Comparative Evaluation of Ammonium Removal Activity and Microbial Community Structure in Field-Derived Nitrifying Inocula

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Abstract

This study evaluated ammonium removal activity and microbial community structure in three field-derived nitrifying inocula with different origins and cultivation histories. Batch NH_3-N removal tests were conducted, and the specific nitrification rate (SNR) was calculated based on mixed liquor volatile suspended solids (MLVSS). Microbial community composition was analyzed using 16S rRNA gene sequencing. The average NH_3-N removal rates of inocula A, B, and C were 19.05, 21.32, and 26.23 mg NH_3-N/L/hr, respectively. The corresponding SNR values were 1.99, 2.51, and 2.62 mg NH_3-N/g MLVSS/hr. Inoculum A showed the highest OTU richness, Shannon diversity, and combined relative abundance of *Nitrosomonas* and *Nitrospira* (3.46%), but exhibited the lowest SNR. In contrast, inoculum C showed the highest NH_3-N removal activity despite a low abundance of nitrification-related microorganisms (0.60%). These results indicate that 16S rRNA-based relative abundance alone does not directly represent actual ammonium removal activity. Therefore, microbial community analysis should be combined with batch activity tests and SNR assessment when evaluating field-derived nitrifying inocula.

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Introduction

Nitrogen removal in wastewater treatment facilities is an important process for maintaining effluent water quality and preventing eutrophication of receiving water bodies. In biological nitrogen removal, nitrification is the oxidation of ammonium nitrogen, in which ammonium is first oxidized to nitrite by ammonia-oxidizing bacteria (AOB) and subsequently oxidized to nitrate by nitrite-oxidizing bacteria (NOB). Because nitrification provides oxidized nitrogen species for subsequent denitrification, deterioration of nitrification can destabilize the overall biological nitrogen removal process (Henze et al., 2008; Grady et al., 2011; Metcalf & Eddy, 2014).

Nitrification is mainly mediated by AOB such as *Nitrosomonas* and *Nitrosospira* and NOB such as *Nitrospira* and *Nitrobacter*. *Nitrosomonas* has commonly been reported as a representative AOB in wastewater treatment processes, while *Nitrospira* is widely recognized as a major nitrite-oxidizing group (Ge et al., 2015; Daims et al., 2015; Gruber-Dorninger et al., 2015). Recent reports of complete ammonia oxidation (comammox) by some *Nitrospira* have further shown that the ecology and function of nitrifying microorganisms are more complex than the conventional AOB/NOB division (Daims et al., 2015; van Kessel et al., 2015).

Nitrifying microorganisms generally grow more slowly than heterotrophic microorganisms and are sensitive to operational disturbances. Temperature, dissolved oxygen, pH, alkalinity, ammonium loading, free ammonia (FA), free nitrous acid (FNA), and sludge retention time are major factors affecting nitrification rates and nitrifying microbial communities (Anthonisen et al., 1976; Blackburne et al., 2007; Ge et al., 2015). Low temperature, insufficient dissolved oxygen, pH decline, or sudden loading fluctuations can reduce nitrifying activity, leading to ammonium accumulation or elevated effluent nitrogen concentrations. Therefore, maintaining stable nitrification and developing recovery strategies are important operational tasks in wastewater treatment facilities.

One possible approach for restoring nitrification or improving start-up stability is the application of external sludge or nitrifying inocula. In practice, activated sludge, municipal wastewater treatment sludge, industrial wastewater treatment sludge, mixed cultures, or commercial microbial products may be used to enhance nitrification. However, field-derived inocula can differ substantially in microbial community structure and physiological activity depending on their origin, operating history, storage conditions, cultivation environment, and degree of mixing. Activated sludge is a complex microbial ecosystem composed of multiple functional guilds rather than a single microbial population, and its community structure can vary according to influent characteristics and operational conditions (Zhang et al., 2012; Saunders et al., 2016; Ju et al., 2014). Therefore, evaluation of nitrifying inocula should consider not only sample origin or name, but also actual ammonium removal activity and microbial community characteristics.

Nitrifying activity can generally be assessed using the temporal decrease in ammonium nitrogen, and the specific nitrification rate (SNR) can be used for more quantitative comparison. SNR represents the ammonium removal rate per unit biomass and is useful for comparing nitrifying activity among different sludges or inocula (Grady et al., 2011; Blackburne et al., 2007). Because simple NH_3-N removal rates can be affected by the solids concentration or biomass contained in each inoculum, MLVSS-based SNR can be used as a suitable functional indicator for comparing field-derived mixed microbial inocula (APHA et al., 2017; Metcalf & Eddy, 2014).

