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Research Article

Changes in Denitrification Potentials and Riverbank Soil Bacterial Structures along Shibetsu River, Japan

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Riverbank soil ecosystems are important zones in terms of transforming inorganic nitrogen (N), particularly nitrate (NO_3^--N), in soils to nitrous oxide (N_2O) gases. Thus, the gasification of N in the riverbank soil ecosystems may produce a greenhouse gas, N_2O, when the condition is favourable for N_2O-producing microbes. One of the major N_2O-producing pathways is denitrification. Thus, we investigated the denitrification potentials along Shibetsu River, Hokkaido, Japan. We sampled riverbank soils from eight sites along the Shibetsu River. Their denitrification potentials with added glucose-carbon (C) and NO_3^--N varied from 4.73 to 181 $\mu g{\cdot}N{\cdot}kg^{-1}{\cdot}h^{-1}$. The increase of the denitrification after the addition of C and N was negatively controlled by soil pH and positively controlled by soil NH_4^+-N levels. Then, we investigated the changes in 16S rRNA bacterial community structures before and after an anaerobic incubation with added C and N. We investigated the changes in bacterial community structures, aiming to identify specific microbial species related to high denitrification potentials. The genus Gammaproteobacteria Aeromonadaceae *Tolumonas* was markedly increased, from 0.0 ± 0.0% to 16 ± 17%, before and after the anaerobic incubation with the excess substrates, when averaged across all the sites. Although we could not find a significant interaction between the denitrification potential and the increase rate of G. Aeromonadaceae *Tolumonas*, our study suggested that along the Shibetsu River, bacterial response to added excess substrates was similar at the genus level. Further studies are needed to investigate whether this is a universal phenomenon even in other rivers.

1. Introduction

Riverbank ecosystems are very important ecological zones in relation to nitrogen (N) cycle. Nitrogen (N) can be a source of water pollution particularly with excess nitrate (NO_3^--N) derived from agricultural systems. During the filtration process performed by riverbank ecosystems, the concentration of NO_3^--N may decrease due to the gasification of NO_3^--N [1]. Denitrification is one of the microbial processes responsible for this gasification process. This process reduces NO_3^--N to gases such as nitrous oxide (N_2O) and di-nitrogen (N_2). Thus, it is important to evaluate the factors controlling denitrification potential in riverbanks along river ecosystems.

While denitrification can potentially reduce NO_3^--N from the riverbank ecosystems, it can also negatively impact the environment because N_2O is a greenhouse gas. The global warming potential of N_2O is reported to be 298 times greater than carbon dioxide (CO_2) [2]. Thus, previous studies concluded that the riverbank ecosystems' denitrification can increase the environmental risk of N_2O emissions [3, 4]. Nitrification process (the oxidation of ammonia to NO_3^--N) is also an important N_2O-producing process when soils are well aerated [5], but denitrification is more important when soil is wetter or submerged with water [6]. Carbon and N contents in soils and the amount of readily available inorganic-N positively control the denitrification potentials because these are substrates for the activity of denitrifying microbial communities [7, 8]. Soil pH also controls denitrification potentials although the relationship between pH and denitrification is markedly influenced by other factors

such as the size of denitrifying microbial communities [9, 10]. Also, temperature, oxygen availability, and physical factors such as hydraulic conductivity of the sediment were reported as controlling factors of denitrification potentials in riverbank ecosystems [3, 11–13]. However, a review article suggested that further studies are needed in this area particularly using three-dimensional approaches (spatial heterogeneity, soil depth, and time courses) [14].

To further understand the factors controlling the variability of denitrification potentials in riverbank ecosystems, microbial approaches are useful. Denitrification processes are mainly performed by a group of microbes called denitrifiers. Microbes capable of denitrification are phylogenetically extremely diverse [15]. For example, many groups in the phylum *Proteobacteria*, such as *Thauera*, *Paracoccus*, *Hyphomicrobium* and *Comamonas*, were reported to be performing denitrification in a wastewater system [16]. Also, denitrifying microbes interact with other microbes in soils. In recent years, 16S rRNA-based bacterial community structural analyses in natural ecosystems, including riverbank ecosystems, are becoming more common [17]. Thus, 16S rRNA approaches may be useful to identify potential denitrifiers in riverbank ecosystems and to identify factors controlling the variability in denitrification potentials. Similar approaches have already taken to identify denitrifying microbial communities in agricultural soils [18, 19]. For example, Ishii et al. [19] performed 16S rRNA analyses of soil DNA extracted from a paddy field and identified several bacterial genus, including *Herbaspirillum* in the phylum *Proteobacteria*, as key players during denitrification process. However, few studies compared multiple sites using the 16S rRNA approaches to find out whether the same bacterial species are controlling the magnitudes of denitrification or not.

Thus, by compiling the heterogeneity of denitrification potentials in different riverbank ecosystems along a river with basic soil characteristics (i.e., soil pH, soil carbon, moisture, and texture) and 16S rRNA, we may be able to identify microbial phyla/families controlling the magnitude of denitrification potential of a soil in a larger scale. The information can be important to evaluate ecosystem disservices of rivers, particularly N_2O emissions. Rivers located in an area with developing farming industry and/or with large cities may require this type of information to develop sustainable future plans.

