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Title: Accurate and precise quantification of atmospheric nitrate in streams draining land of various uses by using triple oxygen isotopes as tracers Authors: U. Tsunogai, T. Miyauchi, T. Ohyama, D.D. Komatsu, F. Nakagawa, Y. Obata, K. Sato, and T. Ohizumi MS No.: bg-2015-627

Dear Prof. Bol:

Thank you very much for your decision on our manuscript (Publish subject to minor revisions). We have revised the manuscript accordingly. All the revisions from the previous version sent to you on May 11 are as follows:

1) The English of the manuscript was thoroughly edited by Editage English editing service (http:// www.editage.jp/) again.

2) We used red instead of pink in Figures 6 and 7.

3) We maximized the axes in Fig 8; used -1 to +5 instead of -2 to +8 for x-axis and -5 to +15 instead of -10 to +30 for y-axis.

4) We made minor improvements on the figures.

5) We added present address of Mr. Y. Obata.

Please find the revised manuscript uploaded, in which the newly added or changed sentences from BGD were presented in blue/green.

We would like to thank you and referees for the helpful comments and suggestions. We trust that the revision is satisfactory response to your comments. Thank you for your consideration.

Sincerely yours, Urumu Tsunogai, PhD

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Accurate and precise quantification of atmospheric nitrate in streams draining land of various uses by using triple oxygen isotopes as tracers

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21 Abstract

Land use in <u>a</u> catchment area has significant impacts on nitrate eluted from the catchment, including atmospheric nitrate deposited onto the catchment area and remineralized nitrate produced within the catchment area. <u>Although</u> the stable isotopic compositions of nitrate eluted from a catchment can be a useful tracer to quantify the land use influences on the sources and behaviour of <u>the</u> nitrate, <u>it is best to</u> determine <u>these</u> for the remineralized portion of <u>the</u> nitrate separately from the unprocessed atmospheric nitrate to obtain <u>a</u> more accurate and precise quantification of the land use influences. In this study, we determined <u>the</u> spatial

distribution and seasonal variation of stable isotopic compositions of nitrate for more than 30 1 2 streams within the same watershed, the Lake Biwa watershed in Japan, in order to use ¹⁷O excess (Δ^{17} O) of nitrate as an additional tracer to quantify the mole fraction of atmospheric 3 nitrate accurately and precisely. The stable isotopic compositions, including Δ^{17} O of nitrate, 4 in precipitation (wet deposition; n = 196) sampled at the Sado-seki monitoring station were 5 also determined for three years. The deposited nitrate showed large ¹⁷O excesses similar to 6 those already reported for mid-latitudes: Δ^{17} O values ranged from +18.6‰ to +32.4‰ with a 7 three-year average of +26.3‰. On the other hand, nitrate in each inflow stream showed small 8 annual average Δ^{17} O values ranging from +0.5% to +3.1%, which corresponds to mole 9 fractions of unprocessed atmospheric nitrate to total nitrate from $(1.8\pm0.3)\%$ to $(11.8\pm1.8)\%$, 10 respectively, with an average for all inflow streams of (5.1 ± 0.5) %. Although the annual 11 average Δ^{17} O values tended to be smaller in accordance with the increase in annual average 12 stream nitrate concentration from 12.7 to 106.2 μ mol L⁻¹, the absolute concentrations of 13 unprocessed atmospheric nitrate were almost stable at (2.3 ± 1.1) µmol L⁻¹ irrespective of the 14 changes in population density and land use in each catchment area. We conclude that changes 15 in population density and land use between each catchment area had little impact on the 16 17 concentration of atmospheric nitrate and that the total nitrate concentration originated 18 primarily from additional contributions of remineralized nitrate. By using the average stable 19 isotopic compositions of atmospheric nitrate, we excluded the contribution of atmospheric nitrate from the determined $\delta^{15}N$ and $\delta^{18}O$ values of total nitrate and estimated the $\delta^{15}N$ and 20 21 δ^{18} O values of the remineralized portion of nitrate in each stream to clarify the sources. We found that the remineralized portion of the nitrate in the streams could be explained by mixing 22 between a natural source having values of $(+4.4\pm1.8)$ % and (-2.3 ± 0.9) % for δ^{15} N and δ^{18} O, 23 respectively, and an anthropogenic source having values of $(+9.2\pm1.3)$ % and (-2.2 ± 1.1) % 24 for $\delta^{15}N$ and $\delta^{18}O$, respectively. In addition, both the uniform absolute concentration of 25 atmospheric nitrate and the low and uniform δ^{18} O values of the remineralized portion of 26 nitrate in the streams imply that in-stream removal of nitrate through assimilation or 27 28 denitrification had small impact on the concentrations and stable isotopic compositions of nitrate in the streams, except for a few streams in summer, having catchments of 29 30 urban/suburban land uses.

1 **1 Introduction**

Nitrate (NO₃⁻) in stream water can be an important source of information for understanding 2 the biogeochemical cycles within the catchment area of the stream (Likens et al., 1970; Durka 3 4 et al., 1994; Swank et al., 2001). In addition, the nitrate concentration in stream water is important to primary production, and an excess of nitrate can lead to eutrophication in 5 6 downstream areas, including receiving lakes, estuaries, and oceans (McIsaac et al., 2001; Paerl, 2009). However, nitrate concentrations in stream water are determined through a 7 8 complicated interplay of several processes within the catchment area including (1) the 9 addition of atmospheric nitrate ($NO_3^{-}(atm)$) through deposition, (2) the production of remineralized nitrate (NO_3 (re)) through microbial nitrification, (3) the removal of nitrate 10 through assimilation by plants and microbes, and (4) the removal of nitrate through 11 denitrification by microbes. In addition to natural processes, anthropogenic processes can 12 have a significant impact on the sources and dynamics of nitrate within each catchment area, 13 particularly those with urban or agricultural catchment zones. Therefore, interpretation of the 14 processes regulating nitrate concentration in stream water is not always straightforward. 15

The ¹⁵N/¹⁴N and ¹⁸O/¹⁶O ratios of nitrate have been widely applied worldwide in the 16 determination of the sources and behaviours of nitrate in stream water (Durka et al., 1994; 17 18 Campbell et al., 2002; Silva et al., 2002; Barnes and Raymond, 2010; Nestler et al., 2011; 19 Lohse et al., 2013). By combining the two isotopic ratios, the relative mole fractions among 20 various nitrate sources such as atmospheric (unprocessed), fertiliser, manure, and sewage plants can be quantified through a simple isotope mass balance approach. Partial removal of 21 nitrate through either assimilation or denitrification, however, results in residual nitrate being 22 enriched with ¹⁵N and ¹⁸O (Böttcher et al., 1990; Granger et al., 2010), which complicates the 23 24 interpretation of the ratios beyond that of the simple isotope mass balance approach. In 25 addition, trace contributions of unprocessed NO_3 (atm) can have a significant impact on the ¹⁸O/¹⁶O ratios of the total nitrate in stream water (Durka et al., 1994; Kendall, 1998; Mayer et 26 al., 2001; Michalski et al., 2004; Tsunogai et al., 2010). Therefore, ¹⁸O/¹⁶O ratios are used as 27 tracers based on assumptions such as (1) the ${}^{18}O/{}^{16}O$ ratios of nitrate in stream water simply 28 reflect the mole fraction of unprocessed $NO_3^{-}(atm)$ within total nitrate (Durka et al., 1994; 29 Williard et al., 2001; Ohte et al., 2004; Campbell et al., 2006; Barnes et al., 2008; Burns et al., 30 2009; Ohte et al., 2010; Tobari et al., 2010; Thibodeau et al., 2013; Zeng and Wu, 2015), (2) 31 32 the mole fractions of unprocessed $NO_3^{-}(atm)$ within total nitrate are minimum for specific

samples (such as soil solution samples) studied (Hales et al., 2007), and (3) the mole fractions 1 2 of unprocessed NO₃ (atm) within total nitrate are uniform in the entire samples studied (Wankel et al., 2006; Johannsen et al., 2008). To verify the reliability of these assumptions 3 and to utilise the ¹⁸O/¹⁶O ratios for quantification of the mole fractions among various nitrate 4 sources based on the isotope mass balance approach, the mole fraction of NO₃⁻(atm) within 5 the total nitrate in stream water must be better understood based on more accurate and more 6 precise quantification rather than <u>on</u> traditional quantification using the ${}^{15}N/{}^{14}N$ and ${}^{18}O/{}^{16}O$ 7 8 ratios of nitrate.

To overcome the limitation in using the ${}^{15}N/{}^{14}N$ and ${}^{18}O/{}^{16}O$ ratios, the ${}^{17}O/{}^{16}O$ ratios of 9 10 nitrate have been used as an additional tracer of NO_3 (atm) in stream water in recent studies (Michalski et al., 2004; Tsunogai et al., 2010; Costa et al., 2011; Dejwakh et al., 2012; Riha et 11 al., 2014; Tsunogai et al., 2014; Rose et al., 2015). Because the oxygen atoms of NO₃ (re) are 12 derived from either terrestrial O₂ or H₂O through usual chemical reactions such as 13 nitrification, NO_3 (re) shows mass-dependent relative variations between ${}^{17}O/{}^{16}O$ and ${}^{18}O/{}^{16}O$ 14 ratios. On the other hand, only unprocessed $NO_3^{-}(atm)$ displays an anomalous enrichment in 15 16 ¹⁷O from the mass-dependent relative variations, reflecting oxygen atom transfers from ozone anomalously enriched in ¹⁷O during the conversion of NO_x to NO₃ (atm) (Michalski et al., 17 18 2003; Morin et al., 2008). By using the Δ^{17} O signature (the magnitude of 17 O excess) defined by the following equation (Miller, 2002; Kaiser et al., 2007), we can distinguish unprocessed 19 NO₃ (atm) (Δ^{17} O > 0) from NO₃ (re) (Δ^{17} O = 0): 20

21
$$\Delta^{17}O = \frac{1 + \delta^{17}O}{\left(1 + \delta^{18}O\right)^{\beta}} - 1,$$
 (1)

where the constant β is 0.5279 (Miller, 2002; Kaiser et al., 2007), $\delta^{18}O = R_{sample}/R_{standard} - 1$, and *R* is the ¹⁸O/¹⁶O ratio of the sample (or the ¹⁷O/¹⁶O ratio in the case of $\delta^{17}O$ or the ¹⁵N/¹⁴N ratio in the case of $\delta^{15}N$) and each standard reference material. Please note that all the nitrate other than <u>the unprocessed NO₃ (atm) is classified into NO₃ (re), including the nitrate</u> produced through natural/anthropogenic processes in <u>the biosphere/hydrosphere/geosphere</u> and <u>that</u> stored in soil, fertiliser, manure, sewage, etc.

In addition, Δ^{17} O is stable during the mass-dependent isotope fractionation processes within surface ecosystems. Therefore, although the atmospheric δ^{15} N or δ^{18} O signature can be overprinted by biogeochemical processes subsequent to deposition, Δ^{17} O can be used as a robust tracer of unprocessed NO₃⁻(atm) to reflect the accurate mole fraction of unprocessed NO₃⁻(atm) within total NO₃⁻ regardless of biogeochemical partial removal processes
 subsequent to deposition by using the following equation:

$$3 \qquad \frac{C_{atm}}{C_{total}} = \frac{\Delta^{17}O}{\Delta^{17}O_{atm}},$$
(2)

4 where C_{atm} and C_{total} denote the concentrations of NO₃⁻(atm) and NO₃⁻ in each water sample, 5 respectively, and $\Delta^{17}O_{atm}$ and $\Delta^{17}O$ denote the $\Delta^{17}O$ values of NO₃⁻(atm) and nitrate (total) in 6 each water sample, respectively. This is the primary merit of using the ¹⁷O/¹⁶O ratio as an 7 additional tracer of NO₃⁻(atm).