In parallel, 16S rRNA gene-based microbial community analysis is widely used to interpret microbial ecology in activated sludge and biological treatment processes. Advances in high-throughput sequencing have enabled culture-independent characterization of microbial composition, including dominant and rare populations, at the phylum and genus levels (Quast et al., 2013; Yoon et al., 2017; Knight et al., 2018). In particular, the detection and relative abundance of nitrification-related taxa such as *Nitrosomonas*, *Nitrospira*, and *Nitrobacter* can provide useful biological indicators for interpreting nitrogen transformation potential in treatment processes (Ge et al., 2015; Gruber-Dorninger et al., 2015).

However, relative abundance obtained from 16S rRNA-based community analysis does not always directly represent process performance or microbial activity. Although 16S rRNA sequencing is useful for identifying the relative composition of microbial taxa, it does not directly measure actual metabolic activity, enzyme activity, functional gene expression, substrate utilization, or physiological adaptation to specific operating conditions (Knight et al., 2018; Pollock et al., 2018; Bokulich et al., 2020). In addition, relative abundance reflects the proportion of taxa within a community and may not correspond to the absolute abundance or actual reaction rate of a functional group. Therefore, integrated evaluation combining nitrification-related microbial abundance with actual NH_3-N removal rate and MLVSS-based SNR is required for field-derived nitrifying inocula.

This study evaluated three field-derived nitrifying inocula with different origins and cultivation histories. Batch NH_3-N removal tests were conducted, and MLVSS-based SNR was calculated to compare ammonium removal activity among inocula. In addition, 16S rRNA gene-based microbial community analysis was performed to

compare sequencing depth, alpha diversity, and phylum- and genus-level community structures. The relationship between the relative abundance of nitrification-related microorganisms, especially *Nitrosomonas* and *Nitrospira*, and actual nitrifying activity was examined to assess the need for combining microbial community analysis with functional activity tests.

Materials And Methods

Test inocula

Three field-derived nitrifying inocula with different origins and cultivation histories were used to compare ammonium removal activity and microbial community structure. The sample origins and names were anonymized, and the inocula are referred to as inocula A, B, and C throughout this manuscript.

Inoculum A was classified as a municipal wastewater treatment plant-derived activated sludge-type inoculum. Inoculum B was classified as a mixed sludge-based nitrifying inoculum prepared from field-derived sludge. Inoculum C was classified as an industrial field-derived sludge inoculum. All three inocula were considered field-derived mixed microbial communities rather than single strains or pure cultures.

Microbial communities in activated sludge and field-derived sludge can vary depending on influent substrate, oxygen conditions, operating history, sludge retention time, and storage conditions. Accordingly, inocula intended for nitrification may differ in microbial community structure and physiological activity (Grady et al., 2011; Saunders et al., 2016; Zhang et al., 2012). Therefore, inocula A, B, and C were treated as field-derived nitrifying inocula with different origins and community characteristics rather than as standardized microbial products. The characteristics of the three field-derived inocula are summarized in Table 1.

Table 1. Characteristics of field-derived nitrifying inocula used in this study

Inoculum	Sample characteristics
A	Municipal wastewater treatment plant-derived activated sludge-type inoculum
B	Mixed sludge-based nitrifying inoculum
C	Industrial field-derived sludge inoculum

Batch ammonium removal test

Batch NH_3-N removal tests were conducted to evaluate ammonium removal activity of each inoculum. Batch activity tests can be used to compare substrate removal capacity and reaction rates of biological sludges or inocula, and nitrifying activity is commonly evaluated using changes in ammonium nitrogen concentration over time (Grady et al., 2011; APHA et al., 2017; Ge et al., 2015).

Each test was performed by supplying an ammonium nitrogen substrate to each inoculum and monitoring changes in NH_3-N concentration over time. NH_3-N was measured at 0, 1, 2, and 3 h. The temporal decrease in NH_3-N concentration was used to calculate the ammonium removal rate of each inoculum and was used as the primary functional indicator of nitrifying activity.

In this study, the decrease in NH_3-N concentration was interpreted as an indicator of ammonium removal activity. However, NH_3-N removal alone does not prove complete nitrification. In a complete nitrification process, decreases in ammonium nitrogen should be accompanied by the formation of nitrite and nitrate. Therefore, NO_2^--N and NO_3^--N formation should be examined to clarify the nitrification pathway more rigorously (Henze et al., 2008; Metcalf & Eddy, 2014; Ge et al., 2015). Accordingly, the results were discussed in terms of ammonium removal activity or potential nitrifying activity rather than complete nitrification. The ammonium removal rate was calculated as follows:

$$RNH_3\text{-}N = (C_0 - C_t) / t$$

where RNH_3-N is the ammonium removal rate (mg NH_3-N/L/hr), C_0 is the initial NH_3-N concentration (mg/L), C_t is the NH_3-N concentration after t h (mg/L), and t is the reaction time (h). The average NH_3-N removal rate was calculated using the concentration change from 0 to 3 h. Descriptive statistics, including mean, standard deviation, minimum, median, and maximum values, were calculated from repeated tests for each inoculum.

