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# Influence of Elution Characteristics of Steelmaking Slags on Major Bacterial Communities in Biofilms

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**Abstract:** Steelmaking slags are prospective base materials for seaweed beds, resulting from a continuous process of biofouling, starting from biofilm formation and leading to growing algae. While focusing on biofilm formation, we investigated specific features of steelmaking slags when utilized as a base for seaweed beds by comparing the bacterial communities in marine biofilms between steelmaking slags and artificially produced ones. Genomic DNA was extracted from the biofilms collected on days 3 and 7, and partial 16S rRNA libraries were generated and sequenced by second-generation next-generation sequencing. The read sequences were analyzed using QIIME 2™, then heatmaps and non-metric multidimensional scaling based on the Bray–Curtis dissimilarity index in the R program. *Rhodobacteraceae* and *Flavobacteriaceae* were the most dominant family members in all samples on both days 3 and 7. However, *Mariprofundus*, comprising iron-oxidative bacteria, was predominantly detected in the samples of steelmaking slags on day 7. This suggested that the growth of *Mariprofundus* was dependent on Fe(II) ion concentration and that steelmaking slags eluted Fe(II) ions more easily than artificial slags. In contrast, *Sulfurovaceae*, sulfur-oxidizing bacteria, were dominantly present in all samples on day 3, but decreased by day 7, regardless of the sulfur content. It was supposed that engine oil-derived sulfur compounds strongly influenced *Sulfurovaceae* growth, whereas slag-derived sulfur compounds did not. Heatmap analysis indicated that the submersion period significantly influenced the bacterial communities, regardless of the differences in the main slag content ratios. Summarizing these results, the elution characteristics of steelmaking slags have the potential to influence the formation of marine biofilms, and this formation is significantly influenced by environmental conditions.

**Keywords:** steelmaking slag; biofilm; bacterial community; second-generation NGS; 16S; iron; sulfur



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## 1. Introduction

Iron and steel slags are industrial byproducts mainly consisting of blast furnace slag, generated during the production of pig iron in blast furnaces, and steelmaking slag, produced in converter or electric furnaces during the manufacturing of strong steels from pig iron or iron scraps, oxygen, lime, and other supplemental materials. Blast furnace slags (total production: 22 Mt in Japan, 2021) are totally recycled as the raw materials for cement (81%), road (11%), concrete (5%), and others, whereas steelmaking slags (total production: 13 Mt in Japan, 2021) are partially recycled as the raw materials for road (44%) and civil engineering (13%) works, while 19% are reused in steel manufacturing [1]. Some studies have evaluated the use of steelmaking slags in the ceramic and biomedical industries [2].

Steelmaking slags also have the potential to restore coral reefs, regenerate seaweed, and protect marine sands [3].

Regenerating seaweed is linked to the process of biofouling. Biofouling initially involves the creation of biofilms composed of marine bacteria that produce extracellular polymeric substrates on the surface of various materials, which is known as micro-biofouling. The next stage involves macro-biofouling, where larger organisms, such as barnacles, young oysters, and algae, become irreversibly attached and subsequently grow on top of the biofilm-covered materials. In our previous study, we investigated the bacterial biome of biofilms formed on three steelmaking slags that emerged in part of Ise Bay, Japan. We concluded that these biofilms were appropriate for the regeneration of seaweed and could aid in seawater purification [4]. We also observed that the slag components differed among the three steelmaking slags, which may have influenced the development of bacterial consortia. However, the key factors that affect the formation of biofilms during marine biofouling remain poorly understood, and it remains unclear which of the slag components plays a predominant role in this process.

In this study, we aimed to identify the factors and/or components that contribute to the formation of biofilms on steelmaking slags in marine environments. We focused on the differences in total sulfur content of three practical steelmaking slags because, in the previous study, sulfur content had influenced the growth of sulfur-oxidative bacteria, which were found to be more abundant in the biofilms formed on steelmaking slags with higher sulfur content [4]. Based on this perception, we made three artificial steelmaking slags consisting of different sulfur contents (0 wt% or 0.4 wt%) and different basicities (1.0 or 1.3). These six steelmaking slags were submerged in Ise Bay, Japan, to facilitate the formation of marine biofilms, and the bacterial communities within these biofilms were analyzed.

