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IDENTIFICATION OF PREPONDERANT MARINE BACTERIA AND THEIR BIOFOULING CHARACTERISTICS ON ADSORBENTS OF DIFFERENT SIZES AND SHAPES IN SEAWATER

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Key words: marine biofouling, bacterial colonization, Confocal Laser Scanning Microscopy (CLSM), Scanning electron microscopy (SEM), seawater.

ABSTRACT

We aimed to investigate biofouling characteristics on the surfaces of adsorbents and reservoirs used for lithium recovery from seawater. Three types of adsorbents (type 1, 2 μm sphere-shaped; type 2, 2 mm circular; and type 3, 2 mm rod-shaped) and a reservoir (polyurethane sponge) were immersed in seawater for 30 days. Biofouling on the surface of adsorbents and the reservoir was initiated by *Vibrio*, *Alteromonas*, and *Pseudoalteromonas*, suggesting that primary colonization of *Gammaproteobacteria* was an important feature of the biofouling on the surface. We observed pore size-dependent bacterial composition on the adsorbents; only *Alteromonas* was found on type 1 adsorbent, while *Vibrio* was mainly identified on types 2 and 3 on day 15; these were succeeded by *Alteromonas* over time. The bacterial composition of adsorbents wrapped in a reservoir was similar to that of the reservoir over time. CLSM and SEM images showed that bacterial distribution on surfaces strongly depended on the shape of the adsorbent.

I. INTRODUCTION

Marine biofouling refers to bacterial adhesion to underwater structures, including ship hulls, leading to surface roughness that increases frictional resistance to flow and the maintenance cost of microbial removal (Yebra et al., 2004; Marhaeni et al., 2011; Schultz et al., 2011). Since such biofouling is a serious problem for the operation and management of marine facilities and industries (Park et al., 2012), its characteristics should be considered. Biofouling is generally separated into four main stages: (1) formation of primary film caused by the adsorption of organic or inorganic macromolecules immediately after immersion, (2) initial colonization that occurs through adherence of bacterial cells on the surface, (3) secondary colonization where unicellular eukaryotic attachment to the substrate is observed, and (4) tertiary colonization, which defines the macrofouling stage where a more complex community with multicellular species on the surface is observed (Yebra et al., 2004; Rana and Matsuura, 2010). In the biofouling formation process, the first colonization when the initial bacterial attachment occurs, is important because it can either accelerate or inhibit the succession to the next stage of fouling (Bowman, 2007).

Numerous studies have been conducted to investigate the bacterial characteristics of biofouling on marine structures or membranes. Lee and Kang (2015) examined the amount of biofouling on porous ceramics, which were used to eliminate red tide organisms for 3 months. Further, morphological and biochemical characterization of biofouling bacteria on ship hulls were performed to control the bacteria in the marine environment (Dhanasekaran et al., 2009). Ultrastructural, chemical, and microbiological analyses of the bacterial population on reverse osmosis (RO) membranes for seawater desalination were also performed to study the effect of water quality on membrane fouling (Khan et al., 2014). This study showed that *Betaproteobacteria* was the major colonizing species of wastewater (WW)-fouled RO membrane, and *Alpha- and Gammaproteobacteria* were mainly found on seawater (SW)-fouled RO membranes. Several physicochemical and biological factors affecting biofouling growth were also investigated. Geographical location, depth, temperature, season, conductivity, salinity, hydrodynamic condition, and organic matter in seawater, as well as competition between species, can play an important role in the biofouling process (Babin et al., 2008). The characteristics of biofouling are strongly dependent on substrate properties such as surface roughness (Scheuerman et al., 1998), surface charge (Kerr et al., 1998), and hydrophobicity. Therefore, the bacterial characteristics that cause fouling should be investigated in a sub-

