Environmental Factors Shape the Epiphytic Bacterial Communities of Gracilariopsis Lemaneiformis

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Research Article

Keywords: Macroalgae host variety, red macroalga Gracilariopsis lemaneiformis, environmental factors, total nitrogen

Posted Date: January 18th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-144624/v1

Version of Record: A version of this preprint was published at Scientific Reports on April 21st, 2021. See the published version at https://doi.org/10.1038/s41598-021-87977-3.

Abstract

Macroalgae host variety of symbionts on their surface which play critical role in their growth and development processes. Nevertheless, the complete understanding of this interaction of epiphytic bacteria and host algae is still in its infancy. This study comprehensively analyses epiphytic bacterial communities composition of red macroalga *Gracilariopsis lemaneiformis* and environmental factors such as nitrogen and phosphorus which shape the composition of epiphytic bacterial communities of *G. lemaneiformis* and variation of epiphytic bacterial communities composition at different geographical location. The composition and structure of epiphytic bacterial communities were characterized using high throughput sequencing data of the V3-V4 hypervariable region of 16S rRNA gene amplicon sequencing. The epiphytic bacterial communities composition data revealed that epiphytic bacteria varied significantly among three different geographic locations i) Nan'ao Island (NA) (ii) Lianjiang County (LJ) and iii) Nanri Island (NR) in China. Redundancy analysis (RDA) showed that the relative abundance of Bacteroidetes, Firmicutes, Verrucomicrobia and Epsilonbacteraeota at NR were strongly positively correlated with total nitrogen (TN), total phosphorus (TP), nitrate nitrogen (NO_3-N), and dissolved inorganic nitrogen (DIN), whereas negatively correlated with nitrite nitrogen (NO_2-N). In addition, the relative abundance of cyanobacteria at NA and LJ were strongly positively correlated with NO_2-N, whereas negatively correlated with TN, TP, NO_3-N, and DIN. Furthermore, the results of Mantel test indicated that the epiphytic bacterial communities composition is significantly correlated with these environmental factors, which are also proved by Pearson correlation analysis. In conclusion, it is proposed that environmental factors such as NO_3-N and DIN play key role in the communities composition of epiphytic bacteria in *G. lemaneiformis*. Our study provides important baseline knowledge for the communities composition of epiphytic bacteria in *G. lemaneiformis* and their correlation among themselves as well as with their surrounding environmental factors.

Introduction

Macroalgae are a major habitat-forming organisms in marine ecosystems and play critical role in building the physical structure of habitats[1,2]. They produce nutrients[3] by releasing extracellular products to the surrounding environment during their growth process[4], forming a unique microenvironment on the surface of algal body[5]. Epiphytic bacteria release nutrient supplements[6] such as vitamins B12[7], and fatty acids[8] and also regulate the growth for the macroalgae[9] and some produce antibacterial compounds[10].In addition to that, epiphytic bacteria regulate and prevent the settlement of other marine organisms. For example the epiphytic bacteria of green algae Ulva reticulata produces bioactive compounds which inhibit the settlement of polychaete *Hydroides elegans*[11] on macroalgal surfaces[8,12].

The study of biodiversity and communities composition of epiphytic bacteria of macroalgae have remained the focus of scientists in last decade using culture dependent analysis. Such as Burke et al.[13] reported Alphaproteobacteria and Bacteroidetes as dominant group and Rhodobacteriaceae, Sphingomonadaceae, Flavobacteriaceae and Sapropiraceae as dominant families of bacteria on the

surface of green macroalgae collected at Bare Island, La Perouse. Similarly, Tujula et al.[14] also reported Alphaproteobacteria and Bacteroidetes as dominant epiphytic microbial communities on the surface of *Ulva australis* collected from Shark Point, Clovelly, NSW, Australia. Additionally, the epiphytic bacteria on *Ulva australis* was found taxonomically and functionally distinct from surrounding seawater[13,15]. These results were further confirmed by Roth-Schulze et al.[1,16]. They demonstrated[1] that most of the OTUs that are abundant on *Ulva* surface are undetectable in the surrounding seawater. They also observed that the epiphytic bacterial communities of *Ulva* spp., are the result of selection of planktonic bacterial communities and interestingly planktonic microbial communities are both taxonomically and functionally distinct regardless of the host or surface-types[16]. Furthermore, Selvarajan et al.[17] also observed similar group of bacteria i.e. Proteobacteria, Bacteroidetes, Firmicutes, Cyanobacteria, Planctomycetes, Actinobacteria and Verrucomicrobia as dominant groups on the surfaces of eight different seaweeds collected from a rocky intertidal zone from Indian Ocean at Cape Vidal, South Africa. This study revealed that the diversity of epiphytic bacteria depends on algal secondary metabolites and elemental deposits responsible for triggering chemotaxis response of epiphytic bacteria to metabolize those substrates.

Recently, it is revealed that the epiphytic bacterial composition of macroalgae are host-specific[16,18] and are regulated by environmental factors[18]. Briefly, they studied spatial diversity of bacterial communities associated with two invasive *Asparagopsis* spp., i.e., *A. taxiformis* and *A. armata* from the north east of the Atlantic Ocean and observed different epiphytic bacterial communities composition of same algal spp isolated from two different locations i.e. continental and island habitat. Moreover, Roth-Schulze et al.[16] found unique taxonomic diversity to each host type such as green, red, brown macroalgae, seagrass, rock and seawater using hierarchy design. The host-specificity of epiphytic microbial communities might be due to extracellular secondary metabolites secreted from host during their life cycle. For example, Lachnit et al.[19] detected chemical compounds from the surface of *Fucus vesiculosus* and tested for bacterial settlement and communities composition, and found that compounds associated with algal surfaces mediated colonization of epiphytic bacteria. Similarly, Nylund et al.[20] demonstrated that surface-bound antibacterial compounds produced by red alga *Bonnemaisonia asparagoides* had a significant effect on the abundance and composition of epiphytic bacterial communities. These studies indicate that host-specificity of the epiphytic bacterial communities are closely correlated to the physicochemical properties of host surface while few studies propose a lottery model for colonization of the algal surface in an attempt to explain the unusual lack of similarity in bacterial communities composition across different algae[13,15,21]. The lottery model[22], originally designed to explain the coexistence of reef fish species that occupy the same ecological niche, may explain the pattern of bacterial colonization on the surface of macroalgae[13]. This model suggests that hosts are colonized by 'suitable' bacteria from the surrounding species pool, resulting in high variability of bacterial communities structure across sites even between individual samples[13,21].

In some cases, environmental factors such as temperature, salinity, light intensity, nitrogen and biogeographic differences may also affect the composition of bacterial communities associated with macroalgae. For example, epiphytic bacteria from member of Rhodobacteraceae family of *F. vesiculosus*

found 20%-50% increase in relative abundance with increasing temperature[23]. Salinity has also been reported to play important role in structuring alga-associated epiphytic bacterial of *F. vesiculosus*[24]. This observation had already been confirmed by study of Zhang et al.[25], they demonstrated that the dominant bacterial communities associated with kelp were different under different salinity conditions. Moreover, Liao and Xu[26] studied the correlation of epiphytic bacterial communities and nitrogen in *G. lemaneiformis* and found significant differences in the epiphytic bacterial communities with the increase of time under different nitrogen concentration. In addition, they found significant differences in the epiphytic bacterial communities at the apex and root of *G. lemaneiformis*. Interestingly, it was observed that the transfer of macroalgae from their natural environment to an aquarium had different epiphytic microbial communities[27] which is due to significant changes in microbial communities associated with *Delisea pulchra* for 15 days of aquarium maintenance.

Previously, it has also been reported that the similarity of the epiphytic bacterial communities composition of same species decreases with the increase of distance[28]. For example, the similarity between epiphytic bacterial communities was smaller for *U. australis* obtained from the two large scale position i.e. 18,000 km apart as compare to the samples obtained from the two small scale position i.e. 500 m apart[1] while the taxonomic composition of epiphytic bacterial communities of *Ulva* varies according to biogeographical locations, as reported that 70% of the detected core functions are independent of host species and biogeographic factors[1].

G. lemaneiformis, is commercially importance red macroalgae. This is not only a source of human food and abalone fodder, but also an ideal material to restore the eutrophication of water body[29,30]. *G. lemaneiformis* has been cultivated on a large scale in southeast coastal areas of China particularly in Nan'ao Island, Nanri Island and Lianjiang County, and has quickly become the third largest economic macroalgae after *Laminaria* and *Porphyra* [31].

The knowledge about the diversity and communities composition of epiphytic bacteria from *G. lemaneiformis* in large-scale artificial algal farms remained limited to a single geographic location[30]. Therefore, keeping in view the commercial importance of *G. lemaneiformis*, we for the first time analyzed the epiphytic bacterial communities of *G. lemaneiformis* from three different geographic areas located in southeast of China (Fig. 1) along with environmental factors effecting its composition.

Results

Measuring the physicochemical factors of environment

The temperature (Temp), pH, salinity (Sal), dissolved oxygen (DO), electrical conductance (EC), total dissolved solids (TDS), total nitrogen (TN), total phosphorus (TP), ammonia (NH_4-N), nitrate (NO_3-N) and nitrite (NO_2-N) levels of seawater samples were measured (Figure 2 & S. Tab. 1). The temperature in NR and NA was significantly higher ($p < 0.05$) than that in LJ (Figure 2a), while the concentration of

dissolved oxygen was significantly lower ($p < 0.05$) than that in LJ (Figure 2b). There were no significant differences ($p > 0.05$) in pH between NR and NA, as well as between NR and LJ (Figure 2a). The salinity and electrical conductance in NR were significantly higher ($p < 0.05$) than that in LJ (Figure 2b, 2c). There were no significant differences ($p > 0.05$) in salinity and electrical conductance between NR and NA, as well as between NA and LJ (Figure 2b, 2c). It was also observed that there were no significant differences in the concentration of total dissolved solids among NR, NA, and LJ (Figure 2c). The concentrations of total nitrogen, total phosphorus, nitrate, and dissolved inorganic nitrogen in NR were significantly higher ($p < 0.05$) than that in NA and LJ (Figure 2d, 2e, 2f). However, the concentration of nitrite in NR was significantly lower ($p < 0.05$) than that in LJ and lower ($p > 0.05$) than that in NA (Figure 2f). It was also observed that there was no significant difference ($p > 0.05$) in the concentration of ammonia between NR and NA, but they were significantly higher ($p < 0.05$) than that in LJ (Figure 2e).

