"Biofouling and Biocorrosion"

Progress Report

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Prepared by:

Guangyi Wang, Ph.D.
Department of Oceanography
University of Hawaii

Prepared for:

Hawaii National Marine Renewable Energy Center
Hawaii Natural Energy Institute
University of Hawaii

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INTRODUCTION

The cost effectiveness of producing electricity with wave energy conversion (WEC) devices and ocean thermal energy conversion (OTEC) plants largely depends on the life–cycle reliability of equipment exposed to ocean conditions. A typical milliliter (ml) of seawater contains 10^3 fungal cells, 10^6 bacteria, and 10^7 viruses, including large populations of biofouling microbial communities, which cause corrosion to all marine installations. Materials that are exposed to seawater can initiate biological and chemical events that trigger the formation of a microbiological complex layer called fouling or biofouling (4). Biofouling has generally been used to describe the biological formation on a surface, which results from the accumulation of microbial communities, including bacteria, protozoa, fungi, and algae (1, 3). Formation of biofouling film may require a few hours or a few weeks, largely depending on substratum composition, and the physical and biological features of the seawater.

Biocorrosion occurs when complex microbial consortia interact with metallic surfaces through the establishment of multispecies biofilms. Biofilms mediate interactions between metal surfaces and the liquid environment, leading to major modifications of the metal-solution interface by drastically changing the types and concentrations of irons, pH, and oxygen levels (2, 10, 12). The mechanism of biocorrosion is complex and insufficiently understood. Thus, ocean energy generating facilities will be vulnerable to biocorrosion, which is already a serious problem for conventional power generation facilities and the offshore oil and gas industry. For example, microbial-influenced corrosion (MIC) alone accounts for 20-30% of all corrosion losses (about $30-50 billions per year) (7, 9).

Corrosion has been one important consideration of ocean energy production and development (13). For example, significant amount of efforts has been made to develop corrosion-resistant materials for OTEC heat exchangers. Likewise, biofouling, which is closely affiliated with corrosion, is another major consideration in the design and development of ocean power generating materials. Biofouling developments can seriously reduce the performance efficiency and capacity, eventually leading to the shut-down of ocean generating systems.

Previous US Department of Energy studies accomplished significant progress and provided critical technological information on the validation of materials for OTEC heat-exchangers (13). Titanium and stainless steel were not recommended for the use of OTEC facilities because of their relatively high costs. Some aluminum alloys were considered as excellent candidates for the use as heat-exchanger materials because they have high thermal conductivity, can be readily drawn or extruded, can be brazed, and are relatively inexpensive. There are some concerns about the susceptibility of some aluminum alloys to localized corrosion attack, mainly in the form of pitting and crevice corrosion (13). Due to the high fabrication costs, alclad alloys were not recommended for use in OTEC heat-exchangers. Furthermore, the biofouling-control technique of monthly brushing with nylon-bristle brushes is undesirable because it increased the corrosion rate of aluminum alloys (13). Daily chlorination at levels of between 70 and 100 ppb for one hour per day was shown to be an effective method of controlling the biofouling of all aluminum surfaces that were exposed to warm seawater. Negligible biofouling was observed for metal surfaces exposed to cold seawater; therefore, no biofouling-control method was required for OTEC condensers (13).

As a member of research team of the Hawaii National Marine Renewable Energy Center, the author of this report (Dr. Guangyi Wang, Department of Oceanography) collaborated with Dr. Lloyd Hihara (Hawaii Corrosion Laboratory, Department of Mechanical Engineering) to work on the center's project tropic "increased reliability and survivability of marine and hydrokinetic renewable energy technologies, including development of corrosive-resistant materials". This report summarizes test results of antifouling coating and antifouling activity screening of natural compounds derived from marine algae and sponges.

MATERIALS AND METHODS

Biofouling test site

Immersion experiments for the biofouling tests were done at the Makai Research Pier. The Hawaii Undersea Research Laboratory, a UH entity funded in part by the State of Hawai'i, provided space at its facilities for the immersion experimental tests.