8 Moreover, additional measurements of the Δ^{17} O values of nitrate together with δ^{15} N and δ^{18} O 9 enable us to exclude the contribution of NO₃⁻(atm) in the determined δ^{15} N and δ^{18} O values 10 and to estimate the corrected δ^{15} N and δ^{18} O values (δ^{15} N_{re} and δ^{18} O_{re}, respectively) for 11 accurate evaluation of the source and behaviour of NO₃⁻(re) (Tsunogai et al., 2010; Tsunogai 12 et al., 2011; Dejwakh et al., 2012; Liu et al., 2013; Tsunogai et al., 2014; Riha et al., 2014), 13 including NO₃⁻(re) produced through anthropogenic processes. The details of the calculation 14 <u>are presented in Section 2.5</u>.

Previous studies have successfully applied the Δ^{17} O tracer to nitrate eluted from arid/semi-15 16 arid watersheds (Michalski et al., 2004; Dejwakh et al., 2012; Riha et al., 2014), forested 17 watersheds (Tsunogai et al., 2010; Tsunogai et al., 2014; Rose et al., 2015), and a large river basin (Liu et al., 2013) to determine mole fractions of unprocessed NO₃ (atm) in total nitrate 18 19 more accurately and precisely than ever before, in addition to the fate of the $NO_3^{-}(atm)$ that had been deposited into each watershed. However, relative changes in the source and fate of 20 NO_3 (atm) in accordance with the changes in land use of catchments have not been studied 21 thus far by using the Δ^{17} O tracer of nitrate. 22

23 In this study, we measured the concentrations and the stable isotopic compositions of nitrate including Δ^{17} O values for more than 30 streams flowing into a lake in Japan with catchments 24 of widely varying land uses within the same watershed, which includes urban, suburban, 25 agricultural (mostly rice paddies), and forested catchments. By using the Δ^{17} O tracer, we 26 quantified both spatial and temporal variations in the concentrations of both $NO_3^{-}(atm)$ and 27 28 NO_3 (re) in streams across the land use settings accurately and precisely to gain insight into the processes controlling the sources, transport, and fate of $NO_3^{-}(atm)$ and $NO_3^{-}(re)$ (Fig. 1). 29 Although NO_3 (re) increases during nitrification within each catchment area, NO_3 (atm) is 30

stable during nitrification, so we were able to evaluate the progress of nitrification within each 1 2 catchment area by using the changes in the concentrations of both $NO_3^{-}(atm)$ and $NO_3^{-}(re)$. In addition to those from the streams, we determined the concentrations and the stable isotopic 3 compositions of nitrate including Δ^{17} O values in precipitation (wet deposition) for comparison 4 to obtain accurate and precise mole fractions of both NO₃⁻(atm) and NO₃⁻(re) within nitrate 5 (total) in each stream. Moreover, by using both the estimated mole fractions of $NO_3^{-}(atm)$ in 6 nitrate (total) and the $\delta^{15}N$ and $\delta^{18}O$ values of NO₃ (atm), we estimated the $\delta^{15}N_{re}$ and $\delta^{18}O_{re}$ 7 8 values for accurate evaluation of the source and behaviour of NO₃ (re) in streams. Furthermore, we determined the concentrations and the stable isotopic compositions of nitrate 9 including Δ^{17} O values in an outflow river of the same lake to evaluate the influences of flow 10 stagnation in the lake on the concentrations of both NO3⁻(atm) and NO3⁻(re) by using the 11 differences between inflows and outflows (Fig. 1). The results presented herein increase our 12 13 understanding of the fate of $NO_3^{-}(atm)$ deposited onto land, particularly the fate of that deposited on urban/suburban and forested catchments (Fig. 1). 14

15

16 2 Experimental Section

17 2.1 Steam water samples

Lake Biwa, located in the central part of the Japanese Islands, is the largest freshwater lake in Japan (Fig. 2). It has a surface area of 670.4 km², a total catchment area of 3174 km², and annual precipitation of around 2000 mm. More than 120 streams flow into the lake, <u>but</u> the Seta River (No. 33 in Fig. 2(b)) at the southern end <u>of the lake</u>, also known as the Yodo River, is the only natural outflow. The average residence time of water in the lake is 5.5 years.

23 Similar to many lakes throughout the world, Lake Biwa has experienced eutrophication in the 24 past. Urbanisation near the lake, beginning in the 1960s, particularly on the southern and 25 eastern shores, likely caused an increase in nutrient loading. Blooms of Uroglena americana and cyanobacteria have occurred since 1977 and 1983, respectively (Hsieh et al., 2011). To 26 clarify the pathways and sources of nitrate that was fed into the lake, the stable isotopic 27 compositions (δ^{15} N and δ^{18} O) of dissolved nitrate were determined in the major streams 28 flowing into the lake (Ohte et al., 2010). Based on the δ^{15} N values of nitrate showing positive 29 30 correlation with the population densities of each catchment area, it was concluded that sewage effluent was the dominant source contributing to the increase in the δ^{15} N values of nitrate. 31

In this study, stream water samples were collected near the mouths of <u>33 inflow streams and 1</u> 1 outflow river (Seta River) of Lake Biwa (Table 1; Fig. 2(b)) during base flow periods four 2 times in 2013, on March 15, June 17, August 5, and October 21, except for stream Nos. 3 and 3 28 in June, which became dry arroyos at that time. The catchments of the studied inflow 4 5 streams occupied 70% of the entire Lake Biwa basin area. The streams were selected to cover those in which the concentrations and stable isotope compositions of nitrate, $\delta^{15}N$ and $\delta^{18}O$, 6 7 had already been determined in 2004–2006 (Ohte et al., 2010). The categories of locations 8 classified by Ohte et al. (2010) were also used in this study to classify the location of each 9 stream (Table 1). Either a bucket or dipper was used to collect samples as far from the bank as 10 possible. Each sample was transferred into a dark polyethylene bottle that was pre-rinsed at 11 least twice with the sample itself and subsequently stored in a refrigerator. Then, the samples 12 were filtered through a pre-combusted Whatman GF/F filter with a 0.7 µm pore size within a 13 few hours after collection, and the filtrate was stored in a different dark polyethylene bottle at 4°C until analysis. 14

15 In this study, we defined the sampling number n, where n = 1, 2, 3, and 4, which represents 16 the sampling in March, June, August, and October, respectively. In addition, we defined one 17 more hypothetical sampling number (n = 5) set just one year later than the n = 1 date. Please 18 note that there are no data for sampling n = 5. Furthermore, we rated the intervals between n = 19 1 and n = 2, n = 2 and n = 3, n = 3 and n = 4, and n = 4 and n = 5 as spring, summer, autumn, 20 and winter, respectively, for the streams in this study.

21 2.2 Wet deposition samples

The Sado-seki National Acid Rain Monitoring Station (38°14'59"N, 138°24'00"E) was 22 established on Sado Island (Fig. 2(a)), at 110 m above sea level, as a monitoring observatory 23 24 of the Acid Deposition Monitoring Network in East Asia (EANET) representing the central 25 Japan area (EANET₂ 2014). Samples of wet deposition were taken at the station by using 26 standard methods for evaluating acid deposition in Japan for the three Japanese financial years (FYs) from April 2009 to March 2012. An automatic wet deposition sampler (US-420, 27 Ogasawara) was used in the collection. All of the deposition samples were stored in 1 L 28 polyethylene bottles under refrigeration until daily recovery. After measuring the volume (i.e. 29 30 precipitation rate), conductivity, and pH, the recovered samples were filtered through a 0.2 µm pore-size membrane filter (Dismic-25CS, ADVANTEC) and stored in a refrigerator until 31 32 analysis.

1 The annual wet deposition rate of nitrate was 19.3 mmol $m^{-2}y^{-1}$ for FY2009, from April 2009 2 to March 2010; 28.0 mmol $m^{-2}y^{-1}$ for FY2010, from April 2010 to March 2011; 27.0 mmol 3 $m^{-2}y^{-1}$ for FY2011, from April 2011 to March 2012; and 24.5 mmol $m^{-2}y^{-1}$ on average from 4 FY2009 to 2011 (EANET, 2014). The annual wet deposition rate of NH₄⁺ was 17.1 mmol 5 $m^{-2}y^{-1}$ on average from FY2009 to 2011 (EANET, 2014).

6 2.3 Analysis

The concentrations of nitrate (NO_3^-) and nitrite (NO_2^-) in each filtrate sample were measured 7 8 by ion chromatography (Prominence HIC-SP, Shimadzu, Japan) within a few days (stream 9 water samples) and within two weeks (wet deposition samples) after each sampling. The error (standard error of the mean) in the determined concentrations of nitrate was $\pm 3\%$. The δ^{18} O 10 values of H₂O in the samples were analysed using the cavity ring-down spectroscopy method 11 by employing an L2120-i instrument (Picarro Inc., Santa Clara, CA, USA) equipped with an 12 13 A0211 vaporizer and auto sampler; the error (standard error of the mean) in this method was 14 ±0.1‰. Both Vienna Standard Mean Ocean Water (VSMOW) and Standard Light Antarctic Precipitation (SLAP) were used to calibrate the values to the international scale. 15

To determine the stable isotopic compositions, nitrate in each filtrate sample was chemically 16 converted to N₂O by using a method originally developed to determine the ¹⁵N/¹⁴N and 17 ¹⁸O/¹⁶O ratios of seawater and freshwater nitrate (McIlvin and Altabet, 2005) and that was 18 19 later modified (Tsunogai et al., 2008; Konno et al., 2010; Yamazaki et al., 2011). In brief, the procedures were as follows. Approximately 10 mL of each sample solution was pipetted into 20 21 a vial with a septum cap. Then, 0.5 g of spongy cadmium was added, followed by 150 µL of a 1 M NaHCO₃ solution. The sample was then shaken for 18–24 h at a rate of 2 cycles/s. Then, 22 the sample solution was decanted into a different vial with a septum cap. After purging the 23 solution using high purity helium, 0.4 mL of the azide/acetic acid buffer was added. After 45 24 min, the solution was made basic by adding 0.2 mL of 6 M NaOH. 25

26 Then, the stable isotopic compositions (δ^{15} N, δ^{18} O, and Δ^{17} O) of N₂O in each vial were 27 determined by using a continuous-flow isotope ratio mass spectrometry (CF-IRMS) system at 28 Nagoya University. The analytical procedures using the CF-IRMS system were the same as 29 those detailed in previous research (Komatsu et al., 2008; Hirota et al., 2010). The obtained 30 values of δ^{15} N, δ^{18} O, and Δ^{17} O for N₂O derived from the nitrate in each sample were 31 compared with those derived from our local laboratory nitrate standards that had been calibrated using the internationally distributed isotope reference materials to calibrate the values of the sample nitrate to an international scale and to correct for both the isotope fractionation during the chemical conversion to N_2O and the progress of oxygen isotope exchange between the nitrate-derived reaction intermediate and water (ca. 20%). In this study, we adopted the internal standard method (Nakagawa et al., 2013; Tsunogai et al., 2014) for the calibrations of sample nitrate. All values in this paper are expressed relative to air (for nitrogen) and VSMOW (for oxygen).