The epiphytic bacterial communities diversity on the surface of *G. lemaneiformis*

A total of 23 samples (two NR1 samples, three NR2 samples, three NR3 samples, three NA2 samples, three NA3 samples, three LJ1 samples, three LJ2 samples, three LJ3 samples) were analyzed using Hiseq sequencing of 16S rRNA gene amplicons, obtaining 1234507 effective sequences with an average of 45722 reads per sample (n=23). Sequences were clustered into operational taxonomic units (OTUs) at the 97% similarity level to generate a total of 6238 OTUs from 23 samples. In all, the OTUs were classified into 11 phyla, 21 classes, 52 orders, 94 families and 196 genera. The Shannon index of the epiphytic bacterial communities at NR was 5.48±0.65 per sample, which was higher ($p > 0.05$) than that at NA (4.83±0.18) and LJ (5.10±0.65) (Figure 3a & S. Tab. 2). The Chao1 index of the epiphytic bacterial communities at NR was significantly higher ($p < 0.05$) than that at NA and higher ($p > 0.05$) than that at LJ (Figure 3b & S. Tab. 2). In addition, observed species index was highest ($p < 0.05$) in the epiphytic bacterial communities at NR compared with that at NA and LJ (Figure 3c & S. Tab. 2). The epiphytic bacterial communities at NR had the highest ($p < 0.05$) PD_whole_tree index compared with that at NA and LJ (Figure 3d & S. Tab. 2).

Geographic comparison of the epiphytic bacterial communities on the surface of *G. lemaneiformis*

Based on the OTUs analysis, an NMD ordination biplot indicated clear clustering of the epiphytic bacterial communities. The stress and RSQ values were 0.14391 and 0.91376, respectively (Figure 4). Overall, it was shown that *G. lemaneiformis* samples at LJ clustered together, and the similar phenomenon was also observed at NA and NR. Notably, the composition of epiphytic bacterial communities was more similar with *G. lemaneiformis* samples at NA and LJ compared with that at NR based on Dimension 1.

The epiphytic bacterial communities on the surface of *G. lemaneiformis* showed considerable changes within three different geographic locations (Figure 5). Bacteroidetes was the most predominant phylum on the surface of *G. lemaneiformis* in NR, with a relative abundance of 44.982%, followed by Proteobacteria (28.941%) and Firmicutes (20.565%). Significantly different the epiphytic bacterial communities were found in of *G. lemaneiformis* collected from NR as compare to *G. lemaneiformis* collected from NA and LJ. For examples, Proteobacteria was found to be the most predominant phylum on the surface of *G. lemaneiformis* in NA and LJ, with a relative abundance of 61.727% and 64.437%, respectively, which was significantly higher than that in NR ($p < 0.05$) followed Bacteroidetes, with a relative abundance of 30.140% at NA and 27.008% at LJ, which was significantly lower than that in NR ($p < 0.05$). However relative abundance of Firmicutes in NR was significantly higher than that in NA and LJ ($p < 0.05$) and the abundance of Deinococcus-Thermus in NA was significantly higher than that in NR and LJ ($p < 0.05$).

Redundancy analysis and heat map

RDA was used to explore the correlations between bacteria at the phylum level in samples of epiphytic bacteria of *G. lemaneiformis* and environmental factors as variables (Figure 6). RDA results showed that epiphytic bacteria at NR, NA and LJ were clustered in a group respectively. RDA1 and RDA2 explained 47.58% and 29.16% of the total variance, respectively. Nitrate nitrogen (NO_3-N), dissolved inorganic nitrogen (DIN), total nitrogen (TN) and total phosphorus (TP) were strongly positively correlated with the relative abundance of Bacteroidetes, Firmicutes, Verrucomicrobia and Epsilonbacteraeota, whereas nitrite nitrogen (NO_2-N) showed negative correlations in the epiphytic bacterial communities of *G. lemaneiformis* at NR. Nitrite nitrogen (NO_2-N) was strongly positively correlated with the relative abundance of Cyanobacteria, whereas nitrate nitrogen (NO_3-N), dissolved inorganic nitrogen (DIN), total nitrogen (TN) and total phosphorus (TP) showed negative correlations with the relative abundance of Cyanobacteria in the *G. lemaneiformis* collected from NA and LJ. RDA results also showed that nitrate nitrogen (NO_3-N) and dissolved inorganic nitrogen (DIN) had a significant influence ($p < 0.05$) on the distribution of macroalgae samples and microorganisms, and similar results were found by Pearson correlation analysis (Figure 7). The Mantel tests were performed to examine the contribution of environmental factors on assembly of communities composition of the epiphytic bacteria from *G. lemaneiformis*. The Mantel tests conducted with both the phylum and genus level found that communities composition correlated significantly with NO_3-N, NO_2-N, DIN, TN, and TP (Table 1). Three significant correlations between temp, pH, DO and the genus communities composition were also detected.

Pearson correlation analysis was conducted in order to investigate that how environmental factors affect the compositions of epiphytic bacterial communities of *G. lemaneiformis*. The relation between the composition of epiphytic bacterial communities on the surface of *G. lemaneiformis* and environmental factors of surrounding seawater was well demonstrated by heat map based on the Pearson correlations

(Figure 7) between species abundance and environmental factors. The epiphytic bacterial communities variation at genus level demonstrate that all epiphytic bacterial composition except *Dokdonia* and *Maribacter* at NR exhibited a positive correlation with NO_3-N, DIN, TN and TP ($0.31 < r < 0.81$), and a negative correlation with NO_2-N ($-0.77 < r < -0.35$). It is noteworthy that the abundance of *Escherichia-Shigella* detected in samples at NR2 displayed extremely significantly positive correlation with NO_3-N, DIN, TN and TP. However, the abundance of all epiphytic bacterial composition except *Flavobacteriaceae*_unclassified, *Hellea*, *Arenicella*, *Leucothrix* and *Caulobacteraceae*_unclassified at NA showed a positive correlation with NO_2-N ($0.10 < r < 0.70$), and a negative correlation with NO_3-N, DIN, TN and TP ($-0.77 < r < -0.11$). Meanwhile, the abundance of all epiphytic bacterial composition except *Sphingomonadaceae*_unclassified and *PhormidesmisANT-LACV5-1* at LJ showed a positive correlation with NO_2-N ($0.06 < r < 0.70$), and a negative correlation with NO_3-N, DIN, TN and TP ($-0.82 < r < -0.05$).

Functional prediction and comparative analysis

Besides, revealing the epiphytic bacterial composition of *G. lemaneiformis*, it is equally important to uncover the functional profile of microbial communities. For that reason, we have to know either taxonomic differences detected in the communities of epiphytic bacteria of *G. lemaneiformis* collected from three different geographic locations would lead to functional changes of the epiphytic bacterial communities. Phylogenetical investigation of communities by reconstruction of unobserved states (PICRUSt) was used to analyze and predict the functional capabilities of epiphytic bacterial communities of *G. lemaneiformis*. The KEGG pathways obtained from breakdown of all predicted metagenomes showed that metabolism was the most abundant functional composition of level 1 for all groups i.e. NR: 50.060%, NA: 51.384%, LJ: 52.717%, respectively (S. Fig. 3). Within the metabolism assignments, relative abundances for all groups showed the highest values for amino acid metabolism, carbohydrate metabolism and energy metabolism (Figure 8 & S. Tab. 4). Amino acid metabolism showed amino acid related enzymes and arginine and proline metabolism as the most predominant for all groups, while phenylalanine metabolism found lowest in all groups (S. Fig. 4). Regarding carbohydrate metabolism, amino sugar and nucleotide sugar metabolism, glycolysis/gluconeogenesis and pyruvate metabolism were the predominant assignment for all groups (S. Fig. 5). In addition, energy metabolism showed oxidative phosphorylation as the most abundant for all groups, while photosynthesis-antenna proteins were lowest in all groups (S. Fig. 6).

Discussion

To our knowledge, this is the first study ever conducted to evaluate the geographic and environmental specificity of the epiphytic bacterial communities of red macroalgae *G. lemaneiformis* cultivated at three different geographic locations by using next generation sequencing. Previous studies focused on spatial and temporal diversity of the epiphytic bacterial communities from macroalgae[14,18,21]. Few reports demonstrated that differences in the epiphytic bacterial communities composition on the surface of

macroalgae from different geographic locations are likely due to environmental selections[18,32]. Environmental selection can produce substantial biogeographic patterns in the global microbe population[33]. This suggests that there are selective mechanisms in place, which determine the assemblage of species that exist in one environment or the other[13]. In order to understand this phenomenon, we investigated the epiphytic bacterial communities composition and their correlation at spatial level and as well as the role of different environmental factors such as nitrogen and phosphorous in shaping epiphytic bacterial communities composition. In our study, the similarity among the epiphytic bacterial communities were higher in *G. lemaneiformis* collected from three different sampling sites which are 300 m apart i.e. NR: 17.5%-66.92% similarity, NA: 12.5%-64.77% similarity, and LJ: 50%-75.07% similarity when compared with the *G. lemaneiformis* collected from NA and LJ which are 500 km apart exhibiting only 7.5% similarity. In addition to that we also found that the similarity of the epiphytic bacterial communities among different sampling sites in any of the three locations was much higher than that between the two most distant locations (NA and LJ, 500 km apart). This correlation fits in the distance-decay of similarity model, in which the decrease in similarity of microbial communities is related to the increase of geographic distance[28] and our findings are consistent with previous study conducted by Roth-Schulze et al.[1] Variations in similarity of epiphytic bacterial communities correlated by distance have also been observed in marine, soil, sediment and plant-associated ecosystems[1].