Aluminum alloys metal coupons

In previous studies (13), aluminum alloys were recommended for use in OTEC heat-exchangers. This study has primarily targeted aluminum alloys for biofouling tests. Three aluminum alloys (Al20242, Al60613, and Al7075) were tested in this project. These aluminum alloys were chosen based upon their unique mechanical and physical features. Aluminum alloy 2024 contains copper and magnesium as the alloying elements. It can be used in applications requiring high strength to weight ratio, as well as good fatigue resistance, but has poor corrosion resistance. Aluminum alloy 6061 is a precipitation hardening aluminum alloy, containing magnesium and silicon as its major alloying elements. It has good mechanical properties and exhibits good weldability and is one of the most commonly used aluminum alloys. Aluminum alloy 7075 has zinc as the primary alloying element. It is strong, with strength comparable to many steels, and has good fatigue strength and average machinability, but has less resistance to corrosion than many other aluminum alloys. Its relatively high cost limits its use to many applications.

Sample coupons were manufactured in the Hawaii Corrosion Laboratory. All coupons were coated with a silicone coating called SiloXel^{TM} developed in the Hawaii Corrosion Laboratory. Uncoated metal coupons were used as a control. Metal coupons were placed on immersion racks and were immersed 2.5 meters below the seawater surface for biofouling test (Fig. 1). One coupon of each aluminum alloy was removed from the immersion tests for SEIM and microbial cultivation and other analyses at designated test times.

Culturable bacteria of biofouling communities

To isolate bacteria from biofilm developed on the metal coupons, biofilm removed from the metal coupon was grounded with autoclaved seawater. The homogenate was diluted with sterile seawater at four dilutions (1:10, 1:100, 1:1000, and 1:10000) (16). For bacterial isolation, 100 µl of each dilution was plated in quadruplicate onto plates of marine agar 2216 (Difco, Detroit, Mich), 2 Gause I agar (per liter, 15 g agar, starch 20 g, KNO_3 1g, K_2HPO_4 0.5g, $MgSO_4 \cdot 7H_2O$ 0.5g, NaCl 0.5g, $FeSO_4$ 0.01g; pH 7.2-7.4). All plates were incubated at 20°C and inspected daily for 3-4 weeks. Pure cultures were obtained after several transfers.

To identify the cultivated bacterial isolates, colony PCR was performed to amplify 16S rRNA genes using the primers U341f /U1406R, A344f /Ekb1241r, A333f/U1406r, U519f /U1406r (Baker et al. 2003). The 16S rRNA genes of most isolates can be amplified using the primers U341F/U1406R. The isolates whose 16S rRNA genes could not be amplified using the primers U341F/U1406R were amplified using one of the following three pairs of primers: A344F/Ekb1241R, A333F/U1406R, and U519/U1406R. A single colony of cultivated isolates was picked with a sterile toothpick, suspended in 50 µl of LB broth, and lysed by heating at 95°C for 10 min. Cell lysates were rapidly cooled on ice and used as a PCR template (Michaud et al. 2004). PCR amplification was performed in the 50 µl reactions containing 5 µl of 10×reaction buffer with 15 mM $MgCl_2$, 2 µl of 2.5 mM dNTPs, picomol of each primers, 2 µl of cell lysates, 0.5 µl of Taq DNA polymerase (5 U/ µl, Promega), and 31.5 µl ddH_2O. PCR conditions were as follows: initial denaturation at 94°C for 10 min, 30 cycles of 94°C for 50 s, 55°C for 50 s, and 72°C for 1 min, and a final extension of 10 min at 72°C. The PCR products were purified using QIAquick PCR Purification Kit (Qiagen) or QIAquick Gel Extraction Kit (Qiagen). The purified PCR products were eluted into sterile H_2O for sequencing analyses. Alternatively, recovered PCR products were cloned into pGEM-T easy vector (Promega) if satisfactory sequence results were not obtained from PCR products.

DNA extraction, PCR, clone library construction, and T-RFLP analysis

Biofilms removed from individual metal coupons were used for the total genomic DNA extraction using the Fast DNA kit (MP Biomedicals). PCR amplifications were performed in a 50-μl reaction for 30 cycles of denaturation at 95°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1.5 min with the total genomic DNA as a template. To construct the 16S rRNA gene library, bacterial forward primer 27F (5'-AGAGTTTGGATCMTGGCTCAG-3') and reverse primer 1492R (5'-CGGTTACCTTGTTACGACTT-3') were used to amplify PCR products with the total genomic DNA as a template. PCR products were cloned into the pGEM-T vector (Promega) and transformed into *Escherichia coli* DH5α competent cells. The transformed cells were plated on LB agar plates containing 100 μg/ml of ampicillin, 80 μg/ml of X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside), and 0.5 mM IPTG (isopropyl-β-D-thiogalactopyranoside).

