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ORIGINAL ARTICLE

Decaying cyanobacteria decrease N_2O emissions related to diversity of intestinal denitrifiers of *Chironomus plumosus*

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ABSTRACT

Nitrous oxide (N_2O) emission of fresh invertebrates has too long been neglected in eutrophic lakes, although the sediments these animals inhabit are presumably hot spots of N_2O emission. Thus, the experiment in this research was designed to gain insight into the influence of cyanobacterial degradation on the N_2O emission by fresh water invertebrates (Chironomus plumosus). *The presence of decaying cyanobacteria in Lake Taihu decreased the N_2O emission rate of* Chironomus plumosus *larvae from the larvae body by almost 400% for the larvae as a whole. The N_2O emission rate decreased by 350% based on readings from studies of their gut, which was mostly due to stimulation of intestinal complete denitrification. The quantitative PCR results showed that intestinal gene abundance of* nirK, nosZ *(encoding the copper nitrite reductase and N_2O reductase, respectively) were significantly increased with the presence of decaying cyanobacteria. In contrast* nirS *(encoding the cytochrome cd_1 heme nitrite reductase) and the total bacteria decreased. In the gut of* Chironomus plumosus, *the diversity and richness of* nosZ *and* nirK *were lower with the cyanobacteria. Phylogenetic analysis of the intestinal function genes (*nosZ *and* nirK*) showed that the* nosZ- *and* nirK*-type denitrifying bacterial sequences were related to different phylotypes. Hence, additional cyanobacteria increased the abundance, but decreased the richness and diversity of intestinal nitrate-reducing bacteria, probably by providing more carbon source in the gut. The data obtained in this study elucidates that the decaying cyanobacteria decreased the emissions of N_2O by the aquatic invertebrates in freshwater sediment and could serve as a valuable resource for nitrogen removal affecting greenhouse gas emissions.*

Key words: Cyanobacterial bloom, nitrous oxide, Chironomus plumosus *larva, gut denitrification,* nosZ.

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INTRODUCTION

The global increase of atmospheric nitrous oxide (N_2O) concentration correlates with enhanced nitrogen fertilization, biomass burning, and industrial processing. N_2O and carbon dioxide (CO_2) are both powerful greenhouse gases that contribute to stratospheric ozone destruction (Duce *et al.,* 2008; Galloway *et al.,* 2008). The atmospheric concentration of N_2O is approximately one-thousandth of CO_2, but its relative efficiency is 216 times greater than that of CO_2 (Jung *et al.*, 2014; Zhu *et al.,* 2013b). Total global N_2O emissions include a wide range of sources from terrestrial to aquatic systems. About 35% of aquatic N_2O emissions come from rivers, estuaries and continental shelves (Seitzinger *et al.,* 2000). Although sediment beds and water column are presumably hot spots of N_2O emission (Wang *et al.,* 2006b; Chen *et al.,* 2011), present estimates defining their effect on global emissions are still under debate. Recently, aquatic animals have also been shown to emit this greenhouse gas (Stief *et al.,* 2009; Svenningsen *et al.,* 2012; Heisterkamp *et al.,* 2013).

Benthic animals are an indispensable constituent of the benthic sediment ecosystem and play an important roles in the water-sediment processes in shallow lakes (Cai *et al.,* 2011). The capacity of N_2O emission or rates of nitrous oxide per individual by benthic animals vary, ranging from 1 pmol individual^{-1} h^{-1} (*Isoperla* sp.) to 700 pmol individual^{-1} h^{-1} (*Bithynia tentaculata*) (Stief *et al.,* 2009). Although these benthic animal emission rates are far lower than nitrous oxide emission by soil-living earthworms (Horn *et al.,* 2006a; Depkat-Jakob *et al.,* 2010) or soil-feeding termites (Ngugi and Brune, 2012), the contribution to the global N_2O emissions by benthic animals cannot be ignored due to their huge quantity and exuberant fecundity. This animal-associated N_2O production is due to the denitrification activity of ingested bacteria in the anoxic gut, and the emission capacity of N_2O depends on feed type, body weight, oxygen availability in gut, nitrate content in sediment, and temperature (Stief *et al.,* 2009; Heisterkamp *et al.,* 2010; Heisterkamp *et al.,* 2013). Furthermore, the contribution of this source to all nitrous oxide emission from aquatic environments can increase with higher nitrate content and lower oxygen in habitat (Stief *et al.,* 2009; Heisterkamp *et al.,* 2010; Stief and Schramm, 2010). This indicates that the habitat environment plays an important role in producing nitrous oxide by gut denitrification. However, due to the complexity of

the lake ecosystem and lack of invertebrate information, N_2O emission of fresh invertebrates has been neglected in eutrophic lakes (Wang *et al.,* 2006b; Chen *et al.,* 2011; Liu *et al.,* 2011).

Lake Taihu is the third largest freshwater lake in China and cyanobacterial blooms have received increasing attention in recent years (Zhong *et al.,* 2010). The development and subsequent decay of massive cyanobacterial blooms in highly polluted regions of Lake Taihu has caused *black spot* events in which dissolved oxygen (DO) was depleted and high total organic carbon (TOC) and ammonium concentrations formed throughout the water column (Zhu *et al.,* 2013a). This phenomenon has lasted for more than one week occurring almost on an annual basis in Lake Taihu; meanwhile, filter- and deposit-feeders such as *Chironomus plumosus* continue to dominate the lake's eutrophic ecosystem.