MLVSS analysis and SNR calculation

MLVSS (mixed liquor volatile suspended solids) was analyzed to normalize differences in biomass among inocula. MLVSS represents the volatile fraction of suspended solids in mixed liquor and is commonly used as an indirect indicator of biologically active biomass in activated sludge and mixed microbial samples (APHA et al., 2017; Metcalf & Eddy, 2014).

Field-derived sludge and mixed inocula may differ in solids concentration, inorganic solids fraction, organic solids content, and microbial composition. Therefore, simple NH_3-N removal rates can be influenced by differences in biomass among inocula. In this study, the MLVSS-based specific nitrification rate (SNR) was calculated to compare ammonium removal activity per unit biomass.

SNR can be used as a biomass-normalized reaction rate for comparing nitrifying activity among different sludges or inocula. This is particularly useful for field-derived mixed microbial samples with different solids

concentrations and biomass levels (Grady et al., 2011; Ge et al., 2015; Blackburne et al., 2007). SNR was calculated as follows:

$$SNR = RNH_3\text{-}N / MLVSS$$

where SNR is the MLVSS-based specific nitrification rate (mg NH_3-N/g MLVSS/hr), RNH_3-N is the ammonium removal rate (mg NH_3-N/L/hr), and MLVSS is the volatile suspended solids concentration of the inoculum (g/L). The calculated SNR values were used to compare nitrifying activity among inocula and to interpret the microbial community analysis results.

Microbial community analysis

Microbial community analysis based on the 16S rRNA gene was used to compare the microbial community structures of the inocula. 16S rRNA gene sequencing is widely used to characterize bacterial communities in environmental samples, including activated sludge and biological wastewater treatment systems (Quast et al., 2013; Yoon et al., 2017; Knight et al., 2018).

The samples used for microbial community analysis corresponded to inocula A, B, and C used in the batch NH_3-N removal tests. The V3-V4 region of the bacterial 16S rRNA gene was targeted. This region is widely used for bacterial community analysis, and the 341F/805R universal primer set has been applied for taxonomic characterization of diverse bacterial groups (Klindworth et al., 2013).

Sequencing reads were processed after quality filtering, removal of non-target amplicons, and chimera sequence removal. Chimera removal is an essential step in 16S rRNA amplicon analysis to reduce erroneous sequences and improve taxonomic accuracy (Edgar et al., 2011; Pollock et al., 2018). High-quality reads were classified using a reference database, and the results obtained from the EzBioCloud microbiome analysis platform based on the PKSSU4.0 database were used. EzBioCloud provides taxonomically curated bacterial information based on 16S rRNA gene sequences and whole-genome assemblies (Yoon et al., 2017).

Operational taxonomic units (OTUs) were clustered using a 97% sequence similarity cutoff. This cutoff has traditionally been used as a species-level approximation in 16S rRNA-based bacterial community analysis (Stackebrandt and Goebel, 1994; Edgar, 2013). OTU number and alpha diversity indices, including ACE, Chao1, Shannon, and Simpson indices, were calculated to compare microbial diversity among inocula.

ACE and Chao1 indices were used to assess species richness, while the Shannon index was used to evaluate both richness and evenness. The Simpson index was used as an indicator related to dominance. These alpha diversity indices are commonly used to evaluate richness, evenness, and dominance in microbial communities (Knight et al., 2018; Pollock et al., 2018; Bokulich et al., 2020).

To evaluate sequencing depth, valid reads, Good's coverage, and rarefaction curves were examined. Good's coverage indicates the extent to which the sequencing data cover microbial diversity in a sample, while rarefaction curves show the relationship between sequencing depth and OTU detection (Pollock et al., 2018; Knight et al., 2018).

Microbial compositions were compared at the phylum and genus levels. Nitrification-related taxa, including *Nitrosomonas*, *Nitrosospira*, *Nitrospira*, and *Nitrobacter*, were specifically examined. *Nitrosomonas* and *Nitrosospira* were interpreted as AOB, while *Nitrospira* and *Nitrobacter* were interpreted as NOB. *Nitrosomonas* and *Nitrospira* have been repeatedly reported as major nitrogen transformation groups in wastewater nitrification processes (Ge et al., 2015; Daims et al., 2015; Gruber-Dorninger et al., 2015).

Because 16S rRNA-based community analysis provides relative abundance information, it does not directly rpresent actual metabolic activity, functional gene expression, or absolute cell abundance. Therefore, the relative abundance of nitrification-related taxa was interpreted together with the batch NH_3-N removal test and MLVSS-based SNR results rather than being treated as a direct indicator of nitrification performance (Knight et al., 2018; Bokulich et al., 2020).

Data analysis

Ammonium removal characteristics were compared using temporal changes in NH_3-N concentration, average NH_3-N removal rate, and MLVSS-based SNR. Removal rates and SNR values were summarized as mean, standard deviation, minimum, median, and maximum values for each inoculum.