A total of 100 random colonies were selected from each clone library, screened using colony-PCR, and grouped on the basis of RFLP patterns using *Hha* I (TaKaRa) (6). Representative clones from each group were used for sequencing analysis. T-RFLP analysis was performed following the method described by Liu et al. (1997). In brief, bacterial forward primer 27F-FAM and reverse primer 1492R were used for PCR amplification with the total genomic DNA as a template. PCR products were purified using QIAquick PCR Purification Kit (Qiagen). The purified PCR products were digested for 1 h with 5 U of *Hha* I. Endonuclease was inactivated by heat as specified by the manufacturer (65°C, 20 min) to terminate the restriction reaction. Fragment analysis was performed at the University of Hawaii DNA Core Sequencing Facility. In the T-RFLP profile, each restriction fragment (T-RF) was considered as an OTU (operational taxonomic unit) (8, 11).

Sequence analysis

Plasmids were sequenced at the University of Hawaii DNA Core Sequencing Facility on an Applied Biosystems 3730XL automated DNA sequencer. Sequence data were edited with the Chromas Lite, version 2 (Technelysium) software package. Chimeric sequences were checked using the Ribosomal Database Project (RDP) II Chimera Check program (http://rdp8.cme.msu.edu/cgis/chimera.cgi). Clone sequences were grouped using DOTUR (15). The 16S rRNA gene sequences with a percent sequence identity of greater than 97 % was placed in the same OTU (Operational Taxonomic Unit). One sequence from each OTU was selected as a representative for further analysis. For preliminary identification, sequences of bacterial 16S rRNA genes were compared with those deposited in the NCBI database (National Center for Biotechnology Information; http://www.ncbi.nlm.nih.gov) and were compared with those in Ribosome Database Project (RDP) using http://rdp.cme.msu.edu/classifier/classifier.jsp.

Scanning Electron Microscope (SEM) analysis

Three different coated aluminum alloy coupons along with uncoated ones were submerged into seawater and collected during 1-week and 4-week incubation period. The microbial community and biocorrosion status of the alloy coupons were analyzed by scanning electron microscope (SEM). All coupons were first fixed with 2.5% (vol/vol) glutaraldehyde for 1 hour and washed twice in 0.1 M of sodium cacodylate buffer (12% sucrose) for 10 minutes, followed by washing once in distilled water. Graded ethanol series (30%-50%-70%-85%-95%) were applied for dehydration of specimen, washing twice at each ethanol concentration step for 10 minutes each. Absolute ethanol was then used to dry specimen 3 times for 10 minutes each. In the final step, 100% ethanol was substituted with hexamethyldisilizane (HMDS) within 3 steps, washing with 1:1, 1:3 ratio of ethanol: HMDS solution once for 10 minutes each, respectively, and 3 times in 100% of HMDS. Coupons were covered loosely and dried overnight before observing. The microbial community and biocorrosion status of coupons were then examined with a HITACHI S-4800 field emission scanning electron microscope operating at 5 kV.

Antifouling Screening

Marine sessile organism such as sponge, soft corals, and seaweeds are known to elaborate chemical defense mechanisms against predation and epibiont growth. Therefore, the utilization of bioactive ingredients derived from marine organisms has long been considered as one promising alternative to heavy metal-based paints for antifouling. These metabolites already exist in the marine environment, and as natural products they would be target-oriented, biodegradable, and environment-friendly. Tissues of five alien marine sponges (*Mycale armata*, *Subrites zeteki*, *Dysidea* sp., *Gelliodes fibrosa*, *Tedania* sp.), which were chopped into small pieces and stored at -80°C for microbial diversity, were freeze dried and used for the extraction of natural compounds. A 30-g sample of dried sponge was extracted, with stirring, for 2 hours in distilled water using previously described method (5). After centrifugation and filtration, the aqueous supernatant was lyophilized and designed. The residue sample from above was further extracted, with stirring, in 300 ml of 95% ethanol. The alcoholic solution was collected by centrifugation. The residual tissue was re-extracted 4 times using the same procedure. The alcoholic extracts were pooled and concentrated, under vacuum, at 35°C. Distilled water (50 ml) was subsequently added to the organic residue before portioning against dichloromethane (10-50 ml). The aqueous phases were collected, lyophilized, re-dissolved in absolute ethanol (50 ml), filtered, and concentrated under vacuum at 35°C. The dichloromethane phases were collected dehydrated with $CaCl_2$, filtered, and concentrated under vacuum.

