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Research Article

Eichhornia crassipes Cleans Wetlands by Enhancing the Nitrogen Removal and Modulating Denitrifying Bacteria Community

The role of floating macrophytes on modulating the microbial nitrogen removal is not well understood. In this study, the cultivation of *Eichhornia crassipes* in eutrophic water may affect the nitrogen (N) fate by modulating the denitrifying bacteria diversity and abundance. The gaseous N losses via denitrification were estimated by ^{15}N stable isotope tracing and the diversity and abundance of denitrifying genes (*nirS*, *nirK*, and *nosZ*) were investigated by molecular tools. The denaturing gradient gel electrophoresis (DGGE) profiles showed that the diversity of denitrifying genes in the treatments with *E. crassipes* was significantly higher than that in the treatment without *E. crassipes*. The real-time PCR (qPCR) results showed the trend of denitrifier abundance in the entire system was in the order of N-ER (nitrate with just root of *E. crassipes*) and A-ER (ammonia with just root of *E. crassipes*) > N-R (nitrate with *E. crassipes*) and A-R (ammonia with *E. crassipes*) > N-W (nitrate without plant) and A-W (ammonia without plant). The gaseous ^{15}N losses via denitrification were significantly and positively related to the abundance of *nirK*, *nirS*, and *nosZ* genes. The results indicated that cultivation of *E. crassipes* in eutrophic water could increase the diversity and abundance of denitrifying bacteria, resulting in more N being removed as gases via denitrification.

Keywords: Denitrification gene; DGGE; ^{15}N Stable isotope tracing; qPCR; Water hyacinth cleaning

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1 Introduction

Nitrogen (N) enrichment in water is recognized as one of the main causes for deterioration of aquatic ecosystems worldwide [1–3]. Large-scale confined cultivation of the floating macrophytes, especially cultivation of *Eichhornia crassipes*, in eutrophic lakes has attracted increasing interests in recent years because macrophytes can assimilate large amounts of nitrogen and phosphorus (P) and, if harvested and shipped away from the catchment, can remove excess nutrients from the waters [4–6].

The transformation and N removal in macrophyte-based aquatic treatment systems comprises several pathways, including suspended solid settling, direct assimilation, biotransformation (e.g. nitrification and/or denitrification), and physicochemical reactions [4]. In the past, much attention was given to direct assimilation of N by *E. crassipes* during the treatment process. However, the role of *E. crassipes* on modulating the microbial nitrogen removal, such as via partial nitrification and/or denitrification, or Anammox and completely autotrophic nitrogen removal over nitrite (CANON) processes, are not well understood [7–11].

Although Anammox and CANON technologies have been frequently used in waste treatment facilities, canonical denitrification other than Anammox and CANON is still believed to account for more than half of nitrogen removal in natural or manual influenced aquatic systems such as lakes and constructed wetlands [12, 13]. Denitrification mainly depends on microbiological processes which are responsible for permanently returning N from soil or water to atmosphere [14, 15]. Numerous studies have reported that generally denitrification accounts for more than half on N removal in aquatic macrophyte-based treatment systems such as constructed wetlands [12, 14, 15].

Significant correlation between denitrification potential rates and denitrifying community patterns in macrophyte-based aquatic treatment systems also suggested a possible role of denitrifying bacterial community structure and abundance in their functioning at an ecosystem level [16, 17]. The research focus so far has been on denitrifier community composition, but there is also a need to quantify abundance in order to link abundance, community composition, and nitrogen removal in a given environment [18]. The denitrifying bacterial community structure and abundance have been suggested as one of the most important factors regulating denitrification processes [16].

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Abbreviations: **ANOVA**, analysis of variance; **CA**, correspondence analysis; **CANON**, completely autotrophic nitrogen removal over nitrite; **COD**, chemical oxygen demand; **DGGE**, denaturing gradient gel electrophoresis; **TN**, total nitrogen

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Previous studies have shown that bacterial abundance, activity, and diversity were enhanced in the plant rhizosphere regions of aquatic macrophyte-based treatment systems [19–21], suggesting that plants enhance the establishment of microorganisms responsible for removing pollutants. The root system of *E. crassipes* suspended in a water column could be a good surface for microbial attachment and biofilm formation [4, 22, 23]. The role of macrophytes as denitrifier hosts could be enhanced by selecting macrophytes with longer roots (100–200 mm) and increasing root densities to 20% v/v of the water column [22, 24]. *E. crassipes* roots can grow from 50 to 1000 mm, with the surface area approximately 2.5 to 8.0 m^2/kg on a dry weight basis [25, 26].

Roots are able to transport oxygen and secrete exudates such as amino acids and sugars [27], which are important in a number of plant-microbial associations. These exudates can influence the structure and function of bacterial communities in the rhizosphere [28]. In addition, roots provide excellent ecological niches for bacteria to colonize [29, 30]. Characterizing the effect of *E. crassipes* and its roots on the diversity and abundance of denitrifiers, therefore, is essential for understanding the effect of cultivation of *E. crassipes* and similar floating macrophytes on denitrification.

Molecular tools such as qPCR and PCR–denaturing gradient gel electrophoresis (PCR–DGGE) have been employed to evaluate the denitrifiers (*nirS*, *nirK*, and *nosZ*) in an aquatic ecosystem [31–33]. In this study, we hypothesize that cultivation of *E. crassipes* improves N removal from eutrophic water by modulating denitrifying bacteria community. The current study employed the molecular approaches and ^{15}N stable isotope tracing method to investigate: 1) effect of *E. crassipes* on abundance and diversity of denitrifying genes including nitrite reductase gene (*nirS* and *nirK*) and nitrous oxide reductase gene (*nosZ*); 2) the influence of abundance and diversity of denitrifying bacteria communities on the fate of nitrogen in eutrophic waters; 3) quantitative relationships between denitrifying genes and the gaseous removal of N from eutrophic water.

