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Monitoring Bacterial Community Dynamics in Abalone (*Haliotis discus hannai*) and the Correlations Associated with Aquatic Diseases

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Abstract: Bacteria are an integral component of their host. However, information about the microbiota living in and around many aquatic animals is lacking. In this study, multiplex bar-coded pyrosequencing of the 16S ribosomal RNA gene was used to monitor the dynamics of abalone, *Haliotis discus hannai*, bacterial communities in the intestine, water from cement culture ponds, and surrounding sea areas. Correlations between the bacterial communities and common aquaculture diseases were also evaluated. A total of 329,798 valid sequences and 15,277 operational taxonomic units (OTUs) from 32 samples were obtained by 454 tag amplicon pyrosequencing. The Shannon indices of the seawater samples ranged from 2.84 to 5.6 and the Shannon indices of the abalone intestine samples ranged from 1.2 to 5.12, which were much lower than those of seawater. The dominant phyla in seawater samples were Proteobacteria, Bacteroidetes, Fusobacteria, Cyanobacteria, etc. The dominant phyla in the abalone intestine varied greatly in different months. The dominant genera in the seawater of the cement culture ponds changed in different months, mainly *Psychrilyobacter* and *Pseudoalteromonas*. The dominant genera in seawater from the open sea vary considerably between months. The dominant genus of bacteria in the abalone intestine during the months when abalones are susceptible to disease is mainly *Mycoplasma* spp. Canonical correspondence analysis revealed that bacterial communities in seawater and the intestine responded differently to environmental variables, with similar microbiota in the same area. pH, dissolved oxygen concentration, and temperature were closely related to the samples from the sea area. Oxidation-reduction potential, salinity, phosphate, nitrate, and ammonia nitrogen concentrations were closely related to the water samples from the artificial pools. These findings may add significantly to our understanding of the complex interactions between microbiota and environmental variables in the abalone intestine as well as in the surrounding seawater.

Keywords: abalone; bacteria; community; disease

1. Introduction

Abalone is an economically important animal with a high market value. Abalone farming has gradually increased, reaching 129,287 mt globally in 2015 [1]. China is one of

the world's leading abalone producers, with 115,937 tons of abalone farmed in China by 2015, accounting for 90% of world abalone production [1]. In China, abalone farming has become an intensive industry with high pool density.

As the abalone farming industry has become more intensive, more and more problems have arisen [2]. The death of abalone juveniles due to disease and environmental factors is a serious threat to the sustainability of the abalone farming industry and has dealt a heavy blow to the industry [3,4]. The health of abalone is largely influenced by the water environment and its intestinal microbial community [5]. Dramatic changes in the structure of the bacterial community can lead directly to the development of disease, causing huge economic losses to aquaculturists [6]. Aquatic microorganisms have a vital role in the environment and are indicators of the condition of ecosystems [7,8]. Bacteria are one of the most abundant microbial communities in aquatic ecosystems and have an essential function in the cycling of nutrients and in the breakdown of pollutants in the aquatic environment [9–11]. Water quality is often affected by changes in these communities due to the huge influence of environmental bacterial communities [12–14].

Traditional culture methods are commonly used to study microorganisms in mollusks, such as abalone and oysters [15,16]. However, relying on this method alone is not sufficient as traditional culture methods can only identify a limited number of organisms [17]. With the development of DNA sequencing technology, an increasing number of people are choosing to use 16S ribosomal RNA (16S rRNA) gene sequencing to study bacterial populations. However, traditional sequencing techniques, including PCR (polymerase chain reaction) and in situ hybridization, are unable to obtain sufficient amounts of sequencing data to fully evaluate bacterial communities [18,19]. The application of high throughput sequencing (HTS) has improved the efficiency of studies of environmental bacterial communities and the assessment of correlations between bacterial communities and aquatic diseases affecting marine animals [20].

To date, studies of the impact of different microbial populations on molluscs in seawater environments have been limited. Thus, the current study was conducted to analyze the bacterial diversity in abalones in different farming environments using pyrosequencing based on 16S rRNA genes. Comparisons between regions and the different months of the year were made. Bacterial diversity and abundance were also compared between diseased and healthy abalones, and their relationship with environmental factors was assessed. The results of the present study will extend our understanding of the patterns of bacterial communities during different seasons, how microbial communities change during the transition from healthy to diseased abalones, and whether correlations with environmental factors are present.

2. Materials and Methods

2.1. Sample Preparation

The abalones were farmed in artificial cement pools and in the sea area of Zhangzhou in Fujian Province, China (23°42′4.54″ N, 117°25′48.22″ E), which is the major abalone producing region in this locale (Figure 1). Abalones were monitored every month from 2012 to 2013. Three to five abalones, with an average shell length of about 3~5 cm, were dissected. The depth of the artificial pools was 1.3 m, and the acreage of each pond was 30 m^2. The abalones were cultured normally and specifically for this experiment. Seawater samples from artificial abalone culture pools were labeled as XBS, and digestive tract samples of abalones from these pools were labeled as XBB. All artificial pools were equipped with recirculating systems. Seawater samples from the sea area were labeled as DSS, and digestive tract samples of abalones from the sea area were labeled as DSB. Environmental factors (pH value, temperature [temp], oxidation-reduction potential [ORP], and dissolved oxygen [DO] content) in the ponds and sea area were tested simultaneously using an in situ water quality analyzer (YSI, Yellow Springs, OH, USA). Additionally, phosphate, nitrate, nitrite, and ammonia nitrogen concentrations were measured following the "Specification of Oceanographic Investigation" issued by the State Oceanic Administration.

