concentration of each dilution. The amplification factor of the standards was 2.99, and the R-squared value was > 0.98. Amplifications to obtain standard curves were performed in triplicate, while test amplifications and negative non-template controls (NTCs) were run in duplicate. All NTCs had no determinable C_q values. The copy numbers

obtained from qPCR reactions were normalized against the total cell numbers obtained by flow cytometry for the corresponding sample.

2.9. Quantitative PCR for SRB

Both the Desulfobacteraceae and Desulfobulbaceae are families comprised of genera associated with SRB. The copy numbers of 16S rRNA genes representing total Desulfobacteraceae and Desulfobulbaceae were, therefore, determined to discern the presence and abundance of certain types of SRB. The qPCR standards for Desulfobacteraceae and Desulfobulbaceae were prepared by first amplifying the gene fragment with the appropriate primer pairs using DNA extracted from *Desulfobacter hydrogenophilus* DSM3380 and *Desulfobulbus elongatus* DSM2908 as bacterial templates. The amplified products were cloned into vectors, and the inserted genes were sequenced to verify that the sequences were perfectly complementary to the primer target region. The qPCR reactions were carried out as described above. The amplification factor of the standards ranged from 1.98 to 2.13 with R-squared values > 0.99. Amplifications to obtain standard curves were performed in triplicate, while test amplifications and NTCs were run in duplicate. All NTCs had no determinable C_q values. Copy numbers obtained from qPCR were normalized against the total cell numbers obtained by flow cytometry for the corresponding sample.

2.10. Statistical analyses

Two-tailed t-tests were carried out to evaluate significant differences in surface roughness, cell counts and copy numbers of Desulfobacteraceae and Desulfobulbaceae among samples. To compare differences in the microbial communities, one-way unordered Analysis of Similarity (ANOSIM) was carried out on Primer-E version 7 for both material types and times. Similarity

percentage analysis (SIMPER) was also carried out using the Bray-Curtis similarity matrix generated from the high-throughput sequencing data and Primer-E version 7 to determine which microbial population contributed most to the differences between material types. All differences were determined to be significant at the 90% confidence level (i.e., $p < 0.10$). The confidence level was set at 90% on the basis that biological samples have a usual baseline variance that may vary with time, and can contribute to outliers. Hence, setting the confidence level at 90% would account for differences among treatment but at the same time, do not lower the comparison power into irrelevance.

3. Results

3.1. Surface characteristics of the different materials

Surface roughness and the extent of hydrophobicity were evaluated for the four types of materials. Polycarbonate exhibited a significantly higher surface roughness than stainless steel ($p = 0.10$) and titanium ($p = 0.08$). Although the root mean square (RMS) value representative of surface roughness for HDPE was higher than that for both metals, the values were not significantly higher (Table 2). All the materials were hydrophilic and had a liquid-surface droplet of contact angle < 90° (Table 2). However, it was observed that both HDPE and polycarbonate were relatively more hydrophilic than SS316 and titanium.

3.2. Cell counts on different materials

In general, the planktonic cell counts in seawater were lower than the cell counts attached to HDPE, polycarbonate and titanium but approximately the same as those attached to SS316. To illustrate, average planktonic cell counts in the seawater during the sampling months were $9.2 \times 10^5 \pm 7.4 \times$

10^5 cells/mL and were significantly lower than the cell counts attached to HDPE, polycarbonate and titanium ($p < 0.02$). However, the average planktonic cell counts in seawater were not significantly different from those attached to SS316 ($p = 0.20$) (Figure 2). Comparisons of the attached cell counts among the different materials suggested that 1 month was enough to appreciate statistically significant differences between SS316 and the rest of materials ($p < 0.01$). The average cell counts on HDPE, polycarbonate and titanium were $2.2 \times 10^6 \pm 9.0 \times 10^5$ cells/mL, $1.9 \times 10^6 \pm 7.6 \times 10^5$ cells/mL, and $7.9 \times 10^5 \pm 1.9 \times 10^4$ cells/mL, respectively. These counts were more than 1-log higher the one attached to SS316 ($7.2 \times 10^4 \pm 5.0 \times 10^3$) cells/mL. At 2nd and 4th month, the cell counts on SS316 remained significantly lower than those attached to the other three types of materials ($p < 0.01$, reaching a maximum value of 2.8×10^5 cells/mL in the 4 months of operation (Figure 2). There were no significant differences in the attached cell counts on HDPE, polycarbonate and titanium throughout the sampling months ($p > 0.10$).

3.3. Variations in microbial communities attached to different materials compared to those in planktonic seawater

The microbial community was assessed using 16S rRNA gene-based amplicon sequencing. The relative abundance of each genus and unclassified microbial group was used for multivariate analysis and represented on a metric multidimensional scaling (mMDS) plot (Figure 3A). Overall, the sample groupings indicate differences in the microbial communities attached to four types of materials compared to those in planktonic seawater. One-way ANOSIM revealed R statistic values of 0.171, 0.436, 0.382 and 0.357 between seawater and SS316, titanium, HDPE or polycarbonate, respectively ($p < 0.10$) (Table 3A).

The first difference between seawater and an attached biofilm microbial community was the number of unique operational taxonomic units (OTUs). To illustrate, at a sequencing depth of

2715 sequences, there was an average of 905 OTUs identified for SS316 throughout the study period, while the other materials had at least 1.2 times higher numbers of OTUs. However, the numbers of OTUs identified in the biofilms of all four materials were, on average, lower than the 1350 unique OTUs detected in the seawater.

