Referee #1

1) Lack of scientific novelty. Quite a few studies have already reported that the manure nitrogen or ammonium becomes isotopically enriched during compost and this enrichment has been attributed to ammonia volatilization and nitrogen transformation. They only novel point is that the current study found that the enrichment was stronger in the top zone than in the side and core zones.

Thank you very much for your comment. There have been only a few published studies on isotopically enriched ammonium during manure composting, and none of them have focused on the individual zones of the piles. The 15N values of the samples from different zones enabled us to interpret how nitrogen transformation occurs between pile turnings. To our knowledge this is the first report focusing on this topic. Therefore, we believe that our manuscript has significant novelty and could provide insight into the processes of manure composting and its nitrogen transformation.

Nevertheless, the manuscript is largely based on qualitative analyses while the underlying mechanisms was not presented, i.e., the mechanisms underlying the decrease in nitrous oxide emission (this should be a major objective of this study according to the abstract) following bulking agent use or the greater enrichment in 15N in the top zone of the manure piles (this should be another major objective of this study according to the abstract and the title). For the compost piles with bulking agent, the inside temperature reached more than 60°C. Normally under such high temperature, nitrification and denitrification or the microbial activities are much low although these processes may take place in some geothermal ecosystems. The decreased emission of nitrous oxide after bulk agent integration may due to decreased nitrification and denitrification. But this needs experiment evidence.

Thank you very much for this helpful comment. We fully agree with you that the temperature is a possible explanation for the mitigation of N2O emission. A previous report suggested that the optimum temperature for nitrification or denitrification was that under a mesophilic condition (Willers et al., 1998), and another report showed that the N2O production rate can be higher under a thermophilic than under a mesophilic condition (Benoit et al., 2015). The high heterogeneity of temperature in different pile zones makes it very difficult to analyze such results. As we have already stated, the mitigation of N2O emission cannot be explained by the present dataset. We added only a few sentences on N2O emission because we did not provide data on N2O in this manuscript. However, we found many interesting phenomena in terms of 15NH4, and therefore we focused on the nitrogen transformation process between the pile turnings.

2) Mistake in methodology. An isotopic mass balance equation is presented as equation (7). The prerequisite to use an isotopic mass balance model is that the isotopic masses in both sides of the equation are balanced. In terms of manure compost, large nitrogen loss (e.g., ammonia volatilization) is usually taking place. For equation (7), ammonia volatilization should at least be included.

Thank you very much for this helpful comment. We agree with you that the isotopic masses on both sides of the equation should be balanced. However, here we cannot put the ammonia volatilization in the equation because we did not measure the 15NH4 of the volatilized ammonia. However, to truly understand this phenomenon we will need to analyze 15NH4 data obtained using the current analysis methods. As a result, the large ammonia volatilization could be one of the major obstacles to a clear explanation of the phenomenon. We believe that our present data suggest some interesting hypotheses about the sequential events between the pile turnings, as stated in the conclusion section.

3) Understandability, clarity and concise. Throughout the manuscript, there are lots of grammar issues which make the paper hard to understand.

Thank you very much for your comment. The original manuscript was already edited by a professional English editing service. We have had the revised manuscript entirely re-edited by this service, and we have attached a certification of this work.
The experiment needs to be more clearly described. In addition, the terms need to be consistent. For example, according to line 19 in page 7580, samples were collected “just before each turning”. However, in the following sections or the figures, it seems that samples were collected “just after the turning”.

Thank you very much for your comment. We fully agree with you that this can cause confusion for the readers. Actually, samples in each zone (pile top, side and core) should be taken BEFORE each turning because the turnings increase homogenization. Therefore we collected samples from each zone BEFORE each turning. We also collected the homogenized samples AFTER each turning, because the homogenized samples were also needed to understand the changes in the compost piles. We thus collected the samples both before and after the turning events.

For another, in line 1-2 of page 7581, “Total N was measured using raw samples by the Kjeldahl method. The C/N ratio was determined using a C/N analyzer (vario MAX CNS; Elementar, Germany)”. So total N was measured using two methods?

Yes, we measured total N in two different ways. A C/N analyzer can miss the ammonium nitrogen, so we considered that it would be best to cross check this parameter using two approaches. We do not believe that this constitutes a limitation of the study design.

