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Characterization of bacterial community in biofilm and sediments of wetlands dominated by aquatic macrophytes

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ABSTRACT

Though aquatic macrophytes play an important role in wetlands, their effects on bacteria community structures in biofilms and sediments are far from clear. In the present study, bacterial communities were investigated in biofilm attached to leaves, stems and roots of aquatic macrophytes (*Myriophyllum verticillatum*, *Nymphoides peltatum* and *Trapa japonica*) and in vertical sediment cores from vegetated and unvegetated areas in a wetland located in Lake Hongze. The densities of microbes and epiphytic algae in biofilms were higher on leaves of *M. verticillatum* than those of two floating macrophytes. Phyla Proteobacteria, Bacteroidetes, Chloroflexi, Firmicutes and Verrucomicrobia were detected in both biofilms and sediments. As revealed by cluster analysis and principal component analysis, differences in structures of microbial communities were detected between biofilms and sediments and between vegetated and unvegetated sediments. The potential roles of nitrifying- and denitrifying- bacteria in sediments with respect to those in biofilms were discussed. These results highlight that the restoration of aquatic macrophytes can increase bacteria diversity and the surface and quantity of biofilms and therefore bacteria diversity. These data provide useful information for further understanding the role of aquatic macrophyte-biofilm system in wetlands in future.

1. Introduction

Aquatic macrophytes, key components of aquatic ecosystems, can absorb nutrients, heavy metals and other inorganic or organic contaminants from the water column and the sediments (Dhote and Dixit, 2009). Aquatic macrophytes are widely grown in wastewater treatment wetlands for improving water quality (Thorén, 2007). However, due to the improper anthropogenic activities aquatic macrophytes have declined significantly and biodiversity in freshwater has decreased, this will be a major driver of future ecosystem change (Hooper et al., 2012).

Biofilms (known as epiphytic microbes) can form on the gas-liquid and solid-liquid interfaces, and potentially constitute an important step in the integration of biogeochemical cycles and dynamics of microbes to ecosystem function (Battin et al., 2003). Aquatic plants are key components in spatial heterogeneity, which is essential for the establishment and development of biofilms in aquatic ecosystems. Aquatic plants represent a special substrate for biofilms, as they can release oxygen, which is beneficial to aerobic bacteria attached to the plant, and promotes the transformation of nitrogen in water (Eriksson, 2001; Thorén, 2007). In fact, plants can also affect the function and structure of bacterial community in sediments, especially in rhizosphere (Herrmann et al., 2008). Despite the important ecological roles of aquatic plant-epibiotic bacteria system, in aquatic ecosystems, the community ecology of these bacteria is far from being understood (He et al., 2014).

The functions of biofilms are always determined by the biodiversity and species of bacteria forming it. The epiphytic bacterial communities were reported to be diverse and host-specific (Crump and Koch, 2008; He et al., 2012), while the growth status and secretions of macrophytes can affect the bacterial composition in biofilms and sediments (Hempel et al., 2008; Jensen et al., 2007). By using high-through sequencing methods (454 pyrosequenc-

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ing), He et al. (2014) found that bacteria community structures in epiphytic biofilm are different from those in surrounding water column. These references provided valuable information that biofilms can be formed on aquatic macrophytes surface and have roles in nitrogen removal. However, to the present authors' knowledge, minimum information is available about the structure and functions of microbial communities on aquatic plants, nor in the rhizosphere in Lake Ecosystems, at the meta-genomic level.

Lake Hongze, the fourth largest freshwater lake of China, is a moderately eutrophic freshwater Lake with an average water depth of 1.4 m. In recent years, many wetlands were constructed by restoration of abundant floating and submersed macrophytes in lake area to improve the water quality and biodiversity. In order to investigate structure of bacterial communities in biofilms from different organs of common floating and submersed macrophytes and in vegetated and unvegetated vertical sediments and to compare nitrifying and denitrifying bacteria composition in biofilms to those in the sediments, 454 pyrosequencing methods were employed to detect the microbial communities in biofilm attached to the leaves, stems (except for *Myriophyllum verticillatum*) or roots of *M. verticillatum*, *Nymphoides peltatum* and *Trapa japonica*, and in sediments from vegetated or unvegetated area in Lake Hongze. The results were expected to improve our understanding the role of aquatic macrophytes in the functions of wetlands.

2. Materials and methods

2.1. Study sites and sampling

M. verticillatum, *N. peltatum* and *T. japonica* were the dominant aquatic macrophytes in the sampling sites of a wetland located in Lake Hongze. Plant samples and sediment samples were collect in July 2013 because aquatic plants always thrive in summer. Plants were collected without brushing, and floating macrophyte *N. peltatum* and *T. japonica* samples were separated into leaves, stems and roots, while submersed macrophyte *M. verticillatum* samples were separated into leaves and roots. The root samples were washed with double distilled H_2O. Three replicates were collected for each plant sample and each replicate contained at least 50 plants. The *M. verticillatum* is rooted in sediments while roots of *N. peltatum* and *T. japonica* always suspend in water column (Supplementary Fig. S1).