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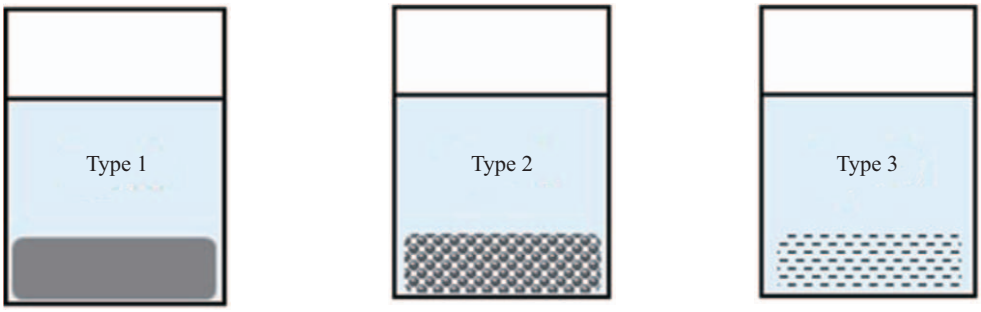


(a) type 1 adsorbent (2-μm sphere-shaped) (b) type 2 (2-mm circular) (c) type 3 (2-mm rod-shaped)

(d) polyurethane sponge that is used as reservoir (e) type 2 adsorbent wrapped in reservoir

Fig. 1. Schematic diagram of Lab-scale biofouling test.

strate dependent manner.

This study aimed to identify the preponderant bacterial species of biofouling on adsorbents immersed in seawater. The adsorbent composed of lithium manganese oxide was developed by the Korea Institute of Geoscience and Mineral Resources (KIGAM) to recover lithium from seawater (Chung et al., 2011; Kim et al., 2013). A pilot plant for lithium recovery using adsorbents was constructed near the Okgye Harbor, Gangneung, Gangwon-do, Republic of Korea. The adsorbents encased in reservoirs of polyurethane sponge were exposed to seawater and prominent biofouling was observed on the adsorbent surface and on their reservoirs, while lithium was recovered from seawater in the pilot plant. Here, we conducted lab-scale biofouling tests to investigate the characteristics of biofouling on the adsorbents and to identify the bacterial species on the adsorbent and reservoir, according to the former's sizes and shapes over time. Three types of adsorbents (i.e., type 1, 2-μm sphere-shaped; type 2, 2-mm circular; and type 3, 2-mm rod-shaped) were immersed in seawater for 30 days. The polyurethane sponge that is used as a reservoir was also exposed to seawater. Microscopy image analysis, including confocal laser scanning microscopy (CLSM) and scanning electron microscopy (SEM), was performed to visualize the bacterial characteristics of biofouling on the adsorbent and reservoir surfaces at the ultrastructural level. This study contributes to understanding the biofouling process of adsorbents in seawater, and subsequently will enhance the efficiency of lithium recovery from seawater.

II. MATERIALS AND METHODS

1. Experimental Setup and Conditions

Lab-scale batch tests were conducted to investigate the bacterial characteristics of fouling on the surfaces of the adsorbent and reservoir (Fig. 1). A total of five test conditions were set up induce biofouling in a 2-L beaker containing seawater sampled from the Okgye Harbor. Three types of lithium adsorbents, 2 μm sphere-shaped pores (type 1); 2 mm circular shaped pores (type 2); and 2 mm rod-shaped pores (type 3), and one commercially available polyurethane sponge used as reservoir were prepared. Polyurethane sponge is an irregular reticular sheet with a pore size of 0.5-2 mm. Each of the three adsorbents and one reservoir was immersed in 2 L seawater; the type 2 adsorbent wrapped in reservoir was additionally set up to examine the effect of reservoir on biofouling on the adsorbent. These five sets of experiments were maintained under controlled temperature (20°C) with aeration for 30 days. Biofilm samples were collected from the surfaces on the days 15 and 30.

2. Identification of Bacterial Species: DNA Isolation, Polymerase Chain Reaction (PCR), and Sequencing

The obtained biofilms were vortex-mixed, and the bacteria-containing samples were spread on marine agar plates and incubated at 30°C for 120 h. Each bacterial colony was selected and cultured at 15-18°C. Total genomic DNA was extracted from the isolates, using a DNA extraction kit (Qiagen, Netherlands), and purified using a genomic DNA purification kit (GeneAll Biotechnology, Korea). The extracted DNA was amplified by PCR using the universal primers, 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′). The PCR mixture

Table 1. The relative abundance of bacterial composition on the surface of three adsorbents at genus level.

