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Microbial Detoxification of Sediments Underpins Persistence of *Zostera marina* Meadows

Yuki Nakashima [1,2], Takumi Sonobe [2], Masashi Hanada [3], Goushi Kitano [4], Yoshimitsu Sonoyama [4], Katsumi Iwai [5], Takashi Kimura [5] and Masataka Kusube [6,*]

[1] Graduate School of Frontier Biosciences, Osaka University, 2-1 Yamadaoka, Suita, Osaka, Japan; u021527c@ecs.osaka-u.ac.jp
[2] Undergraduate Student of Advanced Engineering Faculty, National Institute of Technology, Wakayama College, Gobo, Wakayama, Japan; 2021c13@wakayama.kosen-ac.jp
[3] Promotion of Technical Support, National Institute of Technology Wakayama College, Gobo, Wakayama, Japan; masashi@wakayama-nct.ac.jp
[4] Agri-Light Lab. Inc., Minamata, Kumamoto, Japan; sonoyama@agri-light-lab.co
[5] Study Team for Creation of Waterfront, Yokohama, Kanagawa, Japan; umubeken@nifty.com
[6] Department of Applied Chemistry and Biochemistry, National Institute of Technology Wakayama College, Gobo, Wakayama, Japan; kusube@wakayama.kosen-ac.jp
* Correspondence: kusube@wakayama-nct.ac.jp

Abstract: Eelgrass meadows have attracted a lot of attention not only for their ability to maintain marine ecosystems as feeding grounds for marine organisms, but also for their potential to store atmospheric and dissolved CO_2 as blue carbon. This study comprehensively evaluated the bacterial and chemical composition data obtained from eelgrass sediments of different scales along the coast of Japan to investigate the sediment effect on the acclimatization of eelgrass. Regardless eelgrass habitat, approximately 1% *Anaerolineales*, *Babeliales*, *Cytophagales*, and *Phycisphaerales* were present in the bottom sediment. Sulfate-reducing bacteria (SRB) were present at 3.69 % in eelgrass sediment compared to 1.70 % in bare sediment. Sulfur-oxidizing bacteria (SOB) were present at 2.81% and 1.10% in the eelgrass and bare sediment, respectively. Bacterial composition analysis and linear discriminant analysis revealed that sulfur-oxidizing bacteria detoxified H_2S in the eelgrass meadows and that the larger-scale eelgrass meadows had a higher diversity of sulfur-oxidizing bacteria. Our result indicated that one of the following systems detoxifies the H_2S in eelgrass meadows microbial oxidation by sulfur-oxidizing bacteria, oxidation by O_2 permeated by the physical diffusion of benthos. This study indicated the issues of maintaining sustainable conservation of the eelgrass bed.

Keywords: *Zostera marina*; eelgrass meadows; blue carbon; H_2S detoxification; sulfur-oxidizing bacteria; Sulfur-reducing bacteria; *Chromatiales*

1. Introduction

Zostera marina called Eelgrass is the most widely distributed marine flowering plant in the northern hemisphere. It is a key component of marine ecosystems, providing geological stabilization through the growth of stolon and enables marine organisms to spawn in the eelgrass meadows [1–3]. Eelgrass, with its high photosynthetic capacity, has been reported to absorb approximately 17% of the carbon from seawater and directly from the atmosphere when it exposed to the atmosphere at low tide [4]. Eelgrass meadows in coastal areas are disappearing due to the loss of shallow areas caused by land reclamation, changes in ocean currents, and industrial wastewater with Japan's economic growth [5]. In general, chemical composition analysis of the eelgrass bed sediment has shown that eelgrass meadows had low dissolved oxygen concentrations and high concentrations of toxic reducing substances such as iron, manganese, and sulfides [6]. Sulfides, such as H_2S, are typically toxic to eukaryotic cells even at concentrations as low as 1-10 mmol/L. Eelgrass has been reported to be sulfide tolerant at concentrations up to 4 mmol/L in situ and in vitro as it leaches some of the oxygen produced through photosynthesis from the root tips [7,8]. On the other hand, H_2S above 4 mmol/L is toxic to eelgrass, and excessive accumulation can lead to the decimation of eelgrass meadows. Magnificent and sustainable eelgrass meadows require not only nutrient sources but also sediment conditions that provide H_2S balance. Recent reports have shown that healthy sediment microflora supports huge eelgrass meadows and that each has own unique characteristics [9]. For example, microorganisms involved in the sulfur cycle, such as sulfate reduction and sulfide oxidation, have been detected in the microflora of the eelgrass meadows sediments. The regional nature of these microflora is attributed not only to ocean currents and coastal development, but also to human life and ethnic cultures. However, there are limited research that has investigated the relationship between microflora and chemical components such as sulfide, including regional characteristics [10,11]. Therefore, it is important for the future conservation of eelgrass meadows to determine from comprehensive surveys the factors that are essential for maintaining the expansion of eelgrass meadows. In this study, the eelgrass meadows of different sizes in Japan were systematically characterized by analyzing the microbial communities and chemical components of four geographically distant eelgrass meadows along the Japanese coast.

2. Results

2.1. Microbial Composition in Eelgrass Meadows

The sequencing reads were obtained to fully reflect alpha diversity based on Shannon index (Supplementary Figure S1). The greatest diversity was found in eelgrass at Wakayama coast, and the lowest diversity was found in eelgrass Kanagawa coast. Bacterial diversity in the sediment was not clearly divided between eelgrass meadows and bare sediment. On the other hand, the nonmetric multidimensional scaling method based on the Bray-Curtis distance matrix indicated that the eelgrass meadows sediments and bare soil sediments in Kumamoto clustered significantly apart from the other three sites. Kanagawa and Kumamoto, where the larger eelgrass meadows, showed less difference in diversity compared to bare sediment, while Osaka and Wakayama, where the eelgrass smaller meadows, were clearly plotted far apart from the eelgrass meadows and bare ground.

