Bioresource Technology 157 (2014) 341–345

Contents lists available at ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

Short Communication

Effects of plant biomass on denitrifying genes in subsurface-flow constructed wetlands

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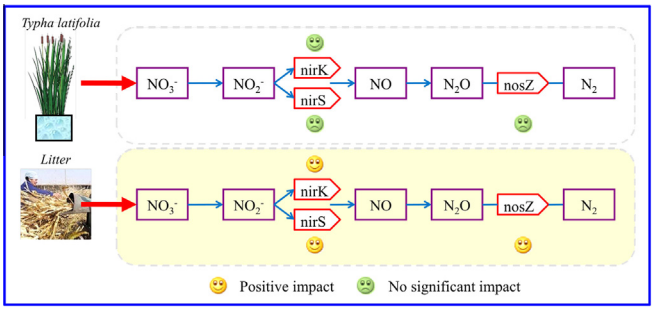
HIGHLIGHTS

- nirS was the dominant denitrifying gene in the CWs treating WWTP effluent.
- The presence of *Typha latifolia* encouraged the growth of nirK containing bacteria.
- Cattail litter stimulated the growth of bacteria containing nirS and nosZ gene.
- Plant biomass indirectly impacted the nitrite reductase via DO and carbon sources.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 14 November 2013
Received in revised form 20 January 2014
Accepted 24 January 2014
Available online 11 February 2014

Keywords:
Subsurface flow constructed wetlands (SSF CWs)
Denitrifying genes
Nitrate removal
Cattail litter
Typha latifolia

ABSTRACT

The effect of *Typha latifolia* and its litter on density and abundance of three denitrifying genes (nirS, nirK and nosZ) were investigated in six laboratory-scale SSF CW microcosms. Results showed that the copy numbers of nirS, nirK and nosZ in wetland microcosms were ranged between 10^8–10^9, 10^6–10^7 and 10^7–10^8 copies g^{-1}, respectively. The presence of *T. latifolia* encouraged the growth of nirK containing bacteria. Addition of cattail litter could greatly stimulate the growth of bacteria containing nirS and nosZ gene. Path analysis illustrated that the presence of plants and litters had no significant direct impact on denitrifying genes, while it affected the denitrifying genes via alteration of dissolved oxygen and carbon sources.

1. Introduction

Excessive nitrogen discharged into water can cause serious ecological problems such as eutrophication, algae blooms and habitat degradation in lakes and rivers. It was reported that eutrophication due to nitrogen pollution is serious in some lakes and reservoirs which are national protected basin in China (Yang et al., 2008). Subsurface flow constructed wetlands (SSF CWs) are a promising approach to further reduce N release from municipal effluent discharges into waterways (Kivaisi, 2001). Given that ammonia was efficiently removed in the nitrification process in wastewater treatment plant (WWTP), nitrate usually becomes the dominant N species in municipal effluent. In order to remove the nitrate, CWs are filled with plant litter, where the litter acts as carbon and energy source for denitrifying microorganisms, which convert nitrate to N gas via microbial denitrification (Wen et al., 2010). Previous studies have demonstrated the feasibility of using cattail litters

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http://dx.doi.org/10.1016/j.biortech.2014.01.137
0960-8524/

as an organic carbon source, and higher nitrate removal can be achieved in the litter added CWs (Ingersoll and Baker, 1998).

Microbial denitrification is the dominant N removal mechanism in litter added CWs, which consists of consecutive reaction steps. Nitrite reductase (nirS and nirK) and nitrous-oxide reductase (nosZ) are considered to be the key functional genes involved in denitrification. The nirS and nirK respectively express the cytochrome cd1-containing and copper containing nitrite reductase, which catalyses the reduction of nitrite to nitric-oxide (Braker et al., 1998). The nirS gene has been shown to be widely distributed, while the nirK was found in only approximately 30% of all known denitrifier species, and they are mutually exclusive in the denitrifying bacteria (Philippot, 2002). The nosZ expresses nitrous oxide reductase, which catalyses the reduction of N_2O to N_2. Cheneby et al. (1998) found that some denitrifying organisms do not possess nosZ and reduce NO_3^- only to nitrous oxide, leading to emissions of greenhouse gas N_2O.

Although previous studies have focused on denitrifying functional genes both in surface flow and SSF CWs (Garcia-Lledo et al., 2011; Chon et al., 2011; Ji et al., 2012), it has not been investigated in the litter added SSF CWs treating WWTP effluent. Furthermore, few studies compared various treatments under controlled conditions to rigorously evaluate the effects of plant and decomposing litter on the abundance of denitrifying functional genes in CWs. The objectives of this study were to (i) quantify the abundance of key functional genes (nirS, nirK and nosZ) present in a wastewater-fed SSF CWs. (ii) To determine the effects of *Typha latifolia* and litter decomposition on denitrifying functional genes.