Relative abundance (%)	Type 1 adsorbent		Type 2 adsorbent		Type 3 adsorbent	
Time	15 day	30 day	15 day	30 day	15 day	30 day
Alteromonas	76.7	92.3		100		100
Pseudomonas			3.33			
Pseudoalteromonas			3.33		10.0	
Vibrio			93.3		90.0	
Others	23.3	7.70				

consisted of 5 μL dNTPs, 5 μL 10× Taq buffer, 1 μL (100 pM) of each primer, 0.5 unit of Taq polymerase (Invitrogen™, Life Technologies, USA), and 3 μL template DNA (20 ng) for a total 50 μL reaction volume. A Bio-Rad thermal cycler (Bio-Rad Laboratories, Hercules, CA, USA) was used for PCR with the following cycling conditions: initial denaturation at 95°C for 5 min; 30 cycles of 95°C for 30 s, primer annealing at 55°C for 30 s, and extension at 72°C for 30 s; and a final elongation at 72°C for 1.5 min. The PCR products were ligated into pGEM®-T Easy (Promega, Madison, WI) for cloning. Plasmid DNA was extracted using plasmid DNA isolation kit (GeneAll Biotechnology) and the recombinant clones were sequenced. All sequences were compared to those available in the National Center for Biotechnology Information (NCBI, USA) database using BLAST.

3. Surface Characterization of Biofouling on the Adsorbents and Reservoir

Biofilms on the surfaces of the adsorbents and reservoir were observed using a CLSM system (LSM510 META NLO, Carl Zeiss Jena GmbH, Germany) and an SEM (S-3500N; Japan) at the KBSI Chuncheon Center (Chuncheon-si, Gangwon-do, Republic of Korea). The biofilm samples were treated with DRAQ5 solution (Biostatus, UK) to stain the nuclei of live cells. Fluorescence signals from the stained DNA of marine bacteria allowed the assessment of the population of colonized microorganisms and the degree of defacement of the microorganisms. Fluorescence-stained sections were examined under an epifluorescence microscope equipped with a laser confocal system, comprising a He/Ne laser with 633 nm excitation, C-Apochromat 40°/1.2 W objective lens, and BP650-710 emission filters. Images were processed with LaserSharp computer software (Bio-Rad Laboratories). The same area of the biofilm was analyzed using an SEM. Samples were prepared by drying and coating them with gold and palladium. Analysis was performed by 2-kV, extra high tension.

III. RESULTS

1. Effects of Adsorbent Pore Size and Shape on the Biofouling Bacterial Species

Fig. 1 depicts the five test conditions for marine biofilm formation on the adsorbents and reservoir developed for this study. The bacterial species obtained from biofilms on the three adsorbents (types 1, 2, and 3) were identified at the genus level (Table 1). Significant differences in bacterial composition among the three adsorbents were observed over time. *Alteromonas* was the predominant group on day 15; their proportion increased from 76.7% to 92.3% over time on the type 1 adsorbent (2-μm sphere-shaped). More than 90.0% of *Vibrio* was detected on day 15 on the type 2 and 3 adsorbents (2-mm circular and 2-mm rod-shaped, respectively), but *Alteromonas* became the predominant group over time (100% on day 30) in both these adsorbents. A small percentage of *Pseudomonas* and *Psuedoalteromonas* were also detected on the type 2 and 3 adsorbents on day 15; however, they disappeared over time (day 30). These results demonstrate that the bacterial species are similar on the type 2 and 3 adsorbents, which have the same size, whereas they are different on the 2 μm type 1 adsorbent, indicating that size of adsorbents influences biofouling more than their shape at early time points.

2. Temporal Changes in Bacterial Species on Fouled Adsorbents and Reservoir: The Role of Reservoir on Biofouling on Adsorbents