The second difference between the microbial communities in seawater and in attached biofilms was the relative abundance of several microbial taxa. Unclassified Bacteria, unclassified Alphaproteobacteria and Bacillariophyta were the predominant groups present in a relative abundance of > 8% on all the materials. Both the unclassified bacteria and the unclassified Alphaproteobacteria were also present in relative abundances of 29.3% and 11.0% of the total microbial community, respectively, in the seawater (Figure 4). However, Bacillariophyta was present in an average relative abundance of only 0.8% in the seawater, and SIMPER analysis revealed that Bacillariophyta was the taxa (Figure 4) that accounted for an average of 6.3% of the difference between the microbial community in seawater and the communities attached to the materials (Supplementary S1). In addition, Candidatus *Pelagibacter* and unclassified Rhodobacteraceae were more abundant in the seawater than in the biofilms attached to all the materials (Figure 4), and they contributed to an additional percentage of approximately 3.2% of the difference between seawater and the attached biofilms.

3.4. Variations in the microbial communities attached to different materials

As observed in Figure 3A, the sample groups showed differences in the microbial communities attached to the HDPE and polycarbonate compared to those attached to titanium or SS316. The microbial communities on HDPE and polycarbonate showed a similarity of 65.2%, but they only shared an average of 53.7% similarity with the microbial communities attached to both types of metal. One-way ANOSIM analyses revealed significant differences between SS316 compared

with HDPE and polycarbonate at the 90% confidence level (Table 3A). To illustrate, the R statistic of SS316 against HDPE and polycarbonate were 0.163 ($p = 0.05$) and 0.133 ($p = 0.10$), while the R statistics of titanium against HDPE and polycarbonate were 0.128 ($p = 0.13$) and 0.149 ($p = 0.11$), respectively. There was also no significant difference between the microbial community attached to SS316 and titanium (One-way ANOSIM, $p = 0.31$).

SIMPER analysis revealed that Bacillariophyta and unclassified Planctomycetaceae were the predominant groups and contributed to a 13% cumulative difference in the microbial communities on metal and plastic (Supplementary S1). These taxa were present at relative abundances of 14.5% and 4.0% on the metals, respectively (Figure 4). In contrast, Bacillariophyta accounted for a relative abundance of 28.6% on the plastic materials while unclassified Planctomycetaceae accounted for < 1% of the total microbial communities attached to the plastics (Figure 4). SIMPER analysis also showed that titanium supported a higher diversity of different microbial populations, specifically, GpIV, *Kangiella*, unclassified Enterobacteriaceae, unclassified Cyanobacteria and *Lutaonella*, which were present in > 2% relative abundance on the titanium (Figure 4) and contributed to approximately 12% of the cumulative difference between the microbial communities on titanium and plastic materials (Supplementary S1).

3.5. Temporal variation of seawater biofilm

Cell counts attached as biofilm on HDPE and polycarbonate remained at the same level of ~10^6 cells/mL throughout the sampling months (Figure 2). In contrast, the 3-month-old biofilm attached to titanium increased by more than 1-log to $1.7 \times 10^7 \pm 9.3 \times 10^4$ cells/mL compared to 1-month-old biofilm ($7.9 \times 10^5 \pm 1.9 \times 10^4$ cells/mL). The mature biofilm from SS316 sampled at the 4^{th} month also had approximately 3-fold higher cell counts than the 1-month and 2-month biofilms from the same material (Figure 2). Multivariate analysis of the relative abundances of

the bacterial genera present in the biofilms sampled throughout the four-month period revealed a temporal succession in the microbial populations (Figure 3B). However, one-way ANOSIM revealed no significant difference in the overall microbial communities among the 4 sampling months (Table 3B).

3.6. Occurrence of opportunistic pathogen genera on different materials

Genera associated with opportunistic pathogens that were within the detection limits of the high-throughput sequencing approach included *Acinetobacter*, *Arcobacter*, *Coxiella*, *Legionella* and *Pseudomonas*. There were no apparent temporal changes in the relative abundance of *Arcobacter*, *Legionella* and *Pseudomonas*, and all were present in relative abundances of ≤ 0.35% of the total microbial community (Figure 5A). This is with the exception of a single instance of an exceedingly high relative abundance of *Pseudomonas* (35.1% of total microbial community) in the 2-month-old biofilm attached to SS316 (Figure 5A). *Acinetobacter* and *Coxiella* were also present at up to ca. 0.36% of the total microbial community on HDPE and SS316 (Figure 5A). In particular, the relative abundance of *Coxiella* remained higher in the biofilm attached to SS316 than in those attached to other materials throughout the 4-month sampling period.

3.7. Occurrence of SRB on different materials

Seawater contains a high sulfate content, which can favor the presence of SRB that are thought to be the main protagonists in biocorrosion. Emphasis was therefore placed on evaluating the presence of SRB attached to different materials using both high-throughput amplicon sequencing and qPCR. The main type of SRB detected in this study included unclassified Desulfobacteraceae, unclassified Desulfobulbaceae and unclassified Desulfovibrionaceae (Figure

5B). The qPCR analyses indicated that there was a 10-fold higher copy number of Desulfobulbaceae detected on SS316 than on the other materials ($p < 0.05$) (Figure 6). The average copy number of Desulfobulbaceae attached to SS316 was 2.1×10^{-3} copies/cell number and was similar to that detected in seawater (2.2×10^{-3} copies/cell number) ($p = 0.85$). In contrast, there was no significant difference in the abundance of Desulfobulbaceae attached to HDPE, polycarbonate and titanium ($p > 0.20$). Desulfobacteraceae was also present in ca. 10-fold higher abundance than Desulfobulbaceae across all samples, but the amounts attached to the different materials were significantly lower than those present in seawater (Figure 6) ($p < 0.10$). To illustrate, the copy number of Desulfobacteraceae present in seawater was 7.4×10^{-1} copies/cell number, while that attached to the SS316 coupons was highest at 9.5×10^{-2} copies/cell number, followed by that on titanium (6.3×10^{-2} copies/cell number), HDPE (3.9×10^{-2} copies/cell number) and polycarbonate (2.6×10^{-2} copies/cell number). The abundance of Desulfobacteraceae present on SS316 was significantly higher than that on HDPE and polycarbonate ($p < 0.05$) but was not significantly different from that on titanium ($p = 0.44$). Similarly, there were no significant differences in the abundances of Desulfobulbaceae on titanium compared with HDPE ($p = 0.51$) or polycarbonate ($p = 0.31$).

