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Investigating microbial activities of constructed wetlands with respect to nitrate and sulfate reduction

Noeon Park[a,c], Jinwook Lee[a], Kyongmi Chon[a], Hojeong Kang[b], Jaeweon Cho[a*]

[a]*Department of Environmental Engineering and Science, NOM Ecology National Research Laboratory, Gwangju Institute of Science and Technology (GIST), Oryong-dong 1, Buk-gu, Gwangju 500-712, Korea*
Tel. +82 62 970-2443, Fax: +82 62 970-2434; email: jwcho@gist.ac.kr
[b]*Department of Civil and Environmental Engineering, Yonsei University, Korea*
[c]*Korea Institute of Science and Technology Evaluation and Planning (KISTEP), 11F Dongwon Industry Bldg., 275 Yangjae-dong, Seocho-gu, Seoul 137-130, Korea*

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ABSTRACT

The biological reduction of both nitrate and sulfate in constructed wetlands, connected to a wastewater treatment plant, was investigated. Three different macrophytes, including *Acorus*, *Nuphar*, and *Typha* plants, were dominant in the free surface flow constructed wetlands; wastewater effluent flows through the *Acorus*, *Nuphar* and *Typha* plants ponds in order. Nitrate was substantially reduced throughout the wetlands, while sulfate was significantly reduced only in the *Typha* wetland, under anoxic conditions. A real-time polymerase chain reaction (RT-PCR) technique was performed to identify and quantify the denitrifying bacteria (DNB) and sulfate reducing bacteria (SRB) in both the wetland effluent and soils, and DNA bands obtained from 16S RNA based PCR experiments were also compared to DNA ladders, which were provided by the corresponding manufacturers. The numbers of the total bacteria in the *Typha* wetland effluent (i.e., a stagnant pond) were higher than those in the other ponds. However, the numbers of total and DNB bacteria extracted from the wetland soils was lower in the *Typha* than in the Acorus wetlands. RT-PCR and acetylene-blocking methods 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. The fractions of both DNB and SRB extracted from the wetlands effluent and soils, against total bacteria, were 2 and 40%, respectively. This work suggests that both nitrate and sulfate ions were effectively reduced by the biological activity of the DNB and SRB present in the wetlands, especially in the *Typha* wetland. The RT-PCR experimental results were shown to be in good agreement with those of the 16S rDNA PCR performed using gel electrophoresis.

Keywords: Denitrifying bacteria; Sulfate reducing bacteria; Total bacteria; Real-time polymerase chain reaction; Constructed wetlands

1. Introduction

Constructed wetlands have been tested for the control of organics, nutrients and heavy metals from discharging either agricultural or wastewater treatment plant effluent [1–5]. The removal of organics by constructed wetlands, in terms of biochemical oxygen demand (BOD) and chemical oxygen demand (COD), have been reported to range from approximate 60 to 90%, and those of anions, including nitrate and sulfate, up to 70%. Heavy metals have also been shown to be efficiently controlled by chemical and biological mechanisms. Recently, the removal of micropollutants (e.g., pharmaceuticals and personal care products) in the wetlands has been investigated [6,7]. Based on previous studies, when wetlands are properly

*Corresponding author.

designed and effectively operated, they have many advantages from both economical and ecological engineering perspectives. Biological degradation is one of the major mechanisms for the removal of both nitrate and sulfate from constructed wetlands. Denitrification by bacteria, from water and soil to the atmosphere, is believed to be an important mechanism for the treatment of wastewater and agricultural fertilizer treatment, and also in the global N-cycle. It should be also noted to emit two greenhouse gases (i.e., NO and N_2O) related to the destruction of ozone layer [8]. The reduction of nitrate by denitrifying bacteria (DNB) was reported to occur in four reaction steps with different enzymes: nitrate (NO_3^-) → nitrite (NO_2^-) → nitric oxide (NO) → nitrous oxide (N_2O) → nitrogen gas (N_2) [9–14]. In these steps, the reduction of nitrous oxide can be blocked by the presence of acetylene. Hence, the production of nitrogen gas can be stopped by the blockage [12,15].