The primary objective of this study was to understand the potential influence of decaying cyanobacterial blooms on N_2O emission of fresh invertebrates in a laboratory setting. Our goal was to simulate the black spot events and estimate the potential influence of decaying cyanobacterial blooms on N_2O emission by benthic animals. Specifically, we focused on *C. plumosus* larva obtained from the highly polluted region of the lake after cyanobacterial scum appears on the water surface in sediment-water columns. We simulated this occurrence in the lab and incubated *C. plumosus* larva in the dark to enhance cyanobacterial degradation. We recorded changes in N_2O emission of benthic animals during the incubation thereby documenting the influence of decaying cyanobacteria on N_2O emission of these invertebrates, which are also known as midge flies. Our study was restricted to the midge fly larva.

METHODS

Microcosms

Sediment was sampled from Meiliang Bay (31°42'65.5''N, 120°20'95.2''E) in a northwest bay of Lake Taihu, and sieved using a 0.5 mm mesh to exclude macro-invertebrates and large detritus and then homogenized with a dough mixer in the laboratory. About 1 L of sediment was respectively transferred into six cylindrical containers (diameter 20 cm, height 20 cm). We added 2 L of aired tap water (containing 2 mg L^{-1} sodium nitrate and 2 mg L^{-1} ammonium chloride) to those containers using intravenous needles. The microcosms were preincubated in the dark at 25°C for 3 weeks maintaining constant nitrate and ammonium concentrations by repeated additions of aliquots from $NaNO_3$ and NH_4Cl stock solutions (NO_3^-N 100 mg L^{-1} and NH_4^+-N 1000 mg L^{-1}, respectively).

The microcosms included 20 *C. plumosus* from Tianjin Yufeng Farm (1000 ind·m^{-2}) based on their densities in a previous study (Cai *et al.,* 2011); they were randomly separated into two groups with three replicates. We then established one group (+C) where cyanobacteria was added and a second group (-C) without cyanobacteria. The lyophilized cyanobacteria obtained along the shore of Meiliang Bay were dispersed into the overlying water of each cyanobacterial microcosm (1000 μg L^{-1}) according to the concentration of chl-*a*, which was similar to that recorded in field observations during previous Lake Taihu black spot events (Wang *et al.,* 2006a). All microcosms were incubated at 25°C and kept in darkness. After two weeks incubation, the surviving *C. plumosus* were collected with a 0.5 mm net. Surviving *C. plumosus* were 17 individuals in microcosm established with cyanobacteria and 19 in microcosm established without cyanobacteria. Physico-chemical characteristics of pore water and overlying water from the control (-C) and cyanobacterial (+C) microcosm are listed in Tab. 1.

N_2O emission rates of whole animals and their guts

Ten *C. plumosus* larvae after two weeks incubation were placed inside a 2 mL glass vial that contained 200 μL of 0.5 mM nitrate concentration to maintain a moist atmosphere. The three vials for different microcosms were capped with butyl rubber stoppers and incubated under anoxic conditions at 21°C. The headspace was periodically analyzed for N_2O using a gas chromatograph coupled to an electron capture detector (GC-ECD) (Ngugi and Brune, 2012). To minimize stress to the insects, measurements were conducted for less than 2 h and the vials were main-

Tab. 1. Physico-chemical characteristics of pore water and overlying water from the control (-C) and cyanobacterial (+C) microcosms.

Samples		NO_2^--N	NH_4^+-N	NO_3^--N	TN	TOC	pH	DO
-C	Overlying water	0.06	0.08	2.01	2.93	23.59	7.56	3.20
	Pore water	0.01	3.96	0.41	5.19	17.03		
+C	Overlying water	0.44	1.52	1.69	4.10	31.14	7.98	0.97
	Pore water	0.02	6.82	0.71	9.08	63.70		

TN, total nitrogen; TOC, total organic carbon; DO, dissolved oxygen. The chemical matter concentrations (mg L^{-1}) were measured in pore water of centrifuged sediments and overlying water passed through 0.45 μm mixed-fibre membrane according to the Chinese standard methods for lake eutrophication surveys. pH and DO in sediment-water surface were measured in situ with Hach HQ11d pH/DO sensor.

tained in a horizontal position during the incubation. The linear increase of nitrous oxide concentration in the incubation vial was used to calculate the nitrous oxide emission rate of the animal, also taking into account the fraction of nitrous oxide that was dissolved in the water phase. The assessment method of N_2O emission in the gut of *C. plumosus* was described by Stief (Stief *et al.,* 2009).

DNA extraction and abundance of *16S*, *nirS*, *nirK* and *nosZ* genes

Ten *C. plumosus* larvae bodies obtained from the initial time and day 15th of incubation, respectively, were washed three times with sterile, double-distilled water, sedated, and surface sterilized with ethanol (70%) to extract complete gut by pressing larva from anterior to posterior with sterilized tweezers. DNA was isolated from the *C. plumosus* complete gut containing the undigested sedimentary particles using the FastDNA SPIN kit (BIO 101, Carlsbad, CA, USA) according to the manufacturer's protocol (Horn *et al.,* 2006a). The quantity and the quality of DNA were determined using a NanoDrop spectrophotometer 2000 (NanoDrop Technologies Inc, Wilmington, DE, USA). The DNA samples were diluted with ultrapure water to a target concentration of ~20 ng/μL template DNA for qPCR.