Thus, in this study, we sampled riverbank soils along Shibetsu River in Hokkaido, Japan, and measured their denitrification potentials along with other chemical properties. Shibetsu area is known as one of the most developed dairy industry zones in Japan and N-related water pollution is an issue in this area [20]. We hypothesized that the variability of the denitrification potentials in riverbank soils along Shibetsu River can be explained by the presence/absence of specific bacterial groups. Also, we hypothesized that the chemical characteristics of the riverbank soils partly influence the magnitudes of denitrification potentials.

2. Materials and Methods

2.1. Riverbank Soil Sampling. Riverbank soil was sampled in September 2016 at eight sites across the Shibetsu River basin, Hokkaido (Figure S1, Table S1). We sampled the soils as evenly as possible along the river but sampling was not possible when the area was managed as private farms (e.g., between E and F). The sites B and G were in stream tributaries to the Shibetsu River. Between the sampling points F and G, Nakashibetsu city is located, and most of the riverbank was solidified in concrete; thus, we did not sample the soil in the city. Soil sampling has performed using a soil auger, and topsoil (0–15 cm depth) and subsoils (15–30 cm depth) were taken separately. For each sampling point, 2–4 soil cores were taken from the 1 m × 1 m zone. The samples were then mixed and placed in a plastic bag. The sampled soils were placed in plastic bags and stored at <4°C until analyses. The basic soil characteristics (pH, ammonium-N, nitrate-N, total C, and total N) were listed in Table S2. The soil type for the sampling points A to F was immature sandy Fluvisol. For the sampling points G and H, the soil was still Fluvisol, but it was not an immature soil. In the vicinity of the sampling points G and H, there was a large peatland zone, but along the Shibetsu River, alluvial deposits formed Fluvisol and our sampling depth (up to 30 cm) did not observe any peat materials [21]. At the sites B, E, and F, we could not sample the subsoils because soils only developed shallowly and rocks were present below 15 cm. The annual rainfall in this area averaged 1158 mm. The average temperature was 5.4°C.

2.2. Riverbank Soil Characteristics. For the riverbank soils at each site, soil moisture contents were measured by oven-drying the soil samples at 105°C for >24 h. For pH_{water}, fresh soil and MilliQ water were mixed in the ratio of 1:2.5, and pH was measured using a pH sensor (AS800, AS ONE Co., Japan). For pH_{KCl}, fresh soil and 10% KCl were mixed in the ratio of 1 : 5, and pH was measured using the same pH sensor. The pH_{water} refers to the acidity of the soil solution whereas the pH_{KCl} refers to the acidity in the soil solution plus the research acidity in the colloids. The total C and total N were measured using 2400 Series II CHNS/O Elemental Analyzer System (PerkinElmer, Inc., Waltham, USA). The inorganic-N concentrations (ammonium-N (NH_4^+-N) and NO_3^--N) were measured by extracting the fresh soil with 10% KCl and filtering through 1 μm (No. 5C, Toyo Roshi Kaisha, Ltd., Japan). The measurements were performed colorimetrically using a flow injection analyzer (Aqualab Co. Ltd., Japan) [22].

2.3. Denitrification Potentials and Their Relationships with Soil Characteristics. Denitrification potentials for the soil samples were measured using an acetylene block method [23] under three different conditions, (1) without any substrate additions, (2) with added NO_3^--N, and (3) with added NO_3^--N and glucose-carbon (glucose-C) with three replicates per sample. This experiment was performed under anaerobic conditions (>99% N_2). First, approximately 2.5 g of fresh soil was placed in 100 ml glass bottle and one of the three solutions, (1) 5 ml of MilliQ water, (2) 5 ml of 0.72 g NO_3^--N·l^{-1}, or (3) 5 ml of 0.72 g NO_3^--N and 0.5 g glucose-C·l^{-1}. Then, the bottles were capped using septa and aluminium caps, and their headspaces were replaced with >99%

N_2 using two needles (one to supply N_2 and another to evacuate atmospheric air). Approximately 10% of the headspace of each 100 ml bottle was replaced with acetylene and the bottles were incubated for 2 hrs at 25°C. The bottles were gently shaken during the incubation.

After the incubation period, 30 ml of the gas inside of each bottle was sampled using a syringe and placed in an evacuated vial for the measurement using a gas chromatograph equipped with an electron capture detector. The denitrification potential for each soil was then expressed as emitted ng N_2O-N·g^{-1} soil hr^{-1}.