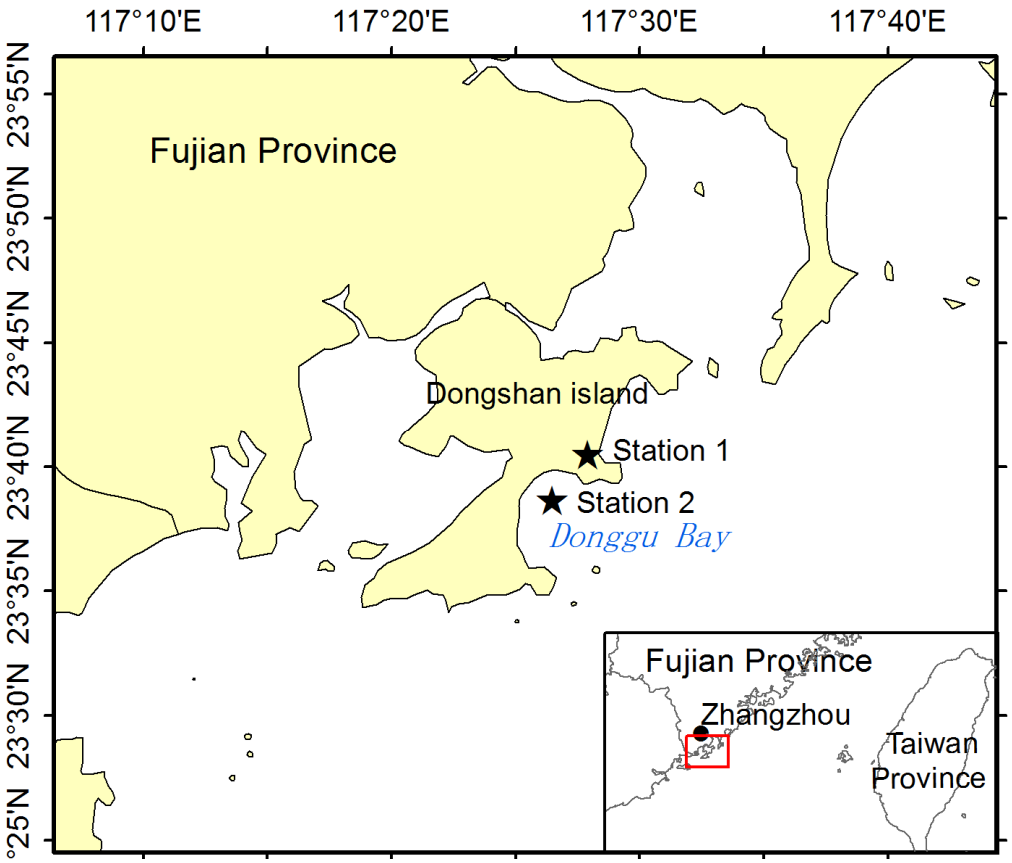

Figure 1. Sampling sites in Fujian Province.

2.2. DNA Extraction and Purification

First, 0.1 g of abalone intestinal contents or mucous membranes were taken from the artificial culture pond and the sea, respectively; 1000 mL of water samples from the artificial culture pond and seawater were filtered. The QIA ampH DNA Stool Mini Kit (Qiagen, Hilden, Germany) was used for the extraction of genomic DNA from filters after filtering water samples and from the abalone intestinal contents. To reduce experimental error, each sample was extracted in duplicate [21]. Afterwards, the DNA purity and concentration were tested using an e-Spect ES-2 device (Malcom, Japan). The extracted DNA was stored at −20 °C.

2.3. 454 Tag Amplicon Pyrosequencing for Analysis of Bacterial 16S rRNA Gene Sequences

We used the forward primer (B-27F) 5′-*CCTATCCCCTGTGTGCCTTGGCAGTCGACT*AGAGTTTGATCCTGGCTCAG-3′(B adapter sequence is italicized and underlined) and the reverse primer (A-533R) 5′-*CCATCTCATCCCTGCGTGTCTCCGACGACT*NNNNNNNNNNTTACCGCGGCTGCTGGCAC-3′ (A adapter sequence is italicized and underlined, Ns represent sample-specific barcode sequences of 10 bases) to amplify the V1-V3 region in the 16S rDNA gene. The total PCR reaction system was 20 µL, including 2 µM each of upstream and downstream primers, 10 ng of the template, 2.5 U of Pfu DNA polymerase (MBI, Fermentas, Waltham, MA, USA), and 5 × PCR reaction buffer. The procedure was carried out for 25 cycles, followed by denaturation at 95 °C for 30 s, followed by denaturation at 55 °C for 30 s, and extension at 72 °C for 1 min. Finally, the extension was performed at 72 °C for 10 min. The PCR products obtained were detected by 2% agarose gel electrophoresis and recovered by cut gel. Quantitative assays were performed using a microfluorometer. The purified amplified fragments were constructed into libraries for sequencing according to the platform's standard operating protocols and sequenced using the GS FLX Titanium platform.

2.4. Data Processing

In the present study, data preprocessing, OTU-based analyses, and hypothesis testing were performed [22]. To improve the quality and reliability of subsequent analyses, the resulting raw data were trimmed to a high quality to remove adapter contaminants and low-quality readings. We set a window of 50 bp, and if the average quality within the window was less than 20 bp, we truncated all sequences at the back end of the base from the front end of the window; splicing the two ends of the sequence according to the overlap, with a maximum mismatch of 3 between the overlaps and a length greater than 10 bp; sequences were split into each sample based on the barcode and primers at the beginning and end of the sequence. The barcode had to be an exact match, and the primers allowed for a 2 bp mismatch, removing sequences with ambiguous bases. UPARSE software (version 7.1 http://drive5.com/uparse/, accessed on 21 October 2021) was used to remove single sequences and chimeras by clustering the sequences in operational taxonomic units (OTUs) based on 97% similarity. We used the RDP classifier (http://rdp.cme.msu.edu/, accessed on 21 October 2021) to annotate each sequence with the species classification, compared to the Silva database (SSU123), setting a comparison threshold of 70%.

