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Nitrogen loading affects microbes, nitrifiers and denitrifiers attached to submerged macrophyte in constructed wetlands

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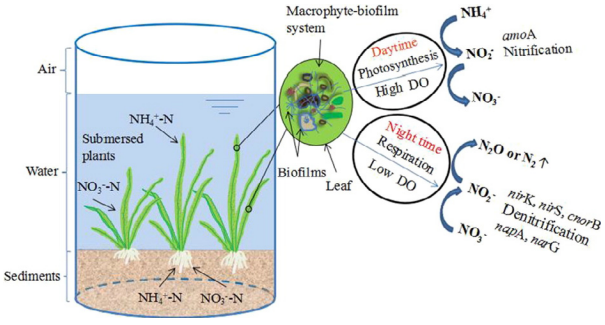
HIGHLIGHTS

- Initial nitrogen supply stimulated biofilm growth and algae density.
- Submerged plants performed better in the removal of nitrogen than artificial plants.
- Nitrogen and the species of plant determined algae composition in biofilm.
- Nitrogen loading stimulated the abundance of nitrifiers and denitrifiers.

GRAPHICAL ABSTRACT



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ABSTRACT

Submerged macrophytes and biofilms are important components of wetlands. However, little is known about the changes of microbes in biofilms attached to submerged macrophytes upon nitrogen loading. This study investigated the changes of microbes, algae, nitrifiers and denitrifiers in biofilms attached to the leaves of artificial plants (AP), *Potamogeton malaianus* (PM), *Vallisneria natans* (VN) and *Hydrilla verticillata* (HV) under varied initial concentrations of total nitrogen (TN). Nitrogen addition increased biofilm biomass and changed dissolved oxygen concentrations and pH values in overlaying water. Epiphytic algal densities showed the same trend at the same N level: AP > PM > VN > HV. As revealed by cluster analysis at phylum level, algae compositions in biofilm from four plants showed some host-specific at 2 and 12 mg L^{-1} TN, but was clustered in the same group at 22 mg L^{-1} TN regardless of plant species. Submerged macrophytes had better performance in total N removal than AP. In general, N application significantly increased the abundance of *amo*A, *nir*K, *nir*S, *nap*A and *cnor*B in biofilm. The abundance of the denitrification genes (*nir*K, *nir*S, *nap*A, narG and *cnor*B) was positively correlated with nitrogen application, while *amo*A was correlated with concentration of dissolved oxygen. These results indicate that N loadings stimulated the growth of biofilms attached to submerged macrophyte and the removal of total N can be partially ascribed to the synergistic interactions of submerged macrophyte and biofilms in wetlands. These results highlight the ecological role of submerged macrophyte-biofilm system in nitrogen removal in wetlands.

1. Introduction

Eutrophication of freshwater has become a global problem due to input of large amounts of nitrogen (N) and phosphorus (P) from agriculture and urban areas (De-Bashan and Bashan, 2010). Eutrophication often causes algae bursts and subsequent decline of submerged macrophyte (Wu et al., 2016). In wetland systems nitrogen is presented in the forms of organic and inorganic N (NO_3^-, NO_2^-, NH_3 and NH_4^+). They can be assimilated by submerged macrophyte, and also cause acute or chronic toxicity to aquatic organisms (*Hydrilla verticillata*) at high concentrations (>1.5 mM) (Wang et al., 2010). Nitrogen is one of

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the key factors affecting the function of constructed wetlands that are dominated by submerged macrophyte.

Biofilm (also known as periphyton) is a microbial community consisting of bacteria, fungi, and algae, as well as other protozoa and metazoan. These microorganisms are combined with the extracellular polymeric substances produced by the microbes in the biofilm (Flemming et al., 2007; Liu et al., 2016b). Microbes are important components of wetland systems and play an important role in the biological processes of nutrient removal through nitrification (oxidizing NH_4^+-N to NO_2^--N and NO_2^--N to NO_3^--N) and denitrification process, which convert NO_2^--N and NO_3^--N into N_2 or N_2O (Li et al., 2014; Hou et al., 2017). Therefore, biofilms are widely used to remove nutrients from freshwater (Pan et al., 2016; Sabater et al., 2002).

Submerged macrophyte growing under water are natural substrates for the growth of biofilm and are commonly used in wetlands. Submerged macrophyte not only absorb nutrients but also provide nutrients and oxygen for biofilms (Bustamante et al., 2011). The response of plants and microorganisms to nutrition enrichment in the water column might include primary production, community structure, and the altered nutrients removal rates; each has an unique role in nutrient cycling with the biofilm (Wu et al., 2016). Respiration in dense stands of submerged vegetation at night may cause a shift from aerobic to anaerobic, which is beneficial to denitrification (Eriksson, 1999). The functional genes (*nar*G, *nap*A, *nir*S, *nir*K, *cnor*B and *nos*Z) related to denitrification have been detected in biofilms attached to the surface of submerged macrophyte (*Potamogeton malaianus, Vallisneria natans, Ceratophyllum demersum* and *Elodea nuttallii*) in wetlands (Zhang et al., 2016a).

Biofilms are important for the removal of nutrients in wetlands, but at a high density, they may limit the growth of submerged macrophyte. Biofilms have a higher nutrient uptake rate than macrophyte and may have a negative influence on *Vallisneria natans* growth in eutrophic water (Song et al., 2015). The biofilm attached to leaf attenuate the incident ray received by plants and cause the decline in photosynthesis of plants (Asaeda et al., 2004). Biofilms can also affect the carbon dioxide absorption and oxygen distribution within the plants (Pang et al., 2016). Submerged plants can excrete allelopathic chemicals against microbes in biofilms, such as algae (Erhard and Gross, 2006). The bacterial and algal community was shown to be somewhat host-specific (Pang et al., 2016). However, minimal information is available about initial N loading on the growth of biofilms and nitrifiers and denitrifiers on submerged macrophyte.