Figure1 shows the results of the 16S metagenomic analysis based on the order level in eelgrass and bare sediment at each collection site. The phyla that contributed to less than 1 % of the total abundance were combined and referred to as “Others”. Bacteroidales, Campylobacterales, Chromatiales and Thiotrichales, as typical bacteria, were present in all eelgrass sediments at greater than 1%. The common bacterial orders of eelgrass and bare ground were Anaerolineales, Babeliales, Cytophagales and Phycisphaerales, marine bacteria that were found to comprise more than 1% of all sediment and marine sediment. Sulfate-reducing bacteria (SRB), which contributed to sulfate reduction, were present at 18.40 % (±7.14 %) in eelgrass sediment compared to 6.66 % (±3.50 %) in bare sediment. This indicated that their presence was approximately twice as abundant in the eelgrass sediment than in the bare sediment. Sulfur-oxidizing bacteria (SOB) were present at 7.14% (±2.16 %) and 4.74% (±1.37 %) in the eelgrass and bare sediment, respectively. There was relatively no difference in the eelgrass sediment at any location, whereas there was no SOB detected in the bare sediment in the Wakayama and Osaka sediment samples.

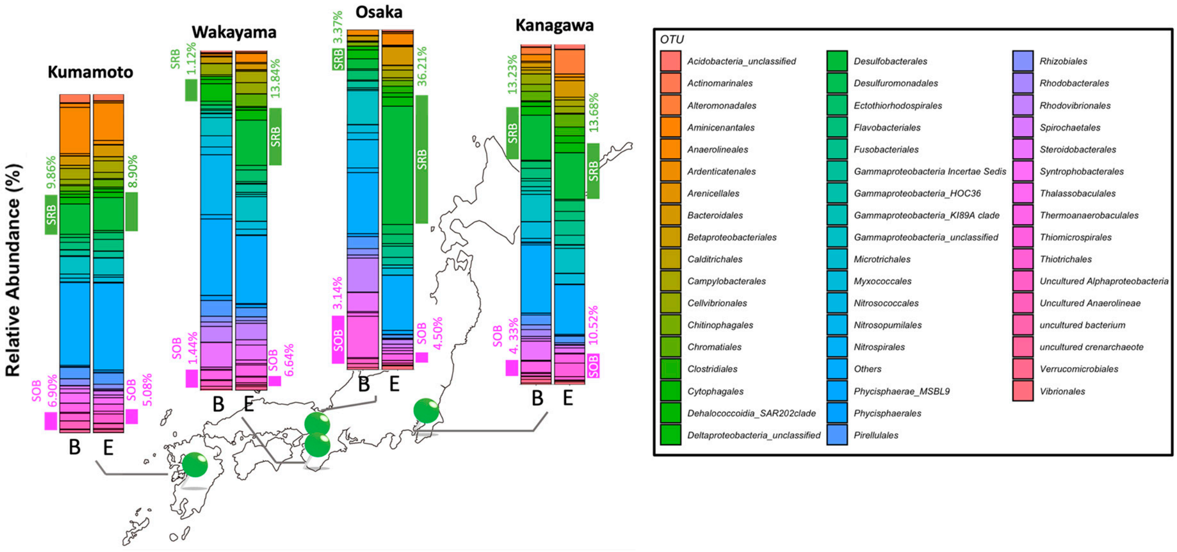

Figure 1. Barplot for microbial community compositions of each eelgrass and bare site based on taxonomic order level on eelgrass sites. E and B show eelgrass and bare meadows, respectively. Taxonomy of the representative sequences with Qiime2 and SILVA database (ver. 138 database clustered to 99 % OTUs). Others shows order that contributed less than 1 % in each sediments.

Linear discriminant analysis using Linear Discriminant Analysis Effect Size (LEFSe) showed that Chlomatiales (LDA score: 4.23, p-value: 0.02) were the dominant microbes in eelgrass sediment and Chitinophagale (LDA score: 4.05, p-value: 0.02) were the main microbes in bare sediment (Supplementary Figure S2(A)). The two governing bacterial orders were positioned distally in terms of phylogenetic relationships (Supplementary Figure S2(B)). Figure 2 shows a heatmap of microbial composition based on order level of more than 1 % in each sediment. The dendrogram, based on the similarity of Euclidean distances at the order level, showed that the microbial communities of all sediments were divided into two groups (yellow: group 1 and green highlight: group 2). Group 1 consisted mainly of aerobic bacteria, such as Gammaproteobacteria, Alphaproteobacteria, Betaproteobacteria, and nitrifying bacteria, while group 2 consisted of anaerobic bacteria, such as SRB and SOB.

Sample clustering showed that the respective coastal bottom sediments were divided into two main clades, eelgrass bed sediments and bare ground sediments, except in the Katakui coast shown as Wakayama_E. Clade 1 consisted of bare ground at the Ozaki coast (Osaka) and Marine Park (Kanagawa) and eelgrass sediment on the Katakui coast. Clade 2 consisted of eelgrass sediment on the Ashikita coast (Kumamoto), Marine Park (Kanagawa), and Ozaki coast (Osaka). Sediments belonging to clade 1 showed a high prevalence of group 1 bacteria, which included rhizobia and nitrifying bacteria, and a low abundance of group 2 bacteria. The Katakui coast (Wakayama) eelgrass sediments showed similar bacterial abundances of both groups. In contrast, in clade 2, the composition of bare sediment was similar to that of the Ashikita (Kumamoto) eelgrass sediment. The Ashikita coast sediments contained the SRB Thiotrichales and Syntrophobacterales. The other type of SRB detected was Desulfobacterales and Desulfuromonadales in the Ozaki (Osaka) eelgrass sediment. However, no SRB were located in the marine park (Kanagawa) eelgrass sediments.

