


Characterization of the denitrifying bacterial community in a full-scale rockwool biofilter for compost waste-gas treatment

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Abstract The potential denitrification activity and the composition of the denitrifying bacterial community in a full-scale rockwool biofilter used for treating livestock manure composting emissions were analyzed. Packing material sampled from the rockwool biofilter was anoxically batch-incubated with ¹⁵N-labeled nitrate in the presence of different electron donors (compost extract, ammonium, hydrogen sulfide, propionate, and acetate), and responses were compared with those of activated sludge from a livestock wastewater treatment facility. Overnight batch-incubation showed that potential denitrification activity for the rockwool samples was higher with added compost extract than with other potential electron donors. The number of 16S rRNA and *nosZ* genes in

the rockwool samples were in the range of $1.64\text{--}3.27 \times 10^9$ and $0.28\text{--}2.27 \times 10^8$ copies/g dry, respectively. Denaturing gradient gel electrophoresis analysis targeting *nirK*, *nirS*, and *nosZ* genes indicated that the distribution of *nir* genes was spread in a vertical direction and the distribution of *nosZ* genes was spread horizontally within the biofilter. The corresponding denitrifying enzymes were mainly related to those from *Phyllobacteriaceae*, *Bradyrhizobiaceae*, and *Alcaligenaceae* bacteria and to environmental clones retrieved from agricultural soil, activated sludge, freshwater environments, and guts of earthworms or other invertebrates. A *nosZ* gene fragment having 99% nucleotide sequence identity with that of *Oligotropha carboxidovorans* was also detected. Some *nirK* fragments were related to NirK from micro-aerobic environments. Thus, denitrification in this full-scale rockwool biofilter might be achieved by a consortium of denitrifying bacteria adapted to the intensely aerated ecosystem and utilizing mainly organic matter supplied by the livestock manure composting waste-gas stream.

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Introduction

Composting is a process of biodegradation of organic matter and is widely used to stabilize nutrients and produce organic fertilizer. In Japan, composting is also a major technique for the management of solid animal waste (e.g., Kuroda et al. 2015). However, a drawback to this approach is that environmentally threatening gaseous compounds are emitted during the composting process. Chief among these is ammonia (NH₃), emission of which has been estimated to account for 52, 24, and 14% of the total nitrogen in poultry, swine, and

cattle feces in Japan (Hojito et al. 2003). Ammonia is a malodorous chemical whose emission is regulated by the Japanese Offensive Odor Control Law (Segawa 2004). It also causes acidification and eutrophication of water (Sutton et al. 2008). According to Hayashi and Yan (2010), intensive livestock farming is the sector of Japanese agriculture that contributes most to the atmospheric nitrogen load. Therefore, treatment of NH_3 volatilized from the livestock manure composting process is a major challenge in animal husbandry.

Biological deodorization is a promising tool for livestock farmers to treat malodorous NH_3 -containing emissions from the manure composting process because of its relatively simple operation and low operational costs compared with other chemical and physical processes. With biofiltration systems, NH_3 removal is achieved partly by absorption and partly by nitrification; that is, half of the incoming NH_3 is converted to nitrites or nitrates ($\text{NO}_2^-/\text{NO}_3^-$) by nitrifying microorganisms and the other half is absorbed into water as ammonium (NH_4^+) in order to achieve an ion balance (Ottosen et al. 2011). Therefore, the percolating water often contains high concentrations of NH_4^+ and NO_2^- or NO_3^- in both laboratory-scale biofilters (Baquerizo et al. 2009) and in full-scale biofilters (Yasuda et al. 2009). In many cases, the effluent percolating water cannot be discarded without further wastewater treatment. On the other hand, nitrogen losses in biofilters have often been reported as in the range of 10 to 50% without the addition of external organic carbon sources (De Clippeleir et al. 2012 and references therein). Nitrogen could be lost from both the packing materials and the percolation water in the biofiltration systems in various gaseous forms through biological and chemical reactions. Denitrification is one of the most well-studied biological reactions contributing to nitrogen loss in gaseous form from both natural and anthropogenic terrestrial environments, and it is considered likely to be the main microbial pathway responsible for the nitrogen removal from both the packing material and percolation water in biofiltration systems. However, no clear details of the microbial aspects of denitrification in biofiltration systems have so far been provided.

Denitrification is a microbial respiration process that reduces nitrate and nitrite to dinitrogen (N_2) with four enzymatic systems: nitrate reductase, nitrite reductase, nitric oxide reductase, and nitrous oxide reductase (Zumft 1997). Denitrifying enzymatic activities are regulated primarily by the availability of electron donors and acceptors. The diversity, abundance, and species composition of denitrifying bacterial communities are also affected by environmental factors such as pH, oxygen, moisture, temperature, salinity, electron donor availability, and NO_3^- concentration (e.g., Jones and Hallin 2010; Philippot et al. 2007). Heterogeneous biofilms form on the surface of the packing material in biofiltration systems (Juhler et al. 2009). Because the presence of oxygen inhibits the denitrification process, the penetration of oxygen into the

biofilm is an important factor in the regulation of denitrification rates. The availability of electron donors, which is another important factor for denitrification, is assumed to differ according to the kind of packing material and the composition of the waste gas. Denitrifying bacterial communities in biofiltration systems are expected to differ from denitrifying bacterial communities found in other natural and anthropogenic anoxic environments because biofiltration systems are generally designed to be intensely aerobic ecosystems.

Rockwool is a manufactured mineral fiber produced from slag or igneous rocks. A biofilter packed with a mixture of rockwool, urethane, zeolite, dried chicken feces, and rice husks has been developed for treating waste gas (Fukumori et al. 1990). This rockwool-incorporating biofilter (rockwool biofilter) is a durable system, but its percolation water contains concentrated nitrogenous compounds and needs to be treated as mentioned above. Denitrification within the biofilter could lower the costs of post wastewater treatment. Although the denitrification potential of this system has been investigated previously (Yasuda et al. 2009), the community composition and the physiology of the denitrifiers in the rockwool biofilter have not been identified.

The objective of this study is to investigate the physiological characteristics of the denitrifying bacterial community in response to different electron donors and the denitrifying bacterial community structure in a full-scale rockwool biofilter. Responses to different electron donors were analyzed by using a ^{15}N tracer method, and responses were compared with those of activated sludge from a livestock wastewater treatment facility to reveal the physiological characteristics of the denitrifying bacterial community. The denitrifying bacterial community was analyzed by using polymerase chain reaction denaturing gradient gel electrophoresis (PCR-DGGE) and sequencing methods targeting *nirK* (encoding a Cu-containing nitrite reductase), *nirS* (encoding a cytochrome *cd1*-containing nitrite reductase), and *nosZ* (encoding nitrous oxide reductase). Because denitrifying ability exists in several phylogenetically diverse groups, genes encoding denitrifying enzymes, such as *nirK*, *nirS*, and *nosZ*, rather than the 16S rRNA gene, are commonly used to analyze denitrifying bacterial communities (e.g., Braker et al. 2012). The localization of the denitrifying bacterial community within the biofilter is also discussed.

