Abundance of denitrifying genes coding for nitrate (narG), nitrite (nirS), and nitrous oxide (nosZ) reductases in estuarine versus wastewater effluent-fed constructed wetlands

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ARTICLE INFO

Article history:
Received 16 October 2008
Received in revised form 24 April 2009
Accepted 26 April 2009
Available online xxx

Keywords:
Functional genes
Real-time PCR
Microbial activity

ABSTRACT

Constructed and estuarine wetlands, influenced by wastewater treatment plants, were investigated, with respect to microbial activity in terms of functional genes. The density and abundance of three denitrifying genes coding for nitrate (narG), nitrite (nirS), and nitrous oxide (nosZ) reductases, in sediment soil samples from wastewater effluent-fed and estuarine wetlands, were quantified using the SYBR green-based real-time polymerase chain reaction (PCR). To assess seasonal effects (i.e., winter (average temperature ~2 °C) versus spring (average temperature ~20 °C)), the densities of denitrifying genes, with respect to the abundance of functional genes, for the two different wetlands were determined. The three functional genes for all the sampling sites ranged from 1.0 × 10^6 to 1.0 × 10^9 copies/g of soil. Without considering seasonal variation, the nitrite-reducing functional genes were dominant over the other two genes in the effluent-fed wetland samples. However, nitrate and nitrite-reducing functional genes were dominant in relatively cold and warm seasons, respectively, in the estuarine wetland samples. Even though robust patterns and conclusions could not be obtained from the limited investigations, patterns with certain trends and needs for potential future research directions were obtained.

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1. Introduction

Wetlands have been widely studied and subsequently identified as highly efficient ecosystems, with respect to nutrient control (Axt and Walbridge, 1999; Lin et al., 2002). Constructed wetlands have also been studied to efficiently control organics, nutrients, and heavy metals from either discharged water from agricultural areas or effluents from wastewater treatment plants (Kadlec and Knight, 1996; Brix and Arias, 2005; Vymazal, 2005; Maine et al., 2006; Song et al., 2006; Park et al., 2008).

The estuarine wetland is a unique ecosystem that plays an important buffering role in the transport of nitrogen from agricultural and other terrestrial-anthropogenic sources into marine ecosystems. The importance of the estuarine wetland is related not only to biodiversity but also functional activities for water quality improvement through nutrient control. In estuarine sediments, microbially driven processes may result in a net removal of nitrogen from the environment. One such ecosystem is the Suncheon estuarine wetland located on the south coast of Korea, where a gradient of nitrate was found to exist due to a connected river that is influenced by a wastewater treatment plant. Bacterial denitrification was also investigated in a wastewater-fed constructed wetland, located in Damyang City, Korea, by examining the abundance of three different functional genes using polymerase chain reaction (PCR).

Microbial denitrification is a respiratory process that consists of four consecutive reaction steps in which nitrate is reduced to dinitrogen gas (Zumft, 1997). The reduction of NO3− to NO2− can be catalyzed by either the membrane-bound nitrate reductase (nar) or the periplasmic nitrate reductase (nap), which is encoded by the narG or the napA gene, respectively (Bru et al., 2007). Denitrifying bacteria are reported to contain one or both of the nitrate reductases (i.e., narG and napA) (Carter et al., 1995; Roussel-Delis et al., 2005). The reduction of NO2− to NO was reported to be catalyzed by either a copper nitrite reductase (encoded by nirK) or a cytochrome cd1 nitrite reductase (encoded by nirS) (Braker et al., 1998, 2000; Henry et al., 2004)). The nirS gene has been shown to be widely distributed, while the nirK was found in only approximately 30% of all known denitrifier species (Coyne et al., 1989). The last step of the denitrification pathway, reduction of N2O to N2, is catalyzed by nitrous oxide reductase, which is encoded by the nosZ gene present in the periplasm (Scala and Kerkhof, 2004).
Real-time PCR has several advantages over other PCR-based quantification methods, including a relatively high accuracy, rapid analysis, and reproducibility. Real-time PCR has been used to quantify the copy numbers of denitrifying functional genes extracted from various environmental samples (Henry et al., 2004, 2006; López-Gutíérrez et al., 2004; Wallenstein and Vilgalys, 2005; Philippot, 2006; Smith et al., 2006; Zhang and Fang, 2006; Geets et al., 2007).