We found considerable variations of epiphytic bacterial communities composition of *G. lemaneiformis* among three different geographic locations. Briefly, the epiphytic bacterial communities of *G. lemaneiformis* from NR, NA, and LJ were mainly composed of *Flavobacteriaceae, Saprospiraceae, Muribaculaceae* (Bacteroidetes), *Hyphomonadaceae, Sphingomonadaceae, Rhodobacteraceae* (Alphaproteobacteria), *Cellvibrionaceae, Thiohalorhabdaceae, Nitrincolaceae, Thiotrichaceae, Halomonadaceae* (Grammaproteobacteria), *Ruminococcaceae, Lachnospiraceae* (Firmicutes), *Microtrichaceae* (Actinobacteria), *Trueperaceae* (Deinococcus-Thermus) and Rare taxa (S. Fig. 1 & S. Tab. 3). These observations are consistent with previously demonstrated studies of marine algal-associated bacterial communities. For example, the epiphytic bacterial communities on the surface of the red alga *Delisea pulchra* was found to be comprised of *Rhodobacteraceae, Sphingomonadaceae, Flavobacteriaceae, Planctomycetaceae* and unclassified Grammaproteobacteria[34], whereas the green alga *U. australis* hosts Alphaproteobacteria, Bacteroidetes, Planctomycetes and unclassified Grammaproteobacteria[13]. *Fucus vesiculosus* has been found to be associated with a high proportion of Alphaproteobacteria, Bacteroidetes, Verrucomicrobia, and Cyanobacteria in summer, while Grammaproteobacteria in winter[35]. The core microbial communities were often defined as the suite of members shared among microbial communities from similar habitats[36]. Discovering core microbial communities is important for understanding the stable and consistent components across complex microbial assemblages. Our study revealed significant differences of the core epiphytic bacterial communities across the three different geographic locations (S. Fig. 2). For instance, *G. lemaneiformis* at NR had Cyclobacteriaceae, Pseudomonadaceae, Sphingobacteriaceae, Xanthobacteraceae, Burkholderiaceae, Beijerinckiaceae, Nocardiaceae, Rhizobiaceae and Micrococcaceae as the dominant

bacterial family, while at NA and LJ Veillonellaceae, Bifidobacteriaceae, Coriobacteriaceae, Porticoccaceae, Bacillaceae and Sandaracinaceae, Staphylococcaceae, Corynebacteriaceae, Blastocatellaceae, RhizobialesIncertaeSedis, Flammeovirgaceae, Shewanellaceae were found dominate bacteria respectively.

Hence, these findings lead to conclusion that these epiphytic bacteria on the surface of different macroalgae may be vital to hosts. For example, cross-kingdom chemical signals derived from members of Halomonas (*Halomonadaceae*), Roseobacter (*Rhodobacteraceae*) and Sulfitobacter (*Flavobacteriaceae*) are beneficial for the thallus development of *Ulva mutabilis* (Chlorophyta)[37]. Few studies reveal that Dimethylsulfoniopropionate (DMSP) plays a key role in the macroalgal-bacterial interactions[38]. Alphaproteobacteria, which are morphologically and metabolically extremely diverse[14], have a critical role in the assimilation of DMSP in the oceans and contribute significantly to the global sulphur cycling[39]. Furthermore, DMSP, usually produced by macroalgae including *Ulva* sp., attract some bacteria[38]. It's worth mentioning that the members of family *Hyphomonadaceae* are widely dispersed in marine environment and play an important role in mineralizing dissolved organic matters in oligotrophic waters[18]. From our finding we assume that the ability of *G. lemaneiformis* to grow normally in three different oligotrophic waters may partly benefit from the mineralization of these microorganisms on its surface. In addition, previous study performed by Holmström et al.[40,41] had suggested multiple bioactive compounds produced by the genus *Pseudoalteromonas* present on the surface of *Ulva lactuca* might play an important role in the chemical defense against biofouling in the marine environment.

Studies on holobionts had shown either host-specificity of epiphytic bacterial communities within different species, or geographic differences of epiphytic bacterial communities between different locations[1,16,18,21,32]. For instance, the epiphytic bacterial communities on the surface of different host species were taxonomically and functionally distinct, and this distinction was not due to the phylogeny of host, but was due to physicochemical properties of host[16]. As Lachnit et al.[42] suggested that the difference in the epiphytic bacterial communities on different algae host are due to the physiochemical properties of macroalgae surface which allow the settlement and colonization of specific bacteria. In terms of geographic diversity, Roth-Schulze et al.[1] demonstrated that the same algal-genus across different regions can harbor different microbial communities at the taxonomic and functional level, which could be due to local geographic conditions and host specificity.

To explore the specificity of the epiphytic bacterial communities associated with *G. lemaneiformis* and its correlation with different environmental factors. Our study demonstrates that the concentrations of TN, TP, NO_3-N and DIN at NR were significantly higher as compare to NA and LJ ($p < 0.05$), while the concentration of NO_2-N at NR was significantly lower as compare to LJ ($p < 0.05$) and NA ($p > 0.05$) besides having similar environmental conditions i.e. temperature, pH, salinity, dissolved oxygen, electrical conductance, and total dissolved solid. It has been reported that changes in environmental conditions, such as nutrient concentration, nutrient ratio and temperature, can affect the physicochemical properties of macroalgae[43]. Subsequently, Van Alstyne[44] suggested that *Ulva lactuca* and *Ulva obscura* grown in

high nitrogen concentration have higher DMSP content than that in low nitrogen concentration. It suggests that different environmental conditions can affect the content of algal-associated compounds. There are increasing evidences which demonstrate that the compounds associated with algal surface can mediate epiphytic bacterial colonization, abundance and communities composition of macroalgae[45–47]. The above studies also show that the differences in the epiphytic bacterial communities composition of macroalgae led by the changes of environmental factors is attributed to the variation of physicochemical properties on the surface of macroalgae. Therefore, we speculate that the variation of the epiphytic bacterial communities of *G. lemaneiformis* is probably related to the differences of algal-associated compounds caused by environmental conditions.

Notably, there were considerable changes of the epiphytic bacterial communities on the surface of *G. lemaneiformis* from three different geographic locations regardless of the taxonomic level (Figure 5, S. Fig. 1 and S. Tab. 3). At the level of phyla (Figure 5), the most predominant phylum associated with *G. lemaneiformis* at NR was Bacteroidetes, while Proteobacteria at NA and LJ. Our findings are similar with previous study of surface-associated bacterial communities on macroalgae, conducted by Burke et al.[13], which reveal that epiphytic bacteria of *U. australis* are dominated by Proteobacteria- and Bacteroidetes[13]. While at family level (S. Tab. 4), the relative abundance of *Muribaculaceae*, *Ruminococcaceae* and *Lachnospiraceae* at NR was significantly higher ($p < 0.05$) than that at NA and LJ, and no significant differences ($p > 0.05$) was observed between NA and LJ. The above results showed that there were significant differences of the epiphytic bacterial communities composition on the surface of *G. lemaneiformis* between NR and NA and also between NR and LJ, but no significant differences were observed between NA and LJ which is contrary to previous reports which reveal that the algal epiphytic bacterial communities varies from different locations[1,32]. The possible explanation for that might be due to secretion of certain compounds on the surface of *G. lemaneiformis* which regulate the epiphytic bacteria communities composition.

Furthermore, in order to have better explanation of our findings, RDA[48] and Pearson correlation analysis[30] are employed. Interestingly, the epiphytic bacterial communities on the surface of *G. lemaneiformis* at NR exhibited a positive correlation with TN, TP, NO_3-N and DIN, and a negative correlation with NO_2-N (Figure 6 and 7). For example, clinically important bacterial genus, *Escherichia-Shigella*, was found on the surface of *G. lemaneiformis* at NR2 and was extremely significant correlated with NO_3-N, DIN, TP and TN (Figure 7). *Escherichia-Shigella*, an enteric pathogen, being released from waste water treatment plant[49] and can secrete toxins to the surrounding environment[50]. The correlation between the abundance of *Escherichia-Shigella* and environmental factors could be of ecological/health concerns[51]. However, the epiphytic bacterial communities on the surface of *G. lemaneiformis* at NA and LJ showed a positive correlation with NO_2-N, and a negative correlation with TN, TP, NO_3-N and DIN. The above results lead to make conclusion that the differences of epiphytic bacterial communities associated with *G. lemaneiformis* from different geographic locations is because of environmental factor rather than different geographical locations, which is consistent with previous study[18]. In that study *Asparagopsis*-associated bacterial communities have been observed to be modulated by environmental conditions.

Moreover, Roth-Schulze et al.[1] suggested that most of the *Ulva*-associated bacterial communities are horizontally derived from the environment as macroalgae *U. australis* isolated from distinct geographic locations have been observed to share only two low-abundance OTUs. We observed different findings that *G. lemaneiformis* from NA and LJ (500 km away from each other) only share 7.5% similarity. It is surprisingly interesting that environmental factors rather than geographic different locations caused changes in the epiphytic bacterial communities of *G. lemaneiformis*, which is contrary to what was previously reported by Roth-Schulze et al.,[1]. Therefore, we hypothesize that the epiphytic bacterial communities of *G. lemaneiformis* is regulated by nitrogen and phosphorus (environmental factors) rather than geographical locations.

