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Effects of root exudates on denitrifier gene abundance, community structure and activity in a micro-polluted constructed wetland

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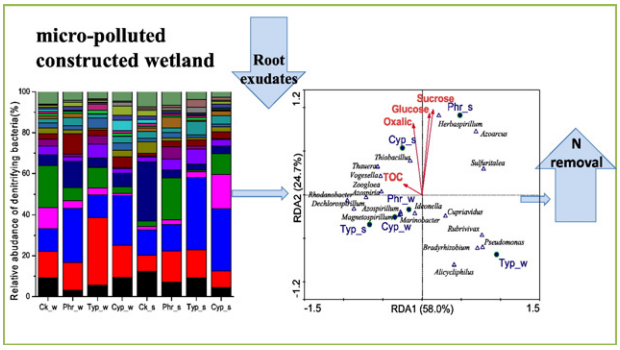
HIGHLIGHTS

- Exudates acted as endogenous carbon sources in micro-polluted CW.
- Exudates and microorganisms varied significantly among plant species and seasons.
- Denitrifier gene abundances were significantly affected by sucrose and glucose.
- Microbial communities were significantly affected by sucrose and oxalic acid.

GRAPHICAL ABSTRACT



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ABSTRACT

In micro-polluted constructed wetland (CW), the low pollutant concentrations and the low COD/N ratios (chemical oxygen demand: total nitrogen in influent), make the biological treatment more difficult. It is expected that root exudates drive microbial-based transformations within plant rhizosphere. In this research, the roles of root exudates of three aquatic plants (*Phragmites australis*, *Typha angustifolia* and *Cyperus alternifolius*) in improving the growth of heterotrophic denitrifying bacteria were determined in a micro-polluted CW. In studied root rhizospheres, the total organic carbon (TOC) released from the plant roots varied significantly among plant species and seasons; the average TOC ranged from 0.1715 to 0.9221 mg g^{-1} root DM d^{-1}, which could fuel a denitrification rate of approximately 156–841 kg NO_3^--N ha^{-1} $year^{-1}$ if all were used by the denitrifying bacteria; the abundances of *nirK*- and *nirS*-encoding bacteria were significantly influenced by the concentration of sucrose and glucose ($0.869 \le r \le 0.933$, $p < 0.05$), and microbial community richness and diversity had response to root exudates. The results revealed that root exudates can act as endogenous carbon sources for heterotrophic denitrifying bacteria and ultimately determine the microbe distribution patterns in micro-polluted CW.

1. Introduction

Nitrogen (N) fertilizers are widely used in farming and planting, so N compounds are flushed from soils during rain, enter nearby lakes, and ultimately cause eutrophication (Beghelli et al., 2016; Li et al., 2015). As both N and carbon (C) pollutants occur at relatively low concentrations (typically <10 mg L^{-1}) in natural water bodies, these waters are termed micro-polluted (Zhou et al., 2016). Constructed wetlands (CWs), as an emerging ecological engineering technology, have shown their attractions and been widely used to treat micro-polluted water due to high N removal efficiency and

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low operation cost. In CWs, the N removal mechanisms are known to include biological (e.g., ammonification, nitrification, denitrification, plant uptake) and physicochemical routes (e.g., ammonia volatilization, and adsorption) (Coleman et al., 2001; Lee et al., 2009). Biological nitrification followed by denitrification is believed to be the major pathway for N removal in CWs (Saeed and Sun, 2012). The proportion of total N (TN) removed by microbial nitrification-denitrification is typically 60%–95% compared to 0.5%–60% assimilated by plants and algae (Li et al., 2015; Meng et al., 2014; Shamir et al., 2001).

Under the natural conditions all forms of N tend to transform into NO_3^--N and exist stably in the surface water as NO_3^--N (Zhou et al., 2016), therefore, NO_3^--N removal by denitrification is believed to be the critical process for N removal. Most denitrification is accomplished by heterotrophic bacteria, when N oxides serve as terminal electron acceptors for respiratory electron transport and organic compounds serve as electron donors, the biochemical reaction is strongly dependent on C availability. Generally, a COD/N (chemical oxygen demand: total nitrogen in influent) ratio of 4 or more is generally considered to the best situation for microorganism reproduction and N removal in CW (Ding et al., 2012). C deficiency is the key limiting factor for N removal under low COD/N, but the excessive C source further restrain the reaction of nitrification by competition for dissolved oxygen. In theory, within the reasonable range, higher COD/N is more favorable for denitrification. But the characteristics of micro-polluted are the low pollutant concentrations and the low COD/N ratios, which make biological treatment more difficult. Plants can convert atmospheric CO_2 into biomass (organic C) through photosynthesis, which might eventually become available to denitrifying bacteria through a number of pathways such as the death and decomposition of plant litter and the secretion of root exudates (Zhai et al., 2013).