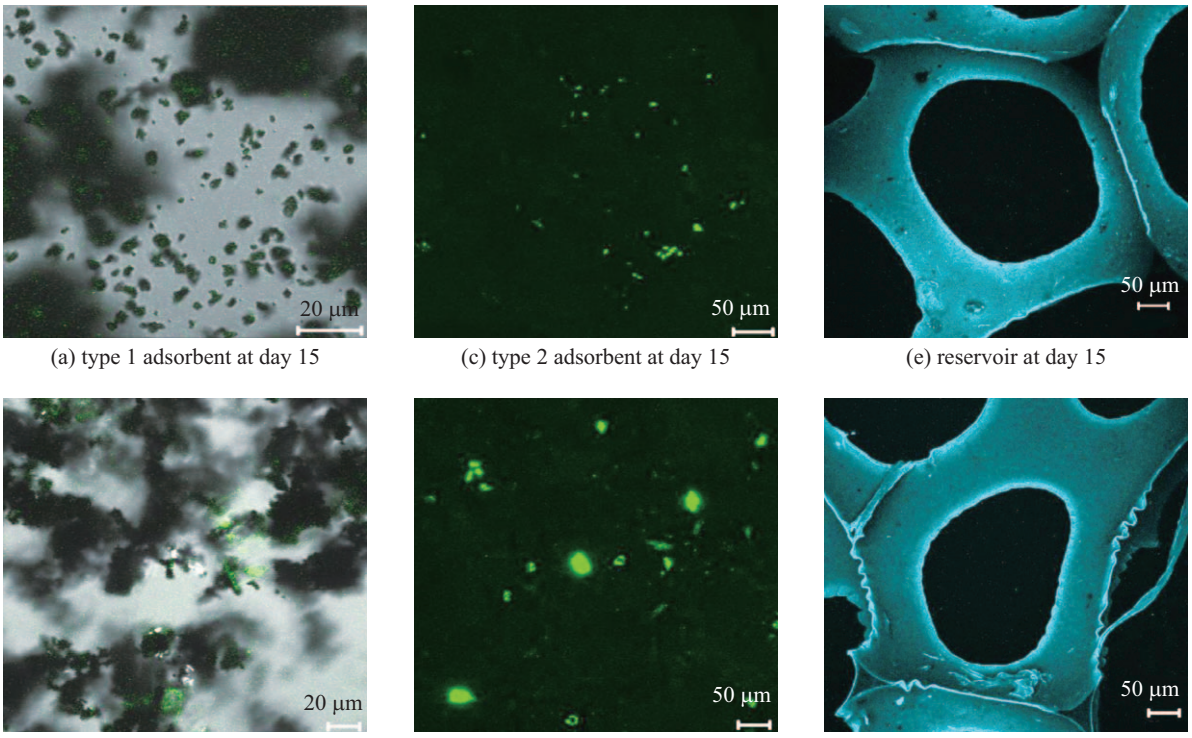
The bacterial species on the surface of the polyurethane sponge reservoir and type 2 adsorbent (2-mm circular) wrapped in the reservoir were identified at the genus level to investigate the effect of reservoirs on biofouling (Table 2). The type 2 adsorbent was selected as a representative adsorbent for lithium recovery for convenience, given that the pore size of the type 1 adsorbent (2-μm sphere-shaped) is too small to be directly exposed to seawater. In the case of the polyurethane sponge, *Vibrio* showed high relative abundance (93.3%), followed by *Psuedoalteromonas* (20.0%), *Marinomonas* (3.33%), and *Pseudomonas* (1.70%) on day 15. Over time, *Alteromonas* was detected (20.0%), and *Psuedoalteromonas* increased to 43.3% whereas *Vibrio* decreased to 36.7%. When the type 2 adsorbent (2-mm circular) wrapped in polyurethane sponge was exposed to seawater, the bacterial composition was similar to that of the type 2 adsorbent alone during the initial period to day 15, with 93.3% *Vibrio* and 6.70% *Pseudomonas*. Notably, this changed to being similar to the bacterial composition of the reservoir over time, with 70.0% *Psuedoalteromonas*, 20.0% Photobacterium, 6.70% *Alteromonas*, and 3.30% *Vibrio*. This result reveals that the biofouling characteristics depend on the outer material (e.g., reservoir) when exposing the adsorbent to seawater wrapped in a reservoir.

3. Visualization of Biofouling on Adsorbent and Reservoir: CLSM and SEM Analysis

The surfaces of type 1 and 2 adsorbents (2-μm sphere-shaped

Table 2. The comparison of the relative abundance among type 2 adsorbent, reservoir, and type 2 adsorbent wrapped in reservoir at genus level.