The major enzyme for denitrification is nitrite reductase, which catalyzes soluble nitrite into nitrogen gas. Two types of nitrite reductase (i.e., cytochrome cd_1- and copper-) have been used to identify the DNB in the environment, but cytochrome cd_1- nitrite reductase (encoded by *nirS*) has been used more than copper nitrite reductase (encoded by *nirK*) due to the limitations of the latter. Sulfate reduction bacteria (SRB) are anaerobic bacteria, which use sulfate as an electron acceptor, with organic matters in the environment being consumed as electron donor. They can live ubiquitously in various environments, including soils and freshwater sediments, and are also related to global carbon and sulfur cycles [16,17]. The final product of the action of SRB is H_2S gas. Primers for six major phylogenetic groups of SRB were developed, and then tested on leachate samples from landfill sites. Methanogenesis can also been influenced by SRB, as they inhibit carbon mineralization in the absence of high concentrations of sulfate. Thus, both DNB and SRB are believed to be important in the ecosystem such as natural and constructed wetlands, from global cycles of important elements, including carbon, nitrogen and sulfur.

Polymerase chain reaction (PCR) has been widely used for the identification and quantification of specific bacteria in biotechnology and molecular biology fields. The fingerprints of bacteria can be identified by amplifying specific regions (i.e., either 16S ribosomal DNA or functional genes) of a template DNA strand. Although several different methods have performed for the identification of microbial activities, the technique of real-time PCR (RT-PCR) is believed to be rapid, sensitive and reproducible [18]. It requires specifically designed forward and reverse primers, containing a fluorescent reporter either in the form of a fluorescent DNA-binding dye or as an oligonucleotide primer. In a previous study, RT-PCR was used to monitor and quantify the nitrifying bacteria in the mixed liquor suspended solids (MLSS) from a municipal wastewater treatment plant [19]; nitrifying bacteria from MLSS were quantified by amplifying 16S rDNA. Meanwhile, DNB were quantified by amplifying the denitrifying nitrite reductase, which is a functional gene [20,2]); in their research, environmental samples, including lake and marine sediment samples, were also used. Furthermore, methanotrophic bacteria and the biodiversity of potential pathogens in constructed wetland sediments were studied using PCR method [22].

The objective of this study was to evaluate constructed wetlands with respect to the controls of nitrate and sulfate with DNB and SRB, using PCR technique.

2. Methods and materials

2.1. Site description and sampling

Three macrophytes, *Acorus*, *Nuphar*, and *Typha* plants, were dominant in the tested free surface-flow constructed wetlands. Secondary wastewater effluent from the Damyang wastewater treatment plant flows into the wetlands (*Acorus*, *Nuphar*, and *Typha* plants ponds in order), with the wetlands effluent connected to Youngsan River, Korea. The surface areas of the *Acorus*, *Nuphar*, and *Typha* ponds are 3450, 50 and 2850 m^2, respectively. The depth of each wetland is approximately 20 cm. The *Typha* wetland contains two different regions: the one conveyed wastewater effluent from the *Acorus* wetland to the river, with a relatively short hydraulic retention time. Meanwhile, the other has a relatively long retention time (i.e., somewhat stagnant pond). From an on-site investigation, the water quality of the final wetland effluent was believed to be influenced mostly by the *Acorus* wetland, with some influence due to the edge of the *Typha* wetland. The hydraulic retention time of the whole wetlands was designed to be approximate 6 h. The soil layers in the *Acorus* wetland are composed of porous media with red mud, which provide habitats for microorganisms [23]. However, other wetlands consist of fine sand layer.

Both the wastewater and wetlands effluents were sampled in June and July 2007, and subjected to ion chromatograph (IC) measurements after pretreatment using a micro-filter with a pore size of 0.45 μm and immediate storage in a refrigerator. The oxidation-reduction potential (ORP) of each sample was measured in the field. For the PCR experiment, a 150 mL sample was pretreated using a 5 μm filter to exclude soil and plant debris, and then concentrated using a freeze-dryer (Il Shin freeze dryer, Il Shin Lab, Korea). The samples were dissolved by the addition of 2 mL phosphate buffer solution. The dissolved organic carbon (DOC) of the wastewater and wetlands samples were measured using a total

organic carbon analyzer (TOC-820, Sievers, CO, US). Wetland soils were also sampled to quantify the numbers of bacteria throughout the wetlands.