2 Materials and methods

2.1 Preparation of eutrophic water containing $^{15}NO_3^-$ or $^{15}NH_4^+$

The eutrophic water used in the experiment was prepared according to the method of Vermaat and Hanif [34, 35]. The ingredients sucrose, acetate and propionic acid (10 mg/L chemical oxygen demand (COD)) were added to 60 L ¼-strong modified Hoagland nutrient solution prepared in tap water. This eutrophic water was prepared to simulate effluent of a poorly performing anaerobic treatment facility. Concentration of COD (10 mg/L) in the eutrophic water was similar to that normally found in water of Lake Taihu, a large fresh water lake in China, which suffered serious eutrophication in recent years [36]. ^{15}N labeled KNO_3 with 9.98% (at. %) ^{15}N (in NO_3^- treatment) or $(NH_4)_2SO_4$ with 10.08% (at. %) ^{15}N (in NH_4^+ treatment) was separately added to the prepared eutrophic water to obtain the concentrations of 5.35 ± 0.48 mg/L NO_3^- and 7.63 ± 0.45 mg/L total nitrogen (TN) in the NO_3^- treatment or 5.60 ± 0.55 mg/L NH_4^+ and 9.06 ± 0.18 mg/L TN in the NH_4^+ treatment.

2.2 Preparation of macrophytes

E. crassipes was collected from a pond in Jiangsu Academy of Agricultural Sciences, Jiangsu province, China, in October 2011. Before starting the experiment, full size individuals of *E. crassipes* that grew under natural light and had a length of approximately 20 cm were collected from the pond for use in the experiment. Each treatment with cultivation of macrophyte received 0.90–0.93 kg of *E. crassipes* (6 to 7 individuals). The *E. crassipes* roots were prepared to reduce the amount of N absorbed by *E. crassipes* by cutting off most stems and leaves, and leaving about 1 cm length of stems. Each treatment with cultivation of macrophyte roots received 0.29–0.30 kg of *E. crassipes* roots (6 to 7 individuals).

2.3 Experimental design

The experimental design was multivariate with four between-subject variables (N-15 labeled nitrate, N-15 labeled ammonium, macrophyte and macrophyte roots with stems chopped off). The experiment consisted of six treatments with three replicates each treatment (Tab. 1).

The experiment was conducted in cubic base PVC containers with closed Plexiglass chamber. Each Plexiglass chamber had a headspace of 45 × 30 × 45 cm. The macrophyte or the macrophyte roots (supported and suspended by foam board) grew in the container filled with 60 L prepared eutrophic water. The shoots of *E. crassipes* extended to the Plexiglass headspace chamber, where gas samples were taken through a sampling port equipped with a rubber septum. The Plexiglass headspace chamber and the cubic base container were connected through a groove (2 cm in width, 4 cm in depth) filled with tap water to ensure gas tightness. To minimize the disturbance of gaseous N from air, the Plexiglass headspace was initially flushed with a mixture of 79% He + 21% O_2 for 20 min through the inlet and outlet at the top of the chamber to replace the air trapped in the headspace before the experiment. Finally, the inlet and outlet were closed. During the experiment, a mixture of 95% O_2 + 5% CO_2 was blown into each closed chamber for 5 min through the inlet every day to maintain the photosynthesis and respiration.

E. crassipes was harvested after 20 days. For molecular DNA extraction, 10–15 g fresh roots were collected randomly from *E. crassipes* plants grown in each container. The N concentration and at.% ^{15}N in shoots and roots of *E. crassipes* were analyzed after drying in an oven at 60°C for 24 h and grinding to pass a 245 μm sieve.

Table 1. Labels for treatments

Treatment	Item sampled	Label
Labeled $^{15}NO_3^-$ solution without cultivation of *E. crassipes*	Water	N-W
Labeled $^{15}NO_3^-$ solution with cultivation of *E. crassipes*	Water, root	N-E
Labeled $^{15}NO_3^-$ solution with cultivation of *E. crassipes* roots	Water, root	N-ER
Labeled $^{15}NH_4^+$ solution without cultivation of *E. crassipes*	Water	A-W
Labeled $^{15}NH_4^+$ solution with cultivation of *E. crassipes*	Water, root	A-E
Labeled $^{15}NH_4^+$ solution with cultivation of *E. crassipes* roots	Water, root	A-ER

Three-liter water samples were collected from each treatment when the macrophytes were harvested. One liter water sample was filtered through a 0.45-μm membrane filter chemically preserved with 1 mL of $HgCl_2$ solution (200 mg/L), and the filtrate was stored at 4°C before analysis. Two-liter water samples were filtered through a 5 μm sterile filter to remove the impurities, and the resultant filtrates were filtered through 0.22 μm Millipore membrane filters using a vacuum air pump; the membranes were stored at −80°C for DNA extraction.

Root detritus in water was collected by passing all 60 L of water through a 245 μm nylon net, and N concentration and at % ^{15}N abundance were analyzed in roots [37]. The most algae developed in the water without cultivation of macrophytes were attached to the wall of the cubic base containers. These algae were collected by scraping with a stainless steel blade, whereas algae in water were collected by passing all 60 L of water through 25 μm nylon net.

2.4 Chemical analysis

The concentrations of NO_3^-, NO_2^-, NH_4^+, and TN in filtered water samples were analyzed using a SEAL AutoAnalyzer 3 (SEAL Analytical, Hampshire, UK). The N concentration of shoots, roots, root detritus and algae was determined by the H_2O_2–H_2SO_4 decomposition method [38], and was quantified by a DigiPREP total Kjeldahl nitrogen system (SCP Science, Canada). Samples were analyzed for ^{15}N content with the help of the Analysis and Test Center of the Institute of Soil Science, Chinese Academy of Sciences. The ^{15}N content analysis of macrophyte roots and shoots, root detritus, and algae was determined using a Flash-EA elemental analyzer coupled to a Delta V isotope ratio mass spectrometer (Thermo Finnigan, Bremen, Germany) [37]. $NH_4^+ - N$, NO_3^-, and NO_2^-N in the water sample were transformed to N_2, NO, and N_2O, respectively, using chemical methods according to Cao et al. [39].