To determine whether samples were deteriorated or contaminated during storage and whether 8 the conversion rate from nitrate to N₂O was sufficient, concentrations of nitrate in the samples 9 were determined each time we analysed isotopic compositions using CF-IRMS based on the 10 N_2O^+ or O_2^+ outputs. We adopted the $\delta^{15}N$, $\delta^{18}O$, or $\Delta^{17}O$ values only when concentrations 11 measured by CF-IRMS correlated with those measured by ion chromatography just after the 12 13 sampling within a difference of 10%. About 10% of the whole isotope analyses showed 14 conversion efficiencies lower than this <u>criterion</u>. Nitrate in these samples was converted to 15 N₂O again and re-analysed for the stable isotopic compositions. None of the samples showed significant nitrate deterioration or nitrate contamination during storage. 16

We repeated the analyses of the δ^{15} N, δ^{18} O, and Δ^{17} O values of nitrate for each sample at least 17 three times to attain high precision. Most of the samples had a nitrate concentration of more 18 than 5.0 μ mol L⁻¹, which corresponded to <u>a nitrate quantity</u> greater than 50 nmol in a 10 mL 19 sample. This amount was sufficient for determining the δ^{15} N, δ^{18} O, and Δ^{17} O values with high 20 precision. For cases of nitrate concentration less than 5.0 μ mol L⁻¹, the sample volume was 21 increased to 30 mL and the number of analyses was also increased. Thus, all isotopic data 22 presented in this study have an error (standard error of the mean) better than $\pm 0.2\%$ for $\delta^{15}N$, 23 $\pm 0.3\%$ for δ^{18} O, and $\pm 0.1\%$ for Δ^{17} O. 24

25 Nitrite (NO_2^{-}) in the samples interferes with the final N₂O produced from nitrate (NO_3^{-}) because the chemical method also converts NO₂⁻ to N₂O (McIlvin and Altabet, 2005). 26 Therefore, it is sometimes necessary to correct for the contribution of NO_2^{-} -derived N₂O to 27 determine the stable isotopic compositions of the sample nitrate accurately. However, in this 28 study, more than 90% of the samples analysed for stable isotopic compositions had NO₂⁻ 29 concentrations lower than the detection limit (0.05 μ mol L⁻¹). Even for the samples having 30 NO_2^- concentrations higher than the detection limit, the NO_2^-/NO_3^- ratio was less than 1%. 31 Thus, in this study, the results were used with no correction. 32

2.4 Calculating average concentration and isotopic compositions in each stream

To clarify the chemical and isotopic characteristics of each stream, we determined both the flow-weighted annual average concentration (\overline{C}_{total}) and <u>the</u> flow-weighted annual average $\delta^{15}N$, $\delta^{18}O$, and $\Delta^{17}O$ values ($\overline{\delta}$) of nitrate for each stream assuming the same flow rate, the same nitrate concentration, and the same isotopic compositions for the interval until the next observation by using Eqs. (3), (4), and (5):

8
$$q = \sum_{n=1}^{4} \left(f_n \cdot \Delta t_n \right), \tag{3}$$

9
$$\overline{C}_{\text{total}} = \frac{\sum_{n=1}^{4} (C_n \cdot f_n \cdot \Delta t_n)}{q},$$
 (4)

$$10 \qquad \overline{\delta} = \frac{\sum_{n=1}^{4} \left(\delta_n \cdot C_n \cdot f_n \cdot \Delta t_n \right)}{\sum_{n=1}^{4} \left(C_n \cdot f_n \cdot \Delta t_n \right)}, \tag{5}$$

11 where C_n and δ_n denote the concentration (C_{total} in Eq. (2)) and isotopic values ($\delta^{15}N$, $\delta^{18}O$, or 12 $\Delta^{17}O$) of nitrate in each stream during each observation n, respectively; f_n denotes the flow 13 rate of each stream during each observation n; and Δt_n denotes the time interval between the 14 observation n and the next observation n+1. When possible, we used the flow rate of each 15 stream <u>that</u> was determined monthly by the Shiga Prefecture (Shiga_prefecture, 2015) for f_n . 16 For small streams with no data for flow rate (n = 13), we used a small and stable flow rate of 17 0.1 m³/s for f_n .

18 2.5 Calculating δ^{15} N and δ^{18} O of remineralized nitrate

To exclude the contribution of $NO_3^{-}(atm)$ from the $\delta^{15}N$ and $\delta^{18}O$ values of nitrate (total) and to clarify the sources and behaviour of $NO_3^{-}(re)$ by using both $\delta^{15}N$ and $\delta^{18}O$ as tracers, we estimated the end-member $\delta^{15}N$ and $\delta^{18}O$ values of the remineralized nitrate portion, $\delta^{15}N_{re}$ and $\delta^{18}O_{re}$, by excluding the contribution of $NO_3^{-}(atm)$ in nitrate (total) (Tsunogai et al., 2010; Tsunogai et al., 2011; Dejwakh et al., 2012; Liu et al., 2013; Tsunogai et al., 2014; Riha et al., 2014) by using Eqs. (6) and (7):

$$1 \qquad \delta^{15} N_{re} = \frac{C_{total} \cdot \delta^{15} N - C_{atm} \cdot \delta^{15} N_{atm}}{C_{total} - C_{atm}}, \tag{6}$$

$$2 \qquad \delta^{18} O_{re} = \frac{C_{total} \cdot \delta^{18} O - C_{atm} \cdot \delta^{18} O_{atm}}{C_{total} - C_{atm}}, \tag{7}$$

where C_{atm} and C_{total} denote the concentrations of NO₃⁻(atm) and nitrate (total) in each water 3 sample, respectively, and $\delta^{15}N_{atm}$, $\delta^{18}O_{atm}$, and $\Delta^{17}O_{atm}$ denote the $\delta^{15}N$, $\delta^{18}O$, and $\Delta^{17}O$ values 4 of NO₃ (atm) in each sample, respectively. The actual values of $\delta^{15}N_{atm}$, $\delta^{18}O_{atm}$, and $\Delta^{17}O_{atm}$ 5 used in this study were determined from the δ^{15} N, δ^{18} O, and Δ^{17} O values of nitrate in the wet 6 7 deposition samples and are reported in Section 3.1 along with the ranges of errors. Please note that the errors in the estimated values of $\delta^{15}N_{re}$ and $\delta^{18}O_{re}$ become larger in accordance with 8 an increase in the C_{atm}/C_{total} ratio due to the propagation law of errors, even <u>if</u> the errors in the 9 values of $\delta^{15}N_{atm}$ and $\delta^{18}O_{atm}$ are the same. 10

11 **2.6** Possible variations in Δ^{17} O during the progress of partial removal and 12 mixing

13 Because we used the power law shown in Eq. (1) for the definition of Δ^{17} O, the Δ^{17} O values 14 are different from those based on the linear definition (Michalski et al., 2002). Please note that 15 our Δ^{17} O values would be (0.1±0.1)‰ higher for the stream water nitrate and (0.9±0.3)‰ 16 higher for the atmospheric nitrate if we had used the linear definition for calculation.

Compared with Δ^{17} O values based on the linear definition, Δ^{17} O values based on the power 17 law definition are more stable during mass-dependent isotope fractionation processes, so we 18 rated the Δ^{17} O values of nitrate as always stable irrespective of any biogeochemical partial 19 20 removal processes subsequent to deposition, such as assimilation or denitrification. On the other hand, Δ^{17} O values based on the power law definition are not conserved during mixing 21 processes between <u>fractions</u> having different Δ^{17} O values, so the C_{atm}/C_{total} ratios estimated 22 using Eq. (2) are somewhat deviated from the actual C_{atm}/C_{total} ratios in the samples. However, 23 in this study, the extent of the deviations of the Catm/Ctotal ratios of the stream nitrate was less 24 than 0.15%, so we disregard this effect in the discussion. 25

1 **3 Results and Discussion**

3.1 Atmospheric <u>n</u>itrate

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The δ^{15} N, δ^{18} O, and Δ^{17} O of atmospheric nitrate NO₃⁻(atm) are shown in Figs. 3(a–c), respectively, as a function of sampling day (local time, UT +_9:00), and the daily depositional flux of NO₃⁻(atm) when each of the wet deposition samples was taken is also shown in Fig. 3(c). The daily depositional flux of NO₃⁻(atm) was calculated from the nitrate concentration and the daily precipitation (Sup. Table S2).

The atmospheric nitrate at the Sado-seki monitoring station showed large ¹⁷O excesses with 8 Δ^{17} O values from +18.6‰ to +32.4‰. Moreover, a clear normal correlation between Δ^{17} O 9 and δ^{18} O was shown ($r^2 = 0.878$) (Fig. 4). A similar trend was reported for atmospheric nitrate 10 aerosols collected for a one-vear period in La Jolla, California (32.7°N, 117.2°W) (Michalski 11 et al., 2003), and similar results also have been obtained in other areas of the world (Kaiser et 12 al., 2007; Morin et al., 2009). Michalski et al. (2003) interpreted that the linear correlation 13 corresponds to the mixing line between tropospheric ozone and tropospheric H₂O, and thus 14 tropospheric OH radicals, with $\Delta^{17}O = 0\%$ and $\delta^{18}O = -5\%$. However, the NO₃^{-(atm)} data 15 obtained at <u>the</u> Sado-seki monitoring station showed a somewhat different trend in the $\Delta^{17}O-$ 16 δ^{18} O plot between summer, from May to October, and winter, from November to April (Fig. 17 4). Although the line fitted to the summer data showed a slope of 2.21 ± 0.22 and an intercept 18 of (+19.7±5.1)‰ in the $\Delta^{17}O-\delta^{18}O$ plot, that of the winter data showed a statistically 19 significant larger slope of 2.89 ± 0.38 and a smaller intercept of $(+3.0\pm9.2)$ %; all errors were 20 in the 2 σ range. Although the winter data included an intercept of -5% reported by 21 Michalski et al. (2003) as the end-member δ^{18} O value of the tropospheric OH radical within 22 the possible error range, the intercept of summer data deviated strongly from the value. 23 24 Because the monitoring station is located in the Asian monsoon area, the major air mass that arrived at the station was different seasonally: Pacific air originated from south-east was 25 26 dominant in summer, whereas continental air originated from the north-west was dominant in winter. The present results imply seasonal and regional changes in the $\delta^{18}O/\Delta^{17}O$ ratios of 27 tropospheric ozone and the OH radical. 28

29 On the basis of both the temporal variation in the daily depositional flux of NO₃⁻(atm), shown 30 in Fig. 3(c), and the Δ^{17} O value, we estimated the monthly average Δ^{17} O value of NO₃⁻(atm) 1 $(\Delta^{17}O(m))$ deposited at <u>the Sado-seki monitoring station for each month (m) from April 2009</u> 2 to March 2012 by using

3
$$\Delta^{17}O(m) = \frac{\sum_{k} \left(C_{k} \cdot V_{k} \cdot \Delta^{17}O_{k} \right)}{\sum_{k} \left(C_{k} \cdot V_{k} \right)},$$
(8)

4 where C_k denotes the concentration of nitrate in each wet deposition sample and V_k denotes 5 the total water volume of each wet deposition sample. Then, we estimated the annual and <u>the</u> 6 three_year average $\Delta^{17}O$ values of NO₃⁻(atm) ($\Delta^{17}O_{avg}$) as +25.5‰ for FY2009, +27.2‰ for 7 FY2010, +25.7‰ for FY2011, and +26.3‰ for the three years by using

8
$$\Delta^{17}O_{avg} = \frac{\sum_{m} \left(D(m) \cdot \Delta^{17}O(m) \right)}{\sum_{m} D(m)},$$
(9)

9 where D(m) denotes the monthly wet deposition rate of nitrate at the Sado-seki monitoring 10 station determined by EANET (EANET, 2014). Because no wet deposition sample for 11 measuring stable isotopes was taken in May 2009 or March 2012, we used the $\Delta^{17}O(m)$ 12 values of May 2010 and March 2011, respectively, for these values. Substituting $\Delta^{17}O$ with 13 $\delta^{15}N$ ($\delta^{18}O$) in Eqs. (8) and (9), we estimated $\delta^{15}N_{avg}$ ($\delta^{18}O_{avg}$) as -4.4% (+78.5‰) for 14 FY2009, -3.8% (+81.8‰) for FY2010, -4.4% (+78.6‰) for FY2011, and -4.2% (+79.8‰) 15 for the three years.