Materials and methods

Full-scale rockwool biofilter

A detailed description of the full-scale rockwool biofilter installed at the Institute of Livestock and Grassland Science, National Agriculture and Food Research Organization (NILGS) in Tsukuba, Japan, was given in a previous study

(Yasuda et al. 2009). The biofilter is 5.8 m in depth, 8.0 m in width, and 3.0 m in height, and the volume of the packing material is 116 m³. Exhaust gas from cattle and swine manure composting is treated by passing it through the biofilter (100–200 s contact time). The biofilter is sprayed with water once per day to maintain the moisture content of the packing material at an average of 47%. The concentration of NH₃ in the compost waste gas fluctuates in the range of 0–150 ppm in accordance with the turning of the composting manure.

Assay of potential denitrification activity by the ¹⁵N tracer technique

A sample of the rockwool packing material taken from 15 cm below the surface of the biofilter was obtained for the measurement of potential denitrification activity. Activity measurements were conducted within 3 weeks of sampling. Activated sludge from the final sedimentation tank of a livestock wastewater treatment facility at NILGS was used for comparison, and the measurements were conducted within 2 months of sampling. Potential denitrification activity was determined by the ¹⁵N tracer technique according to the method of Yoshinaga et al. (2011). Both rockwool and activated sludge samples were suspended in an autoclaved 1 mM phosphate buffer (pH 7.4) and were washed to discard residual organic matter, NH₄⁺, and NO₃⁻ by four rounds of centrifugation, each consisting of 3000×g for 5 min. The washed samples were suspended again at a ratio of 1:19 (*w/v*) for the rockwool samples and 1:199 (*v/v*) for the activated sludge samples. The suspension was sparged with argon gas to eliminate dissolved oxygen. Portions (15 mL) were transferred into 26-mL vials anaerobically, and headspace gases were replaced with helium. The vials were pre-incubated overnight at 28 °C with shaking (120 rpm) in the dark. Subsequently, ¹⁵NO₃⁻ (final conc. 5 mM) was supplied together with one of the following potential electron donors: H₂S (0.32 mM corresponding to 0.65 mM total oxygen demand [TOD]), propionate (0.28 mM), water extracts of compost (1 mM biological oxygen demand [BOD₅]), NH₄⁺ (0.67 mM), and acetate (0.5 mM). The final concentrations of all electron donors except for H₂S were set to 1 mM TOD. Incubation with 5 mM of acetate was also conducted to evaluate the concentration effect. Propionate and H₂S were used because, after NH₃, they are the main malodorous gases emitted from livestock manure composting and introduced into the rockwool biofilter (Ishibashi et al., unpublished data). The compost extract was prepared from a mixture of cow and swine manure sampled from the composting facility of NILGS. The BOD₅ of the compost as a tenfold dilution in distilled water was measured by using a coulometer (OM3100; Ohkura Electric Co., Ltd., Saitama, Japan) after adding a nitrification inhibitor (Formula 2533; Hach, Loveland, CO, USA). The compost extracts for the ¹⁵N assay were obtained by shaking a 1:1.85 suspension

(wet compost:sterilized pure water) for 30 min, centrifugation at 13,000×g for 30 min, then sterilization by filtration through a No. 5A filter paper (Advantec, Tokyo, Japan) followed by filtration through a 0.22-μm membrane filter (Millex GV; Millipore, Bedford, MA, USA). Every day during the 4 days of incubation the N₂ isotopologues (²⁸N₂, ²⁹N₂, ³⁰N₂) in the headspace gas in each vial were quantified by gas chromatography–mass spectrometry as described in Waki et al. (2013). Two replicate incubations were conducted for each treatment. Dry weight of the rockwool samples was determined after drying overnight at 105 °C. The volatile suspended solids (VSS) of the activated sludge were measured according to standard methods (APHA 1998) except for ignition at 600 °C. Suspended solids were determined with a 1:50 suspension (wet rockwool sample:distilled water) according to standard methods (APHA 1998), and total solids did not differ from suspended solids in the rockwool sample. Potential denitrification activities were expressed on the basis of volatile solids (VS) for rockwool and VSS for activated sludge.

Denitrifying bacterial community structure analysis by PCR-DGGE and by sequencing targeting the *nirK*, *nirS*, and *nosZ* genes

Rockwool samples for molecular analysis were obtained from six positions in the biofilter (depths of 50 and 140 cm at three sites: a, b, and c) (Supplementary Fig. S1). Site “a” was near the first compartment of the composting barn and tended to be exposed to waste gas from the initial stage of the composting process (the first 3 weeks). Site “c” tended to be exposed to waste gas from the later stage (approximately 6–9 weeks), and site “b” was in the middle. The DNA samples in this study were the same as those used in a previous study (Yasuda et al. 2010). PCR-DGGE for the *nirK* and *nirS* genes was conducted according to the method of Throbäck et al. (2004) using the primer pairs FlaCu/R3Cu and cd3aF/R3cd, respectively, and *nosZ* genes were analyzed as described in Enwall et al. (2005) using nosZF/nosZ1622R. The amplification was performed in a 50-μL reaction mixture containing 10–20 ng template DNA, each primer, 10× PCR Gold Buffer (Applied Biosystems, Foster City, CA, USA), 0.2 mM of each dNTP, 1.5 mM MgCl₂, bovine serum albumin (BSA; Takara Bio, Shiga, Japan), and 2.5 U AmpliTaq Gold (Applied Biosystems). The concentrations of each primer and BSA were, respectively, 1 μM and 1000 ng/μL for the *nirK* reaction, 1 μM and 400 ng/μL for *nirS*, and 0.5 μM and 600 ng/μL for *nosZ*. The amplification was performed as described in the above references except that the annealing temperatures were 63 °C for *nirK* and 58 °C for *nirS*. PCR products of the four replicate reactions were mixed and concentrated by ethanol precipitation for *nirK* and *nirS* and by a QIAquick PCR purification kit (Qiagen, Hilden, Germany) for *nosZ*. The PCR products were separated by electrophoresis on 2% agarose gels, and

approximately 80 ng was loaded on 7% polyacrylamide gels. DGGE conditions were the same as those in each reference except that 100 V was used for *nirK* and *nirS*, and a denaturant gradient of 45–75% was used for *nosZ*. DGGE bands were stained with SYBR Green I (Molecular Probes, Eugene, OR, USA) for 30 min and were visualized with a UV illuminator (Epi-Light UV FA500; Taitec, Saitama, Japan). The sequences of the *nirK*- and *nirS*-specific bands were determined using the same procedure as that described in Yasuda et al. (2010). The *nosZ* fragments were sequenced with an ABI BigDye Terminator v3.1 Cycle Sequencing kit with AmpliTaq (FS enzyme) (Applied Biosystems) according to the protocols supplied by the manufacturer by using a DNA Engine Dyad PTC-220 Peltier Thermal Cycler (Bio-Rad, Hercules, CA, USA). The determined nucleotide and translated amino acid sequences were compared with those deposited in the National Center for Biotechnology Information (NCBI) database by a BLASTN or BLASTX search. Multiple alignments and phylogenetic analyses were conducted using MEGA ver. 5.1 (Tamura et al. 2011). Trees were constructed by using the neighbor-joining method with the Poisson correction distance based on 138 amino acid sequences (*nirK*), 134 amino acid sequences (*nirS*), or 144 amino acid sequences (*nosZ*). The gene sequences determined in this study were deposited in the DNA Data Bank of Japan (DDBJ) under the following accession numbers: LC095668–72 for *nirK*, LC095665–67 for *nirS*, and LC093888–97 for *nosZ*.