2.5 DNA extraction

Fresh roots of *E. crassipes* (2 g) were transferred into 200 mL of sterile water. Bacteria attached to *E. crassipes* roots were detached by vigorous shaking for 30 min (18.3 Hz, Thermomixer Eppendorf) and filtered through a 5-μm sterile filter to remove the impurities. The resultant filtrates were filtered through 0.22-μm Millipore membrane filters using a vacuum air pump, and the membranes were stored at −80°C for DNA extraction [40, 41].

Water and root sample membranes were cut into pieces with sterile scissors and used immediately for DNA extraction. DNA extraction was performed using an E.Z.N.A.® Water DNA Kit (OMEGA Bio-Tek, Doraville, GA, USA) by following the manufacturer's instructions. The extracted DNA was stored in a −20°C freezer and was further analyzed for the diversity and abundance of *nirS*, *nirK* and *nosZ* genes.

2.6 PCR amplification and DGGE analysis

For the DGGE analysis, the PCR was performed in reaction mixtures including 1 μL of template DNA, 5 μL of 10 × PCR buffer, 1 μL of dNTPs (10 mM each), 1 μL of each primer (20 μM) (Tab. 2), 2 U of Taq polymerase (Takara Bio, Dalian, China) and adjusted to a final volume of 50 μL with sterile deionized water. The touchdown PCR amplification of *nirS* (Cd3Af/R3cd-GC) and *nosZ* (*nosZ*-F/nosZ1622R-GC) was performed as follows: initial denaturation at 94°C for 2 min, followed by 10 cycles, which involved a denaturation step at 94°C for 30 s, annealing at 57°C for 30 s in the initial cycle and at decreasing temperatures by 0.5°C/cycle until a temperature of 52°C was reached in the subsequent cycles. The extension step was performed at 72°C for 1 min. After the touchdown program, 30 cycles at denaturation at 94°C for 30 s, annealing at 53°C for 30 s and elongation at 72°C for 1 min, and following by a final elongation step at 72°C for 10 min were performed. The nirK gene (F1aCu/R3Cu-GC) PCR program was carried out with an initial denaturation at 94°C for 3 min, followed by 32 cycles consisting of denaturation step at 94°C for 30 s, annealing at 58°C for 30 s and elongation at 72°C for 45 s, followed by a final elongation step at 72°C for 10 min, and end at 10°C.

The amplified products were pooled and resolved on DGGE gels using a Dcode system (Bio-Rad, Hercules, USA). PCR products of *nirS*, *nirK and nosZ* were run on 6% w/v polyacrylamide (37.5:1, acrylamide/bisacrylamide) gels with denaturing gradients of 50–75% for 15 h (*nirS*), 50–70% for 12 h (*nirK*) and 50–70% for 15 h (*nosZ*) (100% denaturant contains 7 mol/L urea and 40% v/v formamide). The gels were run in 1× TAE (40 mM Tris-acetate and 1 mM EDTA) at 100 V and 60°C. The gel was silver-stained using the protocol [47]. Polaroid pictures of the DGGE gels were scanned using an Epson Perfection V700 Photo scanner (Seiko Epson, Nagano, Japan), and stored as TIFF files and analyzed with Quantity One software (Version 4.5, Bio-Rad, USA). Digitized information from the DGGE banding profiles was used to calculate the diversity indices such as richness (*S*) was determined from the number of bands in each lane, and Shannon-Wiener index (*H*) was calculated from [48]:

$$H = -\sum P_i \ln P_i \quad (1)$$

where P_i is the importance probability of the bands in a gel lane, calculated as:

Table 2. Primers used for the qPCR and DGGE

Gene		Primer	Thermal profile
nosZ		*nosZ*-F [42]	CGYTGTTCMTCGACAGCCAG
	for qPCR	*nosZ*1622R [42]	CGSACCTTSTTGCCSTYGCG
	for DGGE	*nosZ*1622-GC [43]	GGCGGCGCGCCGCCCGCCCCGCCCCCGTCGCCC -CGSACCTTSTTGCCSTYGCG
nirS		Cd3Af [44]	GTSAACGTSAAGGARACSGG
	for qPCR	R3cd [44]	GASTTCGGRTGSGTCTTGA
	for DGGE	R3cd-GC [45]	GGCGGCGCGCCGCCCGCCCCGCCCCCGTCGCCC- GASTTCGGRTGSGTCTTGA
nirK		F1aCu [46]	ATCATGGTSCTGCCGCG
	for qPCR	R3Cu [46]	GCCTCGATCAGRTTGTGGTT
	for DGGE	R3Cu-GC [46]	GGCGGCGCGCCGCCCGCCCCGCCCCCGTCGCCC- GCCTCGATCAGRTTGTGGTT

$$P_i = \frac{n_i}{N} \quad (2)$$

where n_i is the intensity of a band and N is the sum of intensities of all bands.

2.7 Quantitative real-time PCR

Real-time PCR (qPCR) was performed on ABI 7500 real-time System (Life Technologies, USA). Amplification was performed in 20-μL reaction mixtures by using SYBR® Premix Ex Taq™ (TIi RNaseH Plus) qPCR Kit as described by the suppliers (Takara Bio, Dalian, China). The primers used to amplify each target gene in real-time PCR are listed in Tab. 2. The qPCR amplification of *nirS* (Cd3Af/R3cd) and *nosZ* (*nosZ*-F/nosZ1622R) was performed as follows: initial denaturation at 95°C for 2 min, followed by 35 cycles consisting of denaturation step at 95°C for 5 s, annealing at 54°C for 30 s and elongation at 72°C for 30 s. The data were collected during the 72°C for a 30-s step. The *nirK* gene (F1aCu/R3Cu) qPCR program was carried out with an initial denaturation at 95°C for 2 min, followed by 35 cycles consisting of denaturation step at 94°C for 5 s, annealing at 58°C for 30 s and elongation at 72°C for 30 s. The data were collected during the 72°C for a 30-s step. Data was analyzed using the ABI 7500 software (Version 2.0.6, Life Technologies, USA). The parameter Ct (threshold cycle) was determined as the cycle number at which a statistically significant increase in the reporter fluorescence was detected. The standard curves for real-time PCR assays were developed as described by Henry et al. [41–50] and Zhou et al. [51].