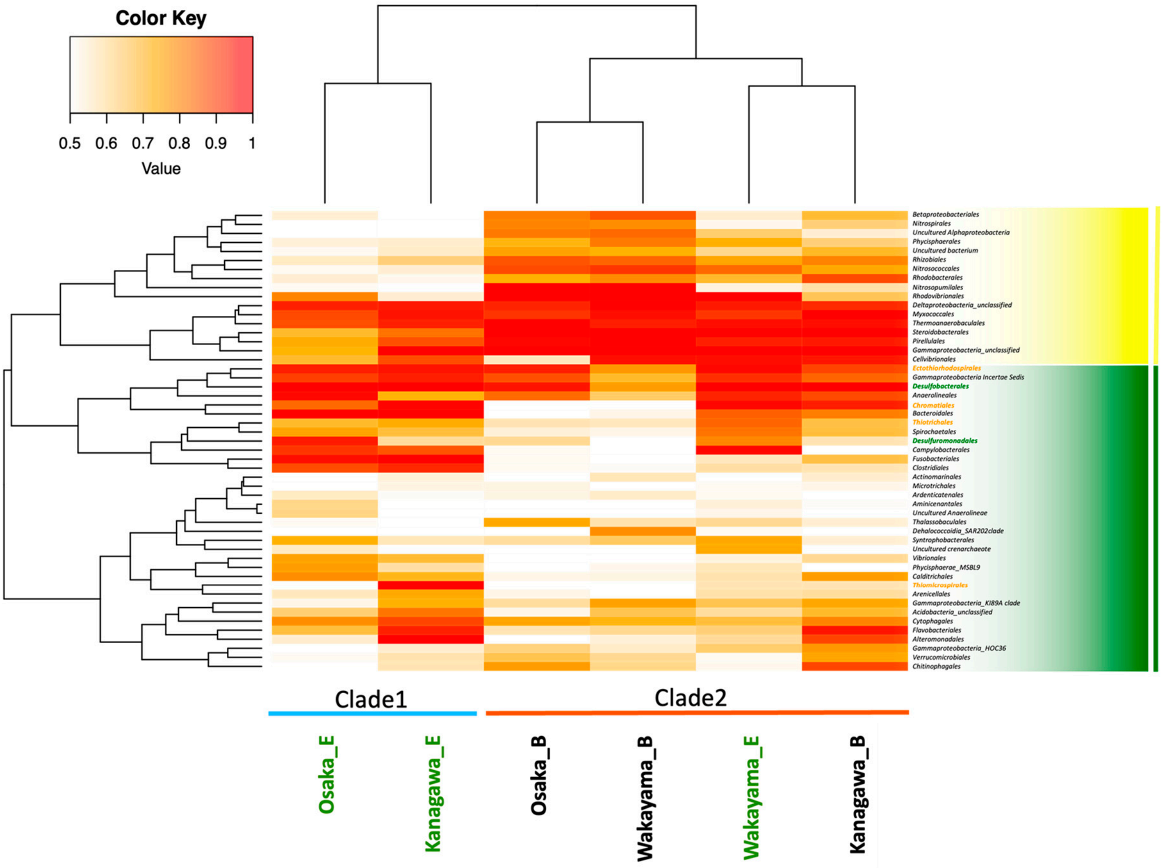

Figure 2. Heatmap of clustering analysis for the relationship between sediment and taxonomic order. The color code indicates abundance of organisms and ranging from white (low abundance) to red (high abundance).

2.2. Determination of Total Bacteria and Chromatiales Population

The total number of bacteria and Chromatiales was calculated using qPCR (Supplementary Figure S3). The total bacterial cells ranged from 3.5E+05 to 2.2E+10 cells/g-sediment and varied broadly between the different sampling sites, except for the Ashikita coast (Kumamoto) sediment, whereby the bare sediment was confirmed to be 10 to 1,000 times more abundant than the eelgrass sediment. Chromatiales had the lowest population (5.2E+03 cells/g-sediment) in the Marine park (Kanagawa) sediment, and the highest population (5.8E+05 cells/g-sediment) in the Ashikita coast (Kumamoto) sediment. Chromatiales accounted for approximately 1.2E-06 to 8.4E-03 of the total number of bacteria. The Chromatiales were approximately 4.7 times higher in the eelgrass meadows sediment than in bare sediments.

2.3. Determination of Chemical Component in Eelgrass Sediment

The DO, ORP, H_2S, TOC, Fe, Ca^{2+}, K^+, and NO_3^- components in each sediment sample are shown in Table 1. Low DO levels were observed in eelgrass sediment at all sites and were in good agreement with ORP behavior. This supports the hypothesis that an anaerobic environment is an important factor for eelgrass. However, H_2S molecules are harmful to plants such as eelgrass, despite existing in all eelgrass bed sediments. The lowest level of H_2S was 2.70 ppm (STD: 1.37) in the Marine park (Kanagawa), while the highest level of 23.29 ppm (STD: 4.34) was measured in Katakui coast (Wakayama). These H_2S concentration levels at 4 sites were on average 11.8 times higher than those in the bare sediments. NO_3^- was highly dependent on the sampling site and showed no correlation with the other chemical components.

Table 1. Concentration of chemical indicators (ORP, DO, H_2S, TOC, Fe, K^+, Ca^{2+} and NO_3^-) for each sediments (n=3). Values in parentheses indicate standard deviations.