The increase of the denitrification potentials due to the addition of glucose-C and NO_3^--N was evaluated using a multiple regression model to investigate the best fitted model, and the smallest Akaike Information Criterion (AIC) was calculated. This approach was commonly used to minimize the number of parameters (soil characteristics) to explain the variables (denitrification potentials). The multiple and adjusted R^2 values were 0.582 and 0.500, respectively. Based on the AIC analyses, the following model was established:

$$\text{Increase in denitrification potential with added C and N} \left(\text{ng}\cdot N\cdot \text{g}^{-1}\cdot \text{hr}^{-1}\right) = 464 + (-69.1) \times \text{pH}_{\text{water}} + 1.32 \times \text{NH}_4 + \left(\text{mg}\cdot N\cdot \text{kg}^{-1}\cdot \text{soil}\right). \quad (1)$$

2.4. 16S rRNA Bacterial Community Structures. For the topsoil samples (0–15 cm, eight samples in total), we investigated the changes in 16S rRNA bacterial community structures before and after the anaerobic incubation with NO_3^--N and glucose-C, with two replications (eight soils × before and after C and N addition × two reps = 32 samples). The incubation procedure and substrate concentrations were the same as the method used for the measurement of denitrification potential except that the incubation length was 48 h, and the number of replication was two for this 16S rRNA experiment.

For the soil samples before and after the anaerobic incubation, soil DNA was extracted using PowerSoil Kit (MO BIO Laboratories, Inc. Carlsbad, USA). Then a PCR was performed using AmpliTaq Gold® 360 Master Mix (Applied Biosystems™, Foster City, USA) and primers to amplify the V4 region of 16S rRNA (amplicon size ≈ 250 bp, forward primer = 515F: 5′-GTGCCAGCMGCCGCGGTAA-3′, reverse primer = 806R: 5′-GGACTACHVGGGTWTCTAAT-3′) and Miseq was used for the analyses. The PCR cycle was 95°C for 10 min, then 25 cycles of 95°C for 30 sec, 55°C for 30 sec, and 72°C for 1 min, followed by 72°C for 7 min.

After the amplification, the PCR products were analysed using a next-generation sequencer (Ion PGM, Thermo Fisher Scientific Inc.). The obtained sequence data were analysed using the software QIIME [24]. There were eight soil samples, and we analysed the bacterial community structures before and after the addition of NO_3^--N and glucose-C in duplicates. Thus, we had 32 samples in total. Using QIIME, we obtained the community structure data with taxonomies, at phylum, order, family, and genus levels.

2.5. Statistics. For the denitrification potential data, two-way ANOVA with *F* test was performed to investigate the effect of sampling sites and the treatments (the addition of NO_3^--N and glucose-C). Post hoc Tukey's test was performed when there was a significant interaction between the sampling sites and the treatments ($p < 0.05$) to evaluate the effect of treatments for each sampling site. Then, the relationship among the denitrification potentials (with NO_3^--N and glucose-C) and other soil characteristics was investigated using linear regressions.

For the increase in denitrification with added C and N (difference between the denitrification potential with C and N, and the denitrification without any substrate addition), we performed a multiple regression with other environmental variables. The environmental variables were pH_{water}, pH_{KCl}, NH_4^+-N, NO_3^--N, total C, and total N. To determine the best fitted model based on the multiple regressions, we used the Akaike's Information Criterion (AIC) approach. Using this approach, we aimed to simplify the model using minimum number of the variables. The smaller the AIC, the better the model is, according to the AIC approach.

For the soil microbial community structure data, significantly increased phyla after the anaerobic incubation with added C and N were identified using *t*-tests. For the significantly increased phyla, they were further studied more in details (at the class/order/genus levels). Also, for the phylum level community data, canonical-correlation analysis (CCA) was performed to investigate the similarity among the communities. The statistical analyses were performed using the software RStudio (version 1.1.383).

3. Results

3.1. Soil Characteristics. The riverbank soils' C and N contents varied from 3.6 to 62.1 g·C·kg^{-1} and 0.40–4.8 g·N·kg^{-1}. The riverbank soil pH_{water} varied from 5.53 to 6.82 and pH_{KCl} varied from 4.27 to 6.43; thus, it was slightly acidic. The inorganic-N contents were 3.0–41.4 mg N·kg^{-1} and 0.7–13.6 mg·N·kg^{-1} for NH_4^+-N and NO_3^--N, respectively.

3.2. Denitrification Potentials and Their Relationships with Soil Characteristics. In the topsoil, the denitrification potentials were generally higher with added C and N, when compared to those without any added substrates (Figure 1(a)). Both sampling sites and the addition of C and N had significant effects on the denitrification potentials (both resulted in $p < 0.001$). Also, there was a significant interaction between the sampling sites and the treatments ($p < 0.001$). For the site A and B, the denitrification potential did not respond to the addition of N but increased with the addition of C and N. For the sites C, D, and H, both the addition of N and addition of C and N resulted in higher denitrification potentials when compared to the controls. The sites E, F, and G did not clearly respond to the addition of substrates.

