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Abundance and expression of denitrifying genes (*narG, nirS, norB,* and *nosZ*) in sediments of wastewater stabilizing constructed wetlands

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ABSTRACT

As expected, the expression of denitrifying genes in a *Typha* wetland (relatively stagnant compared to other ponds), showing higher nitrogen removal efficiency in summer, was affected by temperature. The abundance and gene transcripts of nitrate reductase (*narG*), nitrite reductase (*nirS*), nitric oxide reductase (*norB*), and nitrous oxide reductase (*nosZ*) genes in seasonal sediment samples taken from the *Acorus* and *Typha* ponds of free surface flow constructed wetlands were investigated using quantitative polymerase chain reaction (Q-PCR) and quantitative reverse transcription PCR (Q-RT-PCR). Denitrifying gene copy numbers (10^5-10^8 genes g^{-1} sediment) were found to be higher than transcript numbers-(10^3-10^7 transcripts g^{-1} sediment) of the *Acorus* and *Typha* ponds, in both seasons. Transcript numbers of the four functional genes were significantly higher for *Typha* sediments, in the warm than in the cold season, potentially indicating greater bacterial activity, during the relatively warm season than the cold season. In contrast, copy numbers and expression of denitrifying genes of *Acorus* did not provide a strong correlation between the different seasons.

Keywords: Constructed wetland, Denitrifying genes, quantitative polymerase chain reaction (Q-PCR), quantitative reverse transcription polymerase chain reaction (Q-RT-PCR), Sediments

1. Introduction

Constructed wetlands have been used to remove nitrate from wastewater effluents after conventional treatment processes [1-3]. Denitrification is considered as a major nitrogen removing mechanism, which transforms NO_3^- and NO_2^- to the gases NO, N_2O and N_2 in various constructed wetlands and is a critical process of the nitrogen cycle [4]. Denitrification is carried out by heterotrophic bacteria under anoxic or anaerobic conditions [5]. Denitrifying bacteria use nitrate as an electron acceptor, which is reduced to nitrogen gas. The reduction of NO_3^- to gaseous forms of nitrogen is catalyzed by several reductases [6].

Functional genes that encode key enzymes, such as nitrate, nitrite, nitric oxide, and nitrous oxide reductase in the denitrification pathway, have been exploited as molecular markers for qualitative and quantitative studies of denitrifiers in the environment. The reduction of soluble nitrate to nitrite is catalyzed by either a membrane-bound (*nar*) or a periplasmic nitrate reductase (*nap*), which is encoded by the *narG* or the *napA* gene, respectively [7]. The reduction of soluble nitrite to nitric oxide gas is catalyzed by either a cyto-chrome cd_1 nitrite reductase (encoded by *nirS*) or a copper nitrite reductase (encoded by *nirK*) [8-10]. The reduction of NO to N_2O is catalyzed by two nitric oxide reductases, *cNOR* (cytochrome c electron donor) and *qNOR* (quinol electron donor) [11]. *cNor* is the most commonly observed indenitrifiers. *qNor* has also been found in some denitrifiers but also in non-denitrifying microorganisms with a detoxification function against nitric oxide [12, 13]. The last step of the denitrification pathway, the reduction of nitrous oxide to dinitrogen, is catalyzed by nitrous oxide reductase (encoded by the *nosZ*) which exists in the periplasm [14-16].

Quantitative polymerase chain reaction (Q-PCR) is now widely used in microbial ecology since it is a highly reproducible, sensitive, and robust method to determine functional gene abundances under varying environmental or experimental conditions [17]. Q-PCR has been applied to quantify the copy numbers of denitrifying functional genes in various environmental samples [18-22].

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Combining Q-PCR with an initial reverse transcription (RT) reaction facilitates the quantification of gene transcript numbers in environmental systems, enabling quantitative estimates of the metabolic activity of functional genes in the environment. The application of quantitative reverse transcription PCR (Q-RT-PCR) to environmental samples has been limited to the quantification of functional gene transcripts from aqueous system, individual species and soils [23-25]. This technique has been applied to quantify the denitrification gene transcripts in estuarine sediments [26], to evaluate the denitrifying gene expression in *Thiobacillus denitrificans* under denitrifying conditions [27] and to evaluate the effect of complex carbon sources on the denitrifier mRNA levels in agricultural soils [28]. However, there have been relatively few studies on the abundance and gene expression of denitrifying bacteria using Q-PCR and Q-RT-PCR methods in constructed wetlands. Therefore, little is known about the role of denitrifying bacteria with regard to nitrogen control in constructed wetlands.