2.2. Measurement of ions

Both the nitrate and sulfate ion concentrations of the samples were measured using an IC apparatus (DX-120, Dionex, CA, US), equipped with an AS14 column (4× 250mm, Dionex, CA, US). The loop volumes of the auto sampler and flow rate were 100 µL and 1.0 mL/min, respectively. Standard solutions, ranging from 10 ppm to 10 ppb, made by the serial dilution of concentrated solutions (Dionex, CA, US), were prepared to construct a calibration curve, with sodium as 3.5 mM Na_2CO_3, and 1 mM $NaHCO_3$ were used as the IC mobile phase solution. The operating pressure for measurement was delivered by helium gas.

2.3. PCR experiments

2.3.1. DNA extraction and purification

The wetlands effluent samples were concentrated 50 times using a freeze dryer. Genomic DNA from the concentrated wastewater and wetlands effluent was extracted using a DNA extraction kit (AccuPrep Genomic DNA extraction kit k-3032, Bioneer, Korea), according to the manufacturer's instructions. Total genomic DNA from soil samples was also extracted using a PowerSoil DNA isolation Kit (PowerSoil, Mobio laboratories Inc., CA, US). Extracted genomic DNA from both wetland effluent and soils were then purified using a DNA purification kit (AccuPrep PCR purification kit k-3034, Bioneer, Korea) after 16S rDNA amplification with PCR premix, for the construction of a calibration curve. DNA samples of 100 µL were obtained from the kit, and then stored at 4°C until analysis. The concentration of DNA was measured using a fluorometer (Qubit, Invitrogen, CA, US).

2.3.2. PCR primers

The primers for total bacteria, DNB and SRB were prepared by Xenotech, Daejeon, Korea, based on previous works [17,19,24]. Sterile distilled water was added into the primers to prepare a concentration of 20 µM. These primers had previously been designed and tested using genomic DNA extracted from environmental and cell samples. The primer pairs for the DNB targeted the nitrite reductase gene (*nirS*), which is a key enzyme in dissimilatory denitrification. An approach using 16S rDNA did not seem suitable for the identification of the denitrifying bacteria communities, as denitrification is widespread, even among phylogenetically unrelated groups. For the SRB, five RT-PCR primers were used for identification, as based on the work of Daly et al. [17]. Even though the selected primers were not able to cover the total communities of SRB in the wetlands, they could provide information and quantification of the five major SRB phylogenetic groups. The primer information, including DNA-product sizes and sequences, is summarized in Table 1.

2.3.3. PCR and RT-PCR

The PCR mixture included a PCR premix (AccuPower PCR PreMix kit k-2010, Bioneer, Korea), 20 pmole of each primer and 10 µL of concentrated template DNA. PCR amplification was performed in a mastercycler (centrifuge 5415C, Eppendorf, Hamburg, Germany). The PCR protocol for the total bacteria included: 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, and then held at 25°C for 5 min. The protocol for DNB was as follows: 95°C for 10 min, 15 cycles of 95°C for 30 s, 56°C for 40 s, 72°C for 40 s, 30 cycles of 95°C for 30 s, 54°C for 40 s and 72°C for 40 s, and then held at 25°C for 5 min. Lastly, the protocol for SRB was as follows: 50°C for 3 min, 95°C for 10 min, 45 cycles of 95°C for 60 s, 60°C for 60 s and 72°C for 60 s, and then