In summary, the manuscript needs substantially improvement. The manuscript was rewritten and, we believe, substantially improved through the help of your insightful comments.
Isotopically enriched ammonium shows high nitrogen turnover in the pile top zone of dairy manure compost

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Abstract

δ15N-NH4+ levels of dairy manure compost piles with and without bulking agent (10% w/w) were compared to understand the effects of the use of bulking agent on nitrogen conversion during manure composting. The amounts of δ15N-NH4+ in each of three pile zones (top, side and core) were also compared. At the end of the process, piles with bulking agent showed significantly higher δ15N values (17.7±1.3‰) than piles without bulking agent (11.8±0.9‰), reflecting the significantly higher nitrogen conversion and NH3 loss in the former. The samples from the top zone, especially in the piles with bulking agent, showed very high NH4+ concentrations with significantly high 15N (δ15N: 12.7-29.8 ‰ ) values, indicating that extremely high nitrogen conversion, nitrification-denitrification activity of the microbes and NH3 volatilization occurred in this zone.
1. Introduction

Nitrogen is one of the most abundant major elements in the Earth’s atmosphere. There are two major anthropogenic activities affecting the global nitrogen cycle: energy production and food production (Galloway et al., 2004). Because nitrogen is one of the most important elements for plant nutrition, huge amounts of industrially fixed nitrogen are used as fertilizer to improve the productivity of agricultural crops (Tilman et al., 2002). Current anthropogenic nitrogen input to the environment (160 Tg per year) is already greater than the input from natural biological fixation (110 Tg) on land or in the ocean (140 Tg) (Gruber & Galloway, 2008), and the significance of agricultural nitrogen input on the global nitrogen cycle is expected to increase along with the nutritional needs of a growing population. In the livestock production industry, livestock intake organic nitrogen from their feed, and produce large quantities of organic nitrogen in the form of manure, a byproduct and potential resource which must be handled appropriately to protect the environment (Sharpley et al., 1998). Most of this manure is used as organic fertilizer for efficient nutrient cycling, and thus a proper understanding of nitrogen flow in the manure management system is critically important.

The nitrogen contained in dairy manure exists mostly as organic nitrogen or NH$_4^+$.

Through the composting process, the heat production by degradation of organic matter leads to a significant loss of nitrogen into the atmosphere as gaseous ammonia (NH$_3$) (Dämmgen & Hutchings, 2008). Nitrifiers and other families of microorganisms in the manure also convert this nitrogen as nitrite (NO$_2^-$) or nitrate (NO$_3^-$), and both nitrifiers and denitrifiers can use them as electron acceptors. They reduce these nitrogen oxides into dinitrogen (N$_2$) and return them to the atmosphere in a process called denitrification (Zumft, 1997). Nitrous oxide (N$_2$O), a greenhouse gas, is emitted through the nitrogen conversion in the composting process (Sommer et al., 2009). Because it is known that N$_2$O has very strong greenhouse effects (298-fold greater than the greenhouse effects of CO$_2$ over a 100-year time horizon; IPCC, 2007), and N$_2$O is also known to contribute to ozone layer destruction (Ravishankara et al., 2009), these gas emissions must be mitigated.

With respect to this N$_2$O emission, our previous studies clarified that nitrification
occurs in the compost surface, and compost turning (mixing by machines) and subsequent denitrification can be major sources of N\textsubscript{2}O (Maeda et al., 2013b; Maeda et al., 2010b). Also, we have shown that the appropriate use of bulking agents can reduce the N\textsubscript{2}O emission significantly (Maeda et al., 2013a). However, the mechanism of this N\textsubscript{2}O mitigation is largely unknown. Because bulking agents are generally used to increase the supply of oxygen to the compost piles (Jolanun & Towprayoon, 2010), it is expected that the increase in oxygen increases nitrification and the subsequent N\textsubscript{2}O production.

To solve this contradiction, we compared the level of $\delta^{15}$N-NH\textsubscript{4}+ in these composts, because this parameter can be used to track the level of reaction involving NH\textsubscript{4}+ in the environment (Brooks et al., 1989; Garten Jr, 1992; Yeatman et al., 2001). Because it has already been established that the NO\textsubscript{x} accumulation and the bacterial communities are different in different regions of the pile (Maeda et al., 2010a), we sampled from both the compost side and core independently, and surveyed them into the $\delta^{15}$N-NH\textsubscript{4}+ analysis.

2. Materials and Methods

2.1. Composting experiment

The composting experiment was performed three times at the Hokkaido Agricultural Research Center (Sapporo City, Hokkaido): once from 27 May through 21 July in 2010 (Run 1), once from 15 September through 10 November in 2010 (Run 2) and once from 19 May through 14 July in 2011 (Run 3). The cows were fed orchard grass silage and corn silage, oat hay, alfalfa hay, beet pulp and two types of concentrate mixtures to meet their digestible energy requirements, as recommended by the Japanese Feeding Standard for Dairy Cattle. Lactating Holstein cow excrement and dried grass (Orchard grass; Dactylis glomerata) were used in this study to make the compost.