The objectives of this study were to quantify the abundance and transcripts of key denitrifying functional genes (*narG*, *nirS*, *norB*, and *nosZ*), with respect to controls of nitrate, in constructed wetland, using SYBR green based both Q-PCR and Q-RT-PCR. To our knowledge, this is the first report on the expression of denitrifying functional genes in response to nitrogen removal and seasonal changes in wastewater stabilizing constructed wetlands.

2. Materials and Methods

2.1. Site Description and Sampling

All sediment samples were collected in triplicate from the Damyang surface-flow constructed wetlands (35°18'N, 126°58'E) on September 3rd (25˚C) and November 17th (10˚C), 2009. The wetlands consist of two different ponds, with *Acorus* and *Typha* plants dominating one pond each. The *Acorus* pond was further upstream than the *Typha* pond, each of which acts as tertiary treatment systems between the wastewater effluent and the downstream wetlands system. The water characteristics for each pond have been previously described by Chon et al. [29]. Removal efficiency of total nitrogen (TN) was higher in September than in November, in the *Typha* pond, indicating that the denitrification rate was affected by temperature. The flow rate and hydraulic retention time (HRT) of the whole wetland system was designed to be approximately 1,800 m^3/day and 6 hr, respectively. The whole wetland dimensions were: 120 m × 30 m × 0.13 m (length × width × depth) [22]. The soil layers in the Acorus wetland consist of a porous media, which provide habitats for microorganisms. In contrast, the *Typha* wetland soil has been classified as a loamy sand. Sediment samples were transported to the laboratory in an ice box, and were then homogenized and divided into aliquots and frozen at −80˚C until further molecular analysis.

2.2. DNA Extraction

Soil DNA was extracted in triplicate using the PowerSoil DNA isolation Kit (PowerSoil; Mobio laboratories Inc., CA, USA), according to the manufacturer's instructions. The concentration of DNA was also determined at 260 nm using a Nano-drop spectrophotometer. DNA extracts were stored at −20˚C until further analysis.

2.3. Total RNA Extraction and cDNA Synthesis

Total RNA was extracted from 1.5 g sediment samples using the RNA PowerSoilTM total RNA isolation Kit (PowerSoil; Mobio laboratories Inc., CA, USA), according to the manufacturer's instructions. Extraction of RNA was confirmed by gel electrophoresis. Aliquots of total RNA sample was digested with DNase I (Takara, Japan) according to the provided instructions. After incubation for 30 min at 37˚C, phenol/chloroform extraction was performed to inactivate DNase I. Purified precipitated RNA samples were resuspended in Diethyl Pyrocarbonate (DEPC) treated water. Genomic DNA was confirmed to be removed completely by gel electrophoresis. Extracts were stored at −80˚C prior to use. RNA purity was assessed by the A260/A280 ratio, with range of 1.9-2.1.

2.4. Primers Design

Table 1 lists the details of primers used for the amplification of denitrifying functional genes (*narG*, *nirS*, *norB*, and *nosZ*). All the designed primers were synthesized by Xenotech (Daejeon, Korea).

2.5. PCR Conditions

PCR amplification of denitrifying functional genes from soil samples was performed to prepare Q-PCR standards and to confirm the specificity of amplicons. PCR amplification of *narG*, *nirS*, *norB* and *nosZ* genes was performed at a total volume of 20 μL in a mastercycler (Eppendorf Mastercycler personnel, Germany). The PCR mixture consisted of PCR premix (AccuPower PCR PreMixkit; Bioneer, Korea), 1 μL of 10 pmol of each primer, and 30 ng of

Table 1. Primer Sets Used for Q-PCR

Functional gene	Primer	Primer sequence (5´-3´)	Reference
narG	narG1960m2f	TAY GTS GGG CAG GAR AAA CTG	[30]
	narG2050m2r	CGT AGA AGA AGC TGG TGC TGT	
nirS	niS2F	TAC CAC CCS GAR CCG C GC GT	[8]
	nirS3R	GCC GCC GTC RTG VAG GAA	
norB	cnorB2F	GAC AAG NNN TAC TGG TGG T	[11]
	cnorB6R	GAA NCC CCA NAC NCC NGC	
nosZ	nosZF	AGA ACG ACC AGC TGA TCG ACA	[22]
	nosZR	TCC ATG GTG ACG CCG TGG TTG	

Q-PCR: quantitative polymerase chain reaction.

template DNA.