The DGGE gel images were scanned and intensities of the bands were calculated by using a Luminous Imager (Aisin Cosmos R&D Co., Ltd., Aichi, Japan) (Hanajima et al. 2009). The intensity of each band was transformed to P_i as $P_i = n_i/N$, where n_i is the intensity of band i and N is the sum of all band intensities in a gel lane (Supplementary Table S1). The Shannon index of diversity (H) was calculated as $H = -\sum P_i \ln P_i$ (Magurran 1988). A t test was performed using the TTEST procedure of the SAS statistical package version 9.4 (SAS Institute Inc., Cary, NC, USA) to examine the significance of differences in *nirK* and *nirS* diversity indices. Principal component analysis and correlation analysis were performed using the PRINCOMP and CORR procedures of the SAS statistical package based on the P_i values.

Quantification of the *nosZ* and 16S rRNA genes

For the *nosZ* gene, the most probable number (MPN) method combined with PCR was used as in Braker et al. (2012) with the primer set nosZ2F/nosZ2R (Henry et al. 2006). The PCR product (267 bp) was obtained in a 25- μ L reaction mixture containing template DNA, 0.5 μ M of each primer, 10 \times PCR Gold Buffer, 0.2 mM of each dNTP, 1.5 mM MgCl₂, and 1.25 U AmpliTaq Gold (Applied Biosystems). Triplicate 10-fold serial dilutions were used as template DNA. The thermal cycling conditions were 94 °C for 2 min; followed by

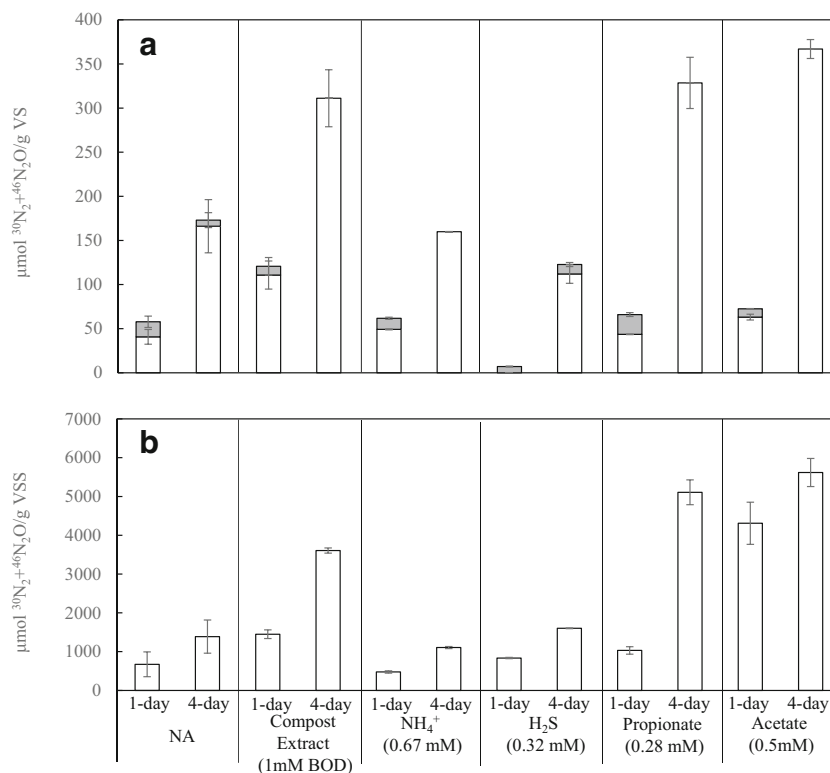
10 cycles of 94 °C for 30 s, 60 °C for 30 s with a touchdown of –0.5 °C per cycle, and 72 °C for 60 s; and 30 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 60 s; and a final extension at 72 °C for 10 min. The MPN was determined from the calculation method of Hurley and Roscoe (1983). Evaluating the MPN–PCR system by using standard PCR products (described below) showed the detection limit to be approximately 6 copies/ μ L. For the 16S rRNA gene, real-time PCR was performed using the primer pairs 357f and 517r (primers p1/p3; Muyzer et al. 1993). Amplification (product size: 194 bp) was performed in a 12.5- μ L reaction mixture containing 0.5 ng template DNA, 0.2 μ M of each primer, 2 \times iQ SYBR Green Supermix (Bio-Rad), and 400 ng/ μ L BSA. Thermal cycling conditions were 95 °C for 3 min; followed by 30 cycles of 95 °C for 20 s, 55 °C for 30 s, and 72 °C for 30 s. Melt curve analysis was performed from 65 °C to 95 °C with a reading made every 0.5 °C, and the samples were held for 10 s per step. The amplification efficiencies were 106% and R^2 of the standard curve was 0.98. Standard PCR products for the 16S rRNA gene and *nosZ* gene assays were prepared by amplification from genomic DNA of *Achromobacter denitrificans* (NBRC15125) using the same primer sets. The DNA concentrations of the standard PCR products were quantified using a Fluorescent DNA Quantification Kit (Bio-Rad) after purification with a QIAquick PCR purification kit (Qiagen).

Results

Potential denitrification activity of the rockwool packing material of the full-scale biofilter

The amounts of ³⁰N₂ and ⁴⁶N₂O produced after 1 and 4 days of batch incubation of samples derived from the rockwool biofilter or activated sludge with the addition of different electron donors are shown in Fig. 1. The potential denitrification activity of the rockwool samples calculated from the 1-day incubation without addition of external electron donors was 0.38 \pm 0.04 μ mol (³⁰N₂ + ⁴⁶N₂O)/g dry/h, or 2.41 \pm 0.24 μ mol (³⁰N₂ + ⁴⁶N₂O)/g VS/h, which was 8.6% of the activity for the activated sludge samples (28.0 \pm 13.3 μ mol ³⁰N₂/g VSS/h). ⁴⁶N₂O was detected in the headspace gas of the rockwool samples after 1 day of incubation. The ratio ⁴⁶N₂O/(³⁰N₂ + ⁴⁶N₂O) was 29.4% without addition of an external electron donor, 8.2% with addition of compost extract, 20.4% with addition of NH₄⁺, 34.0% with addition of propionate, and 12.9% with addition of acetate. ⁴⁶N₂O became undetectable after 4 days of incubation with the addition of these electron donors. Hydrogen sulfide initially inhibited the denitrification activity of the rockwool sample and the only denitrification product after 1 day of incubation was ⁴⁶N₂O. Longer incubation with the addition of H₂S increased the

Fig. 1 $^{30}\text{N}_2$ and $^{46}\text{N}_2\text{O}$ production of **a** rockwool samples and **b** activated sludge after 1 and 4 days of batch incubation with the addition of different electron donors. NA: without addition of an electron donor. Error bars indicate the range ($n = 2$) except for NA, which is the SD ($n = 4$). White bars represent the portions of $^{30}\text{N}_2$, and gray bars represent those of $^{46}\text{N}_2\text{O}$. VS volatile solids, VSS volatile suspended solids



denitrification activity. $^{46}\text{N}_2\text{O}$ was not detected in the headspace gas of any of the activated sludge samples (Fig. 1b). The denitrification activity after 1 day of incubation was higher in rockwool samples with addition of compost extracts than with addition of NH_4^+ , H_2S , propionate, or acetate. The denitrification activity observed with the addition of the compost extracts was twice that of the control with no addition of electron donors. For activated sludge samples, on the other hand, acetate induced the highest denitrification activity after 1 day of incubation, with the addition of acetate increasing the activity 6.4-fold over that of the control. When the amount of acetate was increased tenfold (from 0.5 to 5.0 mM), the production of $^{30}\text{N}_2 + ^{46}\text{N}_2\text{O}$ increased exponentially rather than linearly in the rockwool samples, indicating the growth of the denitrifying population that utilizes acetate (Fig. 2a), while in the activated sludge, the production of $^{30}\text{N}_2 + ^{46}\text{N}_2\text{O}$ increased rather linearly during the incubation period with 5 mM acetate (Fig. 2b). $^{46}\text{N}_2\text{O}$ was also detected after only 1 day of incubation in the rockwool sample with 5 mM acetate and accounted for 8% of the denitrification end products.