The objectives of this study were to (i) quantify the densities of key functional genes (narG, nirS, and nosZ) present in a wastewater-fed and an estuarine wetlands, using real-time PCR, and (ii) to determine the effects of seasons (i.e., winter (average temperature ~2 °C) versus spring (average temperature ~20 °C)) on the densities of denitrifying genes in two different wetlands.

2. Materials and methods

2.1. Samplings

Samplings were conducted at two different sites: the Damyang constructed wetlands and the Suncheon estuarine wetlands. Samples were taken from the Acorus and Typha (the first and last) ponds in the Damyang constructed wetlands, which were connected to the Damyang wastewater treatment plant. The wetland effluent flows to the Youngsan River, Korea, as shown in Fig. 1(a). The flow rate and hydraulic retention time of the entire wetlands were designed to be approximately 1800 m³/day and 6 h, respectively. The average length, width, and depth of the entire wetland were ca. 120, 30, and 0.13 m, respectively (Park et al., 2008). Sediment samples were taken from the both Acorus and Typha wetlands. Nitrogen from the wastewater treatment plant was mostly in nitrate form and ranged 50–60 mg/L as NO₃⁻ (Park et al., 2008). The Suncheon estuary has a coastline length of 39.8 km, which consists of a foreshore area of 21.6 km² and a reed field area of 5.4 km². The Suncheon estuarine wetlands were indirectly affected by effluent from the Suncheon wastewater treatment plant; ammonia and nitrate levels from the treatment plants ranged 17–18 and 0–4 mg/L and those at the estuarine wetlands were 2–3 and 1–3 mg/L, respectively (Park et al., 2008). Sediment samples were collected along the Suncheon estuary, at the estuary head, midway down the estuary, and from the estuary mouth (see Fig. 1(b)). In order to investigate seasonal variations, all sediment samples were taken in triplicate from upper 1 cm layers in mid February and late May. The sediments samples were placed on ice and returned to the laboratory. Samples were then stored at −80 °C prior to further molecular analyses.

2.2. Bacterial strain

Three different types of bacteria were used as controls to test and optimize the amplification of denitrification genes; Escherichia coli JM 109 ATCC 53323, Roseobacter denitrificans ATCC 33942, and Ralstonia eutrophica ATCC 17699 were cultivated in Luria-Bertani broth (Difco) at 37 °C, marine broth (Difco) at 20 °C, and nutrient broth (Difco) at 30 °C, respectively. When the cells were grown to the late exponential phase, genomic DNA was extracted and purified using the Accuprep Genomic DNA extraction kit (k-3032, Bioneer, Korea), according to the manufacturer’s instructions. The concentration of the extracted DNA was measured using a Nano-drop ND-1000 UV−vis spectrophotometer (Nano-Drop Technologies, Wilmington, DE, USA) at 260 nm. A 1 μL of sample volume was used for Nano-drop ND-100 measurements. Prior to determining the concentration of a sample via Nano-drop, a blank was measured with the DNA elution buffer. Three replicate measurements were taken for each sample and averaged. DNA purity was estimated from the A₂₆₀/A₂₈₀ ratio. The A₂₆₀/A₂₈₀ ratio of each DNA sample ranged from 1.7 to 2.0.

2.3. DNA extraction

DNA was extracted from 0.25 g of soil using the PowerSoil DNA isolation kit (PowerSoil, Mobio Laboratories Inc., CA, USA), according to the manufacturer’s instructions. The concentration of DNA was also quantified using a Nano-drop spectrophotometer at 260 nm. DNA extracts were stored at −20 °C for further molecular analyses.