2.5. Macrogenome Sequencing and Functional Annotation

From April to May, when abnormal abalone mortality occurred, three to five abalones were dissected, and the abalone intestine contents were taken. *Mycoplasma* DNA was extracted using a *Mycoplasma* DNA extraction kit (Venor®Gem qEP, Minerva, Germany) and then tested for DNA purity and concentration using an e-Spect ES-2 device (Malcom, Japan). Genomic DNA was interrupted at standard atmospheric pressure (15 psi, 101.3 kPa) nitrogen atomization for 1 min. Genomic DNA was separated into 300 to 800 bp fragments, and the interrupted DNA was recovered and purified using QIAGEN purification columns. The A and B junctions (3′ and 5′ ends are specific) are ligated to the DNA fragments. Single-stranded DNA fragments with A and B junctions made up the sample library and were sequenced using the GS FLX Titanium platform. To improve the quality and reliability of subsequent analyses, the raw data generated were cropped to obtain high-quality data to remove adapter contaminants and low-quality reads. Reads were compared to host DNA sequences using BWA software (http://bio-bwa.sourceforge.net, accessed on 6 January 2022) to remove contaminating reads with high similarity. Clustering analysis was performed using CD-HIT software (http://www.bioinformatics.org/cd-hit/, accessed on 6 January 2022) (parameters: 95% identity: 90% coverage) and the longest gene in each category was selected as the representative sequence to construct a redundancy-free gene set. Pathway annotation was performed using BLASTP (BLAST Version 2.2.28+, http://blast.ncbi.nlm.nih.gov/Blast.cgi, accessed on 6 January 2022), and the genomes were compared with the KEGG database (BLAST comparison parameters were set to an expected e-value of 1×10^{-5}).

2.6. Statistical Analyses

Based on the results of OTUs, the sample sequences were randomly sampled, and the alpha indices such as Chao1, Shannon, Simpson, etc., were calculated. The alpha diversity analysis of variance for multiple group comparisons was obtained using Kruskal–Wallis analysis with R calculations. To determine whether the amount of sequencing data in the sample was sufficient, a random sampling of sequences was used to construct a rarefaction curve based on the number of sequences and the number of OTUs they could represent, to observe the tendency of the rarefaction curve. The relationship between each sample bacterial community and environmental variables was analyzed using canonical correspondence analysis (CCA) based on OTU levels. CCA was based on a single-peaked model. Pearson correlation coefficients in SPSS 17.0 were used to determine the correlation between each sample bacterial community and environmental parameters. p-values < 0.05 were considered to be significant. A graphical abstract is available via the drawing tool https://biorender.com/, accessed on 17 March 2022.

3. Results

3.1. Characterization of the Bacterial Community in Seawater Samples and the Intestine of Abalone

Through 454 pyrophosphate sequencing analyses, 329,798 valid sequences and 15,277 OTUs were obtained from 32 samples collected from artificial cement pools and the sea area (Table S1). As the amount of sequencing increases, the rarefaction curve flattens out and the rank abundance curve shows a long, flat fold, suggesting that more data will only produce a small number of new OTUs (Figures S1 and S2). In contrast, Shannon indicator values varied considerably between 1.2 and 5.6 for the artificial concrete pond and the sea area samples, respectively. The results showed that the Shannon index peaked in May for seawater samples from the open sea, therefore the highest bacterial species richness was recorded in May. In August, the Shannon index for the intestine of abalone cultured in seawater from the open sea was 5.12, indicating that the highest bacterial species richness was found in the abalone intestine in August.

3.2. Analysis of the Bacterial Community of Seawater Samples and Abalone Intestine during Different Months

During the year, the dominant phyla in the seawater samples from the artificial ponds were mainly Proteobacteria (21.83~65.95%), Bacteroidetes (10.90~43.96%), and Fusobacteria (1.09~43.25%). This was followed by Cyanobacteria, Firmicutes, etc. (Table S2). The dominant phyla in the samples from the open sea were mainly Proteobacteria, Bacteroidetes, and Cyanobacteria, followed by Actinobacteria (Table S2). Planctomycetes and Firmicutes were also present in the open sea water, but not in high abundance. The dominant phylum in the abalone intestine varied considerably during different months, with Proteobacteria and Tenericutes predominating in March, accounting for 55.11% of the total (Table S2). In June and August, Proteobacteria, Planctomycetes, and Tenericutes were predominant, with 85.74% to 91.34%. Actinobacteria were also present in the abalone intestine, but not in high proportions.

The bacterial composition at the genus level of the seawater samples from the artificial cement pools is shown in Figure 2. Results show that between November 2012 and March to May 2013, the dominant genus was *Psychrilyobacter*, with a range of 10.87% to 42.61%. Interestingly, in May 2013, the bacterial diversity was 2.84, a significant decrease compared to the other months of the year (Table S1). The dominant genera in the sea water were *Psychrilyobacter*, Unclassified and uncultured. Between June and October 2013, the microbial diversity in the seawater samples increased, with no significant dominant genera. *Vibrio*, *Pseudoalteromonas*, *Mycoplasma*, and *Polaribacter* were present each month, but in low abundance.