Relative abundance (%)	Type 2 adsorbent		Reservoir		Type 2 adsorbent wrapped in reservoir	
Time	15 day	30 day	15 day	30 day	15 day	30 day
Alteromonas		100		20.0		6.70
Pseudoalteromonas	3.33		20.0	43.3		70.0
Marinomonas			3.30			
Pseudomonas	3.33		1.70		6.70	
Photobacterium						20.0
Vibrio	93.3		75.0	36.7	93.3	3.30

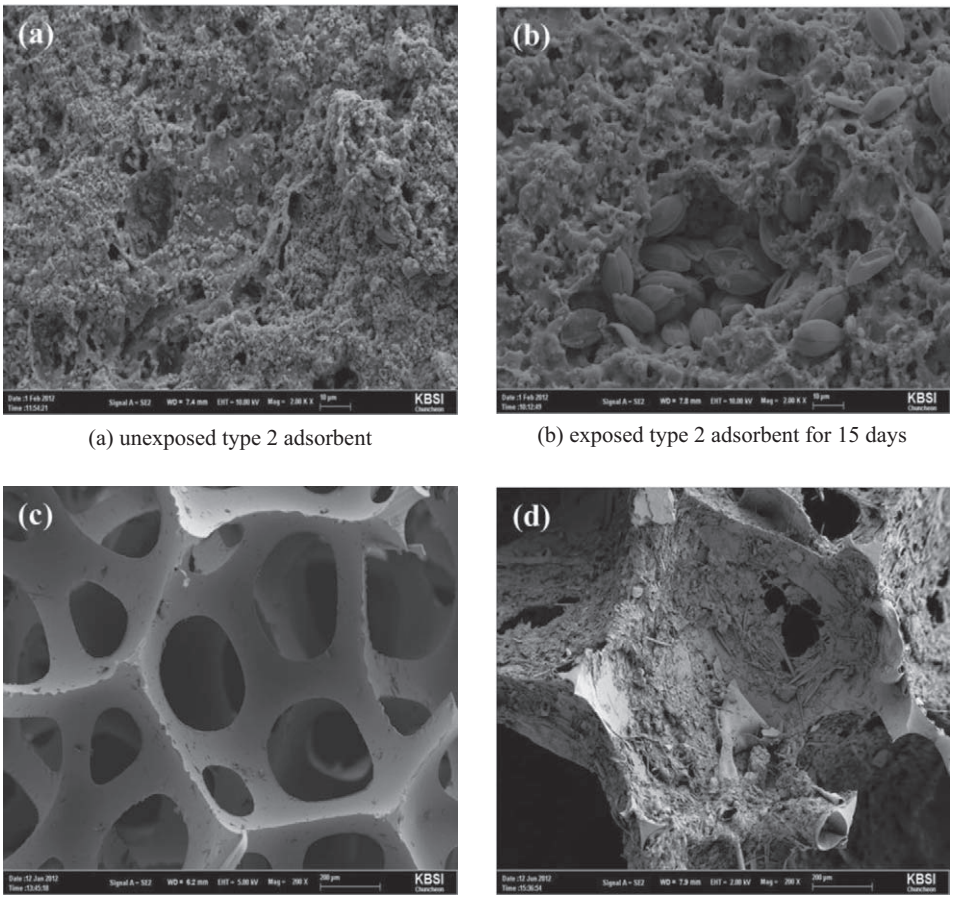


(a) type 1 adsorbent at day 15 (c) type 2 adsorbent at day 15 (e) reservoir at day 15

(b) type 1 adsorbent at day 30 (d) type 2 adsorbent at day 30 (f) reservoir at day 30

Fig. 2. CLSM images of biofouling on the surfaces of adsorbents and reservoir.