Denitrifier abundance was estimated by qPCR of *nirS-*, *nirK*- and *nosZ*-type genes, encoding the cytochrome cd_1 heme nitrite reductase, copper nitrite reductase and N_2O reductase, respectively (Braker *et al.,* 2000; Michotey *et al.,* 2000; Henry *et al.,* 2006). The total bacterial community was quantified using the *16S* rRNA gene (Suzuki *et al.,* 2000). The quantification was based on SYBR Green II chemistry with a total of 30-35 cycles run on a Rotor-Gene6000 real-time System (Corbett Life Science, Mortlake, Australia). Each reaction was performed in a 20 μL volume containing 2 μL of template DNA, 0.2 μM of each primer and 10 μL of SYBR II Premix Ex TaqTM (Takara, Dalian, China). Standard curves were obtained using serial dilutions of a known amount of plasmid DNA containing a fragment of the respective genes, and these were all linear from 10^2 to 10^9 gene copies of template DNA (R^2>0.98). Tab. 2 lists the primers, thermal cycling conditions and efficiencies of qPCR. All qPCR assays were performed in triplicate. The presence of PCR inhibitors in the DNA extracts was tested by mixing a known amount of standard DNA with a DNA extract prior to qPCR. In all cases, no inhibition was detected. A melting curve analysis (65-90°C) and standard agarose gel electrophoresis were performed to confirm amplification specificity (Hamonts *et al.,* 2013). Differences in gene abundance of *16S* rRNA, *nirS, nirK* and *nosZ* genes were evaluated using variance (ANOVA) with values of 0.05 or 0.01 selected for significance.

Cloning, sequencing and phylogenetic analysis

Three PCR products were obtained for constructing clone libraries of the *nirK* and *nosZ* genes with primer pairs, which were used frequently in previous study (Tab. 2). Each PCR product was separated by agarose (2%) gel electrophoresis and purified using an Axygen gel extraction kit (Axygen Biosciences, Union City, CA, USA). The purified DNA fragment was cloned into pMD-19T Vector (Takara) separately according to the manufacturer's instructions. The recombinant plasmids were transformed into competent *Es-*

Tab. 2. Overview of primer sequences, thermal cycling conditions and efficiencies of the qPCR reaction.

Primers	Sequence (5'-3')	Length (bp)	Thermal protocol	Efficiency
Bacterial *16S* rRNA gene			94°C, 4 min	103.7±5.3%
Prok1541R	AAGGAGGTGATCCRGCCGCA		94°C for 30 s,	
Bac1369F	CGGTGAATACGTTCYCGG	172	55°C for 45 min, 30 cycles	
TM1389F	CTTGTACACACCGCCCGTC			
nirK			94°C, 5 min	
F1aCu	ATCATGGT(C/G)CTGCCGCG		94°C for 15 s,	
R3Cu	GCCTCGATCAG(A/G)TTGTGGTT	473	60°C for 1 min,	
			72°C for 1 min, 80°C for 5 s, 35 cycles	102±1.4%
nirS			95°C, 3 min	
cd3af	GT(C/G)AACGT(C/G)AAGGA(A/G) AC(C/G)GG		94°C for 30 s,	
r3cd	GA(C/G)TTCGG(A/G)TG(C/G) GTCTTGA	425	58°C for 45 s,	
			72°C for 45 s, 80°C for 5 s, 35 cycles	99.3±2.1%
nosZ gene			95°C, 3 min	
nosZ-2F	CGCRACGGCAASAAGGTSMSSGT		94°C for 30 s,	
nosZ-2R	CAKRTGCAKSGCRTGGCAGAA	267	59°C for 45 s,	
			72°C for 30 s, 80°C for 5 s, 30 cycles	88±1.8%

The efficiencies of the qPCR were expressed as average value±standard deviation.

cherichia coli DH 5α (Takara). Positive clones were randomly selected and sequenced with the vector-specific primers M13F and M13R (Genomics, Shanghai, China). Diversity statistics were calculated using mothur software (Schloss *et al.,* 2009). The coverage of the clone library was calculated based on the formula of C=[1–(n_1/N)]×100, where n_1 is the number of unique operational taxonomic units (OTUs) and N is the total number of clones in a library. OTUs were defined as groups in which the sequence similarities were greater than 95%. Representative *nirK* or *nosZ* sequences as well as the closest matched sequences identified using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) were aligned using CLUSTAL X 1.83. Phylogenetic trees were constructed based on a 5% cut-off using the neighbor-joining method based on the Jukes-Cantor correction by MEGA version 5. Bootstrap support was calculated (1000 replications).

The partial *nirK* and *nosZ* gene sequences were available under KJ616896-KJ617304 and KJ616761-KJ616860, respectively.

RESULTS

N_2O emission of living *C. plumosus* and its gut

The N_2O emission efflux from the living *C. plumosus* incubated with cyanobacteria decreased significantly ($P<0.01$) (Fig. 1A). Within two hours, the N_2O emission efflux by the living *C. plumosus* was 15.50 pmol ind^{-1} with the presence of cyanobacteria, accounting for 39.8% of N_2O emission efflux in microcosm without cyanobacteria. Consequently, the presence of cyanobacteria decreased the emission rate of N_2O by the living *C. plumosus*, ranging from 16.82 pmol ind^{-1} h^{-1} to 4.25 pmol ind^{-1} h^{-1}, a difference of up to 75%. The emission efflux of N_2O from the *C. plumosus* gut was 25.5 pmol ind^{-1} without cyanobacteria and 12.0 pmol ind^{-1} with cyanobacteria, respectively (Fig. 1B), which accounted for 66 and 77% of total N_2O emission from the living animal, indicating that the gut was the major site for N_2O emission. Compared to results of specimens incubated without cyanobacteria, the emission efflux and rate of N_2O from the gut accounted for 47.1 and 28.6%, respectively, with the presence of cyanobacteria thereby decreasing up to 2.12 and 3.50 times respectively. Hence, the presence of cyanobacteria decreased the emission of N_2O from the gut leading to lower N_2O emissions from living *C. plumosus*.