Table 1
Primer properties

Target	Primer	Product size (bps)	Sequence (5′-3′)
Total bacteria	1055f/1392r	331	ATGGCTGTCGTCAGCT/ ACGGGCGGTGTGTAC
DNB	nirS 2f/nirS 3r	164	TACCACCCCGGAAGCCGCGCGT/GCCGCCGTCAGTGACGAGGAA
SRB	DFM140/842	700	TAGMCYGGGATAACRSYKG/ATACCCSCWWCWCCTAGCAC
	DBB121/1237	1120	CGCGTAGATAACCTGTCYTCATG/GTAGKACGTGTGTAGCCCTGGTC
	DSB127/1273	1150	GATAATCTGCCTTCAAGCCTGG/CYYYYYGCRRAGTCGSTGCCCT
	DCC305/1165	860	GATCAGCCACACTGGRACTGACAGGGGCAGTATCTTYAGAGTYC
	DSV230/838	610	GRGYCYGCGTYYCATTAGC/SYCCGRCAYCTAGYRTYCATC

held at 25°C for 5 min. Aliquots of 10 μL of the reaction mixtures were analyzed by gel electrophoresis, using a 1% wt/vol agarose in trizma base acetic acid and EDTA (TAE) buffer, and then stained for 20 min with ethidium bromide at a concentration of 0.5 mg/L. Amplified DNA bands of the samples were visualized using a UV transilluminator (TFM-20, UVP, Cambridge, UK). The tested bacteria in the samples were identified by comparison to DNA ladders with known DNA fragment sizes.

RT-PCR experiments were performed using the Syber green method (RG 3000, Corbett research, NSW, Australia) to quantify the tested bacteria, with a total samples volume of 25 μL, containing 12.5 μL of premix Ex Taq, 0.5 μL of each primer, 9 μL of a template DNA and 2.5 μL of de-ionized pure water. The temperature profile for the RT-PCR experiments was the same as for the previously described tests. The Rotor gene software, version 6.0 (Corbett research, NSW, Australia), was used for the RT-PCR, with the threshold cycle of each PCR reaction automatically determined by the software. The DNA extracted from the wetland samples was amplified and purified, and was also diluted from 10^{-1} to 10^{-10} for construction of a calibration curve. The standard DNA concentrations of the total bacteria, DNB and SRB collected from the wetlands were 17.32, 8.90 and 8.34 ng/μL, respectively. Negative controls, without template DNA, during the PCR experiments, were used to demonstrate any background contamination.

2.3.4. Acetylene blocking method

In order to determine the dominant denitrification steps in the wetlands, an acetylene blocking method was employed using wetland soil samples from the *Acorus*, *Nuphar* and *Typha* wetlands. The nitrous oxide level was measured in units of mass of N_2O per unit time per unit mass of either dry soil or organic matter mass.

2.3.5. Uncertainty of measurements

Both DOC and PCR experiments were performed in triplicate, with coefficients of variance for all the analyses of less than 10%. Measurements of both the nitrate and sulfate concentrations were performed twice using IC.

3. Results and discussion

3.1. Water characteristics of samples in the constructed wetlands

Table 2 summarizes the characteristics of the wetlands effluent samples, in terms of pH, DOC and ORP values. The pH values of all the samples were within the neutral range. The levels of DOC, with the exception of the *Typha* wetland, slightly increased throughout the wetlands, as opposed to wastewater effluent. The tested wetlands appeared inefficient with respect to the control of effluent organic matter (EfOM) in terms of DOC. The DOC was substantially higher in the *Typha* compared to the other wetlands, which presumably resulted from the leaching of organics from the wetlands sediments under anoxic conditions; humic and fulvic acids have reduction potentials of 0.5 and 0.7 V versus the normal hydrogen electrode, respectively [25]; thus, the organics from wetland soils can be leached under anoxic conditions (refer to ORP values), as listed in Table 2. Fig. 1 demonstrates both the nitrate and sulfate reductions through the wetlands; nitrate ion concentration continuously decreased through the wetlands, and was almost completely removed in the *Typha* wetland. The sulfate ions were efficiently removed only in the *Typha* wetland. Generally, the major removal mechanism of these anions in the environment is known to be microbial metabolisms [26]. It was hypothesized that nitrate/sulfate reducing bacteria were dominantly present in the *Typha* wetland as both anions were efficiently removed, which can be demonstrated using RT-PCR technique.