About 4 t of dairy cow excrement and 400 kg of dried grass were mixed to form the treatment pile (pile 1), while the control pile (pile 2) consisted of dairy cow excrement alone. The compost was piled up on a waterproof concrete floor, and turned once every two weeks with a front loader and manure spreader. Each pile had a volume of 7.5 m\textsuperscript{3} with pile dimensions of 4 m in diameter and 1.8 m in height at the start of the
experiment. The temperatures of the compost piles and the ambient air were measured hourly using a Thermo Recorder RTW-30S (Espec, Japan).

2.2. Chemical analysis of the compost

Fresh samples (About 1 kg) were taken from each zone (the pile top, side, and core) just before each turning. Samples were also taken just after each turning, at the start and the end of the three composting experiments. Samples were homogenized and fresh subsamples were used to measure total solids, volatile solids, inorganic-N, pH and electrical conductivity, or stored at -20°C for total nitrogen determination. Total solids (TS) were measured after drying the samples overnight at 105°C, and dried samples were powdered and used for C/N ratio determination. Volatile solids (VS) were measured after the samples were processed at 600°C for 1 h. Total N was measured using raw samples by the Kjeldahl method. The C/N ratio was determined using a C/N analyzer (vario MAX CNS; Elementar, Germany).

To measure inorganic-N, pH and electrical conductivity, 5 g of fresh compost was placed into a 50 ml polypropylene tube with 40 ml of deionized water, then shaken (200 rpm, 30 minutes) and centrifuged (3,000 g, 20 minutes). The supernatant was collected and NH₄⁺, NO₂⁻-N and NO₃⁻-N were measured using ion chromatography (ICS-1600; Dionex, USA); pH and electrical conductivity (EC) were determined with calibrated electrodes (Horiba, Japan).

2.3. Determination of δ¹⁵N-NH₄⁺ levels and Rayleigh plot analysis

The amount of δ¹⁵N-NH₄⁺ in the extracted samples or trapped NH₃ samples was determined by the diffusion method (Holmes et al., 1998).

One cm diameter GF/D filters (Whatman, UK) were cut into four pieces, acidified with 20 µl H₂PO₄ (0.02 mM) and sandwiched between 2.5 cm diameter 10 mm pore-size Teflon membranes (Millipore, USA). These filter packs were used as an ammonium trap in the samples. Ten ml of the NH₄⁺-N samples (50 µg-N) was placed in 15 ml tubes and 0.5 g of NaCl (ashed at 450°C for 8 h) was added. Then a single filter pack was added to the 15 ml tube, and 0.03 g MgO (ashed at 450°C for 8 h) was added to convert
NH$_4^+$ in the samples into NH$_3$. The 15 ml tubes were incubated at 40°C for 2 weeks
with stirring at 200 rpm. After incubation, the filter pack was removed from the tubes
and dried in a desiccator for 2 days. The dried filter was then recovered and placed in a
tin cup. The tin cup containing the filter was then analyzed by an elemental analyzer
(EA1110, CE Instruments, Ltd., Wigan, UK) coupled with an isotope ratio mass
spectrometer (MAT252; ThermoFisher Scientific KK, Yokohama, Japan) to quantify
δ$^{15}$N-NH$_4^+$ in the samples. Calibration was conducted with IAEA-N1 and IAEA-N$_2$
(NH$_4$SO$_4$), and the precision (1σ) was better than 0.2‰. The δ$^{15}$N of samples was
expressed in parts per thousand deviations from the atmospheric N$_2$ as defined by the
following equation:
\[
\delta^{15}\text{N} \, (\text{‰}) = \left(\frac{R_{\text{sample}}}{R_{\text{standard}}} - 1\right) \times 1000, \quad (1)
\]
where $R_{\text{sample}}$ and $R_{\text{standard}}$ are the $^{15}$N/$^{14}$N ratios of samples and the atmospheric N$_2$,
respectively. Isotopic fractionation factor $\alpha$ was expressed as
\[
\alpha = \frac{R_{B}}{R_{A}}, \quad (2)
\]
where $R_{A}$ and $R_{B}$ are the isotopic ratio of phase A and B, respectively.
Isotopic fractionation can also be described by the enrichment factor $\varepsilon$, which describes
the enrichment of the product relative to that of the substrate, and which is also
expressed per mil (‰).
\[
\varepsilon = (\alpha - 1) \times 1000 \quad (3)
\]
The evolution of the isotopic composition is described by a Rayleigh equation with a
fractionation factor as follows for $^{15}$N:
\[
\frac{R}{R_0} = \left(1 + 10^{-3} \delta^{15}\text{N}\right) / \left(1 + 10^{-3} \delta^{15}\text{N}_0\right) = \left[\left[\text{NH}_4^+\right] / \left[\text{NH}_4^+\right]_0\right]^{\alpha^{-1}}, \quad (4)
\]
where $R$ and $R_0$ are the isotope ratio of samples just before the turning and of the
samples just after the previous turning. Since the piles were homogenized at each
turning event, the amount of ammonium in a sample just after the previous turning
event was taken as the “initial” ammonium.” $\delta^{15}$N and $\delta^{15}$N$_0$ are the respective δ values
for the each NH$_4^+$. $\left[\text{NH}_4^+\right]_0$ and $\left[\text{NH}_4^+\right]$ are the ammonium concentration of the samples
just after the previous turning event and the samples just before the subsequent turning
event, respectively. Using the approximation of $\ln(1+x) \cong x$ with $x \ll 1$, the relationship
between the difference of $\delta^{15}$N values between pile turnings and the reaction rate of the
substrate was obtained from equations (3) and (4) as follows:
\[
\delta^{15}\text{N} - \delta^{15}\text{N}_0 = \varepsilon \ln (1-f), \tag{5}
\]
where \( f \) is the amount of reacted ammonium between the turning events, defined as \( f = (1 - [\text{NH}_4^+] / [\text{NH}_4^+]_0) \).