4. Discussion

The findings in this study demonstrated that different materials have varying extents of surface roughness and hydrophobicity/hydrophilicity. Unlike an earlier study, which showed that there was more microbial colonization on rougher surfaces [34], our study revealed no apparent differences in cell numbers attached to polycarbonate, which has the roughest surface (Table 2),

compared to HDPE and titanium. Earlier studies also demonstrated that relatively hydrophilic surfaces were less prone to cell attachment [35-37]. However, both HDPE and polycarbonate, which are more hydrophilic than titanium and SS316 (Table 2), had higher attached cell numbers than SS316 (Figure 2). In addition, the bacterial cell numbers attached to the different materials showed no significant differences as the biofilms matured with time (Figure 2). This observation is in agreement with that reported earlier [38, 39]. It is likely that as the biofilm matures, bacterial cells condition the surfaces of different materials through the production of extracellular polymeric substances and diminish the role of surface properties in cell attachment.

It is therefore likely that other factors, including the antibacterial effect associated with each type of material, would also affect cell attachment, colonization and biofilm development. To illustrate, in drinking water, plastic materials such as polyethylene were observed to have a higher number of attached total bacterial and viral-like particles than copper [14]. Copper is a heavy metal that was previously shown to inhibit the number of *L. pneumophila* cells in a biofilm matrix [40]. Similarly, stainless steel is an alloy comprising manganese, silicon, chromium, nickel and molybdenum, all of which are heavy metals that could possibly impede cell attachment and development. This likely accounts for the lower cell densities obtained on stainless steel for both the seawater microbial biofilm assessed in this study (Figure 2) and for the drinking water biofilm in other studies [11, 12]. Although titanium is also a metal, its effect contrasted with that of stainless steel. Instead, the total attached cell numbers on titanium were of the same range as those attached to HDPE and polycarbonate (Figure 2). Earlier studies found that pure titanium was non-mutagenic and non-cytotoxic [41, 42] and that comparable cell numbers adhered to titanium and HDPE [42].

Regardless, the microbial community structures attached to the two metallic materials were distinct from those on the two plastic materials. In particular, qPCR revealed that there were higher copy numbers of Desulfobulbacaeae and Desulfobacteraceae when normalized against the total attached cells number on the stainless steel compared to the other materials (Figure 6). Both Desulfobulbaceae and Desulfobacteraceae are members of SRB, which are thought to be the main protagonists for microbial-induced biocorrosion. Their presence on the stainless steel may have accounted for the occurrence of pitting on the SS316 (data not shown) but not on any of the other materials tested in this study. Numerous types of SRB, including *Desulfovibrio vulgaris* [43, 44] and *Desulfobulbus propionicus* [45], are capable of obtaining electrons from stainless or carbon steel by coupling with sulfate reduction to gain maintenance energy. This redox reaction, in turn, causes microbial-induced biocorrosion.

The higher abundance of Desulfobulbaceae and Desulfobacteraceae on stainless steel can be accounted for by the presence of sulfur contents present in the stainless steel alloy. The enrichment of SRB on a conductive matrix was also reported earlier in studies examining the microbial communities on granular activated carbons (GACs) [46, 47]. Similar to stainless steel, GACs are derived from lignite containing sulfur and are bound by van der Waals forces at the molecular level to permit free electron flow (i.e., they are good conductors of electricity). Similarly, the high relative abundance of *Pseudomonas aeruginosa* associated with the stainless steel can be accounted for by the fact that *P. aeruginosa* produces pyocyanins as electron transfer mediators [48, 49] and can, in turn, reduce thiosulfate for its metabolic needs on a conductive matrix such as stainless steel [50, 51]. In contrast, titanium is not a good conductor of electricity, with a relative conductivity of approximately 3% compared to that of copper [52]. Furthermore, based on thermodynamics and kinetics, SRB cannot corrode titanium to obtain energy. These

442 factors may have accounted for the lower abundance of SRB on titanium despite it being a
443 metallic material.

444 In addition to a higher abundance of Desulfobulbaceae and Desulfobacteraceae, there was
445 also high relative abundance of *Acinetobacter* and *Coxiella* associated with stainless steel. Both
446 genera contain species associated with opportunistic pathogens, namely *Acinetobacter*
447 *baumannii* and *Coxiella burnetii*. In an earlier study, bacterial species isolated from drinking
448 water were evaluated for their hydrophilicity and hydrophobicity, and it was observed that the
449 majority of the bacterial isolates, including *Acinetobacter,* were hydrophilic [53]. Since all the
450 materials evaluated in this study are hydrophilic surfaces, it is likely that *Acinetobacter* adheres
451 very well compared to the other isolates due to the hydrophilicity effect. However, qPCR did not
452 show *Acinetobacter baumannii* to be present in any of the samples (data not shown).