To apply the $\Delta^{17}O_{avg}$ values for the three years obtained at <u>the</u> Sado-seki monitoring station as 16 Δ^{17} O of NO₃ (atm) deposited on the studied watershed (i.e. Δ^{17} O_{atm} in Eq. (2)), additional 17 corrections could be needed because the Δ^{17} O value of NO₃ (atm) is a function of the NO_x 18 oxidation channels in the atmosphere, which shift depending on the intensity of sunlight, 19 20 temperature, and oxidant levels (e.g. Michalski et al., 2003; Morin et al., 2008; Kunasek et al., 2008; Alexander et al., 2009; Morin et al., 2012; Savarino et al., 2013). The latitudinal 21 22 difference between the Sado-seki monitoring station (38°15'N, 138°24'E; Fig. 2) and the 23 watershed studied (35°15'N, 136°5'E; Fig. 2) could change the intensity of sunlight and thus the NO_x oxidation channel. Moreover, Tsunogai et al. (2010) reported that nitrate in polluted 24 air masses derived directly from megacities in winter showed slightly larger Δ^{17} O values than 25 26 nitrate in background air masses in the same seasons owing likely to the relative increase in the reaction via NO₃ radicals within the entire NO_3 (atm) production channel to produce 27 NO₃ (atm) in the polluted air mass. The annual average Δ^{17} O value determined in this study 28

1 was lowest in FY2009 when the deposition rate <u>of nitrate</u> was the smallest, at 19.3 mmol 2 $m^{-2}y^{-1}$ (EANET, 2014), whereas the annual average $\Delta^{17}O$ value was highest in FY2010 when 3 the deposition rate was the largest, at 28.0 mmol $m^{-2}y^{-1}$, within the three years of observation. 4 These results also imply that the difference in the deposition rate of nitrate must be also 5 corrected to apply the $\Delta^{17}O_{avg}$ value to $\Delta^{17}O_{atm}$.

Nevertheless, both the annual average and the seasonal variation range of $\Delta^{17}O$ correlated 6 strongly with those determined at the Rishiri monitoring station (45°07'11"N, 141°12'33"E; 7 Fig. 2(a)) in FY2008, at +26.2‰ (Tsunogai et al. 2010), where the wet deposition rate of 8 9 NO_3 (atm) was an average of 40% smaller than that at the Sado-seki monitoring station from 2000 to 2013 (EANET, 2014). Moreover, the values also coincided with those reported for 10 mid-latitudes, such as at La Jolla, at 33°N (Michalski et al. 2003) and at Princeton, at 40°N 11 (Kaiser et al. 2007). We concluded that by allowing 1‰ of error, the standard deviation of the 12 $\Delta^{17}O_{avg}$ values determined <u>at</u> the four different monitoring stations located <u>with</u>in the same 13 mid-latitude range in the past, the obtained $\Delta^{17}O_{avg}$ value of NO₃⁻(atm) can be considered 14 representative for middle latitudes worldwide, including the Lake Biwa watershed basin. 15

In addition, the actual $\Delta^{17}O_{atm}$ values of NO₃ (atm) in each stream water sample can differ 16 from the $\Delta^{17}O_{avg}$ owing to the seasonal variation in the $\Delta^{17}O$ values of NO₃ (atm). In 17 correcting for the seasonal variation, however, it is not adequate to use the $\Delta^{17}O$ values 18 determined for the seasons of sampling, as $\Delta^{17}O_{atm}$ in Eq. (2), because the residence time of 19 water is longer than a few months for most of the catchments in Japan with a humid temperate 20 21 climate (Takimoto et al., 1994; Kabeya et al., 2007). That is, the nitrate in base flow stream water had been stored previously in subsurface runoff and groundwater, for which seasonal 22 Δ^{17} O changes have not been found thus far (Tsunogai et al., 2010; Nakagawa et al., 2013). 23 We concluded that by allowing an additional 1.8% of error, the standard deviation of the six-24 25 month moving average of atmospheric nitrate determined at the Sado monitoring station in this study, the obtained $\Delta^{17}O_{avg}$ value of NO₃ (atm) represented those eluted from the Lake 26 27 Biwa watershed basin.

In summary, we used the three-year average Δ^{17} O value of NO₃⁻(atm) obtained at the Sadoseki monitoring station in this study ($\Delta^{17}O_{avg} = +26.3\%$) as the $\Delta^{17}O_{atm}$ in Eq. (2) to estimate C_{atm} in the streams of the Lake Biwa watershed basin by allowing an error range of 3‰, considering the factor changes of $\Delta^{17}O_{atm}$ from $\Delta^{17}O_{avg}$ described above. About 65% of all of 1 <u>the Δ^{17} O data of NO₃ (atm) obtained at the Sado-seki monitoring station were included in this</u> 2 range of (+26.3±3.0)‰.

In the case of the $\delta^{15}N$ and $\delta^{18}O$ of NO₃ (atm) in each stream water sample (i.e. $\delta^{15}N_{atm}$ and 3 $\delta^{18}O_{atm}$ in Eqs. (6) and (7)), the values differed further from $\delta^{15}N_{avg}$ and $\delta^{18}O_{avg}$ owing to 4 isotopic fractionation during partial removal subsequent to deposition. As a result, while using 5 the three-year average values of $\delta^{15}N$ ($\delta^{15}N_{avg} = -4.2\%$) and $\delta^{18}O$ ($\delta^{18}O_{avg} = +79.8\%$) as 6 $\delta^{15}N_{atm}$ and $\delta^{18}O_{atm}$, we assumed much a larger error range in the values, i.e. $\pm 10\%$ for both 7 $\delta^{15}N$ and $\delta^{18}O$, twice the enrichment factor during assimilation of nitrate. Because the 8 concentration of atmospheric nitrate would be reduced to e^{-2} of the original value if $\delta^{15}N$ and 9 δ^{18} O values increased +10% from their original values through assimilation, it might be 10 11 difficult to detect atmospheric nitrate within total nitrate. Of course, this estimation is less 12 reliable, and we further discuss the appropriateness of these error ranges in Section 3.3. Because of the small C_{atm}/C_{total} ratios of stream water of generally less than 7% (Section 3.2), 13 the error propagated to $\delta^{15}N_{re}$ and $\delta^{18}O_{re}$ was generally small, less than 1‰ and 2‰, 14 respectively, for most of the data presented in this study (Sup. Table S5). 15

16 **3.2 Stream nitrate overview**

The concentrations (C_{total}) and the δ^{15} N, δ^{18} O, and Δ^{17} O values of nitrate in the stream water 17 samples determined for each observation (n = 1, 2, 3, and 4) are presented in Figs. 5(a)_(d). 18 The annual average concentration (\overline{C}_{total}) and the annual average $\delta^{15}N$, $\delta^{18}O$, and $\Delta^{17}O$ values (19 $\overline{\delta^{15}N}, \overline{\delta^{18}O}$, and $\overline{\Delta^{17}O}$, respectively) in each stream estimated by using Eqs. (3), (4), and (5) are 20 shown in the figure as black bars. In this figure, each stream was plotted on the x-axis in the 21 22 order of location, beginning from stream No. 31, which lies southwest of all of the streams (Fig. 2), and proceeding in a clockwise direction. The errors are comparable to the sizes of the 23 symbols in Figs. 5 (a)_(d). The spatially continuous variation of the values of $\overline{\delta^{15}N}$, $\overline{\delta^{18}O}$, and 24 $\overline{\Delta^{17}O}$ imply that the values may represent land use changes in each catchment area. 25

Although the Δ^{17} O values presented significant spatial and temporal variation from +0.0‰ to +6.8‰, the range of the $\overline{\Delta^{17}}$ O values from +0.5‰ to +3.1‰ was typical for nitrate in natural stream water (Michalski et al., 2004; Tsunogai et al., 2010; Liu et al., 2013; Tsunogai et al., 2014; Rose et al., 2015). These results correspond to mole fractions of unprocessed NO₃⁻(atm) to total nitrate from (1.8±0.3)% to (11.8±1.3)%, obtained by using Eq. (2). By using the concentration (C_{total}) and the Δ^{17} O values of nitrate, the NO₃⁻(atm) concentration (C_{atm}) was calculated using Eq. (2) and is plotted in Fig. 5(e). In addition, the annual average concentration of NO₃⁻(atm) (\overline{C}_{atm}) in stream nitrate was calculated and is also presented in Fig. 5(e) as black bars. Please note that the errors in C_{atm} and \overline{C}_{atm} are not presented in Figure 5(e) (See Sup. Table S4 for the respective ranges of errors).

To verify possible secular changes (i.e. long-term non-periodic variation), the estimated \overline{C}_{total} 6 $\overline{\delta^{15}N}$, and $\overline{\delta^{18}O}$ for each stream were compared with those determined by Ohte et al. (2010), in 7 which annual average concentration and annual average δ^{15} N and δ^{18} O values of nitrate (total) 8 were determined for the same streams in 2004 to 2006. Although both concentrations and 9 δ^{15} N and δ^{18} O values in the streams showed significant spatial and temporal variations during 10 2013, as presented in Fig. 5, the annual average values almost correlated with the values 11 determined in 2004 to 2006 (Sup. Fig. S1). The average differences from the values 12 determined in the streams in 2004 to 2006 were +5.3 μ mol L⁻¹ for \overline{C}_{total} , +0.6% for $\overline{\delta}^{15}N$, and 13 +1.6% for $\overline{\delta^{18}O}$, whereas the standard deviation ranges of the differences were 14.9 μ mol L⁻¹ 14 for \overline{C}_{total} , 1.6‰ for $\overline{\delta^{15}N}$, and 2.1‰ for $\overline{\delta^{18}O}$. That is, the differences from the values 15 determined in 2004 to 2006 were smaller than their standard deviation ranges, so the 16 differences were not significant. We concluded that secular changes were minimal for nitrate 17 in the streams, at least for the most recent 10-year period of observations. 18

19 **3.3**

.3 Relationship between Δ^{17} O and δ^{18} O

One of the features in the spatial variation shown in Fig. 5 is the positive correlation between $\Delta^{17}O$ and $\delta^{18}O$. As <u>is</u> clearly represented by the relationship between $\overline{\Delta^{17}O}$ and $\overline{\delta^{18}O}$ (Fig. 6), these values showed linear correlation with <u>an</u> r^2 value of 0.88. Because NO₃⁻(atm) is characterised by highly elevated values of both $\Delta^{17}O$ and $\delta^{18}O$ (Figs. 3 and 4), changes in the mole fraction of unprocessed NO₃⁻(atm) within the total nitrate pool must be responsible for the positive correlation between $\overline{\Delta^{17}O}$ and $\overline{\delta^{18}O}$ for nitrate in the streams.