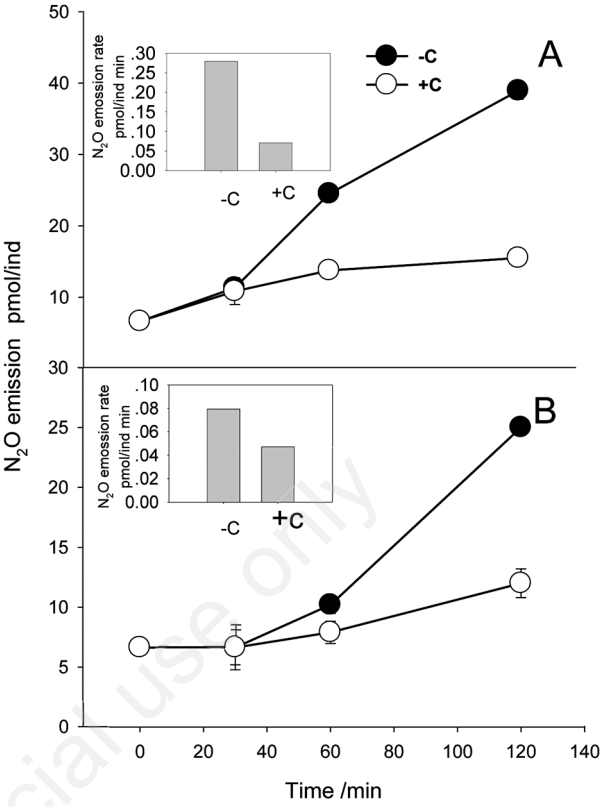

Fig. 1. N_2O emission efflux from the whole animal (A) and the gut (B) with (+C) and without (-C) cyanobacteria. Bars indicate standard error of mean (n=10).

Abundance of intestinal *nirS, nirK, nosZ* and *16S* rRNA

As shown in Fig. 2, quantitative PCR analysis indicated that the abundance of total intestinal bacteria and nitrate-reducing bacteria extensively varied when cyanobacteria was added. The total intestinal bacterial copies significantly decreased with the presence of

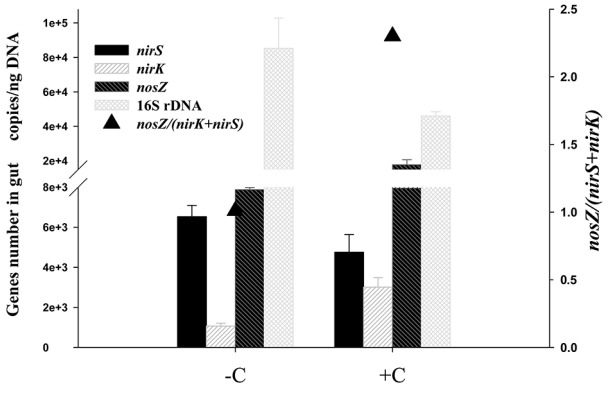

Fig. 2. Abundance of *nirS, nirK, nosZ*, and bacterial 16S expressed as copied numbers per nanogram of total DNA in the gut from the control (-C) and cyanobacterial (+C) microcosms. The ratios of *nosZ* and *nirK+nirS* are represented by closed triangles (▲) in the gut. Bars indicate standard error of mean (n=3).

cyanobacteria, ranging from 8.5×10^5 copies/ng DNA to 4.8×10^4 copies/ng DNA ($P<0.01$). The intestinal nitrite-reducing bacteria were quantified by determining the copy numbers of *nirS* and *nirK* per ng DNA. After 15 days of incubation, *nirS* gene copy numbers were significantly higher (6.5×10^3) in the microcosm with cyanobacteria than *nirK* gene copy numbers (1.1×10^3) in the microcosm without cyanobacteria ($P<0.01$). However, *nirK* gene copy numbers increased in microcosm with cyanobacteria, whereas *nirS* gene copy numbers decreased, leading to *nirS* and *nirK* gene copy numbers approximated after cyanobacteria were added. Differences of nitrite-reductase gene copy numbers were significant between microcosms with or without cyanobacteria based on *t*-test (*nirS*, $P<0.05$; *nirK*, $P<0.01$). The abundance of nitrous oxide-reducing bacteria was followed by quantifying *nosZ* gene copy numbers. Although *nosZ* gene copy numbers were the highest in microcosm without cyanobacteria, the significance differences between *nosZ* and *nirS* gene copy numbers were not detected. In the cyanobacterial microcosm intestinal *nosZ* gene copy numbers even outnumbered *nirS* gene copy numbers and increased to 1.8×10^4, which was significantly higher than that in microcosm without cyanobacteria ($P<0.01$). The ratio of *nosZ* gene copies to the sum of *nirS* and *nirK* gene copies [*nosZ*/(*nirS*+*nirK*)] was higher and strongly affected by cyanobacteria addition in the cyanobacterial microcosm. The *nosZ*/(*nirS*+*nirK*) ratio of 1 means equal copy numbers of nitrite and nitrous oxide reductase genes per ng DNA in microcosm without cyanobacteria; however, the ratio increased to 2.3 in microcosm with cyanobacteria.

Diversity of intestinal *nirK* and *nosZ*

The intestinal *nirS* gene copy numbers obtained from the control and cyanobacterial microcosms were not significantly different, whereas *nirK* and *nosZ* gene, coding nitrite and N_2O reductase respectively, were far outnumbered in the cyanobacterial microcosm than that in the control microcosm. Thus, only the *nirK* and *nosZ* gene clone libraries were constructed to explore denitrifiers community composition.

In this study, 138 *nirK* and 97 *nosZ* sequences were retrieved from 3 *nitK* and *nosZ* clone libraries, respectively (Tab. 3). The numbers of intestinal *nirK* OTUs were 20 and 11 in control and cyanobacterial microcosms, respectively. Within each set of samples, 15 and 14 *nosZ* OTUs were observed. The good coverage values indicated that more than 68% of the *nirK* sequence types and 72% of the *nosZ* sequence types were captured in all the libraries (Tab. 3). The dominant and common *nirK*- and *nosZ*- encoding bacterial denitrifiers in the gut environments were detected. The striking differences in *nirK* and *nosZ* gene diversities were found between the control and cyanobacterial microcosms. Rarefaction analysis indicated that the higher *nirK* and lower *nosZ* diversity/richness indices were from the control microcosm (Fig. 3). The richness indices of *nirK* and *nosZ* were estimated using the S_{chao1} and S_{ACE}, and the result showed that the richness indices of *nirK* and *nosZ* were higher in the control microcosm than that in the cyanobacterial microcosm. The diversity estimators Shannon-Weiner index (*H*) and the Simpson index (*D*) showed a similar result. After 15 days of incubation, the diversity and richness of intestinal *nirK* and *nosZ* sequences were all higher than that at initial incubation due to cyanobacterial addition.

