

Point-by-point responses to the comments

Referee #2

Manure compost is a major source of nitrogenous gases like ammonia (NH₃) and nitrous oxide (N₂O) in the atmosphere, and plays a role on global nitrogen cycle. Especially, N₂O is a highly-efficient greenhouse, and also destroys ozone in the stratosphere. Therefore researches concerning manure compost, especially the emission of nitrogenous gases during manure compost, have important significance. This work is initiated from the phenomenon that the emission of N₂O mitigated when bulking agent was adopted during manure compost, which was found in the authors' previous study.

From the Introduction section, the investigation on the mechanism of N₂O mitigation in dairy manure compost piles with bulking agent through isotope analysis should be the major subject of this paper. However, in the Results and Discussion sections, the authors just focus on the enrichment $\delta^{15}\text{N-NH}_4^+$ at the top of dairy manure compost piles, and attribute this enrichment to high nitrogen conversion, nitrification-denitrification activity and NH₃ volatilization. The mechanism of N₂O mitigation with bulking agent is not interrupted. It is needed a revision to make the subject clear before publication. Besides, some expressions in this paper are unclear and inconsistent, which make it difficult to understand this paper.

Thank you very much for this helpful comment. What we found in previous study was that N₂O emission can be mitigated by the use of bulking agent. Therefore we tried to understand why this occurs. This was our motivation, so we need to state this. Two of the three compost runs used piles exactly the same as in the previous study. We confirmed that N₂O emission was mitigated in these two runs. However these data were already published, so we did not provide the N₂O emission data in this study. We stated that the same compost piles were used in the text, and added some discussion on the N₂O emission.

Specific comments:

1. The authors use “pile with bulking agent”, “pile with dried grass (pile 1)” to describe the dairy manure compost piles. From their previous paper (Maeda et al., 2013a), dried grass is the bulking agent, however, it is not illustrated in the present paper.

Thank you very much for your comment. We stated this in the Materials and Methods section (P.7580, L.9-13).

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 piles (pile 1), while the control piles (pile 2) consisted of dairy cow excrement alone.

2. N₂O mitigation with bulking agent was found in Maeda et al. (2013a). Is similar phenomenon found in the present studies? Are experiments in the two papers the same ones?

Thank you very much for this point. In both studies (this work and the previous one; Maeda et al., (2013a)), we used the data from three independent manure compost piles. Two of them were identical to each other.

The manure compost piles used in the previous study (Maeda et al., (2013a)) were as follows.

Run 1: July 21 through September 17 in 2009

Run 2: May 27 through July 21 in 2010

Run 3: September 15 through November 10 in 2010

The manure compost piles in this study were as follows.

Run 1: 27 May through 21 July in 2010

Run 2: 15 September through 10 November in 2010

Run 3: 19 May through 14 July in 2011

Runs 2 and 3 in the previous work were identical to Runs 1 and 2 in this study. But we did not mention $\delta^{15}\text{N}$ of ammonium in the previous study. A mitigation effect on N₂O emission was observed on at least two of three piles (we did not measure N₂O emission from Run 3). We stated this in the text.

3. Line 16 of Page 7583: “Temporal decrease of $_{15}\text{N}$ value of NH_4^+ were observed in both piles” → “The decrease of $_{15}\text{N}$ value of NH_4^+ in the first two weeks were observed in both piles”

Thank you very much. We have changed the expression as you suggest.

4. Line 19-21 of Page 7583: “The $_{15}\text{N}$ value of NH_4^+ were significantly higher in the piles with bulking agent 17.7-1.3‰ than that of the piles without bulking agent (11.8- 0.9‰.” → “The $_{15}\text{N}$ value of NH_4^+ at the end of experiments were significantly higher in the piles with bulking agent (17.7-1.3‰ than that of the piles without bulking agent (11.8-0.9 ‰.

Thank you very much. We have changed the expression as you suggest.

5. Line 22-25 of Page 7583: Why more organic matter degradation cause higher $_{15}\text{N}$ value of NH_4^+ ? It seems to be inconsistent with the declaration in Line 17-18 that the ammonification of organic N supplies light NH_4^+ .

Thank you very much for your comment. We fully agree with you that these statements are inconsistent. We changed the relevant sentences to make this more clear.

6. Table 1: The authors annotate “C, control; T, treatment; Values followed by different letters indicate significant difference ($P < 0.05$)”, however, there were no “C”, “T” and “letters following values” in the table. The authors should check this table carefully.

Thank you very much for this pointing. We simply deleted the unneeded descriptions from the footnote of Table 1:

~~C, control; T, treatment, Values followed by different letters indicate significant difference ($P < 0.05$).~~

1 **Isotopically enriched ammonium shows high nitrogen turnover in the**
2 **pile top zone of dairy manure compost**

3
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17

18 **Abstract**

19 $\delta^{15}\text{N-NH}_4^+$ levels of dairy manure compost piles with and without bulking agent (10%
20 w/w) were compared to understand the effects of the use of bulking agent on nitrogen
21 conversion during manure composting. The amounts of $\delta^{15}\text{N-NH}_4^+$ in each of three pile
22 zones (top, side and core) were also compared. At the end of the process, piles with
23 bulking agent showed significantly higher $\delta^{15}\text{N}$ values ($17.7 \pm 1.3\%$) than piles without
24 bulking agent ($11.8 \pm 0.9\%$), reflecting the significantly higher nitrogen conversion and
25 NH_3 loss in the former. The samples from the top zone, especially in the piles with
26 bulking agent, showed very high NH_4^+ concentrations with significantly high ^{15}N ($\delta^{15}\text{N}$:
27 12.7-29.8 ‰) values, indicating that extremely high nitrogen conversion,
28 nitrification-denitrification activity of the microbes and NH_3 volatilization occurred in
29 this zone.
30