Surface water (2 L each site, three replicates were collected) was sampled from the macrophytes dominated area. Undisturbed vertical sediment cores were sampled using a Beeker sampling device (Eijkelkamp Agrisearch Equipment, Netherlands). The average depth of sediments in this lake was approximately 30 cm around these sampling sites. Three sediment cores (30 cm) were collected from sampling site without plants (water depth, 0.6 m) or with high density of *M. verticillatum* (water depth, 1.2 m). Core vertical sediment samples were divided into nine layers (3 cm per layer), and the segment layers of 0–3 cm, 10–12 cm and 21–24 cm were selected to represent the surface, middle and bottom layer of the sediment, respectively. The sediment samples were stored in aseptic plastic bags, and all samples were kept in an ice box and were transported to the lab within 24 h. Nutrient concentrations in sediments and surface water were provided as Supplementary materials (Table S1).

2.2. Sample pre-treatment

Approximately 50 g of fresh plant material were transferred into a sterile 500 mL polyethylene bottle containing 400 mL of 50 mM phosphate buffered saline (PBS, pH = 7.4) solution. Epiphytic microbes were detached after 3 min of ultra-sonication, 30 min of shaking (225 r/min) and subsequent ultra-sonication for 3 min (He et al., 2012). The surface area of leaves was calculated by using image software Adobe Photoshop CS3 (Adobe Systems Software Ireland Ltd, American). For bacteria counting, formaldehyde was added in the 5 mL of eluents or surface water samples to a final concentration of 2% formaldehyde, while 5 mL eluent and water samples were added with Lugo's reagent for algae identification. Plant leaves for microscopic observation were fixed in a final concentration of 2% formaldehyde in 50 mM PBS solution.

2.3. Measurements of the bacteria and algae

100 μL eluent or water sample was further mixed with 700 μL 4′,6-diamino-2-phenylindole (DAPI, 10 μg/mL) and incubated in the dark for 30 min. The samples were filtered through a 0.22 μm incubated filter. The number of bacteria on the black membrane was counted under a fluorescence microscope (ZEISS, Germany) and for each slide, thirty random fields were enumerated. 100 μL eluent or water sample was dropped in the middle of the plankton counting chamber (Beijing Purity instrument CO., LTD, China) and then were observed under a fluorescence microscope (ZEISS, Germany). Fifty random fields were counted for each sample. The algae were identified at genus level based on morphological parameters. Triplicates were performed for each sample.

2.4. Scanning electron microscopy (SEM) analysis

Plant leaves were cut into small pieces (5mm × 5 mm). After being dehydrated through a series concentration of ethanol (in sequence 30%, 50%, 70%, 80% and 90%) for 15 min at each concentration, these small pieces were immersed in 100% ethanol twice for 15 min each and then transferred to a freeze-drier. The dried samples were visualized by using a scanning electron microscopy after being sputter coated with gold (S3400, Hitachi, Japan).

2.5. DNA extraction, PCR amplification and 454 pyrosequencing

The biofilm solution or the sediment samples were mixed with ethyl alcohol at 1:2 ratio (v:v) and then centrifuged at 8000 rpm for 10 min. All the condensed samples were collected and stored at −80 °C for DNA extraction. Biofilm and sediment DNA (three replicates per sample) were extracted with the PowerBiofilm DNA Isolation kit and the PowerSoil DNA Isolation kit (MoBio Laboratories, USA) according to manufacturer's protocols, respectively. Three extracted DNA solutions from the same sample were combined for the following analysis. The 16S rRNA gene of bacteria was amplified with the primer 342F (5′-CTACGGGGGGCAGCAG-3′) and 806R (5′-GGACTACCGGGGTATCT-3′) (Mori et al., 2014). PCR reactions were performed in a 20 μL mixture, containing 4 μL of 5 × FastPfu Buffer, 2 μL of 2.5 mM dNTPs, 0.8 μL of each primer (5 μM), 0.4 μL of FastPfu Polymerase, and 10 ng of template DNA. The amplification program consisted of an initial denaturation step at 95 °C for 2 min, followed by 25 cycles, where 1 cycle consisted of 95 °C for 30 s (denaturation), 55 °C for 30s(annealing) and 72 °C for 30 s (extension), and a final extension at 72 °C for 5 min.

Before sequencing, each PCR product was purified with the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, U.S.) and quantified using QuantiFluor™ −ST (Promega, U.S.). Amplicons from different samples were then mixed to achieve equal mass concentrations in the final mixture, which were used for pyrosequencing on a Roche 454 GS FLX+ Titanium platform (Roche 454 Life Sciences, Branford, CT, U.S.) according to standard protocols.

The low quality sequences with average quality score <20 over a 50 bp sliding window and sequences shorter than 200 bp, with homopolymers longer than six nucleotides, and con-