		ORP/mV	DO/ppm	H_2S/ppm	TOC/%	Fe/ppm	K^+/ppm	Ca^{2+}/ppm	NO_3^-/ppm
Eelgrass	Kanagawa	-154.5(201.39)	3.0(2.3)	2.70(1.37)	1.28(0.13)	393.0(19.7)	36.7(2.7)	65.0(5.3)	0.22(0.023)
	Osaka	-175.0(63.25)	1.9(0.9)	12.32(6.68)	2.76(0.42)	671.4(57.5)	58.6(0.9)	108.4(14.7)	95.10(23.92)
	Wakayama	-163.6(54.50)	2.1(0.9)	23.29(4.34)	2.30(0.14)	214.7(25.4)	38.6(0.7)	138.9(6.3)	19.67(9.40)
	Kumamoto	-282.3(6.41)	0.1(0)	16.14(0.20)	4.52(0.18)	798.8(20.1)	57.8(0.7)	115.1(1.4)	0.031(0.025)
Bare	Kanagawa	131.6(68.69)	5.3(1.0)	2.24(1.57)	1.20(0.09)	353.7(12.6)	25.8(4.0)	46.1(9.3)	0.14(0.022)
	Osaka	166.3(1.00)	5.9(0.7)	0.42(0.21)	1.36(0.33)	508.6(28.8)	36.7(2.7)	84.0(4.4)	40.49(12.49)
	Wakayama	46.2(62.65)	9.1(2.0)	1.47(1.45)	1.23(0.57)	144.0(68.5)	31.8(2.8)	116.4(11.9)	24.19(13.58)
	Kumamoto	-261.8(8.49)	0.4(0.2)	18.58(9.96)	3.98(0.25)	777.4(25.8)	52.5(1.1)	130.3(4.3)	0.28(0.40)

Figure 3(A) shows that the eelgrass sediment in Marine Park (Kanagawa) had an overall balanced profile. The eelgrass sediment on the Ozaki coast (Osaka) showed higher than average values for NO_3^-, Fe, and K^+. On the Katakui coast (Wakayama), Fe was lower than the other sites, and Ca^{2+} was higher than in the other sites. The Ashikita coast (Kumamoto) sediments had lower NO_3^- and DO levels than those of the other sites. In contrast, the Marine Park (Kanagawa) bare sediment showed higher positive ORP values than the eelgrass sediment. The Ozaki coast (Osaka) bare sediment was characterized by NO_3^-, K^+, and Fe values that were significantly lower than those of the eelgrass sediments and had positive ORP values. The Katakui coast (Wakayama) bare sediment showed higher DO values than eelgrass sediment. The Ashikita coast (Kumamoto) sediment showed similar profiles between the eelgrass and bare sediment.

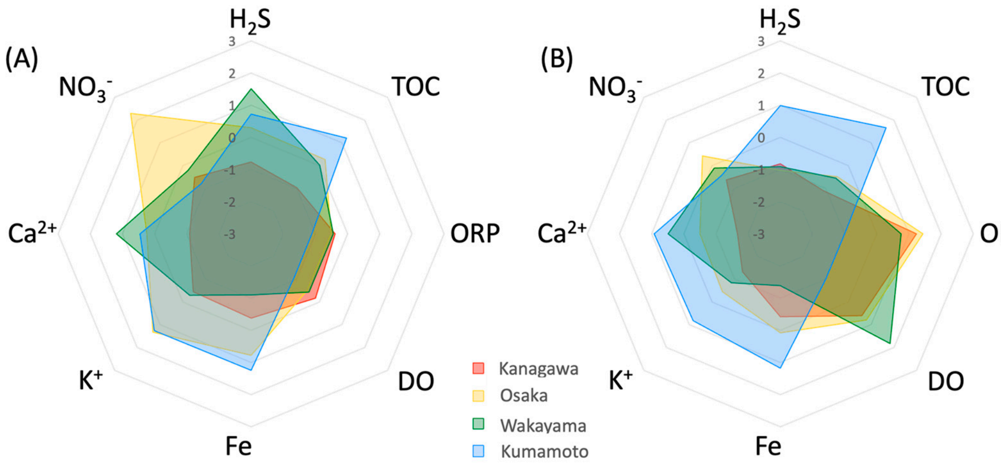

Figure 3. Radar chart of all chemical indicator (ORP,DO,H_2S,TOC,Fe,K^+,Ca^{2+} and NO_3^-) value as Z-score. (A) Eelgrass meadows sediment and (B) bare meadows sediment.

Figure 4 indicates the relationship between the determined chemical components (ORP, DO, H_2S, TOC, Fe, K^+, Ca^{2+}, and NO_3^-) and the principal component analysis. The plot shape indicates eelgrass vegetation conditions, and the color indicates the sampling site. The arrows reflect the orientation of the variables used in the analysis relative to the principal components and the length of the arrows reveals the strength of the correlations with each principal component, which in the PCA1 and PCA2 planar plots reflect approximately 71% of the total data. Evaluation of the overall distribution of the plots showed that the bare sediments formed small clusters at all three sites, except at the Ashikita coast (Kumamoto), and eelgrass sediments formed clusters in all the regions. Overall, the first principal component explained 57.1% of the variance, correlated negatively with DO and ORP, and correlated positively with H_2S, TOC, Ca^{2+}, K^+ and Fe. The second principal component explained 14.5% of the variance with a strong negative correlation with NO_3^-. In the first quadrant, eelgrass meadows and bare sediments mainly from the Ashikita coast (Kumamoto) were plotted, and

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correlated positively with the organic matter content. The H_2S, SOB, and eelgrass sediments from the Katakui coast (Wakayama) also correlated slightly with SOB. In the second quadrant, eelgrass and bare sediments mainly from Marine Park (Kanagawa) were plotted closely to the bare sediments on the Katakui coast (Wakayama). Eelgrass sediments from the Ozaki coast (Osaka) were plotted in the fourth quadrant, and bare sediments were plotted in the fourth quadrant. From these PCA plots, the eelgrass sediments formed a separate cluster from the bare sediments; however, within that cluster, they formed small clusters by region. In contrast, the bare sediments were found to have similar bottom sediment conditions with no regional differences.