## 2. Materials and Methods

### *2.1. Samples*

Three practical slags (Slag-A, Slag-F, and Slag-5-2) were provided by Prof. Ryo Inoue of Akita University, Akita, Japan. $CaCO_3$ was burned at 950 °C for 12 h in an air atmosphere to generate CaO (lime). To generate FeO, Fe and $Fe_3O_4$ powders were mixed in the same molar ratio, and the mixture was then pressed. The mixture was burned at 1100 °C for 10 h in an air atmosphere, cooled down to 600 °C, and placed in a vacuum to air-cool. Three artificial slags (Mock-1, Mock-2, and Mock-3) were produced in accordance with the procedures shown in Table 1. The main components of the slag samples are summarized in Table 2. Aquarium sand and polyurethane-formed sponges were purchased from the Komeri home improvement store (Niigata, Japan). Polyurethane-formed sponges were cut into rectangular cylinders (15 mm × 15 mm × 30 mm).

**Table 1.** Raw materials and synthetic processes of the three artificial slags.

| | | | Artificial Slag | | |
|---|---|---|---|---|---|
| | | | **Mock-1** | **Mock-2** | **Mock-3** |
| Contents of raw materials | | $SiO_2$ [wt%] | 38.2 | 33.3 | 33.0 |
| | | CaO [wt%] | 38.3 | 43.2 | 43.2 |
| | | $Al_2O_3$ [wt%] | 5 | 5 | 5 |
| | | MgO [wt%] | 5 | 5 | 5 |
| | | FeO [wt%] | 13.5 | 13.5 | 13.4 |
| | | CaS [wt%] | 0 | 0 | 1 |
| Synthetic process | Step 1 | | Mixed | Mixed then pressed | |
| | Step 2: Melting | Crucible | Alumina | Molybdenum | |
| | | Temperature | 1600 °C | | |
| | | Reaction time | 5 h | 1 h | |
| | | Atmosphere | Deoxidated and dehydrated air | | |
| | Step 3: Cooling | 1st step | Slowly cooled down until reaching 20–25 °C | Gradually cooled down to 1350 °C (at −400 °C/h) | |
| | | 2nd step | | Transferred onto a copper plate until reaching 20–25 °C | |

**Table 2.** Proportion of main components of real slags and artificial slags.

| Sample | [wt%] | | | | | | | | Basicity * |
|---|---|---|---|---|---|---|---|---|---|
| | Total S | Total Fe | CaO | $SiO_2$ | MgO | $Al_2O_3$ | MnO | $P_2O_5$ | |
| Slag-A | 0.3 | 4.3 | 55.3 | 18.7 | 1.9 | 3.0 | 5.6 | 4.6 | 2.9 |
| Slag-F | n.d. | 12.2 | 52.2 | 14.1 | 2.8 | 3.0 | 4.3 | 2.9 | 3.7 |
| Slag-5-2 | 0 | 4.7 | 37.6 | 22.6 | 6.5 | 4.2 | 12.0 | 5.4 | 1.7 |
| Mock-1 | 0 | 13.5 | 38.3 | 38.2 | 5.0 | 5.0 | 0 | 0 | 1.0 |
| Mock-2 | 0 | 13.5 | 43.2 | 33.3 | 5.0 | 5.0 | 0 | 0 | 1.3 |
| Mock-3 | 0.4 | 13.4 | 43.2 | 33.0 | 5.0 | 5.0 | 0 | 0 | 1.3 |

* Basicity = $CaO/SiO_2$ (in weight), n.d.: not detected.

### 2.2. Submersion Test in Ise Bay

Each sample was inserted into a polyethylene net (mesh size: 16 $mm^2$, Nogyo-ya, Mie, Japan) and fastened onto a stainless-steel holder. Ten test pieces were prepared in Slag-A, Slag-F, Slag-5-2, and Sponge. Twenty test pieces were prepared in Mock-1, Mock-2, Mock-3, and Sand. The holder was submerged 2 m from the surface at a part of the floating dock of Marina Kawage, Mie, Japan (34°47′53.98″ N, 136° 33′44.54″ E) from 27 August 2018 to 3 September 2018. Half of each sample was collected on 30 August, and the remaining half was collected on 3 September. The weather ranged from sunny and hot days (temperatures of up to 34 °C) from 27 August to 31 August to cloudy days (temperatures around 29 °C) from 1 September to 3 September. All collected samples were stored at −80 °C until DNA extraction.