3.2. Application RT-PCR to effluent and soil samples from wetlands

Standard DNA samples for the total bacteria, DNB and SRB were collected from tested wetlands, and used after 16S rDNA amplifications. The calibration curve for the calculated copy numbers samples versus the measured threshold cycle (CT) of diluted each DNA sample is shown in Fig. 2. The values of the regression coefficient (R^2) for all the PCR experiments were above 0.97. The limits of detection (i.e., negative template control test) for

Table 2
Characteristics of the water in the constructed wetlands

	26 June, 2007			09 July, 2007		
	pH	DOC (mg C/L)	ORP (mV)	pH	DOC (mg C/L)	ORP (mV)
WWTP effluent	6.79	5.18	624.8	7.14	5.15	267.5
Acorus	7.01	4.78	243.5	7.27	6.30	191.2
Nuphar	7.14	5.58	123.1	7.34	7.45	172.1
Typha	6.95	36.55	−148.1	7.47	43.00	−32.0
Wetland effluent	7.15	5.83	73.1	7.09	7.10	139.7

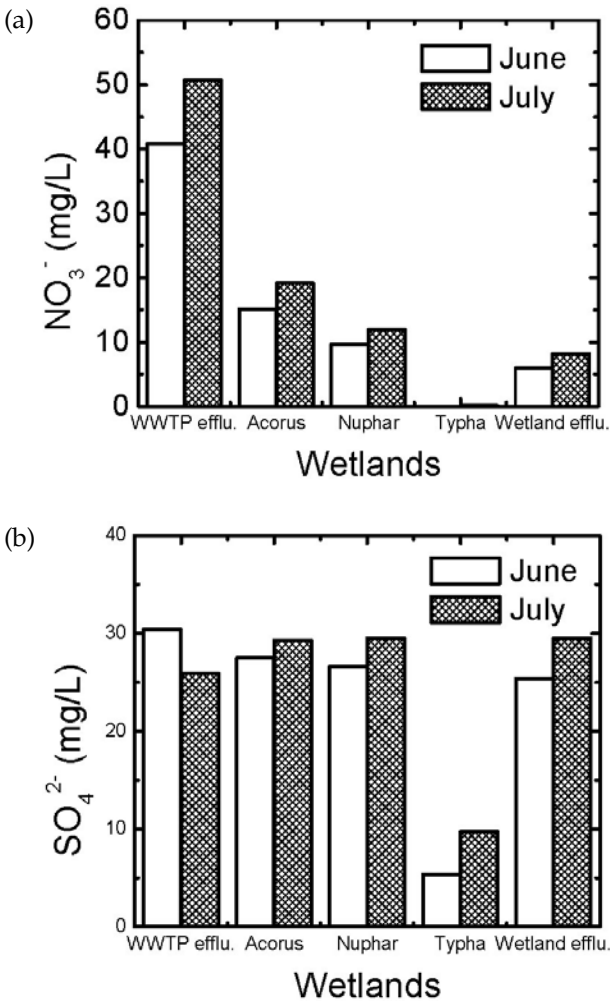

Fig. 1. Nitrate and sulfate concentrations in the constructed wetlands. (a) Nitrate; (b) Sulfate.

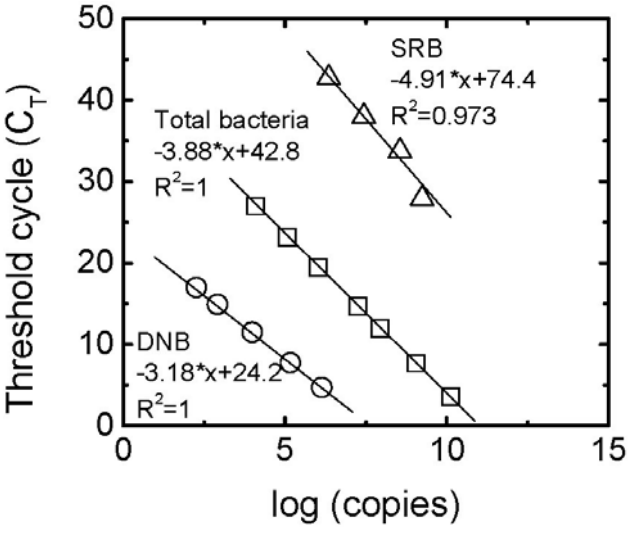

Fig. 2. Threshold cycle measurements against calculated copies of diluted DNA..