2.4 Keeling plot analysis

The basis of the Keeling plot method is conservation of mass. The ammonium concentration of each location of the pile before the pile turnings can be expressed as
\[
c_b = c_a + c_s, \tag{6}
\]
where \( c_b, c_a, \) and \( c_s \) are the ammonium concentration measured in each location of the pile just before the turning, the ammonium concentration just after the previous pile turning, and the additional concentration component produced by the source, respectively. Given conservation of mass, we have
\[
\delta^{15}\text{N}_b c_b = \delta^{15}\text{N}_a c_a + \delta^{15}\text{N}_s c_s, \tag{7}
\]
where \( \delta^{15}\text{N} \) represents the nitrogen isotope ratio of the ammonium in each sample. By combining equations (6) and (7), we arrive at
\[
\delta^{15}\text{N}_b = c_a (\delta^{15}\text{N}_a - \delta^{15}\text{N}_s) (1/c_b) + \delta^{15}\text{N}_s. \tag{8}
\]

2.5 Statistical analysis

The chemical component data were analyzed by ANOVA using the general linear model procedure described by SAS (SAS Institute, 2001). Tukey’s multiple range comparison tests were used to separate the means. A value of \( P<0.05 \) was considered statistically significant.
3. Results

3.1. Composting experiments

The temperature of the piles with bulking agent (10% w/w) exceeded 60°C throughout the entire experiment (Fig. S1), while the piles without bulking agent showed significantly lower temperature (below 50°C). The initial weight was 4,543±137 kg in the piles with bulking agent and 4,136±124 kg in those without bulking agent, and the final turning these values dropped significantly to 1,413±99 kg and 1,960±291 kg, respectively (Table 1). The total solids of the piles with and without bulking agent after the composting process were 43.8±11.3% and 23.5±1.8%, respectively. The C/N ratios of the piles with and without bulking agent dropped significantly from 23.8±3.3 to 12.8±0.8 and from 22.8±1.2 to 15.6±2.6, respectively. These parameters all indicate that the organic matter degradation rate was much higher in the piles with bulking agent.

Pile top samples (2.8-7.4 mg-N/g TS; pile 1) and core samples (1.0-14.6 mg-N/g TS; pile 1) contained higher ammonium concentrations than the pile side samples (0.1-1.8 mg-N/g TS; pile 1) (Fig. 1A-C). High NO₂⁻ accumulation was also observed in the pile top samples (0.03-3.8 mg-N/g TS; pile 1), but not in the pile core samples. NO₃⁻ was also detected in the pile top and side samples, but the concentrations were low (0-0.29 mg-N/g TS; pile 1). Although similar trends were observed for pile 2 (Fig. 1D-F), the amount of NH₄⁺ in the top region of pile 2 was generally lower (0.15-2.2 mg-N/g TS) than that in pile 1. Accumulations of NO₂⁻ (0.08-2.2 mg-N/g TS) and NO₃⁻ (0.02-0.7 mg-N/g TS) were also detected in both the top and side samples of pile 2.

3.2. δ¹⁵N of NH₄⁺ in mixed samples

δ¹⁵N-NH₄⁺ values of the mixed samples just after the pile turning events are shown in Fig. 2. All compost runs showed a similar tendency. The initial δ¹⁵N-NH₄⁺ values were 5.8±2.5‰ and 7.4±3.8‰ for the piles with and without bulking agent, respectively. These values dropped slightly between weeks 0 and 2, to 4.4±2.8‰ and 6.1±2.3‰ for piles with and without bulking agent in all runs, although these changes were not statistically significant. After week 4, these values increased significantly, and at the
end of the experiments they reached 17.7±1.3‰ and 11.8±0.9‰ for the piles with and
without bulking agent, respectively. Also, the piles with bulking agent showed higher
values than the piles without bulking agent, and this difference was statistically
significant.