1 **1. Introduction**

2 Nitrogen is one of the most abundant major elements in the Earth's atmosphere.
3 There are two major anthropogenic activities affecting the global nitrogen cycle: energy
4 production and food production (Galloway et al., 2004). Because nitrogen is one of the
5 most important elements for plant nutrition, huge amounts of industrially fixed nitrogen
6 are used as fertilizer to improve the productivity of agricultural crops (Tilman et al.,
7 2002). Current anthropogenic nitrogen input to the environment (160 Tg per year) is
8 already greater than the input from natural biological fixation (110 Tg) on land or in the
9 ocean (140 Tg) (Gruber & Galloway, 2008), and the significance of agricultural
10 nitrogen input on the global nitrogen cycle is expected to increase along with the
11 nutritional needs of a growing population. In the livestock production industry, livestock
12 intake organic nitrogen from their feed, and produce large quantities of organic nitrogen
13 in the form of manure, a byproduct and potential resource which must be handled
14 appropriately to protect the environment (Sharpley et al., 1998). Most of this manure is
15 used as organic fertilizer for efficient nutrient cycling, and thus a proper understanding
16 of nitrogen flow in the manure management system is critically important.

17 The nitrogen contained in dairy manure exists mostly as organic nitrogen or NH_4^+ .
18 Through the composting process, the heat production by degradation of organic matter
19 leads to a significant loss of nitrogen into the atmosphere as gaseous ammonia (NH_3)
20 (Dämmgen & Hutchings, 2008). Nitrifiers and other families of microorganisms in the
21 manure also convert this nitrogen as nitrite (NO_2^-) or nitrate (NO_3^-), and both nitrifiers
22 and denitrifiers can use them as electron acceptors. They reduce these nitrogen oxides
23 into dinitrogen (N_2) and return them to the atmosphere in a process called denitrification
24 (Zumft, 1997). Nitrous oxide (N_2O), a greenhouse gas, is emitted through the nitrogen
25 conversion in the composting process (Sommer et al., 2009). Because it is known that
26 N_2O has very strong greenhouse effects (298-fold greater than the greenhouse effects of
27 CO_2 over a 100-year time horizon; IPCC, 2007), and N_2O is also known to contribute to
28 ozone layer destruction (Ravishankara et al., 2009), these gas emissions must be
29 mitigated.

30 With respect to this N_2O emission, our previous studies clarified that nitrification

1 occurs in the compost surface, and compost turning (mixing by machines) and
2 subsequent denitrification can be major sources of N₂O (Maeda et al., 2013b; Maeda et
3 al., 2010b). Also, we have shown that the appropriate use of bulking agents can reduce
4 the N₂O emission significantly (Maeda et al., 2013a). However, the mechanism of this
5 N₂O mitigation is largely unknown. Because bulking agents are generally used to
6 increase the supply of oxygen to the compost piles (Jolanun & Towprayoon, 2010), it is
7 expected that the increase in oxygen increases nitrification and the subsequent N₂O
8 production.

9 To solve this contradiction, we compared the level of $\delta^{15}\text{N-NH}_4^+$ in these composts,
10 because this parameter can be used to track the level of reaction involving NH₄⁺ in the
11 environment (Brooks et al., 1989; Garten Jr, 1992; Yeatman et al., 2001). Because it has
12 already been established that the NO_x⁻ accumulation and the bacterial communities are
13 different in different regions of the pile (Maeda et al., 2010a), we sampled from both the
14 compost side and core independently, and surveyed them into the $\delta^{15}\text{N-NH}_4^+$ analysis.

15 16 **2. Materials and Methods**

17 **2.1. Composting experiment**

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

26 **About 4 t of dairy cow excrement and 400 kg of dried grass were mixed to form the**
27 **treatment pile (pile 1), while the control pile (pile 2) consisted of dairy cow excrement**
28 **alone.** The compost was piled up on a waterproof concrete floor, and turned once every
29 two weeks with a front loader and manure spreader. Each pile had a volume of 7.5 m³
30 with pile dimensions of 4 m in diameter and 1.8 m in height at the start of the

1 experiment. The temperatures of the compost piles and the ambient air were measured
2 hourly using a Thermo Recorder RTW-30S (Espec, Japan).

3 4 **2.2. Chemical analysis of the compost**

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

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

21 22 **2.3. Determination of $\delta^{15}\text{N-NH}_4^+$ levels and Rayleigh plot analysis**

23 The amount of $\delta^{15}\text{N-NH}_4^+$ in the extracted samples or trapped NH₃ samples was
24 determined by the diffusion method (Holmes et al., 1998).

25 One cm diameter GF/D filters (Whatman, UK) were cut into four pieces, acidified with
26 20 μl H₃PO₄ (0.02 mM) and sandwiched between 2.5 cm diameter 10 mm pore-size
27 Teflon membranes (Millipore, USA). These filter packs were used as an ammonium
28 trap in the samples. Ten ml of the NH₄⁺-N samples (50 $\mu\text{g-N}$) was placed in 15 ml tubes
29 and 0.5 g of NaCl (ashed at 450°C for 8 h) was added. Then a single filter pack was
30 added to the 15 ml tube, and 0.03 g MgO (ashed at 450°C for 8 h) was added to convert