Phylogenies of intestinal *nirK* and *nosZ*

Three *nosZ* gene clone libraries were prepared, and a total of 97 *nosZ* gene sequences were analyzed. In all, 25 OTUs were recovered based on one amino acid residue cut-off (Tab. 3). The combined phylogenetic tree based on the *nosZ* amino acid sequences showed the distinct distribution of OTUs among the three libraries (Fig. 4). These OTUs shared 85-99% similarities with known GenBank *nosZ* sequences detected from a variety of sedimentary environments and gut analysis after dissection (Heylen *et al.,* 2006; Bai *et al.,* 2012; Poulsen *et al.,* 2014). Most of 25 *nosZ* OTUs (96%) occurred in only one or two libraries, whereas

Tab. 3. Biodiversity and predicted richness of the *nirK* and *nosZ* sequences in gut samples from the control (-C) and cyanobacterial (+C) microcosms.

Genes	Samples	Clones	OTUs	C (%)	S_{chao1}	S_{ACE}	*H*	1/*D*
nirK	G0	57	11	94.74	11.50	12.88	1.38	2.24
	-C	41	20	68.29	35.60	94.70	2.63	12.42
	+C	39	11	87.18	14.33	17.10	1.98	6.18
nosZ	G0	28	9	89.29	8.75	14.26	1.63	4.20
	-C	33	15	72.73	27.00	44.93	2.40	10.78
	+C	36	14	80.56	22.00	23.03	2.46	12.35

OTUs, operational taxonomic units; OTUs of the nirK *and* nosZ *sequences were determined as described in the text. The coverage (C), Shannon-Weiner (*H*), Simpson (*D*), and* S_{ACE} *and* S_{Chao1} *richness estimators were calculated with the OTU data. G0 represented the gut before incubation, -C and +C represented the gut samples from the control (-C) and cyanobacteria (+C) microcosm at 15 days respectively.*

only one OTU occurred in all libraries. The number of OTUs recovered from individual libraries ranged from 2 OTUs from the gut at Day 0 to 3 and 8 OTUs each from the control and cyanobacterial microcosm at Day 15 (Fig. 5). These indicated that distinctive *nosZ* gene phylotypes appeared in the gut between the control and cyanobacterial microcosm. Phylogenetic analyses revealed that all sequences fell into five clusters (Bellini *et al.,* 2013), and the community composition of nitrous-oxide-reducing bacteria was affected by the presence of cyanobacteria (Fig. 4). As shown in Fig. 6, the *nosZ* sequences at initial time of incubation were predominantly (82.1%) affiliated within cluster I with the representative of *Zoogloea*, while at 15 days the relative abundance decreased to 45.5 and 25% in the control and cyanobacterial microcosm respectively. Cluster IV with the representative of *Ochrobactrum* (6.1%) belonging to alphaproteobacteria was only detected in the control microcosm, whereas cluster V mostly related to DSCN-S25 (61.1%) which was the dominant genus in the cyanobacterial microcosm.

For *nirK* library, 40-57 clones were randomly selected and sequenced. Based on a 5% cut-off, 29 OTUs out of 138 clones were identified (Tab. 3). Most (86.2%) of the 29 *nirK* OTUs occurred in only one or two libraries, whereas four OTUs occurred in all libraries. The number of intestinal OTUs recovered from individual libraries ranged from 4 OTUs at initial time to 11 and 5 OTUs at 15 days of incubation in the control and cyanobacterial microcosm, respectively (Fig. 5). These results indicated that the distinctive *nirK* gene phylotypes appeared in the gut between the control and cyanobacterial microcosms. Phylogenetic analyses revealed that all sequences fell into three clusters (Heylen *et al.,* 2006), and the community composition of nitrite-oxide reducing bacteria was affected by the presence of cyanobacteria (Fig. 4). Among the 138 *nirK* sequences retrieved, 95.9% fell within Cluster I and these sequences can be further classified into three subclusters (Ia, Ib, Ic) (Fig. 4). As shown in Fig. 6, the *nirK* sequences at initial time were predominantly (91.2%) affiliated within Ib, while the relative abundance decreased up to 53.7 and 32.5% at 15 days of incubation in the control and cyanobacterial microcosms, respectively. Subcluster Ia (45%) was the largest cluster in the gut obtained from the cyanobacterial microcosm, whereas subcluster Ic (24.4%) was the second largest cluster from the control microcosm. The *nirK* sequences affiliated with Cluster II (2.5%) were only detected in the cyanobacterial microcosm, whereas *nirK* sequences affiliated with Cluster III, low relative abundance, were detected in both the control and cyanobacterial microcosms.