The thermal cycling conditions for *narG* amplification were 10 min at 95˚C, 6 cycles of 95˚C for 30 sec, 63˚C for 30 sec with a touchdown of −1˚C by cycle, and 72˚C for 30 sec, 35 cycles with annealing at 58˚C. The conditions for *nirS* included 95˚C for 10 min, 35 cycles at 95˚C for 30 sec, 61˚C for 30 sec, and 72˚C for 30 sec; followed by a final extension step at 72˚C for 10 min. The *norB* PCR amplification conditions included 95˚C for 10 min, 10 cycles at 95˚C for 30 sec, 30 sec of primer annealing with a touch down from 61 to 55˚C, and 72˚C for 30 sec, 30 cycles with annealing at 55˚C. PCR amplification of *nosZ* included 10 min at 95˚C, 35 cycles of 95˚C for 30 sec, 60˚C for 30 sec, and 72˚C for 30 sec; followed by a final extension step at 72˚C for 5 min. All the PCR products were analyzed on 1.5% (w/v) ethidium bromide-stained agarose gels to ensure the correct size fragment was amplified.

2.6. Q-PCR and Q-RT-PCR Standard Curves

The PCR amplicons were purified using the AccuPrep PCR purification kit (k-3034; Bioneer, Korea). The purified PCR products were ligated into the yT&A cloning vector (RBC, Twain) using the protocol provided in the yT&A Cloning kit and transformed into HIT-DH5 α (RH617; RBC, Twain) cells. Transformants were selected on Luria-Bertani agar plates containing ampicillin (70 μg/mL), 20 μL of 5-bromo-4-chloro-3-indolyl-δ-D-galactoside (X-gal) (40 mg/mL), and 8 μL of isopropyl-beta-D-thiogalactopyranoside (IPTG) (40 mg/mL). A single white colony containing the recombinant plasmids was inoculated into 200 μL Luria-Bertani (LB) broth containing ampicilin (70 μg/mL), and incubated at 37˚C for 4−5 hours. The plasmid DNA was prepared by the Gene All quick plasmid kit (GeneAll Biotechnology, Korea) according to the manufacturer's protocol. The plasmid concentrations were measured by a Nano-drop Spectrophotometer using the absorbance at 260 nm. Plasmids containing the fragments were linearized using the XbaI (Takara, Japan) restriction enzyme. The presence of the appropriate insert in the recombinant plasmids was verified by PCR amplification using the corresponding primers. Serially diluted linearized plasmid with 10^3 to 10^8 gene copies μL^{-1} was used as standards. Standard curves for the *narG*, *nirS*, *norB*, and *nosZ* assays were generated by plotting the threshold cycle (Ct) values against log10 of the gene copy numbers. The amplification efficiency (E) was calculated from the slope of the standard curve through the following formula: $E = (10^{-1/\text{slope}}) - 1$.

Q-RT-PCR standards were produced by *in vitro* transcription of the target functional genes. The target gene amplicon was ligated into the yT&A cloning vector (RBC, Twain) and transformed into HIT-DH5α (RH617, RBC) cells. Transformants were selected on LB agar plates containing 70 μg/mL ampicillin, 20 μL of 40 mg/mL X-gal, and 8 μL of 40 mg/mL IPTG. A single white colony containing the recombinant plasmids was inoculated into 200 μL LB broth with 70 μg/mL ampicilin, and incubated at 37˚C for 4−5 hours. The plasmid DNA was then extracted and purified using the Gene All quick plasmid kit (GeneAll Biotechnology, Korea). The plasmid concentrations were assessed by measuring absorbance at 260 nm using a Nano-drop Spectrophotometer. The presence of the appropriate insert in the recombinant plasmids was verified by PCR with the insert reverse primer and the vector primer M13F. The amplicons were purified using the AccuPrep PCR purification kit (k-3034; Bioneer, Korea) and used for by *in vitro* transcription using the MEGAshortscriptTM kit (Ambion). The *in vitro transcription reaction* (20 μL) contained template DNA, 75 mM of each ribonucleotide, 10 × reaction buffer and 1 × T7 enzyme mix. The reaction was incubated at 37˚C for 4 hr. The DNA template was removed from the reaction by treatment with 1 μL of TURBO DNase (Ambion) at 37˚C for 5 min. The reaction was terminated by adding 115 μL nuclease-free water and 15 μL 3M sodium acetate. Phenol:chloroform extraction followed by alcohol precipitation were performed to remove all enzymes and most of the free nucleotides from the reactions. RNA concentration was measured using a Nano-drop Spectrophotometer at 230 nm. The transcripts were loaded and analyzed in 1% agarose gel after heating with Gel loading Buffer (Ambion) for 3 min at 95˚C. All RNA samples were stored in aliquots at −80˚C until use. Each RNA was individually reverse transcribed, and the cDNA from RT reaction was used to create a serial dilution, which was subsequently used as template for the standard curve.