Microbial community analysis results were first summarized using valid reads, Good's coverage, OTU number, species found, ACE, Chao1, Shannon, and Simpson indices to evaluate sequencing depth and alpha diversity. Relative abundances of dominant microbial groups were then compared at the phylum and genus levels. Because microbial groups such as Proteobacteria, Bacteroidetes, and Chloroflexi are involved in organic matter removal, nitrogen transformation, floc formation, and microbial interactions in activated sludge and biological treatment processes, major phylum- and genus-level compositions were used to interpret the community characteristics of each inoculum (Zhang et al., 2012; Saunders et al., 2016; Ju et al., 2014).

Finally, the relative abundance of nitrification-related taxa, especially *Nitrosomonas* and *Nitrospira*, was compared with MLVSS-based SNR to evaluate the relationship between microbial community structure and actual ammonium removal activity. Because 16S rRNA-based relative abundance does not directly represent reaction rate or metabolic activity, the primary focus of this study was whether the abundance of nitrification-related taxa

agreed with the functional activity indicator, SNR (Knight et al., 2018; Bokulich et al., 2020).

Results And Discussion

Ammonium removal characteristics of the inocula

Temporal changes in NH_3-N concentration were analyzed under batch conditions to compare ammonium removal activity among the three field-derived nitrifying inocula. Repeated test data were used for each inoculum, with 225 tests for inoculum A, 253 tests for inoculum B, and 141 tests for inoculum C. Batch activity tests can be used to compare substrate removal capacity and reaction rates of biological sludges or inocula, and nitrifying activity is commonly evaluated using changes in ammonium nitrogen concentration over time (Grady et al., 2011; APHA et al., 2017; Ge et al., 2015).

The average NH_3-N removal rates of inocula A, B, and C were 19.05 ± 3.83, 21.32 ± 9.15, and 26.23 ± 5.59 mg NH_3-N/L/hr, respectively. Based on the average removal rate, inoculum C showed the highest ammonium removal activity, followed by inoculum B and inoculum A. The median removal rate of inoculum C was also the highest at 27.33 mg NH_3-N/L/hr, while those of inocula A and B were 20.00 and 19.67 mg NH_3-N/L/hr, respectively. The ammonium removal rates and MLVSS-based SNR values are summarized in Table 2.

Table 2. Summary of NH_3-N removal rates and MLVSS-based SNR values for the inocula.

Inoculum	n	NH_3-N removal rate mean ± SD (mg NH_3-N/L/hr)	NH_3-N removal rate range (mg NH_3-N/L/hr)	MLVSS mean ± SD (mg/L)	SNR mean ± SD (mg NH_3-N/g MLVSS/hr)
A	225	19.05 ± 3.83	6.33-24.67	9,880 ± 2,043	1.99 ± 0.50
B	253	21.32 ± 9.15	6.00-47.67	8,459 ± 1,974	2.51 ± 0.78
C	141	26.23 ± 5.59	8.00-35.67	10,342 ± 2,597	2.62 ± 0.61

Inoculum A showed the lowest average NH_3-N removal rate but a relatively small standard deviation, indicating comparatively low variability among repeated tests. In contrast, inoculum B showed a higher average removal rate than inoculum A, but its removal rate ranged widely from 6.00 to 47.67 mg NH_3-N/L/hr. This suggests that ammonium removal activity in inoculum B may have varied considerably depending on test period, cultivation status, or inoculum composition. Nitrifying microorganisms are generally slower growing than heterotrophs and are sensitive to operational conditions such as temperature, dissolved oxygen, pH, alkalinity, ammonium loading, and sludge retention time (Anthonisen et al., 1976; Grady et al., 2011; Metcalf & Eddy, 2014).

Inoculum C exhibited the highest mean and median removal rates, indicating the strongest NH_3-N removal activity under the batch conditions used in this study. However, because this study evaluated ammonium removal mainly based on NH_3-N decrease, the observed removal cannot be interpreted as complete nitrification without caution. In a typical nitrification pathway, NH_3-N removal should be accompanied by the formation of NO_2^--N and NO_3^--N. Therefore, oxidized nitrogen species should be analyzed to more clearly confirm the nitrification pathway (Henze et al., 2008; Metcalf & Eddy, 2014; Ge et al., 2015).

Comparison of MLVSS-based specific nitrification rate

Because simple NH_3-N removal rates can be affected by the amount of biomass contained in each inoculum, MLVSS-based SNR was calculated to compare ammonium removal activity per unit biomass. MLVSS is commonly used as an indirect indicator of biologically active biomass in activated sludge or mixed liquor and can serve as a biomass normalization factor when comparing reaction rates among different sludges or inocula (APHA et al., 2017; Metcalf & Eddy, 2014).