To identify antifouling activities of algal extracts, red algae *Acanthophora spicifera*, *Laurencia nidifica*, *Gracilaria salicornia*, *Gracilaria coronopifolia*, and *Hypnea musciformis* were collected for the extraction of natural compounds. The algal samples were rinsed with sterile artificial seawater in order to remove surface microflora, lyophilized, and stored in -20°C before use. Extraction was performed as previously described by Bazes et al. (2009). The dried algae were suspended, with stirring, in 95% ethanol. The alcoholic fraction was collected by centrifugation. The resulting pellet was re-extracted 4 times using the same procedure. The alcoholic extracts were combined and evaporated under the previously described condition. Distilled water was added and partitioned with dichloromethane used the above procedure. All the extracts (alcoholic or dichloromethane extracts) were stored at -20°C before use.

Bioassays

Flexibacteraceae bacterium strain was obtained from the marine biofilm and identified in the PI's group. Antibacterial evaluation of the extracts and fractions was performed in 96-well plates as previously described in Plouguerne (2006). Samples of cultures grown overnight (2×10^8 cells/mL) were incubated with extracts and biocides at the concentration of 25, 50, 100, 200 and 300 µg/mL for 48 h at 20 °C. All inhibition assays were carried out in triplicate. Growth was monitored by measuring OD600 with Synergy HT Multi-Mode Microplate Reader (BioTek) and the percentage of inhibition was calculated for each concentration:

$$\%\text{inhibition} = (ODc\text{-}ODt)/ODc \times 100$$

where, ODc is the mean optical density of the bacterial controls and ODt is the mean optical density of the test samples. Control testing with the solvents was performed for every assay and showed no inhibition of the microbial growth.

RESULTS AND DISCUSSION

SEM analysis of biofilm and corrosion

Metal coupons of three aluminum alloys submerged in seawater for 1 and 4 weeks were subject to SEM analysis. Bacteria, protozoa, fungi and algae (diatoms) were identified among the biofouling communities developed on the alloys using the morphological approach (Fig. 2). In addition, different morphologies of biofouling communities were noticed in the biofouling communities (data not shown). The composition of these biofilms associated with different metal coupons need to be confirmed using molecular approaches. Rod-shaped bacteria were found among the fouling communities of biofilms derived from all three aluminum alloy coupons (Fig. 2H). However, these bacteria did not constitute the first layer of biofilm as noticed in previous study (13). Much more fouling communities were observed on uncoated metal coupons in both testing times (week 1 and 4) than on those uncoated ones. Therefore, our results suggested that the coating SiloXel™ can significantly reduce the fouling community

development. Corrosion was observed in week one on uncoated A2024, not on the coated one. Interestingly, no uniform biofilm development was observed on any of these tested coupons as observed in the previous study (13). Thus, results of this study did not support the uniform-corrosion mechanism. This discrepancy likely ascribed to the following reasons: 1) higher resolution of SEM was used in this study; and 2) submerged environments were different (controlled vs natural). Currently, we are still analyzing SEM results derived from different metal coupons.

Cultured bacterial communities

A total of 56 isolates were cultured from the biofilms developed on A2024. After ADRA and sequencing analyses, 8 unique bacterial strains were identified (Table 1). They belonged to 3 phyla and 3 bacterial classes. Strains 1 and 3 were (98 and 97%, respectively) closely affiliated with marine bacterial strain *Psudoaltermonas* sp. 1tb4 derived from the coral *Montastrea annularis* and strain 5 with *Phtobacterium* sp. r33 from the coral *Montipora* sp.. Stains 8, 9 and 10 were related to the bacteria isolated from the dinoflagellates *Scrippsiella trochoidea*, macroalgae, and the alga *Delisea pulchra*, respectively. Interestingly, strain 6 was related to (95%) *Bacillus* sp. SMB9 isolated from marine biofilm. Finally, strain 2 was related to uncultured bacterium *Bacillus* sp.clone b44 from seawater. Overall, most of the culture-able bacterial strains belong to members of biofouling communities. Some of these strains have been cultured for the first time from the biofilms and can be used for future antifouling assays.