2.7. Calculation of Copy Numbers

The copy number for Q-PCR standards were calculated by assuming a molecular mass of 660 Da for double-stranded DNA and 330 Da for single-stranded RNA. The calculation was done with the following equations: Copy number = concentration of standard (g/μL) × Avogadro constant (6.02×10^{23} copies/mole)/MW (g/mol)

2.8. Q-PCR and Q-RT-PCR Analysis

The Q-PCR assay was performed on the Rotor-Gene 6000 (Corbett research, NSW, Australia). Q-PCR assays were performed within a single plate to generate both a DNA standard curve, a cDNA standard curve, and to quantify the numbers of denitrifying functional genes (*narG*, *nirs*, *norB*, and *nosZ*) from the environmental DNA and cDNA templates. Each assay was conducted in a 72-well rotor with each standard, no template controls (NTC), and three replicate DNA and cDNA samples. Both triplicate DNA and cDNA samples were amplified in triplicate within individual Q-PCR assays. Amplification reactions were performed in a volume of 20 μL and the reaction mixture contained 10 μL 2 × SensiMixPlus (Quantace, Norwood, MA), 0.25 μM of each primer, 1 μL of template DNA/cDNA, and RNase-free water.

The thermal cycling steps of the Q-PCR for *narG* amplification were 10 min at 95˚C, 6 cycles of 95˚C for 10 sec, 63˚C for 15 sec with a touchdown of −1˚C by cycle, and 72˚C for 20 sec, and 81˚C for 15 sec (acquisition data step); 35 cycles with annealing at 58˚C. The *nirS* PCR amplification conditions included 95˚C for 10 min, 35 cycles at 95℃ for 10 sec, 61℃ for 15 sec, and 72˚C for 20 sec. The PCR conditions for *norB* amplication were 10 min at 95˚C, 10 cycles of 10 sec at 95˚C, 15 sec of primer annealing with a touch down from 57 to 53˚C, primer extension of 30 sec at 72˚C, and an additional 40 cycles with a constant annealing temperature of 53℃. PCR amplification of *nosZ* included 10 min at 95˚C, 40 cycles of 95˚C for 10 sec, 60˚C for 15 sec, and 72˚C for 30 sec. The specificity of each PCR assay was confirmed

using both melting curve analysis and agarose gel electrophoresis. Two independent Q-PCR assays were performed on each of the three replicate soil DNAs. Unknown DNA/cDNA samples were quantified against standard curves using RotorGene 6000 series softwear v. 1.7 (Corbett research, NSW, Australia). The coefficient of determination (r2), the slope, and the y-intercept value for each standard curve were determined. In addition, the amplification efficiency (E) was estimated using the slope of the standard curve through the following formula: $E = (10^{-1/\text{slope}}) - 1$. In order to test for an inhibitory effect of soil DNA on PCR amplification, Q-PCR was conducted by spiking soil DNA with control plasmid DNA. In addition, serial dilutions of soil DNA were quantified and compared.

Two-step Q-RT-PCR was carried out. RT reaction was performed by using PrimeScript™ RT reagent Kit (Takara, Japan), according to the manufacturer's instructions. The initial 10 μL volume RT reaction was performed as follows: 1 μL of RNA standard or 1 μL of environmental RNA, 25 pmol of oligo dT primer, 50 pmol of Random 6 mers, 0.5 μL of PrimeScript TM RT Enzyme Mix, and 2 μL of PrimeScript TM Buffer was made up to a final volume of 10 μL with RNase Free dH_2O. The reaction was incubated at 37°C for 15 min, followed by inactivation of the reaction at 85°C for 5 sec. 1 μL of the first strand cDNA synthesized in this reaction was amplified by Q-PCR using the same reaction and cycling conditions as described above.