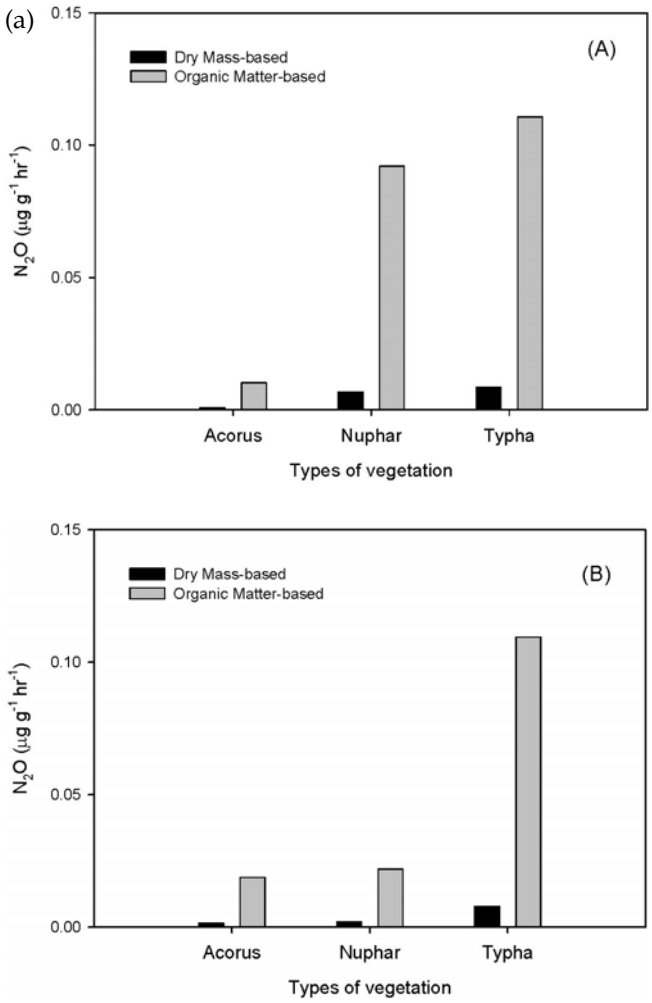

Fig. 3. Nitrous oxide emitted from the different wetland soils taken in (A) June and (B) July, as measured based on dry soil and organic matter masses.

the total bacteria, DNB, and SRB were 1.5, 0.1 and 0.1×10^3 copies, respectively. There might be the potential for inhibition of the PCR experiments, for example, humic substance and DNA contaminations [27]. However, the calibration curve provided good correlation, implying there was virtually no interference in the quantification of the DNA samples extracted from wetlands in this work.

Quantitative evaluation of the total bacteria, DNB and SRB extracted from wetlands effluents and soils was performed using RT-PCR experiments (see Table 3). As mentioned, five primers were tested to identify SRB in the wetlands. However, only one primer (i.e., DCC) was detected for the wetland soils. The copy numbers of the total bacteria ranged from 1.0×10^4 to 3.5×10^5 throughout the wetlands, and the *Typha* wetland exhibited the highest values among all the wetlands. The stagnant *Typha* wetland was believed to provide bacterial habitats, as based on the quantification data. In June, only DNB were

Table 3
Number of total bacteria, DNB and SRB
(a) Copies

	26 June, 2007			09 July, 2007		
	Total bacteria	DNB	SRB	Total bacteria	DNB	SRB
WWTP effluent	$1.1\pm0.5\times10^4$	ND	ND	$1.1\pm0.1\times10^3$	ND	ND
Acorus	$1.1\pm0.4\times10^4$	ND	ND	$1.3\pm0.04\times10^3$	$1.1\pm1.5\times10^2$	ND
Nuphar	$0.1\pm0.7\times10^3$	ND	ND	$8.4\pm1.3\times10^2$	ND	ND
Typha	$3.5\pm0.3\times10^5$	$1.8\pm0.1\times10^3$	ND	$5.8\pm0.06\times10^3$	$1.1\pm1.0\times10^2$	ND
Wetland effluent	$1.5\pm0.3\times10^4$	ND	ND	$1.6\pm2.1\times10^3$	ND	ND