2.4. Primers design

Table 1 lists the information on the primers selected for the amplification of the different genes encoding 16S rDNA, membrane-bound nitrate reductase (narG), cytochrome cd1 containing nitrite reductase (nirS), and nitrous oxide reductase (nosZ). The nosZ primer was designed using GenBank accession numbers of AF016055–AF016059 (Scala and Kerkhof, 1998). Redesigned nosZ primers were identified for specificity using the Blast and Fasta nucleotide database search tools (Pearson and Lipman, 1988;
values versus log 10 of the gene copy numbers. The amplification
were prepared using serially diluted plasmid DNA with 10^3 to 10^8
was verified by PCR with the corresponding primers. Standards
presence of the appropriate insert in the recombinant plasmids
the manufacturer's instructions. The plasmid concentrations were
for 4–5 h. The plasmid DNA was then extracted and purified using

2.5. PCR conditions

PCR amplification of denitrifying functional genes extracted
from both pure cultures and soil samples was performed to prepare
the real-time PCR standards and to confirm the specificity of ampli-
cons. PCR amplification of 16S rDNA, narG, nirS, and nosZ genes
was conducted at a total volume of 20 μL in a mastercycler (Eppendorf
Mastercycler personnel, Germany).

The PCR mixture included PCR premix (AccuPower PCR PreMix
kit, Bioneer, Korea), 1 μL of 10 pmol of each primer, and 30 ng of
template DNA. The PCR was programmed for 16S rDNA amplifi-
cation using various procedures, including 50 °C for 3 min, 95 °C
for 10 min, 45 cycles of 95 °C for 30 s, 50 °C for 60 s, and 72 °C
for 20 s. The PCR conditions for narG amplification were 2 min at 50 °C,
10 min at 95 °C, then, 6 cycles, consisting of 95 °C for 15 s, primer
annealing at 63–58 °C for 30 s, and extension at 72 °C for 30 s,
34 cycles with annealing at 58 °C. The PCR conditions for nirS
amplification were 10 min at 95 °C, and 15 cycles of 95 °C for 30 s, 56 °C
for 40 s, 72 °C for 40 s, then, 30 cycles of 95 °C for 30 s, 54 °C for 40 s,
and 72 °C for 40 s. Lastly, PCR amplification of nosZ was conducted
using the following conditions: 5 min at 94 °C, 35 cycles at 94 °C
for 60 s, 61 °C for 60 s, 72 °C for 75 s, and the final extension step
at 72 °C for 5 min. All the PCR amplification experiments included
positive controls containing template DNA from reference bacteria
as well as negative controls. All the PCR products were analyzed on
1.5% (w/v) ethidium bromide-stained agarose gels.

2.6. Standard curves

The amplicons were purified using the AccuPrep PCR purifica-
tion kit (K-3034, Bioneer, Korea). The purified PCR products were
ligated into the pT&A cloning vector (RBC, Twain) and transformed
into Luria-Bertani agar plates containing ampicillin (70 μg/mL).
Transformants were selected on the LB broth with ampicillin (70
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L LB broth with ampicillin (70

2.7. Real-time PCR assays

The real-time PCR assay was performed on the Rotor-Gene 3000
(Corbett research, NSW, Australia). Amplification reactions were
performed in a volume of 25 μL and the reaction mixture con-
tained 12.5 μL SYBR Premix Ex Taq™ (TaKaRa, Japan), 0.5 μM of
each primer, 18 ng of total DNA, and RNase-free water.

The thermocycling steps of the real-time PCR for 16S rDNA
amplification included 95 °C for 30 s, 45 cycles at 95 °C for 15 s,
53 °C for 20 s, and 72 °C for 20 s. The PCR conditions for the
narG amplification were 30 s at 95 °C, 6 cycles of 95 °C for 15 s, 63 °C
for 30 s, and 72 °C for 20 s, then, 34 cycles with annealing at 58 °C.
The nirS PCR amplification conditions included 30 s at 95 °C, 15
cycles of 95 °C for 15 s, 66 °C for 20 s, 72 °C for 20 s, 30 cycles of
95 °C for 15 s, 60 °C for 20 s, and 72 °C for 20 s. Lastly, PCR
amplification of nosZ included 30 s at 95 °C 15 cycles of 95 °C
for 15 s, 59 °C for 20 s, 72 °C for 20 s, 30 cycles of 95 °C for 15 s,
57 °C for 20 s, and 72 °C for 20 s. The specificity of each PCR assay
was confirmed using both melting curve analysis and agarose gel
electrophoresis. All the measurements were performed in triplic-
ate.