Figure 3 shows the bacterial composition at the genus level in the seawater samples from the open sea. Results show that the microbial community composition of the seawater samples was stable in different months, with *Chloroplast*_norank being the dominant genus in the seawater samples between November 2012 and March 2013, with a range of 13.93% to 23%, and *Loktanella* increasing in abundance and becoming the dominant genus in April 2013. Between May and July 2013, *Nautella* was the most abundant genus with 10.65%, followed by BD1-5_norank and *Vibrio*. Between September and October, seawater was dominated by Candidate_division_TM7_norank (14.63%), *Arcobacter* (10.68%), and *Psychrilyobacter* (8.56%). *Vibrio* and *Polaribacter* were also present but in low abundance. In October, *Prolixibacter* had the highest abundance at 12.43%. This was followed by *Pseudomonas*, *Salinisphaera*, *Psychrilyobacter*, and *Acinetobacter*.

Over the year, the Shannon index for seawater samples ranged from 2.84 to 5.6 and from 1.2 to 5.12 for abalone intestine samples, indicating that the bacterial diversity of seawater was much higher than that of the abalone intestine. It is noteworthy that the *Mycoplasma* dominated (accounting for 80.62~94.16% of the total) during the abalone susceptibility period (April, May, July, and October) compared to the microbial composition in seawater (Figure 4). Although *Mycoplasma* was also present in seawater for most of the year, their abundance was low. Metagenomic analysis of *Mycoplasma* in the intestine of

abalone that died unnaturally between April and May suggested that *Mycoplasma* may be associated with the development of infectious diseases (Figure 5). However, whether *Mycoplasma* is associated with abalone health needs further study.

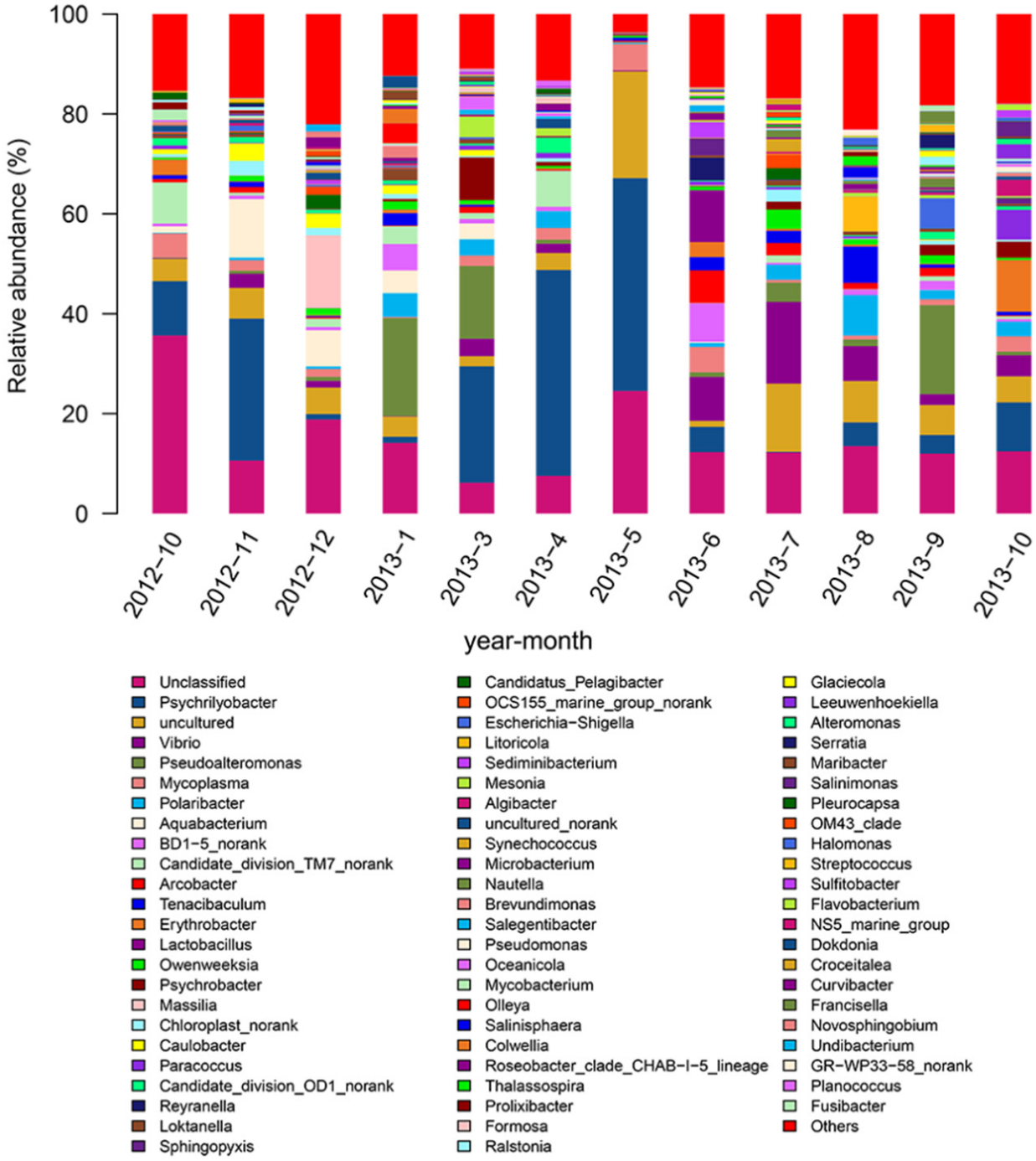

Figure 2. Genus-level abundance of bacterial community in seawater samples from artificial cement ponds for abalones in different months. The vertical coordinates indicate the relative abundance of different bacterial species. The horizontal coordinate represents the year and month. Different colors represent different bacterial species.