Denitrifying bacterial community composition in the full-scale rockwool biofilter

The number of copies of both 16S rRNA and *nosZ* genes in rockwool biofilter samples from six different positions were enumerated. The number of 16S rRNA genes was in the range of $1.64\text{--}3.27 \times 10^9$ copies/g dry or $0.84\text{--}1.57 \times 10^5$ copies/ng

DNA, and the number of *nosZ* genes was in the range of $0.28\text{--}2.27 \times 10^8$ copies/g dry or $0.92\text{--}9.60 \times 10^3$ copies/ng DNA. The proportions of *nosZ* genes to 16S rRNA genes were in the range of 1.1–8.5%.

Profiles of DGGE targeting *nirK*, *nirS*, and *nosZ* genes in samples of the rockwool packing material are shown in Fig. 3. Principal component analyses revealed differences in the localization of the DGGE band profiles within the biofilter depending on the genes for denitrifying enzymes (Fig. 4). For *nirK* and for *nirS*, there was a tendency for upper and lower samples to be separated (band profiles were separated by sampling depth), whereas for *nosZ* gene fragments, band profiles were separated by sampling position. Shannon index of diversity was significantly higher for *nirK* than for *nirS* ($P < 0.05$, $n = 6$) (Table 1). The diversity indices of *nirK*, *nirS*, and the lower samples of *nosZ* were greater at site “b” than at sites “a” or “c.” The indices also tended to be higher at the lower sites than upper samples for *nirK*, and vice versa for *nirS*. The diversity indices increased in magnitude in the order of site “a” (smallest) through “c” (largest) only for upper samples of *nosZ*.

Nucleotide sequences obtained from several intense bands in the DGGE profiles were translated to amino acid sequences and placed into phylogenetic trees along with sequences deposited in the NCBI database (Fig. 5). The amino acid sequences corresponding to those of the *nirK* bands were mainly divided into four phylogenetic groups (Clusters A–D; Fig. 5a). Bands K4 and K5 fell into Cluster A. Sequences of both of

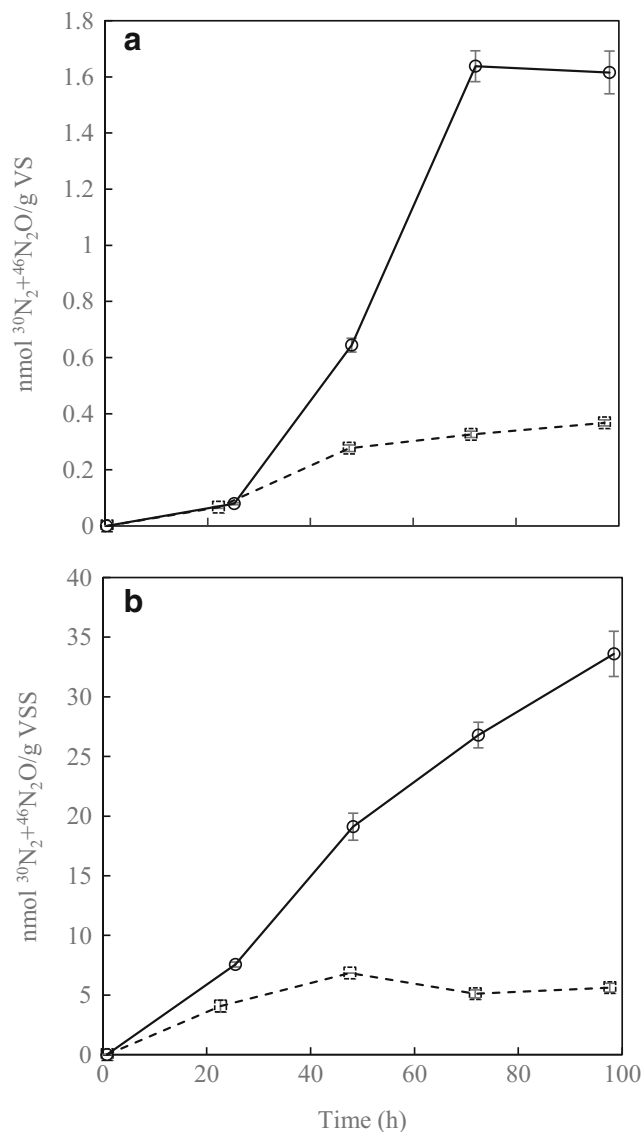


Fig. 2 Time courses of the sum of $^{30}\text{N}_2$ and $^{46}\text{N}_2\text{O}$ production in batch incubations of **a** rockwool samples and **b** activated sludge with addition of 0.5 mM (white square) or 5 mM (white circle) of acetate. Error bars indicate the range ($n = 2$). VS volatile solids, VSS volatile suspended solids

these bands did not exactly match those of any known denitrifier isolates. Bands K4 and K5 had 96% nucleotide sequence identity and 99 and 98% amino acid sequence identities, respectively, with that of the uncultured denitrifying bacterium K36 isolated from a constantly aerated granular sludge reactor (Cyzdik-Kwiatkowska et al. 2014). The nucleotide and amino acid sequence identities of Band K3, which appeared in all rockwool samples except that from sample position a-140, were both 99% with the bacterium clone K114 obtained from an enrichment culture of methane oxidation coupled with denitrification under micro-aerobic conditions (Liu et al. 2014). Band K2 was closely related to Cluster VI categorized by Braker et al. (2012) and was grouped together

in Cluster C with *Mesorhizobium* sp. and clones from activated sludge, agricultural soil, and estuarine water. The sequence of Band K2 had 86% nucleotide and 89% amino acid sequence identities with uncultured paddy soil clone K1_15. Band K1 was grouped together in Cluster D with clones from soil, estuarine sediment, and estuarine water. Among them, Band K1 had 97% nucleotide and amino acid sequence identities with the estuary water column clone YRE-KB59 (Li et al. 2014).