The slope value of the least–squares-fitted line between $\overline{\Delta^{17}O}$ and $\overline{\delta^{18}O}$ (Fig. 6) also supports this hypothesis. By extrapolating the least–squares-fitted line to the region of NO₃⁻(atm) having a $\Delta^{17}O$ value of +26.3‰, we obtained $\delta^{18}O = (+86\pm7)\%$, which also corresponds with the average $\delta^{18}O$ value of NO₃⁻(atm) of +79.8‰ obtained in Section 3.1. We concluded that the $\overline{\delta^{18}O}$ values also primarily reflect the mole fraction of NO₃⁻(atm) within nitrate.

Without Δ^{17} O data and without some assumptions, it was difficult to decide the major factor 1 controlling the δ^{18} O values of the stream nitrate. However, by adding Δ^{17} O data, as presented 2 above, it became apparent that the changes in the mole fraction of unprocessed $NO_3^{-}(atm)$ 3 within the total nitrate pool were primarily responsible for the δ^{18} O variation between the 4 streams. These results further support our hypothesis, presented in Section 1, that 5 interpretations on the stable isotopic compositions of nitrate ($\delta^{15}N$ and $\delta^{18}O$) made without 6 Δ^{17} O values can often <u>be</u> misleading. <u>When</u> using stable isotopic compositions, particularly 7 the δ^{18} O value, of nitrate in freshwater environments to trace its sources and fate, the 8 determination of Δ^{17} O values is essential. 9

By extrapolating the linear correlation between $\overline{\Delta^{17}O}$ and $\overline{\delta^{18}O}$ to $\overline{\Delta^{17}O} = 0\%$, we obtained the 10 δ^{18} O value of (-2.9±1.2)‰ as the average δ^{18} O value of the remineralized portion of nitrate 11 (NO₃⁻(re)) in the streams. Although the δ^{18} O value was substantially ¹⁸O-depleted compared 12 with that produced through microbial nitrification in soil during in vitro incubation 13 experiments in past studies (Mayer et al., 2001; Burns and Kendall, 2002; Spoelstra et al., 14 2007), it correlated strongly with the δ^{18} O values of NO₃ (re) determined recently by using 15 the linear relationship between Δ^{17} O and δ^{18} O of nitrate eluted from forested watersheds, such 16 as NO_3^{-} (re) in the groundwater of cool-temperate forested watersheds at (-4.2 ± 2.4) %, where 17 the $\delta^{18}O(H_2O)$ was around -13% (Tsunogai et al. 2010), and NO₃ (re) in stream water in a 18 cool-temperate forested watershed at (-3.6 ± 0.7) %, where the $\delta^{18}O(H_2O)$ was around -11% 19 (Tsunogai et al. 2014). Moreover, the δ^{18} O value of NO₃ (re) obtained in this study, 20 (-2.9 ± 1.2) %, is close to the possible lowermost δ^{18} O value of NO₃ (re) produced through 21 microbial nitrification under H_2O of (-7.8 ± 1.0) % (the average and the standard deviation of 22 the δ^{18} O values of H₂O of the streams; Sup. Table S3) (Buchwald et al., 2012). Furthermore, 23 the δ^{18} O value of NO₃⁻_{re} correlates strongly with that obtained through in vitro incubation 24 25 experiments in recent studies that simulated temperate forest soils (Fang et al., 2012). We concluded that the δ^{18} O value of NO₃ (re) produced through nitrification in the temperate 26 watershed having a $\delta^{18}O(H_2O)$ value of (-7.8 ± 1.0) % was (-2.9 ± 1.2) % and that we should 27 use such a low δ^{18} O value for the NO₃ (re) produced through nitrification in the watershed. 28 Understanding the relationship between Δ^{17} O and δ^{18} O of nitrate shown in Fig. 6 is highly 29 useful for determining the δ^{18} O value of NO₃ (re) in each watershed (Tsunogai et al. 2010). 30

31 Although the Δ^{17} O values of nitrate were stable during partial biogeochemical processing_a 32 such as partial removal through assimilation or denitrification, the δ^{18} O values of nitrate could

1 vary through the isotopic fractionation processes within each catchment area. Nevertheless, the δ^{18} O values of nitrate in the streams plotted on the mixing line between the NO₃ (atm) that 2 had been deposited in the watershed and NO₃⁻(re) having δ^{18} O and Δ^{17} O values close to those 3 produced through nitrification in the catchments. Thus, we concluded that the range of 4 isotopic fractionations owing to partial removal through assimilation or denitrification 5 6 subsequent to deposition of $NO_3^{-}(atm)$ or production of $NO_3^{-}(re)$ within each catchment area 7 was generally small for the major portion of nitrate eluted from the watershed. If such isotopic fractionations were significant for the portion of NO_3 (atm) in total nitrate, the data should 8 plot on the ¹⁸O-enriched side of Fig. 6, especially for those data enriched in $NO_3^{-}(atm)$ (i.e. 9 those showing high Δ^{17} O values). This result also supports our assumption in Section 3.1 that 10 the actual $\delta^{15}N$ and $\delta^{18}O$ values of NO₃ (atm) in each stream water sample ($\delta^{15}N_{atm}$ and 11 $\delta^{18}O_{atm}$ in Eqs. (6) and (7)) correlate with the $\delta^{15}N_{avg}$ and $\delta^{18}O_{avg}$ estimated at <u>the</u>Sado-seki 12 monitoring station within an error of $\pm 10\%$. 13

14 **3.4** δ^{15} N values of remineralized nitrate in streams

To trace the source of the ¹⁸O-depleted $NO_3^{-}(re)$ eluted from the watershed into the lake, the 15 annual average δ^{15} N and δ^{18} O values of the remineralized portion of nitrate ($\overline{\delta^{15}}$ N_{re} and $\overline{\delta^{18}}$ O_{re}) 16 in each inflow stream were estimated using Eqs. (6) and (7) and are plotted as a function of 17 18 population density in Fig. 7(c) and Fig. 7(d), respectively. The original δ^{18} O is also in Fig. 7(d). Because of the large δ^{18} O differences of about 80% between the nitrate in streams and 19 the NO₃⁻(atm), the $\delta^{18}O_{re}$ values were a few % lower than each original $\delta^{18}O$ value in total 20 nitrate (Fig. 7(d)). On the contrary, because of the small δ^{15} N differences of less than 15‰ 21 22 between the total nitrate in streams and NO_3 (atm), as well as the small C_{atm}/C_{total} ratios in the streams, most of the $\delta^{15}N_{re}$ values showed small deviations of less than 1‰ from each 23 corresponding original δ^{15} N value in the total nitrate in most of the streams, so the original 24 $\overline{\delta^{15}N}$ values are not presented in Fig. 7(c). Although the annual average $\overline{\delta^{18}O}_{re}$ values were 25 low and almost uniform, from -4.0% to -0.1%, as implied in the linear correlation between 26 $\overline{\Delta^{17}O}$ and $\overline{\delta^{18}O}$ in Fig. 6, $\overline{\delta^{15}N}_{re}$ showed larger variation from +1.7% to +10.9%. 27

Moreover, $\overline{\delta^{15}N_{re}}$ showed positive linear correlation with the population density in logarithmic scale (r²=0.64, p<0.001) (Fig. 7(c)). A similar trend was reported for the $\delta^{15}N$ values of total nitrate (= NO₃⁻(atm) + NO₃⁻(re)) in past studies in this watershed (Ohte et al., 2010) and others (Mayer et al., 2002). We further verified that the remineralized portion of nitrate

1 (NO₃⁻(re)) was responsible for the positive correlation between the δ^{15} N values of total nitrate 2 and population density that <u>has been found often in various streams in the world</u>.

Both the concentrations and the isotopic compositions shown in Fig. 7 clearly demonstrate 3 that most portions of the nitrate eluted from the catchments with lower population densities of 4 less than 100 km⁻², showing δ^{15} N values of (+4.4±1.8)‰ and δ^{18} O values of about 5 (-2.3 ± 0.9) %, were produced through nitrification in naturally occurring soil organic matter 6 7 (Kendall et al., 1995; Ohte et al., 2010). In the latter half of this section, we discuss the source of the ¹⁵N-enriched NO_3^{-} (re) eluted from the catchments with higher population densities of 8 more than 1000 km⁻², showing δ^{15} N values of (+9.2±1.3)‰ or more and δ^{18} O values of about 9 10 (-2.2 ± 1.1) %.

Denitrification in riverbed sediments adjacent to riparian zones or groundwater bodies 11 (McMahon and Böhlke, 1996) can increase the δ^{15} N value of stream nitrate. However, if such 12 post-production alternation were responsible for the ^{15}N enrichment of $NO_3^{-}(re)$ and thus the 13 total nitrate, the values of $\delta^{18}O_{re}$ in addition to those of $\delta^{15}N_{re}$ would be increased (Granger et 14 al., 2008). Moreover, the absolute concentration of NO3 (atm) (Catm) would decrease in 15 accordance with the progress of denitrification, but the observed C_{atm} was almost uniform 16 irrespective of population density. Of course, the initial C_{atm} could vary between the streams. 17 However, to explain the observed uniform Catm, unrealistic assumptions are needed for Catm, 18 such as the initial C_{atm} being higher in accordance with higher population density. The low 19 and uniform $\delta^{18}O_{re}$ values (Fig. 7(d)) as well as the uniform C_{atm} irrespective of population 20 density (Fig. 7(b)) imply that denitrification in riverbed sediments was minor for the nitrate in 21 the streams. Rather, the NO_3^{-} (re) must be enriched in ¹⁵N from its initial production through 22 nitrification within the catchments with high population densities. In addition, the small 23 differences in δ^{18} O values of NO₃ (re) between those values irrespective of the population 24 densities in the catchment area (Fig. 7(d)) imply that the essential parameters for determining 25 the δ^{18} O values of nitrate during nitrification, such as the δ^{18} O values of H₂O and the pH of 26 soils (Buchwald et al., 2012; Fang et al., 2012), should be similar among them. 27

Based on the $\delta^{15}N$ values of total nitrate eluted from catchments with high population densities, as well as the positive correlation between the $\delta^{15}N$ values of total nitrate and <u>the</u> population densities, Ohte et al. (2010) proposed sewage effluent as the dominant source contributing to the increase in the $\delta^{15}N$ values of total nitrate eluted from such catchments. The $\delta^{15}N$ and $\delta^{18}O$ values of NO₃⁻(re) newly estimated in this study, (+9.2±1.3)‰ or more

and (-2.2 ± 1.1) %, respectively, also imply that the dominant source contributing to the 1 increase in the δ^{15} N values of total nitrate had been produced through nitrification in which 2 the source N of the nitrate had already been enriched in ¹⁵N. Although the δ^{15} N and δ^{18} O 3 values of total nitrate in sewage effluent determined in past studies (Aravena et al., 1993; 4 Widory et al., 2005; Wankel et al., 2006; Xue et al., 2009) were a few % higher than the $\delta^{15}N$ 5 and δ^{18} O values of NO₃ (re) eluted from the high population density catchments, δ^{15} N_{re} = 6 $(+9.2\pm1.3)$ % and $\delta^{18}O_{re} = (-2.2\pm1.1)$ %, the slight deviations in the reported $\delta^{15}N$ and $\delta^{18}O_{re}$ 7 8 values from our results can be explained by several factors, such as (1) a slight contribution of 9 NO_3 (atm) and (2) the progress of denitrification subsequent to production. We concluded that sewage effluent was the most probable pollution source of nitrate to explain the observed 10 concentrations and isotopic compositions of nitrate eluted from the catchments with high 11 population densities, particularly for those of more than 1000 km^{-2} . 12