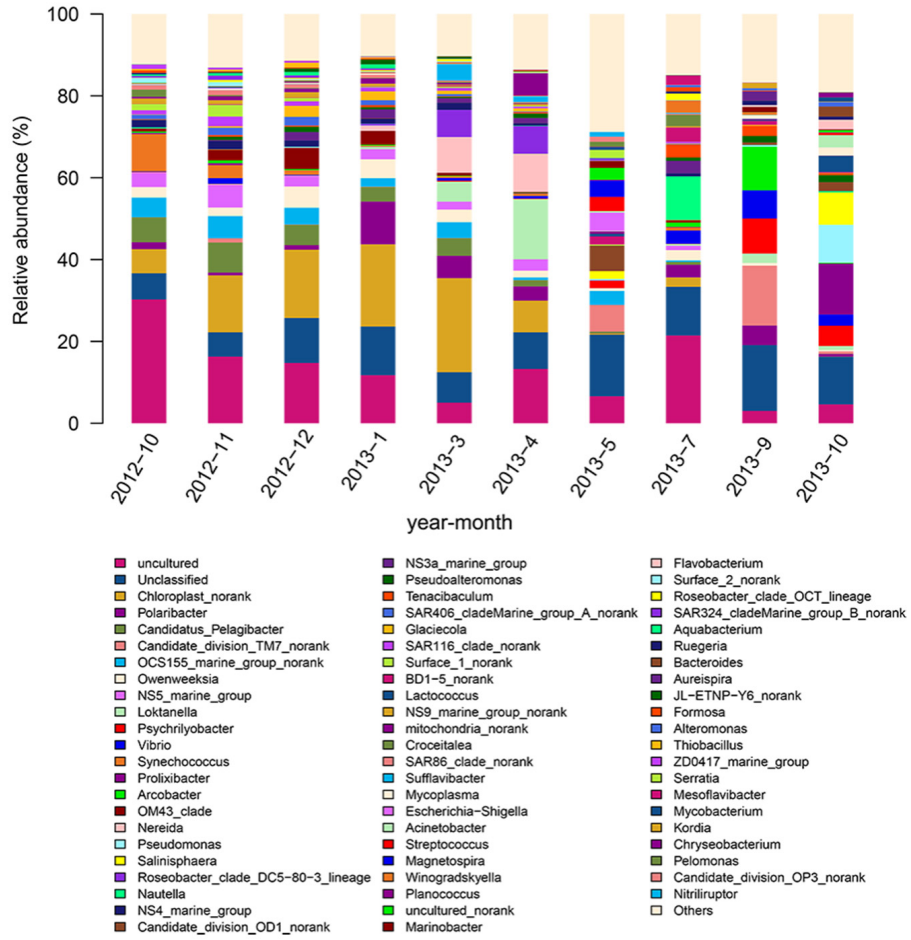

Figure 3. Genus-level abundance of bacterial community in samples of open sea seawater in different months.

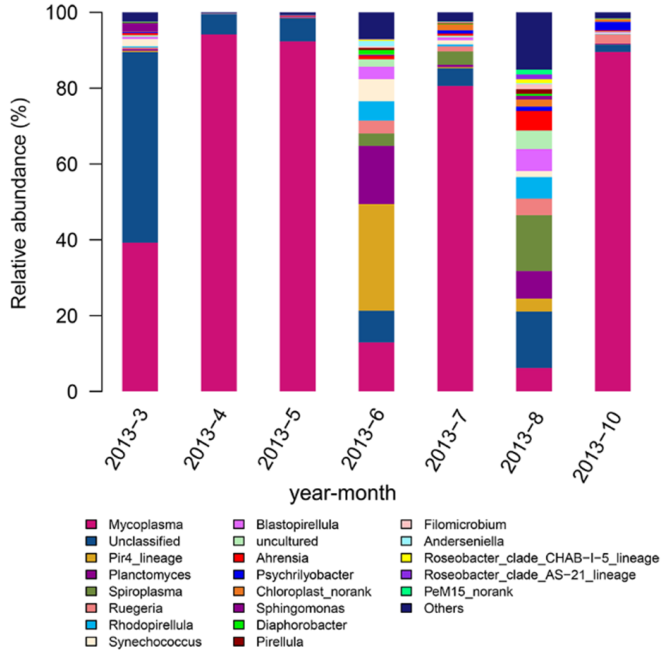

Figure 4. Genus-level abundance of bacterial community in the intestine of abalone cultured in the open sea area in different months.