453 For the genus *Coxiella*, *C. burnetti* is the only member of this genus currently isolated
454 and characterized. This species is an obligate intracellular parasite that is commonly detected in
455 seawater by molecular methods [54], which may explain their occurrence in the seawater
456 biofilm. It has been postulated that being obligate intracellular parasites [55], species within this
457 genus may be protected from external environmental factors, including the antibacterial effect of
458 stainless steel, and may successfully establish itself as one of the bacterial populations in the
459 biofilms on all the tested materials. However, it remains unknown why this genus would attach
460 preferentially to stainless steel compared to the other materials.

461 **5. Conclusions**

462 Although this study did not assess the impact on cell numbers and microbial community
463 structure of pipe materials in combination with other factors (e.g., the presence of chlorine

disinfectant and/or varying shear force), it is one of the few studies providing a comprehensive evaluation of the differences in seawater microbial biofilms as a result of attachment to a material substratum. The findings from this study suggest that certain material types, for example polycarbonate and HDPE, are preferred by bacterial to attach and establish biofilm. This is evidenced from the higher cell numbers attached to both materials compared to SS316. Despite a lower number of cells attached on SS316, there was a selective enrichment of sulfate-reducing Desulfobacteraceae and Desulfobulbaceae on SS316 compared to the other materials. This may make the stainless steel piping network relatively prone to biocorrosion. The use of stainless steel as a piping material in most industrial applications involving seawater usage may not be as ideal compared to titanium.

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631 **Table 1.** Primers used for quantitative PCR.

Target	**Primer name**	**Sequences (5'-3')**	**Reference**
Desulfobacteraceae (16S rRNA genes)	DSBAC357F	GTGAGGAATTTTGCGCAATGG	[56]
	519R	GWATTACCGCGGCKGCTG	[57]
Desulfobulbaceae (16S rRNA genes)	519F	CAGCMGCCGCGGTAATWC	[57]
	DSB706R	ACCGGTATTCCTCCCGAT	[58]
Acinetobacter baumannii (*ompA* gene)	*ompA*-F	TCTTGGTGGTCACTTGAAGC	[59]
	ompA-R	ACTCTTGTGGTTGTGGAGCA	

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634 **Table 2.** Surface characteristics of the tested materials.

	Average root mean square, RMS, of surface (n = 3) ± standard deviation	**Average liquid-solid contact angle (n = 6) ± standard deviation**
HDPE	0.24 ± 0.18 [a]	63.9° ± 1.8 [o]
Polycarbonate	0.53 ± 0.20 [b]	61.6° ± 1.7 [o]
Titanium	0.19 ± 0.11 [a]	80.2° ± 1.5 [o]
Stainless steel SS316	0.19 ± 0.06 [a]	75.6° ± 0.9 [o]

635 a, b Homogenous subgroups by two-tailed t-test method with a significance level of 0.10

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Table 3. ANOSIM R statistic values and p-values between the **(A)** different materials, and **(B)** sampling months. Values in bold refer to significant differences between the sample pairs at the 90% confidence level.

A.	Seawater	HDPE	Polycarbonate	Titanium	SS316
Seawater		**0.382 (p = 0.008)**	**0.357 (p = 0.011)**	**0.436 (p = 0.016)**	**0.171 (p = 0.065)**
HDPE			-0.139 (p = 0.967)	-0.128 (p = 0.133)	**0.163 (p = 0.054)**
Polycarbonate				0.149 (p = 0.108)	**0.133 (p = 0.100)**
Titanium					0.037 (p = 0.305)
SS316					

B.	1-month	2-month	3-month	4-month
1-month		0.041 (p = 0.309)	-0.005 (p = 0.485)	0.125 (p = 0.115)
2-month			0.058 (p = 0.228)	0.087 (p = 0.146)
3-month				-0.009 (p = 0.404)
4-month				