Molecular analysis of biofouling bacterial communities

Both morphological and cultivation-dependent approaches have limited power in identify bacteria from natural environments. To investigate the differences of bacterial composition for biofilms, molecular approaches are used to analyze biofilm samples derived from the metal coupons. Due to the limited amount of biofilm sample from individual coupons, extraction of sufficient total genomic DNA was a challenge. At the same time, polysaccharides of biofimls made it difficult to extract high quality of DNA. Three DNA extraction methods were used to extract total genomic DNA from the biofilm samples. DNA analysis indicated that FastDNA kit was good choice for this study because it gave good quality and high yield of DNA from biofim samples (data not shown).

T-RFLP results revealed interesting bacterial communities associated biofilms. Firstly, biofilms contain bacteria from seawater, but also include those not identified in the seawater. Secondly, bacterial composition for biofilm samples derived coupons submerged for one week was different from those from coupons submerged for 4 weeks. This observation indicated the consistent development of fouling bacterial communities of biofilm on these metal coupons. Finally, bacterial composition of biofilms derived from uncoated coupons was different from those from those coated coupons. This observation is consistent with that of SEM results. Results of T-EFLP analysis further confirmed that the coasting can affect the bacterial development on the metal coupons. Currently, analysis of bacterial communities associated with biofilms from different aluminum alloys are in progress.

Antifouling analysis

Extracts from marine sponges and algae have been shown to be a good resource for antifouling compounds (5, 14). Partially-purified chemicals from these extracts have been applied in antifouling painting with very promising results. Extracts derived from alien Hawaiian marine sponges and marine algae were tested for the inhibition activity of *Flexibacteraceae* sp. The results are summarized in Table 2. Extracts from both marine sponges and algae displayed interesting antifouling activity based on the growth inhibition of the marine

bacterium *Flexibacteraceae* sp. (Table 2). Generally speaking, dichloromethane extracts derived from both marine sponges and algae displayed higher antifouling activities than aqueous ones. Also, extracts from sponges had higher antifouling activities than those from algae. Because of time limitation, antifouling assays for the sponge specie *Tedania* sp. and two algal species *Hypnea musciformis* and *Laurencia nidifica* were not completed. Inhibition of phytoplankton growth (*Cylindrotheca closterium*) still needs to be perfrmed to confirm antifouling activities of these extracts.

SUMMARY

This report adds new technological information for the development of resistant materials for the use of ocean energy facilities. Although previous studies (1983-1987) funded by the Department of Energy had provided important information for OTEC materials and biofouling research, conclusions of this study further provide critical information for ocean renewable energy industry. Clearly, the coating SiloXel™ can significantly improved the antifouling capability of aluminum alloys Al20242, Al60613, and Al7075 and is recommended to be used for the antifouling painting on ocean energy facilities in the Hawaiian waters. In addition, results of this study slightly deviated from the previous report in the feature and corrosion-mechanism of aluminum alloys. Further studies on the aspects of biofouling and biocorrosion on aluminum alloys are strongly recommended. Furthermore, extracts from marine sponges and algae displayed interesting antifouling activities and can be applied in the antifouling painting with more detailed studies. Particularly, extracts used in this study were extracted from alien marine species and can be produced in large quantity. Development of these extracts for the use of ocean energy generating industry will be an interesting and promising R&D project.