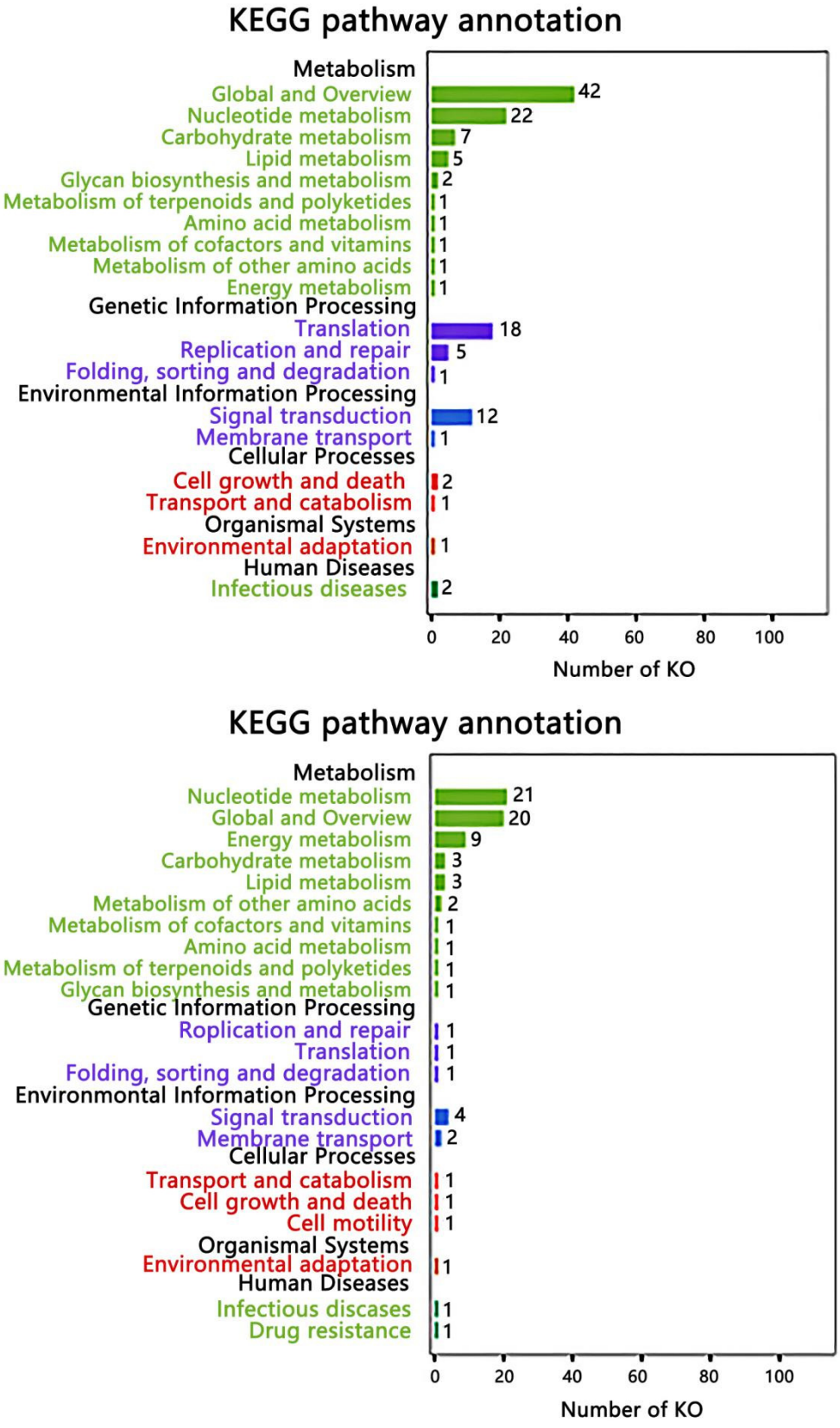

Figure 5. Metagenomic analysis of *Mycoplasma* in intestines of diseased abalones. The horizontal coordinates represent the number of KO pathways, and the vertical coordinates represent the names of the KEGG pathway.

3.3. Comparison of the Number of OTUs in the Intestine of Abalone in Different Months

The number of OTUs in the abalone intestine during different months is shown in Figure 6. The results show that the number of unique OTUs in the abalone intestine was lower during the disease period (April, May, and July) than in June, and the lowest number of unique OTUs in April was 6. The highest number of unique OTUs was 301 in June. This result indicates that the microbiota in the abalone intestine changes when disease occurs.

