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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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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.

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 NO_3^- to NO_2^- 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-Delif et al., 2005). The reduction of NO_2^- 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 N_2O to N_2, is catalyzed by nitrous oxide reductase, which is encoded by the *nosZ* gene present in the periplasm (Scala and Kerkhof,

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1998, 1999; Stres et al., 2004; Throbäck et al., 2004; Horn et al., 2006).

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-Gutiérrez 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 m^3/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 NO_3^- (Park et al., 2008). The Suncheon estuary has a coastline length of 39.8 km, which consists of a foreshore area of 21.6 km^2 and a reed field area of 5.4 km^2. 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. Soil 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 eutropha* 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-100 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

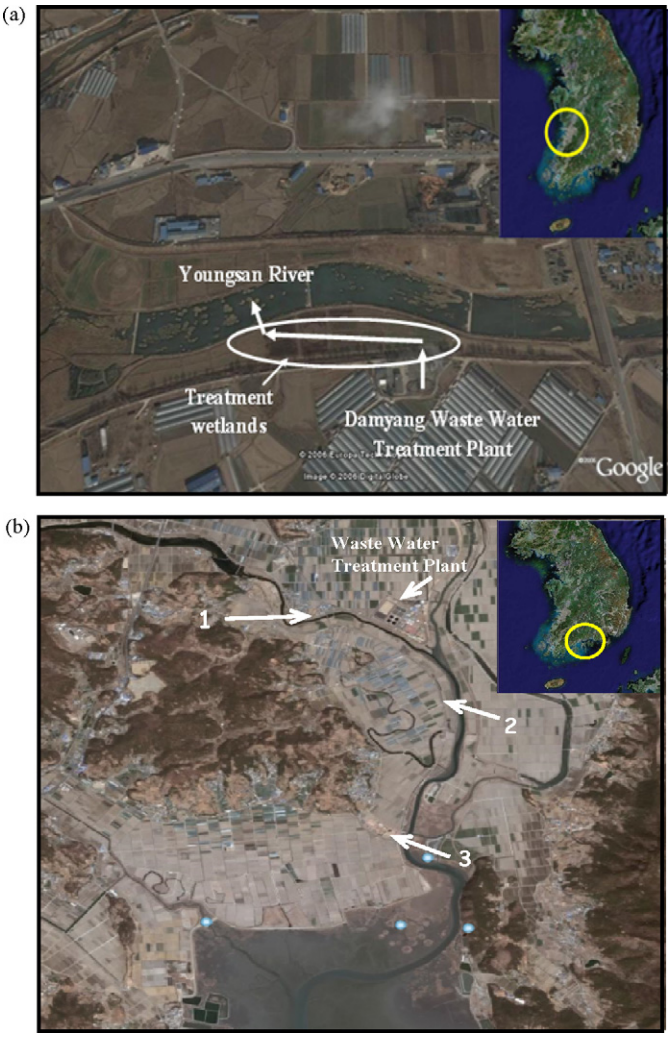

Fig. 1. Sampling sites: (a) the Damyang constructed and (b) the Suncheon estuarine wetlands.

measured with the DNA elution buffer. Three replicate measurements were taken for each sample and averaged. DNA purity was estimated from the A_{260}/A_{280} ratio. The A_{260}/A_{280} 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;