2.9. Data Analysis

Two independent Q-PCR assays were carried out on each of the three replicate soil DNA/RNA extracts. One way analysis of variance (ANOVA) was performed, using SPSS v. 15., for each wetland soil in order to observe differences in functional gene copy numbers and their transcripts between sites and sampling dates. Data were log or square root transformed to meet the ANOVA premises. Means were compared using the least significant difference (LSD) test at $p < 0.05$.

3. Results and Discussion

3.1. Production of Standard Curves for Q-PCR and Q-RT-PCR

Standard curves for Q-PCR and Q-RT-PCR were generated by preparing 10-fold dilutions of four plasmids and four cDNA solutions containing *narG*, *nirS*, *norB*, and *nosZ* genes, respectively. All standard curves exhibited linearity between $10^2 - 10^8$ copies for Q-PCR and $10^3 - 10^8$ copies for Q-RT-PCR, and had high correlation coefficients of $r^2 > 0.99$ for Q-PCR and Q-RT-PCR. The calculated Q-PCR and Q-RT-PCR efficiencies for *narG*, *nirS*, *norB*, and *nosZ* genes were 96%, 95%, 108%, and 80% (Fig. 1(a)) and 98%, 100%, 82%, and 89%, respectively (Fig. 1(b)).

3.2. Q-PCR Quantification of Denitrifying Genes (*narG, nirS, norB, and nosZ*)

Q-PCR assays were used to investigate the abundance of *narG*, *nirS*, *norB*, and *nosZ* genes in the sediment samples taken from *Acorus* and *Typha* wetlands in September and November 2009, using the primer sets described in Table 1. The density of *nirS*, *norB*, and *nosZ* in *Acorus* and *Typha* sediments ranged between 10^7 and 10^8 copies g^{-1} of sediment according to sampling date, whereas *narG* gene density was between 10^5 and 10^6 copies g^{-1} of sediment (Fig. 2).

The copy numbers of *narG* and *nirS* genes found in the *Acorus* system were higher than those found in the *Typha* wetland in both September and November ($p < 0.05$), while the gene copy numbers for *norB* and *nosZ* found at *Typha* system were higher than those found at *Acorus* in both September and November ($p < 0.05$). The higher abundance of *narG* and *nirS* gene copy numbers in *Acorus* is in good agreement with our previous study. We confirmed that the first (NO_3^- to NO) and second (NO to N_2) half denitrification procedures were dominant in the *Acorus* and *Typha* wetlands, respectively, during the summer season by using Q-PCR and acetylene-block techniques [31].

Gene copy numbers of *nirS* and *norB* were significantly higher in *Typha* sediments in September than in November, but there was no significant difference determined for *narG* and *nosZ* gene copy numbers in *Typha* sediments between September and November. Gene copy numbers of *narG* were significantly higher in *Acorus* sediments in September than in November, but *nosZ* gene copy numbers were significantly greater in *Acorus* sediments in