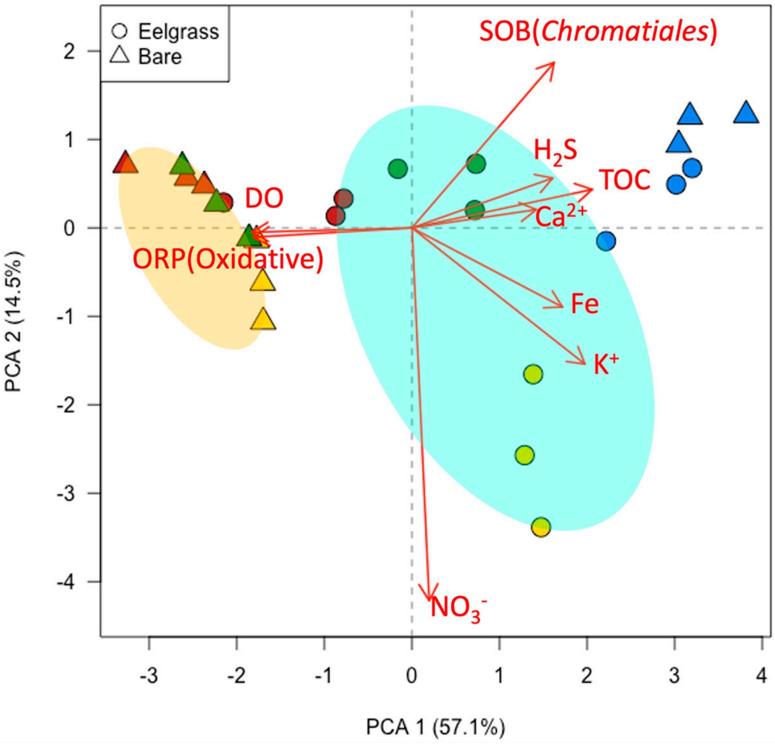

Figure 4. PCA plot of chemical and bacterial profiling in each sediment. Circle plots indicate eelgrass meadows and triangle plots indicate bare sediments. Red, yellow, green and blue show as Marine Park Kanagawa, Ozaki coast Osaka, Katakui coast Wakayama and Ashikita coast Kumamoto Prefecture, respectively.

3. Discussion

3.1. Characteristics of Bacterial Composition in Eelgrass Sediments

This study provides insights into the microbial composition and chemical profiling in geographically distant eelgrass meadows along the Pacific coast. While the microbial composition and chemical composition of local eelgrass meadows have been analyzed regionally from previous study, it has become evident that the microbial and chemical profiles in sediment vary depending on the scale and coast region (Nishijima et al., 2015, Miyamoto et al., 2023, Muwawa et al., 2021). Eelgrass bed creation is an important coastal environment for biodiversity and a decarbonized society. However, eelgrass bed creation has sometimes been difficult to scale up and sustain. The objective of this study is to comprehensively investigate the bacterial and chemical composition of eelgrass sediments in geographically distinct coastal areas of Japan in order to identify similarities and regional characteristics of eelgrass sediments for the sustainable creation of eelgrass beds. Previous studies surveyed the Japanese coastal areas using a combination of bacterial composition analysis and chemical composition determination [25–27]. In another report for the Japanese coast, a multifaceted investigation was conducted by geographical and biochemical assessment to “visualize”

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a causal structural network of symbiotic bacterial communities as photosynthetic bacteria and gut bacteria living in the bottom mud of thriving eelgrass [9]. Furthermore, since eelgrass actively releases photosynthetic products and absorbs nutrients from marine sediment through its stolon, eelgrass survival is strongly related to factors in the rhizosphere. Therefore, the bacterial composition in the rhizosphere of geographically separated eelgrass and bare sediments was evaluated in this study. These bacterial composition in eelgrass sediment were indicated similarities to previously reports which were investigated worldwide. In particular, SRB (*Desulfobulbaceae, Desulfovibrionaceae, Desulfuromonadaceae* or *Desulfobacteraceae* families) and SOB (*Sulfurimonas* family) were detected in eelgrass beds along the west and east coasts of the United States, Yellow Sea, Baltic Sea, Irish Sea and Mediterranean Sea, which showed good agreement with our results. Therefore, these SRBs and SOBs are characteristic bacteria in eelgrass bed sediments [28,29]. The SRB were approximately twice as abundant in the eelgrass sediment as in the bare sediment (Figure 1), suggesting that H_2S production by sulfate reduction may be more active in eelgrass sediment than in the bare sediment as previously reported, and also in the sites covered in this study. Sediments from eelgrass and bare sediment on the Ashikita coast (Kumamoto) showed very similar bacterial compositions. This was due to the homogenization of marine sediments caused by the inflow of sediment from the mountains as a result of the landslide that occurred in July 2020. A heat map, used to examine the bacterial flora in more detail, revealed the proportion of each bacterial order. This indicated a high proportion of sulfate-reducing bacteria as a common feature in eelgrass sediments (Figure 2). The eelgrass sediments in Marine Park (Kanagawa) contained below average percentages of SRB, *Desulfobacterales* and *Desulfuromonadales* on the Ozaki coast (Osaka), *Thiotrichales* and *Desulfuromonadales* on the Katakui coast (Wakayama) and *Thiotrichales* and *Syntrophomonadales* in Ashikita (Kumamoto). *Thiotrichales* and *Syntrophobacterales* were the main SRB. The SRB were more abundant in eelgrass sediments than in bare sediments, however a different order of SRB was detected, suggesting that the types of SRB present reflect the characteristics of eelgrass sediments in each region. In contrast, the presence of SOB, which oxidizes hydrogen sulfide to sulfate ions or single sulfur, was 2.6 times higher in eelgrass sediments than in bare sediments. The main SOB was as follows: *Thiomicrospirales*, *Flavobacteriales*, *Ectothiorhodospirales* and *Chromatiales* in the Marine Park (Kanagawa), *Ectothiorhodospirales* and *Chromatiales* on the Katakui coast (Wakayama), *Flavobacteriales* on the Ashikita coast (Kumamoto), and *Ectothiorhodospirales, Chromatiales* and *Flavobacteriales* on the Ozaki coast (Osaka). The composition of these bacterial flora was in some respects consistent with the report by Miyamoto et al. introduced earlier [9]. However, our results clearly indicate a marine-specific bacterial flora that is characteristics of the region, supporting the regional nature of eelgrass bed sediments.