2.8 ^{15}N recovery calculation

^{15}N recovery (%) = (amount ^{15}N in sample/total ^{15}N added) × 100%

2.9 Statistical analyses

The data were analyzed with one-way analysis of variance (ANOVA) using SPSS 16.0 to check for quantitative differences between samples. $p < 5\%$ was considered to be statistically significant. Correspondence analysis (CA) for community ordination was conducted using CANOCO 4.5 for Windows using relative band intensity data obtained from the Quantity One analysis [51]. The band information derived from DGGE, as well as Shannon–Wiener index (H) and richness (S) were then analyzed with Univariate test (SPSS 16.0). The number of copies of denitrifier genes (*nirK*, *nirS*, and *nosZ*) was analyzed using the ABI 7500 software. Total number of copies of denitrifier genes (*nirK*, *nirS*, and *nosZ*) in various treatment systems (combined root and water samples).

3 Results and discussion

3.1 ^{15}N recovery in water, macrophyte, algae and root detritus derived from $^{15}NO_3^-$ or $^{15}NO_4^+$ in water

The mass balance of the added $^{15}NH_4^+$ or $^{15}NO_3^-$ indicated that nearly all (99–100%) of the $^{15}NO_3^-$ or $^{15}NH_4^+$ added to water was transformed during the experimental period when *E. crassipes* plants or just roots were cultivated in the water (Tab. 3). The ^{15}N recovery of summed up $^{15}NO_3^-$, $^{15}NO_2^-$, and $^{15}NH_4^+$ was 54.5 ± 4.5% in water without plants in the $^{15}NO_3^-$ treatments and 40.5 ± 2.5% in non-plant water in the $^{15}NH_4^+$ treatments.

The rhizomes of *E. crassipes*, after removing the stems and leaves, still clonally propagated in the containers. In addition, during the experimental period, algae developed in the non-plant water, but not in the water planted with whole *E. crassipes* or just *E. crassipes* roots. In non-plant water, the N-15 recoveries in algae were 19.2 ± 3.4% in the $^{15}NO_3^-$ treatments and 30.3 ± 4.2% in the $^{15}NH_4^+$ treatments.

The highest N-15 recovery in whole *E. crassipes* (shoots + roots) occurred in the A-E treatment (77.7 ± 6%) and the lowest was in the N-ER treatment (34.5 ± 2%). In general, the N-15 recoveries in the whole *E. crassipes* were higher than that in *E. crassipes* without stems and leaves, which was in agreement with the macrophyte biomass (data not shown).

Un-recovered N-15-labeled $NH_4^+ - N$ accounted for 22.2 ± 2.3% of the added $^{15}NH_4^+ - N$ in water with cultivation of *E. crassipes* (A-E treatment), and 45.7 ± 6.6% in water with cultivation of just *E. crassipes* roots (A-ER treatment). The proportion of un-recovered N-15-labeled nitrate was found to be the highest (56.9 ± 4.7%) in water with cultivation of just *E. crassipes* roots (N-ER treatment) and the lowest (22.2 ± 2.3%) in water with *E. crassipes* (A-E treatment).

3.2 DGGE fingerprints of *nirS, nirK*, and *nosZ* genes

The community structures of *nirK* (Fig. 1a), *nirS* (Fig. 2a), and *nosZ* (Fig. 3a) genes were analyzed by DGGE. DGGE profiles with three replicates (only one was shown) for each sample indicated good

Table 3. Mass balance of added ^{15}N in various treatment systems

Treatment	Water %	Shoots %	Roots %	Algae %	Root detritus %	N unaccounted for %[a)]
N-W	54.5 ± 4.5	ND	ND	19.2 ± 3.4	ND	25.7 ± 3.6
N-E	0	45.3 ± 5.6	19.0 ± 5.4	ND	4.4 ± 1.4	31.3 ± 4.0
N-ER	0	20.1 ± 3.2	14.4 ± 2.1	ND	8.5 ± 0.5	56.9 ± 4.7
A-W	40.5 ± 2.5	ND	ND	30.3 ± 4.2	ND	29.2 ± 2.6
A-E	0	55.1 ± 7.7	20.6 ± 3.6	ND	2.0 ± 0.5	22.2 ± 2.3
A-ER	0.3 ± 0.2	23.6 ± 2.3	16.1 ± 1.6	ND	14.4 ± 0.7	45.7 ± 6.6

ND, no data; N-W, labeled $^{15}NO_3^-$ without cultivation of macrophytes; N-E, labeled $^{15}NO_3^-$ water with cultivation of macrophytes; N-ER, labeled $^{15}NO_3^-$ water with cultivation of macrophyte roots; A-W, labeled $^{15}NH_4^+$ water without cultivation of macrophytes; A-E, labeled $^{15}NH_4^+$ water with cultivation of macrophytes; A-ER, labeled $^{15}NH_4^+$ water with cultivation of macrophyte roots.
Results are presented as means ± SDs, $n = 3$.
[a)] Estimation by stable isotope ratio, including nitrogen-containing gases ($^{15}N_2O$ and $^{15}N_2$).