\[ \delta^{15}N-\text{NH}_4^+ \] values were also determined for the pile top, side and core samples, and
are shown in Fig. 3. The data were expressed as the difference from the mixed samples
taken after the pile homogenization. The values for the pile top samples (9.6-22.5‰)
were higher than those for the side samples (9.2-11.3‰) in both the piles with and
without bulking agent. The core samples showed low \[ \delta^{15}N-\text{NH}_4^+ \] values in week 2
(1.7±1.0‰ and 4.7±2.0‰ for the piles with and without bulking agent, respectively),
reflecting the newly formed “light” \( \text{NH}_4^+ \)-N, which was supplied by the degradation of
organic-N in the manure. On the other hand, the heaviest \( \text{NH}_4^+ \) (25.4±6.8‰) was also
observed in the pile core samples at the end of the experimental period. This
phenomenon was observed only from the piles with bulking agent.
4. Discussion

The stable isotope $\delta^{15}$N value of NH$_4^+$ in dairy manure compost with and without bulking agent was studied to clarify the mechanism of the significant N$_2$O mitigation achieved using a bulking agent. A decrease in the $\delta^{15}$N value of NH$_4^+$ in the first two weeks of composting was observed in both piles, although this result was not observed in the previous study (Kim et al., 2008). The discrepancy can be attributed to the supply, in the present experiments, of newly formed “light” NH$_4^+$ by the ammonification of organic N, which has a low value ($\alpha=\sim1.000$) of isotopic fractionation (Högberg, 1997).

The weight decrease in the piles with bulking agent (4,543±137 kg to 1,413±99 kg) was greater than that in the piles without bulking agent (4,136±124 kg to 1,960±291 kg), indicating that a relatively large amount of “light” NH$_4^+$ was supplied to the piles with bulking agent. The $\delta^{15}$N value of NH$_4^+$ at the end of the experiments was significantly higher in the piles with bulking agent (17.7±1.3‰) than in those without bulking agent (11.8±0.9‰) (Fig. 2), indicating that the nitrogen transformation rate after the supply of newly formed ammonium was much higher in the piles with bulking agent.

In a previous work, we demonstrated that the use of bulking agent clearly reduced the greenhouse gas N$_2$O emission (up to 62.8%) when using the exact same scale and methods of dairy manure composting as used in the present study (Maeda et al., 2013a). Runs 2 and 3 in the previous work were identical to Runs 1 and 2 in this study. However, the present study did not provide a detailed explanation for this result. Our initial hypothesis, that the use of bulking agent reduced nitrogen transformation by nitrification-denitrification process, leading to lower N$_2$O emission, was not supported by the present data. One possible explanation for the difference in the mitigation of N$_2$O emission is the difference of temperature between the treatments, since it is known that the optimum temperature for the nitrifiers in the manure is around 35-40°C, and much lower nitrification activity can be observed above 50°C (Willers et al., 1998). The optimum temperature for denitrification and N$_2$O production can be higher than these values (Benoit et al., 2015), but denitrification requires the presence of NO$_2^-$ or NO$_3^-$ for electron acceptor. The use of a bulking agent enabled oxygen supply into the pile, which could have enhanced the oxidation of ammonium (nitrification), but the high
temperature inside the piles (>60°C) inhibited nitrification activity. Piles without a bulking agent showed lower temperature (30-40°C), which could have enhanced the nitrification, denitrification and N2O emission in the piles without bulking agent. However, the higher nitrogen turnover achieved by other nitrogen transformations, such as NH3 volatilization, assimilation and re-degradation of the bacterial cells, could have contributed to the higher δ15N value of NH4+ observed in the piles with bulking agent.

Because significantly different concentrations for not only NH4+ but also NO2- and NO3- were observed every two weeks (Fig. 1), it was suggested that the reactions proceeded in a different manner in each of the pile regions studied. To examine this possibility, we collected samples from each location (pile top, side and core), and confirmed that the NH4+ concentration was clearly higher in the top region of the samples just before the first turning event than in the more homogenous samples after the last turning event (Fig. 1). This result might be attributable to the high temperature of pile core, especially in the piles with bulking agent (>60°C). The high temperature causes an internal convective airflow even if the piles are not aerated (Barrington et al., 2003; Lynch & Cherry, 1996; Yu et al., 2005), and this air flow can cause the transportation of NH3-N from the specific zone where significant ammonification of organic-N occurs. δ15NH4+ levels were also determined for these samples, and we found that the 15N value of NH4+ was significantly enriched in the top pile samples (Fig. 3). This finding indicated that the nitrogen turnover rate was very high in the top pile zone, where significantly high NH4+ and NO2- concentrations were observed. The high NH4+ concentrations in the pile top could only be explained by the transformation from the pile core, as stated above, but the NH4+ in the pile core generally showed depleted δ15NH4+ (Fig. 3). We therefore performed a Keeling plot analysis to explain the phenomenon (Fig. 4A). If there were a single “heavy” 15NH4+ source, we would expect to see a significant regression line between the 15NH4+ values and inverse ammonium concentration. However, we did not see such a line, indicating that the nitrogen turnover and isotope fractionation occurred independently in each location. In turn, this means that the nitrogen turnover rate was extremely high in the pile top samples, which showed high NH4+ concentration with highly enriched δ15N values. We can think of two
possible explanations for the highly enriched $\delta^{15}\text{NH}_4^+$. One is that the enrichment was due to extremely high nitrification-denitrification activity in these samples, and the other is that it was due to high loss of nitrogen in the gaseous NH$_3$ state.