DISCUSSION

Benthic invertebrates are often widespread in freshwater and marine environment and play an important role

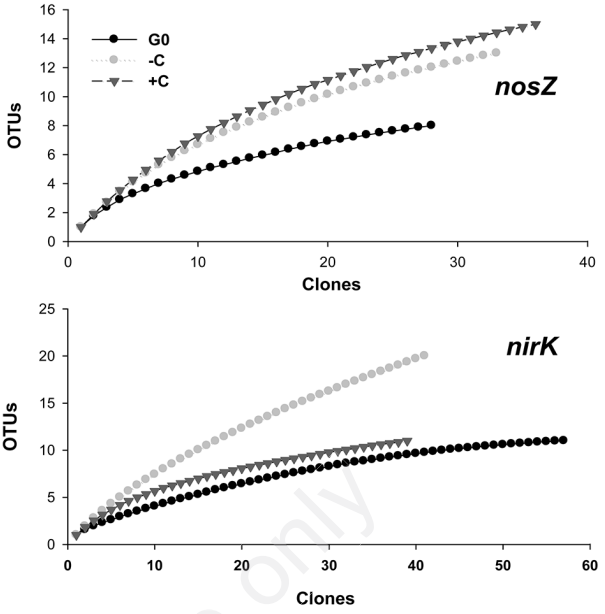

Fig. 3. Rarefaction curve of *nosZ* and *nirK* gene clone libraries in gut samples. G0 represents the gut before incubation; -C and +C represent the gut samples from the control (-C) and cyanobacterial (+C) microcosm at 15 days, respectively. OUTs, operational taxonomic units.

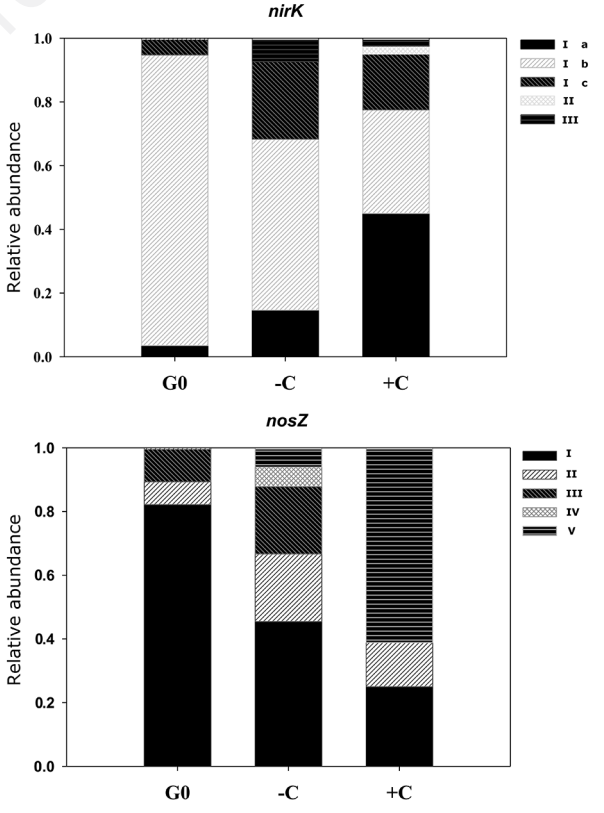

Fig. 4. Relative abundance of *nirK* and *nosZ* gene clone libraries in gut samples. The names of different groups were defined in Tab. 3.

in the water-sediment processes (Cai *et al.*, 2011). Previous studies focused primarily on distribution, species richness and function (Covich *et al.,* 1999; Bellini *et al.,* 2013; Korovchinsky, 2013; Rogers *et al.,* 2013; Pérez-Bilbao *et al.,* 2014). However, recently some researchers have confirmed N_2O emission from benthic invertebrates ranging from predators to filter-and deposit-feeders (Stief *et al.,* 2009; Heisterkamp *et al.,* 2010; Svenningsen *et al.,* 2012; Heisterkamp *et al.,* 2013; Poulsen *et al.,* 2014).

In this study, the gut was the main site for the N_2O emission in *Chironomus plumosus* larvae accounting for 66-77% of whole body (Stief *et al.,* 2009; Poulsen *et al.,*