In the subsoil, the addition of C and N also significantly increased the denitrification potentials, when averaged across the sampling sites, but the significance was relatively weaker ($p < 0.05$) when compared to the topsoil (Figure 1(b)). The sampling sites also had a significant influence on the

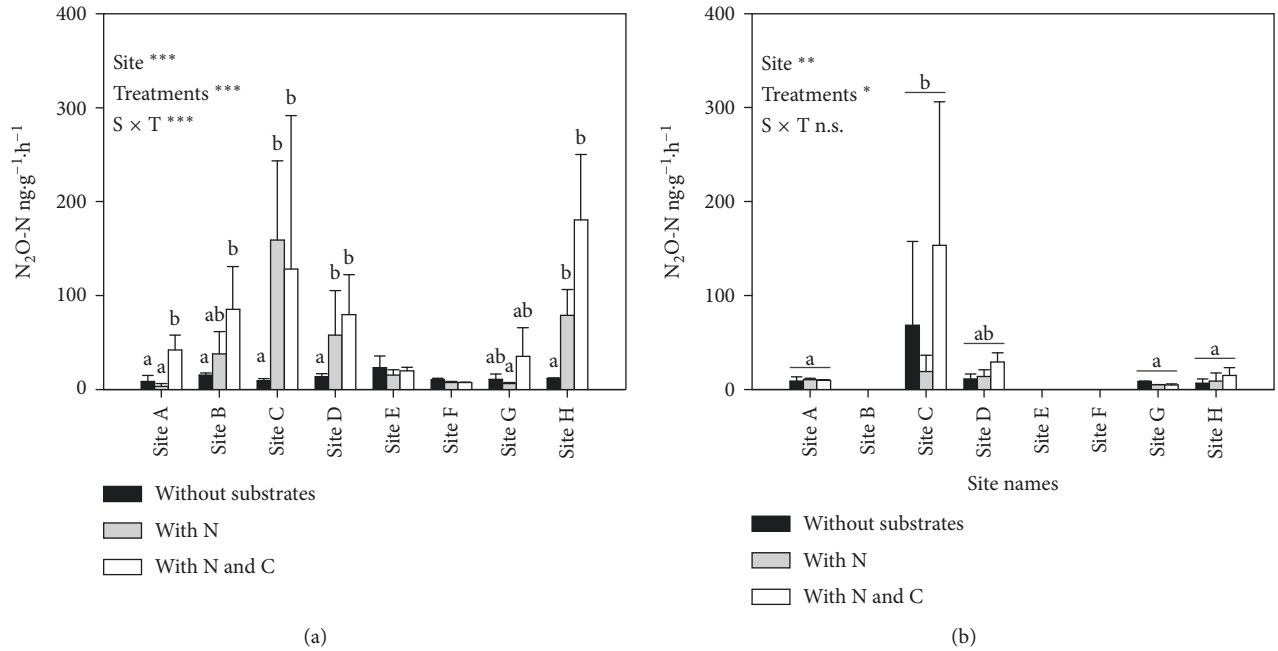

Figure 1: Denitrification potentials for the topsoils (0–15 cm) and subsoils (15–30 cm). Small letters define significant differences among three substrate treatments ("control," "with N," and "with C and N"), for each sampling site (A–H), for the topsoils. For the subsoils, the small letters define significant differences among the different sites. The error bars were standard deviations ($n = 3$). (a) Topsoil and (b) subsoil n.s. = not significant, $^{*} = p < 0.05$, $^{**} = p < 0.01$, $^{***} = p < 0.001$.

denitrification potentials in the subsoil too ($p < 0.01$), but there was no interaction between the sampling sites and the treatments. The site C had relatively higher denitrification potentials when compared to other subsoils, but the variability among the replications was also larger when compared to other subsoils.

The denitrification potentials with added C and N correlated positively to the soil moisture at the sampling ($p < 0.001$) and correlated negatively to soil pH_{water} ($p < 0.01$) (Figures S2(a) and S2(b)). However, total C, total N and inorganic-N (NH_4^+-N and NO_3^--N) contents were not correlated to the denitrification potentials with glucose and KNO_3 (Figures S2(c)–S2(f)).

3.3. The Effect of C and N Addition (Increase in Denitrification) and Environmental Variables. The increase in denitrification with substrates (denitrification potential with C and N minus denitrification potential without any substrate addition) was negatively correlated with pH_{water}, when all parameters' correlations were tested (Figure S3). Based on the multiple regression model, the smallest AIC was achieved when we used pH_{water} and NH_4^+-N as the environmental parameters (1).

3.4. 16S rRNA Bacterial Community Structures. Soil microbial community structures were measured before and after the addition of C and N. The difference was characterized by an increase in *Proteobacteria*, at the phylum level, from 44 ± 8% (before) to 65 ± 14% after the addition of C and N, when averaged across the sampling site (error = s.d.) (Figure S4).

When the changes within *Proteobacteria* phylum (class and order levels) were compared before and after the incubation with C and N, the major increase of this phylum was supported by the increase of the class *Gammaproteobacteria* from 9.3 ± 8.5% (before C and N addition) to 27 ± 15% (after C and N addition) (Figure S5). Within *Gammaproteobacteria*, the increase of an order *Aeromonadales* was conspicuous (relative abundance within the whole bacterial community = 0.0 ± 0.0% and 17 ± 18%, without and with C and N addition, resp.) (Figure S6). Within the order *Aeromonadales*, the genus Aeromonadaceae *Tolumonas* was dominated (relative abundance within the whole bacterial community = 0.0 ± 0.0% and 16 ± 17%, without and with C and N addition, resp.) (Figure 2).