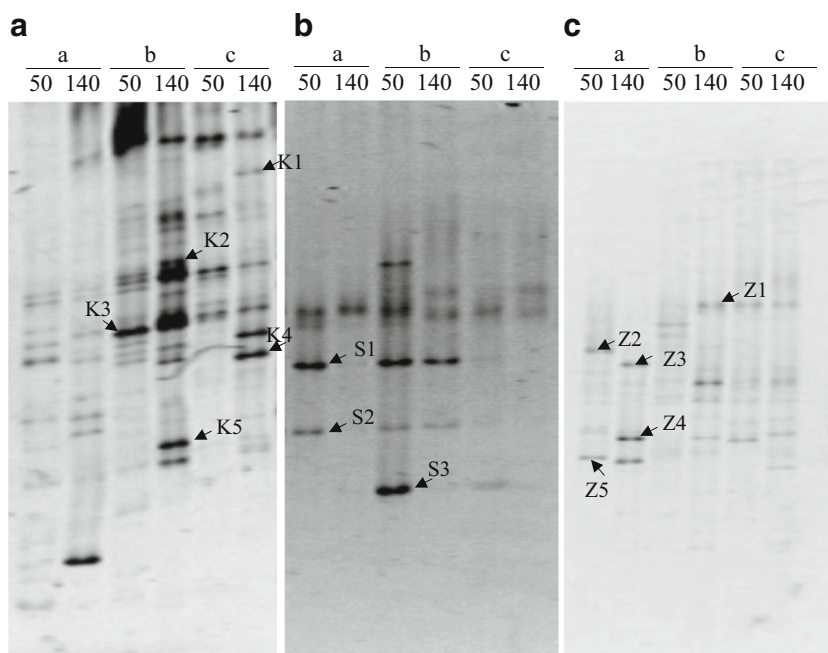
The nucleotide sequences of the three obtained *nirS* bands did not exactly match any known denitrifier isolates. However, all three deduced NirS sequences had amino acid residue Arg-391 at which a hydrogen bond was formed with a d_1 heme (Fülöp et al. 1995). The nucleotide and amino acid sequences respectively had 88–92% and 94–99% identities with clones retrieved from agricultural soil, river sediment, and wastewater treatment systems. Band S3 was grouped together with Cluster I categorized by Braker et al. (2012) (Fig. 5b). This cluster contains mostly uncultured agricultural soil clones. Bands S1 and S2 were also closely related to this cluster.

A phylogenetic tree of the deduced NosZ amino acid sequences is shown in Fig. 5c. The sequences obtained from Bands Z1–Z5 were grouped into four clusters (Clusters A–D). Among them, Clusters A, B, and C were all related to the α class of *Proteobacteria*. Band Z1 had 88% nucleotide and 93% amino acid sequence identities with uncultured earthworm gut bacterium clones (Depkat-Jakob et al. 2010), and Band Z2 had 90% nucleotide and 96% amino acid sequence identities with an uncultured bacterium clone isolated from the guts of larval chironomids in lake sediment (Stief et al. 2009). These ingested clones were generally considered to be soil-derived. The sequences of Bands Z1 and Z2 were clustered together with *Hyphomicrobium nitratorans* (Cluster A). Band Z3 had 99% nucleotide and 100% amino acid sequence identities with *Oligotropha carboxidovorans* in the α class of *Proteobacteria* (Volland et al. 2011). Band Z5 had 88% nucleotide and 87% amino acid sequence identities with the uncultured denitrifying microbial fuel cell clone Z_t3_29 (Vilar-Sanz et al. 2013), and 88% nucleotide and 94% amino acid sequence identities with the uncultured clone ZC37 retrieved from agricultural soil amended with maize mucilage (Mounier et al. 2004). These were clustered together with sequences of the order *Rhizobiales* of the α class of *Proteobacteria*. Band Z4 had 87% nucleotide and 89% amino acid sequence identities with *Castellaniella defragrans* in the β class of *Proteobacteria* (Kämpfer et al. 2006).

Discussion

The potential denitrification activity of the rockwool samples without the addition of an external electron donor was

Fig. 3 DGGE profiles of **a** *nirK*, **b** *nirS*, and **c** *nosZ* gene fragments from the rockwool packing material from the full-scale biofilter. Letters and numbers above each lane represent the sampling positions within the biofilter (Supplementary Fig. S1). Arrows indicate sequenced bands



estimated to be 0.38 $\mu\text{mol } (^{30}\text{N}_2 + ^{46}\text{N}_2\text{O})/\text{g dry/h}$ or 10.6 $\mu\text{g N/g dry/h}$ in this study. Our previous study showed much lower values for the potential denitrification activity of rockwool samples (0.59–1.95 $\mu\text{g N/g dry/h}$; Yasuda et al. 2009). One possible explanation is the difference in pretreatment procedures. The acetylene blockage technique that was used in the previous study could also result in an underestimation of denitrification in general (Butterbach-Bahl et al. 2013). The potential denitrification activity of the rockwool samples measured in this study was lower than that of a woodchip denitrification bed (11.2–20.3 $\mu\text{g N/g dry/h}$; Warneke et al. 2011a), larger than that of agricultural soils (<0.6 $\mu\text{g N/g dry/h}$; Enwall et al. 2005), and within the range of forest soils (0.084–14 $\mu\text{g N/g dry/h}$; Katsuyama et al. 2008). The potential denitrification activity of the activated sludge measured in this study was higher than but the same order of magnitude as the value obtained in a previous study (16.7 $\mu\text{mol } ^{30}\text{N}_2/\text{g VSS/h}$; Waki et al. 2013). The activity for the full-scale biofilter rockwool samples was therefore assumed to be approximately one order of magnitude lower than that of activated sludge.

The number of *nosZ* gene copies estimated in this study was similar to that of soils collected from grasslands, forests, agricultural fields, and orchards (10^3 copies/ng DNA; Bru et al. 2011), agricultural soil (10^7 – 10^8 copies/g dry; Domeignoz-Horta et al. 2015), paddy soil (10^3 copies/ng DNA; Bannert et al. 2011), a quartz-sand filter for a water treatment system (10^6 – 10^8 copies/g dry; Bai et al. 2013), and a gas-phase biofilter (10^3 copies/ng DNA; Yang et al. 2014). The number of *nosZ* gene copies was lower than that for a microbial fuel cell cathode (10^4 – 10^5 copies/ng DNA; Vilar-Sanz et al. 2013) and slightly higher than agricultural soil (10^6 – 10^7 copies/g dry; Kong et al. 2010). The 16S rRNA gene numbers were also similar to those of soils (10^4 – 10^5 copies/ng DNA; Bru et al. 2011) and agricultural soils (10^9 copies/g dry; Domeignoz-Horta et al. 2015), and were lower than in a quartz-sand filter for a water treatment system (10^9 – 10^{10} copies/g dry; Bai et al. 2013). Both the copy numbers of the *nosZ* and 16S rRNA genes were in the range of copies found for denitrification beds (10^6 – 10^{10} copies/g dry for both; Warneke et al. 2011b), but the proportions of *nosZ* to 16S rRNA obtained from the rockwool samples were

Fig. 4 Plots from principal component analyses of DGGE bands of **a** *nirK*, **b** *nirS*, and **c** *nosZ* gene fragments. Letters and numbers within the plots represent the sampling positions as in Fig. 3