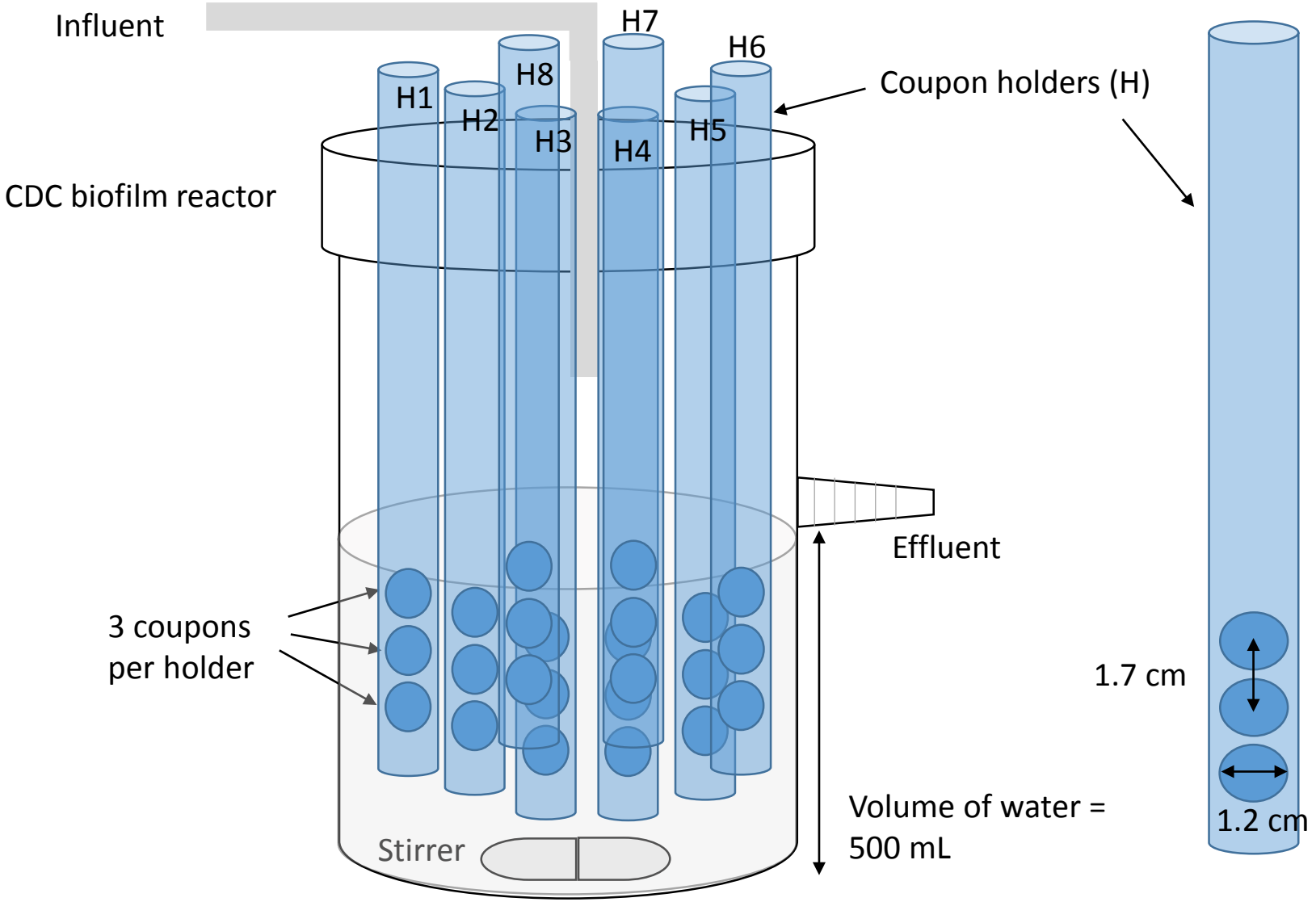

Figure 1. Illustration of the CDC biofilm reactor.

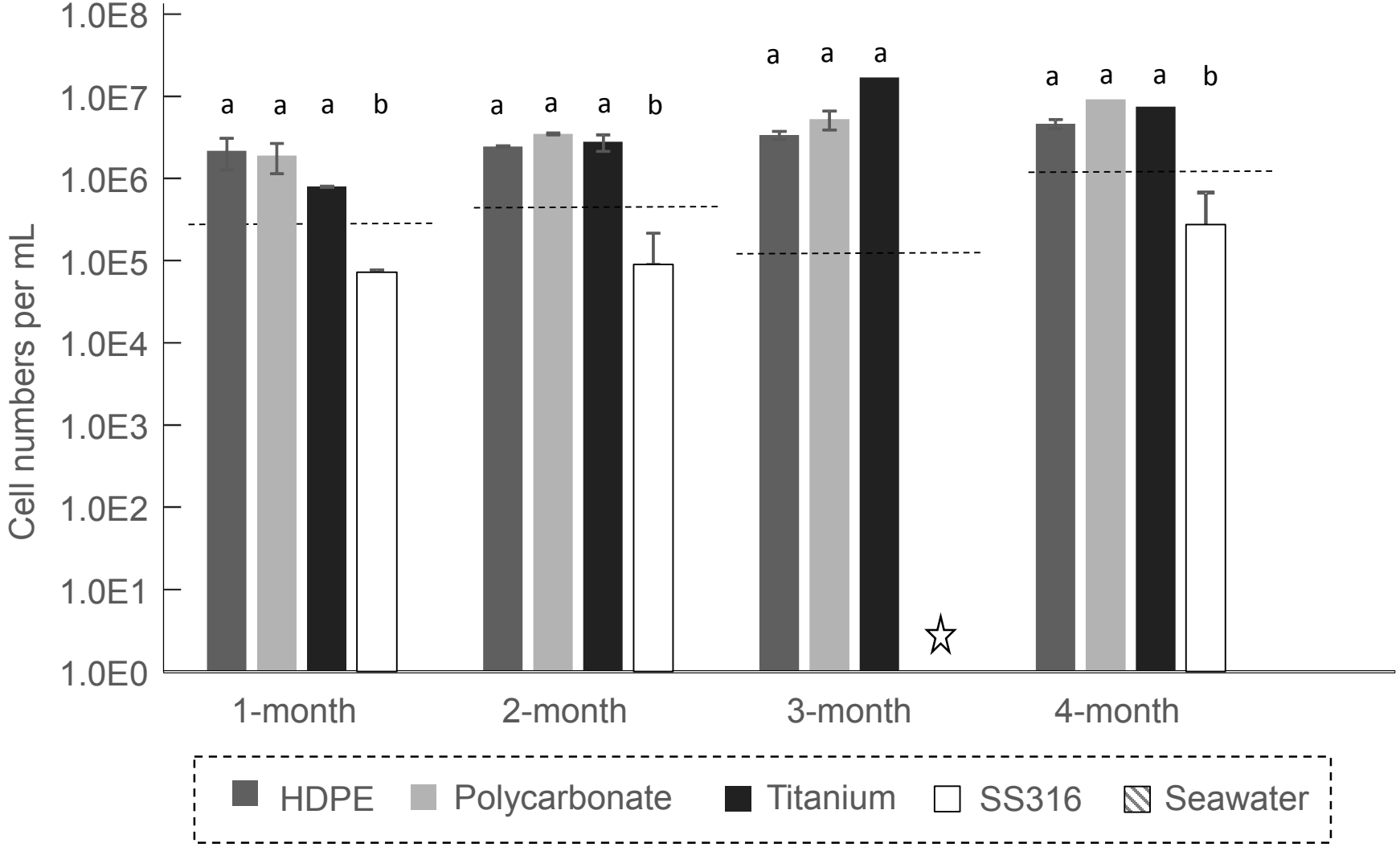

Figure 2. Enumeration of total cell counts attached to different types of materials over a duration of four months. The star denotes no measurements obtained for that sampling month for SS316 due to sampling error. a, b denote homogenous subgroups by two-tailed t-test method with a significance level of 0.10. Dash lines correspond to the cell numbers per mL of seawater at that month. Star denotes no measurements were obtained for that sampling month for stainless steel.

