The indication of soil microorganism to wetland restoration in Three River Source Area

Jiankang Ling¹,², Xufeng Mao¹,²*, Xiaoyan Wei¹,³, Wenjia Tan⁴, Yi Wu¹,², Yanchun Zhang¹,², Xianxia Bao¹,² and Lingling Tong¹,²

¹ Academy of Plateau Science and Sustainability, School of Geographical Science, Qinghai Normal University, Xining, 810008, China
² Qinghai Key Laboratory of Physical Geography and Environmental Processes, MOE Key Laboratory of Tibetan Plateau Land Surface Processes and Ecological Conservation, Xining, 810008, China
³ School of Economics and Management, Qinghai Normal University, Xining, 810008, China
⁴ Department of Ecological Environment Protection 810008, Xining, China

*Corresponding author: 2025106@qhnu.edu.cn

Abstract. Post-assessment of wetland restoration projects offers insight into project efficiency, effectiveness and sustainability. In order to evaluate the wetland restoration in three river source area, Qinghai Province, 16SrRNA high-throughput sequencing, OTU analysis, Alpha diversity analysis, etc., were used to analyze the soil microorganisms in the three restoration measures. Then, the species distribution and species richness cluster information were obtained. Compared with the wetland without any restoration measures, the results showed that the soil health index in three plots was higher or lower, which was specifically reflected in: replanting plot > rodent control plot > enclosure plot. Finally, the health status of alpine meadow wetland was comprehensively analyzed. The results in this work can offer reference for the restoration and protection of meadow wetland in Qinghai-Tibet Plateau.

1. Introduction

The Qinghai-Tibet Plateau, known as the "roof of the world", is the largest plateau in China and the highest in the world. Its unique geographical location and climate have created a vast grassland area on the Qinghai-Tibet Plateau, of which alpine meadow accounts for the largest proportion [1]. According to the annual total ecosystem service value offered by natural grassland resources in Qinghai-Tibet Plateau, the contribution rates of alpine meadow, mountain meadow and alpine grassland to grassland ecosystem service value were 62.52%, 14.14% and 12.92%, respectively. Therefore, the ecological status of alpine meadow is directly related to the ecological quality of natural grassland in Qinghai-Tibet Plateau [4]. It is particularly important for the ecological restoration of alpine meadow [5, 6]. The study on local soil microorganism is helpful to better understand the habitat condition and ecological restoration effect.

Soil microorganism is a general term of microorganisms in soil that can not be seen or recorded directly by naked eyes. It mainly includes bacteria, fungi, viruses, protozoa, eukaryotes, etc., yet the living roots of plants are not included in it. Previous studies have shown that soil is a natural medium,
which is most suitable for microbial growth and reproduction. On a global scale, there are tens of thousands of microbial species in the soil, and the number is even more amazing [7]. A wide variety of soil microorganisms are essential for the biogeochemical cycling of biosphere elements and inorganic ions [8]. Therefore, it is of great significance to study the composition, species and number of soil microorganisms. Apart from a small number of soil microorganisms can be cultivated in the laboratory, most of them are difficult to use traditional methods. With the popularity of high-throughput sequencing and the progress of metagenomics research technology, people can gain a new understanding for soil microorganisms through emerging technologies. Metagenome refers to the total amount of genetic material contained in the whole environment [9]. Its development overcomes the issue that it is impossible to cultivate microorganisms in the laboratory. By analyzing the genetic information of all the microorganisms in the soil for bioinformatics analysis, the diversity of microorganisms in the environment was studied. In this study, the second generation high-throughput sequencing technology was used to obtain the soil microorganisms information of each sample plot under different restoration measures, and then the soil microorganisms status was comprehensively analyzed to offer suggestions for the protection of alpine meadow.