3.5. Denitrification Potentials and Soil Microbial Community Structures. We have not found a clear relationship between the 16S rRNA bacterial community data and the magnitudes of the denitrification potential. The results from the CCA analyses suggested that sites B, C and E showed minor changes in their community structures whereas other sites showed more significant and similar changes in their community structures, after the addition of C and N (Figure S7). However, the magnitudes of the community structure changes due to the addition of C and N and the denitrification potentials were not related. Also, at the genus level, the magnitudes of the increase in Aeromonadaceae *Tolumonas* (Figure 2) were compared to the denitrification potentials in the topsoil (Figure 1(a)) but there was no significant relationship between them.

4. Discussion

4.1. Denitrification Potentials and Their Relationships with Soil Characteristics. The denitrification potentials were higher with added glucose-C and NO_3^--N, when compared to those

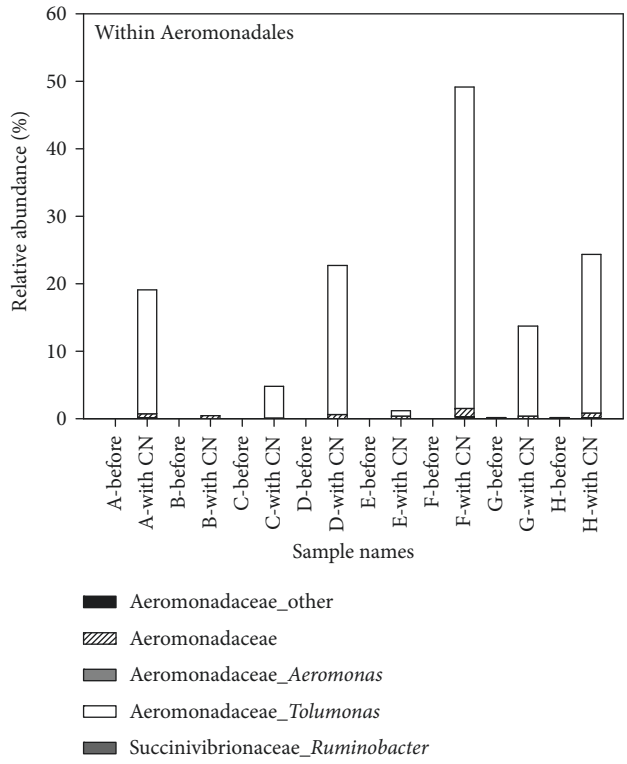

Figure 2: Changes in relative abundance of classes within the order *Aeromonadales* (within *Proteobacteria Gammaproteobacteria*), based on 16S rRNA analysis before and after the addition of C and N. The capitals A to H define the sampling sites. The word "before" describes the data before the addition of C and N whereas "with C and N" suggested the data from the soils with added C and N.

with added NO_3^--N or those without any added substrates (Figure 1). The range of the denitrification potentials with added C and N was similar to previous studies [3, 25–28]. The range of the denitrification potentials without C and N was also similar to the previous study measuring denitrification potentials in the same river [29].

The increased rates of denitrification potentials with the addition of glucose-C and NO_3^--N, compared to the denitrification potentials without the addition of C and N, markedly varied among the samples (Figure 1). It is difficult to fathom reasons behind these variations with our limited sample numbers; however, this phenomenon is important to understand the factors controlling the gaseous loss of N from this ecosystem, including ecosystem disservice such as N_2O emissions. A previous study concluded that the denitrification potentials in a riverbed sediment (South Platte River Basin, Colorado, US) positively responded to the addition of acetate-C (ranging from 0 to 650 μmol·l^{-1}), but the response rates decreased with increasing sampling depth (sampling depth = 0.61, 1.22, and 2.44 m). The current study showed similar results with shallower depth range (0–15 and 15–30 cm) with multiple sites because the shallower soils (Figure 1(a)) clearly responded to the addition of NO_3^--N and glucose-C + NO_3^--N, when compared to the deeper soils (Figure 1(b)). Thus, even along the same river, the impact of nutrient loading on denitrification and limiting factors of denitrification potentials can be variable. The movement and mixture of ground and surface water have been reported as a factor controlling the variability of denitrification potential in Mississippi river [30]. Also, different plants have reported to have different impacts on denitrification potentials in their root zone soils [31]. Thus, the vertical and lateral movements of water and vegetation types may explain some of the variabilities in denitrification potentials found in the current study [32]. Further studies in this area may reveal some key parameters to predict the impact of nutrient loading on the riverbank area, and the parameters may be useful to plan future land use along a river.

Denitrification potentials with added C and N correlated positively to the soil moisture at the sampling ($p < 0.001$) and correlated negatively to soil pH_{water} ($p < 0.01$) (Figures S2(a) and S2(b)). However, total C, total N, and inorganic-N (NH_4^+-N and NO_3^--N) contents were not correlated to the denitrification potentials with glucose and KNO_3 (Figures S2(c)–S2(f)). Thus, in our experiment, the soil pH and moisture condition relatively more strongly controlled the denitrification potentials than the nutritional condition (total C, total N, and inorganic N). Moisture status in soils was previously reported as main factors controlling soil denitrification potentials in the riparian area [33, 34]. For example, a previous report stated that with increasing soil moisture from 15% to 65% (gravimetric), denitrification potentials of the riparian zone soils sampled in Gwynns Falls Watershed, Baltimore, US, linearly increased from approximately zero to 500 ng·N·g^{-1}·h^{-1} [33]. This relationship was similar to what we found in the current study although the moisture range was narrower (30–55%) (Figure S2(a)).