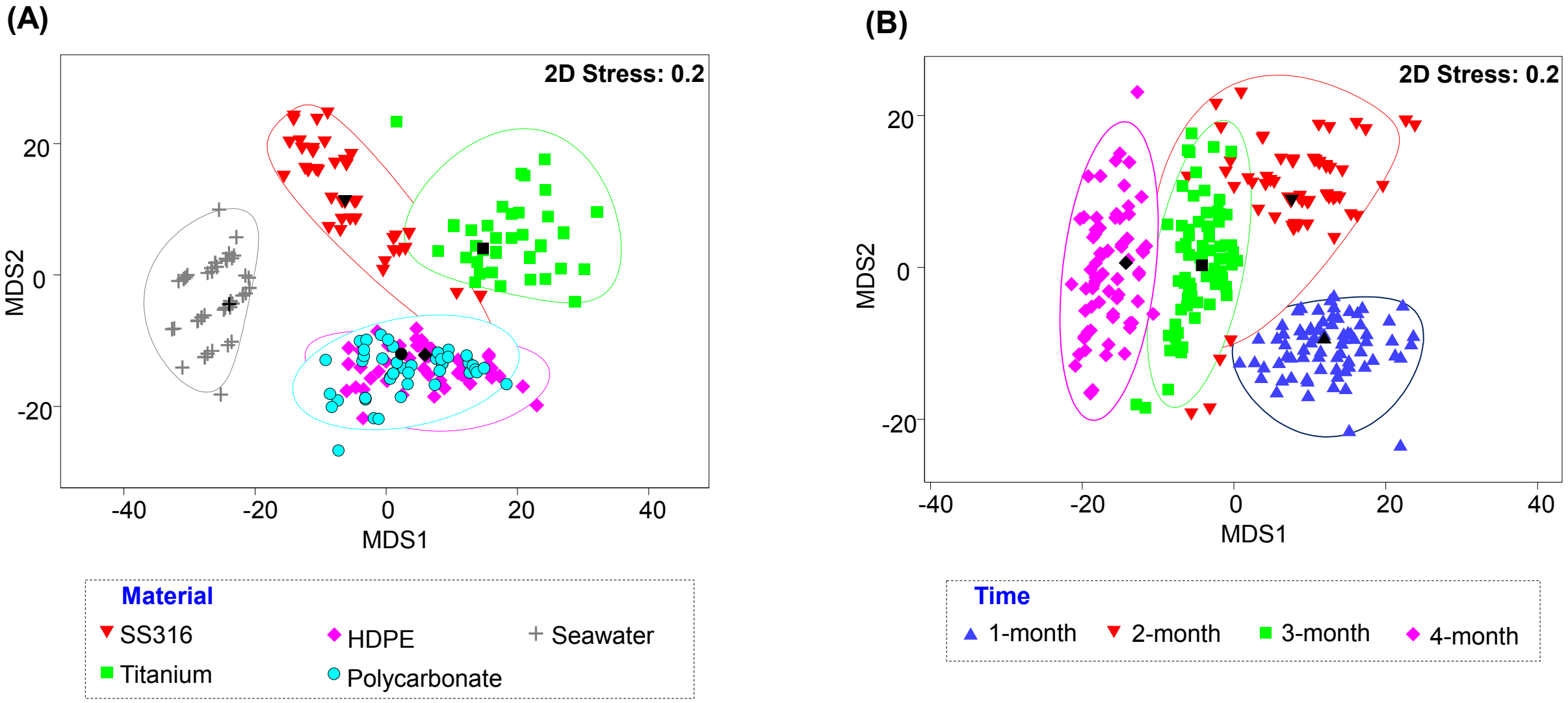

Figure 3. Bootstrapped metric multidimensional scaling (MDS) plots of microbial communities in relation to **(A)** the type of material and **(B)** the temporal effect.