and 2-mm circular) after exposure to seawater were investigated by CLSM. The type 3 adsorbent (2-mm rod-shaped) was excluded from image analysis because it showed bacterial composition similar to that of the type 2 adsorbent. Fig. 2 presents the relative quantity of the bacterial species on the surfaces of the adsorbents and reservoir over time. The stronger green fluorescence indicates more populated bacterial species. Small, scattered signal patterns were observed on the surface of the type 1 adsorbent (Fig. 2(a)) during the initial period to day 15. This scattered signal represents small bacterial communities. After 30 days, the number of bacterial species increased; however, they floated between the particles rather than attaching to the them (Fig. 2(b)). Random, irregular, circular clusters were observed on the surface of the type 2 adsorbent (Figs. 2(c) and (d)). The size of the clusters increased from approximately 1-25 μm over time, probably due to bacterial multiplication. Since the polyurethane sponge used as reservoir produces a bright fluorescent signal, no observable increase in signal was detectable over time (Figs. 2(e) and (f)). SEM analysis was conducted with the type 2 adsorbent and polyurethane sponge. Fig. 3 shows the surface of unexposed type 2 adsorbent (a), exposed type 2 adsorbent for 15 days (b), unexposed polyurethane sponge (c), and exposed polyurethane sponge for 30 days (d). The SEM images indicate that the surface is completely clogged compared to the surface of unexposed adsorbent and polyurethane sponge over time. Energy dispersive spectrometry (EDS) results suggest that these surfaces might be covered with crystalline sea salt such as Na, Cl, and Mg, as well as bacterial species (data



(a) unexposed type 2 adsorbent (b) exposed type 2 adsorbent for 15 days

(c) unexposed reservoir (d) exposed reservoir for 30 days

Fig. 3. SEM images of biofouling on the surface of adsorbents and reservoir. All images are examined under magnification 1000×.

not shown). The CLSM results indicate that different materials (e.g., shape of type 1 and 2) could affect bacterial distribution on the surface of an adsorbent.

IV. DISCUSSION

Biofouling on the surface of adsorbents and the reservoir for 30 days was led by bacterial colonization of *Vibrio*, *Alteromonas*, and *Pseudoalteromonas*, which are known as *Gamma-proteobacteria* (Tables 1 and 2). Although A*lpha-proteobacteria*, *Gamma-proteobacteria*, and *Cyanobacteria* are represented in coastal and open seawater (Brown et al., 2009; Gilbert et al., 2012; Du et al., 2013), *γ-proteobacteria* were abundant members of the attached communities on the adsorbents in our study. Bacterial community composition of biofilms was markedly different from that of the surrounding seawater, as reported in previous studies (Jone et al., 2007; Lee et al., 2008; Lee et al., 2016). Despite the relatively low abundance of *Gamma-proteobacteria* (approximately 12.0%) in Antarctic seawater, *Gamma-proteobacteria* on the surface was about 2.6 to 6.3 times higher than in seawater during the early stage of biofilm development (up to day 7) (Lee et al., 2016). These results suggest that the primary colonization by *Gamma-proteobacteria* was an important feature of the bacterial fouling on the surface of adsorbents and the reservoir, and these acted as pioneer bacteria that attach to the surface during the early colonization step (Lee et al., 2008; Huang et al., 2011).

This study shows that the bacterial species present on fouled surfaces strongly depends on the physical characteristics of the exposed material and the exposure time. When the three adsorbents (i.e., type 1, 2-μm sphere-shaped; type 2, 2-mm circular; type 3, 2-mm rod-shaped) were compared in our study, their sizes were found to affect the composition of the bacterial species more than their shapes. The type 2 and 3 adsorbents have the same size (2 mm), and showed similar bacterial composition on their surfaces, which differed from that on the type 1 adsorbent (2 μm). *Vibrio* and *Alteromonas* were the dominant bacteria on the surfaces of the type 2 and 3 adsorbents on days 15 and 30, respectively, whereas only *Alteromonas* were detected throughout the entire experimental period on the surface of the type 1 adsorbent. This difference in abundance patterns with size might depend on the biochemical characteristics of the bacterial species responsible for biofouling. In general, *Alteromonas*, *Pseudoalteromonas*, and *Vibrio* are dominant bacterial groups frequently found in marine environments (Satheesh et al., 2016). Since *Vibrio* are especially known to have extremely fast growth