The average MLVSS concentrations of inocula A, B, and C were 9,880 ± 2,043, 8,459 ± 1,974, and 10,342 ± 2,597 mg/L, respectively. Inoculum C had the highest average MLVSS, while inoculum B had the lowest. Therefore, comparison based solely on NH_3-N removal rates could include the effect of biomass differences among inocula. For this reason, MLVSS-based SNR was used as the main activity indicator in this study.

MLVSS-based SNR values of inocula A, B, and C were 1.99 ± 0.50, 2.51 ± 0.78, and 2.62 ± 0.61 mg NH_3-N/g MLVSS/hr, respectively. Inoculum C also showed the highest SNR after biomass normalization, followed by inoculum B and inoculum A. Detailed descriptive statistics of MLVSS-based SNR are presented in Table 3. These trends are illustrated in Fig. 1.

Table 3. Descriptive statistics of MLVSS-based SNR for the inocula

Inoculum	n	Mean	SD	Minimum	Median	Maximum
A	225	1.99	0.50	0.62	1.97	3.74
B	253	2.51	0.78	0.66	2.45	4.59
C	141	2.62	0.61	0.67	2.67	3.81

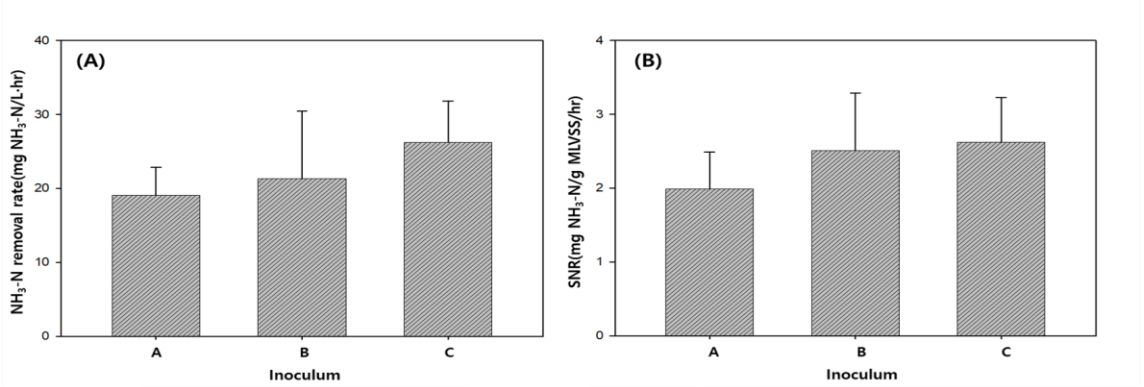

Fig. 1: Comparison of ammonium removal activity among inocula A, B, and C. (A) NH_3-N removal rate and (B) MLVSS-based specific nitrification rate (SNR).

Inoculum B exhibited the widest SNR range, from 0.66 to 4.59 mg NH_3-N/g MLVSS/hr. This suggests that nitrifying activity in inoculum B was not fixed at a constant level, but may have varied according to the composition or cultivation history of the mixed microbial community. In mixed microbial communities, functional guilds may be selectively activated or suppressed depending on substrate conditions and operational environments, and nitrifiers are particularly sensitive functional groups (Ge et al., 2015; Daims et al., 2015).

Inoculum A showed the lowest mean SNR but a relatively narrow range of variation, indicating a more stable but lower activity level. In contrast, inoculum C showed the highest mean and median SNR, suggesting that it maintained high ammonium removal activity even after biomass normalization. These results indicate that SNR should be considered together with simple concentration-based removal rates when evaluating the nitrification performance of field-derived inocula (Grady et al., 2011; APHA et al., 2017).

Microbial diversity and community structure of the inocula

16S rRNA gene-based microbial community analysis showed that valid reads for inocula A, B, and C were 25,481, 22,185, and 21,720 reads, respectively. Good's coverage values were 98.7%, 99.6%, and 99.1%, respectively, indicating that the sequencing depth was sufficient to characterize the major microbial communities of each inoculum. Good's coverage and rarefaction curves are commonly used to evaluate whether sequencing depth is adequate for microbial community analysis (Knight et al., 2018; Pollock et al., 2018; Bokulich et al., 2020). Sequencing statistics and alpha diversity indices are summarized in Table 4.

Table 4. Sequencing statistics and alpha diversity indices of the inocula.

Inoculum	Valid reads	Species found	OTUs	Good's coverage	ACE	Chao1	Shannon	Simpson
A	25,481	881	1,690	98.7%	1,935.4	1,831.8	5.807	0.012
B	22,185	236	487	99.6%	550.4	519.9	3.436	0.080
C	21,720	403	785	99.1%	924.9	866.8	3.867	0.074

Alpha diversity analysis showed that inoculum A had the highest OTU richness (1,690 OTUs) and Shannon index (5.807) among the three inocula. In contrast, inoculum B had the lowest diversity, with 487 OTUs and a Shannon index of 3.436. Inoculum C showed an intermediate level of diversity, with 785 OTUs and a Shannon index of 3.867. The Shannon index reflects both richness and evenness, with higher values indicating more diverse and evenly distributed communities (Knight et al., 2018; Pollock et al., 2018). The Simpson index was lowest in inoculum A (0.012) and higher in inocula B and C (0.080 and 0.074, respectively), suggesting that inocula B and C were more strongly dominated by a limited number of taxa than inoculum A.

