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Metagenomic analysis reveals microbial diversity and function in the rhizosphere soil of a constructed wetland

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Microbial communities play a critical role in the degradation of effluent contaminants in constructed wetlands. Many questions remain, however, regarding the role of microbial communities in rhizospheric soil. In this study, we used metagenomic analysis to assess microbial community composition and function in a constructed wetland receiving surface water. The diversity of the microbial community of rhizosphere soil was found to be significantly greater than that of the wetland influent water. This enhancement is likely due to the availability of diverse habitats and nutrients provided by the wetland plants. From function annotation of metagenomic data, a number of biodegradation pathways associated with 14 xenobiotic compounds were identified in soil. Nitrogen fixation, nitrification and denitrification genes were semi-quantitatively analysed. By screening of manganese transformation genes, we found that the biological oxidation of Mn$^{2+}$ (mainly catalysed by multicopper oxidase) in the influent water yielded insoluble Mn$^{4+}$, which subsequently precipitated and were incorporated into the wetland soil. These data show that the use of metagenomic analysis can provide important new insights for the study of wetland ecosystems and, in particular, how biologically mediated transformation or degradation can be used to reduce contamination of point and non-point source wastewater.

Keywords: metagenomic; microbial community; water purification; rhizosphere; constructed wetland

1. Introduction

Wetlands have demonstrated a capacity to remove a variety of conventional and toxic pollutants from regional waterbodies.[1] Because of this ability, constructed wetland treatment systems are generally created to improve the quality of wastewater using natural biogeochemical processes mediated by specialized soil microorganisms.[2] Previous studies have shown that the abundant rhizosphere microorganisms in constructed wetlands play a crucial role in removing certain contaminants from water.[3,4] In the rhizosphere, where plant roots interact with soil and associated biota, microorganism activity and abundance are significantly enhanced, with plant roots providing favourable habitat for the growth of microbes.[5–7] Despite a recognition of the importance of the root-soil microorganisms, there remains a gap in the understanding of the interactions between microbial ecology and the cycling of nitrogen, carbon or heavy metals in constructed wetlands, and how those interactions impact performance optimization of the treatment systems.[8]

The majority of previous studies used 16S rRNA-based or specific function gene-based techniques to examine microbial community composition or function in wetlands.[9–11] These techniques, though highly useful, do not provide information on whole community physiology. With the rapid development of molecular techniques, metagenomic methods have proved increasingly valuable. The methods are based on high-throughput sequencing, and have allowed for identification and characterization of both composition and function of microbial communities.[12–14] Using a metagenomic technique, it may be possible to understand, in greater detail, how microbial communities transform and/or degrade the nutrients/pollutants in wetlands. In addition, the application of this technique may allow the use of microbial community composition and function as biological indicators of wetland health since microorganisms play a key role in wetland biogeochemical processes, and respond quickly to environmental disturbances.[11]

In this study we used a metagenomic approach, based on high-throughput sequencing, to explore microbial community composition and function in influent water (as a reference matrix) and reed rhizosphere soil of a constructed wetland. The objective of this study was to elucidate the potential role of microorganisms found in reed rhizosphere soil from a constructed wetland.

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2. Materials and methods

2.1. Sampling site

The influent and effluent water samples, and rhizosphere soil sample were obtained from the Shijiuyang water source ecological wetland in Jiaxing City, China (30°46′45″N, 120°42′29″E) (Figure 1(a)). This constructed wetland is the largest urban drinking water source treatment system (treating surface water) in China and has operated for more than four years. Wetland performance has been stable and effective to date.[15] Water and soil samples were collected from the wetland on 18 July 2012; ambient temperature at the time of collection was 25°C. The water samples, obtained from the wetland influent and effluent (1 L for each), were collected in sterile plastic containers. For the rhizosphere soil samples (Figure 1(b)), experimental manipulations were conducted within a 5 × 5 m square plot located at the centre of the wetland, in an area that was dominated by wetland reeds. About 50 g soil samples were collected approximately 30 cm below the reed soil surface by first removing the upper inorganic/organic layers and then gathering the soil with a clean trowel into sterile containers. Soil samples were homogenized in the laboratory. The properties of the water and soil samples were determined using standard methods described previously.[16,17]

2.2. DNA extraction

Approximately 200 ml of influent water were syringe-filtered through a 0.2 μm membrane (47 mm diameter, Milipore, Billerica, MA, USA). Total DNA from the influent water was extracted using a PowerWater DNA isolation kit (MO-BIO Laboratories, Inc., Carlsbad, CA, USA) according to the manufacturer’s instructions. Approximately 0.5 g of homogenized rhizosphere soil was used to extract DNA with a PowerSoil DNA isolation kit (MO-BIO). Three replicate DNA extractions of individual water or soil samples were performed. All DNA samples were stored at −80°C in a freezer for further molecular analysis.