13 **3.5 Seasonal variation**

Although the annual average values of Δ^{17} O and δ^{18} O in each stream, $\overline{\Delta^{17}}$ O and $\overline{\delta^{18}}$ O. 14 respectively, showed linear correlation, as presented in Fig. 6, the same results were not 15 always attained for those in each season. Particularly for those values obtained during June 16 and August (i.e. summer), some of the streams showed significant deviations in δ^{18} O of more 17 than a few ‰ from the hypothetical mixing line between NO₃ (atm) ($\Delta^{17}O = +26.3$ ‰ and 18 $\delta^{18}O = +79.8$ ‰) and NO₃ (re) ($\Delta^{17}O = 0$ ‰ and $\delta^{18}O = -2.9$ ‰) (Fig. 8). Even though the 19 values of Δ^{17} O and δ^{18} O of NO₃ (atm) showed seasonal variation, as presented in Figs. 3 and 20 4, the large deviations from the mixing line <u>could not be explained</u> based on the seasonal 21 changes in NO₃ (atm). Rather, we must assume some seasonal changes in the biogeochemical 22 nitrogen cycles within each catchment area to explain the relationship, because unlike the 23 Δ^{17} O values, the δ^{18} O values of nitrate could vary during biogeochemical processing within 24 each catchment area. As a result, we can evaluate the seasonal changes in the biogeochemical 25 processing within each catchment area by using the seasonal changes in the relationship 26 between Δ^{17} O and δ^{18} O shown in Fig. 8. 27

The increases in the number of data deviated from the hypothetical mixing line, especially those plotted on the ¹⁸O-enriched region (i.e. vertically upward direction in the figures), by more than a few ‰ from the hypothetical mixing line in June and August imply that partial nitrate removal through assimilation or denitrification was active within each catchment area in <u>these months</u>. The spatial differences in the ¹⁸O enrichment also support this hypothesis. As
presented in Fig. 8 by the orange squares, ¹⁸O enrichment was common in samples obtained
<u>from</u> the southern streams having high population densities in <u>their</u> catchment areas. We can
assume elevated loading of both nutrients and organic matter of anthropogenic origin in these
catchments, both of which naturally enhance assimilation and denitrification.

6 On the contrary, most samples obtained during March and October were distributed on the hypothetical mixing line between $NO_3^{-}(atm)$ and $NO_3^{-}(re)$, as presented in Fig. 8. We 7 8 concluded that the range of isotopic fractionation subsequent to production, such as partial 9 removal through assimilation or denitrification, in winter was generally small for the major portion of nitrate eluted from the watershed and fed into the lake. Therefore, the annual 10 average values (i.e. $\overline{\delta^{18}O}$ and $\overline{\Delta^{17}O}$) of the streams distributed on the hypothetical mixing line, 11 12 as shown in Fig. 6, because the nitrate influx in winter occupied a major portion of the annual 13 nitrate influx. Active removal of nitrate from the streams through denitrification/assimilation 14 in summer was also responsible for the small relative importance of nitrate influx into the lake in summer. In conclusion, the relationship between $\Delta^{17}O$ and $\delta^{18}O$ of nitrate eluted from a 15 catchment area is a useful indicator for evaluating the biogeochemical processing within the 16 17 catchment area, including the seasonal change.

18 **3.6** Spatial and temporal Δ^{17} O variation

19 By using the δ^{18} O values of nitrate as <u>a</u> tracer, Ohte et al. (2010) found that the mole fractions 20 of unprocessed NO₃⁻(atm) within the total nitrate pool were high in the northern streams of 21 the watershed in winter, from November to late April. Our present results shown in Fig. 5 22 further verify the past results <u>by adding</u> more robust evidence through the use of the Δ^{17} O 23 tracer for NO₃⁻(atm).

Based on the high accumulation rate of snow in the catchment zones of the northern streams, 24 Ohte et al. (2010) concluded that high loading of unprocessed $NO_3^{-}(atm)$ via snow in the 25 26 catchment zones increased the stored unprocessed $NO_3^{-}(atm)$ in the snowpack, which was 27 subsequently released into the streams during the melting seasons. This process enhanced the 28 mole fraction of unprocessed $NO_3^{-}(atm)$ within the total nitrate pool during the melting 29 season, as was also reported for streams worldwide (Kendall et al., 1995; Ohte et al., 2004; 30 Piatek et al., 2005; Ohte et al., 2010; Pellerin et al., 2012; Tsunogai et al., 2014). However, the contribution of nitrate from anthropogenic sources could be smaller in this area because of 31

1 lower population densities in the catchments (Table 1). Because a major portion of the 2 possible anthropogenic nitrate in the catchments must be occupied by $NO_3^-(re)$ (Ohte et al., 3 2010), a lower $NO_3^-(re)$ supply from anthropogenic sources in each catchment area could 4 elevate the mole fraction of unprocessed $NO_3^-(atm)$ within the total nitrate pool, even if the 5 absolute concentration of $NO_3^-(atm)$ (C_{atm}) was uniform in the streams.

To determine the C_{atm} variability among the streams, the C_{atm} values estimated in this study were plotted as a function of population <u>density</u>, <u>as shown in Fig. 7(b)</u>. The C_{atm} was almost uniform at $(2.3\pm1.1)_{\mu}$ mol L⁻¹ irrespective of changes in the population density of <u>the</u> catchment areas. However, a clear C_{total} enrichment trend was noted in accordance with <u>increasing</u> population <u>density</u> of the catchments (Fig. 7(a)). Similar C_{total} enrichment trends have been reported in previous studies (Ohte et al., 2010).

The northern streams such as Nos. 3, 4, and 5 were enriched in Catm, showing Catm annual 12 average values of (5.3±1.1), (2.9±0.7), and (4.3±0.9) μ mol L⁻¹, respectively, and Δ^{17} O values 13 of +3.1%, +1.9%, and +2.9%, respectively. These results support the previous observation of 14 the streams determined by using the δ^{18} O tracer. Similar C_{atm} enrichment of about 3 µmol L⁻¹ 15 or more, however, was also found in streams in other areas, such as Nos. 14 ($C_{atm} = (3.3 \pm 1.0)$) 16 μ mol L⁻¹), 25 ((3.2±0.8) μ mol L⁻¹), and 21 ((4.2±0.9) μ mol L⁻¹), but these streams showed 17 lower Δ^{17} O values of +0.9‰, +1.5‰, and +2.0‰, respectively, and thus low mole fractions 18 19 of unprocessed NO_3 (atm) within total nitrate. We concluded that the difference in the 20 addition of anthropogenic nitrate composed of NO₃ (re) in the catchments was primarily 21 responsible for the difference in the mole fraction of unprocessed NO₃ (atm) within the total 22 nitrate pool, as well as the Ctotal variation in accordance with the population densities of the 23 catchment areas, as illustrated in Fig. 1. That is, a small contribution of anthropogenic nitrate in the catchments of the northern rivers was primarily responsible for the low C_{total} and thus 24 the high mole fraction of unprocessed $NO_3^{-}(atm)$ within the total nitrate pool, or the C_{atm}/C_{total} 25 ratio, in the northern streams of the watershed. 26

Although the difference in the accumulation rate of snow between each catchment zone was not the major factor controlling the C_{atm}/C_{total} ratios, the concentrated release of NO₃ (atm) stored in the snowpack during the melting seasons should be one of the important factors determining the C_{atm} variation among the streams. Most of the C_{atm} -enriched streams, such as Nos. 3, 4, 5, 14, and 25, originated from <u>a</u> forested catchment at <u>a</u> high elevation of more than 800 m above sea level; thus, we can anticipate heavy snowpack in winter <u>in</u> each headwater

1 region. Moreover, the maximum C_{atm} values in these streams were found in March, which is 2 the season of snowmelt (Fig. 5). On the contrary, most of the C_{atm}-depleted streams, such as Nos. 29 ((0.9±0.3) μ mol L⁻¹), 19 ((0.8±0.3) μ mol L⁻¹), 23 ((0.5±0.2) μ mol L⁻¹), and 30 3 $((0.6\pm0.2) \mu mol L^{-1})_{a}$ originated from low elevations having urban and suburban catchment 4 5 areas (Table 1). As a result, the concentrated release of stored $NO_3^{-}(atm)$ in the snowpack to 6 the forest floor in the catchment zone during the melting seasons is strongly responsible for 7 the C_{atm} enrichment of some of the streams, particularly that in the streams during the month 8 of March, as presented in Fig. 1.

9 The only exception is stream No. 21, located in the southernmost part of the watershed, which 10 showed a high annual average C_{atm} of (4.2±0.9) µmol L⁻¹. This small stream originates from a 11 low elevation of about 200 m and has a small catchment area of 4 km². In addition, although 12 the other C_{atm} -enriched streams showed the maximum C_{atm} in March, C_{atm} in stream No. 21 13 was highest in August, showing an extraordinarily high value of more than 10 µmol L⁻¹. It is 14 unlikely that NO₃⁻(atm) stored in the snowpack in winter was the major source of NO₃⁻(atm) 15 in this stream.

16 The catchment zone of stream No. 21 had the highest population density of the catchments of the streams studied (Table 1). About one-third of the catchment includes residential areas. 17 18 Artificial drainage systems in urban or residential areas and agricultural lands in humid 19 temperate regions are usually designed to drain rainwater efficiently into streams (Takimoto 20 et al., 1994). As a result, a significant portion of the NO_3 (atm) deposited into the catchment area was deposited onto paved surfaces and was then drained directly into the stream via 21 22 storm sewers without penetrating the ground. Thus, no interaction with soils occurred, as presented in Fig. 1. Because biogeochemical interactions within soils are the major sink for 23 NO_3 (atm) and thus for ¹⁷O excess of nitrate (Nakagawa et al., 2013; Tsunogai et al., 2014), 24 25 the development of such sewage systems in urban/suburban areas is largely responsible for 26 the high C_{atm} in stream No. 21. Similar bypassing effects of NO₃ (atm) from soil contact by paved surfaces have been suggested in urban/suburban watersheds by using $\delta^{18}O$ values of 27 nitrate as a tracer (Burns et al., 2009; Kaushal et al., 2011). We further verified that the 28 sewage systems in urban/suburban catchments changed the fate of the $NO_3^{-}(atm)$ deposited 29 30 onto land to some extent.

The observed uniform C_{atm} irrespective of population <u>density</u> and headwater elevation shown in Fig. 7 implies that the influences of <u>snowpacks</u> and paved surfaces were still minor in determining the C_{atm} values in the streams. Rather, the observed stable C_{atm} implies that most of <u>the NO₃ (atm)</u> in the streams had been stored in groundwater/subsurface runoff in the watershed, <u>which had similar C_{atm} concentrations</u> and then gushed to the surface at <u>the</u> respective headwater zones <u>with</u> various elevations and land uses as presented in Fig. 1.

5 When using the δ^{18} O tracer, it was difficult to determine the precise absolute concentration of 6 NO₃⁻(atm) (C_{atm}) in each stream water_a as presented in this study_a and to determine whether 7 the absolute concentration of NO₃⁻(atm) was stable among the streams. <u>However, by</u> using 8 the Δ^{17} O values, we can determine the precise C_{atm} in each stream for each season <u>and</u> thus 9 clarify the fate of NO₃⁻(atm).