2. Materials and methods

2.1. Overview on study area
The experimental study area is located at 2000 meters southeast of Dawu town, Maqin county, Guoluo prefecture and Longbao town, Yushu city, China. The geographical coordinates are 100º12'58.3"E, 34º27'53.9"N, 96º35'17.0"E, 33º09'47.2"N. It is a national key research and development project (2016YFC0501900). The sample plot is located in the core area of three river source national nature reserve, with an average annual rainfall of 423~565mm, with only alternating cold and warm seasons. It belongs to a typical alpine meadow wetland ecosystem.
In August 2018, alpine meadow wetlands with the same degree of degradation were randomly selected as the research samples in Maqin county and Longbao town. The selected sampling sites include rodent control plot, rodent control + non-woven fabric plot, enclosure plot, enclosure + non-woven fabric plot, grass planting plot, native marsh wetland, mild degradation sample plot, moderate degradation sample plot, severe degradation sample plot, sprinkler irrigation sample plot, enclosure sample plot and moderate degradation contrast sample plot. Three 0-30cm soil samples were taken from each sample plot by S-type point distribution method, and the soil samples were combined with different sampling points and divided into bags according to the depths of 0-10cm, 10-20cm and 20-30cm. They were put into sterile and airtight plastic bags and refrigerated at -20℃ to bring them back to the laboratory. A total of 36 samples were obtained in this sampling. In order, 27 samples between M110-M930 were numbered Q3-Q29, and 9 samples between L110-L330 were numbered Q30-Q38.

2.2. Research method

2.2.1. 16SrRNA functional gene predictive analysis. In this study, the primers were used to amplify the V3+V4 Illumina gene of 16SrRNA gene in soil samples for microbial sequencing and information analysis. The most common primers were 338F: 5’-ACTCCTACGGGAGGCAGCA-3’ and 806R: 5’-GGACTACHVGGGTWTCTAAAT-3’.

2.2.2. OTU analysis. OTU (Operational Taxonomic Units) is an operational taxon, which is the same mark set for a taxon (strain, species, genus, grouping, etc.) for the convenience of analysis in phylogenetic research or population genetics research. In bioinformatics analysis, each sequence obtained by sequencing comes from a bacterium. In order to know the number of strains and genera in a sample sequencing result, it is necessary to cluster the sequence. Through the classification operation, the sequence is divided into many groups based on their similarity, and a group is an OTU. Generally,
all sequences are divided into OTUs according to 97% similarity.

2.2.3. Alpha diversity analysis. Alpha diversity is used to analyze the internal complexity of a single sample, which reflects the richness and diversity of a single sample. There are many measurement indexes: Chao1, ACE, Shannon and Simpson.

Chao1 index is used as an index to measure species richness in ecology. It is mainly used to estimate the OTU number index in the community. The larger the value is, the more species the index will be. The formula is:

\[
\text{Chao1} = \frac{S_{\text{obs}} + n_1(n_1 - 1)}{2(n_2 + 1)}
\]

Among them, \(\text{Chao1}\) is the estimated OTU; \(S_{\text{obs}}\) is the observed OTU; \(n_1\) is the number of OTUs with only one sequence (i.e., "single"); \(n_2\) is the number of OTUs with only two sequences (i.e., "double precision"). The larger the Chao1 index, the higher the community richness. The effects of Ace index and Chao1 index were similar.

Shannon index and Simpson index are used to measure species diversity. Shannon index indicates the degree of community diversity. The higher the value, the higher the diversity. The Simpson index is the microbial diversity index in the sample, and the higher the value, the lower the community diversity. It was affected by species richness and community evenness. In the same species richness, the greater the evenness of each species in the community, the greater the diversity of the community. The larger the Shannon index and the smaller the Simpson index, the higher the species diversity.

2.2.4. Information analysis process. Based on the Overlap relationship between PE reads, the double ended sequence data obtained by Hiseq sequencing were merged into a sequence Tags, and the Reads quality and Merge effect were quality controlled and filtered. FLASH v1.2.7 software is used to splice the Reads of each sample through Overlap, and the splicing sequence is Raw Tags data. Then, Trimmomatic v0.33 software is used to filter Raw Tags splitted to get high quality Clean Tags. Finally, UCHIME v4.2 software is used to identify and remove chimeric sequences to get the final Effective Tags.