Replicate samples from the same *Gracilaria* species found at the same location showed certain variability in the epiphytic bacterial communities composition (17.5%-66.92%, 12.5%-64.77%, 50%-75.07% Bray-Curtis similarity for NR, NA and LJ, respectively) which is similar with previous studies which have shown high level of intraspecies variability of epiphytic bacterial communities associated with macroalgae[1,15]. This could be due to the depth level of sequencing[14], or as reported in lottery model in which random variation can be seen in the recruitment of the epiphytic bacterial communities on algae surface[22] which was in actual developed for the coexistence of reef fish species share the same niche. Briefly this hypothesis asserts that recruitment of species having same tropic abilities in any of the ecosystem is stochastic fashion i.e. who so ever gets there first wins the space, but they must share similar ecologies[52,53]. Furthermore, in order to draw a clear hypothesis regarding the environmental factor and their role in the selection of the epiphytic bacterial communities of macroalgae, we suggest a study for the comparative epiphytic bacterial communities composition of other algae in the presence of similar environmental factors in future.

Moreover, it is worth noting that there were considerable changes in the composition of epiphytic bacterial communities on *G. lemaneiformis* at three different geographic locations, however the functional composition of epiphytic bacterial communities remained similar. There is an emerging consensus that the bacterial community composition on macroalgae is mainly driven by functional genes rather than taxonomic composition[15,17]. In our study, the functional capabilities of epiphytic bacterial communities on *G. lemaneiformis* at different locations were similar, which indicate similar functions of these bacterial communities at different locations. As regard the fundamental factor that causing the differences in the composition of bacterial communities was microenvironment established by the physiological and biochemical properties of the algal host[3,17].

We found bacterial genes associated with these amino acids including glycine, alanine, arginine, proline, glutamic, and aspartic acids which majorly contribute in algal proteins[54] (S. Tab. 4). This could be an explanation for higher percentage of bacterial genes assigned to amino acid metabolism in our study (Figure 8). In addition, abundant functional genes related to carbohydrate metabolism found which are believed to be involved in the mineralization of dissolved organic matter under oligotrophic environment of coastal water (Figure 8). Similar to our finding, Selvarajan et al.[17] reported higher abundance of

bacterial functional genes associated with carbohydrate metabolism in all seaweeds at intertidal zones of Mission Rocks, Cape Vidal, Leven Point, South Africa. The limitation of current study is that all the inferences are based on predicted functional characters by PICRUSt annotation. It is not a complete substitute for metagenomic research, so there could be inherent inaccuracies in interpreting functional biogeography in certain ecosystem.

Conclusion

Current study demonstrates that epiphytic bacterial communities associated with *G. lemaneiformis* varied significantly at three different geographic locations. The relative abundance of epiphytic bacterial communities at NR was strongly positively correlated with total nitrogen, total phosphorus, nitrate, and dissolved inorganic nitrogen, whereas at NA and LJ it was strongly positively correlated with nitrite. Therefore, it is proposed that environmental factors such as nitrate and dissolved inorganic nitrogen play key role in the epiphytic bacterial communities composition of *G. lemaneiformis* regardless their geographic locations. This is the first study ever conducted to provide insight into the epiphytic bacterial communities composition of *G. lemaneiformis* at three different geographic locations and the environmental factor influence their composition.

Materials And Methods

Collection location and sampling

Individual thalli from *G. lemaneiformis* were collected from three different geographic locations, Nan'ao Island of Guangdong Province (NA, 117°6'40"E, 23°29'9"N), Nanri Island of Fujian Province (NR, 119°33'13"E, 25°13'29"N) and Lianjiang County of Fujian Province (LJ, 119°43'35"E, 26°23'31"N) in the month of January and February 2018. The selection of sampling seasons was based on the vigorous growth of *G. lemaneiformis* in this period. At each location, three sampling sites (ca. 300 m apart) were randomly selected. Three replicate surface (0.5 m depth) water were collected at each sampling site in 1.0 L and used for physicochemical analyses. At NA, three replicate *G. lemaneiformis* were collected at two sampling sites except NA1 (due to inclement weather). At NR and LJ, three replicate *G. lemaneiformis* were collected at each sampling site. The NR1.1 sample was missing due to the experiment. Full details of the sampling information can be found in S. Tab. 5. All algae samples were stored in sterile polyethylene bags along with seawater. Both algae and seawater samples were transported to the laboratory in cold environment.

Determination of physicochemical factors

Temperature, salinity, pH, dissolved oxygen (DO), electrical conductance (EC) and total dissolved solids (TDS) of seawater samples were measured using In-Situ SMARTPOLL MP (U.S.A). Briefly, seawater samples were filtered on a 0.45 µm mixed cellulose ester microporous filter membrane (MF-Millipore

HAWP04700, USA) within 2 hours. Ammonia nitrogen (NH_4-N), nitrate nitrogen (NO_3-N), nitrite nitrogen (NO_2-N), total nitrogen (TN) and total phosphorous (TP) of seawater samples were analyzed according to the standard methods as described by AQSIQ[55].

Preprocessing of sample and elution of epiphytic bacteria

The elution of epiphytic bacteria from *G. lemaneiformis* following the methods described by Burke[56]. Briefly, prior to the extraction, the algae samples were washed three times with filter-sterilized seawater to remove loosely associated microorganisms, microalgae and phytoplankton. Five grams wet weight of *G. lemaneiformis* were placed into 250 mL conical flask containing 100 mL of calcium- and magnesium-free artificial seawater (CMFSW, 0.45 M NaCl, 10 mM KCl, 7 mM Na_2SO_4, 0.5 mM $NaHCO_3$ and 10 mM EDTA) and 1 mL filter-sterilized rapid multienzyme cleaner (3M, Shanghai, China). Samples were incubated in oscillating concentrator (DHZB-500, Jintan Science Analysis Instrument Co., Ltd.) for 3 hours at 25℃ and 180 rpm. To elute the epiphytic bacteria on the surface of *G. lemaneiformis* completely, samples were sonicated (JY92-□N, Ningbo Scientz Biotechnology Co., Ltd.) for 10 minutes (50W, 5s/5s)[57]. Algae material was removed by sterilized tweezers and the remaining solution centrifuged at 8,000 rpm for 20 minutes. The supernatant was removed from the upper layer, and about 1.0 mL of liquid was left at the bottom of the centrifuge tube to be mixed with the bacterial precipitate and transferred to the new centrifuge tube and centrifuged at 12,000 rpm for 5 minutes to completely remove the supernatant. The obtained bacterial precipitate was thoroughly washed once with 1×TE buffer, the bacterial precipitates were dissolved in 1 mL 1×TE buffer and stored provisionally in a freezer at -20℃ for DNA extraction.

The detailed DNA extraction procedures

The DNA extraction of epiphytic bacteria from *G. lemaneiformis* was carried out by the procedure described in our previous study[58]. Briefly, the bacterial precipitate samples were taken out from the refrigerator and centrifuged at 12,000 rpm for 5 minutes to remove the supernatant. 100 μL TE buffer was added to each centrifuge tube and suspended the bacterial precipitate thoroughly. 60 μL 10% SDS solution and 10 μl 20 $mg \cdot ml^{-1}$ proteinase K were added to each centrifuge tube and mixed gently and incubated at 37℃ for 1 hour. After incubation, 100 μl 5 $mol \cdot L^{-1}$ NaCl solution were added to each centrifuge tube and the centrifuge tube was turn upside down for more than 10 times. 80 μl CTAB/NaCl solution was added to each centrifuge tube followed by mixed gentle mixing and incubation at 65℃ for 10 minutes. Then, 700 μl Chloroform: Isoamyl Alcohol (24:1) were added and mixed the samples gently and centrifuged at 12,000 rpm for 2 minutes. The supernatant was collected and transferred to a new 1.5 mL sterile polypropylene centrifuge tube, followed by addition of equal volume of Phenol: Chloroform: Isoamyl Alcohol (25:24:1) samples were mixed gently and centrifuged at 12,000 rpm for 2 minutes. The supernatant was transferred to a new 1.5 mL sterile Eppendorf EP tube. DNA was precipitated by mixing the supernatant with 2X cold absolute ethanol. Precipitated DNA was collected by centrifugation at 12,000 rpm and 4℃ for 15 minutes, the supernatant was removed thoroughly. Pelleted DNA was washed

with 300 µl of 70% cold ethanol and recollected by centrifugation at 12,000 rpm and 4℃ for 15 minutes and to supernatant was discarded. Furthermore, residual supernatant was removed by transitory centrifugation for few seconds. Pelleted DNA was dried for 5-10 minutes in clean beach and resuspended in 100 µl TE buffer solution. DNA concentration and purity were measured using a NanoDrop ND-1000 photometer (Thermo Scientific). DNA integrity was checked on Agarose Gel Electrophoresis and stored at -20℃.

PCR amplification of 16S rRNA gene and sequencing

Amplification of the 16S rRNA gene was carried out using the primers (16S rRNA V3-V4 341F: 5'-CCTAYGGGRBGCASCAG-3' and 16S rRNA V3-V4 806R: 5'-GGACTACNNGGGTATCTAAT-3') with a standard PCR protocol[59]. The PCR amplification program was performed as following: 95℃ for 5 min, 27 cycles, where 1 cycle was consisted of 95℃ for 30 s (denaturation), 55℃ for 30 s (annealing), 72℃ for 45 s (extension), and a final extension at 72℃ for 7 min. The quality of PCR products was verified by 1% agarose electrophoresis gel. PCR products were purified with a PCR fragment purification kit (TaKaRa Biotech, Japan). The Illumina high-throughput sequencing (Hiseq 2500 PE250) technique was used to study the diversity of epiphytic bacteria associated with *G. lemaneiformis*. Sequencing and bioinformatic analysis were performed at the Total Genomics Solution, Shenzhen, China. The sequence data reported in this study have been deposited in the NCBI GenBank database under the accession number SRP198594. All SRA data are available: https://www.ncbi.nlm.nih.gov/sra/PRJNA543182.