1 NH_4^+ in the samples into NH_3 . The 15 ml tubes were incubated at 40°C for 2 weeks
 2 with stirring at 200 rpm. After incubation, the filter pack was removed from the tubes
 3 and dried in a desiccator for 2 days. The dried filter was then recovered and placed in a
 4 tin cup. The tin cup containing the filter was then analyzed by an elemental analyzer
 5 (EA1110, CE Instruments, Ltd., Wigan, UK) coupled with an isotope ratio mass
 6 spectrometer (MAT252; ThermoFisher Scientific KK, Yokohama, Japan) to quantify
 7 $\delta^{15}\text{N-NH}_4^+$ in the samples. Calibration was conducted with IAEA-N1 and IAEA-N2
 8 (NH_4SO_4), and the precision (1σ) was better than 0.2‰. The $\delta^{15}\text{N}$ of samples was
 9 expressed in parts per thousand deviations from the atmospheric N_2 as defined by the
 10 following equation:

$$11 \quad \delta^{15}\text{N} (\text{‰}) = ((R_{\text{sample}}/R_{\text{standard}}) - 1) \times 1000, \quad (1)$$

12 where R_{sample} and R_{standard} are the $^{15}\text{N}/^{14}\text{N}$ ratios of samples and the atmospheric N_2 ,
 13 respectively. Isotopic fractionation factor α was expressed as

$$14 \quad \alpha = R_B/R_A, \quad (2)$$

15 where R_A and R_B are the isotopic ratio of phase A and B, respectively.

16 Isotopic fractionation can also be described by the enrichment factor ε , which describes
 17 the enrichment of the product relative to that of the substrate, and which is also
 18 expressed per mil (‰).

$$19 \quad \varepsilon = (\alpha - 1) \times 1000 \quad (3)$$

20 The evolution of the isotopic composition is described by a Rayleigh equation with a
 21 fractionation factor as follows for ^{15}N :

$$22 \quad R/R_0 = (1+10^{-3} \delta^{15}\text{N}) / (1+10^{-3} \delta^{15}\text{N}_0) = ([\text{NH}_4^+]/[\text{NH}_4^+]_0)^{\alpha-1}, \quad (4)$$

23 where R and R_0 are the isotope ratio of samples just before the turning and of the
 24 samples just after the previous turning. Since the piles were homogenized at each
 25 turning event, the amount of ammonium in a sample just after the previous turning
 26 event was taken as the “initial” ammonium.” $\delta^{15}\text{N}$ and $\delta^{15}\text{N}_0$ are the respective δ values
 27 for the each NH_4^+ . $[\text{NH}_4^+]_0$ and $[\text{NH}_4^+]$ are the ammonium concentration of the samples
 28 just after the previous turning event and the samples just before the subsequent turning
 29 event, respectively. Using the approximation of $\ln(1+x) \cong x$ with $x \ll 1$, the relationship
 30 between the difference of $\delta^{15}\text{N}$ values between pile turnings and the reaction rate of the

1 substrate was obtained from equations (3) and (4) as follows:

$$2 \quad \delta^{15}\text{N} - \delta^{15}\text{N}_0 = \varepsilon \ln(1-f), \quad (5)$$

3 where f is the amount of reacted ammonium between the turning events, defined as $f = (1$
4 $- [\text{NH}_4^+] / [\text{NH}_4^+]_0)$.

5

6 **2.4 Keeling plot analysis**

7 The basis of the Keeling plot method is conservation of mass. The ammonium
8 concentration of each location of the pile before the pile turnings can be expressed as

$$9 \quad c_b = c_a + c_s, \quad (6)$$

10 where c_b , c_a , and c_s are the ammonium concentration measured in each location of the
11 pile just before the turning, the ammonium concentration just after the previous pile
12 turning, and the additional concentration component produced by the source,
13 respectively. Given conservation of mass, we have

$$14 \quad \delta^{15}\text{N}_{bc_b} = \delta^{15}\text{N}_a c_a + \delta^{15}\text{N}_s c_s, \quad (7)$$

15 where $\delta^{15}\text{N}$ represents the nitrogen isotope ratio of the ammonium in each sample. By
16 combining equations (6) and (7), we arrive at

$$17 \quad \delta^{15}\text{N}_b = c_a (\delta^{15}\text{N}_a - \delta^{15}\text{N}_s) (1/c_b) + \delta^{15}\text{N}_s. \quad (8)$$

18

19 **2.5 Statistical analysis**

20 The chemical component data were analyzed by ANOVA using the general linear model
21 procedure described by SAS ([SAS Institute, 2001](#)). Tukey's multiple range comparison
22 tests were used to separate the means. A value of $P < 0.05$ was considered statistically
23 significant.

24

1 **3. Results**

2 **3.1. Composting experiments**

3 The temperature of the piles with bulking agent (10% w/w) exceeded 60°C
4 throughout the entire experiment (Fig. S1), while the piles without bulking agent
5 showed significantly lower temperature (below 50°C). The initial weight was
6 4,543±137 kg in the piles with bulking agent and 4,136±124 kg in those without bulking
7 agent, and the final turning these values dropped significantly to 1,413±99 kg and
8 1,960±291 kg, respectively (Table 1). The total solids of the piles with and without
9 bulking agent after the composting process were 43.8±11.3% and 23.5±1.8%,
10 respectively. The C/N ratios of the piles with and without bulking agent dropped
11 significantly from 23.8±3.3 to 12.8±0.8 and from 22.8±1.2 to 15.6±2.6, respectively.
12 These parameters all indicate that the organic matter degradation rate was much higher
13 in the piles with bulking agent.

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

23

24 **3.2. δ¹⁵N of NH₄⁺ in mixed samples**

25 δ¹⁵N-NH₄⁺ values of the mixed samples just after the pile turning events are shown in
26 Fig. 2. All compost runs showed a similar tendency. The initial δ¹⁵N-NH₄⁺ values were
27 5.8±2.5‰ and 7.4±3.8‰ for the piles with and without bulking agent, respectively.
28 These values dropped slightly between weeks 0 and 2, to 4.4±2.8‰ and 6.1±2.3‰ for
29 piles with and without bulking agent in all runs, although these changes were not
30 statistically significant. After week 4, these values increased significantly, and at the

1 end of the experiments they reached $17.7 \pm 1.3\text{‰}$ and $11.8 \pm 0.9\text{‰}$ for the piles with and
2 without bulking agent, respectively. Also, the piles with bulking agent showed higher
3 values than the piles without bulking agent, and this difference was statistically
4 significant.