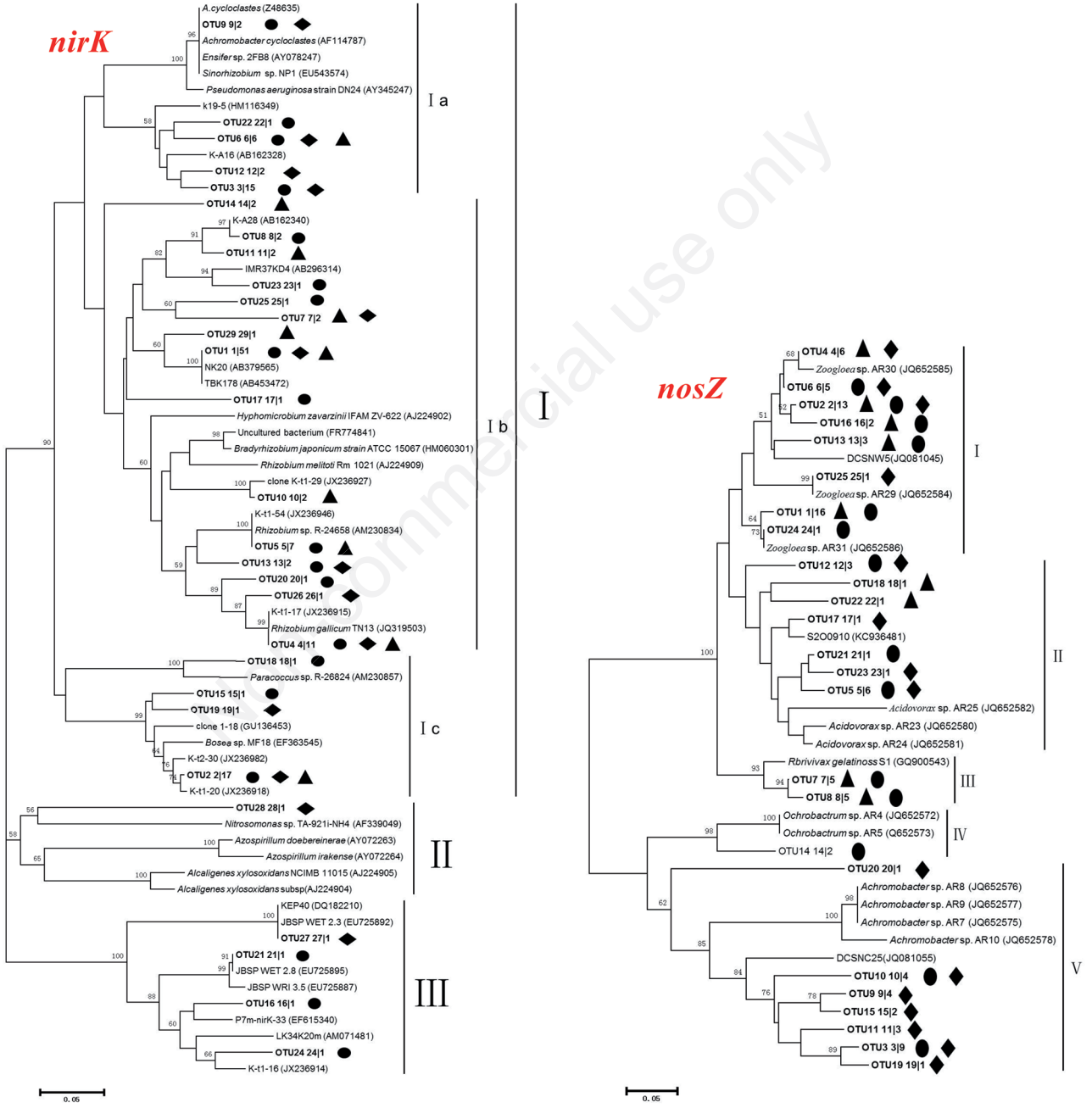

Fig. 5. Phylogenetic analysis of *nosZ* and *nirK* reconstructed from deduced 67 and 156 amino acid sequences, respectively. Bootstrap values greater than 50% (1000 replicates) are shown. The scale bar indicates the number of changes per sequence position. The origins of sequences are indicated by closed triangles (▲, G0), closed circles (●, -C), and closed rhombus (♦, +C). For definitions of symbols, see Tab. 3.

2014), with remaining emissions possibly caused by bacteria on the surface of *C. plumosus* (Heisterkamp *et al.,* 2013; Stief, 2013). The *in vivo* emission of N_2O by the gut of *C. plumosus* larvae provided the main denitrification production, accounting for 30-68%, indicating that N_2O was derived primarily from the uncompleted denitrification (Horn *et al.,* 2006b; Poulsen *et al.,* 2014). Although acetylene inhibition was not detected, the presence of decaying cyanobacteria decreased the N_2O emission rate by the gut of *Chironomus plumosus* larvae up to 60%, which was mostly due to stimulation of intestinal complete denitrification. The decaying cyanobacteria changed the physico-chemical characteristics of the sediment and the overlying water (Tab. 1); for example, decaying cyanobacteria decreased O_2 availability and nitrate concentration while increasing carbon availability and pH, which influences the N_2O:N_2 ratio of denitrification. In this study, the dissolved oxygen (DO) concentration (3.2 mg L^{-1}) in the water column of the control microcosm was higher than 2.9 mg L^{-1}, leading to DO concentration, which was higher in the gut. In contrast, DO was close to 0 mg L^{-1} in the cyanobacterial microcosm (Stief *et al.,* 2009). Nitrate concentrations in the water column from the microcosms were all lower than 15.5 mg L^{-1} (Tab. 1). The nitrate concentration in the gut of *C. plumosus* larvae increased linearly with the NO_3^- concentration in the water column of the laboratory microcosms (Stief *et al.,* 2010). The higher NO_3^- concentrations and lower DO usually resulted in more N_2O due to suppression of Nos activity, which represents the enzyme responsible for the conversion of N_2O to N_2 (Stief *et al.,* 2009; Heisterkamp *et al.,* 2010; Stief and Schramm, 2010; Saggar *et al.,* 2013). It was generally considered that the ratio of N_2O:N_2 would decrease with increasing carbon (C) availability, and more organic matter without significant NO_3^- input would lead to low N_2O emissions. Due to cyanobacterial degradation TOC in pore water of sediment increased to 63 mg L^{-1}, and the labile C:NO_3^- ratio increased from 41 to 89. The change in the N_2O:N_2 ratio from denitrification due to the changing labile C:NO_3^- ratio could be explained by changes in enzyme status and/or the diffusion rate of NO_3^- into denitrifying microsites (Saggar *et al.,* 2013).

In the control microcosm the ratio of *nosZ* to total bacteria was not significantly higher than *nirS* indicating that N_2O-reducing bacteria did not outnumber denitrifiers in the gut, thereby agreeing with previous research (Ihssen *et al.,* 2003; Stief *et al.,* 2009; Poulsen *et al.,* 2014). One-third of all denitrifiers, defined as *nirS*-or *nirK*-containing microorganisms, lack the genetic potential for N_2O reduction and thus are major contributors to microbial N_2O production (Harter *et al.,* 2014). Our data suggest that the addition of cyanobacteria changed the denitrifier microbial community composition by promoting the growth of N_2O-reducing bacteria (containing a *nosZ* gene) relative to *nirS*- and *nirK*-containing denitrifiers in the gut (Fig. 2). This could be explained by the fact that among N-reductase enzymes, Nos was more severely inhibited by oxygen resulting from the decaying cyanobacteria than Nar, Nir and Nor (Saggar *et al.,* 2013). The presence of decaying cyanobacteria also increased the abundance of *nirK*-type denitrifiers. This observation is in line with earlier studies indicating that *nirK*-type rather than *nirS*-type