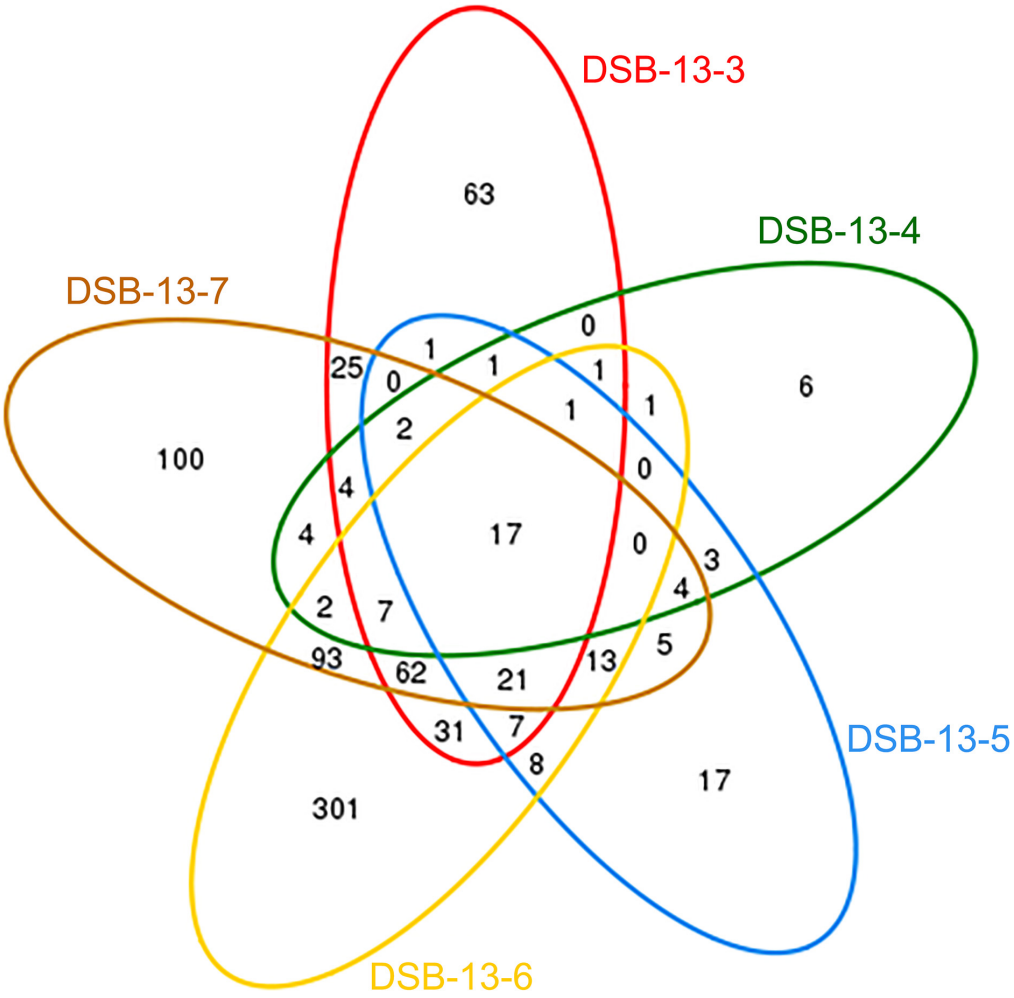

Figure 6. Venn diagram of abalone intestine samples in different months. Sample names: Subgroup name–year–month.

3.4. Relationship between the Bacterial Community and Environmental Factors

The relationships between the bacterial communities in each sample and environmental variables were investigated using canonical correspondence analysis (CCA) based on the OTU level. The samples from different sources were distributed in different regions of the CCA biplot, which suggests that the microflora from the same area were similar (Figure 7). The plot illustrates that pH, DO concentration, and temperature were closely related to the samples from the open sea area (including seawater samples and abalone samples), including DSS-13-7, DSS-13-10, DSB-13-7, DSB-13-10, and DSB-13-5. ORP, salinity, and phosphate, nitrate, and ammonia nitrogen concentrations were closely related to the water samples from the artificial cement pools (XBS-13-3, XBS-13-4, XBS-13-5, XBS-13-6, XBS-13-8, XBS-13-9, and XBS-13-10) (Figure 7).

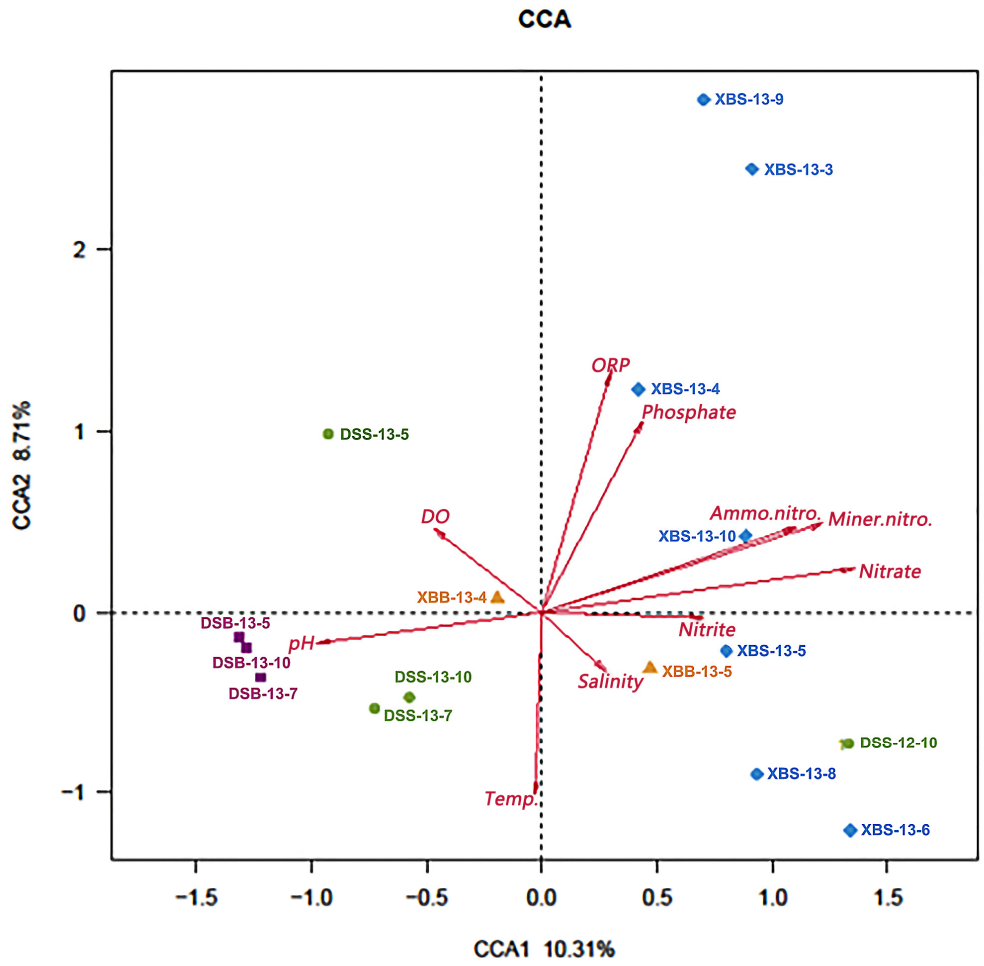

Figure 7. CCA biplot of the distribution of bacterial communities in relation to environmental factors. Numbers in the figure indicate sample names: Subgroup name–year–month; different colors or shapes indicate sample groups in different environments; arrows indicate environmental factors; the angle between samples and environmental factors represents positive and negative correlations between samples and environmental factors (acute angle: Positive correlation; obtuse angle: Negative correlation; right angle: No correlation).