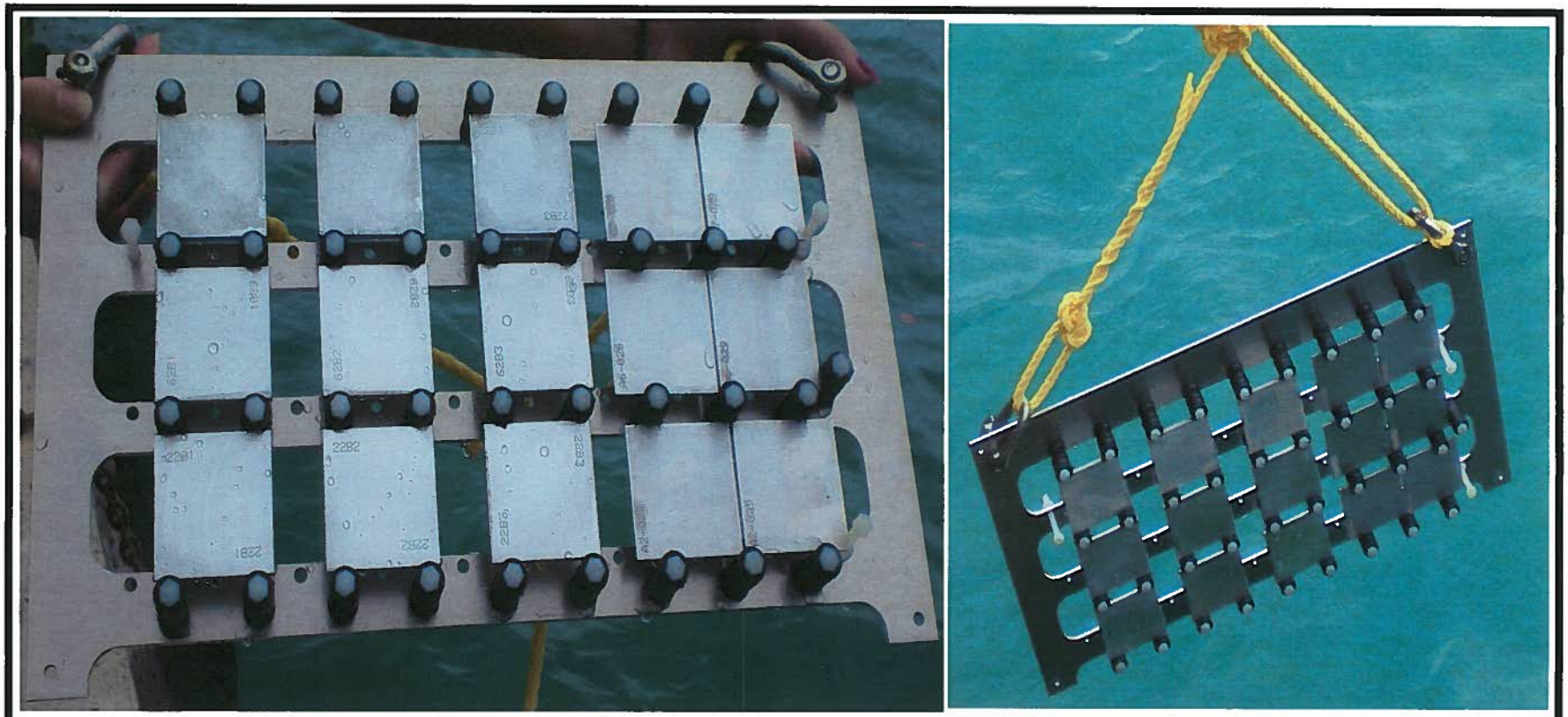

Figure 1. Aluminum alloy coupons of Al20242, Al60613, and Al7075 treated with SiloXel™ silicone coatin were mounted on an immersion rack (left panel). Immersion rack carrying coupons was on its way for immersion test at the Makai Research Pier (right panel).