5 $\delta^{15}\text{N-NH}_4^+$ values were also determined for the pile top, side and core samples, and
6 are shown in Fig. 3. The data were expressed as the difference from the mixed samples
7 taken after the pile homogenization. The values for the pile top samples ($9.6\text{-}22.5\text{‰}$)
8 were higher than those for the side samples ($9.2\text{-}11.3\text{‰}$) in both the piles with and
9 without bulking agent. The core samples showed low $\delta^{15}\text{N-NH}_4^+$ values in week 2
10 ($1.7 \pm 1.0\text{‰}$ and $4.7 \pm 2.0\text{‰}$ for the piles with and without bulking agent, respectively),
11 reflecting the newly formed “light” $\text{NH}_4^+\text{-N}$, which was supplied by the degradation of
12 organic-N in the manure. On the other hand, the heaviest NH_4^+ ($25.4 \pm 6.8\text{‰}$) was also
13 observed in the pile core samples at the end of the experimental period. This
14 phenomenon was observed only from the piles with bulking agent.

15

4. Discussion

The stable isotope $\delta^{15}\text{N}$ value of NH_4^+ in dairy manure compost with and without bulking agent was studied to clarify the mechanism of the significant N_2O mitigation achieved using a bulking agent. A decrease in the $\delta^{15}\text{N}$ 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 ($\alpha \sim 1.000$) of isotopic fractionation (Högberg, 1997). The weight decrease in the piles with bulking agent ($4,543 \pm 137$ kg to $1,413 \pm 99$ kg) was greater than that in the piles without bulking agent ($4,136 \pm 124$ kg to $1,960 \pm 291$ kg), indicating that a relatively large amount of “light” NH_4^+ was supplied to the piles with bulking agent. The $\delta^{15}\text{N}$ value of NH_4^+ at the end of the experiments was significantly higher in the piles with bulking agent ($17.7 \pm 1.3\%$) than in those without bulking agent ($11.8 \pm 0.9\%$) (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_2O 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_2O emission, was not supported by the present data. One possible explanation for the difference in the mitigation of N_2O 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 $35\text{--}40^\circ\text{C}$, and much lower nitrification activity can be observed above 50°C (Willers et al., 1998). The optimum temperature for denitrification and N_2O 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

1 temperature inside the piles ($>60^{\circ}\text{C}$) inhibited nitrification activity. Piles without a
2 bulking agent showed lower temperature ($30\text{-}40^{\circ}\text{C}$), which could have enhanced the
3 nitrification, denitrification and N_2O emission in the piles without bulking agent.
4 However, the higher nitrogen turnover achieved by other nitrogen transformations, such
5 as NH_3 volatilization, assimilation and re-degradation of the bacterial cells, could have
6 contributed to the higher $\delta^{15}\text{N}$ value of NH_4^+ observed in the piles with bulking agent.

7 Because significantly different concentrations for not only NH_4^+ but also NO_2^- and
8 NO_3^- were observed every two weeks (Fig. 1), it was suggested that the reactions
9 proceeded in a different manner in each of the pile regions studied. To examine this
10 possibility, we collected samples from each location (pile top, side and core), and
11 confirmed that the NH_4^+ concentration was clearly higher in the top region of the
12 samples just before the first turning event than in the more homogenous samples after
13 the last turning event (Fig. 1). This result might be attributable to the high temperature
14 of pile core, especially in the piles with bulking agent ($>60^{\circ}\text{C}$). The high temperature
15 causes an internal convective airflow even if the piles are not aerated (Barrington et al.,
16 2003; Lynch & Cherry, 1996; Yu et al., 2005), and this air flow can cause the
17 transportation of $\text{NH}_3\text{-N}$ from the specific zone where significant ammonification of
18 organic-N occurs. $\delta^{15}\text{NH}_4^+$ levels were also determined for these samples, and we found
19 that the ^{15}N value of NH_4^+ was significantly enriched in the top pile samples (Fig. 3).
20 This finding indicated that the nitrogen turnover rate was very high in the top pile zone,
21 where significantly high NH_4^+ and NO_2^- concentrations were observed. The high NH_4^+
22 concentrations in the pile top could only be explained by the transformation from the
23 pile core, as stated above, but the NH_4^+ in the pile core generally showed depleted
24 $\delta^{15}\text{NH}_4^+$ (Fig. 3). We therefore performed a Keeling plot analysis to explain the
25 phenomenon (Fig. 4A). If there were a single “heavy” $^{15}\text{NH}_4^+$ source, we would expect
26 to see a significant regression line between the $^{15}\text{NH}_4^+$ values and inverse ammonium
27 concentration. However, we did not see such a line, indicating that the nitrogen turnover
28 and isotope fractionation occurred independently in each location. In turn, this means
29 that the nitrogen turnover rate was extremely high in the pile top samples, which
30 showed high NH_4^+ concentration with highly enriched $\delta^{15}\text{N}$ values. We can think of two

1 possible explanations for the highly enriched $\delta^{15}\text{NH}_4^+$. One is that the enrichment was
2 due to extremely high nitrification-denitrification activity in these samples, and the
3 other is that it was due to high loss of nitrogen in the gaseous NH_3 state.