2.3. Metagenomic analysis

Prior to metagenomic analysis, we pooled equal amounts of triplicate water or soil DNA samples. The DNA chains of two samples were initially treated to be short fragments (200 bp size) for constructing paired-end libraries. Cluster generation was performed on an Illumina eBot (Illumina Inc., San Diego, CA, USA) automated cluster generation system and the Illumina Hiseq 2000 sequencing system was used for sequencing (90 bp pair-end reads). Similar to our previous study,[18] the raw sequence reads were initially filtered to remove adapters and ambiguous reads. The filtered reads (~2 Gbps for each data set) were assembled using velvet software.[19] The assembled contigs were uploaded to the online MG-RAST (Meta Genome Rapid Annotation using Subsystem Technology, v3.2.2) for taxonomic classification and function annotation.[20] Microbial taxonomy was determined using the rRNA data set, with M5RNA as the reference database. The best-hit classification method was adopted with the following parameters: minimum identity confidence value, 80% and minimum alignment length, 50 bp. For function annotation, we focused on the metabolism of xenobiotic compounds, N and heavy metals (iron and manganese). For xenobiotic compounds and N, SEED subsystems and Kyoto Encyclopedia of Genes and Genomes (KEGG) annotation in MG-RAST with parameters [21] (minimum alignment length, 15 amino acids and E-value cutoffs, 1e-5) were used to identify sequences to different functional groups (SEED) or metabolic pathways (KEGG). Metabolic genes present in water and soil were identified and the abundances of those gene sequences were calculated. For Fe and Mn oxidation or reduction genes, we used Genebank as the reference database to search all the major genes involved in Fe and Mn transformation.

To compare community composition and function between the water and soil samples, Statistical Analysis of Metagenomic Profiles (STAMP) bioinformatics software [22] was adopted. Statistical significance was
calculated using a two-tailed Fisher’s exact test, and the differences between proportions were analysed using the asymptotic approach at a 95% confidence interval.

Two metagenomic data sets are deposited at NCBI Sequence Read Archive under the accession numbers SRX377439.

3. Results and discussion

Based on influent and effluent water quality, this constructed wetland provides effective treatment of surface water (Table 1). Organic, inorganic and heavy metal contaminant concentrations had a significant decrease. A small portion of nutrients/pollutants entering a wetland are typically removed via direct assimilation by plants. Most of contaminant treatment, however, occurs via microorganisms, especially rhizosphere soil microbes.

To elucidate the potential role of microbes in rhizosphere soil of the constructed wetland, we used a metagenomic technique based on Illumina sequencing, with influent water serving as the reference matrix. A total of 1,524,600 sequences with an average length of 149 bps (soil), and 794,465 sequences with an average length of 156 bps (water), were obtained after the velvet assembly. The two assembled metagenomic data sets were then uploaded to MG-RAST. Microbial community composition was assessed using 3483 (0.2%) identified rRNA sequences in the soil data set (2%) and 2106 rRNA sequences in the water data set (0.3%). Microbial community function was assessed using 780,016 protein sequences (51.2%) with known functions in the soil data set and 495,703 protein sequences (62.4%) in the water data set.