ND: not detected

(b) Copies/g soil

	26 June, 2007			09 July, 2007		
	Total bacteria	DNB	SRB	Total bacteria	DNB	SRB
Acorus soil	$2.7\pm0.7\times10^8$	$1.6\pm0.0003\times10^7$	$9.0\pm0.4\times10^4$	$1.1\pm0.04\times10^8$	$3.4\pm0.0007\times10^7$	$1.3\pm0.1\times10^3$
Nuphar soil	$1.1\pm0.04\times10^8$	$2.9\pm0.0002\times10^7$	$4.3\pm0.1\times10^5$	$3.2\pm0.02\times10^4$	$2.3\pm0.07\times10^4$	ND
Typha soil	$7.9\pm0.3\times10^4$	$3.2\pm0.2\times10^4$	$3.4\pm0.2\times10^3$	$1.0\pm0.03\times10^8$	$3.5\pm0.1\times10^6$	$8.9\pm0.5\times10^3$

(a) Wetland effluent samples. (b) Wetland soil samples.

Table 4
Fractions of DNB and SRB against total bacteria in the various wetlands (%)

	26 June, 2007		09 July, 2007	
	DNB/Total bacteria	SRB/Total bacteria	DNB/Total bacteria	SRB/Total bacteria
WWTP effluent	ND	ND	ND	ND
Acorus	ND	ND	8.2	ND
Nuphar	ND	ND	ND	ND
Typha	0.5	ND	1.9	ND
Wetland effluent	ND	ND	ND	ND
Acorus soil	6.0	0.03	30.9	0.001
Nuphar soil	26.3	0.4	72.8	ND
Typha soil	40.4	0.4	35.6	0.09

quantified in the *Typha* wetland. Meanwhile, they were identified in both *Acorus* and *Typha* wetlands in July. Although the effluent samples had been concentrated 50 times, no SRB were identified in the effluent samples, which required another method, such as cell centrifugation, for the quantification of all the bacteria in the wetlands. The DNB DNA (i.e., *nir*) copies extracted from the wetland soils were also quantified in this work. Unlike the effluent samples, the DNA copies extracted from the *Acorus* wetland soil were higher than those from the *Typha* wetland soil. From this result, only the bacteria responsible for the specific denitrification step (i.e., NO_2^- to NO) appeared to be dominant in the *Acorus* wetland soil over in the *Typha* wetland soil. Therefore, the other bacteria responsible for the other steps (NO to N_2O to N_2) might show a different pattern in wetland soils. In order to demonstrate this, an acetylene-blocking method was performed using the wetland soils. Fig. 3 shows measured nitrous oxide masses. Overall, the denitrification rates (i.e., NO to N_2O) were highest in the *Typha* wetland soil. RT-PCR and acetylene-blocking methods 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.

Based on these results, the fractions of the bacterial populations in the wetlands are summarized in Table 4. For effluent samples, DNB in the *Typha* wetland exhibited ratios between 0.5 and 2% against total bacteria. For soil samples, both DNB and SRB exhibited ratios of between 6% and 72% and 0.001 and 0.4% against total bacteria,