### 2.3. DNA Extraction and Next-Generation Sequencing

One sample (from Slag-A, Slag-F, Slag-5-2, and Sponge) or two samples (from Mock-1, Mock-2, Mock-3, and Sand) were pooled into a sterile microtube and crushed using a sample crusher (Taitec, Saitama, Japan) for 1 min at 4000 rpm. After centrifuging the crushed samples (1 s at 8000× *g*), the supernatants were transferred to a new tube and centrifuged for 20 min at 15,000 × *g* to recover microbial cells. Bacterial genomic DNA extraction was performed using a DNA purification kit (Promega, Madison, WI, USA) according to the manufacturer's instructions. The concentrations of purified DNA solutions were measured using a fluorescence-based DNA detection system (Thermo Fisher Scientific, Waltham, MA, USA). Partial 16S rRNA subunit sequences were amplified using the PCR conditions reported in our previous study [4]. Next-generation sequencing was performed on the PCR products using MiSeq (Illumina, San Diego, CA, USA) to read partial 16S rRNA subunit sequences. The sequence data were provided as two fastq files. These data were registered in the Sequence Read Archive (SRA) of the National Library of Medicine (NIH), National Center for Biotechnology Information (NCBI). The accession number is PRJNA973798.

### 2.4. Sequencing Data Analysis

The fastq data files were input and processed using QIIME 2™ (version 2021.4) [5] to determine the bacterial biomes in the biofilms formed on the samples. The DADA2 plugin [6] was used for filtering chimeric sequences and trimming the forward primers from the first 59 characters of forward sequence data, as well as the reverse primers from the first 54 characters of reverse sequence data. Denoised datasets were summarized in the featured table, which included representative sequence data obtained through the following process. Representative sequence data were used to identify the bacterial taxonomy by the trained classifier that was developed in the QIIME 2 system based on the data of SILVA 138 SSU Ref. NR 99 [7]. After the identified representative sequences were counted and categorized in each sample, each portion of the taxonomic categories was determined as the proportion per total number of sequences. Subsampled sequence data (11,465 reads in each sample), which removed mitochondria-derived, chloroplast-derived, and eukaryote-derived sequences, were used to analyze heatmaps and non-metric multidimensional scaling (NMDS) based

on the Bray–Curtis dissimilarity index in the R program ver.4.2.0 [8]. In the NMDS of the R program, the operational taxonomic units of the three practical slags (Slag-A, Slag-F, and 5-2) and three artificial slags (Mock-1, Mock-2, and Mock-3) were extracted to calculate the relationship between the compositions of the bacterial communities and the differences in the components of these slags.

## 3. Results

### *3.1. Biofilm Bacterial Composition*

#### 3.1.1. Major Bacterial Families

Chloroplast was removed from the family categories, and minor family categories were integrated into the others. Minor family categories were selected based on the rule that the proportion of the target category was equal to or less than 3% in one of all samples. The number of major family members was 14 and 12 on days 3 and 7 (Figures 1 and 2), respectively. Six family members (*Thiotrichaceae*, *Saprospiraceae*, *Flavobacteriaceae*, *Rhodobacteraceae*, *Woeseiaceae*, and *Halieaceae*) were the same in the day 3 and 7 samples. Except for the Sponge sample, *Rhodobacteraceae* and *Flavobacteriaceae* were the most abundant and second most abundant in the 3- and 7-day samples, respectively; however, their proportion increased during the submersion period. *Alcanivoracaceae1* was ranked as a major family found only in the samples from day 3, and the genus detected was *Alcanivorax*. In contrast, *Cyanobiaceae* (alternative name: *Synechococcaceae*) and *Hyphomonadaceae* were relatively dominant in all of the day 7 samples. *Mariprofundaceae* was only detected in Slag-5-2 (0.5%) and Slag-A (1.8%) on day 3, and in Slag-5-2 (1.5%), Slag-A (0.3%), Slag-F (3.9%), and Sponge (0.1%) on day 7. *Mariprofundus*, as a genus of *Mariprofundaceae*, was detected only in the practical slags.