2. Methods

2.1. Design and operation of the SSF CW

Six sequencing batch SSF CW microcosms, each with a bulk volume of 0.045 m^3 (length: 0.3 m, width: 0.3 m, height: 0.5 m) and a pore volume of 12 L, were set up in this study. Six sets of systems: unplanted control (W1), litter added microcosms (W2: 100 g; W3: 200 g), planted microcosms (W4: 22 plants m^{-2}; W5: 44 plants m^{-2}) and planted plus litter added microcosms (W6: 100 g litter, 22 plants m^{-2}) were established. All the microcosms were filled with washed gravel (ϕ 8–13 mm, porosity = 0.4) and three of them (W4, W5 and W6) were planted with cattail (*T. latifolia*). The seedlings of plants from the watercourses in vicinity of a laboratory were transplanted at an initial height of 20 cm In March 2011. The cattail litters (cut into 1–1.5 cm lengths) were collected from wetland microcosms in laboratory in November, 2010. The wetland microcosms were located in an air-conditioned greenhouse at a temperature of 25 ± 1 °C since 2005. Details of the wetland microcosm design have been given in the previous studies (Wen et al., 2010; Chen et al., 2011).

Before the start of the experiment, the microcosms were fed in batches with secondary effluent for 6 months until the plant shoots and microorganisms were well established. After an acclimation period of 6 months, cattail litter was mixed with gravel homogeneously and then compacted with a tamping rod at 4–5 cm increments during loading and filled the columns to a height of 40 cm. The details of litter loading have been given in a previous paper (Chen et al., 2011). The wetland microcosms were fed with the secondary effluent from a neighboring WWTP and the nitrate–N concentrations were 7.2 ± 3.1 mg L^{-1}. The six microcosms operated as a batch system with pulse loading, which were filled with wastewater at the start of each cycle and were gravity drained within 1 h prior to the next cycle. The operating cycle of wastewater filling and draining was 5 days, and there were 18 cycles (90d). All treatments (W1–W6) were replicated in two periods. According the previous research, the experiment was divided into three stages: an initial stage (days 1–30, cycles 1–6), a middle stage (days 31–70, cycles 7–14) and a terminal stage (days 71–90, cycles 15–18). Herein, cycles 2 (C2), 10 (C10) and 18 (C18) were chosen as typical cycles that represented the three stages, respectively.

2.2. DNA extraction

After cycles 2 (10d), 10 (50d) and 18 (90d), approximately 200 g of gravel and litter were collected from the top (5 cm), middle (20 cm) and bottom (40 cm) sections of the wetland microcosms. The three samples were combined for DNA extraction. Before DNA extraction the gravels/litters were vigorously shaken at 225 r min^{-1} for 3 h in sterile glass bottles in order to release the attached biofilm into the liquid phase. The precipitate was collected in bottles, after twice centrifuging at 5000 g for 20 min, for further analysis. Total genomic DNA was extracted from the gravels and litters using E.Z.N.A.® Soil DNA Kit (OMEGA bio-tek).

2.3. Real-Time PCR assays

Real-Time PCR assays were carried out in order to quantify the key functional genes nirS, nirK and nosZ using primers: nirSCd3aF/nirSR3cd, nirK876/nirK1040 and nosZ2F/nosZ2R respectively (Table S1; Warneke et al., 2011). The real time PCR were performed on an ABI STEPONE PLUS detection system (Applied Biosystems, USA) according to the manufacturer's instructions using SYBR-green based detection. QPCR was performed using a two-step amplification procedure and the thermal cycling conditions consisted of 2 min at 95 °C followed by 40 cycles of 10 s at 95 °C, and 40 s at 60 °C. All samples were run in triplicate. A duplicate 10-fold dilution series of standard DNA was used to calculate a standard curve. The standard curves for the bacterial and functional genes had R^2 values of 0.991–0.999 and the amplification efficiencies were 84–110%.

2.4. Statistical analysis

Paired-Samples T-test was used to analysis the effect of plants (W1 vs. W4) on copy numbers of nirS and nirK. *T*-tests and multiple regressions were performed using SPSS version 19.0 software, and were considered significant at the 0.05 level. Path analysis using AMOS version 21 (SPSS, IBM) was conducted to compare the direct (presence of plant or litter) and indirect paths (i.e. DO, organic matter) on denitrifying genes.

3. Results and discussion

3.1. Denitrifying functional genes isolated from gravel

3.1.1. Copies of total bacteria and denitrification genes

The copy numbers of total bacteria and denitrification genes in wetland microcosms were shown in Fig. 1. As shown in Fig. 1a, the copy numbers of 16S rRNA in W1–W6 microcosms ranged between 10^8 and 10^{10} copies g^{-1} gravel, higher than the numbers reported in the previous study (Baptista et al., 2008). As shown in Fig. 1c–e, the copy numbers of nirS, nirK and nosZ in wetland microcosms were respectively 10^8–10^9, 10^6–10^7 and 10^7–10^8 copies g^{-1}, relatively higher than the numbers reported in the previous studies elsewhere (Garcia-Lledo et al., 2011; Chon et al., 2011). This was likely due to the available carbon sources (i.e. sugars, amino acids and volatile fatty acids) from cattail litter which can serve as carbon and energy source for denitrifying bacteria growth (Wen et al., 2010).