Denoising of sequence data

Perl script was used to split the offline data into different sample data according to Barcode sequence, and Barcode sequence and PCR primer sequence were cut off[60]. Splicing of PE Reads followed the following steps[61]: (1) The bases below the mass value of 20 at the end of the read were filtered and a 50 bp window was set. If the average mass value in the window was less than 20, the back-end base was cut off from the window and the read below 50 bp after quality control was filtered. (2) According to the overlap relationship between PE reads, a pair of reads was merged to form a sequence, and the minimum overlap length was 10 bp. (3) The allowable maximum mismatch ratio of overlap region of the splice sequence was 0.2 and non-conforming sequences were screened. (4) Samples were distinguished according to the barcode at both ends of the sequence and the primer, and the sequence direction was adjusted. The mismatch number allowed by barcode was 0, and the maximum primer mismatch number was 2. The Tags sequences obtained after the above processing are compared with the database (Gold database, http://drive5.com/uchime/uchime_download.html) to detect the chimera sequences, and finally the chimera sequences were removed to get the final Effective Tags[62].

OTU and species communities analysis

Uparse software (Uparse v7.0.1001, http://drive5.com/uparse/) was used for performing 97% identify clustering of all Effective Tags sequences of all samples to form OUT[63]. The representative OTU sequences was selected and compared with GreenGene (16S chloroplast, mitochondria, http://greengenes.secondgenome.com/), RDP (16S, http://rdp.cme.msu.edu/), Silva (18S, http://www.arb-silva.de) and Unite (ITS, http://unite.ut.ee/index.php) database by using the assign_taxonomy.py (http://qiime.org/scripts/assign_taxonomy.html) script and RDP Classifier method[64] in Qiime to obtain species annotation information (the default confidence threshold was above 0.8). OTU and its Tags annotated as chloroplast or mitochondria and not annotated to the boundary level were removed. Perl script was used to carry out statistics of Effective Tags data of each sample, low-frequency Tags data, Tags annotation data, data annotated by chloroplast and mitochondria, and the number of OTU obtained for each sample[65]. At the same time, R software was used to carry out statistics of the annotation proportion of each classification level of OTU and the relative abundance of species in each classification level. R software was used to draw the relative abundance heatmaps of OTU level and genus level, and cluster analysis between samples and species was also performed.

Alpha and Beta diversity analysis

Based on the uniform OTU abundance table, alpha_diversity.py (http://qiime.org/scripts/alpha_diversity.html) script in the QIIME software package was used to calculate four diversity indexes (Observed species, Chao, Shannon, PD_whole tree)[66]. Among them, the calculation of PD_whole tree index required the phylogenetic tree data of OTU. Jackknifed_beta_diversity.py (http://qiime.org/scripts/jackknifed_beta_diversity.html) script in the QIIME software package was used to calculate three beta diversity distances (Bray Curtis, Weighted Unifrac, Unweighted Unifrac) based on the homogeneous OTU abundance table[67]. For nMDS, R software was used for nMDS analysis, and drawing was based on the uniform OTU abundance table[68].

Correlation analysis of environmental factors

For redundancy analysis (RDA), the RDA function in the vegan package was used for ranking analysis[48]. Through envfit function, r^2 and P values of the influence of each environmental factor on species distribution could be calculated, and then RDA analysis could be performed by screening out environmental factors with significant influence. By means of the ‘bioenv’ function in the vegan package, the environmental factor or combination with the largest correlation with the species matrix (Spearman) could be screened out, and the environmental factor obtained by screening could be analyzed by targeted RDA. For Spearman correlation, Spearman correlation values of species and environmental factors were calculated by using the ‘Corr. test’ function of psych package in R and its significance was tested[30]. The pheatmap function in pheatmap package was used for visualization. The vegan package in R was used for the Mantel test[69]. Vegdist function was used to transform the distance matrix for ‘species matrix’ and

‘environmental factor data matrix’, and then Spearman correlation analysis was conducted for the two types of matrices with the Mantel function to obtain r and P values.

Functional annotation analysis

Based on 16SrDNA sequencing, PICRUTs (http://huttenhower.sph.harvard.edu/galaxy/root?tool_id=PICRUSt_normalize) was used to predict the function of microbial communities[70]. The functional abundance table of each level was obtained. R software was used to draw the heatmap of functional abundance spectrum. R software was used for species, functional consistency analysis and mapping.

Statistical analysis of data

The physicochemical factors of seawater and the alpha-diversity of epiphytic bacterial communities were analyzed using one-way ANOVA. The significant difference analysis of data was carried out using SPSS 19.0 software, with significant threshold set to 0.05. The difference among the epiphytic bacterial communities at the three different geographic locations were performed by the Permutational Multivariate Analysis of Variance (PERMANOVA) with Adonis function from the vegan package in R software[30].

Declarations

Competing interests

The author(s) declare no competing interests.

Data Availability Statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary Material.

Author Contributions

The authors’ contribution to the manuscript were as following: PP, HD and MA design the experiment. PP conducted the experiment, PP and HL collected the samples and analyzed the data. PP, HD, MA, HW and XL wrote and revised the manuscript. All authors discussed the results and agreed for its submission for publication.

Funding

This research was supported by the National Natural Science Foundation of China (41806168 and 41976125), the China Agriculture Research System (CARS-50), and Key Special Project for Introduced Talents Team of Southern Marine Science and Engineering Guangdong Laboratory (Guangzhou) (GML2019ZD0606).

Acknowledgments

We thank Prof. Weizhou Chen of Shantou university and boffin and Qi Lin of Fisheries Research Institute of Fujian for their help with sampling, Prof. Hui Wang for data analysis and Prof Edmond Sanganyado for providing assistance in English editing.

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Tables

Table 1 Mantel correlations highlight the relationship shared between environmental factors and communities composition at phylum and genus level. Bold-faced entries indicate significant vales in which $p < 0.01$.

Environmental factors	*Phylum*		*Genus*	
	Mantel score (r)	P-*value*	*Mantel score (r)*	P-*value*
Temp (°C)	0.071	0.172	**0.325**	**0.001**
pH	0.036	0.308	**0.388**	**0.001**
Sal (ppt)	0.046	0.291	0.093	0.133
DO ($mg \cdot L^{-1}$)	0.065	0.173	**0.372**	**0.001**
EC ($\mu s \cdot cm^{-1}$)	0.037	0.327	0.095	0.155
TDS (ppm)	0.038	0.327	0.074	0.153
NH_4-N ($mg \cdot L^{-1}$)	-0.097	0.227	0.051	0.271
NO_3-N ($mg \cdot L^{-1}$)	**0.352**	**0.002**	**0.299**	**0.002**
NO_2-N ($mg \cdot L^{-1}$)	**0.625**	**0.001**	**0.732**	**0.001**
DIN ($mg \cdot L^{-1}$)	**0.308**	**0.007**	**0.249**	**0.008**
TN ($mg \cdot L^{-1}$)	**0.330**	**0.004**	**0.445**	**0.001**
TP ($mg \cdot L^{-1}$)	**0.395**	**0.002**	**0.460**	**0.001**