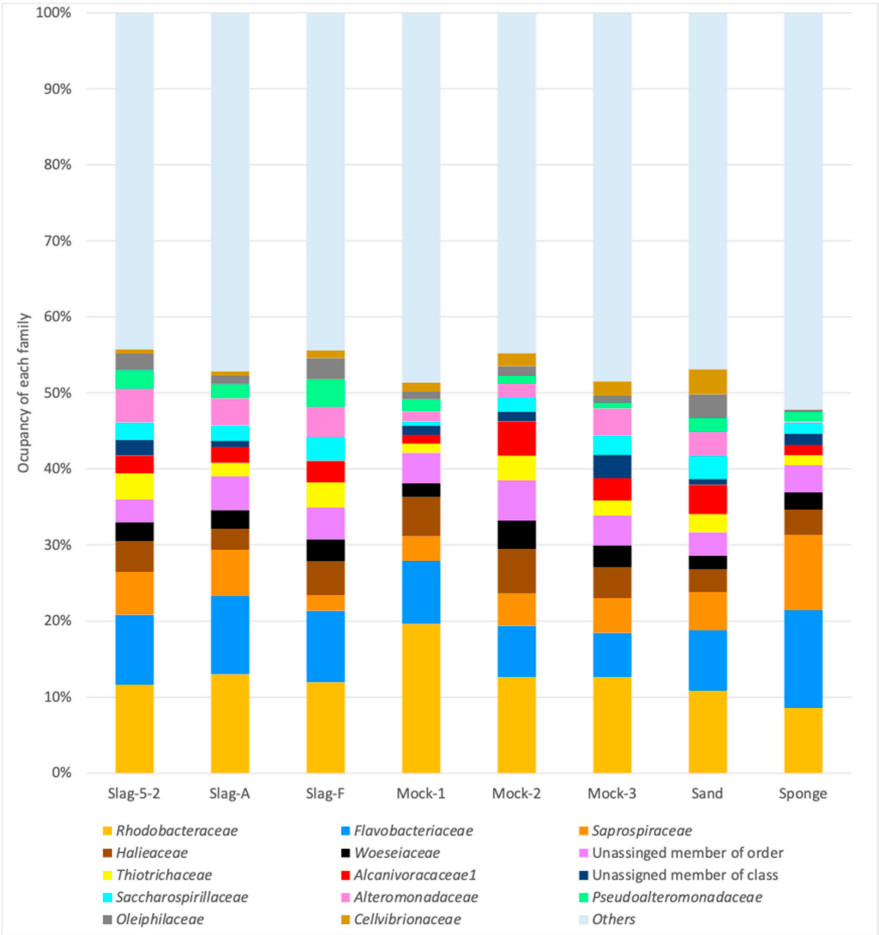


**Figure 1.** Major bacterial communities in marine biofilms formed on submerged samples on day 3.

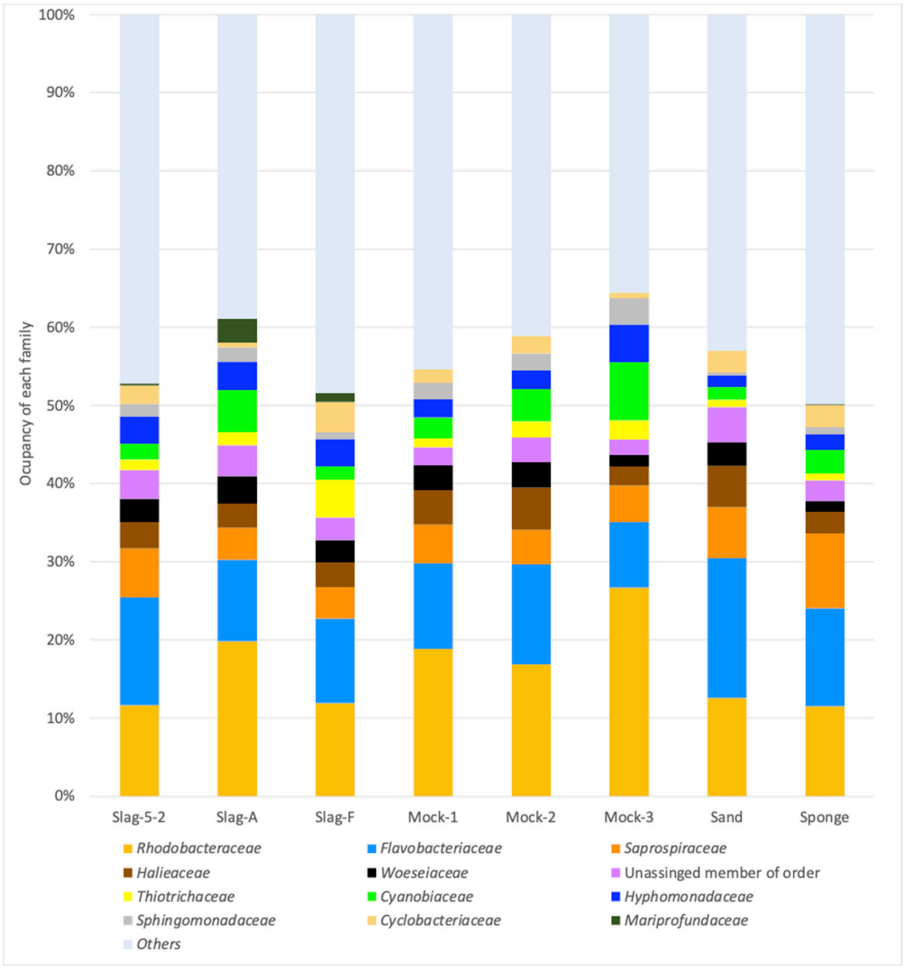


**Figure 2.** Major bacterial communities in marine biofilms formed on submerged samples on day 7.

### 3.1.2. Heatmap Analysis

The top 80% of family members are described in the logarithmic converted heatmap from the R program (Figure 3). The color density indicates the abundance of the target family. *Flavobacteriaceae*, *Rhodobacteraceae*, *Saprospiraceae*, *Woeseiaceae*, and *Halieaceae* were the dominant families among all samples on days 3 and 7. *Saccharospirillaceae*, *Oleiphilacease*, *Vibrionaceae*, *Alteromonadaceae*, *Cellvibrionaceae*, *Rickettsiaceae*, an uncultured group categorized in *Oligoflexales*, *Alcanivoracaceae1*, *Colwelliaceae*, and *Pseudoalleromonadaceae* were dominant only in the day 3 samples. In contrast, *Rhodothermaceae*, *Bdellovibrionaceae*, *Sphingomonadaceae*, *Cyanobiaceae*, *Fracisellaceae*, *Hphomonadeceae*, *Thiotrichaceae*, *Cyclobacteriaceae*, and an unknown family were dominant in the day 7 samples, except in Slag-F. *Sulfurovaceae* and *Desulfocapsaceae* were not detected in Slag-F on day 7.