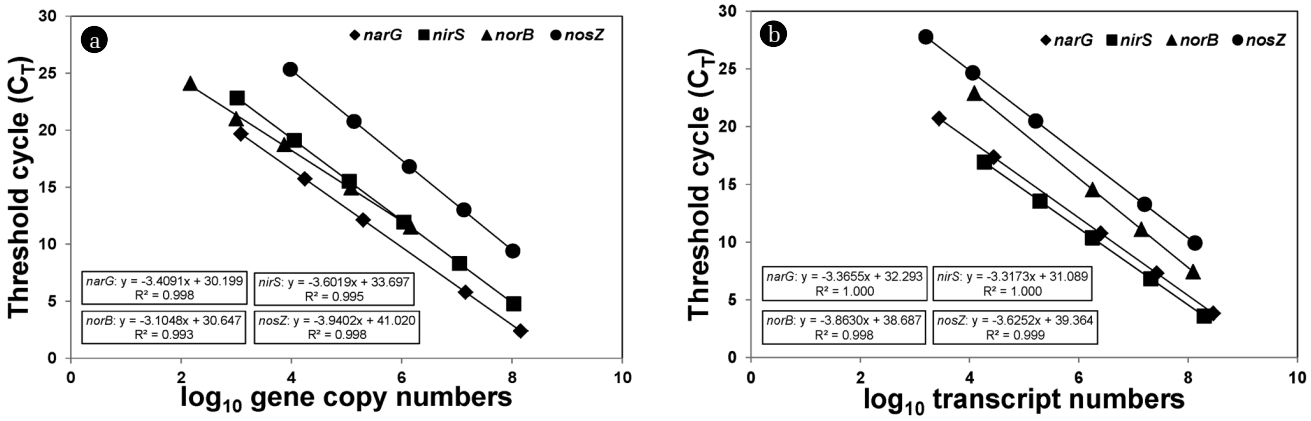

Fig. 1. Standard curves of *narG, nirS, norB,* and *nosZ* Q-PCR assays obtained by calculating (a) gene copy numbers versus threshold cycle, and (b) transcript numbers versus threshold cycle.