10 **3.7** Differences in outflows from inflows

The concentrations and δ^{15} N, δ^{18} O, and Δ^{17} O values of nitrate in the outflow river (Seta River; 11 No. 33) are also presented in Fig. 5. In a manner similar to the inflow streams (i.e. by using 12 Eqs. (3) to (5)), we estimated the annual average concentration of total nitrate in the outflow 13 river (\overline{C}_{total}) to be 13.3 µmol L⁻¹, the annual average $\delta^{15}N$ value ($\overline{\delta^{15}N}$) to be +13.1‰, the 14 annual average δ^{18} O value ($\overline{\delta^{18}O}$) to be +1.5‰, and the annual average Δ^{17} O value ($\overline{\Delta^{17}O}$) to 15 16 be +0.9‰, as presented in Fig. 5. Moreover, in a manner similar to that used for the inflow streams (i.e. by using Eqs. (2), (6), and (7)), we estimated the annual average concentration of 17 NO₃⁻(atm) in the outflow river (\overline{C}_{atm}) to be (0.4±0.1) µmol L⁻¹ (Fig. 5(e)), the annual average 18 $\delta^{15}N_{re}$ value ($\overline{\delta^{15}N_{re}}$) to be (+13.7±0.6)‰, and the annual average $\delta^{18}O_{re}$ value ($\overline{\delta^{18}O_{re}}$) to be 19 (-1.2 ± 0.9) % (Sup. Table S5). Similar to <u>those</u> for inflows, the Δ^{17} O values were typical for 20 21 nitrate in natural stream waters.

The striking features of the outflow in comparison with the inflows were the depletions in the 22 outflow of both C_{total} and C_{atm} as well as the enrichment in ¹⁵N (Fig. 5). Because the 23 denitrification/assimilation processes remove both nitrate and NO_3 (atm) and preferentially 24 consume ¹⁴N during the removal, the process of denitrification/assimilation in the lake water 25 column can be strongly responsible for the removal of both nitrate and NO₃ (atm) and for the 26 ¹⁵N enrichment of nitrate in the outflow compared with the inflow. If this were the case in 27 Lake Biwa, the total nitrate efflux would have to be smaller than the total nitrate influx. To 28 29 verify this hypothesis quantitatively and to evaluate the influences of the stagnant flow in the 30 lake on nitrate, we estimated the total influx through all of the inflow streams for nitrate and NO₃ (atm), ΔN_{in} , and ΔA_{in} , respectively, and the total efflux for nitrate and NO₃ (atm), ΔN_{out} , 31

1 and ΔA_{out} , respectively, as well as the flow-weighted average $\delta^{15}N$, $\delta^{18}O$, and $\Delta^{17}O$ values of

- 2 all inflows and outflows to discuss their changes in the lake.
- 3 The ΔN_{in} and ΔA_{in} in each interval between the observation n and the next observation n + 1
- 4 (i.e. each season) and the flow-weighted average $\delta^{15}N$, $\delta^{18}O$, and $\Delta^{17}O$ values of the inflows 5 ($\delta(n)$) during each interval between the observation n and the next observation n + 1 were 6 determined by using the following equations, assuming the same flow rate, the same nitrate
- 7 concentration, and the same isotopic compositions for the interval until the next observation:

$$8 \qquad \alpha = \frac{Q_{in}}{\sum_{i} q_{i}},\tag{10}$$

9
$$\Delta N_{in}(n) = \sum_{i} C_{i} \cdot f_{i} \cdot \Delta t_{i} \cdot \alpha, \qquad (11)$$

$$10 \qquad \delta(\mathbf{n}) = \frac{\sum_{i} \delta_{i} \cdot \mathbf{C}_{i} \cdot \mathbf{f}_{i} \cdot \Delta \mathbf{t}_{i}}{\sum_{i} \mathbf{C}_{i} \cdot \mathbf{f}_{i} \cdot \Delta \mathbf{t}_{i}}, \tag{12}$$

11
$$\Delta A_{in}(n) = \Delta N_{in}(n) \cdot \frac{\Delta^{17}O_{in}(n)}{\Delta^{17}O_{atm}},$$
(13)

12
$$\Delta N_{in} = \sum_{n=1}^{4} \Delta N_{in}(n), \qquad (14)$$

13
$$\Delta A_{in} = \sum_{n=1}^{4} \Delta A_{in}(n), \qquad (15)$$

14
$$\delta = \frac{\sum_{n=1}^{2} \delta(n) \cdot \Delta N_{in}(n)}{\sum_{n=1}^{4} \Delta N_{in}(n)},$$
(16)

where Q_{in} denotes the annual gross influx of water into the lake; C_i and δ_i denote the concentration and isotopic values ($\delta^{15}N$, $\delta^{18}O$, or $\Delta^{17}O$) of nitrate in each stream i during each observation n, respectively; f_i denotes the flow rate of each stream i during each observation n; and Δt_n denotes the time interval between the observation n and the next observation n + 1.

For Q_{in}, we used the annual influx of water estimated by Kunimatsu et al. (1995), in which influx via streams and via groundwater were included. To include the influx of nitrate via groundwater and the other minor streams not measured in this study in the calculations, we

- 1 used the correction factor α in Eq. (10), whereby we assumed that both the average 2 concentration and average isotopic compositions of the inflows determined in this study 3 represented those of all inflows into the lake, while assuming an error range of 20% in α . 4 Under the Q_{in}, the correction factor α used in this study became 1.9±0.4.
- By using the aforementioned equations, we estimated the total influx of nitrate to the lake (ΔN_{in}) for each interval, together with the average $\delta^{15}N$, $\delta^{18}O$, and $\Delta^{17}O$ values of nitrate during each interval, as presented in Table 2. Moreover, by using the values of ΔN_{in} during each interval, as well as their $\delta^{15}N$, $\delta^{18}O$, and $\Delta^{17}O$ values, we estimated the total influx of $NO_3^-(atm)$ to the lake (ΔA_{in}) and the average $\delta^{15}N_{re}$ and $\delta^{18}O_{re}$ values for each interval, as presented in Table 2, by using Eqs. (13), (6), and (7). Furthermore, we estimated the annual total influx and the various annual average influx values, as shown in Table 2.
- The annual average Δ^{17} O value of inflows estimated by using Eq. (16) was +1.3‰, which corresponds to <u>an</u> average mole fraction of NO₃⁻(atm) within total nitrate of (5.1±0.5)% <u>by</u> Eq. (2). We concluded that about 5% of the total nitrate in the inflows originated directly from the atmosphere; therefore, the remainder of the nitrate <u>had a</u> remineralized origin (NO₃⁻(re)) <u>and was</u> likely produced through nitrification within the catchments, as discussed in Section 3.4. In addition, we estimated the annual total influx of nitrate to the lake (ΔN_{in}) to be (199±40) Mmol and that of NO₃⁻(atm) (ΔA_{in}) to be (10.1±2.0) Mmol.
- 19 Moreover, we estimated the total efflux of nitrate and $NO_3^{-}(atm)$ from the lake via the 20 outflows (ΔN_{out} and ΔA_{out}) for each interval by using Eqs. (10) to (16) in which ΔN_{in} was replaced with ΔN_{out} , and ΔA_{in} was replaced with ΔA_{out} . Additionally, Q_{in} was replaced with 21 22 Qout, which is the annual gross efflux of water. To include the minor effluxes of nitrate to 23 ΔN_{out} , such as <u>those</u> via canals, we used the correction factor γ instead of α in Eqs. (10) and 24 (11), whereby we assumed that both the concentration and isotopic compositions of the natural outflow determined for each season in this study represented all outflows. For Qout, we 25 26 used the annual efflux of water from Lake Biwa estimated by Kunimatsu et al. (1995), which included the efflux via a natural river (Seta River, No. 33) and that via canals. Under the Qout, 27 28 the correction factor γ used in this study became 1.1±0.2.
- 29 Compared with the annual ΔN_{in} and <u>the</u> annual ΔA_{in_s} both the annual ΔN_{out} and <u>the</u> annual 30 ΔA_{out} were significantly smaller by about 66% and 78%, respectively. Hence, Lake Biwa acts 31 as a net sink for both nitrate and NO_3^- (atm), as previously implied from the ¹⁵N enrichment in 32 outflows. Considering that nitrate constituted about 70% of the total fixed N pool in the

inflows and about 40% of the total fixed N pool in the outflows (Shiga prefecture, 2015), 1 2 Lake Biwa also acts as a net sink for fixed N. Similar results were obtained in previous 3 studies that discussed the fixed N input/output of the lake (Tezuka, 1985, 1992; Kunimatsu, 1995; Yamada et al., 1996). As implied by the significant ¹⁵N enrichment in the remineralized 4 portion of nitrate $(\delta^{15}N_{re})$ in the outflow, (+13.7±0.6)‰, compared to the inflow, 5 (+5.6±0.3)‰, partial removal of nitrate through either assimilation or denitrification is 6 strongly responsible for the (8.1±1.1)‰ increase in $\delta^{15}N_{re}$ as well as the net removal of both 7 nitrate and $NO_3^{-}(atm)$ from the lake. 8

On the contrary, the δ^{18} O differences in the remineralized portion of nitrate (δ^{18} O_{re}) between 9 the inflows and outflows were significantly smaller than those of $\delta^{15}N_{re}$, at an annual average 10 of only (1.6±1.8)‰ (Table 2). If the nitrate in the outflows is the residual nitrate of 11 assimilation/denitrification in the lake, $\delta^{18}O_{re}$ should <u>also</u> increase (<u>Granger et al., 2004;</u> 12 Granger et al., 2008). The much smaller $\delta^{18}O_{re}$ difference implies that nitrate supplied directly 13 14 from inflows occupied a small portion of the nitrate in the outflows and that most of nitrate with high δ^{15} N values in the outflows was produced through nitrification in the lake water 15 column in which the fixed N was enriched in ¹⁵N. Isotopic fractionations during fixed N 16 cycling in the lake, such as during denitrification or assimilation, and the subsequent removal 17 of ¹⁵N-depleted organic N during sedimentation (Fig. 1) are likely responsible for the ¹⁵N 18 enrichment of the total fixed N. That is, most of nitrate fed into the lake via the inflows was 19 20 removed at least once from the lake water column and was involved in the total fixed N cycling in the lake, in which the ¹⁵N-enriched nitrate in the outflow was produced (Fig. 1). 21 The stagnation of flow in the lake (around 5 years) encouraged primary production and thus 22 23 the net removal of total fixed N through either denitrification or sedimentation, which resulted in ¹⁵N enrichment of the total fixed N pool compared with that in the inflows. Further studies 24 on N cycling in the lake are needed to verify these results. 25

26

27 4 Concluding Remarks

In this study, we applied the Δ^{17} O tracer of nitrate to determine accurate and precise mole fractions of unprocessed NO₃⁻(atm) within the total nitrate value for more than 30 streams in the Lake Biwa watershed basin. Although the nitrate concentration <u>varied</u> from 12.7 to 106.2 μ mol L⁻¹ among the inflow streams and the mole fraction of NO₃⁻(atm) within <u>the</u> total nitrate also <u>varied</u> from 1.8% to 11.8%, the absolute concentration of NO₃⁻(atm) (C_{atm}) in

each stream water was almost stable at (2.3 ± 1.1) µmol L⁻¹ irrespective of the changes in 1 population density and land use among the catchment areas. We concluded that changes in 2 population density and land use among the catchment areas had little impact on C_{atm} and that 3 the total nitrate concentration was determined primarily by the extent of the additional 4 5 NO_3 (re) contribution, which was mostly from anthropogenic sources. When relying on only the δ^{15} N and δ^{18} O tracers of nitrate, it was difficult to determine the precise C_{atm} in <u>the</u> stream 6 water and whether C_{atm} was uniform among the streams. By using the $\Delta^{17}O$ values, we were 7 8 able to estimate accurate and precise Catm in each stream for each season; thus, we could 9 clarify the fate of the $NO_3^{-}(atm)$ deposited into the catchments.