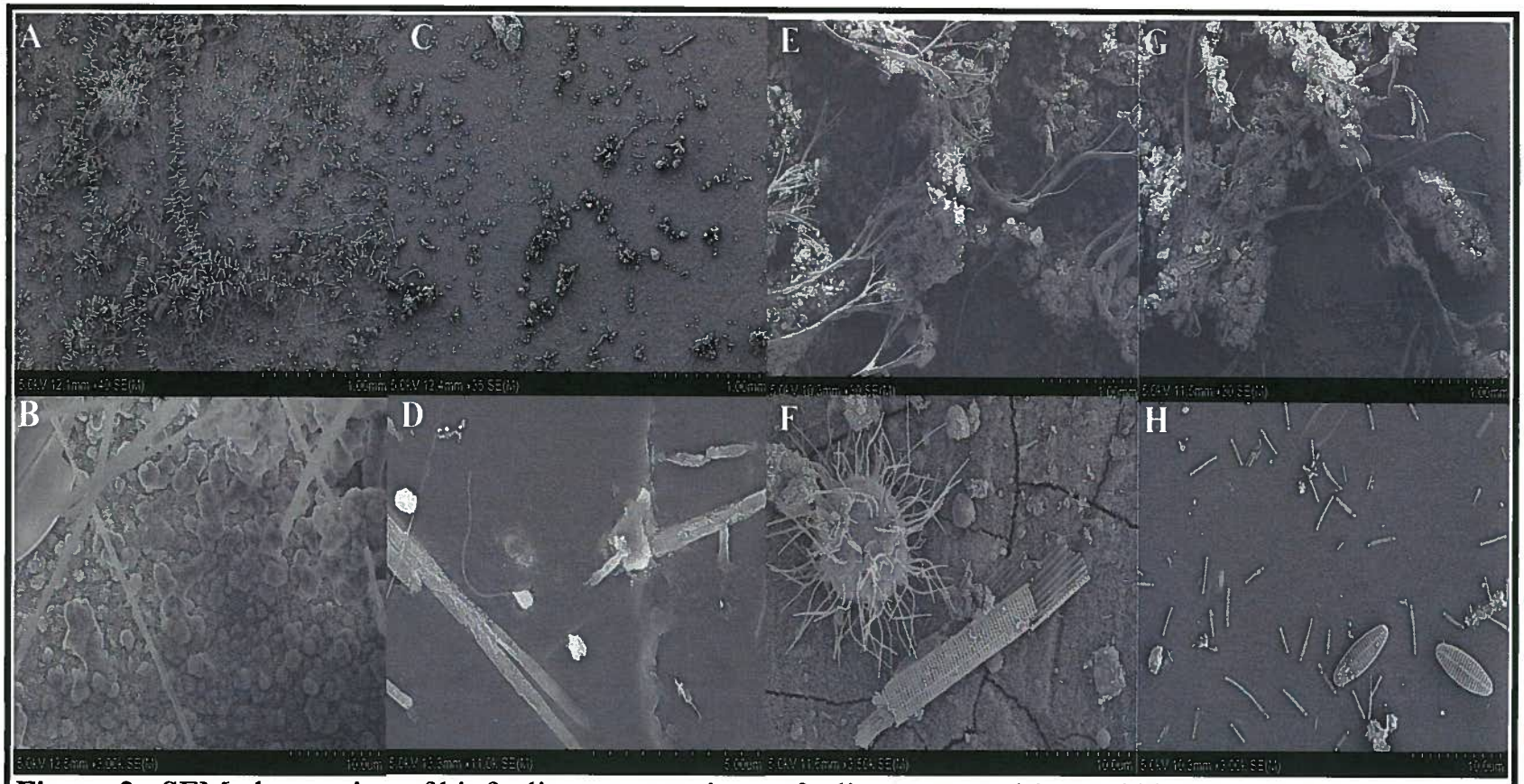

Figure 2. SEM observation of biofouling community on fouling communities and biocorrosion on A2024. views A, B, C, & D for one-week submersion and E, F, G, & H for four-week submersion. Views A, B, E & F were biofouling communities of uncoated A2024 and views C, D, G, & H for those of coated A2024.