3. Results and analysis

A total of 3,026,592 pairs Reads were obtained from 36 samples. After splicing and filtering, 2,548,382 Clean Tags were generated. Each sample produced at least 58,279 Clean Tags, with an average of 67,063 Clean Tags.

3.1. Alpha diversity analysis

3.1.1. Alpha diversity index statistical analysis. As shown in Table 1, Coverage of each sample has reached 99%, indicating that almost all sample sequences have been detected and reached saturation state. It also reflects the authenticity of the composition and diversity in soil microbial flora. The Coverage index is close to 100%, which proves the reliability of the experimental data. The higher the OTU coverage index, the higher the probability of species detection. The index reflects whether the sequencing results represent the real situation of microorganisms in the sample.

Of all the samples, the Chao1 value in M920 is the largest, 1253.1143, indicating that the microflora richness in the soil of 10-20cm depth in M9 sample plot is the largest. The Shannon value in M230 is the largest, 6.0101, and the Simpson value is 0.0056, belonging to the lowest value hierarchy, indicating that the microbial community diversity in the soil of 20-30cm depth in M2 sample plot is the highest.
Table 1. Alpha diversity index statistics

| Sample ID | OTU   | ACE   | Chao1  | Simpson | Shannon | Coverage |
|-----------|-------|-------|--------|---------|---------|----------|
| L110      | 739   | 915.3169 | 924.6707 | 0.0685 | 4.3311 | 0.9962   |
| L120      | 605   | 686.1096 | 689.6418 | 0.0807 | 4.0272 | 0.9974   |
| L130      | 985   | 1,049.7058 | 1,048.5938 | 0.0129 | 5.3604 | 0.9975   |
| L210      | 1,113 | 1,172.8721 | 1,194.5316 | 0.0151 | 5.6311 | 0.9972   |
| L220      | 1,034 | 1,101.7448 | 1,123.3214 | 0.0108 | 5.5398 | 0.9972   |
| L230      | 1,020 | 1,091.1112 | 1,110.1163 | 0.0131 | 5.372  | 0.9971   |
| L310      | 1,062 | 1,138.0702 | 1,161.75  | 0.0101 | 5.5415 | 0.9971   |
| L320      | 1,113 | 1,183.7225 | 1,190.8919 | 0.013  | 5.4427 | 0.997    |
| L330      | 1,110 | 1,188.1238 | 1,196.9712 | 0.0108 | 5.304  | 0.9969   |
| M110      | 1,111 | 1,158.165  | 1,163.5612 | 0.0266 | 5.3854 | 0.9973   |
| M120      | 1,122 | 1,173.7079 | 1,182.9785 | 0.0162 | 5.6666 | 0.9971   |
| M130      | 972   | 1,055.1828 | 1,093.9167 | 0.0084 | 5.6074 | 0.9967   |
| M210      | 1,121 | 1,178.9185 | 1,198.6947 | 0.0072 | 5.8326 | 0.9963   |
| M220      | 1,169 | 1,210.1079 | 1,229.6375 | 0.0059 | 5.9896 | 0.9971   |
| M230      | 1,163 | 1,199.0532 | 1,212.88  | 0.0056 | 6.0101 | 0.9975   |
| M310      | 1,048 | 1,099.9142 | 1,132.0   | 0.0143 | 5.5521 | 0.9975   |
| M320      | 1,153 | 1,204.5247 | 1,234.0143 | 0.0061 | 5.9653 | 0.9973   |
| M330      | 1,078 | 1,188.1238 | 1,196.9712 | 0.0096 | 5.7123 | 0.9971   |
| M410      | 1,169 | 1,209.3848 | 1,246.2881 | 0.0066 | 5.9249 | 0.9978   |
| M420      | 1,104 | 1,144.8383 | 1,158.1216 | 0.0058 | 5.939  | 0.9977   |
| M430      | 1,032 | 1,094.1828 | 1,109.6024 | 0.0081 | 5.7    | 0.9971   |
| M510      | 1,120 | 1,164.4363 | 1,180.6375 | 0.0073 | 5.783  | 0.9975   |
| M520      | 1,119 | 1,178.4443 | 1,194.8571 | 0.0384 | 5.2063 | 0.9977   |
| M530      | 1,102 | 1,180.4786 | 1,204.9286 | 0.0077 | 5.7921 | 0.9966   |
| M610      | 751   | 865.4409 | 877.3636 | 0.0454 | 4.5872 | 0.9965   |
| M620      | 698   | 805.9746 | 817.5294 | 0.0396 | 4.7988 | 0.9967   |
| M630      | 702   | 809.1552 | 856.8182 | 0.0409 | 4.7538 | 0.9971   |
| M710      | 1,060 | 1,132.3526 | 1,162.2727 | 0.011  | 5.5722 | 0.9969   |
| M720      | 1,058 | 1,133.1371 | 1,160.2561 | 0.022  | 5.3206 | 0.997    |
| M730      | 1,117 | 1,185.9579 | 1,211.53  | 0.0156 | 5.5733 | 0.9952   |
| M810      | 1,138 | 1,192.3471 | 1,225.8889 | 0.0121 | 5.7002 | 0.9973   |
| M820      | 1,100 | 1,157.6435 | 1,176.2209 | 0.0077 | 5.7314 | 0.997    |
| M830      | 1,036 | 1,142.5954 | 1,174.3333 | 0.0268 | 5.026  | 0.996    |
| M910      | 1,149 | 1,191.1219 | 1,205.0964 | 0.0133 | 5.6748 | 0.9977   |
| M920      | 1,192 | 1,230.3378 | 1,253.1143 | 0.0065 | 5.9695 | 0.9977   |
| M930      | 1,230 | 1,273.5957 | 1,302.8   | 0.0054 | 5.9675 | 0.9975   |