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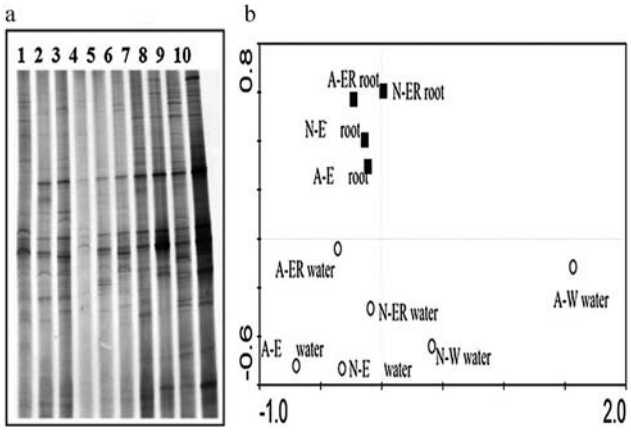

Figure 1. (a) DGGE fingerprints of *nirK* gene in various treatment systems (1, N-W water; 2, N-E water; 3, N-ER water; 4, A-W water; 5, A-E water; 6, A-ER water; 7, A-E root; 8, A-ER root; 9, N-E root; 10, N-ER root), (b) CA ordination diagram of *nirK* gene communities generated by *nirK* DGGE banding patterns.

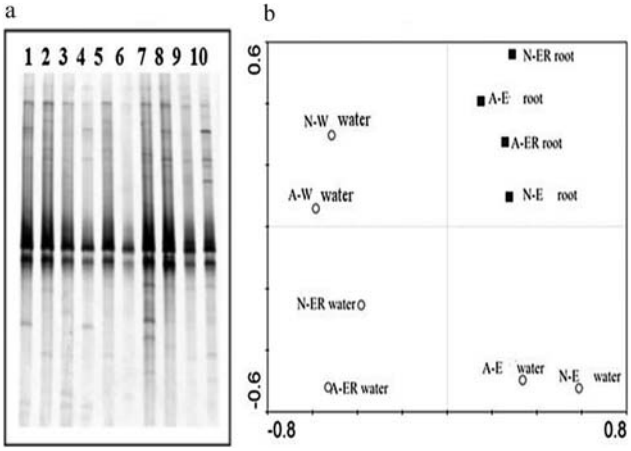

Figure 2. (a) DGGE fingerprints of *nirS* gene in various treatment systems (1, N-W water; 2, N-E water; 3, N-ER water; 4, A-W water; 5, A-E water; 6, A-ER water; 7, A-E root; 8, A-ER root; 9, N-E root; 10, N-ER root), (b) CA ordination diagram of *nirS* gene communities generated by *nirS* DGGE banding patterns.

reproducibility. The bands in the root samples were consistently more diverse than those in the water samples. Detailed comparison showed that various gene types had a differential response to the presence of *E. crassipes* roots. Statistics results of Shannon–Wiener index and richness indicated that *E. crassipes* could significantly increase the diversity of the *nirS* ($p < 0.05$), *nirK* ($p < 0.001$), and *nosZ* ($p < 0.001$) genes.

The CA ordination diagrams showed that *nirK* (Fig. 1b) and *nirS* (Fig. 2b) communities shared relatively similar structures based on the DGGE patterns of water and root samples. There was high similarity among water samples or root samples, but low similarity when the comparison was made between them. The *nirK* and *nirS* Shannon values and richness of the root samples were significantly ($p < 0.05$) higher than those of water samples regardless of cultivation of *E. crassipes*. The highest richness of *nirS* (12.7 ± 1.5) and *nirK* (19.0 ± 0.0) were found in the A-E and N-ER root samples,

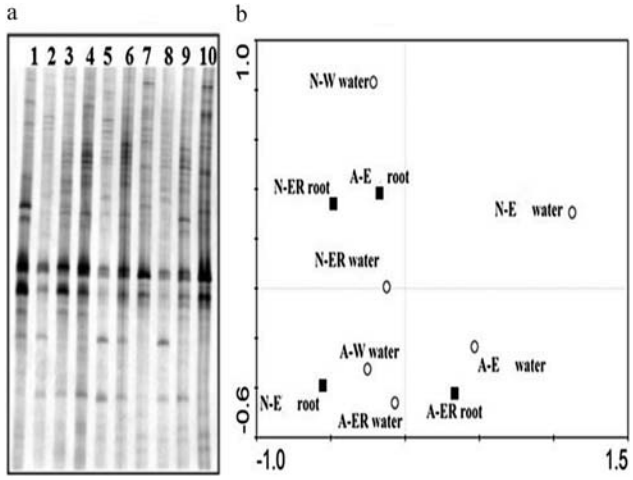

Figure 3. (a) DGGE fingerprints of *nosZ* gene in various treatment systems (1, N-W water; 2, N-E water; 3, N-ER water; 4, A-W water; 5, A-E water; 6, A-ER water; 7, A-E root; 8, A-ER root; 9, N-E root; 10, N-ER root), (b) CA ordination diagram of *nosZ* gene communities generated by *nosZ* DGGE banding patterns.

respectively. In contrast, the lowest richness of *nirK* (7.0 ± 1.0) and *nirS* (6.3 ± 0.6) appeared in the ammonium treatment control (A-W) (Tab. 4).

The CA ordination diagram of *nosZ* gene (Fig. 3b) communities, based on DGGE patterns, was not similar to the CA ordination diagram of *nirS* and *nirK* genes. The results of richness (*S*) of *nosZ* gene revealed significant ($p < 0.05$) differences among the treatments. The Shannon–Wiener index of *nosZ* showed that the index of roots regardless of the presence or absence of shoots (2.4 ± 0.2, N-E root; 2.1 ± 0.2, N-ER root) was significantly higher ($p < 0.05$) than that of water (1.8 ± 0.1, N-W water; 1.7 ± 0.3, N-ER water) in $^{15}NO_3^-$ treatments. However, the Shannon–Wiener index of *nosZ* in the $^{15}NH_4$ treatment showed that the index of roots (with the shoots attached, 1.8 ± 0.2, A-E root) was significantly lower ($p < 0.05$) than that of water with cultivation of either whole *E. crassipes* or just *E. crassipes* roots (A-E water, 2.1 ± 0.3, A-ER water, 2.03 ± 0.21), but significantly higher ($p < 0.05$) than that of water samples without *E. crassipes* (1.8 ± 0.1, A-W water).