Previous researches showed that approximately 5–25% of the total photosynthetically fixed C could be transferred to the rhizosphere through root exudates (Bais et al., 2006; Haichar et al., 2014), which can induce and stimulate the growth of specific bacterial groups and create well-defined bacterial communities around the rhizosphere (Liu et al., 2016; Tanaka et al., 2012). In the rhizosphere of terrestrial plant, root exudates are composed of lots of chemical constituents, such as low molecular weight organic acids (LMWOAs), sugars, amino acids, and other secondary metabolites (e.g., phenolic compounds, flavonoids, tannins, and alkaloids) (Vranova et al., 2013). Meanwhile, soluble sugars and LMWOAs, for instance, acetic acid, oxalic acid and succinic acid, which added in some studies were consider to the major C sources for denitrification (Elefsiniotis et al., 2004; Ruiz-Rueda et al., 2009), Therefore, it is important to elucidate the availability of exudates, and particularly their effects on the activity of denitrifying bacteria.

Many research aimed to detect microbial composition supported by organic C or root exudates (Table S1, Supplementary material), but to the best of our knowledge, the detailed root exudates composition and abundances in the rhizosphere of aquatic plants remain unknown, there is no report about the effect of exudates on the community structure and abundance of rhizosphere denitrifying bacteria in CW. In order to add to the pool of information on root exudates effects on microbial denitrification and provide knowledge about the potential N removal mechanism in CW, we analyzed the effects of root exudates on denitrifier gene abundance, community structure and activity in micro-polluted CW. This main objectives of this paper were oriented as: I) to study the organic acids and soluble sugars secreted by three aquatic plants (*Phragmites australis* (Phr), *Typha angustifolia* (Typ) and *Cyperus alternifolius* (Cyp)) in Dengbeiqiao CW; II) to examine the development of nir-encoding denitrifying bacteria influenced by root exudates; III) to evaluate the potential contribution of root exudates as organic C source for denitrification in CWs.

2. Materials and methods

2.1. Site description and sample collection

Dengbeiqiao wetland (26°01′44″N, 100°09′13″E) is a surface-flow CW located in Dali in south-western China. This area has a low-latitude monsoon climate, average winter and summer temperatures of 12.6 °C and 22.3 °C, respectively. The average inflow of TN, ammonium (NH_4^+-N), nitrate (NO_3^--N) and COD were 1.79, 0.24, 0.71 and 4.41 mg L^{-1}, respectively.

Based on the relative abundance and widespread distribution of aquatic plants in the CW, the top three aquatic macrophytes, Phr, Typ and Cyp were selected for the study. In summer (August 4–5, 2015) and winter (December 15–16, 2015), each plant species was collected from 3 random sample points and then mixed together while keeping the roots intact. The plant root soil was carefully cleaned and stored at −20 °C for microbiological analysis, and root-free bulk soil was collected for use as the control sample (Ck). Water samples were collected from wetland inflows and outflows every 10 days during summer (from 1 July to 30 August) and winter (1 December to 30 January), the TN, NH_4^+-N, and NO_3^--N contents as well as COD were analyzed in the laboratory. All parameters were determined with a flow injection analysis instrument (QC8500, Hach, USA) per the manufacturer's instructions.

2.2. Exudate collection and analysis

Plant roots were carefully and gently washed with ultra-pure water to remove all adhering soil and dead plant tissues. Then, the plants were cultivated in ultra-pure water for 6 h, and the extract was concentrated by rotary evaporation under a dry vacuum at 40 °C. The total organic carbon (TOC), organic acids and soluble sugars were analyzed with a TOC analyzer (multi 3100, Analytik Jena, Germany) and by high-performance liquid chromatography (E2695, Waters, USA); the methods were described in detail by Wu et al. (2016). The contents of the following organic acids were measured: oxalic acid, malic acid, malonic acid, citric acid, acetic acid, and propionic acid, and the detected sugars were glucose and sucrose.

2.3. DNA extraction

The total genomic DNA of each sample was extracted directly from the membranes using a PowerSoil DNA Isolation Kit (MoBio, USA) according to the manufacturer's instructions. The genomic DNA was extracted by 1.2% agarose gel electrophoresis and stored at −20 °C for further use.

2.4. qPCR analysis

A Funglyn Biotech FTC-3000 real-time PCR system (Canada) was used to quantify the *nirK* genes with primers F1aCu (5′-ATCATGGTSCTGCCGCG-3′)/R3Cu (5′-GCCTCGATCAGRTTGTGGTT-3′), and the *nirS* genes were quantified with primers cd3aF (5′-GTSAACGTSAAGGARACSGG-3′)/R3cd (5′-GASTTCGGRTGSGTCTTGA-3′). The total amount of the reaction mixture was 25 μL, comprising 12.5 μL of SYBR Premix Ex Taq™II (Takara, Japan), 5 μL of template DNA (1–10 ng), and 0.3 μL or 1.0 μL of forward and reverse primers (10 mM) for the *nirK* genes and *nirS* genes, respectively; nuclease-free water was added to yield a total of 25 μL. The qPCR cycles were as follows: 10 min at 95 °C and 40 cycles of 10 s at 95 °C, 30 s at 55 °C, and 30 s at 72 °C. The threshold cycles (Ct) obtained in each PCR run were compared with those of the known standard DNA concentrations, and the expression of a glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the endogenous control to normalize the array. Standard curves were obtained following the procedure described by Di et al. (2010).