rates (Zettler et al., 2013), they were abundantly detected at initial period (up to day 15) on the surfaces of the type 2 and 3 adsorbents. Thereafter, the dominant bacterial group changed to *Alteromonas*, which are capable of adhering to surfaces via biofilms, and were commonly found in the stagnant state on day 30 (Teasdale et al., 2009; Badawy et al., 2015). Members of the genera *Alteromonas* and *Pseudoalteromonas* produce exopolysaccharides (EPS) under certain conditions, and these EPS may be involved in their ability to colonize surfaces (Bowman, 2007; Martins et al., 2014). *Alteromonas* species has been described as an important genus in particle (or aggregate)-associated bacteria mainly found on surface colonists in coastal marine systems (Dang and Lovell, 2000). The interesting result that only *Alteromonas* were found on the type 1 adsorbent instead of *Vibrio* during the entire experimental period (for 30 days) can be attributed to the effect of the pore size (2-μm) of the adsorbent. Since 2-μm adsorbents (type 1) have relatively higher surface area than 2-mm adsorbents (types 2 and 3), the former can retain seawater for a longer time compared to other adsorbents; subsequently, they can reach the stagnant stage more rapidly.

One explanation for the alteration in the dominant bacterial group from day 15 to day 30 is that biofilm maturation occurs by synergistic or competitive interactions among the existing bacteria and the recruitment of new colonized species (Dang and Lovell, 2000). The process of succession was characterized as the sequence of pioneer-driven colonization of bacteria followed by subsequent colonization by secondary microorganisms (Dang and Lovell, 2000; Jones et al., 2007). In a previous study, succession of bacterial communities was investigated during the early stage of biofilm formation in seawater (Lee et al., 2016). The same author suggested that the contribution of EPS, produced by *Gamma-proteobacteria, Pseudoalteromonas prydzensis*, could influence the bacterial communities during biofilm development (Lee et al., 2016). EPS play an important role in bacterial adhesion onto surfaces by modifying their physicochemical properties such as charge and hydrophilicity (Neu and Marshall, 1990; Wingender et al., 1999). Further studies should be conducted to confirm that successional colonization on the surface of adsorbents could be associated with the characteristics of EPS production with different sizes of adsorbents.

The result obtained from the comparison of the bacterial composition among the three sets (i.e., type 2 adsorbent, polyurethane sponge used as reservoir, and type 2 adsorbent wrapped in reservoir) indicated that the outer material contributes significantly to characteristics of biofouling. While the identified bacteria of these three sets was mainly *Vibrio* during the initial time to 15 days, the bacterial composition of the type 2 adsorbent wrapped in reservoir changed to that of the reservoir over time, but not to that of the type 2 adsorbent. Since polyurethane sponge is permeable to seawater circulation, the relative abundance of bacteria having adherent properties, including *Alteromonas* and *Pseudoalteromonas*, remained at relatively higher ratio than bacteria having floating properties, such as *Vibrio* (Cooksey and Wigglesworth-Cooksey, 1995). The different shape of adsorbents led to the difference in bacterial distribution pattern, as the CLSM images suggested. Once bacterial DNA reacts with a fluorescent dye, the fluorescence intensity is proportional to the bacterial DNA contents (i.e., quantity of bacterial species) (Dheilly et al., 2010). Our CLSM images show that the intensity of green fluorescence emitted from the samples increases over time (Figs. 2(a)-(d)). More importantly, although the same bacterial species (*Alteromonas*) were identified after 30 days, there is a great difference in the distribution pattern of either interspersion type (scatter) or cluster type. Since the polyurethane sponge naturally emits blue-green fluorescence, the bacterial distribution pattern on the reservoir could not be analyzed from the CLSM images. However, most bacteria seemed to be found along the structures of adsorbents and increased over time. SEM images also revealed that the bacteria attached along a structure of the reservoir and penetrated the pore on the surface of the reservoir over time; subsequently clogging the pores (Fig. 3(d)).

Our study revealed that the effective size of adsorbents can affect bacterial composition, which causes biofouling, and the bacterial distribution pattern on the adsorbents and reservoir was determined by material characteristics such as shape. The reservoir-wrapped adsorbent strongly influenced the kind of bacteria that adhere to the adsorbent. This finding will have meaningful contributions towards the accurate evaluation of sitespecific identification of biofouling bacteria and their characteristics and will help determine an antifouling strategy for lithium recovery from seawater.

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DISCLOSURE STATEMENT

The authors confirm that there is no conflict of interest.

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