Previously Casciotti et al. (2003) reported that biological ammonium oxidation by beta-proteobacterial ammonium oxidizing bacteria (AOB; four *Nitrosomonas* and one *Nitrosospira* species) has an isotopic effect that ranges from 14.2-38.2‰. Another family of ammonium oxidizers, ammonium oxidizing archaea (AOA), also show isotopic fractionation during their activity, and this fractionation ranges from 13-41‰ (Santoro & Casciotti, 2011). Because the pH and availability of ammonia is one of the critical drivers partitioning these two ammonium oxidizers (Hatzenpichler, 2012), and manure compost shows high pH values and contains very high NH$_4^+$ concentration in general, AOB rather than AOA seems to be the main oxidizer in the compost (Yamamoto et al., 2012). Because significant amounts of the bacterial *amoA* gene, which is required for ammonium oxidation by AOB, have been detected in both the pile top and side, but not in the pile core (Maeda et al., 2010b), the contribution of this gene is a possible explanation for the “heavy” $^{15}\text{NH}_4^+$, especially in pile top samples. Therefore we performed a Raleigh plot analysis on our $^{15}\text{NH}_4^+$ data and tried to explain these enriched values with nitrification by the microbes (Fig. 4B). However, only some plots were included in the area attributable to nitrification, and thus nitrification alone could not be the driving factor for these “heavy” $^{15}\text{NH}_4^+$-N.

The isotope fractionation for NH$_3$ volatilization and nitrification are similar, 1.029 and 1.015-1.035 (Högberg, 1997), respectively. In addition, it has been clearly established that high NH$_3$ volatilization contributes to the enriched $\delta^{15}\text{NH}_4^+$ during cattle manure storage (Lee et al., 2011). Another study reported that NH$_4^+$ can easily exist in a gaseous state at high pH environment, and the temperature can also influence the fractionation (Li et al., 2012). The $\delta^{15}$N values of volatilized NH$_3$ from compost piles on the same scale were very low (-17.9--13.5‰, unpublished data), and thus it would seem that NH$_3$ volatilization would likely have contributed to these “heavy” NH$_4^+$ in the pile top, at least in part.

On the other hand, the significant increase in $\delta^{15}\text{NH}_4^+$ in the latter stage of the process
cannot be explained by NH$_3$ volatilization, because most of this occurs during their initial stage of the process, as we showed previously (Maeda et al., 2013a). Although the relative contributions of NH$_3$ volatilization and nitrification/denitrification to these $\delta^{15}$NH$_4^+$ increases are not clear, it is well known that nitrification occurs mainly during the latter stage of the process (Sanchez-Monedero et al., 2001), and the nitrification seems to contribute this increase significantly. Interestingly, highly enriched $\delta^{15}$NH$_4^+$ could be observed from the pile core zone at the end of the experiment in runs 1 and 2. This phenomenon cannot be explained by NH$_3$ volatilization because of its location in the piles, and thus it could be achieved solely by the nitrification-denitrification process. It is well known that high nitrification can occur in the latter stage of the composting process (Bernal et al., 2009; Parkinson et al., 2004), and the amoA gene could be detected from the compost core even in the latter stage of the composting process; therefore, high nitrogen conversion by microbes seems likely to have occurred in the compost core, and this could contribute to the sharp increase of the $\delta^{15}$NH$_4^+$ of the mixed samples.

5. Conclusion

The $\delta^{15}$NH$_4^+$ measurement of the samples collected from each location of the pile suggested an explanation for what occurred between the turnings. A plausible sequence of events between the pile turnings (Fig. 5) is as follows:

(i) Ammonification of organic N supplies a large amount of “light” ammonium in the compost core, where high organic matter degradation activity can be achieved.

(ii) This “light” ammonium is transported to the pile top zone by the upstream airflow generated by heat in the compost core zone.

(iii) Significant nitrification, denitrification and NH$_3$ volatilization occur in the pile top zone, leading to highly enriched $\delta^{15}$NH$_4^+$ in this zone, but these phenomena probably do not occur at significant levels in the pile side zone.