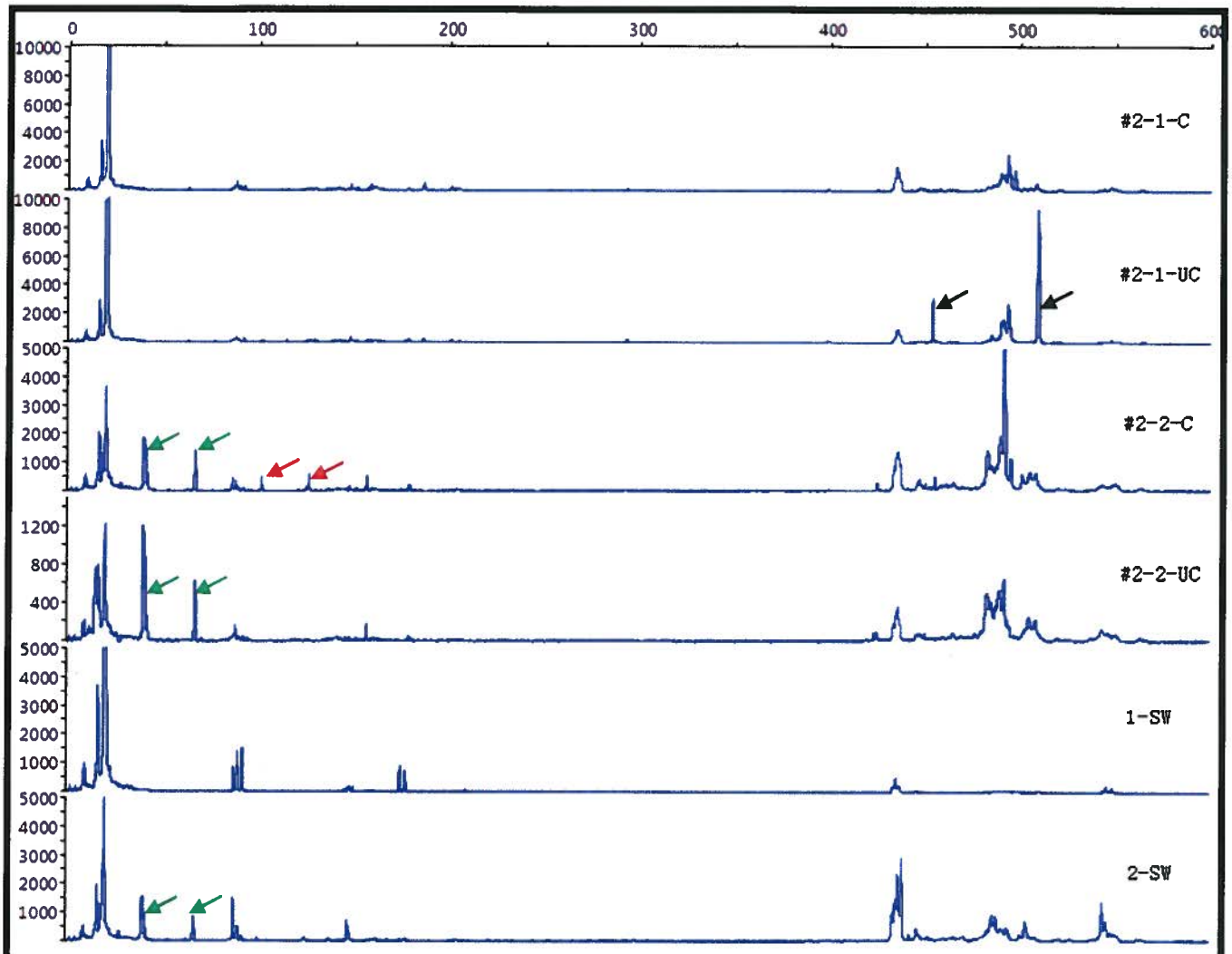

Figure 3. Diversity of bacterial communities associated with biofilms derived from A2024. #2 Stands for coupon number; numbers '1' and '2' stands for sample collected at one- and 4-week submersions; C and UC represents coated and uncoated coupons, respectively; and SW stand for seawater sample.

Table 1.- Cultured bacterial from biofilm developed on A2024.

"Species" ID	Phylum	Class	Closest relative (Accession no.)	Sequence identity (%)
1	*Proteobacteria*	*γ-Proteobacteria*	*Pseudoalteromonas* sp. 1tb4 (FJ952825)	98
2	*Firmicutes*	*Bacillales*	Uncultured *Bacillus* sp. clone b44 (GQ452904)	96
3	*Proteobacteria*	*γ-Proteobacteria*	*Pseudoalteromonas* sp. 1tb4 (FJ952825)	97
5	*Proteobacteria*	*γ-Proteobacteria*	*Photobacterium* sp. r33 (AB470939)	96
6	*Firmicutes*	*Bacillales*	*Bacillus* sp. SMB9 (DQ868675)	95
8	*Bacteroidetes*	*Sphingobacteria*	*Flexibacteraceae* bacterium DG1232 (DQ486479)	93
10	*Proteobacteria*	*γ-Proteobacteria*	*Ferrimonas marina* strain A4D-4 (AB193751)	97
11	*Proteobacteria*	*γ-Proteobacteria*	Gamma proteobacterium D319 (FJ440987)	98

Table 2.- Antifouling activity (EC50 µg/ml) test of crude extract from marine sponges and algae based on the inhibition of the marine bacterium *Flexibacteraceae* sp.

Marine organism	Species	Aqueous fraction	Dichloromethane fraction
Sponges	*Mycale armata*	542	194
	Subrites zeteki	561	205
	Dysidea sp.	604	261
	Gelliodes fibrosa	592	212
	Tedania sp.	n.d	n.d
Algae	*Acanthophora spicifera*	861	302
	Gracilaria salicornia,	721	342
	Gracilaria coronopifolia	742	298
	Hypnea musciformis	n.d	n.d
	Laurencia nidifica	n.d	n.d

*n.d stand for 'not determined'

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