Soil pH negatively correlated to denitrification potentials in the current study (pH ranged approximately 5.5 to 7.0, Figure S2(b)). A similar trend was observed when soil pH was compared with the increase in denitrification potentials after the addition of C and N substrates (Figure S3). Contrastingly, Čuhel et al. [35] reported a positive correlation between soil pH and denitrification potentials in grassland soils (pH ranged 5.5 to 7.7). There was another report showing pH did not influence the denitrification potential in soils (pH ranged 5 to 8) [32]. In the study by Čuhel et al. [35], denitrification potentials ranged between 200 and 1200 ng·N·g^{-1}·hr^{-1}. The range was similar in the study by Liu et al. [36] (200 to 2000 ng·N·g^{-1}·hr^{-1}). However, the maximum denitrification potential in the current study was 170 ng·g^{-1}·hr^{-1}, relatively smaller than the previous reports focusing on the effect of pH on denitrification potentials (Figure S2(b)). The sampling areas in the current study are often flooded; thus, a limited number of denitrifying microbes might be adapted to this area, compared to the soils tested in the previous studies. This might be a reason for the contrasting response of denitrification potentials to pH, although further studies are needed. Also, historical pH changes were reported to be more important than short-term changes of pH as a controlling factor of denitrification potentials [37]. We note that our soil sampling was one-off, and we do not know temporal changes in soil pH over seasons or years. Also, it is still uncertain why pH varies along the river. Previous studies showed that the land-use

change in the area surrounding a river and vegetation types influenced the pH in riverbed area, and the vegetation types were determined due to seasonal flooding events [38, 39]. Further studies are needed in this area, along Shibetsu River.

Soil NH_4^+-N levels were shown as an important factor controlling the magnitudes of the increase in denitrification potentials after the addition of glucose-C and NO_3^--N, in the current study (1). It is difficult to fathom reasons behind this, but the soils tested in the current study had relatively higher amount of NH_4^+-N when compared to NO_3^--N. In the current study, the increase in denitrification potentials after the addition of glucose-C and NO_3^--N was tested under an anaerobic condition, thus nitrification-derived N_2O was likely to be minor. Possibly, high soil NH_4^+-N levels suggest high mineralization of organic-N and relatively higher soil microbial activities, increasing the activity of denitrifiers.

At the field level, the amount of NH_4^+-N might be a limiting factor of denitrification because NH_4^+-N provides substrate (NO_3^--N) for denitrifiers via nitrification. Nitrous oxide emissions are observed both from nitrification and from denitrification; thus, both processes can be a cause of environmental impacts from the riverbed system.

4.2. 16S rRNA Bacterial Community Structures. The difference between the soil microbial community before and after the C and N addition was characterized by a marked increase in *Proteobacteria*, at the phylum level (Figure S4). However, for the soils C and E, the changes in their bacterial communities due to C and N addition were relatively minor compared to other soils. Addition of C sources and the consequent increase in the phyla *Proteobacteria* in soils had been previously reported [27]; thus, *Proteobacteria* had been recognized as a fast grower responding to an addition of readily available substrates.

When the changes within *Proteobacteria* phylum (class and order levels) were compared before and after the incubation with C and N, the major increase of this phylum was supported by the increase of the class *Gammaproteobacteria* (Figure S5) and an order *Aeromonadales* (Figure 2). The class *Gammaproteobacteria* is known as one of denitrifier groups thus might be an important group to determine the capacity of rivers to remove excess-N. Another previous study also showed that *Gammaproteobacteria Enterobacteriaceae* was the dominate family when glucose was added to a forest soil under anaerobic conditions [28]. The order *Aeromonadales* is known as aquatic inhabitants in fresh water [29] and has a denitrifying capacity (reduction of NO_3^--N to nitrite) [30]. Also, *G. Aeromonadales Tolumonas lignolytica* was reported as a genus which utilizes lignin as a sole C source, and it was isolated from a tropical soil [31].