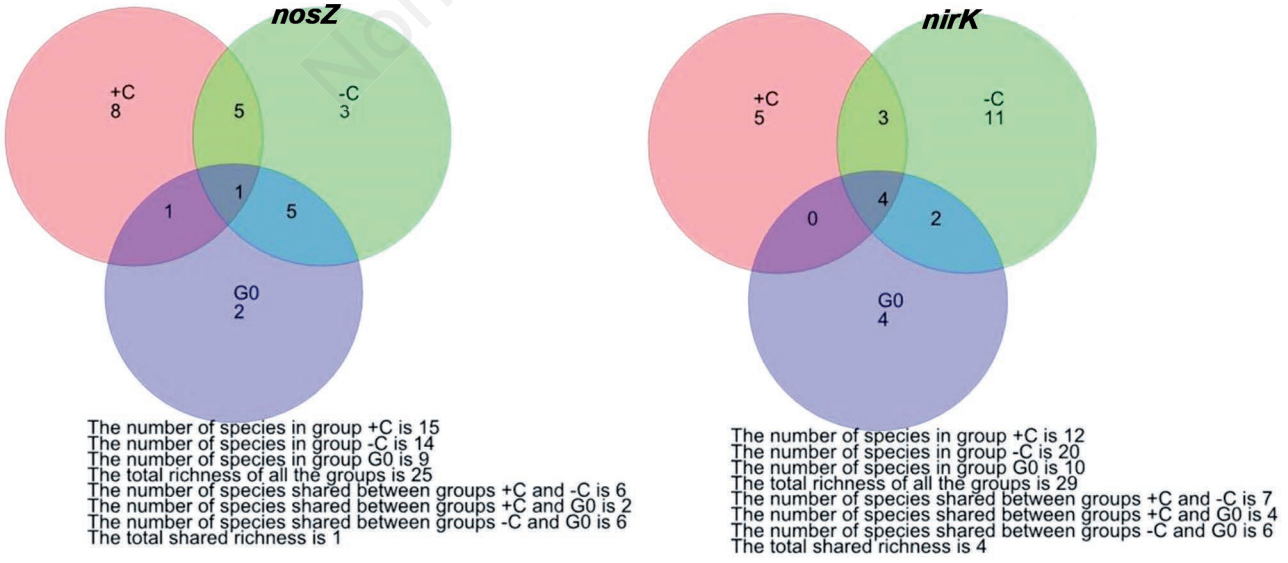

Fig. 6. Shared OTUs of *nosZ* and *nirK* within and between the gut samples. Venn diagram displays the number of shared OTUs in the gut microbiota of at least 50% of the individuals in a host species group. For definitions of the abbreviations, see Tab. 3.

denitrifiers dominate nutrient-rich habitats (Chen *et al.,* 2010; Yoshida *et al.,* 2010). N_2O formation and release from the gut of *C. plumosus* have been shown to be strongly linked to the abundance of N_2O-reducing bacteria. We could deduce that the addition of cyanobacteria also increased the activity of the intestinal *nosZ* gene, resulting in enhanced growth and activity of microorganisms capable of complete denitrification.

The sequences related to known denitrifying bacteria had commonly been found in gut of *C. plumosus* (Stief *et al.,* 2009; Poulsen *et al.,* 2014), termite (Ngugi and Brune, 2012), shell (Heisterkamp *et al.,* 2013) and mostly affiliated with alpha-, beta-, gamma-proteobacteria (Yu *et al.,* 2014). In this study, the *nosZ* gene sequences obtained were mostly related to betaproteobacteria, and the others were alphaproteobacteria, whereas based on *nirK* sequences alpha-, beta-, gamma-proteobacteria were obtained. This result was consistent with a previous report (Poulsen *et al.,* 2014). The genera *Pseudomonas* and *Stenotrophomonas* belonging to Gamma-proteobacteria were ubiquitous in sediment and waste plants, while not detected in the gut due to primer partial (Cutruzzola *et al.,* 2003; Bartacek *et al.,* 2010; Bellini *et al.,* 2013; Desloover *et al.,* 2014; Zheng *et al.,* 2014). The community composition of nitrite- and nitrous-oxide-reducing bacteria in the gut was indeed affected by the presence of cyanobacteria. The richness and diversity of the intestinal *nosZ* and *nirK* genes decreased with the presence of cyanobacteria due to eliminating some of the denitrifying bacteria though environmental changes (Bellini *et al.,* 2013; Saggar *et al.,* 2013; Poulsen *et al.,* 2014). The additional intestinal *nirK* phylotypes covered a broad taxonomic spectrum in the control microcosm. In contrast, the estimated phylotype richness of the *nosZ* gene was higher in the cyanobacterial microcosm based on whether or not these phylotypes have particularly high or low metabolic activities (Bergaust *et al.,* 2011; Poulsen *et al.,* 2014). The *nosZ* gene sequences (61.1%) obtained from the cyanobacterial microcosm was mostly related to DSCN-C25, which was similar to *Paracoccus* sp. BW001 provided with biodegradation of pyridine and had the potential ability to reduce NO_2^- to NO, and then to N_2 (Bai *et al.,* 2008; Bai *et al.,* 2013). While the dominant community was *Zoogloea* sp. in the control microcosm, their denitrification capability remains under debate (Bellini *et al.,* 2013).

CONCLUSIONS

The information acquired in this study confirms that decaying cyanobacteria can decrease N_2O emission from *C. plumosus* to overlying water or atmosphere by increasing the intestinal denitrifier abundance and changing the denitrifier community composition. It can also be shown that complete denitrifiers in the *C. plumosus* gut can cope better with the dynamic conditions than nitrite reducers because they have more cell-specific phylotypes due to more carbon in gut during cyanobacteria decaying. In order to confirm the findings of this study and further advance our understanding on the impact of cyanobacteria on the nitrogen cycling microbial community and invertebrate N_2O emissions, field studies with different invertebrates over longer time periods are needed.

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