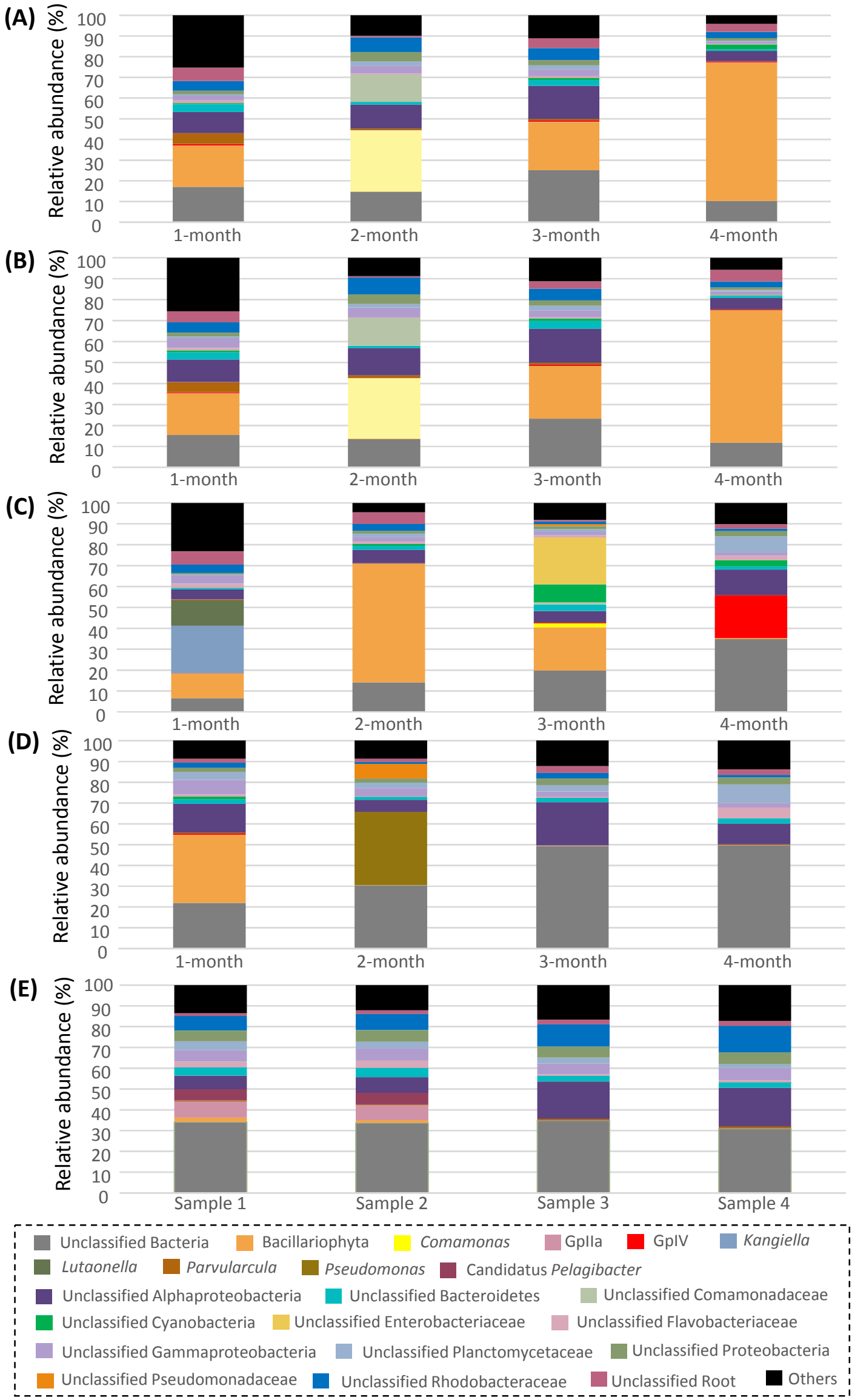

Figure 4. The relative abundances obtained by high-throughput sequencing, representing the predominant microbial taxa attached to (A) HDPE, (B) polycarbonate, (C) titanium, (D) SS316, and in (E) seawater