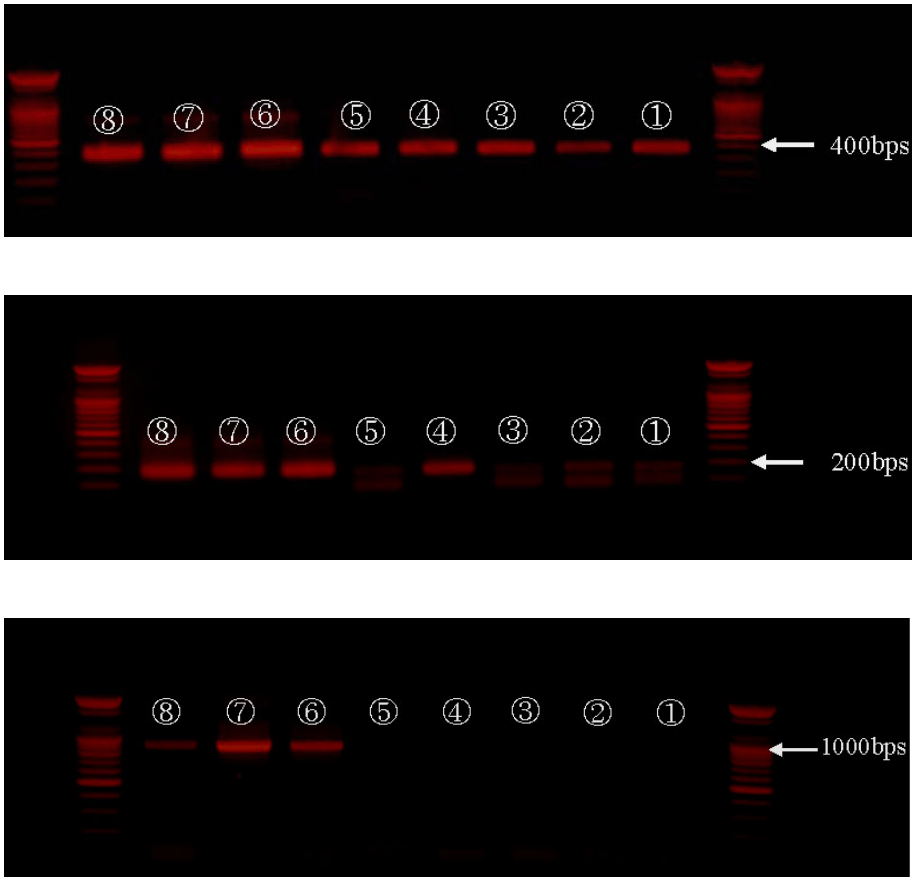

Fig. 4. Bacterial DNA bands of the PCR products after the RT-PCR; (a) Total bacteria (331 bps), (b) DNB (164 bps) and (c) SRB (860 bps). Data were measured for DNA samples taken in June. WWTP effluent (①), *Acorus* (②), *Nuphar* (③), *Typha* (④), wetland effluent (⑤), *Acorus* soil (⑥), *Nuphar* soil (⑦) and *Typha* soil (⑧).

respectively. Both DNB and SRB in the wetland soils exhibited broader ratios than those of the effluent samples. Overall, the numbers of DNB were higher than those of SRB, as the wetlands were governed under anoxic (over anaerobic) conditions, as based on the measured ORP values.

3.3. Comparison and verification of DNA samples between RT-PCR and PCR

The DNA products obtained after the RT-PCR experiments were compared with 16S rDNA experiments results to investigate non-specific bindings of DNA and primers. The DNA bands of the total bacteria, DNB and SRB, after the RT-PCR assays, could be identified (see Fig. 4), using the known DNA product sizes of 331, 164 and 860 bps, respectively, as listed in Table 1. The total bacteria were identified through all the tested wetlands; soil samples appeared as being strongly illuminated compared to the water samples. However, DNB were distinctly illuminated in both *Typha* wetland effluent and soil samples. The DNA products for DNB in the wastewater effluent, *Acorus* and *Nuphar* ponds water samples, and wetland effluent were not distinctly identified; it should be noted that the DNB in these samples were not identified using RT-PCR due to limitation of detection. SRB were only identified in the soil samples, but not in the effluent water samples, which showed good agreement between the RT-PCR and PCR results.

4. Conclusions

Free water surface type wetlands, including *Acorus*, *Nuphar* and *Typha* plants in order, were biologically investigated using both 16S rDNA and RT-PCR techniques. Nitrate was able to be effectively reduced throughout all the tested wetlands, but sulfate was effectively reduced only in the *Typha* wetland. Based on the RT-PCR measurements, the number of total bacteria in the *Typha* wetland (a stagnant pond) effluent was higher than in any other ponds. However, the numbers of total and DNB bacteria extracted from the wetland soils was lower in the *Typha* than in the *Acorus* wetlands. RT-PCR and acetylene-blocking methods 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. This work suggested that both nitrate and sulfate were effectively reduced by the biological activities of DNB and SRB in the wetlands, especially in the *Typha* wetland. The RT-PCR experimental result also showed good agreement with those of the PCR, which were performed with simultaneous gel electrophoresis.

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