4 Previously [Casciotti et al. \(2003\)](#) reported that biological ammonium oxidation by
5 beta-proteobacterial ammonium oxidizing bacteria (AOB; four *Nitrosomonas* and one
6 *Nitrosospira* species) has an isotopic effect that ranges from 14.2-38.2‰. Another
7 family of ammonium oxidizers, ammonium oxidizing archaea (AOA), also show
8 isotopic fractionation during their activity, and this fractionation ranges from 13-41‰
9 ([Santoro & Casciotti, 2011](#)). Because the pH and availability of ammonia is one of the
10 critical drivers partitioning these two ammonium oxidizers ([Hatzenpichler, 2012](#)), and
11 manure compost shows high pH values and contains very high NH_4^+ concentration in
12 general, AOB rather than AOA seems to be the main oxidizer in the compost
13 ([Yamamoto et al., 2012](#)). Because significant amounts of the bacterial *amoA* gene,
14 which is required for ammonium oxidation by AOB, have been detected in both the pile
15 top and side, but not in the pile core ([Maeda et al., 2010b](#)), the contribution of this gene
16 is a possible explanation for the “heavy” $^{15}\text{NH}_4^+$, especially in pile top samples.
17 Therefore we performed a Raleigh plot analysis on our $^{15}\text{NH}_4^+$ data and tried to explain
18 these enriched values with nitrification by the microbes ([Fig. 4B](#)). However, only some
19 plots were included in the area attributable to nitrification, and thus nitrification alone
20 could not be the driving factor for these “heavy” $^{15}\text{NH}_4^+\text{-N}$.

21 The isotope fractionation for NH_3 volatilization and nitrification are similar, 1.029
22 and 1.015-1.035 ([Högberg, 1997](#)), respectively. In addition, it has been clearly
23 established that high NH_3 volatilization contributes to the enriched $\delta^{15}\text{NH}_4^+$ during
24 cattle manure storage ([Lee et al., 2011](#)). Another study reported that NH_4^+ can easily
25 exist in a gaseous state at high pH environment, and the temperature can also influence
26 the fractionation ([Li et al., 2012](#)). The $\delta^{15}\text{N}$ values of volatilized NH_3 from compost piles
27 on the same scale were very low (-17.9~-13.5‰, unpublished data), and thus it would
28 seem that NH_3 volatilization would likely have contributed to these “heavy” NH_4^+ in the
29 pile top, at least in part.

30 On the other hand, the significant increase in $\delta^{15}\text{NH}_4^+$ in the latter stage of the process

1 cannot be explained by NH₃ volatilization, because most of this occurs during their
2 initial stage of the process, as we showed previously (Maeda et al., 2013a). Although
3 the relative contributions of NH₃ volatilization and nitrification/denitrification to these
4 $\delta^{15}\text{NH}_4^+$ increases are not clear, it is well known that nitrification occurs mainly during
5 the latter stage of the process (Sanchez-Monedero et al., 2001), and the nitrification
6 seems to contribute this increase significantly. Interestingly, highly enriched $\delta^{15}\text{NH}_4^+$
7 could be observed from the pile core zone at the end of the experiment in runs 1 and 2.
8 This phenomenon cannot be explained by NH₃ volatilization because of its location in
9 the piles, and thus it could be achieved solely by the nitrification-denitrification process.
10 It is well known that high nitrification can occur in the latter stage of the composting
11 process (Bernal et al., 2009; Parkinson et al., 2004), and the *amoA* gene could be
12 detected from the compost core even in the latter stage of the composting process;
13 therefore, high nitrogen conversion by microbes seems likely to have occurred in the
14 compost core, and this could contribute to the sharp increase of the $\delta^{15}\text{NH}_4^+$ of the
15 mixed samples.

16

17 **5. Conclusion**

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

- 21 (i) Ammonification of organic N supplies a large amount of “light” ammonium in the
22 compost core, where high organic matter degradation activity can be achieved.
- 23 (ii) This “light” ammonium is transported to the pile top zone by the upstream airflow
24 generated by heat in the compost core zone.
- 25 (iii) Significant nitrification, denitrification and NH₃ volatilization occur in the pile top
26 zone, leading to highly enriched $\delta^{15}\text{NH}_4^+$ in this zone, but these phenomena
27 probably do not occur at significant levels in the pile side zone.
- 28 (ix) The nitrification rate exceeds the denitrification rate, leading to accumulation of
29 NO₂⁻ in the pile top and side, which in turn contributes to significant denitrification
30 and N₂O emission just after the turning events.

1 On the other hand, the $\delta^{15}\text{NH}_4^+$ measurement of piles with and without bulking agent
2 did not explain why N_2O emission could be mitigated by the use of bulking agent, and
3 thus further studies are needed.

4 **Author Contribution**

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

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15 Forestry and Fisheries (MAFF), Japan. This work was also supported by a Grant-in-Aid
16 for Young Scientists (B) to K.M. and a grant from the Global Environment Research
17 Fund (B-094) of the Ministry of the Environment, Japan to N. Y.

1 **Figure Captions**

2 **Fig. 1.**

3 NO₂⁻ (white), NO₃⁻ (grey) and NH₄⁺ (black) content of the compost samples from each
4 location (top, side and core) of the pile and the sample just after the turnings (Mixed).
5 These contents were determined every two weeks, just before/after the turning events.
6 A-C indicate the pile 1 of the compost runs 1-3, and D-F indicate the pile 2 of the
7 compost runs 1-3, respectively. The error bars indicate the standard deviation (n=3).

8

9 **Fig. 2.**

10 $\delta^{15}\text{N}$ of NH₄⁺ of the mixed samples just after the turning events. The black bars indicate
11 the compost with bulking agent (10% w/w), and the white bars indicate the compost
12 without bulking agent. A-C indicate the compost runs 1-3. The error bars indicate the
13 standard deviation (n=2).

14

15 **Fig. 3.**

16 $\delta^{15}\text{N}$ of NH₄⁺ of the samples from each compost location (pile top, side and core). The
17 values were expressed as the difference from the mixed samples just after the turning
18 events. Black bars indicate the compost with bulking agent (10% w/w), and the white
19 bars indicate the compost without bulking agent. A-C indicate the compost runs 1-3.
20 The error bars indicate the standard deviation (n=3).