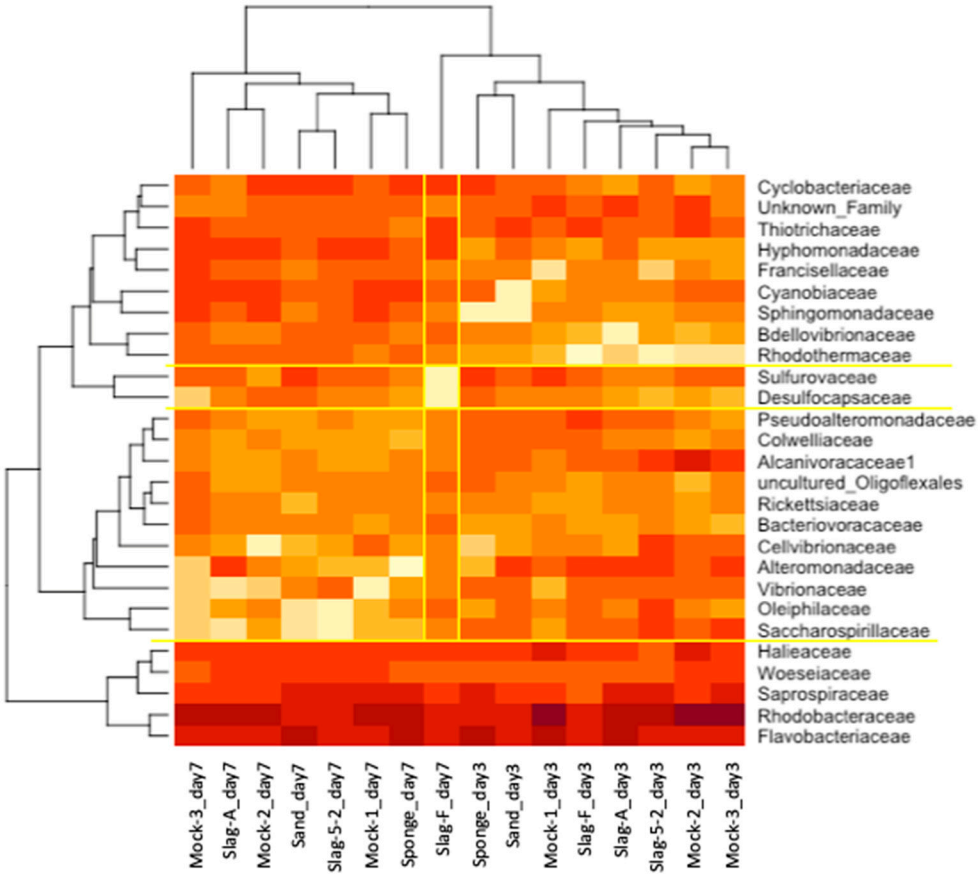


**Figure 3.** Heatmap analysis of the most abundant (80%) bacterial families among all submerged test samples.

### *3.2. Influence of Slag Content on Bacterial Consortiums*

Figure 4 shows the relative distances of the bacterial communities among all samples. On day 3, Mock-1 was located far from the other five slag samples. On day 7, Slag-5-2, Mock-1, and Mock-2 were located close to each other but far from Slag-F, Slag-A, and Mock-3, which were also at a similar distance. The significances (Pr > r) were above 0.2 for $SiO_2$, CaO, $Al_2O_3$, MgO, total Fe, MnO, and basicity, while the significance of the submerging period was 0.005.

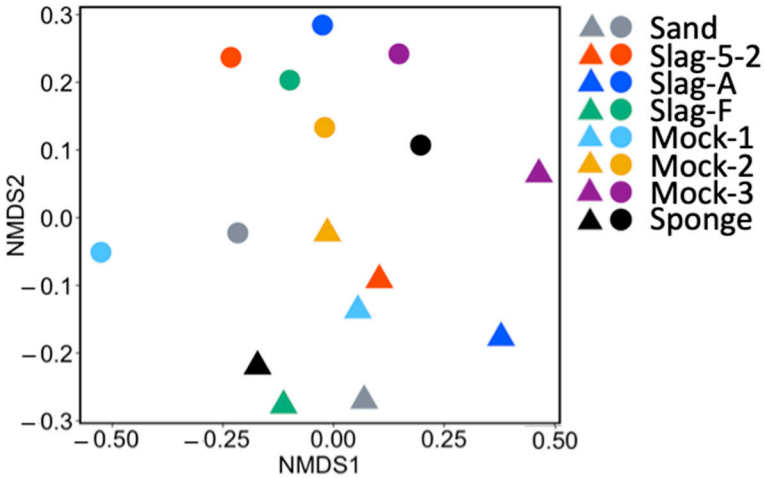


**Figure 4.** Dissimilarity of bacterial communities in the biofilms among all submerged samples. The circles and triangles indicate the samples collected on days 3 and 7, respectively.

## 4. Discussion

Biofilm formation represents the early stage of marine biofouling, and it significantly influences the growth of algae. This is because the extracellular polymeric substrates within the biofilm serve as both nutrient sources and foundations for algae development. This is why we focused on biofilm formation on steelmaking slags in the ocean. According to a report by Li et al. which summarized the chemical composition and mineral constituents of steelmaking slags, CaO (lime) was the richest component, and FeO was also relatively high, depending on the type of slag [9]. In marine environments, the deposition of lime, also known as "white turbidity", often causes concern among fishermen. However, white turbidity was not observed in this study or in our previous one, providing valuable information regarding the suitability of steelmaking slags for application in seaweed beds.