Figures

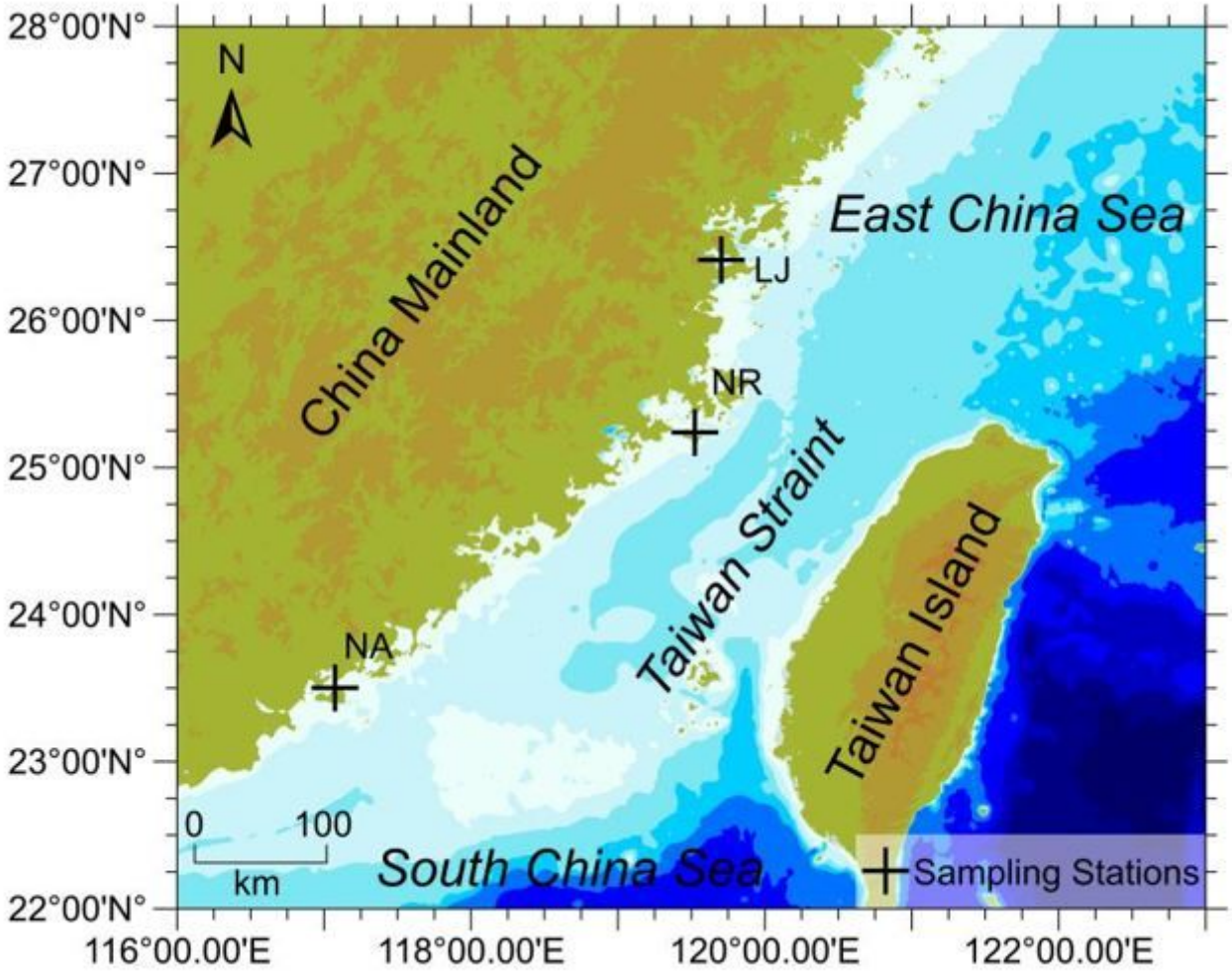

Figure 1

Sampling of G. lemaneiformis from three different geographic locations. The red circle represents Nan'ao Island, the blue circle represents Nanri Island, and the green circle represents Lianjiang County. Note: The designations employed and the presentation of the material on this map do not imply the expression of any opinion whatsoever on the part of Research Square concerning the legal status of any country, territory, city or area or of its authorities, or concerning the delimitation of its frontiers or boundaries. This map has been provided by the authors.

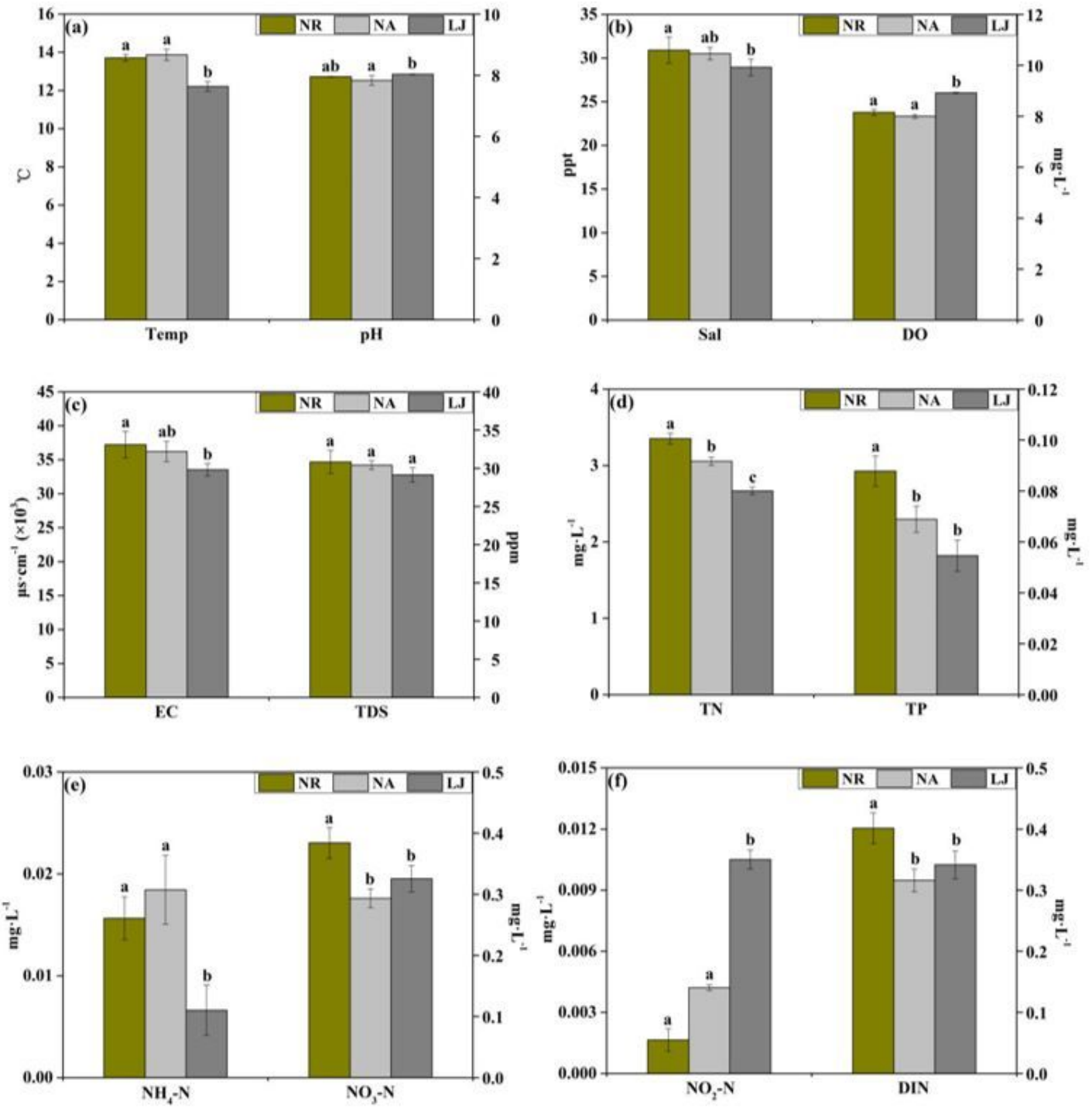

Figure 2

Average (±SD of nine replicates) value of environmental factors in seawater surrounding the G. lemaneiformis from three locations. In each piece of data, y-axis on the left corresponds to the environmental factors on the left, and y-axis on the right corresponds to the environmental factors on the right. Different letters (a, b, c) denote significant ($p < 0.05$) differences in mean value between NR, NA, and LJ in seawater samples.

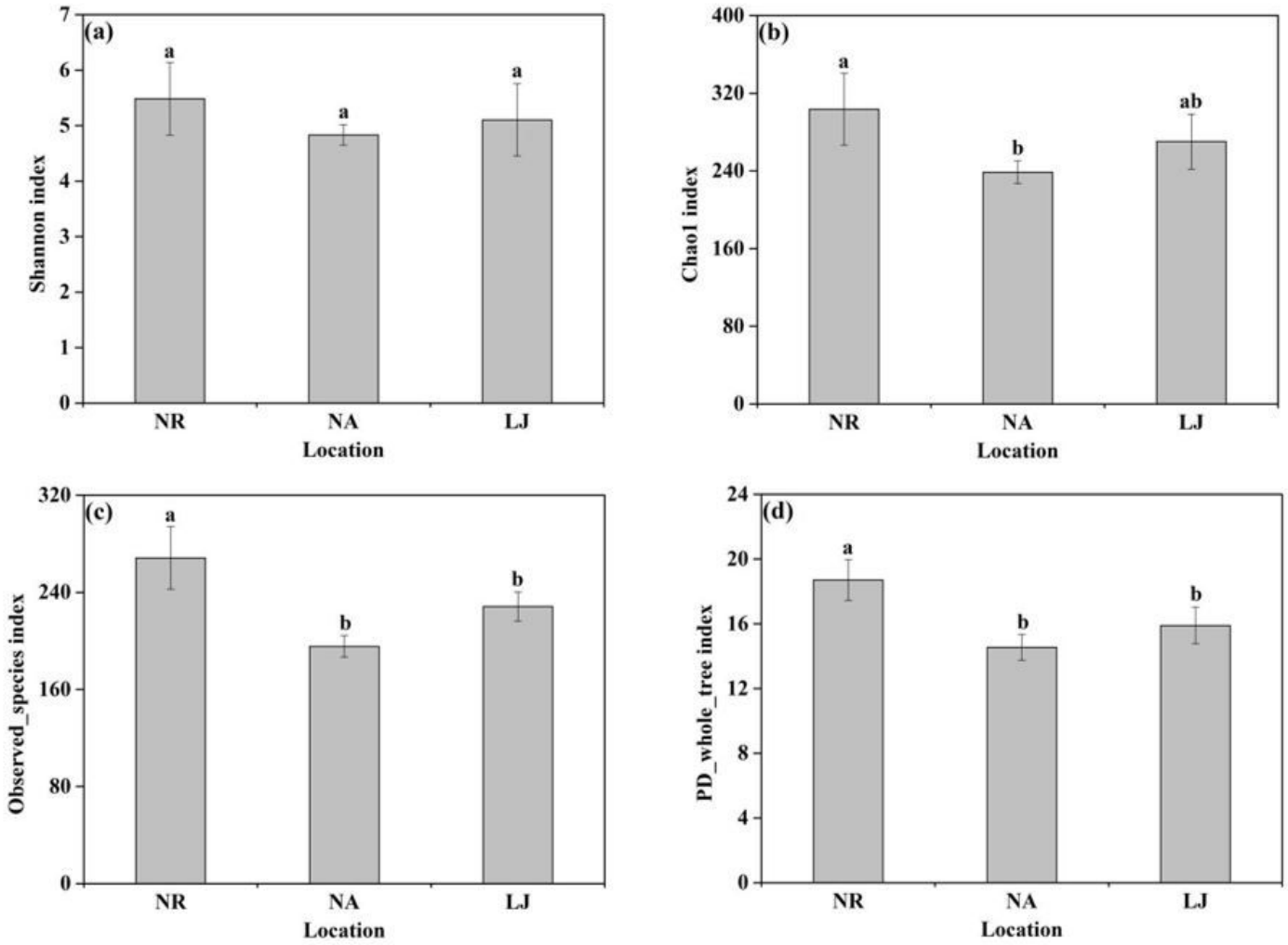

Figure 3

Shannon index (a), Chao1 index (b), Observed_species index (c) and PD_whole_tree index (d) showed the α-diversity of epiphytic bacterial communities on G. lemaneiformis at NR, NA, and LJ. Different letters (a, b) denote significant ($p < 0.05$) differences in mean value between NR, NA, and LJ in G. lemaneiformis samples.