21

22 **Fig. 4.**

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

28

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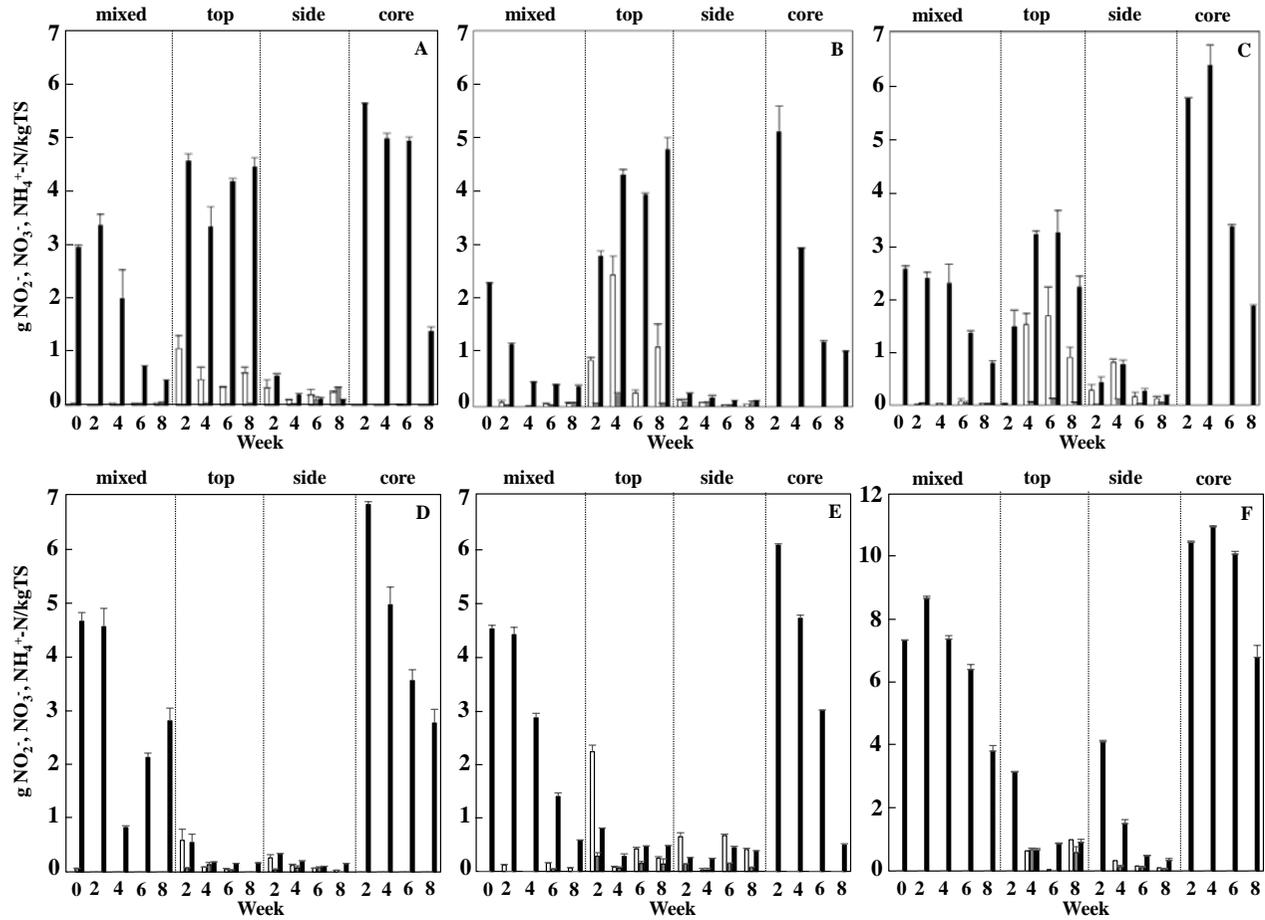
Time	B.A.	Run	Weight kg	TS %	VS %TS	EC mScm ⁻¹	pH	NO ₂ ⁻ -N mg kg ⁻¹ TS	NO ₃ ⁻ -N mg kg ⁻¹ TS	NH ₄ ⁺ -N mg kg ⁻¹ TS	TKN g-N kg ⁻¹ TS	C/N			
I	-	1	4,280	20.5 (0.2)	84.7 (0.3)	2.6	0.0	8.4 (0.1)	0.0	0.0	68.9 (1.8)	4,646.3 (164.7)	27.6 (0.3)	24.2 (0.2)	
I	-	2	4,060	22.7 (0.6)	82.3 (1.1)	3.1	0.0	8.8 (0.1)	0.0	0.0	0.0	0.0	3,497.5 (51.0)	24.2 (0.6)	22.2 (0.2)
I	-	3	4,070	17.8 (0.4)	82.1 (0.4)	2.7	0.0	8.0 (0.1)	0.0	0.0	0.0	0.0	7,347.8 (7.6)	26.2 (0.4)	22.0 (0.4)
I	+	1	4,700	28.3 (0.1)	87.0 (0.3)	2.9	0.0	8.3 (0.1)	0.0	0.0	40.1 (0.9)	2,929.3 (34.0)	20.8 (1.0)	23.0 (0.1)	
I	+	2	4,480	31.2 (0.7)	87.1 (0.8)	3.2	0.0	8.8	0.0	0.0	0.0	0.0	2,288.0 (10.4)	21.6 (0.7)	27.5 (1.7)
I	+	3	4,450	22.6 (0.3)	86.5 (0.6)	3.0	0.0	7.7	0.0	0.0	0.0	0.0	5,840.3 (126.1)	20.4 (0.4)	21.0 (0.3)
F	-	1	1,710	24.8 (0.6)	70.0 (2.9)	2.5	0.0	9.3	0.0	0.0	0.0	0.0	1,353.1 (75.1)	32.3 (1.7)	13.0 (0.1)
F	-	2	2,280	24.3 (0.2)	75.1 (0.4)	2.6	0.0	9.5 (0.1)	61.2 (8.6)	0.0	0.0	0.0	451.1 (0.6)	26.7 (0.6)	16.3 0.0
F	-	3	1,890	21.4 (0.4)	77.2 (0.5)	3.0	0.0	9.2 (0.1)	0.0	0.0	0.0	0.0	3,817.1 (177.4)	28.9 (1.1)	17.5 (0.2)
F	+	1	1,190	52.7 (0.9)	69.1 (0.6)	5.0	0.0	9.5	0.0	44.3 (1.6)	52.6 (0.2)	460.9 (3.3)	30.0	0.0	12.0 (0.1)
F	+	2	1,480	47.6 (0.4)	73.7 (0.9)	4.3	0.0	9.0	0.0	57.4 (7.5)	60.4 (2.5)	375.5 (21.7)	29.1 (0.1)	13.6 (0.2)	
F	+	3	1,570	31.1 (1.0)	71.9 (1.6)	4.9	0.0	9.5 (0.1)	53.9 (6.7)	49.5 (12.1)	1,809.8 (97.8)	29.2 (0.2)	12.7 0.0		