From the taxonomic occupancy data (Figures 1 and 2), we found that *Rhodobacteraceae* and *Flavobacteriaceae* were the predominant families in all samples on days 3 and 7. *Rhodobacteraceae* is a family found abundantly in marine environments [10] and has been reported as a key member in the formation of biofilms during early seawater colonization in the Eastern Mediterranean [11,12]. *Flavobacteriaceae* is also commonly found in marine ecosystems [13] and is capable of degrading polysaccharides and proteins. *Alcanivorax* is a hydrocarbon-degrading bacterium involved in oil degradation that usually exists in low numbers in non-polluted seawater; however, it quickly becomes a dominant population in oil-polluted waters [14,15]. *Synechococcaceae* is categorized within the phylum Cyanobacteria, which has the ability to photosynthesize in water environments [16]. In this study, *Alcanivorax* was dominant in samples collected on day 3 but negligible in those collected on day 7. We hypothesized that the process of biofilm formation was as follows: *Rhodobacteraceae* was mainly related to the formation of primary biofilms. Then, *Flavobacteriaceae* and other dominant bacteria gathered and grew on *Rhodobacteraceae*, providing carbon and amino acids as nutrient sources for biofilm production. Moreover, the submerged location was assumed to be rich in oil and oil-derived hydrocarbons because of the presence of ships in the area during the test period; therefore, the genus *Alcanivorax* could dominate (day 3). However, the internally formed biofilms would provide a poor environment for oil and oil-derived hydrocarbons, resulting in *Alcanivorax* diminishing on day 7.