4. Discussion

In order to determine the density of bacterial cells, many different methods have been developed. However, each method has advantages and disadvantages. It has been shown that only a small proportion of environmental bacteria can be detected by the culture medium, and most cannot be detected [23]. Pyrophosphate sequencing of PCR-amplified 16S rDNA is a next-generation sequencing method that can generate thousands of sequences from multiple samples simultaneously [24,25]. Although the 454 platform is more costly compared to the Hiseq platform, the 454 platform can detect a greater number of bacterial classifications than the Hiseq platform. The Roche 454 system has a long read length, with GS FLX Titanium XL+ sequencers capable of reading lengths of 800–1000 bp. It has been shown that this technique can be used for sequencing repetitive or palindromic sequences, due to its long read length [26].

Microorganisms play an important role in aquaculture, and understanding the characteristics of microbial communities in aquatic ecosystems and within aquatic organisms contributes to the success of abalone farming. High-throughput sequencing technology can better visualize the microbial communities associated with abalone farming. Results showed that Proteobacteria and Bacteroidetes are the dominant bacteria and most efficient colonizers in abalone farming waters, which is consistent with previous studies [27]. In

contrast to terrestrial animals, microorganisms in the aquatic environment have a significant impact on the bacterial composition of aquatic animals due to their constant contact with the surrounding aquatic environment [28–30]. Studies have shown that the microbial composition of the abalone intestine varies considerably in different months, but the main dominant phyla in the intestine of most months are Proteobacteria and Tenericutes. A similar phenomenon was observed at the genus level. The microbial composition of the seawater and abalone intestine varied between months. In addition, the microbial composition of the seawater and abalone intestine differed considerably. The above findings suggest that although some taxa are shared between the seawater and the abalone intestine, the relative abundance (phylum or genus level) of these bacterial taxa differ between the seawater and the abalone intestine. This may be because aquatic ecosystems differ from other systems in that constant inputs of organic matter and other human interventions always influence the composition of bacterial communities in aquatic ecosystems [29,31,32]. In contrast, the intestine microbial assemblage of abalone is not passively collecting bacteria from the environment to adapt to the changing environments [33].

The microbial community in the abalone intestine is altered when the abalone becomes ill or suffers abnormal mortality. In this study, the number of unique OTUs in the abalone intestine decreased and the number of *Mycoplasma* increased abnormally during the month of abnormal mortality. This finding suggests that the structure of the abalone intestine microbiota undergoes large changes when diseases emerge. The progression and outcome of infectious diseases depend on the dynamics of host–pathogen interactions, and recent studies using high-throughput sequencing technologies have provided new insights into the evolution of bacterial pathogens during colonization and infection [34]. This study showed that *Mycoplasma* accounted for upwards of 94.16% of the total flora in the month of the onset of the disease. This suggests that perhaps the presence of *Mycoplasma* is associated with the onset of disease. Most previous studies on bacteria in abalone have focused on culture methods and specific pathogens, such as *Vibrio* spp. [35–37]. In addition, it has been recommended that *Vibrio* is the most dominant genus in circulating system farms and that *Vibrio* may be active in the host gut and facilitate the digestion of feed [38,39].

Numerous microbial and viral diseases can infect one or more stages of the abalone life cycle. Surprisingly, results from the present study suggested that *Vibrio* spp., which has always been considered to be the main pathogen in diseased mollusks based on traditional methods of identification, constituted only a small percentage of the micro-ecological habitat. Although *Vibrio* spp. were present for most of the year and their conditional pathogenicity cannot be ignored, they were not the most important group during periods of the disease. The microbial community inherent in the abalone's intestine remains relatively stable and becomes more abundant when disease occurs.

The gut microbiota plays pivotal roles in the development, immunity, digestion, and overall health of the host. In recent years, extensive investigations have shown that environmental factors have an impact on the composition and activity of the intestinal flora and thus on the health of the host [40]. As a result, water quality has been assessed in many countries [41,42]. Some studies have shown that environmental factors have a more significant impact on intestinal flora than the intestinal environment [43]. Notably, the results of this experiment suggest that pH affects the microbiological community composition in the abalone intestine. This is relevant as surface ocean pH may decrease by 0.1–0.3 units by the end of the 21st century [44].

Ocean acidification is one of the new and significant environmental threats facing aquaculture [45]. Mollusks are extremely vulnerable to the effects of ocean acidification because their early developmental stages are particularly sensitive to changes in pH [46]. Ocean acidification reduces the survival of larvae [47,48]. Thus, the effects of pH on the microbiota of abalones should be of concern. In the current study, ORP, salinity, phosphate, nitrate, and ammonia nitrogen concentrations were closely related to the water samples. It was previously suggested that ORP was closely related to microbial communities and that oxygen could enable heterotrophs to inhibit sulfate-reducing bacteria [49].

As seawater environmental factors change with climate change, the spread and growth of bacteria will likely be enhanced [50]. Such changes will likely cause the host microecosystem to become unbalanced, and disease outbreaks will likely follow. Therefore, studying the effects of environmental factors on the growth and development of abalone is crucial to the conservation and restoration of abalone resources. In addition, it is essential to understand the influence of environmental variables such as pH, ORP, temperature, and DO concentration on the composition of the intestinal microbiota and the occurrence of disease.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/w14111769/s1, Figure S1: The rarefaction curves for seawater samples and abalone intestine samples; Figure S2: The rank abundance curve for seawater samples and abalone intestine samples; Table S1: Structural diversity indices of bacterial communities in seawater samples and abalone intestine samples. Table S2: Bacterial community structure at the phylum level in seawater samples and abalone intestine samples.

Author Contributions: T.Z. and R.W. designed the study. T.Z. and R.W. performed the study. T.Z., R.W. and H.Z. developed the data analysis strategy. T.Z., H.Z. and J.W. (Juan Wang) analyzed the data. T.Z., R.W., X.L., J.W. (Jiangyong Wang), Y.H., H.M., B.L. and X.M. assisted with the analytic tools. T.Z. and R.W. wrote the manuscript. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

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