4.3. Linking Denitrification and Bacterial Community Structures. In this study, we have not found a clear relationship between the changes in the 16S rRNA bacterial community structures before and after an anaerobic incubation with glucose-C and NO_3^--N and the changes in the denitrification potentials (Figure 1, Figure S7). Contrastingly, there have been a few previous papers described a specific genus responsible for denitrification activity in specific conditions. For example, *Betaproteobacteria Burkholderiales Oxalobacteraceae Herbaspirillum* was reported to be a key player of denitrification activity in rice paddy soil under anaerobic condition with added C and N [19]. They tested the variabilities in soil's bacterial community structure and denitrification potentials using one rice paddy soil (with different regimes of C and N additions). Thus, they did not compare soils from multiple locations like the current study. The relationship between the bacterial community structure and denitrification potentials may be site specific. A similar experiment using a soil sampled from a potato (*Solanum tuberosum* L.) field reported that *Gammaproteobacteria Pseudomonadales Pseudomonadaceae Pseudomonas* was the majority of the isolated denitrifiers [18]. However, this study was also observing one soil from one farm and they used isolated bacterial communities on nutrient agar. Thus, the direct comparison of this study and the current study may be difficult. Another experiment using an arable soil suggested that the majorities of isolated denitrifiers were *Betaproteobacteria Burkholderiales Burkholderiaceae Burkholderia*, *Pseudomonas*, *Gammaproteobacteria Xanthomonadales Xanthomonadaceae*, *Bacilli Bacillales Bacillaceae*, and *Actinobacteria Streptomycetales Streptomycetaceae* [15]. This study also observed isolated denitrifiers on nutrient agar, unlike the current study, but we note that many of the previous studies highlighted a group of *Gammaproteobacteria* as a key player in denitrification process.

Our finding suggested that during the optimum condition for denitrification, a specific genus of bacteria may increase in a specific section of the Shibetsu river ecosystem. This was based on the increase in *Gammaproteobacteria Aeromonadales*, observed after the incubation of the sampled soils with glucose-C and NO_3^--N (Figure S6). The short-term (48 h) incubation of soils with glucose-C and NO_3^--N stimulates the growth of fast-growing bacteria and *Gammaproteobacteria* is known as an extremely fast-growing group although its abundance in natural soils is normally low when substrates are not available [40]. However, our data suggested that although the specific family of bacteria increased with C and N, it is not necessarily they correspond to the magnitude of the increase in the denitrification potential. *Gammaproteobacteria* is also known as a metabolically flexible group of bacteria; thus, they perform denitrification only when conditions are favourable. For example, the lack of phosphorus is known as a factor controlling the denitrification performance of a group of *Gammaproteobacteria* [41]. Thus, in future, we should observe the availability of nutrients other than C and N to fully understand the factors controlling the denitrification potentials of riverbank soils. Our study observed the whole bacterial community including nondenitrifying bacteria. Contrastingly, a previous study focused on soil DNA related to the function of denitrification enzymes (*nirK*, *nirS*, and *nosZ* genes or nitrite and nitrous oxide reductase encoding genes, resp.) [42]. They found that the microbial communities harbouring these genes were responsible for denitrifier-derived nitrous oxide emissions from a waterlogged soil. Thus, one

limitation of our study is the lack of information on the relationship between the whole bacterial community structures and the bacterial community-harbouring denitrification genes. A further limitation of our study is also due to the lack of information about the amount of bacterial biomass. Future studies should focus the relationship between the increase of bacterial biomass and denitrification potential.

Additionally, the numbers of our sampling points and replication to analyse DNA were relatively small to discuss the detailed mechanisms behind the relationship between denitrification and soil bacterial community structures. Further studies are needed in larger scale with more detailed samplings.

5. Conclusion

Denitrification potentials' variability along the riverbank soils of Shibetsu river, Hokkaido, Japan varied from 7.43 to 181 $\mu g{\cdot}N{\cdot}g^{-1}{\cdot}h^{-1}$ with added glucose-C and nitrate-N substrates. Added C and N increased the denitrification potentials in some soils but not for others. There was a negative relationship between the soil pH and the denitrification potential; thus, further investigation is needed to understand the mechanisms controlling the pH of riverbank soils. Total C, N, and inorganic-N contents in the sampled soils did not correlated to the denitrification potentials. The soil bacterial community data, based on the 16S rRNA analyses, taken before and after the anaerobic incubation with added C and N suggested that a bacterial family *Gammaproteobacteria* Aeromonadaceae *Tolumonas* markedly increased in most of the riverbank soils we sampled. However, the magnitudes of the increase in G. Aeromonadaceae *Tolumonas* were not related to the denitrification potentials. Further studies are needed to understand the microbial factors controlling the denitrification across a river since riverbank soil often provides an important ecosystem service to remove excess N.

Data Availability

All of the original data related to the current study are available through contacting the corresponding author (Yoshitaka Uchida; uchiday@chem.agr.hokudai.ac.jp).

Conflicts of Interest

There are no conflicts of interest.