The genus *Mariprofundus* comprises aerobic, neutrophilic, chemolithoautotrophic, and marine iron-oxidizing bacteria [17–19] and is related to microbiologically influenced corrosion [20]. In this study, *Mariprofundus* was predominantly detected in the three practical steelmaking slags (Slag-5-2, Slag-A, and Slag-F) on day 7. This indicated that the three practical steelmaking slags produced a large amount of Fe(II) ions during the submersion period, resulting in *Mariprofundus* gradually growing dominant and causing surface corrosion of these slags. In contrast, although the three artificial slags (Mock-1, Mock-2, and Mock-3) contained 13%–14% of the total iron content, *Mariprofundus* was not detected in the biofilms of these slags. It was supposed that the difference in the growth of *Mariprofundus* between the practical steelmaking slags and the artificial ones could be attributed to the difference in the oxidative state of the iron compounds among these slags. The practical slags would have contained readily oxidized iron compounds, such as ferric oxides, whereas the artificial slags would have contained robust oxidized iron compounds, such as ferrous oxides. Futatsuka et al. reported that elution behaviors of elements in steelmaking slags are strongly dependent on the phase under artificial seawater conditions [21]; thus, this study suggests that specific bacteria, such as iron-oxidative bacteria, strongly influence the elution characteristics of the target elements in steelmaking slags, more so than the impact of element occupancy.

In our previous study, biofilms formed on three practical steelmaking slags showed potential for the bioremediation of sulfur-rich seawater [4]. This was because the biofilms were rich in *Helicobacteraceae*, which is involved in marine sulfide oxidation [22], and *Desulfobulbaceae*, which is the main sulfate-reducing bacteria in coastal environments [23]. However, in this study, neither family was detected in the biofilms of the samples on day 3 or day 7. On the other hand, *Sulfurovaceae*, another sulfur-oxidizing family, was present in

all samples on day 3 at around 2%, regardless of sulfur content, decreasing to around 1% on day 7 (0% in Slag-F). The only genus detected in the *Sulfurovaceae* family was *Sulfurovum*, a typical anaerobic sulfur-oxidizing bacterium [24]. Thus, considering the lack of *Sulfurovum* during the submersion period and the abundance of *Alcanivorax* on day 3, we postulated that these bacteria utilized oil-derived sulfur compounds as opposed to slag-derived ones. Furthermore, contaminated oil carried sulfur-oxidating bacteria, such as *Alcanivorax*.

In the current study, polyurethane-formed sponge (Sponge) and aquarium sand (Sand) were also submerged in Ise Bay, and their biofilm bacterial communities were analyzed. When the dissimilarities of major bacterial family members in the Sponge and Sand biofilms were compared with those of the steelmaking slags (Slag-A, Slag-F, and Slag-5-2) and artificial slags (Mock-1, Mock-2, and Mock-3), there were no differences among them (Figure 4). According to Chattopadhyay's report, artificial plastics serve as carbon sources for microbes, including bacteria and fungi, in marine environments. Indeed, several bacteria have been shown to deteriorate some plastics. In the review report, some *Pseudomonas* strains degraded polyurethanes [25]. In this study, *Pseudomonas* was only detected in Sand and Sponge on day 3 (Table 3), suggesting that *Pseudomonas* had been eliminated from the biofilm of the Sponge during the submerging period and did not degrade the Sponge. Liang et al. researched the bacterial communities of biofilms formed on polyurethane-, epoxy resin-, and polydimethylsiloxane-coated glass that had been submerged in Zhoushan, China, for 28 days. Their results showed that the epoxy resin-coated glass had a more diverse bacterial community in its biofilm than the polyurethane- and polydimethylsiloxane-coated glass; however, the biomass of each coating was smaller than that of the non-coated glass [26]. Based on these reports, it is supposed that the biodegradation of artificial polymers progresses very slowly during the maturation of marine biofilms (around one month). Additionally, marine biofilm-forming bacteria exhibit a preference for the surface of glass over artificial polymers, including polyurethanes. Furthermore, the amount of biofilms formed does not correlate with the trend in biodegradation.