3.3 Abundance of *nirS, nirK* and *nosZ* genes

To better analyze the correlation of the abundance of denitrifying bacteria and gaseous removal of N from water, the total copy numbers of *nirK*, *nirS*, and *nosZ* genes in the treatment systems (from 60 L water column or the sum of 60 L water column and all the roots) were calculated (Tab. 5). The lowest copy numbers of *nirK*, *nirS*, and *nosZ* genes were found in the control group (no plants, A-W and N-W). The highest abundance of *nirK* gene appeared in N-ER, being 42.1 and 21.5 times (Tab. 5) higher than that in N-W and A-W, respectively. The highest copy number of *nosZ* gene was also found in N-ER, being 28.3 and 626 times (Tab. 5) higher than that in N-W and A-W, respectively. In contrast, the highest quantity of *nirS* gene copies was found in A-ER. The decreasing trend of denitrifier abundance was N-ER and A-ER > N-R and A-R > N-W and A-W. However, the response of *nirK*, *nirS*, and *nosZ* to different N forms ($^{15}NO_3^-$ or $^{15}NH_4^+$) and plant cultivation (whole plant or just roots) differed slightly (Tab. 5). In addition, the numbers of gene copies were greater for *nirK* than *nirS*

Table 4. Richness (*S*) and Shannon–Wiener index (*H*) of *nirK*, *nirS* and *nosZ*

	nirK		*nirS*		*nosZ*	
Treatments	*S*	*H*	*S*	*H*	*S*	*H*
N-W water	10.7 ± 0.58^{cd}	1.94 ± 0.33^{abc}	7.00 ± 1.00^{cd}	1.16 ± 0.32^{ab}	10.33 ± 1.15^{ab}	1.83 ± 0.14^{a}
N-E water	9.00 ± 0.0^{de}	1.8 ± 0.1^{bc}	9.7 ± 1.2^{abc}	1.26 ± 0.4^{ab}	7.7 ± 3.1^{b}	1.6 ± 0.2^{a}
N-ER water	10.7 ± 0.6^{cd}	1.8 ± 0.6^{abc}	8.7 ± 1.5^{bcd}	1.18 ± 0.2^{ab}	11.0 ± 0.0^{ab}	1.7 ± 0.3^{a}
A-W water	7.00 ± 0.0^{ef}	1.7 ± 0.1^{c}	6.3 ± 0.6^{d}	0.93 ± 0.0^{ab}	12.3 ± 0.6^{ab}	1.8 ± 0.3^{a}
A-E water	7.6 ± 0.6^{ef}	1.6 ± 0.0^{c}	8.7 ± 0.6^{bcd}	1.07 ± 0.3^{ab}	12.0 ± 1.0^{ab}	2.1 ± 0.3^{a}
A-ER water	10.0 ± 1.7^{cde}	2.1 ± 0.1^{abc}	2.7 ± 0.6^{e}	0.59 ± 0.0^{ab}	12.3 ± 1.2^{ab}	2.0 ± 0.2^{a}
A-E root	12.0 ± 1.0^{bc}	2.2 ± 0.0^{abc}	12.7 ± 1.5^{a}	1.51 ± 0.4^{b}	9.7 ± 1.5^{ab}	1.7 ± 0.4^{a}
A-ER root	14.0 ± 1.0^{b}	2.2 ± 0.4^{abc}	10.0 ± 1.0^{abc}	1.31 ± 0.5^{a}	12.0 ± 1.0^{ab}	2.1 ± 0.3^{a}
N-E root	17.0 ± 1.0^{a}	2.5 ± 0.1^{ab}	11.0 ± 1.7^{ab}	1.63 ± 0.1^{ab}	12.7 ± 2.1^{ab}	2.1 ± 0.2^{a}
N-ER root	19.0 ± 0.0^{a}	2.6 ± 0.1^{a}	12.3 ± 0.6^{a}	1.58 ± 0.2^{a}	15.0 ± 3.0^{a}	2.4 ± 0.2^{a}

Results are presented as means ± SDs, $n = 3$.

genes in all treatment systems. The *nirK*/*nirS* ratio was relatively similar, with the absolute abundance of denitrification genes ranging from 4.0 to 8.6, with the highest ratio being 11.9 in N-ER.

3.4 Correlations between *nirS, nirK*, and *nosZ* genes and gaseous N removal

Bivariate analysis indicated that un-recovered N (gaseous N removal) significantly correlated with the *nirK* ($r = 0.77$, $p < 0.01$), *nirS* ($r = 0.60$, $p < 0.01$), and *nosZ* ($r = 0.91$, $p < 0.001$) copy numbers. Figure 4 shows a clear relationship between the denitrifier genes and gaseous N removal, which suggested that all three genes provided good markers of denitrification in the *E. crassipes* treatment systems.

3.5 The effect of *E. crassipes* on gaseous N production in eutrophic waters

Phytoremediation may be a low cost and effective option [52] to assimilate over loaded nitrogen from aquatic ecosystems, though it has complex effects on eutrophic waters and may not be universally applicable to all systems [53, 54]. The principals of nitrogen amendments depended on two aspects: biological assimilation and its associated microbial denitrification [54]. Due to extreme complicated environmental influence on the effective microbial functioning, the relationship between host macrophytes and microbial communities was still not adequately understood, especially in cases of quantitatively applying such knowledge to remediate eutrophic waters, though the roles of denitrifying bacteria in aquatic ecosystems were extensively investigated to estimate the rate of denitrification under different conditions. Our results revealed quantitative and positive effects of *E. crassipes* on the conversion of dissolved N (NH_4^+ and NO_3^-) to gaseous N ($N_2 + N_2O$) via nitrification and/or denitrification, which were evidence in the production of N-15 labeled N_2O during the experiment.