At the phylum level, inoculum A showed a community structure clearly distinct from those of inocula B and C. Proteobacteria was the most dominant phylum in inoculum A, accounting for 61.37%, followed by Bacteroidetes (11.18%), Acidobacteria (5.86%), Planctomycetes (5.09%), and Chloroflexi (2.81%). The nitrification-related phylum Nitrospirae was also detected at 1.55%. In activated sludge ecosystems, Proteobacteria, Bacteroidetes, Chloroflexi, and Actinobacteria have been reported as major microbial groups associated with organic matter removal, nitrogen transformation, floc formation, and other process functions (Zhang et al., 2012; Saunders et al., 2016; Ju et al., 2014).

In contrast, Bacteroidetes, Proteobacteria, and Chlorobi were the dominant phyla in inocula B and C. In inoculum B, Bacteroidetes, Proteobacteria, Chlorobi, and Chloroflexi accounted for 36.01%, 26.22%, 18.28%, and 5.81%, respectively. In inoculum C, Bacteroidetes, Proteobacteria, Chlorobi, and Chloroflexi accounted for 38.95%, 28.19%, 15.18%, and 6.63%, respectively, showing a community structure highly similar to that of inoculum B. The dominant phylum-level compositions of the inocula are shown in Table 5.

Table 5. Major phylum-level relative abundances of the inocula.

Phylum	Inoculum A (%)	Inoculum B (%)	Inoculum C (%)
Proteobacteria	61.37	26.22	28.19
Bacteroidetes	11.18	36.01	38.95
Chlorobi	1.40	18.28	15.18
Chloroflexi	2.81	5.81	6.63
Acidobacteria	5.86	0.49	1.81
Planctomycetes	5.09	-	0.60
Verrucomicrobia	2.61	4.62	3.71
Nitrospirae	1.55	-	-

Bacteroidetes and Chloroflexi have been associated with polymeric organic matter degradation, extracellular polymeric substance utilization, and floc or biofilm structure in activated sludge and biofilm systems (Kindaichi et al., 2004; Saunders et al., 2016). In mixed nitrifying communities, heterotrophic populations can coexist with nitrifiers by utilizing soluble microbial products (SMP) or biomass-derived organics released during nitrifier growth (Kindaichi et al., 2004; Ni et al., 2011). Therefore, the high abundance of Bacteroidetes and Chlorobi in inocula B and C suggests that these inocula had stronger mixed microbial and organic matter-degrading characteristics rather than being dominated solely by nitrifying taxa.

At the genus level, clear differences among the inocula were also observed. In inoculum A, *Methylotenera* (7.12%), CBTK_g (7.08%), AB186832_g (5.63%), and PAC002280_g (4.89%) were among the dominant genera, and the nitrification-related genera *Nitrosomonas* and *Nitrospira* were detected at 1.90% and 1.55%, respectively. In inoculum B, FJ947131_g (19.21%), Ignavibacteriaceae_uc (12.22%), AJ853611_g (11.15%), *Caldimonas* (9.21%), and *Thauera* (5.60%) were dominant. Inoculum C showed a similar genus-level pattern to inoculum B, with FJ947131_g (21.79%), Ignavibacteriaceae_uc (11.39%), AJ853611_g (10.39%), and *Caldimonas* (10.08%) as dominant taxa. The dominant genus-level compositions are summarized in Table 6, and the community composition is illustrated in Fig. 2.

Table 6. Major genus-level relative abundances of the inocula.

Genus A	A (%)	Genus B	B (%)	Genus C	C (%)
Methylotenera	7.12	FJ947131_g	19.21	FJ947131_g	21.79
CBTK_g	7.08	Ignavibacteriaceae_uc	12.22	Ignavibacteriaceae_uc	11.39
AB186832_g	5.63	AJ853611_g	11.15	AJ853611_g	10.39
PAC002280_g	4.89	*Caldimonas*	9.21	*Caldimonas*	10.08
U46750_g	1.98	JN087872_g	5.68	U46750_g	3.43
Nitrosomonas	1.90	*Thauera*	5.60	JN087872_g	3.40
Nitrospira	1.55	U46750_g	4.42	CBTK_g	3.16