3.1.2. Soil microorganism species diversity. Figure 1 shows the OTU number of 36 samples in 12 sample plots. UCLUST in QIIME software is used to cluster Tags at 97% similarity level to obtain OTUs. OTUs are annotated based on SILVE (bacteria) and UNITE (fungus) taxonomic databases. Obviously, the number of OTUs in different plots is different. The total number of OTUs obtained in this experimental sample is 1530. The abscissa is the sample name, and the ordinate is the number of OTUs.
3.1.3. Analysis on dilution curve results. As shown in Figure 2, when sampling Reads are below 20,000, most curves show a sharp increase with the increase of sequencing reads, indicating that a large number of different microbial communities are found in the soil sample. A small part of the curve is more general, and the two curves in the whole process are flat state. Between 20,000 and 40,000, the sequence number of the curve increases slightly, indicating that a small amount of bacteria is still detected in the soil samples of the environment. When Reads in sample exceed 40,000, the curve shows an upward trend, which indicates that the microorganisms detected in this test has not reached saturation yet, and more sequencing has a greater contribution to the discovery of new OTU margins.
3.2. Microorganism composition analysis

3.2.1. Species distribution histogram. Based on the taxonomic analysis results, the community structure composition in different samples at different taxonomic levels can be known. Figure 3 shows the species distribution histogram at genus level, and the abscissa is the sample name and the ordinate is the proportion of species in the sample (i.e., the species distribution histogram in each sample). One color represents a species, and the length of the patches indicates the proportion of species in relative abundance. For the best view, only the top ten species in abundance level are shown, and other species are merged into Others for display in the Figure. It can be seen from the Figure 3 that more than 50% of the samples are other species and species without taxonomic annotation, and the top ten species are relatively small.

![Species distribution histogram at genus level](image)

3.2.2. Species abundance clustering heat map. Heat map is a graphic display method that represents the data size by the color depth in the graph, and clusters the species with high abundance and low abundance in blocks, and reflects the similarity and difference of each sample community composition by different colors and similarity degrees. The graph is drawn by R language, and finally the heat map clustering analysis is carried out at the door classification level. In the abundance clustering heat map, the horizontal clustering is the sample information, and the vertical clustering is the species information. The left cluster tree is species clustering tree, and the upper cluster tree is sample clustering tree. The color represents the species abundance, and the longitudinal clustering represents the similarity of the different species abundance in each sample. The closer the distance between two species is, the shorter the branch length is, indicating that the abundance of these two species is more similar in each sample. Horizontal clustering shows the similarity of species richness in different samples. The closer the distance between the two samples is, the shorter the branch length is, indicating that the species richness in the two samples is more similar.