Table 5. Total number of copies of denitrifier genes (*nirK*, *nirS* and *nosZ*, $\times 10^6$) in various treatment systems (combined root and water samples)

Treatment	*nirK*	*nirS*	*nosZ*
N-W	136 ± 15^{a}	33.8 ± 1.8^{a}	102 ± 5.1^{a}
N-E	2660 ± 122^{b}	412 ± 30.7^{b}	1070 ± 92.9^{c}
N-ER	5730 ± 432^{d}	482 ± 39.7^{c}	2890 ± 223^{e}
A-W	262 ± 2.4^{a}	39.3 ± 0.4^{a}	4.62 ± 0.3^{a}
A-E	2610 ± 102^{b}	375 ± 22.7^{b}	521 ± 44.0^{b}
A-ER	4690 ± 4.1^{c}	543 ± 37.5^{d}	2030 ± 68.7^{d}

N-W, labeled $^{15}NO_3^-$ without cultivation of macrophytes; N-E, labeled $^{15}NO_3^-$ water with cultivation of macrophytes; N-ER, labeled $^{15}NO_3^-$ water with cultivation of macrophyte roots; A-W, labeled $^{15}NH_4^+$ water without cultivation of macrophytes; A-E, labeled $^{15}NH_4^+$ water with cultivation of macrophytes; A-ER, labeled $^{15}NH_4^+$ water with cultivation of macrophyte roots.
Results are presented as means ± SDs, $n = 3$.

The un-recovered N-15 labeled nitrogen was reasonably assumed due to denitrification processes [12, 34]. In all nitrate treatments, the losses of nitrogen were 82 and 122% higher in treatments with cultivation of *E. crassipes* roots (cutting off most stems and leaves) than in treatments with cultivation of whole *E. crassipes* and the treatments with no *E. crassipes*, respectively. This observation supported our hypothesis that retarded growth of macrophytes may leave more substrates available for denitrifier. In ammonia treatments, the losses of nitrogen in treatments with cultivation of *E. crassipes* roots were also 2.05 and 1.57 times higher than the treatments with cultivation of whole *E. crassipes* and the treatments without macrophytes, respectively. *E. crassipes* uptake was responsible for 65 and 43% of total N-15 labeled NO_3^- in the treatment of N-E and N-ER, respectively, while we could account for a mean of 78 and 54% of the total N-15 labeled NH_4^+ absorbed by *E. crassipes*, respectively. One possible explanation was that water hyacinth assimilated NH_4^+ in favor to NO_3^- [55, 56]. Most of NH_4^+−N in water was absorbed by *E. crassipes*, and the proportion of the nitrogen loss in the ammonia treatments was lower than nitrate treatments. This may suggest that after weakening the growth of macrophytes, microorganisms attached to the root surface and in water still removed substantial amount NH_4^+−N and NO_3^-−N in the treatments, though it may not obviously have practical application.

It was of clear evidence that the cultivation of *E. crassipes* improved nitrogen removal in eutrophic waters. Although the rhizosphere of aquatic plants may be less clearly defined than that of terrestrial plants because of diffusion of nutrients in water, there was still a zone impacted by plant roots in aquatic environment [57]. Within the zone, physical, chemical, and biological conditions differed from the surrounding environment [58]. This finding was consistent with what reported in literature [59, 60]. Furthermore, despite being separated in space, aboveground (stems and leaves) and belowground (roots) organisms influence each other. Literature reported that the mechanical damage of aboveground parts induced responses in underground parts by impacting the levels of nutritional compounds

CLEAN Soil Air Water

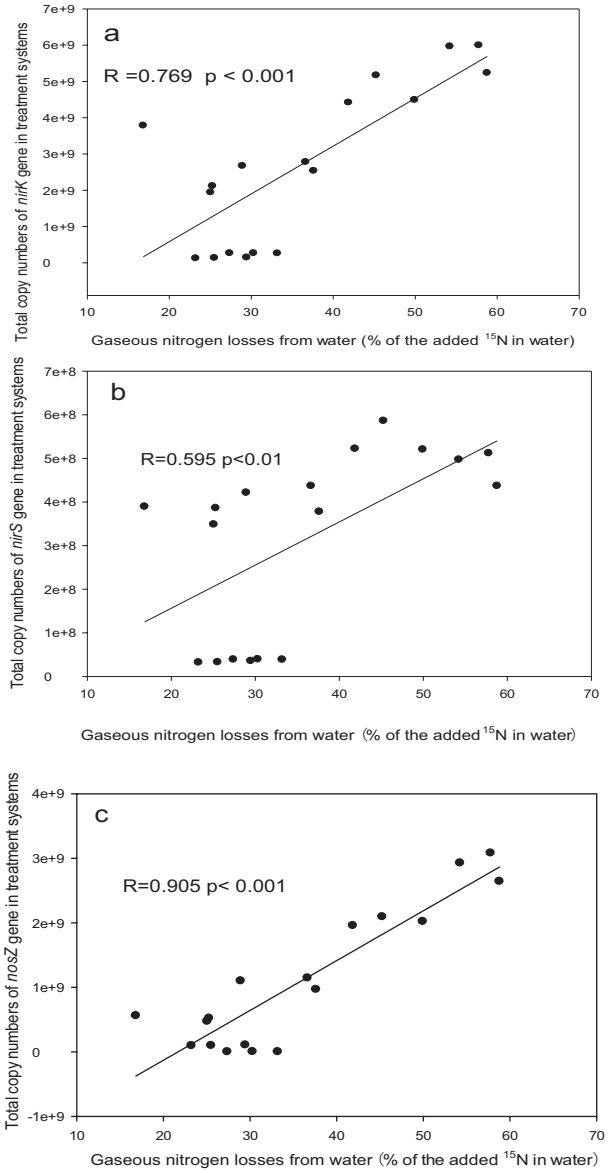

Figure 4. Relationship between total copy numbers of *nirK* (a), *nirS* (b), and *nosZ* genes (c) in the treatment systems (combined root and water samples) and gaseous N removal from water (the percentage of un-recovered nitrogen calculated from N balance).