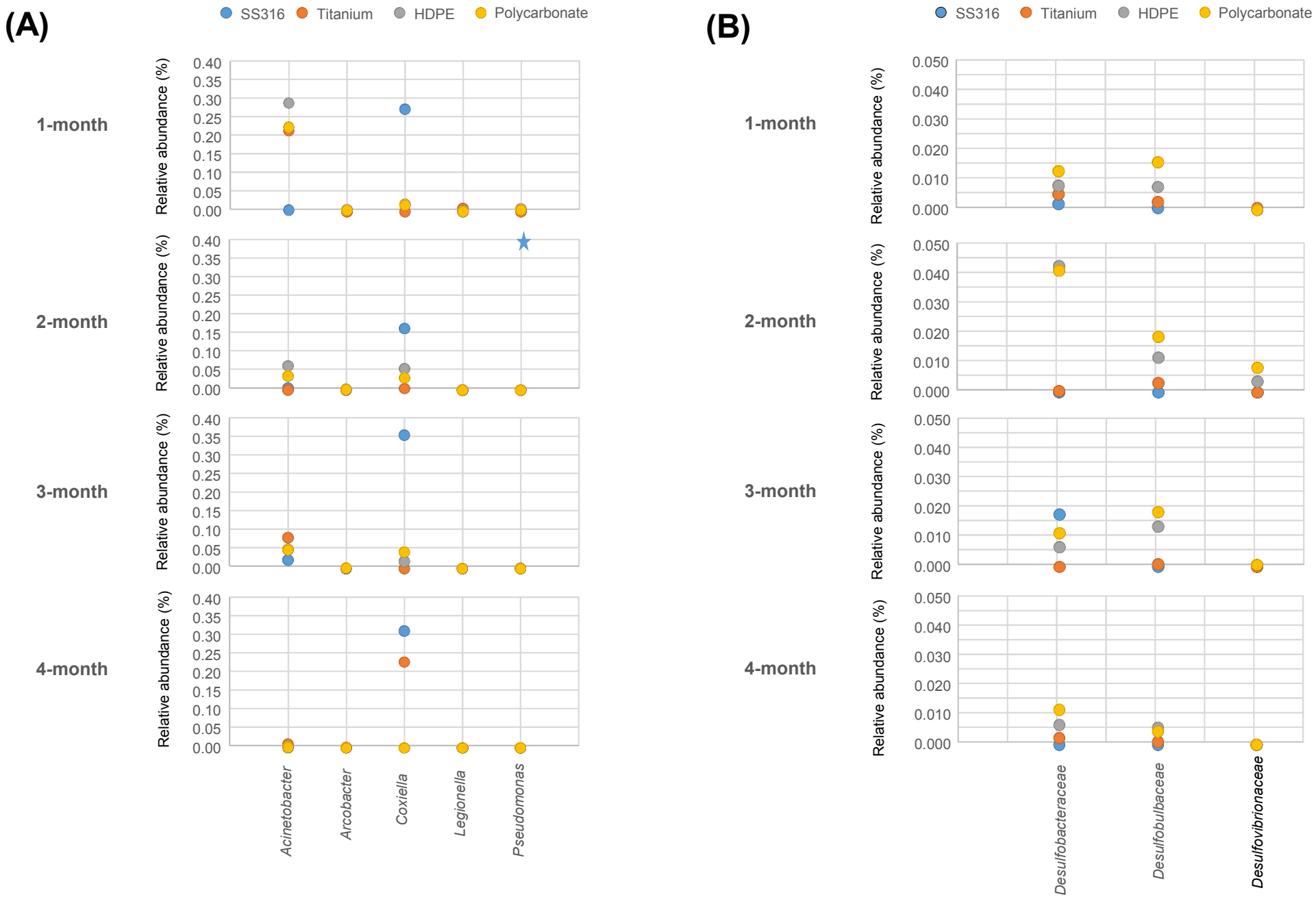

Figure 5. The relative abundances obtained by high-throughput sequencing representing **(A)** genera associated with opportunistic pathogens, and **(B)** selected sulfate-reducing bacteria identified at the family taxonomical level. The star denotes that the relative abundance of *Pseudomonas* was exceedingly high for SS316 at the 2-month sampling event and fell out of the range shown on the y-axis.

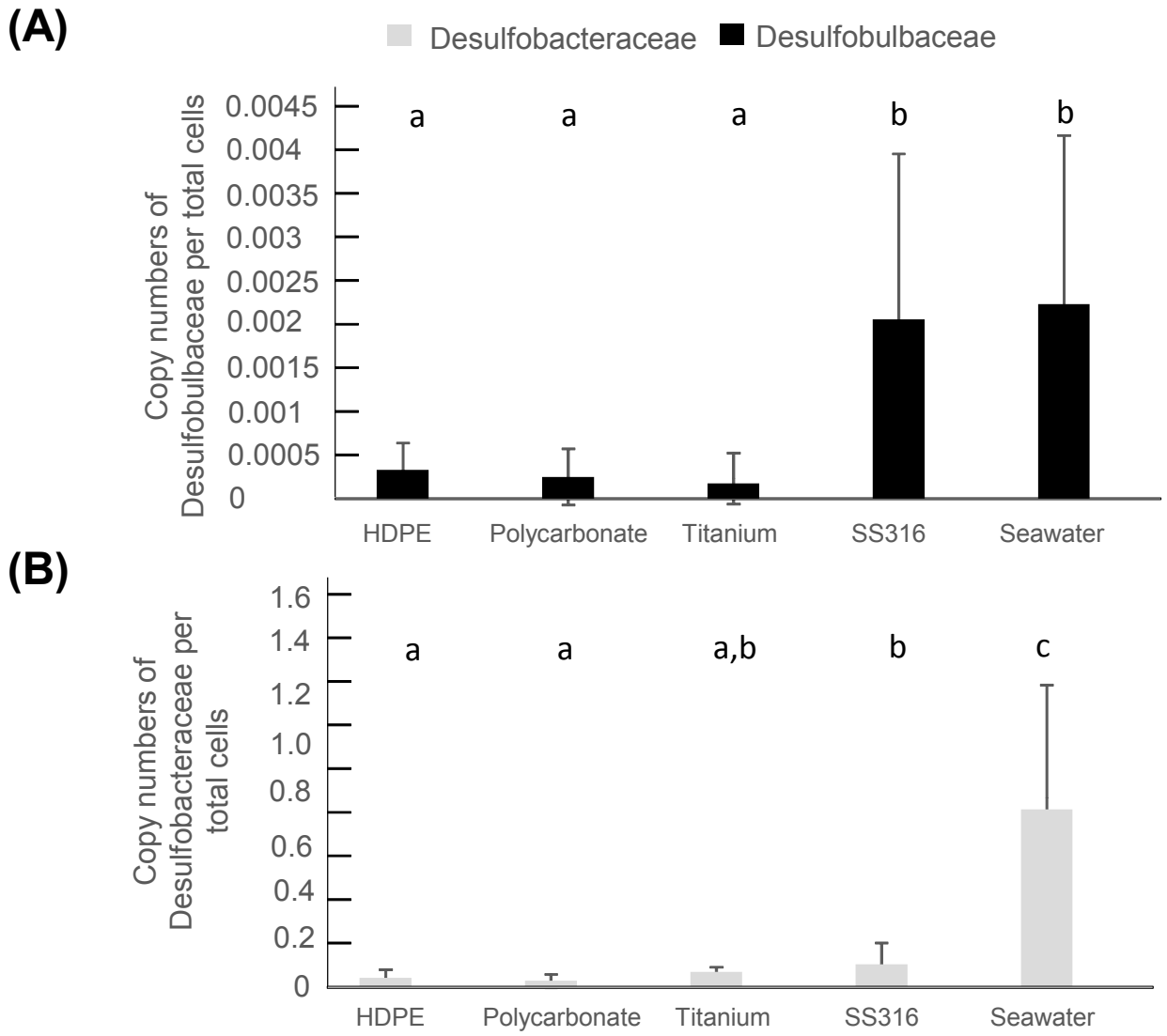

Figure 6. Copy numbers of 16S rRNA genes representative of Desulfobacteraceae and Desulfobulbaceae obtained by quantitative PCR and normalized against total cells. Both Desulfobacteraceae and Desulfobulbaceae comprise genera representative of sulfate-reducing bacteria. a, b, c denote homogenous subgroups by two-tailed t-test method with a significance level of 0.10.