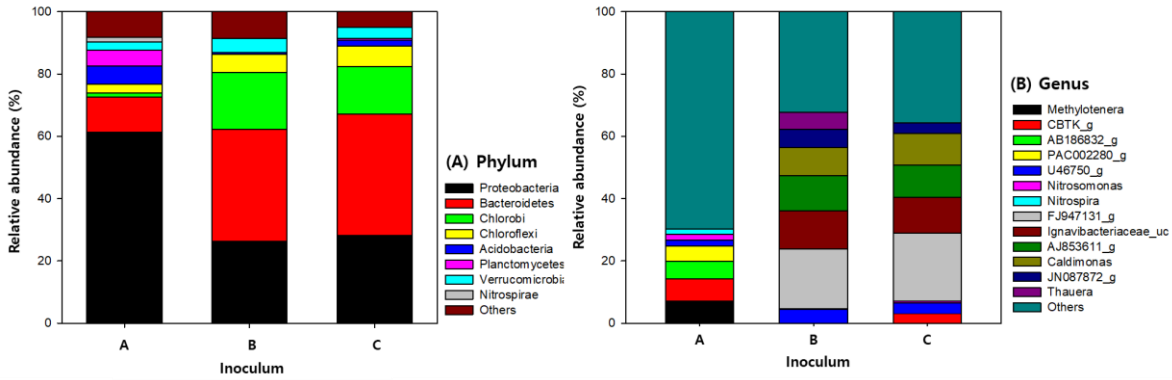

Fig. 2. Taxonomic composition of microbial communities in inocula A, B, and C based on 16S rRNA gene sequencing. (A) Phylum-level composition. (B) Genus-level composition.

Overall, genus-level results indicate that inoculum A had a detectable nitrification-related community, with both *Nitrosomonas* and *Nitrospira* present at relatively higher abundances. *Nitrosomonas* is a representative AOB, while *Nitrospira* is generally recognized as an important NOB involved in nitrite oxidation; both groups are important in biological nitrification (Daims et al., 2015; Ge et al., 2015; Gruber-Dorninger et al., 2015). In contrast, inocula B and C showed low relative abundances of these nitrification-related genera, suggesting that their nitrification potential could not be inferred solely from the presence of typical nitrifying taxa.

Relationship between nitrification-related taxa and SNR

The relative abundance of nitrification-related taxa was compared with MLVSS-based SNR to examine the relationship between microbial community structure and actual ammonium removal activity. In this study, *Nitrosomonas* was selected as the main AOB and *Nitrospira* as the main NOB for comparison. These taxa have been repeatedly reported as key nitrogen transformation groups in wastewater nitrification processes (Daims et al., 2015; Ge et al., 2015; Gruber-Dorninger et al., 2015).

In inoculum A, *Nitrosomonas* and *Nitrospira* were detected at 1.90% and 1.55%, respectively, giving a combined relative abundance of 3.46%, the highest among the three inocula. In inoculum B, *Nitrosomonas* was detected at 0.29%, while *Nitrospira* was not detected among the major taxa, resulting in a combined abundance of 0.29%. In inoculum C, *Nitrosomonas* and *Nitrospira* were detected at 0.53% and 0.07%, respectively, with a combined abundance of 0.60%. The relationship between nitrification-related taxa and SNR is summarized in Table 7 and illustrated in Fig. 3.

Table 7. Relative abundance of nitrification-related taxa and MLVSS-based SNR.

Inoculum	*Nitrosomonas* (%)	*Nitrospira* (%)	Combined nitrification-related taxa (%)	Mean SNR (mg NH_3-N/g MLVSS/hr)
A	1.90	1.55	3.46	1.99
B	0.29	0.00	0.29	2.51
C	0.53	0.07	0.60	2.62

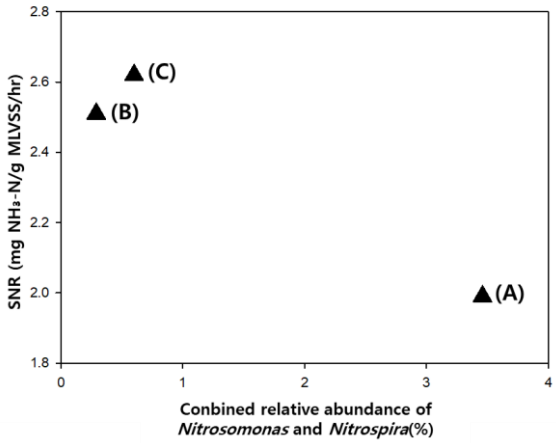

Fig. 3. Relationship between the combined relative abundance of *Nitrosomonas* and *Nitrospira* and MLVSS-based specific nitrification rate (SNR) in inocula A, B, and C.

Based solely on the relative abundance of nitrification-related taxa, inoculum A would be expected to have the highest nitrification potential. Inoculum A contained both *Nitrosomonas* and *Nitrospira* at relatively higher abundances, suggesting the presence of functional groups involved in ammonia oxidation and nitrite oxidation. However, the actual MLVSS-based SNR of inoculum A was 1.99 mg NH_3-N/g MLVSS/hr, the lowest among the three inocula, whereas inocula B and C showed higher SNR values of 2.51 and 2.62 mg NH_3-N/g MLVSS/hr, respectively.