3. Results and discussion

3.1. Production of standard curves for real-time PCR

Standard curves for real-time PCR were generated using previ-
ously diluted plasmid solutions containing the cloned 16S rDNA,
narG, nirS, and nosZ genes. All standard curves exhibited high cor-
relation efficiencies with R^2 > 0.99 and similar slopes (see Fig. 2).
Standard curves were used as the reference to extrapolate and
calculate the concentrations of environmental DNA samples. The
efficiency of PCR amplification of the 16S rDNA, narG, nirS, and nosZ
genes was 100%, 92%, 94%, and 108%, respectively.

Table 1
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<thead>
<tr>
<th>Genes</th>
<th>Cultures</th>
<th>Forward primers</th>
<th>Reverse primers</th>
<th>References</th>
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<tr>
<td>16S rDNA</td>
<td>Escherichia coli JM 109 (ATCC 53323)</td>
<td>ATGGCTGTCTCCTGACCT</td>
<td>ACTGGGCGGTCTGCTAC</td>
<td>Harms et al., 2003</td>
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<td>narG</td>
<td>Escherichia coli JM 109 (ATCC 53323)</td>
<td>TATGCTGGCCAGGARAAACTGCGTAGAAGAAGCTGGTGC</td>
<td>CTGAGAAGAAGCTGTCCTTGT</td>
<td>López-Gutiérrez et al., 2004</td>
</tr>
<tr>
<td>nirS</td>
<td>Roseomonas nitroreducens (ATCC 33942)</td>
<td>TACCCACCGGARCCGGCGT</td>
<td>GCCCCGCGTCTCAGAAAA</td>
<td>López-Gutiérrez et al., 2004</td>
</tr>
<tr>
<td>nosZ</td>
<td>Pseudomonas stutzeri (ATCC 17699)</td>
<td>AGAACGACACGCTGATGAC</td>
<td>TCCATGTTGAGCGCCTGTT</td>
<td>López-Gutiérrez et al., 2004</td>
</tr>
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Altschul et al., 1990). All designed primers were synthesized by
Xenotech (Daejeon, Korea).

Please cite this article in press as: Chon, K., et al., Abundance of denitrifying genes coding for nitrate (narG), nitrite (nirS), and nitrous oxide (nosZ) reductases in estuarine versus wastewater effluent-fed constructed wetlands. Ecol. Eng. (2009), doi:10.1016/j.ecoleng.2009.04.005
estuarine wetlands, which have similar patterns to those per ng of DNA. The three functional genes at all the sites ranged from 1.0 × 10^6 to 1.0 × 10^9 copies per g of soil and estimations of the three denitrifying genes per g of dry soil revealed that the densities of the narG, nirK, and nosZ genes were similar to those obtained in other studies (Henry et al., 2006; Kandeler et al., 2006).

### 3.3. Relative abundance of the different denitrifying genes (narG, nirS, and nosZ)

The ratios of the denitrifying genes to 16S rDNA extracted from total bacteria were determined to evaluate the relative abundance of denitrifiers compared to total bacteria. The relative contributions of the nirS gene to total bacteria were the highest for all samples taken from the Damyang constructed wetland in both February and May, as shown in Fig. 5(a). This trend was similar to the trend observed in the amount of gene copies per unit masses of DNA and soil. The relative abundance of the three functional genes was higher and lower for Acorus than for Typha in February and May, respectively, indicating that during the warm season denitrification related bacteria were more dominant than the others in the Typha pond (i.e., the last part of the whole wetlands). This result is in agreement with our previous study, which showed that nitrate was successfully removed especially by the Typha pond (Park et al., 2008). The relative abundance of the three functional genes significantly decreased for Acorus in May compared to February.