3.1. Microbial composition

From the results of taxonomic classification of rRNA sequences, three microbial domains were found in the rhizosphere soil: bacteria, archaea and eukaryota (Figure 2). Archaea were not present in the water sample. Among the domains, bacteria were predominant in two samples with 83.8% (soil) and 86.2% (water), respectively, suggesting that bacteria were primarily responsible for nutrient/pollutant removal. At the phylum level, 29 bacterial phyla were identified in soil and 20 phyla were identified in water (soil, 50% of all taxa in soil, 65% in water) was the most abundant phylum in both samples. Proteobacteria are important in natural and artificial environments, providing some basic functions related to global transformation of elements. This phylum also closely interacts with eukaryotes, occasionally assuming the roles of symbiont and/or pathogen.[23,24] The Proteobacteria in two samples consisted of five classes: Alphaproteobacteria, Betaproteobacteria, Gammaproteobacteria, Deltaproteobacteria and Epsilonproteobacteria. Among these, Gammaproteobacteria were dominant in both samples and are frequently observed in wetland soil.[25–27] Firmicutes was the second abundant phylum in soil (13%), but was less abundant in water (1%). Crenarchaeota and Eur-yarchaeota, the two major groups of known archaea, were only identified in soil. Eukaryotic phyla, including fungi, algae and protozoa, were found in soil and water. All of these major taxonomic groups play key roles in wetland function. Algae transform inorganic carbon into organic carbon. Prokaryotes, including bacteria and archaea, assimilate and mineralize organic matter. Protozoa feed on bacteria and algae.[28]

Table 1. Water properties of influent and effluent in the constructed wetland.

<table>
<thead>
<tr>
<th>Sample</th>
<th>DO (mg/l)</th>
<th>COD (mg/l)</th>
<th>TN (mg/l)</th>
<th>NH₃-N (mg/l)</th>
<th>Mn (mg/l)</th>
<th>Fe (mg/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Influent</td>
<td>7.28</td>
<td>5.98</td>
<td>5.65</td>
<td>4.50</td>
<td>1.36</td>
<td>0.25</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1.01</td>
</tr>
<tr>
<td>Effluent</td>
<td>7.16</td>
<td>6.25</td>
<td>5.03</td>
<td>3.91</td>
<td>0.85</td>
<td>0.18</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.65</td>
<td></td>
</tr>
</tbody>
</table>

In water and soil, 239 and 391 genera were identified, respectively. While there were 130 genera present in...
significant differences in the genera of microorganisms between soil and water samples \((p < 0.05)\). Genera were determined using rRNA sequences from two metagenomic data sets.

Both samples, some notable taxonomic differences were also apparent. Statistical analysis with STAMP demonstrated that 14 genera were significantly different in proportion (Figure 3). Twelve of these genera were bacteria and the remaining two (\textit{Thalassiosira} and \textit{Cryptomonas}) were algae.

The greater microbial diversity of the rhizosphere soil (alpha diversity, 193) relative to influent water (diversity, 162) is likely due to the migration of many influent taxa to rhizosphere soil where they enhanced the diversity of the indigenous microbes already present in the soil. The wetland ecosystem because of the presence of plant roots, rhizomes and other organic structures, provides new habitat opportunities for prokaryotic and eukaryotic organisms. In addition, the wetland soil is rich in nutrients (some of which are root exudate) which encourage rapid growth and development of a new microbial community.[29,30] Previous studies have shown that deposition of root-derived nutrients to rhizosphere soil promotes a significant increase in the density of microbial communities in rhizosphere soil compared with that of unplanted wetland communities, a phenomenon often referred to as the ‘rhizosphere effect’.[31,32] Data from our study show this effect was also reflected in the microbial diversity.

### 3.2. Microbial function

Rhizosphere soil microbial communities are involved in biogeochemical processes and their activities are crucial to the wetland function.[33] To investigate the microbial community function, we analysed the identified protein sequences from soil and water metagenomic data sets. Hierarchical analysis of SEED Subsystems in MG-RAST revealed that the basic functions of microbes in soil and water were similar (Figure 4). The sequences associated with N or aromatic compound metabolism accounted for around 1–2% of all annotated sequences. In this study we focused on the microbial metabolism of xenobiotic, N and heavy metal.