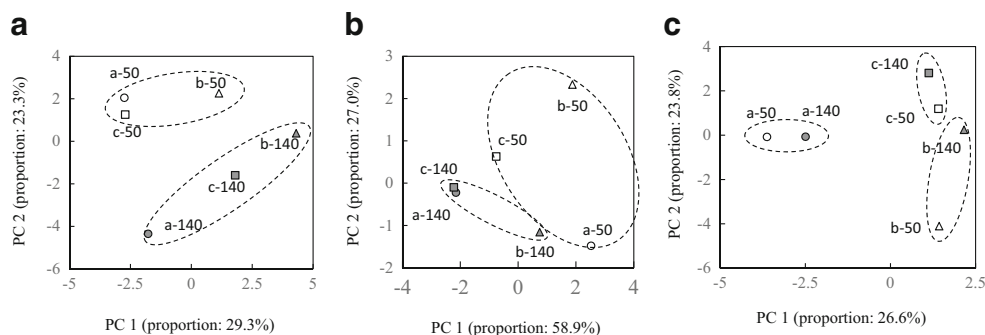


Table 1 Shannon diversity index (*H*) derived from DGGE profiles of each sample

Target genes	samples					
	a-50	a-140	b-50	b-140	c-50	c-140
nirK	1.90	2.06	2.68	2.77	2.01	2.37
nirS	1.31	0.61	1.87	1.29	1.30	0.69
nosZ	1.32	1.51	1.59	2.14	2.05	1.93

estimated to be lower than for the denitrification beds (mostly were in the range of 5.2–58%; Warneke et al. 2011b). Thus, it appears that the total and the *nosZ*-possessing denitrifying bacterial populations in the rockwool biofilter are similar to those of natural and agricultural soils.

The responses of the rockwool samples to the different electron donors were compared to the responses of the activated sludge. With activated sludge, addition of acetate induced the greatest increase in the potential denitrification among the electron donors after 1 day of incubation in this study. Acetate is considered to account for 5–10% of the total chemical oxygen demand in municipal wastewater (Henze et al. 1994), for example, although the composition of wastewater influent varies depending on the kinds of industry, and it is considered to be a key compound involved in the removal of NO_3^- in wastewater treatment plants (Thomsen et al. 2007). Some acetate utilizers have been identified as closely related to *Acidovorax* and *Thauera* spp. within the families of *Comamonadaceae* and *Rhodocyclaceae* (Lu et al. 2014 and references therein). *Azoarcus*- and *Thauera*-related species in the β class of *Proteobacteria* are also reported to consume acetate with NO_3^- (Thomsen et al. 2007). With the rockwool samples, on the other hand, denitrification activity was well induced by the addition of compost extract, indicating that dust in the composting waste-gas stream is an important source of organic matter for the denitrifying bacteria.

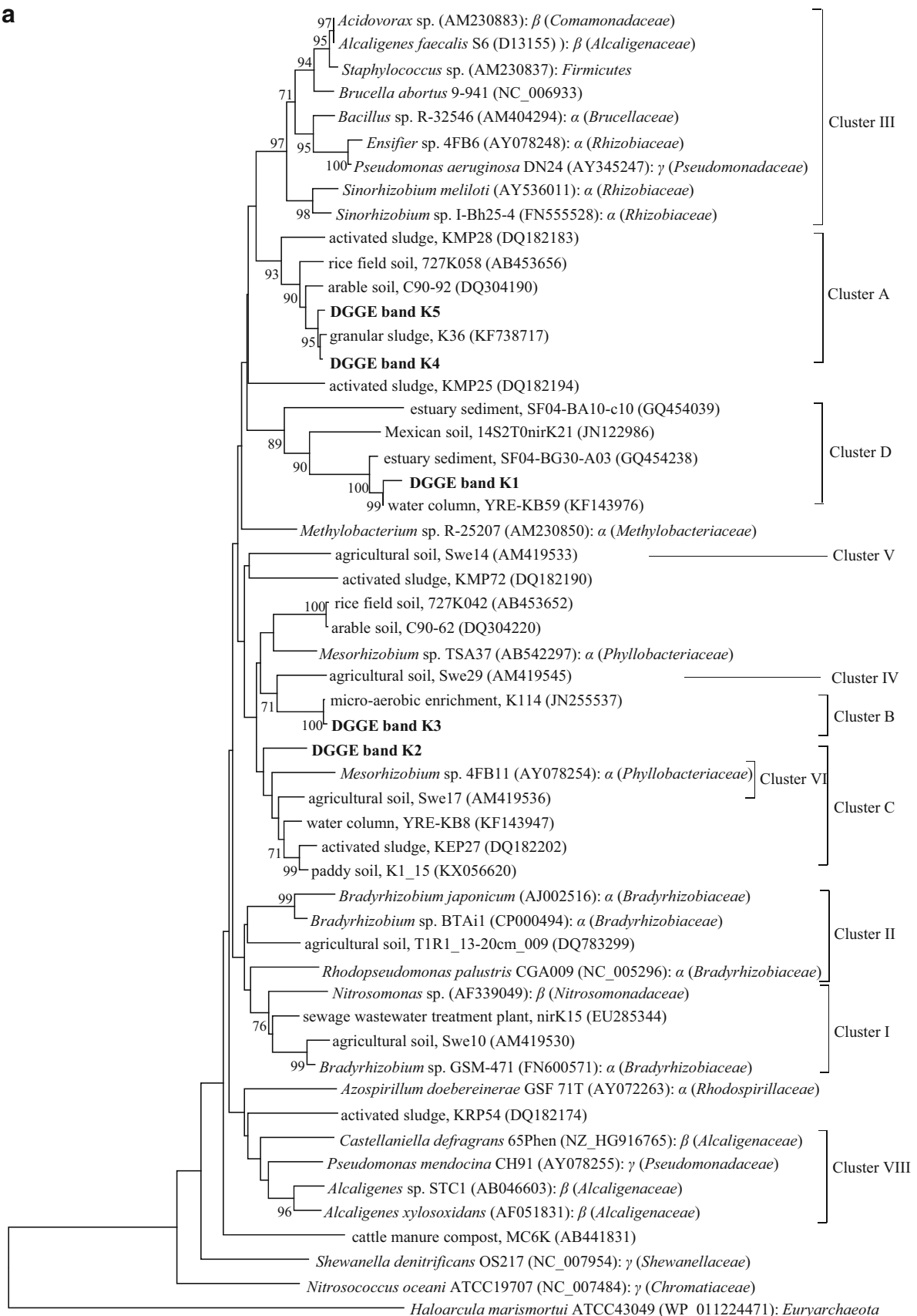
Sequencing of the DGGE bands suggested some link between the denitrifying bacterial community and the possible electron donors for denitrification within the rockwool biofilter. The *nosZ* bands Z2 and Z5 had the lowest Pearson correlation coefficient with the first principal component (Supplementary Table S1) and characterized the samples of site “a.” The closest relatives of these bands were environmental clones retrieved from the gut contents of chironomid larvae or from agricultural soil amended with maize mucilage as described above (Mounier et al. 2004; Stief et al. 2009), which are assumed to be environments with high availability of easily degradable organic carbon. Site “a” tended to be exposed to waste gas from the initial stage of the composting process (Supplementary Fig. S1), and it could be hypothesized that this site is supplied with dust containing a relatively greater quantity of undegraded organic matter than is present in the later stage of composting.