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).

2

1 **Maeda et al., Fig. 1**

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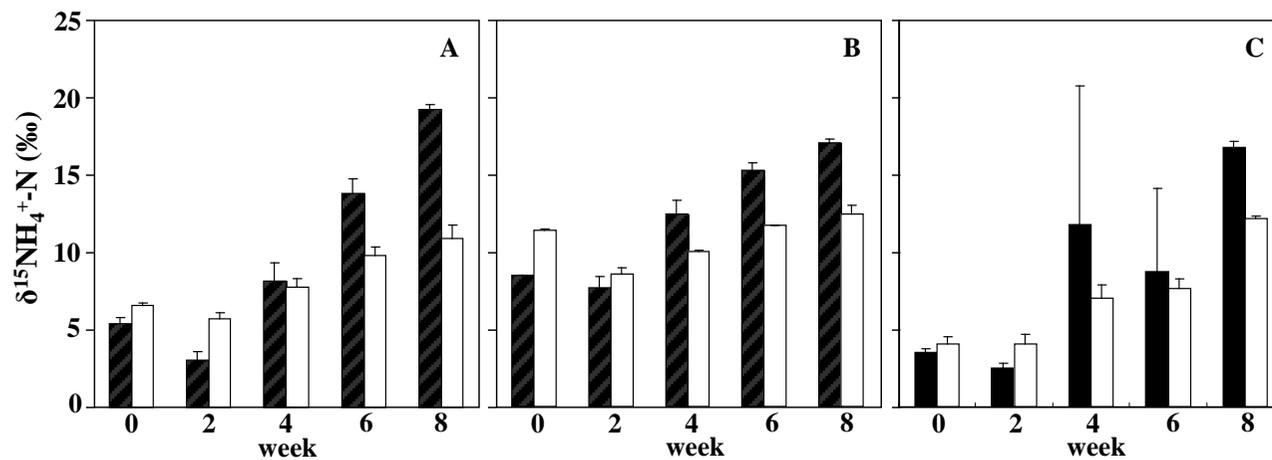
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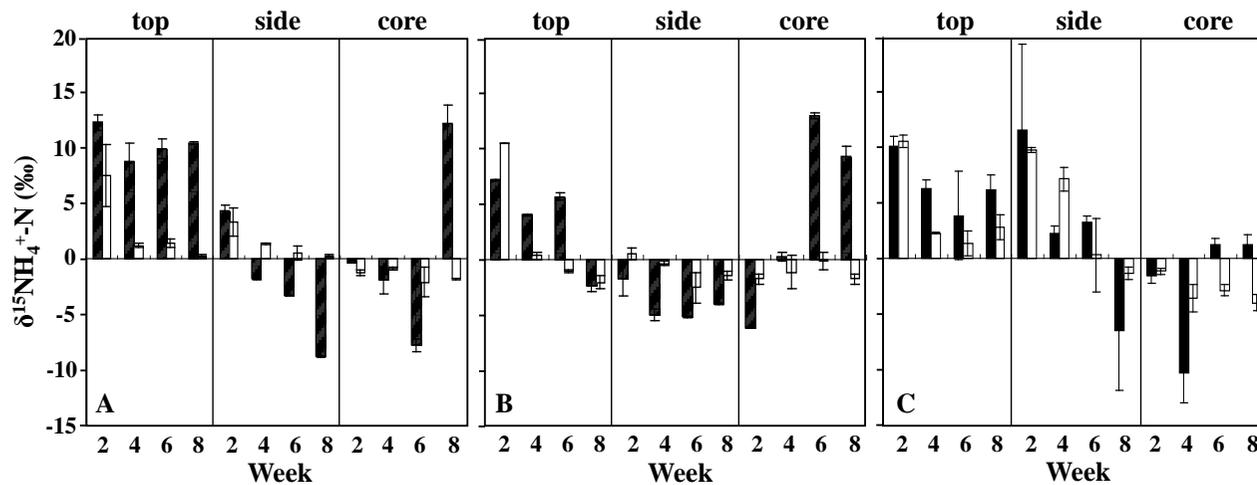
NO₂⁻ (white), NO₃⁻ (grey) and NH₄⁺-N (black) content of the compost samples from each location (top, side and core) of the pile and the sample just after the turnings (Mixed). These contents were determined every two weeks, just before/after the turning events. A-C indicate the pile 1 of the compost 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).