3.2. Detoxification System in Eelgrass Sediments

In general, H_2S is reported to inhibit of initial grow and seeds maturing for plants under anaerobic conditions in waterlogged soils. In particular, the eelgrass bed sediment on the seafloor is a reducing environment with low oxygen and nutrients, and thus H_2S accumulation by SRB is likely to occur [30,31]. According to previous studies, hydrogen sulfide in marine sediments is toxic to eelgrass at concentrations over 4 mM [8]. Therefore, it is necessary to detoxify the irregularly produced hydrogen sulfide to maintain eelgrass sediment. Thus, in terms of microbial H_2S oxidation, the higher the diversity of SOB, the more efficient the detoxification of H_2S. The *Chromatiales* bacterial population, as a representative SOB, was estimated by the absolute quantification method using qPCR (Figure 3). Marine park (Kanagawa) had the highest SOB diversity among all the sites, and the total bacterial population was higher in eelgrass sediment than in bare sediment. Figure 6 summarizes the H_2S formation and detoxification pathways in the eelgrass meadows ecosystem. The H_2S is formed under anaerobic conditions by the sulfate reduction of SRB from SO_4^{2-} and detritus, which are abundant in seawater and sediment. Detoxification of H_2S is performed by the following four pathways: firstly by the microbial oxidation in the eelgrass rhizosphere by the SOB; secondly is chemical oxidation by the oxygen generated from the eelgrass roots; thirdly is the chemical oxidation by NO_3^- present in the seawater; and finally is the chemical oxidation by Fe and its compounds. This ecosystem is similar to the results of rice meadows improvement studies that have been reported for many years. Since H_2S accumulated in rice meadows causes reduced nutrient uptake and various diseases in rice plants, there are examples of countermeasures to precipitate and remove H_2S by Fe_2O_3 [32–34]. Our results indicated that each eelgrass meadows had a different detoxification system (Figures 4 and 5). There was a detoxification system with NO_3^- along the Ozaki coast (Osaka), indicative of an urban coastal environment. The Katakui (Wakayama) and Ashikita coasts (Kumamoto) were significantly affected by the SOB detoxification systems. These coastal areas are close to mountainous areas and have relatively high inflow from the mountains. The inflow of spring water and sediment from mountains is linked to the natural supply of minerals and microorganisms. However, the Marine Park (Kanagawa) showed unique environment. Dissolved oxygen was supplied to the bottom sediment of the eelgrass meadows that had established an anaerobic environment. Despite the relatively large size of the eelgrass meadows, the system that enabled an extensive supply of oxygen can be attributed to the activities of Benthos. Benthos, such as bivalves and lugworms, are more abundant in the bottom sediment of the eelgrass meadows in Marine Park (Kanagawa) than in other eelgrass meadows, and the physical agitation of the bottom sediment is thought to be responsible for the detoxification of H_2S by delivering oxygen to the reduced bottom sediment. We suggest that knowledge of the sediment factors affecting healthy eelgrass meadows will enable the selection and implementation of H_2S detoxification systems that are appropriate for the site.

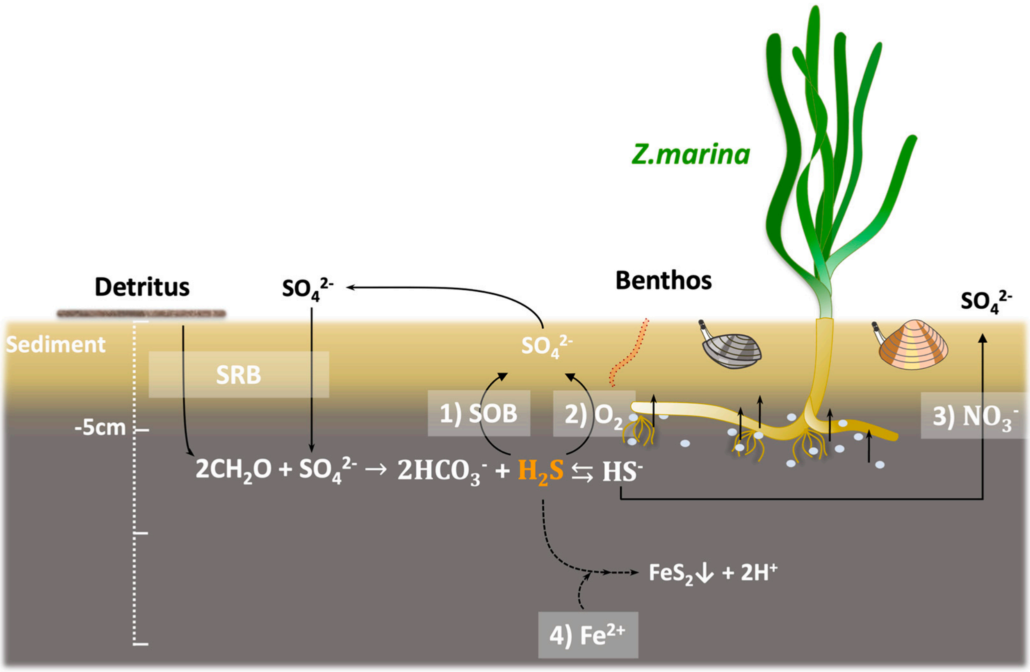

Figure 5. Sulfate reduction and sulfide cycle in eelgrass rhizosphere with biological, botanical and chemical detoxification. Detoxification shows follow as: 1) microbial sulfide by SOB, 2) photo-oxidation by root discharge of O_2 produced by the eelgrass's own photosynthesis and 3) direct chemical oxidation by iron ions and iron compounds.