Fig. 5 Phylogenetic relations between the partial translated amino acid sequences of the **a** *nirK*, **b** *nirS*, and **c** *nosZ* genes obtained from the DGGE bands from the rockwool biofilter packing material and related sequences deposited in the National Center for Biotechnology Information database. Bootstrap values (%) of more than 70% for 1000 replicates are indicated. Sequences from this study are shown in bold. The amino acid sequences of *Haloarcula marismortui* for NirK, *Colwellia psychrerythraea* for NirS, and *Haloarcula marismortui* for NosZ were used as the respective outgroups. Clusters with roman numerals are ordered according to Braker et al. (2012), and other clusters grouped in this study are ordered alphabetically. Dotted lines in (b) indicate the possibility of being the part of the corresponding cluster according to the phylogenetic positions determined in this study. Accession numbers of the International Nucleotide Sequence Database are indicated in parentheses. The phylogenetic positions as the phylum or the class for *Proteobacteria* with the family in parenthesis based on 16S rRNA genes sequences are also shown

The K2 band of the *nirK* gene fragment was grouped together with *Mesorhizobium* sp. as mentioned above. In a study of denitrification under micro-aerobic conditions, one of the acetate-degrading denitrifiers was related to *Mesorhizobium* sp. (Costa et al. 2000). In the present study, 4-day incubation with acetate increased denitrification activity in the rockwool samples. It is possible that acetate-degrading denitrifiers were present in the rockwool biofilter samples, even though their population was small. Band K3 showed 99% nucleotide sequence identity with a clone originating from an enrichment culture of bacterial communities involved in methane oxidation coupled to denitrification under micro-aerobic conditions. This band had the highest Pearson correlation coefficient with the second principal component (Supplementary Table S1) and characterized the upper samples revealed by the principal component analysis. The livestock manure composting waste gas loaded into the rockwool biofilter in our study contains methane with an average concentration of 430 ppm (range: 0–1800 ppm) (Yasuda et al. 2009). In addition, Bands K4 and K5, which appeared with high intensity in the lower samples, had high nucleotide sequence identity (96%) with that of the gene isolated from a constantly aerated granular sludge reactor, as mentioned above. These results may imply that the rockwool biofilter was supportive of denitrifiers that usually live in a micro-aerobic environment. It has been reported that penetration of oxygen into the biofilms in a gas-phase biofilter is higher than the penetration into the biofilms of wastewater treatment systems. For example, the analysis of oxygen profiles in biofilms in wastewater treatment systems show that oxygen penetrates less than around 150 μm into the biofilm (Wuertz et al. 2004). However, Juhler et al. (2009) reported that oxygen could be consumed in biofilms thicker than about 250 μm in a biotrickling filter for a piggery. Zhu et al. (2001) also described high penetration of oxygen within a biofilm in a gas-phase biofilter.

Addition of external electron donors and extended anoxic incubation promoted denitrification and N_2O reduction activity in batch incubation. The temporal differences in N_2O emissions in batch incubation may indicate both the late induction of N_2O

a



0.1

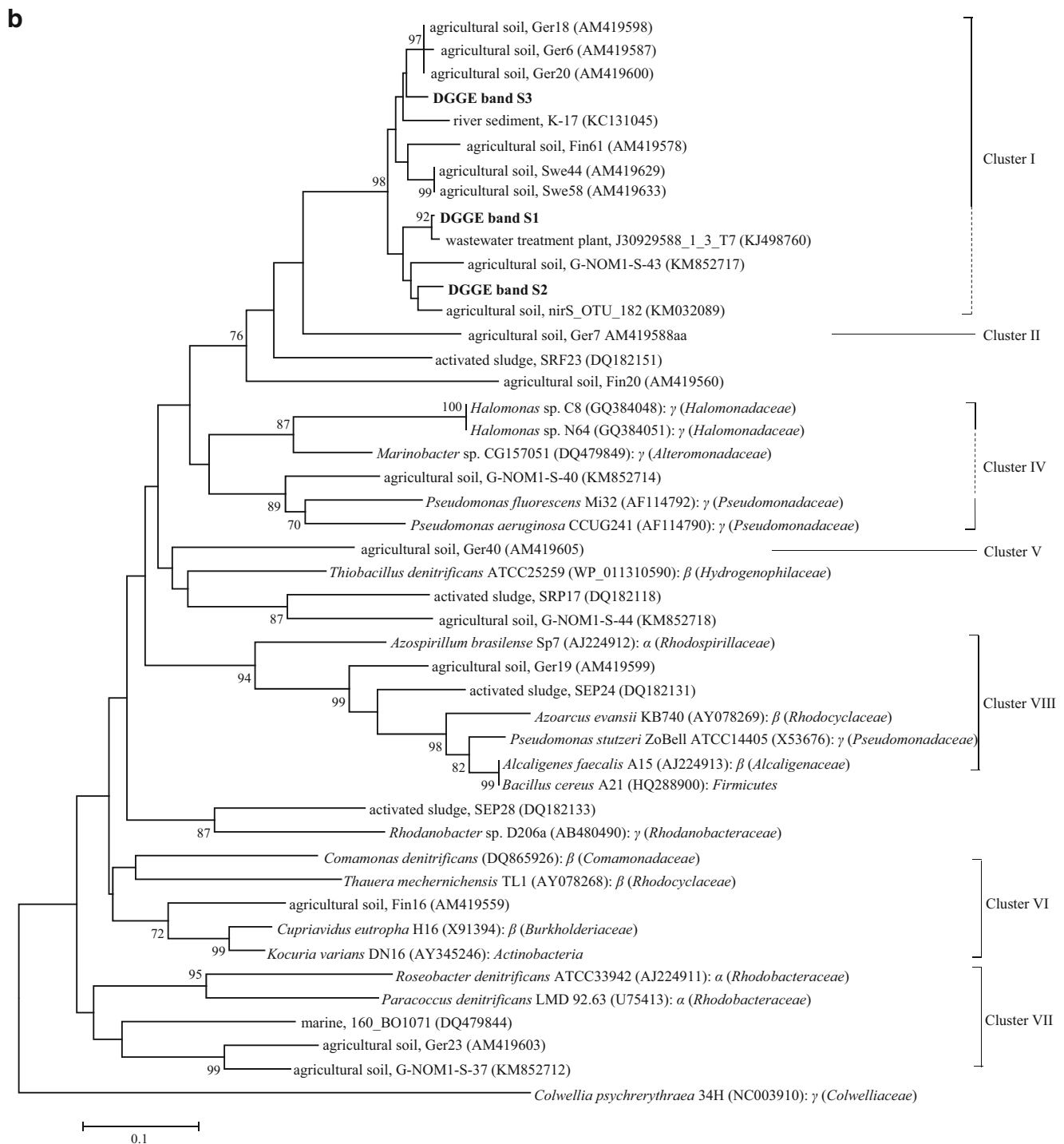


Fig. 5 (continued)

reductase as conditions shift from oxic to anoxic (Stief et al. 2009) and a low availability of electron donors (Morley and Baggs 2010). In the analysis of *nosZ*, Band Z3 in Cluster B was close to *nosZ* of *O. carboxidovorans*. This species is a facultative lithotroph that can utilize carbon monoxide as its sole energy source, as well as a variety of organic substrates such as acetate, pyruvate, and lactate (Volland et al. 2011), but not sugars

or amino acids. Primers for the denitrification genes used in this study have been widely used for monitoring denitrifying bacterial communities in various environments. Recently, the existence of atypical *nosZ*-possessing N_2O reducers (*nosZ* clade II) has been shown, and differences in N_2O affinity between typical denitrifiers (those of *nosZ* clade I) and those belonging to *nosZ* clade II have been reported (Sanford et al. 2012; Yoon et al.