(ix) The nitrification rate exceeds the denitrification rate, leading to accumulation of NO$_2^-$ in the pile top and side, which in turn contributes to significant denitrification and N$_2$O emission just after the turning events.
On the other hand, the \( \delta^{15}\text{NH}_4^+ \) measurement of piles with and without bulking agent did not explain why \( \text{N}_2\text{O} \) emission could be mitigated by the use of bulking agent, and thus further studies are needed.

**Author Contribution**

K.M. and S.T. designed the experiments. K.M., M.Y. and M.F. carried out the experiments. K.M., S.T. and S.H. analyzed the results. K.M., K.N. and N.Y. wrote the paper.

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Figure Captions

Fig. 1. NO$_2^-$ (white), NO$_3^-$ (grey) and NH$_4^+$ (black) content of the compost samples from each location (top, side and core) of the pile and the sample just after the turnings (Mixed). These contents were determined every two weeks, just before/after the turning events. A-C indicate the pile 1 of the compost runs 1-3, and D-F indicate the pile 2 of the compost runs 1-3, respectively. The error bars indicate the standard deviation (n=3).

Fig. 2. $\delta^{15}$N of NH$_4^+$ of the mixed samples just after the turning events. The black bars indicate the compost with bulking agent (10% w/w), and the white bars indicate the compost without bulking agent. A-C indicate the compost runs 1-3. The error bars indicate the standard deviation (n=2).

Fig. 3. $\delta^{15}$N of NH$_4^+$ of the samples from each compost location (pile top, side and core). The values were expressed as the difference from the mixed samples just after the turning events. Black bars indicate the compost with bulking agent (10% w/w), and the white bars indicate the compost without bulking agent. A-C indicate the compost runs 1-3. The error bars indicate the standard deviation (n=3).

Fig. 4. Keeling plot (A) and Raleigh plot (B) of the $\delta^{15}$NH$_4^+$. The error bars indicate the standard deviation (n=2). Black symbols indicate the compost with bulking agent (10% w/w) and white symbols indicate the compost without bulking agents. The gray zone indicates the area that can be explained by ammonium oxidation by AOB (ammonia oxidizing bacteria; $\varepsilon$=14.2-38.2‰) or AOA (ammonium oxidizing archaea; $\varepsilon$=13-41‰).
References


5452-5458, 2008.