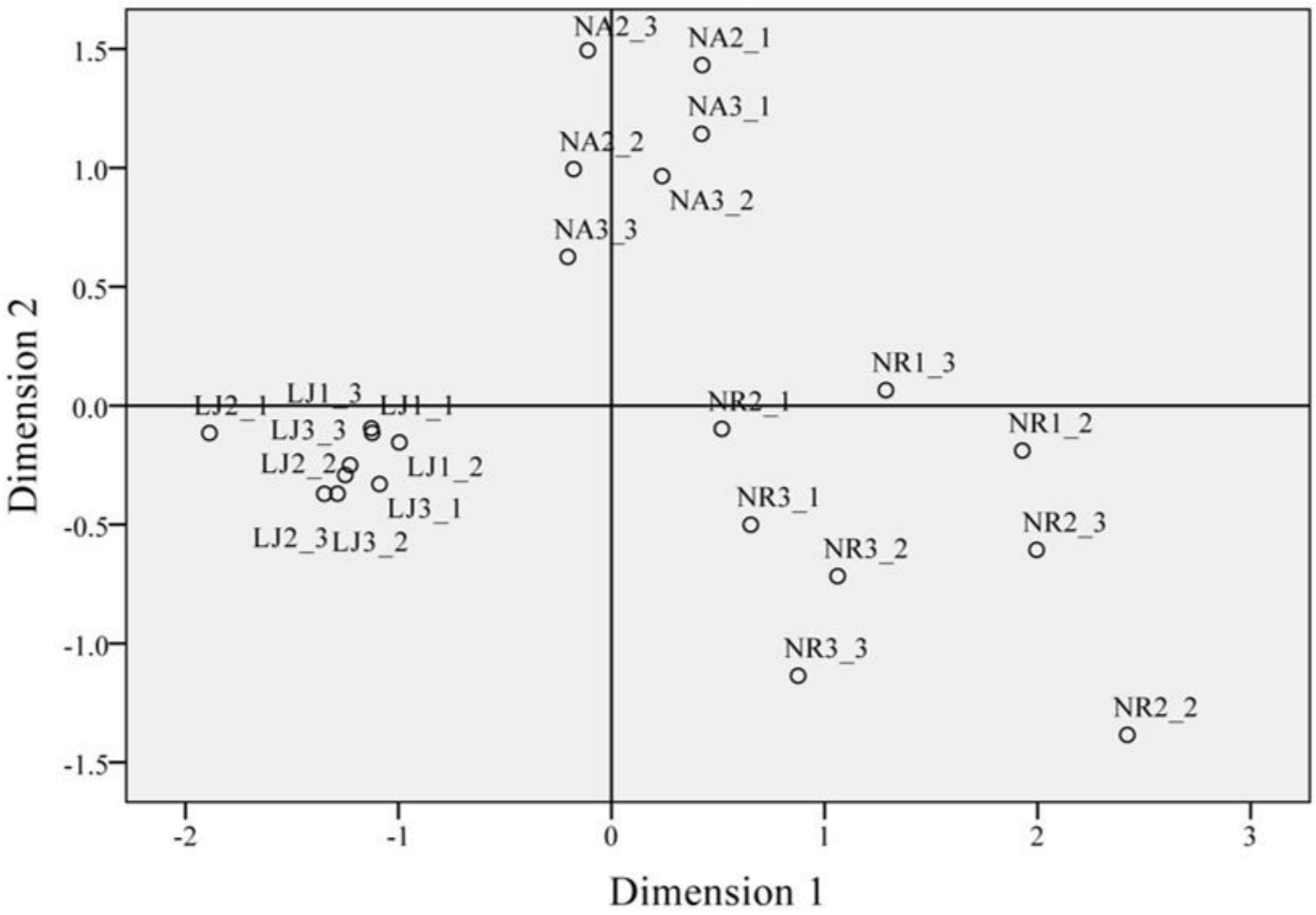

Figure 4

Non-metric multidimensional scaling (nMDS) of based on Bray-Curtis measure. nMDS showed the differences and similarities of epiphytic bacterial communities on G. lemaneiformis at NR, NA and LJ.

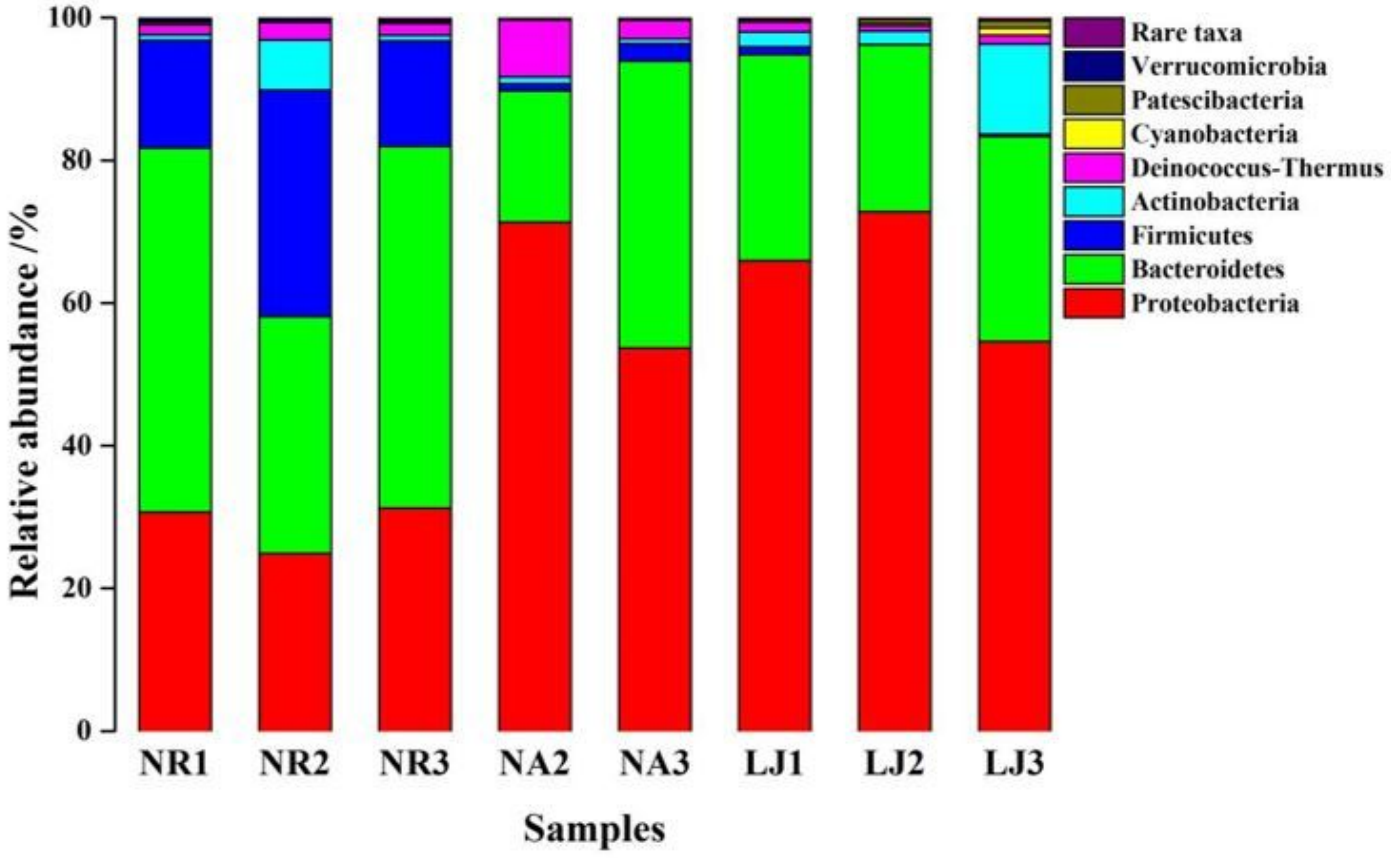

Figure 5

The epiphytic bacterial communities composition at phylum level on G. lemaneiformis at NR, NA, and LJ. Species richness represented less than 0.5% of the total bacteria in all samples were grouped into Rare taxa.