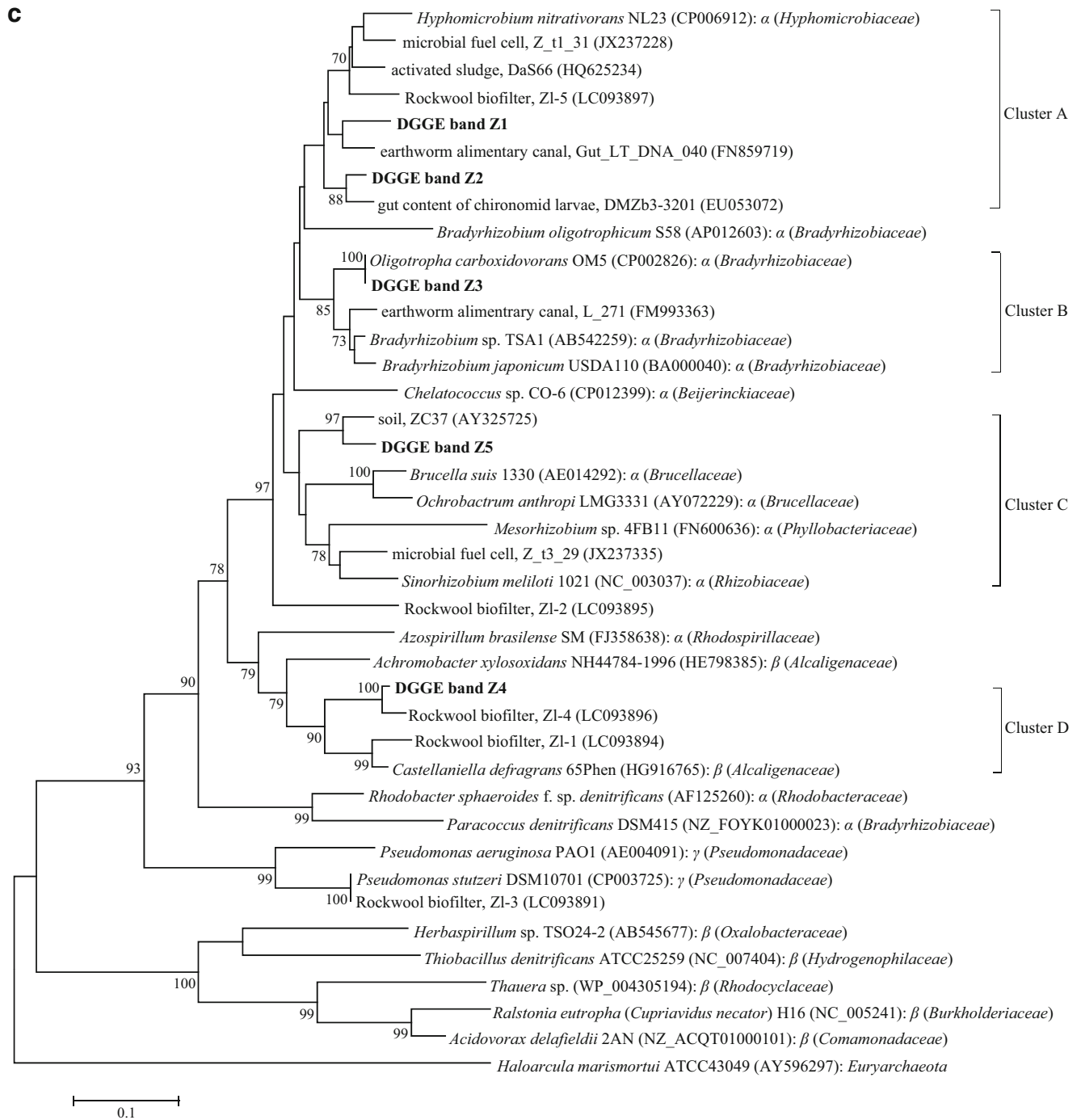


Fig. 5 (continued)

2016). The concentration of N_2O in the inlet gas of the rockwool biofilter was around 4 ppm (Yasuda et al. 2009). At this concentration, it is possible for N_2O reducers belonging to *nosZ* clade II to outcompete *nosZ* clade I (Yoon et al. 2016). The contribution of *nosZ* clade II to N_2O reduction needs to be further studied for a better understanding of the microbial ecology in the biofilter environment.

The results of this study provide microbial evidence that denitrification in the full-scale rockwool biofilter occurs by

utilizing mainly organic matter found in the exhaust gas stream from the composting process. Denitrification might also be linked to the degradation of organic compounds in the biofilter (Juhler et al. 2009; Kristiansen et al. 2011). The application of anoxic autotrophic nitrogen removal achieved by a combination of ammonium-oxidizing bacteria and anammox bacteria is a promising technology to increase the capacity of the biofilter to eliminate nitrogen from the biofiltration systems (De Clippeleir et al. 2012). The

competition between anammox and heterotrophic bacteria for microbial habitat as well as competition between anammox and nitrite-oxidizing bacteria for nitrite might become a subject for controlling microbial activity in the systems treating gaseous emissions from pile-type livestock manure composting. In the rockwool biofilter examined in this study, horizontally separated sites were exposed to composting gases from different composting stages. In addition, water is not circulated, and water is supplied from the top of the biofilter whereas gas is supplied from the bottom, which can result in the vertical stratification of nitrate concentrations (Yasuda et al. 2009). These operational conditions of the composting and biofiltration facilities might result in the horizontal and vertical distributions of the denitrifying bacterial community observed within the biofilter. Therefore, changes in the types of composting facilities may also affect the microbial consortia in biofiltration systems.

In conclusion, the potential denitrification activity of samples taken from the full-scale biofilter was approximately one-order of magnitude lower than that of activated sludge from a livestock wastewater treatment plant. The differences in the response of potential denitrification activity to the addition of external electron donors indicate the intrinsic differences between the physiological properties of the denitrifying bacterial community in the rockwool biofilter packing material and that in the activated sludge. The number of 16S rRNA and *nosZ* genes in the rockwool samples was in the range of $1.64\text{--}3.27 \times 10^9$ and $0.28\text{--}2.27 \times 10^8$ copies/g dry, respectively. DGGE analyses targeting the *nirS*, *nirK*, and *nosZ* genes indicated that the distribution of *nir* genes was spread in a vertical direction and the distribution of *nosZ* genes was spread in a horizontal direction within the biofilter. The corresponding enzymes were mainly related to those from the α and β classes of *Proteobacteria* and to environmental clones retrieved from agricultural soil, activated sludge (from wastewater treatment plants), the freshwater environment, and ingested bacteria in the guts of earthworms and other invertebrates. A *nosZ* gene that had 99% nucleotide sequence identity with that of *O. carboxidovorans* was also detected. Some *nirK* fragments were related to those from micro-aerobic environments. Denitrification in the full-scale rockwool biofilter might occur by a denitrifying bacterial community adapted to the intensely aerated ecosystem and utilizing mainly organic matter supplied from the livestock manure composting gas.

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Compliance with ethical standards

Ethical statement This research does not contain any studies with human or animal subjects.

Conflict of interest The authors declare that they have no conflict of interest.

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