(e.g. sugars and amino acids) or other compounds (e.g. hormones) that were involved in induced defense [61, 62]). The cutting off stems and leaves of *E. crassipes* in aquatic environment had similar response as that in terrestrial environment, and influenced the behavior of denitrifiers bacterial communities in the rhizosphere.

Another explanation for observed differences between treatments with and without stems and leaves of the macrophytes was of the competition of differential N resources between macrophyte and denitrifying bacteria [63, 64]. The decomposition of root detritus, which was produced in higher amount in the treatments with cutting off *E. crassipes* stems and leaves, may also contribute to the increase of *nirK* and *nosZ* abundance in the environment. It had been well documented that the decomposition of root detritus could supply carbon and energy source for denitrifying bacteria [65], thus raise the heterotrophic activity, which indirectly favored denitrification by lowering oxygen concentrations [66].

3.6 The modulation of *E. crassipes* on *nirS*, *nirK*, and *nosZ* diversity and abundance

The suspended root system of *E. crassipes* could be a good surface for microbial attachment [4, 22, 23], in which denitrification occurred [24]. As the CA ordination diagram of water and root samples indicated that the *nirK* and *nirS* communities shared relatively similar structures. The higher *nirK* and *nirS* Shannon indices and richness were observed on the root samples than that on water samples. Furthermore, the qPCR results revealed that the highest abundances of *nirS* and *nirK* genes were occurred in the treatments of N-ER and A-ER, respectively. These results suggested that cultivation of *E. crassipes* roots enhanced denitrifiers diversity and abundance. One possible explanation was that *E. crassipes* roots provided a surface substratum for enrichment of higher diversity of *nirK*- and *nirS*-bearing denitrifying bacteria. Nitrous oxide reductase, encoded by the *nosZ* gene to catalyze the reduction of N_2O to N_2 [67] responded differently to the cultivation of the macrophytes. The CA ordination diagram of *nosZ* gene communities was not in consistent similarity with *nirS* and *nirK* genes, *E. crassipes* roots were not change the diversity of communities of *nosZ*-bearing denitrifying bacteria other than the increased abundance of the bacterial communities. The highest diversity and abundance of *nosZ* gene occurred in N-ER treatment. Considering N_2O being a greenhouse gas, to minimize the N_2O emission while to maximize N_2 production may be desirable. The presence of *E. crassipes* root, thereby, may be favorable for the production of N_2, resulting in permanent removal of N from aquatic ecosystem. The production of high ratio of N_2/N_2O seemed most commonly occurred under high anaerobic environment [68–70].

The abundances and diversity of *nirK* were higher than that of *nirS* in the water and roots samples, which were consistent with the reports in literature [31–33]. This indicated that *nirK* might be more sensitive than *nirS* gene in this experiment, and *nirK* bearing denitrifiers were better adapted to the *E crassipes* mediated environment than the *nirS* bearing denitrifiers. Previous studies also found the similar phenomena in different environmental conditions [51, 71]. Together, this suggested that denitrifiers harboring *nirK* played a greater role in N-removal from the system compared with denitrifiers harboring *nirS*, though there was no functional difference between *nirK* and *nirS* genes, which encode nitrite reductase, have co-evolved to produce two independent pathways and no denitrifier was known to contain both pathways [72, 73].

3.7 Correlations between *nirS*, *nirK*, and *nosZ* genes and gaseous N removal from water

Although it has been difficult to link the changes of denitrifying bacterial abundance and diversity to gaseous N removal, there are great concerns about whether modification in abundance and community composition or loss of diversity will adversely affect gaseous N removal [74–77]. Numerous studies reported changes in the abundance and composition of functional microbial communities involved in N_2O emissions in different environment [59, 60]. In this study, un-recovered N (gaseous N losses) was significantly ($p < 0.01$) correlated with the abundance of *nirK*, *nirS*, and *nosZ* genes. The results confirmed that denitrification made the equal important

contribution to gaseous N losses from eutrophic water, and the presence of the floating macrophyte, in this case, *E. crassipes* further enhanced the processes.

The correlation results suggested that quantitative DNA-based functional group information could provide very important information regarding the pattern and rate of N denitrification processes in the aquatic environment, though it may less closely relate with enzyme expression from mRNA observation [78]. It also implied that denitrification gene abundances reflected quantitative relationship with the rates of denitrification and the processes of N biogeochemical cycling. This highlighted that the denitrifying bacteria populations were important in mediating N biogeochemical processes in eutrophic water bodies in the presence of macrophytes.

4 Concluding remarks

This study suggested that microbial denitrification, modulated by macrophytes, was an equally important mechanism for driving N removal from eutrophic water if subjected to phytoremediation technology with confined cultivation of the macrophytes *E. crassipes*. Cultivation of *E. crassipes* in eutrophic water could increase the diversity and abundance of denitrifier, resulting in more gaseous N losses by microbial denitrification. This study represented an important step in establishing the relationship between gaseous N losses and the distribution of denitrifier genotypes, with consequences for N biogeochemistry and for planning, or making decisions for phytoremediation. Nevertheless, both mRNA and proteins will be further studied because any regulations of transcription, translation or post-translational steps should be taken into account and better to explain the mechanisms of improved nitrogen removal in the eutrophic waters with the cultivation of the floating macrophytes.

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