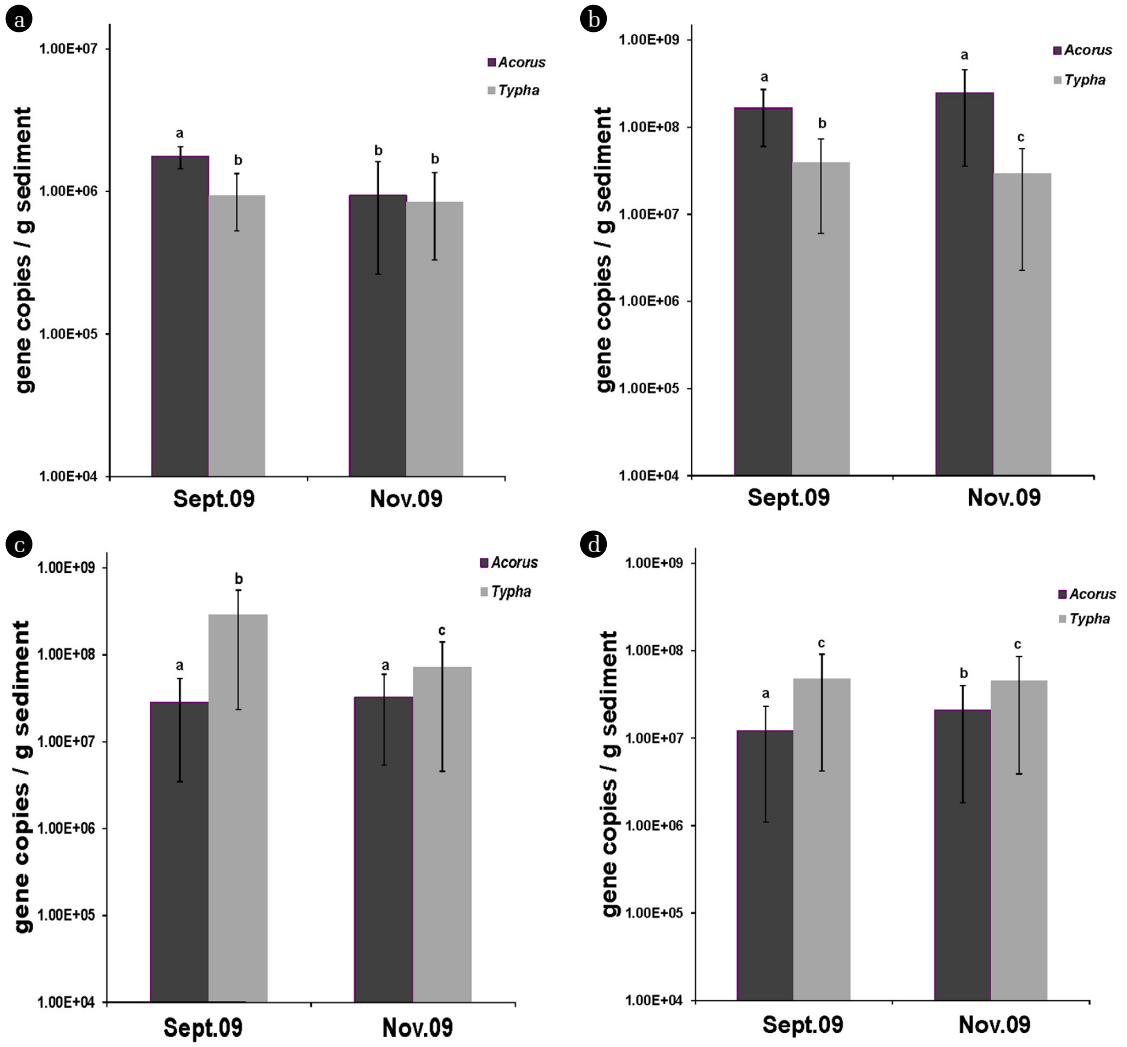

Fig. 2. Variation in gene abundance (copy number g^{-1} sediment) of *narG* (a), *nirS* (b), *norB* (c), *nosZ* (d) in Damyang constructed wetlands in September and November 2009. Error bars indicate standard errors of the two independent PCRs of the three replicate DNA extractions. Significantly different values ($p < 0.05$) between sites and sampling dates are marked by lowercase letters (a to c).

November than in September. There were no significant differences determined for *nirS* and *norB* gene copy numbers in *Acorus* sediments between September and November. The copy numbers of denitrifying genes of *Acorus* and *Typha* wetlands did not seem to strongly correlate with seasonal variation. This result is in agreement with our previous study, which showed little correlation of denitrifying genes (*narG*, *nirS*, and *nosZ*) with seasonal variation [22]. It should be noted that the density of functional genes in environmental samples may not be always linked to bacterial activity, contrary to our expectation.

3.3. Q-RT-PCR Quantification of Denitrifying Genes Transcript Numbers (*narG*, *nirS*, *norB*, and *nosZ*)

Q-RT-PCR assays were used to investigate variation in the levels of *narG*, *nirS*, *norB*, and *nosZ* gene expression using RNA extracted from *Acorus* and *Typha* sediments in September and November 2009. The gene transcripts of *narG*, and *nosZ* in *Acorus* and *Typha* sediments ranged between 10^3 and 10^8 trancripts g^{-1} of sediment according to sampling date, whereas *nirS* and *norB* gene transcripts ranged between 10^5 and 10^7 transcripts g^{-1} of sediment (Fig. 3).

Transcript numbers for *narG*, *nirS*, *norB*, and *nosZ* genes were significantly higher in *Typha* sediments in September than in November, indicating that denitrifying activities are higher during the warm season than during the cold season. It shows good agreement with our previous results representing higher nitrogen removal efficiency at the *Typha* pond of the same wetlands in summer. There was no significant difference observed for *nirS* and *nosZ* transcript numbers in *Acorus* sediments between September and November. However, the *narG* transcript numbers were significantly greater in *Acorus* sediments in November than in September, and the *norB* transcript numbers were sig-