The abundance of sequences associated with specific xenobiotic compound metabolism from KEGG annotation is illustrated in Figure 5. Sixteen types of xenobiotic compound biodegradation were identified in two metagenomic data sets, with substantial overlap between soil (14) and water (14) data sets. Most compounds were persistent organics such as polycyclic aromatics, pesticides and pharmaceutical compounds. Among various xenobiotic degradation genes, those associated with benzoate biodegradation were much abundant in two samples, most likely because benzoate is a key intermediary in the microbial metabolism of various aromatic compounds.[34] For atrazine biodegradation, the presence of hydroxyatrazine ethylaminohydrolase,
N-isopropylammelide isopropylaminohydrolase and cyanuric acid amidohydrolase indicates that the atrazine pathway in the wetland was atrazine to hydroxyatrazine to N-isopropylammelide to cyanuric acid.[35] Regarding chlorocyclohexane and chlorobenzene degradation, we identified mono- and di-oxygenase and maleylacetate dehalogenase, which suggest oxidation, followed by dechlorination, of chlorocyclohexane and chlorobenzene were biologically mediated.

The abundance of sequences associated with N metabolism is shown in Figure 6. Compared with the water sample, genes related to N fixation were much more abundant in soil, which is not surprising since biological nitrogen fixation is known to occur in association with the rhizospheres of many herbaceous angiosperms.[36] In addition to N sources from the soil, atmospheric N can also be converted to ammonia through microbial biochemistry. From the metagenomic analysis, a complete set of nitrification (amo, hao) and denitrification (nar, nir, nor, nos) genes were observed in both water and soil samples. For nitrification, the oxidation of ammonia to hydroxylamine by prokaryotes is a key process in the global N cycle.[37] However, we identified only a few ammonia monoxygenases in the soil sample. These enzymes were all associated with Nitrosomonas eutropha, indicating N. eutropha might be primarily responsible for ammonia oxidation in wetland rhizosphere soil. Sequences of ammonia-oxidizing archaea were not detected, suggesting only a minor role in nitrification in soil. Denitrification is a microbial respiratory process within the N cycle responsible for returning fixed N to the atmosphere.[38] Denitrification genes in both water and soil samples were more abundant than nitrification genes, suggesting a predominant role for denitrification among the three N processes (nitrogen fixation, nitrification and denitrification). The sequences associated with denitrification were phylogenetic diverse, with most being affiliated with Proteobacteria, Bacteroidetes and Chloroflexi.

The influent water contained high concentrations of Fe and Mn, which decreased significantly following wetland treatment (Table 1). The concentration reductions were mainly attributed to the transformation from soluble Fe$^{2+}$ and Mn$^{2+}$ to insoluble Fe$^{3+}$ and Mn$^{4+}$. The rhizosphere is a ‘hotspot’ for Fe and Mn transformation. Because ferrous iron oxidase was not found in the influent water, most of the ferrous iron was chemically oxidized. However, some ferric iron reductases were observed in two metagenomic data sets. The extent of ferric iron reduction depends on the biological role played by Fe species. A number of sequences encoding manganese-oxidizing multicopper oxidase were observed in the soil sample. Multicopper oxidase is essential for manganese oxidation. Several species of phylogenetically distant bacteria are able to oxidize Mn$^{2+}$ to form insoluble manganese oxides with the contribution of multicopper oxidase.[39,40] Therefore, it appears...
that multicopper oxidase played a vital role in manganese oxidation in rhizosphere soil.

4. Conclusion

To investigate the potential role of microbes in the rhizosphere soil of a constructed wetland, we used metagenomic analysis to explore microbial community composition and function. The results showed that the microbial richness and diversity in rhizosphere soil are higher than in influent water, supporting the proposition that rhizosphere soil supports significant microbial growth. Bacteria were the dominant domain (83.3%), with Gammaproteobacteria being the most abundant phylum in the rhizosphere soil. For microbial function annotation, a number of biodegradation pathways of xenobiotic compounds were observed in rhizosphere soil. Nitrification and denitrification processes were the primary transformation route in the N cycle. This study found that soluble Fe$^{2+}$ and Mn$^{4+}$ from influent water were converted to Fe$^{3+}$ and Mn$^{3+}$ in rhizosphere soil by chemical oxidation (Fe$^{2+}$) and biological oxidation (Mn$^{2+}$, employing multicopper oxidase), respectively.

In conclusion, microbial communities growing in the rhizosphere soil of constructed wetlands play a major role on the removal of both nutrients and non-essential contaminants from wastewater. The presence of a stable microbial community is a critical factor in maximizing the efficacy of removal and pollutant reduction.

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