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Supplementary Materials

Table S1: the locations (latitudes and longitudes) of the sampling sites and their basic soil characteristics. Table S2: characteristics of the sampled soils for each sampling depth. We could not obtain the 15–30 cm samples for the sites B, E, and F because the soil was shallow and rocks were present at the depth > 15 cm. The values were shown as averages ± standard deviations ($n = 3$). Figure S1: sampling sites of the riverbank soils. The size of the circles corresponds the magnitude of denitrification potential with excess carbon and nitrate, under an anaerobic condition. The data of water lines, water areas, and contour lines by Geospatial Information Authority of Japan (2016) were used for the base map. Figure S2: the relationships between denitrification potentials with added C and N (DEA) and other soil characteristics, namely (a) soil moisture at sampling, (b) pHwater, (c) total C, (d) total N, (e) nitrate-N contents, and (f) ammonium-N contents. Significant linear relationships were observed for the moisture and pHwater. The open circles were the topsoils (0–15 cm), and the filled circles were subsoils (15–30 cm). Figure S3: correlation plots among the increase in the denitrification potentials after the addition of carbon and nitrogen substrates (DiffCN_cont, $ng{\cdot}N{\cdot}g^{-1}{\cdot}hr^{-1}$) and environmental parameters. The $NH4^{+}$-N and $NO_3{}^{-}$-N values were expressed in mg $N{\cdot}g^{-1}$ soil. The total C and N values were expressed in $g{\cdot}kg{\cdot}soil^{-1}$. The histogram, kernel density overlays, absolute correlations, and significance asterisks (∗, ∗∗, and ∗∗∗ for <0.05, <0.01, and <0.001, respectively) were shown on the figure. Figure S4: changes in relative abundance of bacteria phylum, based on 16S rRNA analysis. The capitals A to H define the sampling sites. The words "before" and "with CN" mean the data before and after the addition of C and N substrates, respectively. Figure S5: changes in relative abundance of classes within the phylum Proteobacteria, based on 16S rRNA analysis. The capitals A to H define the sampling sites. The words "before" and "with CN" mean the data before and after the addition of C and N substrates, respectively. Figure S6: changes in relative abundance of orders within the class Gammaproteobacteria, based on 16S rRNA analysis. The capitals A to H define the sampling sites. The words "before" and "with CN" mean the data before and after the addition of C and N substrates, respectively. Figure S7: the results from the canonical-correlation analysis of the 16S rRNA community data, before and after the addition of C and N substrates. The alphabets A to H suggest the sampling sites and the alphabets with or without "_CN" are the samples after or before the addition of C and N substrates, respectively. (*Supplementary Materials*)

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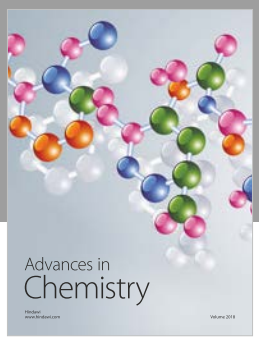

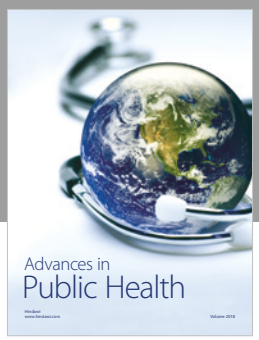

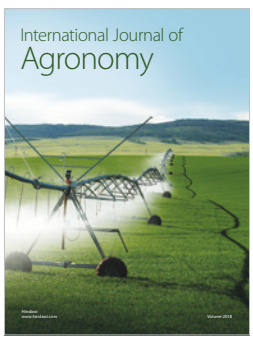

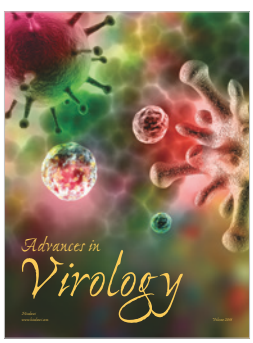

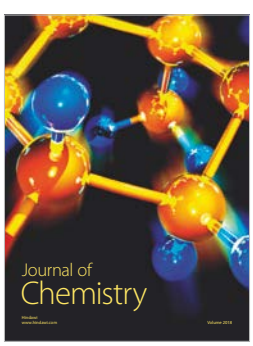

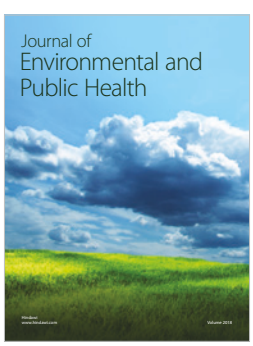

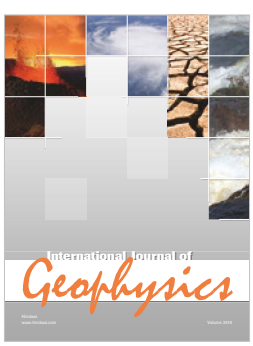

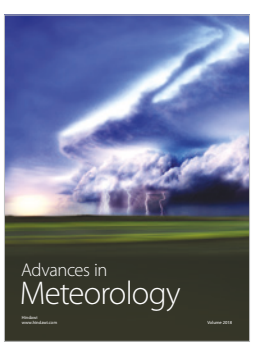

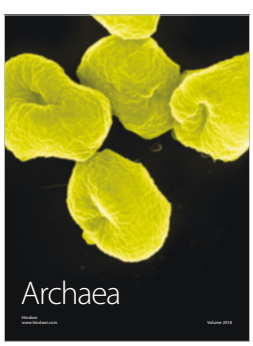

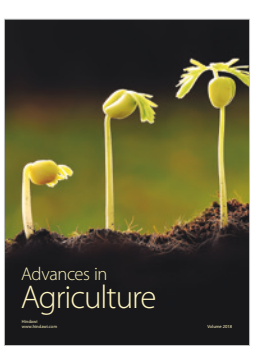

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