In particular, inoculum C showed the highest NH_3-N removal rate and SNR despite having only 0.60% combined abundance of nitrification-related taxa. This indicates that the relative abundance of nitrification-related microorganisms obtained by 16S rRNA gene sequencing does not directly represent actual ammonium removal activity. Although 16S rRNA-based community analysis is useful for determining relative microbial composition, it does not directly quantify absolute abundance, physiological activity, functional gene expression, or process-level reaction rates (Knight et al., 2018; Pollock et al., 2018; Bokulich et al., 2020).

This discrepancy may be explained by several factors. First, nitrifying microorganisms present at low relative abundance may still exhibit high cell-specific activity, whereas taxa present at higher relative abundance may have lower physiological activity at the time of testing. Second, 16S rRNA-based results represent relative abundance and do not directly reflect total bacterial abundance or absolute abundance of nitrifiers in each inoculum (Knight et al., 2018; Bokulich et al., 2020). Third, storage and cultivation history, adaptation to ammonium substrate, pH, alkalinity, and dissolved oxygen conditions may have influenced the observed NH_3-N removal rates (Anthonisen et al., 1976; Blackburne et al., 2007; Ge et al., 2015). Fourth, microbial groups not classified as conventional nitrifiers may have indirectly contributed to nitrifying activity by affecting organic matter degradation, microbial interactions, and oxygen or substrate availability (Kindaichi et al., 2004; Ni et al., 2011).

The fact that inocula B and C showed community structures similar to each other but higher SNR values than inoculum A is also noteworthy. At the phylum level, Bacteroidetes and Chlorobi were relatively abundant in inocula B and C, and the genus-level dominant taxa were also similar. These patterns indicate that inocula B and C had microbial ecological characteristics distinct from those of inoculum A. In particular, inoculum C showed the highest SNR despite a low relative abundance of typical nitrification-related taxa, suggesting that the actual nitrification performance of field-derived inocula may be strongly influenced by cultivation history and physiological status rather than by the relative abundance of typical nitrifiers alone.

Overall, these results demonstrate that both microbial community analysis and functional activity tests are required for evaluating field-derived nitrifying inocula. The relative abundance of nitrification-related taxa can provide useful information on biological composition and nitrification potential, but actual NH_3-N removal activity should be assessed together with reaction rate-based indicators such as MLVSS-based SNR. This approach is consistent with current recommendations that microbial community data should be interpreted together with process-level functional data (Knight et al., 2018; Bokulich et al., 2020).

Because this study evaluated ammonium removal mainly based on NH_3-N decrease, future work should include NO_2^--N and NO_3^--N production to more clearly confirm the completeness of nitrification. In addition, DO, pH, alkalinity, and temperature data, together with qPCR, RT-qPCR, or functional gene analyses, would help clarify the relationship between nitrification-related relative abundance and actual activity (Daims et al., 2015; Ge et al., 2015; Bokulich et al., 2020).

Conclusion

This study evaluated ammonium removal activity and microbial community structure in three field-derived nitrifying inocula by combining batch NH_3-N removal tests, 16S rRNA gene-based microbial community analysis, and MLVSS-based specific nitrification rate (SNR) assessment.

The average NH_3-N removal rates of inocula A, B, and C were 19.05, 21.32, and 26.23 mg NH_3-N/L/hr, respectively, and the corresponding MLVSS-based SNR values were 1.99, 2.51, and 2.62 mg NH_3-N/g MLVSS/hr. Inoculum C showed the highest ammonium removal activity even after biomass normalization. Because nitrification activity can be affected by pH, alkalinity, dissolved oxygen, ammonium loading, and microbial physiological status, the observed activity differences may reflect differences in origin and cultivation history among the inocula (Anthonisen et al., 1976; Ge et al., 2015).

Microbial community analysis showed that inoculum A had the highest combined relative abundance of *Nitrosomonas* and *Nitrospira* (3.46%), but the lowest SNR. In contrast, inoculum C showed the highest SNR despite a low combined abundance of nitrification-related taxa (0.60%). These results indicate that 16S rRNA-based relative abundance alone cannot directly determine actual ammonium removal activity, consistent with previous reports that this method does not directly represent metabolic activity or functional gene expression (Knight et al., 2018; Bokulich et al., 2020).

Therefore, performance evaluation of field-derived nitrifying inocula should combine microbial community analysis with batch NH_3-N removal tests and MLVSS-based SNR assessment. Future studies should include NO_2^--N, NO_3^--N, DO, pH, and alkalinity data to more clearly interpret nitrification completeness and inoculum-specific activity differences.

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Conflict Of Interest

The authors declare that there is no conflict of interest regarding this study.

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