According to the composition and relative abundance of the 36 samples, it can be seen from Figure 4 that the species heat map analysis showed that there were significant differences in the dominant bacterial communities in each sample point. The dominant species at different depths in the same sample point are mostly consistent. For example, the dominant bacteria in Maqin No.6 are mainly...
Ignavibacterium, Nitrospinae, Bacteroidetes, Aminicenantes, etc. However, the dominant bacteria in Maqin are mainly Lactobacillus, GAL15, Gemmatimonadetes and Planctomycetes, which showed a trend of aggregation and relatively concentrated. The depth of some sample points also affects the distribution of bacteria. For example, Saccharibacteria is the dominant bacteria in Lombard 3 between 0-10cm, and Gemmatimonadetes is the dominant bacteria in Lombard 3 between 10-30cm.

![Figure 4. Species abundance clustering heat map](image)

3.3. Recovery of soil microbial-related indexes

The recovery of soil factors closely related to soil microorganisms is shown in Figure 5. In 2017, the health scores of soil factors before recovery showed a downward trend, with only a few factors showing a slight increase. Based on the vegetation restoration, the indexes of the replant plots with the largest recovery rate in 2017 decreased the most. In the rodent control plots with the worst vegetation restoration, there were two factors that increased the health score, which was the most that increased the health level of soil factors among the three plots [10]. In 2018, the scores of replant and rodent control increased apart from the enclosure plot. Similarly, the soil nutrients of the enclosed sample plots with the largest vegetation recovery in 2018 decreased more compared with the vegetation restoration. Secondly, the vegetation restoration of rodent control plot was inferior to that of enclosure plot, higher than that of replant plot, and the recovery range of soil factors in rodent control plot was between the two.

![Figure 5. Restoration of soil-related factors in different restoration plots](image)
4. Conclusion

The composition of soil microbial community is complex, and it is characterized by large number and variety. Currently, there are few studies on soil microbial high-throughput sequencing for wetland restoration evaluation. In this paper, 16SrRNA gene amplification technology was used to investigate the soil microbial environment in alpine meadow wetland system, and the changes of soil factors under different treatment measures were sorted out. Finally, the following conclusions were drawn:

First, it can be found that the soil microorganism richness in sample 9 was the highest through different Chao1 index sizes. Based on Shannon index and Simpson index analysis, the soil microorganism community diversity of No.2 sample site was the largest.

Second, there was a certain difference in the number of environmental soil OTU between different points, amounting to 1530. Compared with the number of OTUs in the degraded plots, the number of OTUs in the sample plots after the measures were taken was larger, indicating that the microbial species abundance in the soil was higher. This may be related to the increase in the number of animals and plants after the measures were taken.

Third, by analyzing the species abundance cluster heat map, it can be found that there are totally different dominant species in different sites of the same sample plot, and the functions of each species are different. If different flora correspond to different restoration measures in the later stage of wetland restoration, the natural or man-made restoration of wetland will get twice the result with half the effort.

Fourth, after one year's artificial restoration, it was found that both the replanting plot and the sunstroke prevention plot achieved harvest apart from the reduction of the health score under the closure measures. The results showed that the order of restoration measures was as follows: replanting plot > rodent control plot > enclosure plot. The following restoration measures could be combined with each other to carry out appropriate replanting under the premise of enclosure and rodent control.

In this study, the three restoration measures can have a positive impact on soil factors. However, the three measures effects are different, and thus the selection of restoration measures becomes particularly important. Through the analysis of microorganism information in three different soil environments, the healthy and normal soil microorganism composition was obtained, which offered a basis for regulating and developing related products in other areas or soil microorganism communities and promoting ecological restoration of plateau meadow wetlands.

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