Abundant N is released from agricultural production systems into the surrounding water bodies, especially during the rainy seasons. Therefore, submerged macrophyte in wetlands are frequently subjected to varied N supply. In this study, a factorial experimental design was used to simulate the impact of three initial N loadings on algae, denitrifiers and nitrifiers attached to three types of submerged macrophyte: *Potamogeton malaianus* (PM), *Vallisneria natans* (VN) and *Hydrilla verticillata* (HV), while artificial plants (AP, abiotic plants) were used as control. The study was conducted in constructed wetland systems to test the following hypotheses: 1) N loading stimulates biofilm growth; and 2) N loading increase N-cycle genes (*amo*A, *nir*K, *nir*S, *nar*G, *nap*A and *cnor*B) abundance. The changes in aquatic environmental conditions, N removal, biofilm and algae densities, and six N transformation-related genes were monitored in the constructed wetlands grown with submerged macrophyte after loading with three levels of N.

2. Materials and methods

2.1. Experimental design and setup

The experiment was conducted with a 3 × 4 factorial design of three N levels (TN = 2, 12 and 22 mg L^{-1}, NH_4^+-N:NO_3^--N at the ratio of 1:5, TP = 0.02 mg L^{-1}) and four plants: artificial plants (AP, a plant that is similar to the other plants and is made of non-toxic plastic), *P. malaianus* (PM), *V. natans* (VN) and *H. verticillata* (HV). The wetlands were constructed with a plastic tank (V = 100 L, D = 60 cm) containing 70 L of de-chlorinated tap water and 7 cm depth of sediment. The sediment was collected from Wulongtan Lake (0–6 cm), Nanjing, China (32°02′59″ N, 118°45′47.94″ E). The concentration of key nutrients and physicochemical properties of the sediments were described in the previous study (Zhang et al., 2014). Healthy plants of *P. malaianus, V. natans* and *H. verticillata* (from Gaochun aquatic plant cultivation base, Nanjing, China, 31°55′32″ N, 118°41′47.26″ E) with similar length and biomass were selected, cleaned and then cultured in the plastic tank, where artificial plants were used as control. Biofilms attached to leaves of plants were scraped gently with a soft toothbrush as described in the paper of Pan et al. (2000). The density of the submerged macrophyte was 75 g m^{-2}, while the artificial plants occupied 70% of the water space. The experiment was conducted in triplicate for 11 days in August and 36 tanks were used in this study. After acclimation for one week, N solution was added to the tanks to the designated levels.

Fresh plant leaves were collected to examine biofilms from each tank on the 3th, 5th, 7th, 9th and 11th day after treatment with N, while pH, DO, TN, NH_4^+-N, and NO_3^--N in the overlaying water were analyzed.

2.2. Environmental parameters and nitrogen determination

Environmental parameters including pH and DO in the overlaying water were monitored using portable meters (DR2800, HACH, USA). Concentrations of TN, NH_4^+-N, and NO_3^--N were determined using an AA3 AutoAnalyzer 3 HR (SEAL, Germany AutoAnalyzer 3 HR).

2.3. Microbe and algae densities

Leaf samples (5 g) were placed into a sterile 100 mL polyethylene bottle containing 70 mL of 50 mM phosphate-buffered saline (PBS, pH = 7.4) solution. The biofilms were detached from the plant leaves in three steps: 3 min of ultra-sonication, 30 min of shaking (225 r/min), and 3 min of additional ultra-sonication (He et al., 2012). Three extracts from the same sample were combined and fixed with 2% formaldehyde. Then, 100 μL of suspension samples were stained with 700 μL of 4′,6-diamidino-2-phenylindole (10 μg mL^{-1}) in the dark for 30 min, and filtered through a 0.22 μm black membrane filter. The bacteria on the black membrane were counted under a fluorescence microscope (ZEISS, Germany). The algae were identified at the genus level at 50 random fields under a fluorescence microscope (ZEISS, Germany) with a plankton counting chamber (Beijing Purity instrument CO., LTD, China) after fixing and diluting. Detailed analysis of the steps and methods were described in a previous report (Pang et al., 2016).

2.4. Scanning electron microscopy (SEM) analysis

Leaf samples collected from the four plants exposed to 12 mg L^{-1} TN on the 11th day were fixed with glutaraldehyde (2.5% in 50 mmol L^{-1} sodium cacodylate). Upon fixation, the samples were incubated in an osmium tetroxide solution (1% in 50 mmol L^{-1} sodium cacodylate buffer) in the dark. After immersing in a serial concentration of ethanol (in sequence: 20%, 40%, 60%, 70%, 80% and 90% ethanol) for 15 min at each concentration, the fixed samples were further dehydrated twice with 100% ethanol for 15 min. The dried samples were observed under SEM (S3400, Hitachi, Japan) after sputter coating with gold.

2.5. DNA extraction and real time PCR amplification

Plant samples were mixed with ethyl alcohol at 1:2 ratio (v:v) and passed through a sieve with a mesh size of 50 μm to remove plant debris, then centrifuged at 8000 rpm for 8 min. The pellets were used for DNA extraction with the Power Biofilm™ DNA Isolation kit (MoBio Laboratories, USA). Three DNA extracts from each set of samples were mixed thoroughly for further analysis. The abundance of the genes for ammonia monooxygenase (*amo*A), periplasmic nitrate reductase