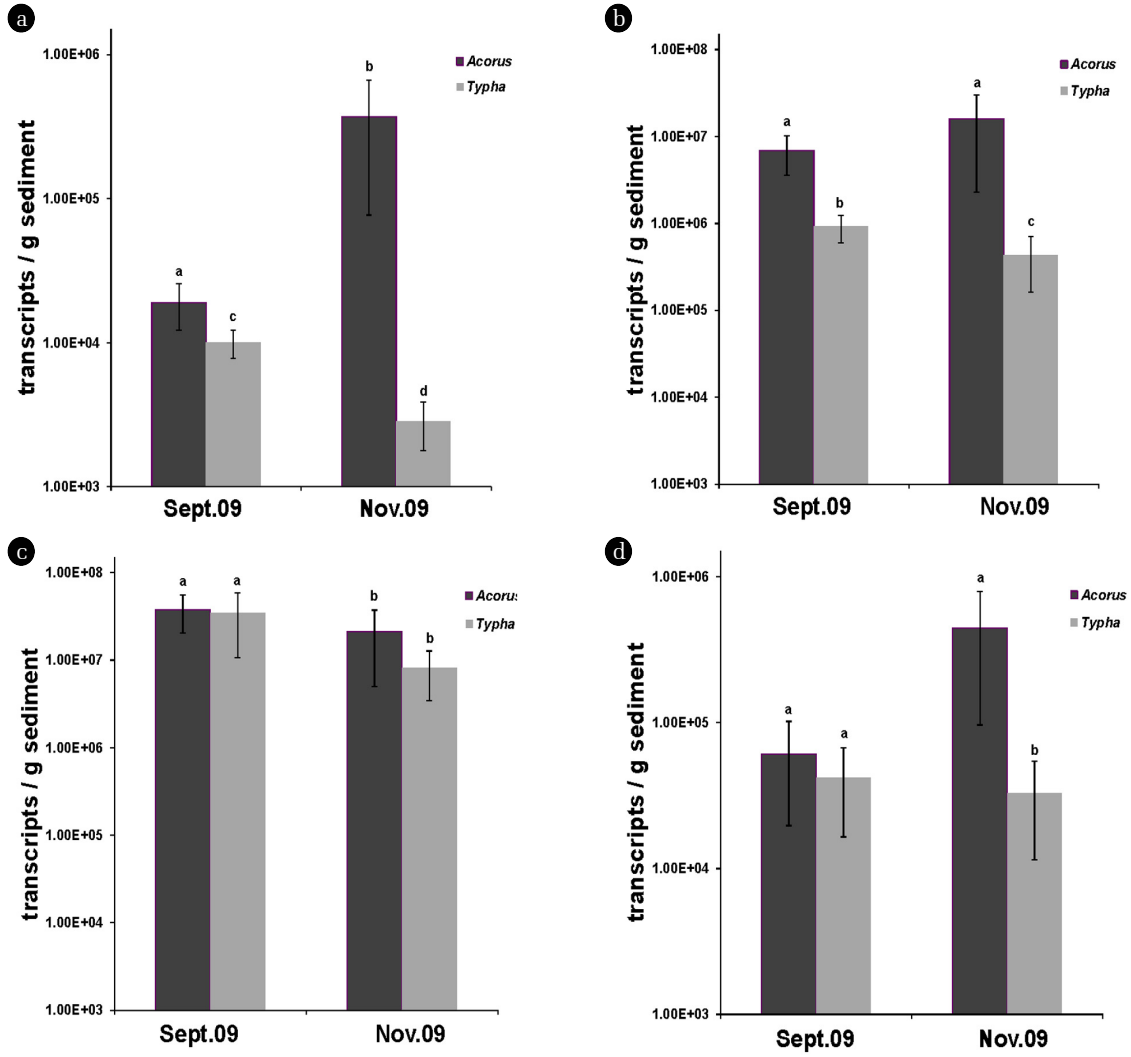

Fig. 3. Variation in gene transcripts numbers (transcripts g^{-1} sediment) of *narG* (a), *nirS* (b), *norB* (c), *nosZ* (d) genes in Damyang constructed wetlands in September and November 2009. Error bars indicate standard errors of the two independent PCRs of the three replicate cDNAs. Significantly different values ($p < 0.05$) between sites and sampling dates are marked by lowercase letters (a to c).

nificantly higher in *Acorus* sediments in September than in November. The *narG* and *nirS* transcript numbers were significantly higher at *Acorus* than at *Typha* in both September and November. Gene copy numbers and transcript numbers of denitrifying functional genes in *Acorus* wetland were not correlated strongly with the seasonal differences. These results are in good agreement with the nitrate data shown in our previous study [29] indicating that there was no significant difference in nitrate removal efficiency in *Acorus* wetland between September and November.

4. Conclusions

SYBR based Q-PCR and Q-RT-PCR were developed and applied to quantify the abundance and expression of denitrifying genes (*narG*, *nirS*, *norB*, and *nosZ*) in Acorus and Typha wetlands in summer and winter. Denitrifying gene copy numbers (10^5-10^8 genes g^{-1} sediment) were higher than transcript numbers (10^3-10^7 transcripts g^{-1} sediment) of the *Acorus* and *Typha ponds* in both seasons. Transcript numbers of the four key functional genes were significantly higher in the *Typha pond* in September than in November, potentially indicating greater bacterial activity during the warmer season than the colder one. It shows good agreement with our previous results representing higher nitrogen removal efficiency at the *Typha pond* of the same wetlands in summer. In contrast, the transcript numbers of denitrifying genes of *Acorus* did not seem to correlate strongly with seasonal differences. Integrated studies on the diversity and distribution of denitrifying functional genes can be further implemented to enhance our understanding of bacterial denitrification in constructed wetlands receiving wastewater effluent.

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