**Table 3.** Abundance ratio of *Pseudomonas* in each biofilm sample on days 3 and 7.

| Sample | Day 3 [%] | Day 7 [%] |
|---|---|---|
| Slag-5-2 | 0 | 0 |
| Slag-A | 0 | 0 |
| Slag-F | 0 | 0 |
| Mock-1 | 0 | 0 |
| Mock-2 | 0 | 0 |
| Mock-3 | 0 | 0 |
| Sand | 0.1 | 0 |
| Sponge | 0.1 | 0 |

Generally, steelmaking slags are composed of lime (calcium oxide), silica (silicon oxide), ferric oxide, alumina (aluminum oxide), manganese oxide, and magnesium oxide. Sulfur and phosphorus oxides are known major components of steelmaking slags. These components vary in content, which depends on the production process and resources of steelmaking [1]. In this study, three practical steelmaking slags and three artificially produced slags were used in marine submersion tests, and the bacterial composition of the biofilms formed in these slags was investigated. Statistical analysis based on amplicon sequence variants indicated that the main components of slags had little effect on the formation of biofilms on steelmaking slags in the ocean within a week. In contrast, a comparison of major taxa plot analysis indicated that the Fe(II)-elution characteristic of slags affected the growth of iron-oxidative bacteria. Over a longer period, steelmaking slags can actually promote the growth of iron-oxidative and sulfur-oxidative bacteria [27]. The fact that the growth of these bacteria was significantly affected by environmental conditions such as weather, location, temperature, and seawater also indicated that measures need to be taken to control these factors to prevent or mitigate damage to underwater structures.

Tsukidate et al. reported that artificial biofilms formed by isolated marine *Sulftobacter* sp. and *Pseudomonas* sp. facilitated the continuous elution of Fe(II) ions from pH-controlled iron and steel slags [28]. Thus, it is supposed that the formation of biofilms on steelmaking slags can drive the release of Fe(II) ions from the slags, regardless of the differences in the main components, which can promote the growth of seaweed beds. Considering the prospect of utilizing steelmaking slags as a base for seaweed beds, steelmaking slags would serve as a valuable source of essential nutrients that enrich biofilm formation, a crucial stage of biofouling in marine environments.

In both the current and previous studies, we employed the partial 16SrRNA DNA sequence library to analyze the bacterial communities of each biofilm formed on the sample. This method carries the risk that the amplification efficiency will differ among the target sequences from several organisms. Hence, the abundance of the bacterial community is expected to change under certain conditions such as PCR primers and PCR reagents. A better method to realize the specific bacterial community will be shotgun metagenomic sequencing. We should consider this risk and progress future work in order to succeed in applying steelmaking slags in the basement of seaweed beds.

## 5. Conclusions

To identify some advantages of practical steelmaking slags as the base for seaweed beds in a marine environment, we focused on the early stage of marine biofouling, the formation of biofilms, and compared the major bacterial communities of biofilms formed among three practical steelmaking slags and three artificial slags by analyzing the partial 16SrRNA-DNA amplicon sequence library. *Mariprofundus*, a marine iron-oxidative bacterium, was predominantly detected only in the biofilms of three practical steelmaking slags on day 7, which suggests that practical steelmaking slags can elute iron ions to seawater more easily than three artificial slags. Considering that iron ions are in short supply in marine environments and that iron is an essential element for marine alga [29], practical steelmaking slags are appropriate candidates for use as the basement of seaweed beds.

**Author Contributions:** Conceptualization, A.O. and N.H.; methodology, A.O., Y.M. and R.T.; validation, A.O., Y.M. and R.T.; investigation, A.O., Y.M. and R.T.; resources, M.S.; data curation, A.O., T.O., R.O. and R.T.; writing—original draft preparation, A.O.; writing—review and editing, Y.M. and R.T.; visualization, A.O. and Y.M.; project administration, N.H.; funding acquisition, N.H. All authors have read and agreed to the published version of the manuscript.

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**Data Availability Statement:** The read metagenomic data have been registered in the Sequence Read Archive (SRA) of the National Library of Medicine (NIH), National Center for Biotechnology Information (NCBI). The accession number is PRJNA973798.

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