4. Materials and Methods

4.1. Sampling Sites and Sample Collection

Eelgrass and bare sediments were collected from four coastal sites (Figure 6): Marine Park Kanagawa Prefecture (35.3378N, 139.6370E), Katakui coast Wakayama Prefecture (33.9398N, 135.0850E), Ozaki coast Osaka Prefecture (34.3727N, 135.2455E), and Ashikita coast Kumamoto Prefecture (32.2994N, 130.4761E). The large eelgrass meadows were located at Marine Park (Kanagawa) and the Ashikita coast (Kumamoto) and measured 77,804 (600 m long) and 44,200 m^2 (700 m long), respectively. Katakui (165 m^2 and 5 m long at Wakayama) and Ozaki coasts (1,200 m^2

and 10 m long in Osaka) were dotted with patchy small eelgrass meadows. Each sediment sample was collected at 6 cm depth or less from the sea bottom by coring (6 cm in inner diameter, 20 cm in height, polyvinyl chloride tube), and oxidation reduction potential (ORP) and dissolved oxygen (DO) were measured at the coring site. Each sediment samples were immediately ice-cooled in a cooler and frozen in a -5 °C freezer within 3 hours and then transported frozen to the laboratory. Approximately 30 g of the frozen sediment samples were transferred to sterile 50 mL plastic tubes and stored in a -20 °C until DNA extraction and metagenomic amplicon sequencing.

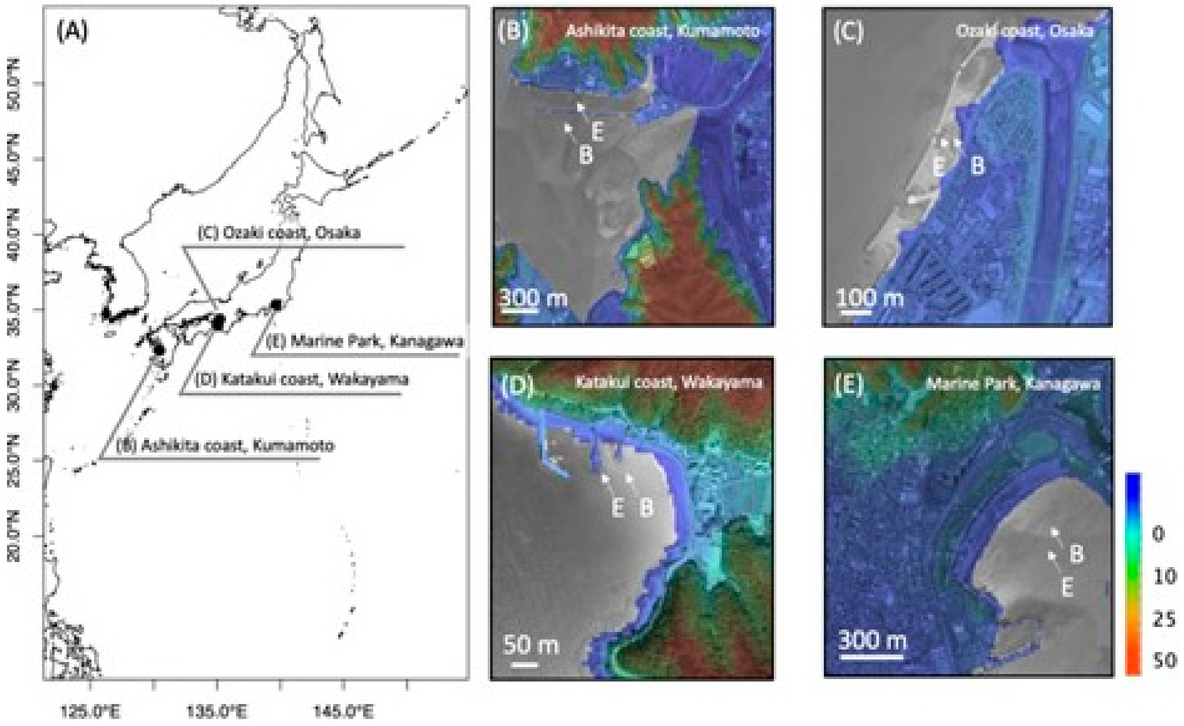

Figure 6. Eelgrass meadow map of sample collection site. (A) Wide map of the collection area. (B, C, D and E) Local maps of E: eelgrass meadow and B: bare meadow. The color scale on the right represents elevation according to the elevation data of the Geospatial Information Authority of Japan.

4.2. 16S Metagenomic Sequencing

Genomic DNA from the collected sediment was extracted using the ISOIL for Beads Beating kit (NIPPON GENE Co.). Extracted DNA was amplified using the 515F (5′-GTGCCAGCMGCCGCGGTAA-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′) primers targeting the v4 hypervariable region of the 16S rRNA gene. Gene RED PCR Mix Plus was used for amplification under the following conditions: 95 °C for 5 min; 30 cycles of 95 °C for 20 s, 65 °C for 20 s, 72 °C for 5 s; and 72 °C for 10 min[12,13]. Agarose gel electrophoresis confirmed the presence of DNA and DNA libraries were constructed using the MiSeq Reagent Kit V2 (500 cycles)(Illumina Co.). The resultant libraries were quantified using a dsDNA HS Assay Kit on a Qubit 2.0 Fluorometer (Thermo Fisher Co.). The DNA library was sequenced as paired-end sequences with an insert size of 380 bp using the Illumina MiSeq.