Moreover, additional measurements of the $\Delta^{17}O$ values of nitrate together with $\delta^{15}N$ and $\delta^{18}O$ 10 enabled us to exclude the contribution of NO₃ (atm) from the determined $\delta^{15}N$ and $\delta^{18}O$ 11 values and to use the corrected δ^{15} N and δ^{18} O values, δ^{15} N_{re} and δ^{18} O_{re}, to evaluate the source 12 and behaviour of NO₃ (re) in each stream. Based on the correction, we successfully estimated 13 the δ^{15} N and δ^{18} O values of NO₃ (re) in the streams to be (+4.4±1.8)‰ and (-2.3±0.9)‰, 14 respectively, for NO_3^{-} produced through nitrification in naturally occurring soil organic 15 matter and $(+9.2\pm1.3)$ % and (-2.2 ± 1.1) %, respectively, for NO₃⁻ supplied from 16 anthropogenic sources, most of which were sewage effluent. In addition, the low and uniform 17 annual average $\delta^{18}O_{re}$ values of NO₃ (re) in the streams implied that denitrification in the 18 riverbed sediments was minor in the streams. 19

Furthermore, we clarified the seasonal changes in the range of isotopic fractionation through partial nitrate removal via assimilation or denitrification by using the relationship between $\Delta^{17}O$ and $\delta^{18}O$ of nitrate in the streams. The changes were small in winter in all of the catchment areas but large in summer in some catchments. Therefore, the relationship between $\Delta^{17}O$ and $\delta^{18}O$ of nitrate eluted from a catchment area is a powerful indicator for evaluating the biogeochemical nitrogen cycles within a catchment area, including the seasonal changes.

Based on the annual influx and efflux of both nitrate and $NO_3^-(atm)$ in Lake Biwa newly estimated in this study, we found that Lake Biwa <u>is</u> a net sink for both nitrate and $NO_3^-(atm)$. Additionally, we found significant ¹⁵N_enrichment ((+8.1±1.1)‰) in the remineralized portion of nitrate in the outflow compared with those in <u>the</u> inflows, whereas <u>the</u> ¹⁸O_enrichment was only (+1.6±1.8)‰. We concluded that most of <u>the</u> nitrate fed into the lake via the inflows <u>was</u> removed at least once from the lake water column and <u>was</u> involved in the total fixed N cycling in the lake, <u>by</u> which the ¹⁵N-enriched nitrate in the outflow <u>was</u> produced (Fig. 1). The stagnation of flow in the lake encouraged primary production and thus net removal of
 total fixed N through either denitrification or sedimentation, which resulted in ¹⁵N enrichment
 of the total fixed N pool compared with that of the inflows.

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- 6

No.	Name	Loc.#	Basin Area [*]	Population Density [*]	Land Use ^{\$}	No	. Name	Loc.#	Basin Area [*]	Population Density [*]	Land Use ^{\$}
			(km^2)	$({\rm km}^{-2})$					(km^2)	$({\rm km}^{-2})$	
Inflow											
31	Tenjin	West	10	539	Forest	14	Seri	East	74	462	Forest
30	Mano	West	23	1048	Forest	15	Inukami	East	102	109	Forest
29	Wani	West	17	186	Forest	16	Ajiki	East	15	1002	Agr
28	U	West	7	66	Forest	17	Uso	East	84	411	Agr
1	Kamo	West	47	89	Forest	18	Bunroku	East	14	595	Agr
2	Ado	West	306	27	Forest	19	Nomazu	East	7	758	Agr
3	Ishida	North	60	84	Forest	20	Echi	East	211	110	Forest
4	Momose	North	13	65	Forest	27	Hino	South	226	338	Forest
5	Chinai	North	51	44	Forest	26	Yanomune	South	42	859	Agr
6	Ohura	North	39	98	Forest	25	Yasu	South	391	324	Forest
7	Oh	North	20	55	Forest	24	Yamaga	South	6	2540	Agr
8	Yogo	North	7	141	Forest	23	Sakai	South	2	979	Agr
9	Chonoki	North	10	412	Agr	22	Hayama	South	34	2048	Agr
10	Та	North	36	301	Agr	34	Kusatsu	South	48	370	Agr
11	Ane	North	372	61	Forest	21	Nagaso	South	4	3174	Res
12	Yone	North	15	2047	Agr	32	Fujinoki	South	4	1805	Forest
13	Amano	North	111	226	Forest						
						Ou	Outflow				
						33	Seta	South	3848	323	Forest

[#] Category of location classified by Ohte et al. (2010).

2

^{*} Data source: Ohte et al. (2010).

[§] <u>Dominant</u> land use in the respective catchment <u>of a</u>gricultural land (Agr), <u>forest</u>, <u>or r</u>esidential (Res). See Table S1 for the specific contents.

- 1 Table 2 Estimated gross influx/efflux of total nitrate (ΔN) and atmospheric nitrate (ΔA) via
- 2 inflows/outflows during each observation interval, together with the average δ^{15} N, δ^{18} O, and
- Δ^{17} O values of total nitrate and remineralized portions of nitrate (δ^{15} N_{re} and δ^{18} O_{re}) in the

4 in	flows/outflows	during	each	interval
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	Spring	Summer	Autumn	Winter	Annual
	(n=1 to 2)	(n=2 to 3)	(n=3 to 4)	(n=4 to 5)	(n=1 to 5)
Duration (days)	94	49	77	145	365
Inflow					
ΔN_{in} (10 ⁶ mol)	69 ± 14	3 ± 1	13 ± 3	114 ± 23	199 ± 40
ΔA_{in} (10 ⁶ mol)	6.4 ± 1.3	0.1	0.8 ± 0.2	2.8 ± 0.6	10.1 ± 2.0
$10^3 \delta^{15} N$	+4.0	+6.8	+5.6	+5.6	+5.1
$10^3 \delta^{18}$ O	+6.1	-0.8	+3.3	-1.5	+1.4
$10^3 \Delta^{17} O$	+2.5	+0.8	+1.7	+0.6	+1.3
$10^3 \delta^{15} N_{re}$	$+4.8\pm0.7$	$+7.1\pm0.2$	$+6.3\pm0.5$	$+5.9\pm0.2$	$+5.6\pm0.3$
$10^3 \delta^{18} O_{re}$	-1.5 ± 1.8	-3.2 ± 0.5	-2.0 ± 1.2	-3.5 ± 0.4	-2.8 ± 0.9
Outflow					
$\Delta N_{out} (10^6 \text{ mol})$	24 ± 5	6 ± 1	5 ± 1	32 ± 6	67 ± 13
$\Delta A_{out} (10^6 \text{ mol})$	1.4 ± 0.1	0.1	0.2	0.4 ± 0.1	2.2 ± 0.4
$10^3 \delta^{15} N$	+7.3	+11.4	+10.4	+18.0	+13.1
$10^3 \delta^{18}$ O	+3.4	+4.8	+3.0	-0.7	+1.5
$10^3 \Delta^{17} O$	+1.6	+0.4	+1.4	+0.4	+0.9
$10^3 \delta^{15} N_{re}$	$+8.1\pm0.6$	$+11.7\pm0.4$	$+11.2\pm0.9$	$+18.3 \pm 0.4$	$+13.7 \pm 0.6$
$10^3 \delta^{18} O_{re}$	-1.5 ± 1.4	$+3.6\pm0.6$	-1.2 ± 1.2	-1.9 ± 0.5	-1.2 ± 0.9



Figure 1. Schematic diagram showing the biological processing of atmospheric nitrate $(NO_3^{-}(atm))$ and remineralized nitrate $(NO_3^{-}(re))$ in the watershed with catchments of <u>various</u> land uses and in the lake water column.



Figure 2. (a) Map showing the location of <u>the Lake Biwa watershed basin in Japan and Sado</u>
Island, where the Sado-seki National Acid Rain Monitoring Station is located. (b) Map
showing the boundary of the Lake Biwa watershed basin (dashed line) and the locations of the
inflows (represented by numbers) and <u>the outflow</u> (Seta River, No. 33) studied in this paper
(modified from Ohte et al. 2010).



Figure 3. Temporal variations in the values of (a) δ^{15} N, (b) δ^{18} O, and (c) Δ^{17} O of nitrate in wet deposition recorded at the Sado-seki National Acid Rain Monitoring Station. The errors were smaller than the sizes of <u>the symbols</u>. <u>The daily</u> depositional flux of nitrate when each of the wet deposition samples was taken <u>is</u> also presented (c).



Figure 4. Relationship between $\Delta^{17}O$ and $\delta^{18}O$ for atmospheric nitrate in wet deposition. The errors were smaller than the size of <u>the</u> symbols. The blue circles represent <u>data for</u> winter, from November to March, whereas the orange squares represent <u>data for</u> summer, from April to October. The blue solid and orange dashed lines represent the least-squares fitted lines to the data of winter and summer, respectively.

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Figure 5. Distribution of (a) total concentrations, (b) $\delta^{15}N$, (c) $\delta^{18}O$, and (d) $\Delta^{17}O$ for nitrate, 3 and (e) atmospheric nitrate concentrations in inflow streams by the various station numbers and the outflow station No. 33 in the Lake Biwa watershed in March (blue diamonds), June (green squares), August (red triangles), and October (orange circles) 2013 together with the annual averages for each river (black bars). The errors were smaller than the sizes of the symbols except <u>for (e)</u>.



1

Figure 6. Relationship of the annual average values of $\Delta^{17}O$ and $\delta^{18}O$ of NO₃⁻ in the inflow streams. The symbols represent the location of each river (west: yellowish green diamonds; north: green circles; east: red triangles; south: orange squares). A hypothetical mixing line between atmospheric nitrate (NO₃⁻(atm)) and remineralized nitrate (NO₃⁻(re)) is shown together with the end_member value of NO₃⁻(re) (large white square) and the 20% and 30% mole fractions of NO₃⁻(atm) on the line. The errors were smaller than the sizes of <u>the</u> symbols.



Figure 7. Annual average concentration of (a) nitrate (C_{total}) and (b) NO₃⁻(atm) (C_{atm}) in each inflow stream plotted as a function of the population density in each catchment, together with (c) the annual average value of δ^{15} N and (d) that of δ^{18} O for remineralized NO₃⁻ (NO₃⁻(re)). The annual average δ^{18} O values of nitrate (total) are also presented in (d). The symbols represent the location of each river (west: diamonds; north: circles; east: triangles; south: squares). The errors larger than the sizes of <u>the</u> symbols <u>are</u> presented <u>by</u> error bars. Some of the streams in (b) <u>are indicated</u> by number.



2 Figure 8. Temporal changes in the relationship between the values of $\Delta^{17}O$ and $\delta^{18}O$ of <u>the</u> 3 total nitrate in the stream water. The symbols are the same as those in Figure 6. A 4 hypothetical mixing line between atmospheric nitrate (NO₃⁻(atm)) and remineralized nitrate 5 (NO₃⁻(re)) is also shown together with the end_member value of NO₃⁻(re) (large white 6 square). The errors were smaller than the sizes of <u>the</u> symbols.