Table 1 Chemical components of compost samples

<table>
<thead>
<tr>
<th>Time</th>
<th>B.A.</th>
<th>Run kg</th>
<th>TS  %</th>
<th>VS %TS</th>
<th>EC mS cm⁻¹</th>
<th>pH</th>
<th>NO₂⁻N mg kg⁻¹ TS</th>
<th>NO₃⁻N mg kg⁻¹ TS</th>
<th>NH₄⁺N mg kg⁻¹ TS</th>
<th>TKN g-N kg⁻¹ TS</th>
<th>C/N g-N kg⁻¹ TS</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>-</td>
<td>1 4,280</td>
<td>20.5</td>
<td>(0.2)</td>
<td>84.7 (0.3)</td>
<td>2.6</td>
<td>0.0</td>
<td>8.4 (0.1)</td>
<td>0.0</td>
<td>68.9 (1.8)</td>
<td>4,646.5 (164.7)</td>
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<tr>
<td>I</td>
<td>-</td>
<td>2 4,060</td>
<td>22.7</td>
<td>(0.6)</td>
<td>82.3 (1.1)</td>
<td>3.1</td>
<td>0.0</td>
<td>8.8 (0.1)</td>
<td>0.0</td>
<td>0.0</td>
<td>3,497.5 (51.0)</td>
</tr>
<tr>
<td>I</td>
<td>-</td>
<td>3 4,070</td>
<td>17.8</td>
<td>(0.4)</td>
<td>82.1 (0.4)</td>
<td>2.7</td>
<td>(0.1)</td>
<td>8.0 (0.0)</td>
<td>0.0</td>
<td>0.0</td>
<td>7,347.8 (7.6)</td>
</tr>
<tr>
<td>I</td>
<td>+</td>
<td>1 4,700</td>
<td>28.3</td>
<td>(0.1)</td>
<td>87.0 (0.3)</td>
<td>2.9</td>
<td>0.0</td>
<td>8.3 (0.1)</td>
<td>0.0</td>
<td>0.0</td>
<td>2,929.5 (34.0)</td>
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<td>I</td>
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<td>31.2</td>
<td>(0.7)</td>
<td>87.1 (0.8)</td>
<td>3.2</td>
<td>0.0</td>
<td>8.8 (0.0)</td>
<td>0.0</td>
<td>0.0</td>
<td>2,288.0 (10.4)</td>
</tr>
<tr>
<td>I</td>
<td>+</td>
<td>3 4,450</td>
<td>22.6</td>
<td>(0.3)</td>
<td>86.5 (0.6)</td>
<td>3.0</td>
<td>0.0</td>
<td>7.7 (0.0)</td>
<td>0.0</td>
<td>0.0</td>
<td>5,840.5 (126.1)</td>
</tr>
<tr>
<td>F</td>
<td>-</td>
<td>1 1,710</td>
<td>24.8</td>
<td>(0.6)</td>
<td>70.0 (2.9)</td>
<td>2.5</td>
<td>0.0</td>
<td>9.3 (0.0)</td>
<td>0.0</td>
<td>0.0</td>
<td>1,353.1 (75.1)</td>
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<tr>
<td>F</td>
<td>-</td>
<td>2 2,280</td>
<td>24.3</td>
<td>(0.2)</td>
<td>75.1 (0.4)</td>
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<td>(0.1)</td>
<td>9.5 (0.1)</td>
<td>61.2 (8.6)</td>
<td>0.0</td>
<td>451.1 (0.6)</td>
</tr>
<tr>
<td>F</td>
<td>-</td>
<td>3 1,890</td>
<td>21.4</td>
<td>(0.4)</td>
<td>77.2 (0.5)</td>
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<td>0.0</td>
<td>9.2 (0.1)</td>
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<tr>
<td>F</td>
<td>+</td>
<td>1 1,190</td>
<td>52.7</td>
<td>(0.9)</td>
<td>69.1 (0.6)</td>
<td>5.0</td>
<td>(0.1)</td>
<td>9.5 (0.0)</td>
<td>44.3 (1.6)</td>
<td>52.6 (0.2)</td>
<td>460.9 (3.3)</td>
</tr>
<tr>
<td>F</td>
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<td>47.6</td>
<td>(0.4)</td>
<td>73.7 (0.9)</td>
<td>4.3</td>
<td>0.0</td>
<td>9.0 (0.0)</td>
<td>57.4 (7.5)</td>
<td>60.4 (2.5)</td>
<td>375.5 (21.7)</td>
</tr>
<tr>
<td>F</td>
<td>+</td>
<td>3 1,570</td>
<td>31.1</td>
<td>(1.0)</td>
<td>71.9 (1.6)</td>
<td>4.9</td>
<td>(0.1)</td>
<td>9.5 (0.1)</td>
<td>53.9 (6.7)</td>
<td>49.5 (12.1)</td>
<td>1,809.8 (97.8)</td>
</tr>
</tbody>
</table>

B.A., bulking agent; I, initial; F, final; TS, total solids; VS, volatile solids; EC, electrical conductivity; NO₂⁻N, nitrite-nitrogen; NO₃⁻N, nitrate-nitrogen; NH₄⁺N, ammonium-nitrogen; TKN, total Kjeldahl nitrogen; C/N, carbon/nitrogen ratio. The values represent the average (standard deviation).
NO$_2^-$ (white), NO$_3^-$ (grey) and NH$_4^+$-N (black) content of the compost samples from each location (top, side and core) of the pile and the sample just after the turnings (Mixed). These contents were determined every two weeks, just before/after the turning events. A-C indicate the pile 1 of the compost runs 1-3, and D-F indicate the pile 2 of the compost runs 1-3, respectively. The error bars indicate the standard deviation (n=3).
Maeda et al., Fig. 2

$\delta^{15}$N of NH$_4^+$ of the mixed samples just after the turning events. The black bars indicate the compost with bulking agent (10% w/w), and the white bars indicate the compost without bulking agent. A-C indicate the compost runs 1-3. The error bars indicate the standard deviation (n=2).
δ\(^{15}\)N of NH\(_4^+\)-N of the samples from each compost location (pile top, side and core). The values were expressed as the difference from the mixed samples just after the turning events. The black bars indicate the compost with bulking agent (10% w/w), and the white bars indicate the compost without bulking agent. A-C indicate the compost runs 1-3. The error bars indicate the standard deviation (n=3).
Keeling plot (A) and Raleigh plot (B) of the δ¹⁵NH₄⁺-N. The error bars indicate the standard deviation (n=2). Black symbols indicate the compost with bulking agent (10% w/w) and white symbols indicate the compost without bulking agents. The gray zone indicate the area which can be explained by ammonium oxidation by AOB (ammonia oxidizing bacteria; 14.2-38.2‰) or AOA (ammonium oxidizing archaea; 13-41‰).
Summary of the events between the pile turnings

Maeda et al., Fig. 5

NH₃ emission

Nitrification/denitrification

Ammonification/transportation

^{14}\text{NH}_4^+\text{-N}

^{15}\text{NH}_4^+\text{-N}

\text{NO}_2\text{-N}

\text{NO}_3\text{-N}