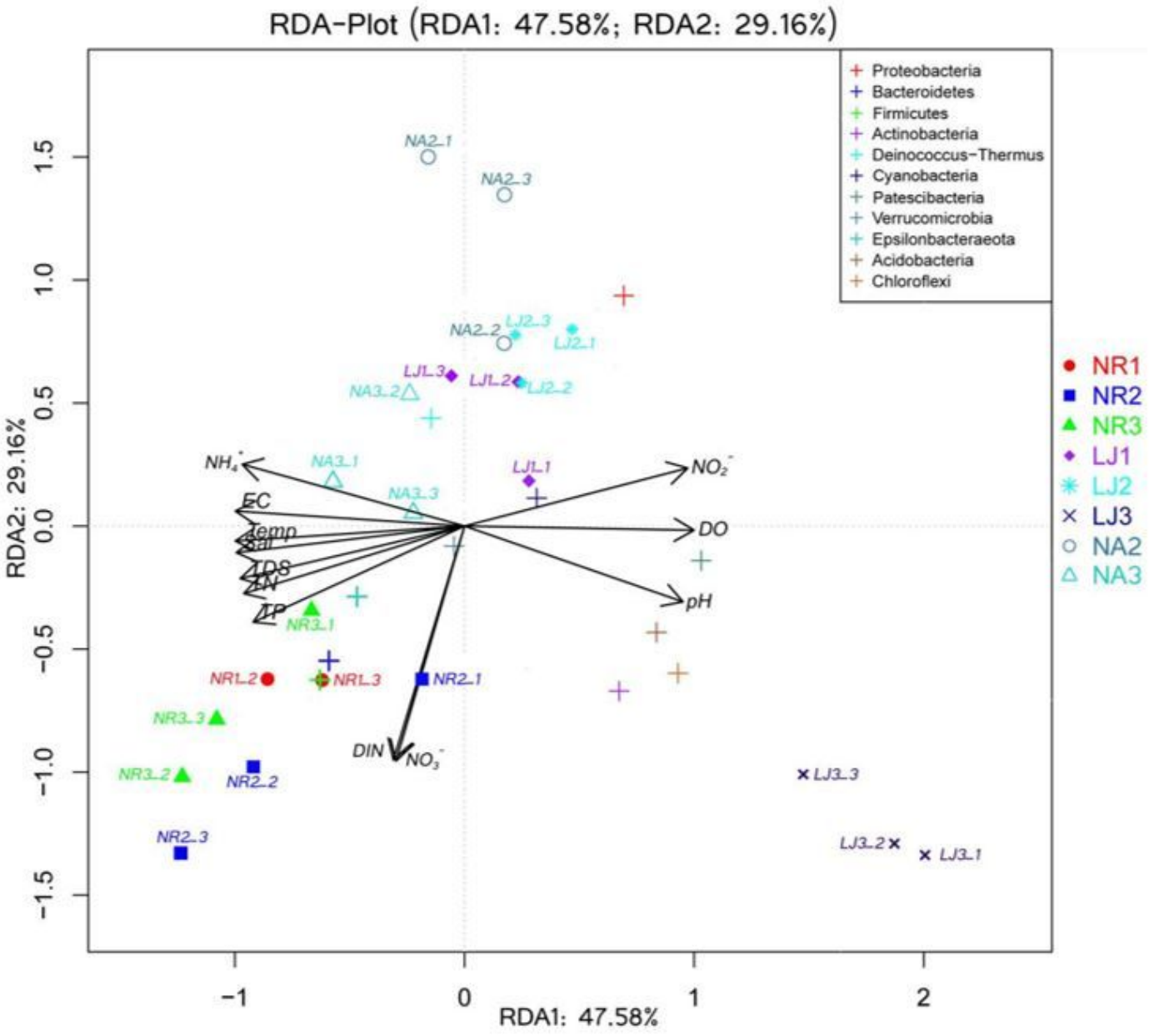

Figure 6

Redundancy analysis (RDA) of phyla (different color plus sign) in samples of epiphytic bacteria on G. lemaneiformis and environmental factors (black arrows) at NR, NA, and LJ.

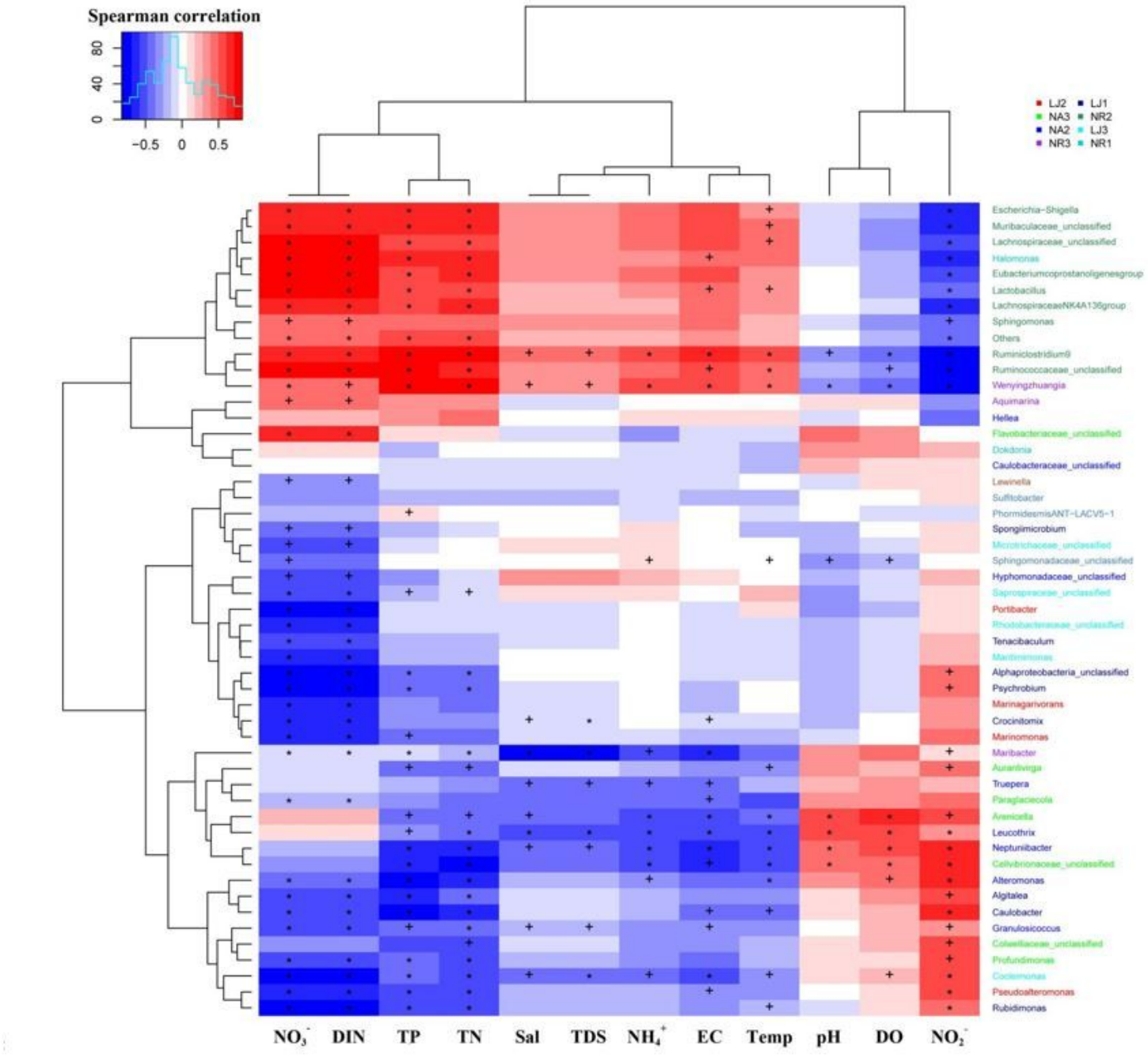

Figure 7

Heat maps indicated correlation between top 50 bacterial genera in species abundance of epiphytic bacteria on G. lemaneiformis and environmental factors in seawater. X and Y cladogram depict environmental factors and species clustering tree, respectively. Values of Pearson's correlation coefficients are color coded in the heat map legends. The species on the right of figure are color coded from different samples. '+' represents significant differences ($p < 0.05$), '*' represents extremely significant differences ($p < 0.01$).

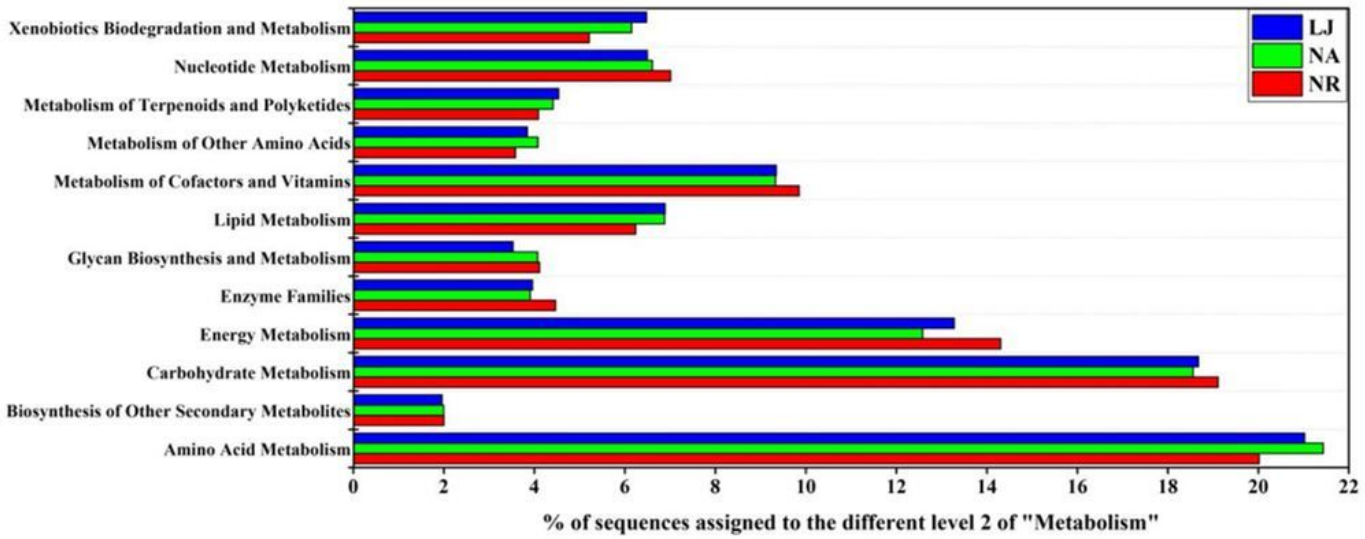

Figure 8

Percentage of metagenomic sequences of functional composition of Level 2—Metabolism—of the epiphytic bacterial communities on G. lemaneiformis at NR, NA, and LJ.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryMaterials.doc