For the Suncheon estuarine wetland, the relative contributions of the narG and the nirS to total bacteria were the highest for all the sampling sites in February and May, respectively (see Fig. 5(b)). The most dominant functional genes appear to vary from season to season, which is potentially accompanied by changes in bacterial activities. The relative abundance of the narG, nirS, and nosZ genes did not exhibit significant changes throughout the sampling sites.

Real-time PCR assays were used to determine the copy numbers of narG, nirS, and nosZ genes in the sediment samples taken from both the Damyang constructed and Suncheon estuary wetlands in February and May 2008, using the primer sets described in Table 1. In the wastewater effluent-fed constructed wetlands, the gene copy numbers per ng of DNA of the nirS gene were higher than those of the narG and nosZ genes in both February and May at all the sampling sites (see Fig. 3(a)). This trend implies that there is a greater abundance of genes for the nitrite-reducing genes than for the nitrate and nitrous oxide reducing genes. There was no major difference in the copy numbers of all of the genes between the Acorus (the front part of the whole wetlands) and Typha (the last part of the wetlands) ponds in both February and May, implying that the former and latter wetland ponds had almost the same denitrifying capabilities, indicated by the number of copies of functional genes per ng of DNA. Gene copies of the narG and nosZ genes slightly decreased from February to May.

In the estuarine wetland samples, Fig. 3(b) depicts the copy numbers of functional genes per ng of DNA extracted from estuary head to mouth in the Suncheon estuarine wetland. The most abundant genes at all sites in February and May were narG and nirS, respectively. This suggests that nitrate and nitrite-reducing genes dominated over the other genes in relatively cold and warm temperatures, respectively. The nirS genes were more abundant in all the samples from the wastewater-fed wetlands in both seasons. Moreover, the gene copy numbers of the narG and nirS genes significantly decreased and increased, respectively, at all the sites from February to May. However, the nosZ copy numbers did not change at all the sites in different seasons.

Fig. 4 shows the copy numbers of functional genes per g of dry soils taken from both the Damyang constructed and the Suncheon
tested in May. However, the relative abundances of the three functional genes increased from the estuary head toward the mouth, in February, which indicates that the estuarine wetland (i.e., mouth) has higher denitrification activities (or abundance of genes) than the riparian sites. The relative abundance of the three denitrifying genes ranged from 0.0% to 3.0%, which is in good agreement with previous culture-based studies where the ratio of denitrifying bacteria to total bacteria was reported to range from 0.1% to 5.0% (Tiedje, 1988; Chéneby et al., 2000).

Lastly, it should be noted that the presence of functional genes in environmental samples does not necessarily indicate that the corresponding bacteria present in the environment will display the expected activities (Philippot and Hallin, 2005). Thus, mRNA-based real-time PCR can be used to identify the denitrifying activities that are associated with the functional genes investigated in the work.

4. Conclusions

In this study, denitrifying genes from soils at the two different wetlands (constructed versus estuarine) were amplified and quantified using SYBR-based real-time PCR. Nitrate-reducing functional genes (i.e., nirS) were dominant in the wastewater effluent-fed wetlands, as measured using gene copies per unit mass of soil and extracted DNA, when wetland pond sites and seasonal variation were not accounted for. The same trend was observed for the relative contribution of functional genes to the total bacteria based on 16S rDNA. Meanwhile, for the estuarine wetland sites, the nitrate (narG) and nitrite (nirS) functional genes were relatively dominant during cold and warm seasons, respectively. Even though robust patterns and conclusions could not be obtained from the limited investigations, patterns with certain trends and needs for potential future research directions were obtained.

Acknowledgement

This research was supported by a grant from the National Research Laboratory Program by the Korea Science and Engineering Foundation (NOM ecology Lab: ROA-2007-000-20055-0).

References