4.3. Microbial Community Composition and Diversity Analysis

Raw reads were processed using the Qiime2 software (ver.2021.8). The DADA2 algorithm was used for reducing noise and for trimming the low-quality regions of the raw reads. The acceptable or passed reads were used for further analysis [14]. The sequences were clustered into operational taxonomic units (OTUs) with 99 % sequence similarity by VSEARCH and chimeras were removed by the USEARCH software (ver. 6.1.544) [15]. Taxonomic OTUs were linked by mapping the 16S rRNA gene amplicons to the Silva database (ver.138) which was used a reference database. LEFSe (Python ver. 3.9.10) was performed with bacterial abundance at order level to identify bacterial species significantly present in eelgrass sediment and bare sediment. LEFSe was performed using the parameters of p-value of $\leq$ 0.05 and an LDA (linear discriminant analysis) score values of 3.0 and below (python ver. 3.9.10) [16]. To investigate the differences between eelgrass and bare condition in the b diversity of microbial community composition, the PERMAMOVA(permutational multivariate analysis of variance) test was estimated by calculating the Bray-Curtis which was based on the relative abundance[17,18].

4.4. qPCR Analysis for Total Bacteria and Sulfur-Oxidizing Bacteria Population in Sediments

qPCR assays were performed using a Rotor-Gene Q (QIAGEN Co.) under the following reaction conditions: 12.5μL QuantiTect SYBR Green PCR Master Mix (QIAGEN Co.), 1 μL of each primer, and extracted DNA were added to a final volume of 25 μL(Ref). Total bacteria and sulfur-oxidizing bacteria in each sediment were determined by quantitative PCR using the extracted DNA, according to the manufacturer’s instructions. The quality of the extracted DNA of each sample was verified using a Qubit2.0 Fluorometer (Thermo Fisher Scientific Co.). The 16S rRNA gene for total bacteria and the specific 16S rRNA gene for *Chromatiales* as sulfur-oxidizing bacteria were amplified according to the thermocycling conditions described in Table2. The 16S rRNA gene was amplified using the universal bacterial primers Eub519F (5′-CAGCAGCCGCGGTRATA-3′) and U785R (5′-GGACTACCVGGGTATCTAAKCC-3′). Specific 16S rRNA gene for *Chromatiales* was amplified using the primers CHR986F (5′-AGCCCTTGACATCCTCGGAA-3′) and CHR1392R (5′-ACGGGCGGTGTGTAC-3′) (Michael 2014). The 16S rRNA, from *Escherichia coli* DH5alpha (JCM1649^T), and the specific 16S rRNA gene, from *Thiocapsa subramanianensis* (JCM14886^T), were cultured in LB media and PRENNIG’s medium1, respectively. The SYBR green staining method was used to determine the number of cells in the culture media[19]. Respective calibration curves were prepared using DNA extracted by boiling at 95 °C for 15 min. Absolute quantification was performed by Rotor-GeneTM Q Software based on ‘Crossing Point’ value that defines the cycle number at which the fluorescence signal of the sample exceeds a background fluorescence value [19,20]. Ct value was calculated the intersection of the generally used threshold value and the amplification curve in the software.

Table 2. qPCR programs for bacterial cell counts.

Primer Name	Target Organisms	Denaturation	Annealing	Extension
Eub519F/ U785R	Total bacteria	5 sec at 95 ℃	30 sec at 60 °C	30 sec at 60 °C
CHR986F/CHR1392R	*Chromatiales*	30 sec at 95 °C	60 sec at 55 °C	120 sec at 72 °C

4.5. Chemical Components Analysis

In all collected sediments, the geochemical indicators such as ORP, DO, H_2S, Fe, K^+, Ca^{2+} and NO_3^- were determined. The ORP and DO were measured in 6 cm of sediment at each sampling site. The ORP was measured with a portable water quality meter LAQUA D-210C and D-210PC (HORIBA Co.) and the DO was measured using a digital dissolved oxygen meter PDO-520 (FUSO Co.). Spectrophotometric analysis was used to determine the H_2S concentrations using the methylene blue method with 670 nm [21]. In Fe content determination in all sediments were conducted with atomic absorption spectrometer (Z-2300, HITACHI Co.) after extracted with 4.5 mL of 0.5 M hydrochloric acid at room temperature for 24 h [22]. The K^+ and Ca^{2+} were determined with ion chromatography method using HPLC system (LC-20AP system, SHIMADZU Co.) equipped with a Shim-pack IC-C4 column (150 mm 4.6 mm, SHIMADZU Co.). All sediments were washed with distilled water and filtered through a 0.45 mm membrane filter. The eluent was 3 mM $(COOH)_2$ and detection were performed by electrical conductivity. After treating all the specimens according to the brucine method, the NO_3^- concentration was determined at a wavelength of 410 nm [23].

4.6. Total Organic Component Analysis

Thermogravimetric analysis was conducted to measure the organic content of the marine sediment. Each sediment was heated at 100 °C to remove moisture and increased 600 °C to vaporize organic contents. The organic matter content was calculated and compared with the sediment weight obtained at 100 and 600 °C. The mass lost after heating can be considered as the total mass of the organic content. Total organic content analyses were performed on all collected sediments [24].

4.7. Statistical Analysis and Data Visualization

Statistical analysis and data visualizations were conducted using the R studio software (ver. 2021.09.0) with the biplot and ggplot2 packages. PCA plots using physicochemical measurements and sulfur-oxidizing bacteria counts collected from eelgrass beds and bare soil at four sites visualized correlations regarding H_2S detoxification by persistent eelgrass beds.

5. Conclusions

The conservation of eelgrass meadows has been studied in terms of seed germination and growth, however it is important to work with H_2S detoxification systems to create a sustainable environment. Considering biodiversity, the coexistence of benthic organisms and eelgrass is desirable, as in the Marine Park (Kanagawa), but there is growing concern that providing too many organisms all at once will affect the existing environmental balance. PCA plots with statistical analysis of bacterial flora and physico-chemical analysis showed that the H_2S detoxification system with SOB and Fe is an effective system and can be adapted to the eel grass meadows protection.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org, Figure S1: Diversity analysis between each sediments; Figure S2: Characterization of microbiome in eelgrass and bare sediment by LEFSe analysis and LDA; Figure S3: Bar plot of the total number of bacteria and the number of *Chromatiales* for each sediment.

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