1 **Maeda et al., Fig. 2**



2
3
4 $\delta^{15}\text{N}$ 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
5 the white bars indicate the compost without bulking agent. A-C indicate the compost runs 1-3. The error bars indicate the standard
6 deviation (n=2).
7

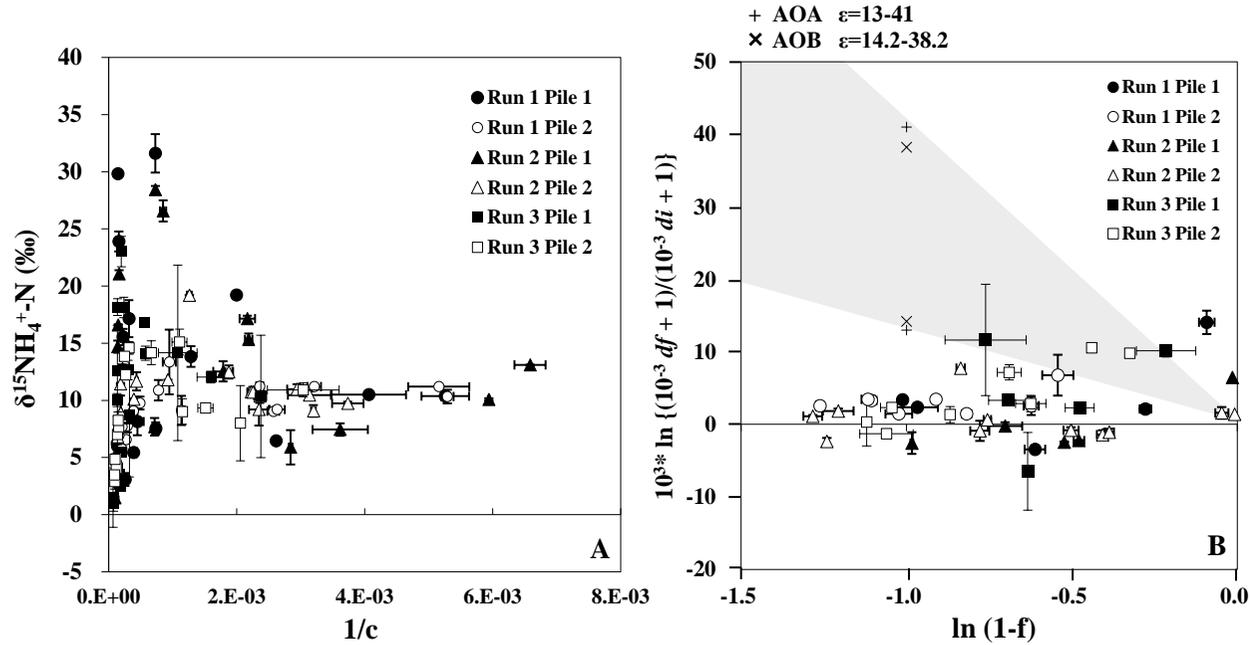
1 Maeda et al., Fig. 3



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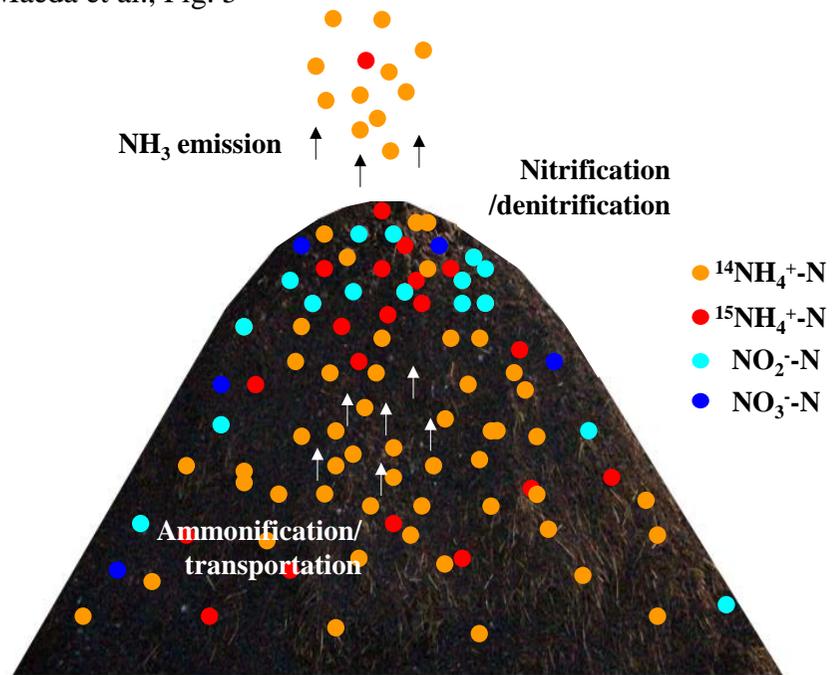
$\delta^{15}\text{N}$ of $\text{NH}_4^+\text{-N}$ of the samples from each compost location (pile top, side and core). The values were expressed as the difference from the mixed samples just after the turning events. The black bars indicate the compost with bulking agent (10% w/w), and the white bars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

1 Maeda et al., Fig. 4



2
 3
 4 Keeling plot (A) and Raleigh plot (B) of the $\delta^{15}\text{NH}_4^+\text{-N}$. The error bars indicate the standard deviation (n=2). Black symbols indicate the
 5 compost with bulking agent (10% w/w) and white symbols indicate the compost without bulking agents. The gray zone indicate the area
 6 which can be explained by ammonium oxidation by AOB (ammonia oxidizing bacteria; 14.2-38.2‰) or AOA (ammonium oxidizing
 7 archaea; 13-41‰).
 8

1 Maeda et al., Fig. 5



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Summary of the events between the pile turnings